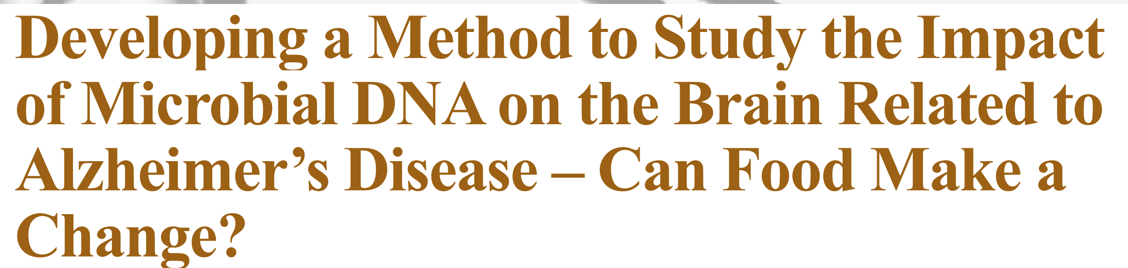

Developing a Method to Study the Impact of Microbial DNA on the Brain Related to Alzheimer’s Disease – Can Food Make a Change?

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Developing a Method to Study the Impact of Microbial DNA on the Brain Related to Alzheimer’s Disease – Can Food Make a Change?

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To all my beloved family

The five grains, the five animals, the five fruits, the five vegetables are called food if they are used for sustenance. They are called medicine if they are used for treatment of diseases or illness.
五穀、五畜、五果、五菜，用之充飢則謂之食，以其療病則謂之藥。

- Grand Simplicity of Inner Canon of Huangdi, Yang Shangshan;《黃帝內經太素》楊上善

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Abstract

Understanding the gut-brain-microbiota axis can help us provide a new tool to manage conditions such as Alzheimer's. However, a major challenge is selectively amplifying the bacterial DNA which is present at a much lower level than the host DNA. In this thesis, we aim to develop a methodology to extract, purify and quantify the bacterial DNA in the brain tissue of mice. The methodology that can be used for further application studying gut-brain-microbiota axis, which in this case is comparison of the microbial DNA composition in mice with Alzheimer's disease vs. healthy littermates fed with natural foods. A major hurdle to this approach is to identify the possible bacterial contamination during the extraction step and to eliminate the host DNA. Brain is a unique and challenging organ to study. The amount of lipids content is second abundance to adipose tissue among others and the solid blood-brain-barrier (BBB) delicately protects the central nerve system (CNS) from microbe's invasion. The extreme low amount of microbial DNA compared to host DNA after the extraction complicate the efficiency to characterize the microbiota community in the brain tissue. Therefore, in this study we evaluated the bacterial DNA extraction efficiency from murine brain samples and a human saliva, using two commercially available kits (Qiagen DNeasy Blood & Tissue Kits and Promega Wizard® Genomic DNA Purification Kit). While the host DNA depletion was performed using NEBNext Microbiome DNA Enrichment kit. The purified and enriched DNA was further analyzed using gel electrophoresis and finally sequenced by next-generation sequencing targeting the V4 region of the 16S ribosomal RNA gene. The percentage of microbiome DNA component increased in Qiagen compared to Promega method with host DNA depletion. DNA extraction with host DNA depletion influences microbial community composition underlying the need for careful selection of DNA extraction kit and usage of microbiome DNA enrichment to improve recovery from a range of bacterial taxa. Overall, the bacterial community profile of brain tissue was not well characterized in this study which needed further investigation.

Key words: DNA extraction, Microbiome DNA enrichment, Host DNA depletion, 16S rRNA sequencing, Alzheimer's disease, Gut-brain axis, Microbiota, Microbial DNA, Black currant

Popular Summary

Apart from our 30 trillion cells which constitute our body, every individual cell harbors 39 trillion little mighty guys that are invisible to human's naked eyes including bacteria, viruses, yeasts, protozoa, and fungi. They can contribute to our health, or they can sometimes be pathogenic and make us sick. They started to build their communities within our body once we were exposed to this world out of our mother's womb and expanded their communities with time. Every person has their unique mighty guy composition. Their composition can vary depending on our genetics, where we live, what we eat, and what we do.

Majority of microorganisms reside in our gut forming gut flora which is significantly dependent on what we eat. Sometimes bacteria or their compounds travel around our body including the brain, our bodies most secure place. They would usually be stopped by the gut-blood and blood-brain-barrier. Yet, some of them can break through the lines under specific circumstances. For instance, it has been shown that production of a specific protein by-product called amyloid-beta causing Alzheimer's disease, which makes our beloved one lose their precious memories, has been linked to changes in gut microbiota composition and also a damage to the blood-brain-barrier that may allow bacteria to come to the brain.

Therefore, we want to find out what kind of bacteria can break the blood-brain-barriers. In order to achieve this, we set out to develop a method to study the presence of bacterial genetic material in murine brain. Through this approach we would be able to better understand the bacterial community in the brain, taking us closer to understanding this process in humans and how we can be more mindful about prevention of diseases like Alzheimer's.

Populärvetenskaplig Sammanfattning

Förutom de 30 biljoner celler som utgör vår kropp, hyser varje individ 39 biljoner små mäktiga mikroorganismer som är osynliga för människans blotta ögon inklusive bakterier, virus, jästsvampar, protozoer och svampar. De kan gynna vår hälsa, men också vara patogena för att göra oss sjuka. De började bygga sina samhällen i vår kropp när vi exponerade den för denna värld ur vår mammas mage och expanderade med tiden. Varje person har sin unika mäktiga bakteriekomposition. Deras sammansättning kan variera beroende på vår genetik, var vi bor, vad vi äter och vad vi gör.

Majoriteten av mikroorganismerna finns i vår tarmbildande tarmflora som är mycket beroende på vad vi äter. Ibland färdas bakterier eller deras föreningar runt vår kropp inklusive hjärnan, vår säkraste plats i kroppen. De skulle vanligtvis stoppas av tarm-blod- och blod-hjärnbarriären. Ändå kan vissa av dem bryta igenom linjerna under specifika omständigheter. Det har till exempel visat sig att produktionen av en specifik proteinbiprodukt som kallas amyloid-beta som orsakar Alzheimers sjukdom, som gör att vår älskade förlorar de värdefulla minnena, har kopplats till förändringar i tarmmikrobiotans sammansättning och även en skada på blodet -hjärnbarriär som kan tillåta bakterier att komma till hjärnan.

Därför vill vi ta reda på vilken typ av bakterier som kan bryta blod-hjärnbarriärerna. För att uppnå detta siktar vi på att utveckla en metod för att studera närvaron av bakteriellt genetiskt material i murin hjärna. Genom detta tillvägagångssätt skulle vi kunna bättre förstå bakteriesamhället i hjärnan, vilket tar oss närmare förståelsen av denna process hos människor och hur vi kan vara mer uppmärksamma på att förebygga sjukdomar som Alzheimers.

Abbreviations

Aβ	Amyloid beta
AD	Alzheimer’s disease
BBB	Blood-brain barrier
CNS	Central nerve system
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EOAD	Early-onset Alzheimer’s disease
GI	Gastrointestinal
GBA	Gut-brain axis
MGB	Microbiota-gut-brain
LOAD	Late-onset Alzheimer’s disease
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFAs	Short chain fatty acids

Introduction

Microbiome

Microorganisms exist as different communities within human body. There are such as skin-, oral-, gut- microbiota etc. Every microbiota is unique to every individual, but it varies systematically along with time and across body habitats [1]. Bacteria from microbiome can be both health-promoting, neutral or even have a harmful effect on host health status.

Next Generation Sequencing

Approach to studying the microbiome in gut and other organs method is completely different since the composition of organs are varied.

DNA sequencing has been established to decode the information for the hereditary and biochemistry properties of life on earth contained in the polynucleotide chains [2]. Next Generation Sequencing, NGS is a DNA sequencing of 16S rRNA gene technology that revolutionized genomic research commonly used in metagenomic studies of complex microbial communities [3]. The massively parallel or deep sequencing of NGS allows millions of fragments to be sequenced in a single run to complete an entire human genome sequencing within a day. In contrast to first-generation sequencing such as Sanger sequencing, requires over a decade to complete since it sequences single genes at a time and can only produce one forward and reverse read [4].

NGS offers fast turnaround time with lower cost and only require small input amounts of starting materials. However, it is highly dependent on DNA quality. Several methods for sample preservation and DNA extraction have been developed, while the standardization of protocol for lipid-rich tissue with low amount of bacterial DNA is still under developmental process.

The brain has the second abundance of lipid content just behind adipose tissue, and the lipids make up 50 % of the brain dry weight [5]. The various lipids and lipid intermediates are of importance of maintaining the structure and function of the brain. The composition of lipids in the brain also makes DNA extraction from it more difficult than from the stool due to the lipids interfering with tissue disruption or influencing the chemistry of the DNA isolation buffer [6]. Apart from the difficulties of microbiome extraction from the fatty tissue, the immense host and microbe's ratio decreases sensitivity for microbiome detection and hampers microbiome identification in the brain [7, 8]. The contamination derived from the DNA extraction kits and other laboratory reagents is another hurdle for analysis [9]. Therefore, in order to eliminate the influence from the host DNA,

the separation of the microbe's DNA from the host using the DNA enrichment method to collect the largest number of it is critical for the analysis and will be evaluated in this study.

Gut-brain microbiome

The gut-brain microbiome has drawn public's attention recently. The gut microbiota is thought to drive the brain disorders by producing various metabolites [10], so did the inflammatory mediators, released by oral microbiota situated in oral cavity being the closest region to the brain, migrate to the distant organs or tissues such as brain through bloodstream [11, 12]. Besides sending signals and producing metabolites, bacteria can also escape from their niche and travel to brain directly [13]. A novel study suggests an increasing bacteria population in AD brain tissue from temporal cortex in frozen and fixed post-mortem comparing to the normal one [14]. The finding indicates that the brain might not be as sterile as we think, and high levels of bacteria in the brain may contribute to the AD development.

Food, disease, and the microbiome

"You are what you eat" is commonly used in the health-related conversations about the connection between food and body. It has been widely shown that microbiota composition can be changed by different diets and food supplements [15]. The experiments showed that large, temporary microbial shifts happened within just 24 hours by altering the diet indicating that diet plays a significant role in shaping the microbiome [16]. Unhealthy food, for instance ultra-processed foods, presence of potentially toxic substances such as polycyclic aromatic hydrocarbons that alter the key bacterial genera result in numerous chronic diseases including inflammatory bowel diseases, obesity, and type 2 diabetes [17]. Thereupon, it is of importance to take in the quality of food and emphasize minimally processed plant foods, black currants for instance used in this study, that increase and maintain the abundance of different bacterial genera to provide pronounced effects on immune status and metabolic health by combining multi fibers [18, 19].

Black currant

Black currants grow in more temperate areas of Northern Europe and Northern Asia and has been a popular snack in Europe for centuries. The strong flavor berries are full of antioxidants such as anthocyanins that give black currants prominent dark color and reduce oxidative stress and cell damage in the body by protecting it from free radicals' attack [20]. Black currant with positive effect on metabolic health, which was been hypothesized, coincided with gut microbiota composition changes in mice, but also in an age-dependent manner [21].

Alzheimer's Disease

An overview

Alzheimer's disease and other dementias is the third cause of deaths for people who were 70 years and older in Sweden, and at the fourth place of death for the elderly in the world [22]. Alzheimer's disease is a progressive neurodegenerative disorder that is the most common cause of dementia. According to World Health Organization, WHO, it contributes to 60-70 % of cases. The progression of the disease does not only impair the individual's life quality from physical, psychological, social to economic but also for their caregivers, families, and entire society [23] being a huge impact on the nation's health system. The cost of care for patients with AD is high and related to the severity of cognitive function and the presence of behavioral disturbances [24].

Cause of Alzheimer's disease

Alzheimer's disease is the result of multiple factors such as excessive extracellular accumulation of Amyloid-beta 42 plaques, intracellular hyperphosphorylated Tau tangles, generation of reactive oxygen species due to mitochondria dysfunction and genetic mutations [25]. AD is mostly characterized by the formation of abnormal proteins amyloid-β (Aβ) plaques and tau tangles. The abnormal proteins result in damages in the brain hindering of communication between nerve cells which eventually cause cell death of neurons. The shrinkage of brain, symptoms like memory loss, language problems, and unpredictable behavior begin to appear after irreversible damages in the brain gradually developed to a certain extent in years [26]. Recent studies have shown that the deposition of Aβ is to protect against microbial inflammation [27] and the formation of misfolding proteins are promoted by bacterial DNA [28]. Dysbiosis in both gut and oral microbiota was proposed to induce and accelerate the formation of Aβ and tau tangles [13].

The abundance of *Proteobacteria* is highly enriched in the feces of patients with Alzheimer's. In contrast, the relative proportion of phylum *Firmicutes* is significantly reduced, which influences the central nervous system (CNS) immunity and neuroendocrine [29]. Other studies show decreased *Firmicutes*, increased *Bacteroidetes*, and decreased *Bifidobacterium* in AD subjects [30]. Since the link between AD and gut microbiota is confirmed [31], AD patients may have different types of bacteria compared to the healthy people not only in the gut but also in their brain.

A wealth of evidence shows that brain mitochondrial dysfunction is involved in the development of neurodegenerative disease [32] due to the changes of physiological activities in the brain by the intervention of microbial metabolites [33]. Emerging evidence suggesting that the cross-talk between gut microbiota and host mitochondria plays a pivotal role during health and disease [34]. The mitochondrial dysfunction and associated oxidative stress, is linked to the changes in the gut microbiota [35] and the root of connection between diabetes, a metabolic disease increases the risk of developing Alzheimer's [36], and coexisting disorders in the brain [37].

The brain microbiota and Alzheimer's disease

The aforementioned has suggested the impact of oral and gut microbiota to the brain, therefore, nutritional tools such as changes in diet, probiotics, and prebiotics for altering the gut microbiome therapeutically are being considered. To study mitigating the progression of Alzheimer's via the gut-brain axis in vivo, a brain microbial DNA quantification approach will be adopted. It is also the ultimate goal for this study to shed more light on if food can really make a change of the composition of microbiota in the brain, in the context of AD pathophysiology and introducing different diet patterns in mice.

Aims of the Thesis

The primary aim of this thesis was to establish an effective and reliable method to yield highest microbial DNA from the brain tissues to set a groundwork for further study. One of the objectives was to study the microbial profile in rodents' brains with different diets related to counteracting Alzheimer's disease development. Hereby using black currant counteracted the adverse effect induced by high fat diet to imitate the disease development and alleviation. Finally, the healthy mice brain microbial profile was characterized, and the approach was proved repeatable.

I. Develop a brain microbial DNA extraction for mice.

II. Investigate how the brain microbial DNA composition described and characterized by using different DNA extraction kits.

Methodology

Materials

Animals

Adult Apoe-/- C57BL/6J middle-aged (22 weeks) male mice were housed in standard laboratory conditions at the Department of Biology, Lund University and acclimatized for two weeks before the experiment. They were fed at 8-week-old with high-fat diet (40 % calories from saturated fat, modified from D12450J) supplemented with black currant powder at a dose of 8 % (*w/w*) fiber on a dry weight basis resulted in 32 % black currant in the diet. After 8 weeks, the mice were sacrificed by heart puncture under anesthesia with isoflurane (Abbott Scandinavia AB, Sweden). The brain tissues were perfused with 5mL sterile phosphate buffer saline (PBS) before removal. The right- and left-brain hemispheres were divided and immediately frozen on dry ice. All experimental procedures to obtain the mice brains used in this study were performed by experienced person according to ethical approval (number: 9874-20) by the Malmö-Lund Ethics Committee on Animal. The brain tissues were kept at -40 °C until the analysis.

In addition, the saliva sample for DNA extraction optimization was taken from an adult female and used as a positive control.

Chemicals

The Milli-Q® Lab Water Solutions system (Germany) was used to obtain purified water for all the analytical procedures. Tris-EDTA buffer (pH 7.4) was obtained from Sigma-Aldrich (Germany). Invitrogen™ DEPC-treated molecular biology grade water free from DNase and RNase for sample preparation was purchased from Thermo Fischer Scientific (USA). Agarose I powder (Thermo Fischer Scientific, USA) was mixed with 1x diluted Tris-acetate-EDTA, TAE buffer from 50x stocked solution purchased from Thermo Fischer Scientific (USA) to make 1 % agarose gel for electrophoresis analysis.

DNA extraction kits

i. DNeasy Blood & Tissue Kits (Qiagen)
ii. Wizard® Genomic DNA Purification Kit (Promega)

Methods

Samples from Mice Brain Tissue

A total of 2 fresh-frozen mice brain tissues in a 1.5 mL microtube (*Figure 4A*) were disrupted using TissueLyser II (Qiagen, Germany) *(Figure 4B)* through 29.5 Hz speed shaking with 5 mm stainless steel beads (Qiagen, Germany) for 5 minutes followed by DNA extraction to increase the concentration of total DNA. The disruption of tissue was achieved by placing the brain tissue with 1 stainless steel bead with 180 µL tissue lysing ATL buffer (Qiagen, Germany) or 600 µL Nuclei lysis solution (Promega, USA) respectively.

Genomic DNA Extraction

DNA extracted from the lysed mice brain tissue was adapted according to the manufacturer's instructions using Qiagen DNeasy Blood & Tissue Kits (Germany) and Promega Wizard® Genomic DNA Purification Kit (USA) respectively with some modifications and briefly mentioned in following session.

Qiagen DNeasy Blood & Tissue Kits

After tissue lysis, the lysate was precipitated with 200 µL AL solution and 200 µL graded ethanol (*Figure 1*). The lysate was transferred to the spin column and washed with AW1 and AW2 buffer. Finally, DNA was eluted with 200 µL AE solution at room temperature for 5 minutes and another 5 minutes elution with 100 µL AE solution at room temperature to collect maximum yield of DNA by centrifugation at 8,000 x *g* for 1 minute. The extracted DNA were stored at -20 °C before performing microbiome DNA enrichment.

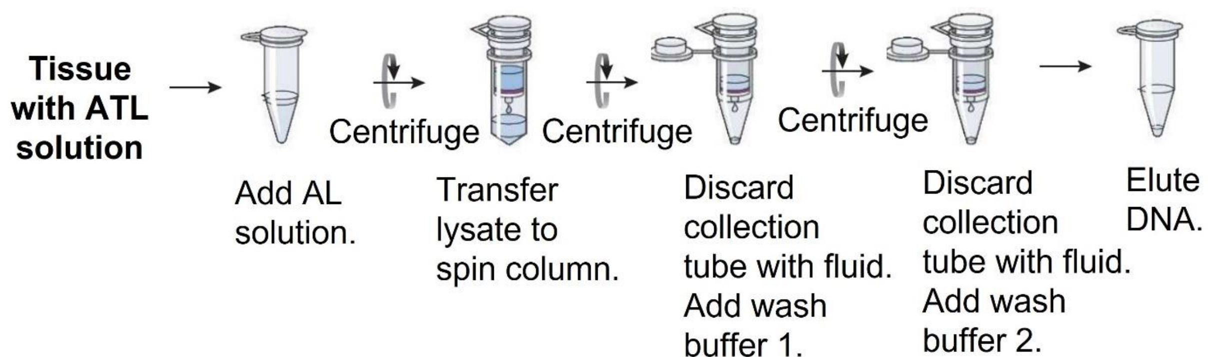


Figure 1 Workflow of Qiagen DNeasy Blood & Tissue Kits (Qiagen, *DNeasy Mini and 96 rocedures,* 2022).

Promega Wizard® Genomic DNA Purification Kit

After tissue lysis, the lysate was incubated at 65 °C for 30 minutes. The protein was separated from the DNA with 200 μL protein precipitation solution (*Figure 2*). The DNA was suspended and collected after centrifugation at 16,000 x *g* for 4 minutes. The lysate was transferred to the 1.5 mL microtube and was precipitated with 600 μL graded isopropanol at room temperature. The supernatant with DNA and isopropanol was gently mixed by inversion until the visible mass of DNA appeared in white thread-like strands. The small white pallet DNA was visible after centrifuged at 16,000 x *g* for 1 minute at room temperature. Then the DNA pellet was rinsed with 600 μL graded 70 % ethanol and air-dried for 15 minutes. Finally, DNA was rehydrated and collected with 100 μL rehydration solution by incubating at 65 °C for 1 hour. The extracted DNA were stored at 4 ℃ before performing microbiome DNA enrichment.

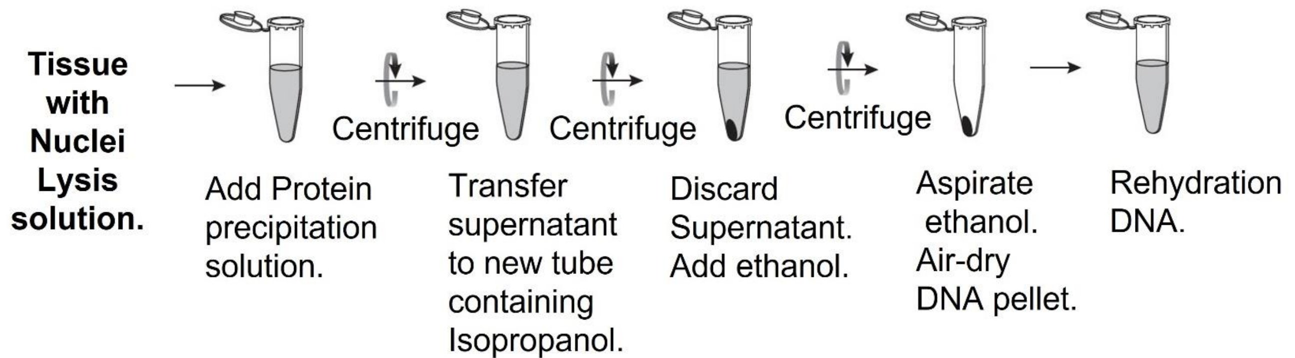


Figure 2 Workflow of Promega Wizard® Genomic DNA Purification Kit (Promega, Isolation of Genomic DNA from Animal Tissue and Tissue Culture Cells, *Wizard® Genomic DNA Purification Kit Quick Protocol FB022,* 2022).

Microbiome DNA Enrichment

The bacterial DNA separated from the host DNA from the mice brain tissue was conducted according to the manufacturer's instructions using NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs, USA). Briefly, after prebinding MBD2-Fc protein to magnetic beads, the methylated host DNA was captured by the MBD2-Fc bound magnetic beads. Then, the isolated microbial DNA was collected. The microbial DNA was purified by AMPure XP beads (Beckman coulter, USA).

Prebind MBD2-Fc Protein to Magnetic Beads

The required MBD2-Fc-bound magnetic beads was calculated according to the extracted DNA concentration. The optimized affinity is per μL MBD2-Fc-bound magnetic beads combined with 6.25 ng/μL DNA. 1X diluted NEBNext Bind Buffer was prepared by adding 1 part 5x buffer into 4 parts DEPC water and kept on ice for later use. Calculated MBD2-Fc protein was added to resuspended NEBNext Protein A Magnetic Beads in 1.5 mL microtube and mixed completely homogeneous by pipetting for 10 times. The bead-protein mixture was placed on a mixer for 10

minutes at room temperature. After being briefly spun, the beads were clung on the wall by placing on the magnetic rack for 2-5 minutes. The beads were then washed with 1 mL prepared 1x Bind buffer by pipetting 10 times after removing the supernatant, placing the tube on a mixer for 3 minutes at room temperature then briefly spinning and placing on the magnetic rack for 2-5 minutes to collect the beads. The washing step was repeated twice. Finally, the same amount of 1x Bind Buffer was added to resuspend the beads by pipetting a few times.

Capture Methylated Host DNA

The amount of extracted DNA used for MBD2-Fc-bound magnetic beads calculation was added to the prepared prebound beads. 5x Bind Buffer was added for a final concentration of 1x. The beads and the buffer were mixed in the microtube by pipetting 10 times and the tube was placed on the mixer for 15 minutes at room temperature.

Collect Enriched Microbial DNA

After briefly spun the tube, the beads were gathered by placing on the magnetic rack for 5 minutes. The supernatant containing the microbial DNA was transferred into a clean microtube without disturbing the beads.

AMPure XP Bead Cleanup

1.8x volume of resuspended AMPure XP Beads were added to the tube with microbial DNA and incubated for 5 minutes at room temperature (*Figure 3*). After briefly spinning the tube, the beads were gathered by placing on the magnetic rack for at least 5 minutes. The supernatant was removed without disturbing with the beads contained microbial DNA. The beads were washed twice by adding 400 µL freshly prepared 80 % ethanol to the tube and incubated at room temperature for 30 seconds while placing on the magnetic rack. All visible liquid was removed, and the beads were air-dried for up to 5 minutes while placing on the magnetic rack with the lid open. The DNA target was eluted from the beads by mixing with 50 µL of 1x TE buffer by pipetting for 10 times and incubating for 2 minutes at room temperature without standing on the magnetic rack. After briefly spinning the tube, the beads were collected by placing the tube on the magnetic rack for 5 minutes or when the solution was clear. The eluate contains purified microbial DNA was transferred to a new microtube and stored at -20 °C until the Polymerase Chain Reaction (PCR) analysis.

Figure 3 Workflow of AMPure XP Bead Cleanup in final microbiome DNA enrichment step (Beckman Coulter Life Sciences, *AMPure XP Workflow*. 2022).

Evaluation of DNA Yields

The concentration of dsDNA was measured before and after performing DNA enrichment using fluorescence spectroscopy Qubit 4 fluorometer (Thermo Fischer Scientific, USA). Qubit dsDNA HS (high sensitivity, 0.2 to 100 ng) Assay kit was used according to the manufacturer's protocol; a sample volume of 2 µL was added to 198 µL of a Qubit working solution and incubated for 2 minutes before the measurement.

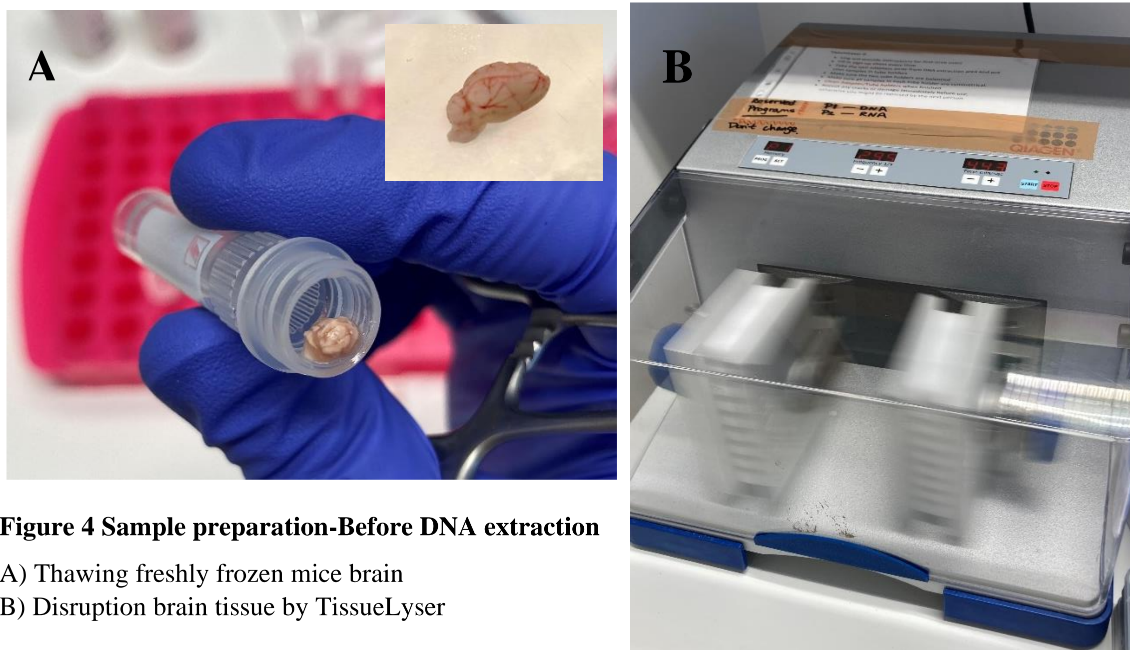


Figure 4 Sample preparation-Before DNA extraction

A) Thawing freshly frozen mice brain
B) Disruption brain tissue by TissueLyser

Amplification of DNA by PCR Analysis

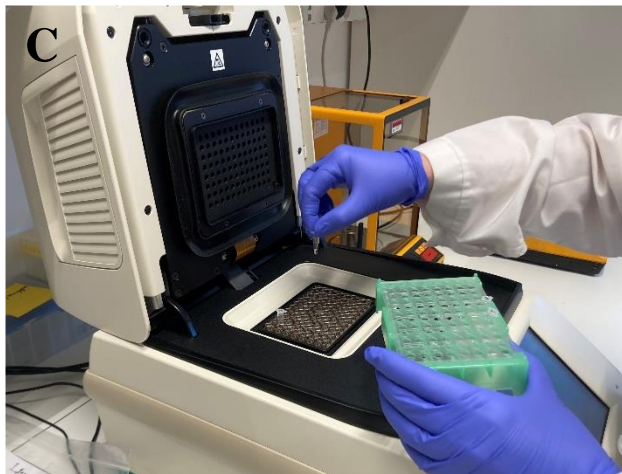
The Polymerase chain reaction (PCR) using 2.5 µL sample with 12.5 µL KAPA HiFi HotStart Ready Mix (Roche, USA), 5 µL forward-tailed specific primer [38] (515F 5'-GTGCCAGCMGCCGCGGTAA-3'), and 5 µL reverse-tailed specific primer [38] (806R 5'-GGACTACHVGGGTWTCTAAT-3') was carried out for the amplification of the 16S rRNA for the following sequencing analysis in Bio-Rad C1000 Touch™ Thermal Cycler with CFX96™ Real-Time System (*Figure 5C*). The V4 region was targeted for amplification by PCR with primers 515F-806R by Kozich et al [38].

The PCR program was under conditions for pre-denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds; annealing step at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, then followed by a final extension step at 72 °C for 5 minutes.

Indices were then added in a subsequent PCR using an Illumina Nextera kit with the PCR program under conditions for pre-denaturation at 98 °C for 30 seconds, followed by 8 cycles of denaturation at 98 °C for 10 seconds; annealing step at 55 °C for 20 seconds, and extension at 72 °C for 20 seconds, then followed by a final extension step at 72 °C for 5 minutes. This step was performed before the out-of-house sequencing by Clinical Microbiome in Denmark.

Evaluation of PCR products by Running Agarose Gel Electrophoresis

To ensure the PCR efficacy for amplified 16S rRNA V4 region (length, ca. 250 bp), the assay using agarose gel electrophoresis was conducted. The PCR products were visualized on 1 % agarose gel after supplemented with SYBR safe staining using Invitrogen™ SYBR™ Safe DNA Gel Stain (Thermo Fischer Scientific, USA) at 250 volts in 1x Tris-acetate-EDTA buffer (Thermo Fischer Scientific, USA) for 25 minutes. The Invitrogen™ 50 bp DNA Ladder (Thermo Fischer Scientific, USA) was used as a molecular standard. The gel was examined, and the image was captured by imager Azure c150 (Azure Biosystems, USA) *(Figure 5D).*



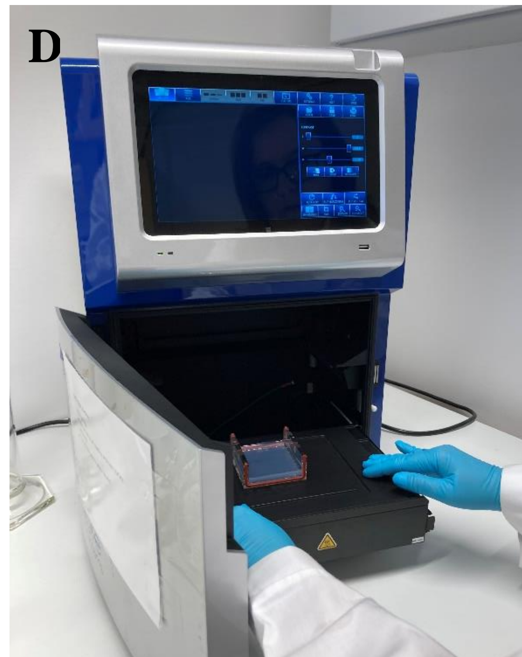


Figure 5 Sample preparation-After DNA extraction

C) Attached primers on DNA template by PCR
D) Visualized the agarose gels by imager contained a UV light box linked to a camera to evaluate the amplicon quality

Next Generation Sequencing (NGS)

Illumina MiSeq DNA sequencing instrument platform deliver high-throughput sequencing data. Sequencing was done on an Illumina MiSeq desktop sequencer using the MiSeeq Reagene Kit v3 (Illumina) for 2x 300 bp paired-end sequencing by Clinical Microbiome in Denmark.

Quality control of raw sequencing data

Primer sequences in raw reads were found and removed by Clinical Microbiome using Cutadapt. Reads that were match without primer or with ambiguous bases (e.g. Ns), and reads that were shorter or longer than expected length after the number of sequencing (300 bp) were discarded as well as the length of the primers. The sequencing data was processed using the Illumina analysis pipeline.

Bioinformatic and Statistic analysis

NGS data was processed using Quantitative Insights Into Microbial Ecology (QIIME II) software [39], which is an open-source bioinformatics pipeline for analyzing the raw microbiome DNA sequencing data, and in-house pipeline. QIIME II analyzed and interpreted the nucleic acid sequence to reconstruct the microbial community phylogenetically and assigned the taxonomy in graphics and statistics such as the taxonomic bar and beta diversity heatmap in this study. The histograms were created using Microsoft Excel 365.

Results and Discussion

Concentration of extracted and enriched DNA

The purity of isolated nucleic acid to perform molecular analysis and represent actual existing genetic diversity is the prerequisite for molecular approaches. The lysis started with (i) ATL solution (Qiagen) and Nuclei lysis buffer of (Promega) treatment with mechanical disruption of the tissues with stainless beads followed by serial washing and DNA recovery steps. The highest amount of genomic DNA was extracted from Promega kit (*Figure 6)* (detailed description in *Suppl. Table 1*). The concentration differences can be due to the extracting solutions different in two kits. Since the contamination of host DNA can reduce the microbial signals for detection in further analysis, high-quality and quantity microbial DNA with host DNA depletion by using microbiome DNA enrichment kit was performed. The mechanism behind microbial enrichment is to trap mammalian DNA by using fused Fc fragment of human IgG with methylated CpG-specific binding protein MBD2 binding rapidly to protein A combine to 60-90 % methylated cytosines at CpG sites. In contrast, it is rare to find methylation at CpG sites in microbial species [40]. The enrichment efficiency was slightly increased in Qiagen kit compared to Promega kit shown also in *Figure 6.*

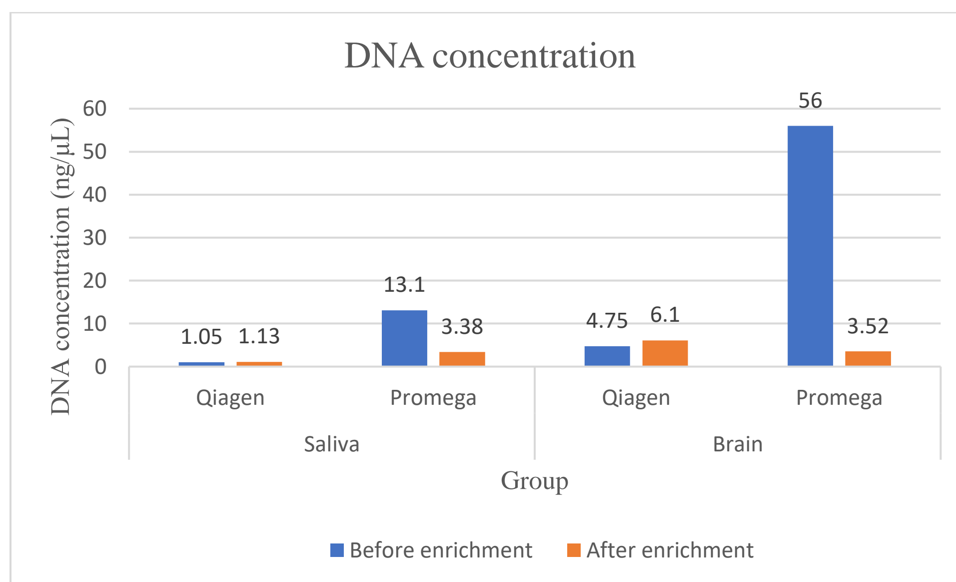


Figure 6 Comparison of the yield of extracted DNA before and after microbiome DNA enrichment between tissues and kits.

The enrichment efficiency was calculated from the DNA concentration measured by using Qubit fluorescent meter to better evaluate the yield among the different extraction kits. The formula was shown in *Figure 8.*

$$\frac{DNA\ concentration\ after\ enrichment}{DNA\ concentration\ before\ enrichment} \times 100\ \% \ = Enrichment\ efficiency$$

Figure 7 The formula of enrichment efficiency

Figure 8 shows Qiagen DNA extraction kit with DNA enrichment resulted in higher enrichment efficiency both in saliva and brain tissue sample than Promega kit. From the DNA extraction point of view, Promega has better yield. While from the sequencing result discussed later was not clear enough to draw a conclusion which kit is best suited. Therefore, further studies in comparing one or two more kits to find another optimal extraction method for brain tissue was suggested. Overall, the enrichment step increased the relatively amount of bacterial DNA. Since the impact of microbial DNA recovery by the NEBNext microbiome kit was lack of reported data [41], it is uncertain how much microbial DNA might be lost and caused biases.

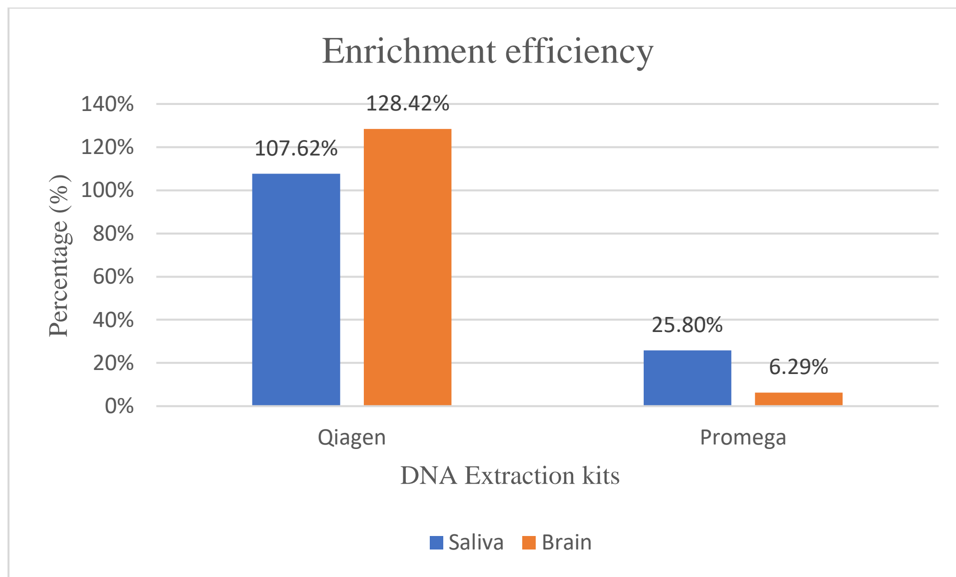


Figure 8 Comparison of the microbiome DNA yield and abundance between two extraction kits in two different types of samples.

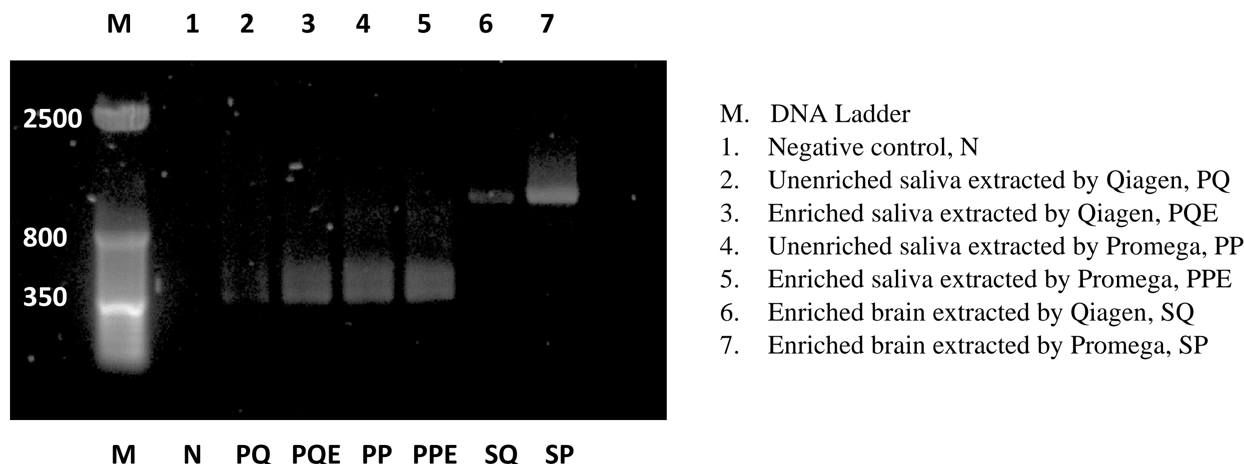


Figure 9 Agarose gel electrophoresis of 16S rRNA partial gene PCR-amplified from 2.5 µL of 1 sample with nuclease-free water, 2 samples with both host and microbial isolates, and 4 samples with only microbial isolates for 35 cycles. PCR amplified products were analyzed using 1 % agarose gel at Volte 250 for 25 minutes.

The extracted DNA of sufficient purity before and after the enrichment with PCR-mediated amplification was quantified in a 1 % agarose gel (*Figure 9*). Smear and dim bands can be seen in the agarose gel but over 250 bp, the length of the target 16S rRNA V4 region, which was possibly insufficient quantity of DNA for amplification. Clear bands can be possibly obtained by adjusting different PCR cycles and temperatures to increase the amplicon concentration for intense signals. However, nested PCR strengthens the evidence for the existence of variety of bacterial species and increase the sensitivity of PCR by using two sequential amplification reactions to target the desired segments, and to reduce non-specific binding is taken into consideration in future studies [42, 43]. Different combinations of primers may provide better coverage of other species [44]. Combining 16S rRNA gene variable regions enable high-resolution microbial community profiling was suggested [45] such as including V3 region as V2-V3 regions have higher resolution for lower-rank taxa [46], or V1-V3 regions have higher phylotype richness [47].

The outcome of microbiome DNA content extraction from the brain comparing to other tissues can be very likely different as brain consist of more than 50 % of lipids. The age and gender of the mice might affect the amount of bacterial DNA depending on their immune system and inflammatory processes in their gut. The menstrual cycle in the female mice makes them have fluctuating hormone level compared to male mice, that might be further explored as a factor that might affect gut-blood-barrier functions.

Overview of generated sequences per sample type

Strict quality filtering was recommended and performed by Clinical Microbiome, and it was found that barely, no read pairs passing their common quality filtering procedure for the DNA samples (*Table 1, Figure 10)*, suggesting that the samples contained very little bacterial DNA. The ideal

read pairs number of samples supposed to be similar to positive control, PC. However, the reads quality was good, suggesting the DNA in the samples were good for sequencing.

Table 1 Summary of the number of read pairs generated for the different sample types. IQR: Interquartile range. (provided by Clinical Microbiome, note 'Quality control and Sequencing data' in the 'Methods' section)

Sample type	No. of samples	No. read pairs provided (median, IQR)
DNA	7	5416 (2637, 5546)
PC	1	67612 (67612, 67612)
NC	1	574 (574, 574)

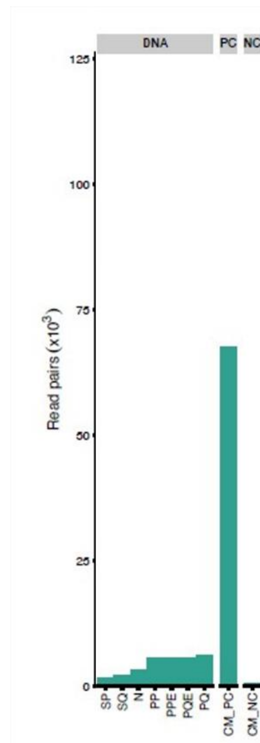


Figure 10 Overview of the number of raw read pairs generated for the different samples (provided by Clinical Microbiome, note 'Quality control and Sequencing data' in the 'Methods' section)

Analysis of putative taxonomy unit in brain samples

Figure 11 shows hierarchical relationships combined heat level of all taxa from phylum to genus level detected in samples. The abundance of genera in SQ and PC is consistent with the taxonomic bar (*Figure 12*) although majority of the species were not assigned due to low read pairs. Without deep sequencing, it is not precise enough to allocate the right taxa for a species especially between close relationships species. Apart from the sequencing using Qiagen kit could be detected, other samples were either blank or with unassigned taxonomy. Unassigned taxonomy is likely due to the represent bacteria did not exist in the database. In this case, however, most likely was the

numbers of bacterial DNA too little to be assigned since the machine had no enough information to classify them and put them into the right category.

Since the read pairs were little, it suggested that the analysis from the Qiagen kit could be either false positive, or there was weak taxonomic signal of a consistent bacterial colonization in murine brain samples. The later assumption was accepted as the reads quality was good enough to perform sequencing as previous mentioned. In the taxonomic bar (*Figure 12)*, however, apart from SQ, rest of samples seem not to have abundant signals or just to be random signals such as shown totally blank in PPE.

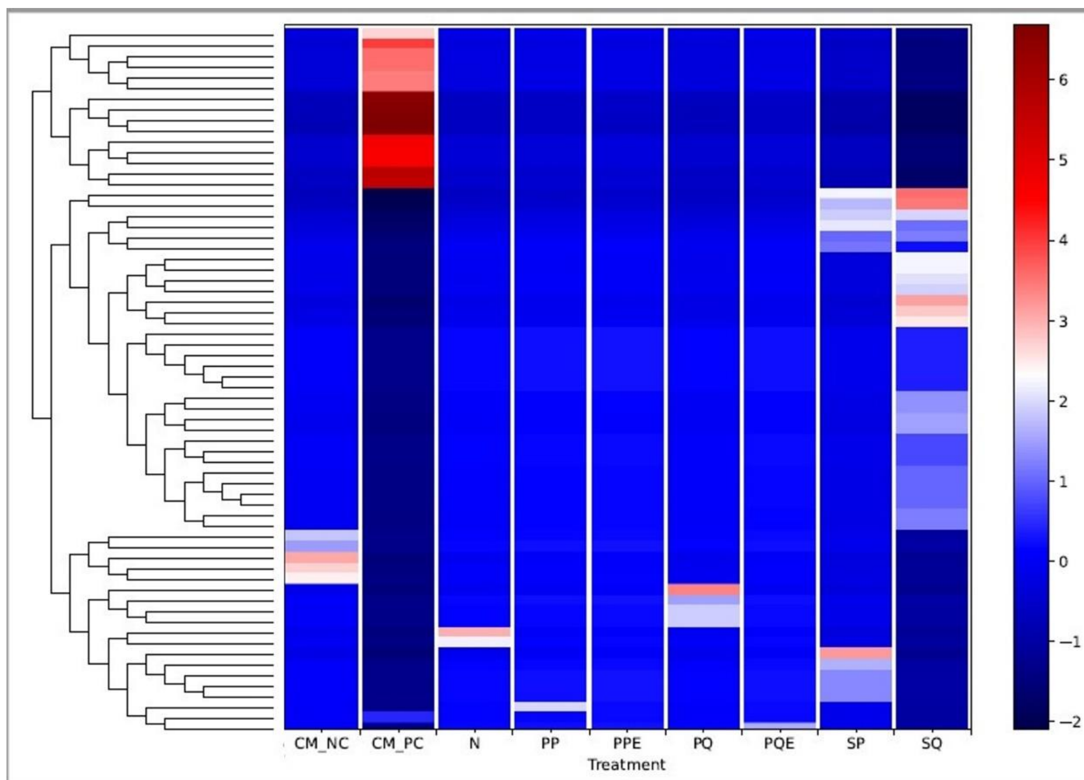


Figure 11 Heatmap cluster analysis of the community composition between samples. Red represents more abundant genera in the corresponding sample, and blue represents the less abundant genera.

Human oral microbial communities has high complexity, however, at the phylum level, Firmicutes (36 %), Proteobacteria (31 %), Bacteroidetes (18 %), Fusobacteria (9 %), and Actinobacteria (5 %) predominantly compose of the human oral saliva microbiome community [48]. It is consistent with the findings of Firmicutes, Proteobacteria and Actinobacteria seen in *Figure 12* in the saliva sample extracted by using Qiagen kit. Among these bacteria, Actinobacteria can be seen in the same sample with an enrichment step. Bacteroidetes and Firmicutes are representing 90 % of gut microbiota [49]. Both Proteobacteria and Actinobacteria are baseline gut microbiome at phylum level [50] and varies contaminant genera sourced from DNA extraction kits and laboratory reagents belong to these phylum as well *[9]*. Methylobacterium and genus substantially under

Enterobacteriaceae detected in negative control (*Suppl. Table 2*) was aligned with existing findings of contaminated by exogenous DNA.

The contamination of external DNA occurred everywhere from the environment, our dander to the laboratory reagents critical impact sequence-based microbiome analyses. Besides including negative control samples concurrently, to minimize the artifacts when applying sequence-based techniques, the strict and standard procedure must be followed to create a low biomass environments [9].

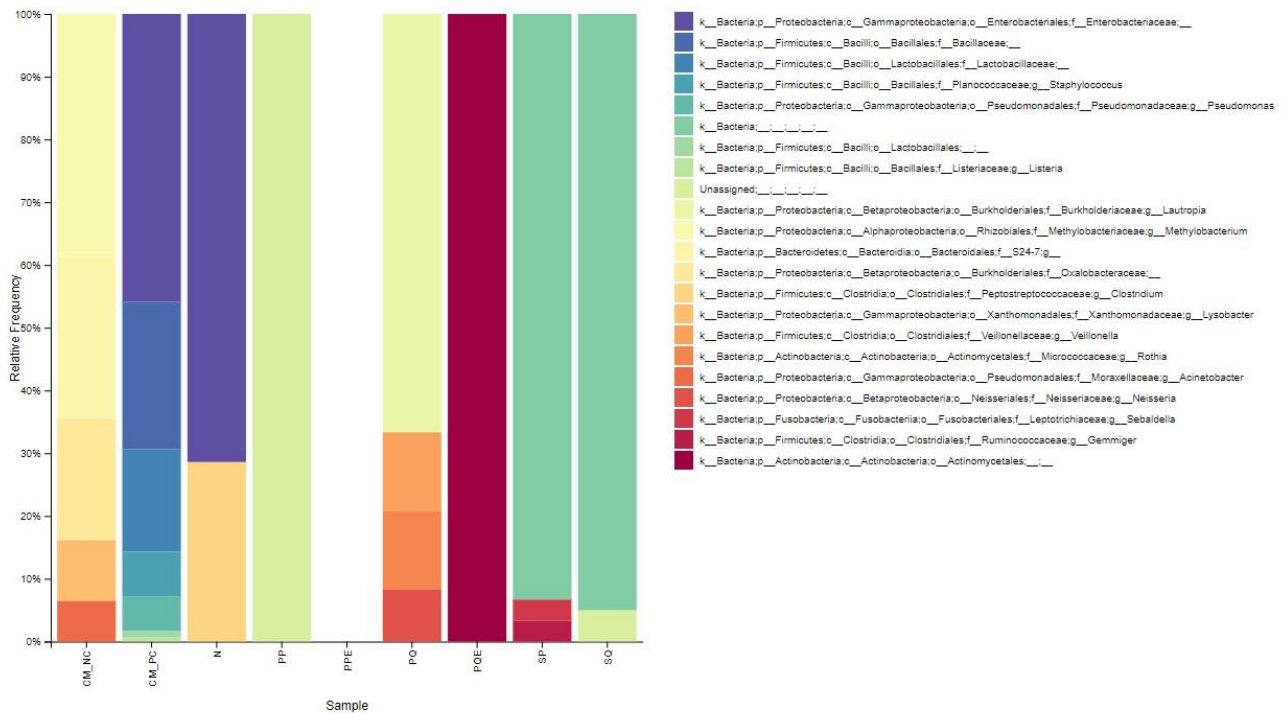


Figure 12 Taxonomic bar shows the variety and relative abundance of the bacterial genera. At phylum level, PPE, Positive Promega enriched with nothing; PP, Positive Promega with nothing; PQE, Positive Qiagen enriched with Actinobacteria; SQ, Sample Qiagen with nothing, SP, Sample Promega with Firmicutes, Fusobacteria; PQ, Positive Qiagen with Proteobacteria, Firmicutes, Actinobacteria; N, Negative control with Proteobacteria and Firmicutes (*Suppl. Table 1*)

Due to the study being of an exploratory nature with small sample size, the results should be interpretated with caution. Extraction quantity differences observed likely reflected the different solutions used in the kits. With host DNA depletion step, the purity of DNA was adequate for further analysis. While the culprit of the sequence results may lie in the PCR process as both extraction step and enrichment step did not yield unreasonable data. The low microbial biomass usage can lead to misinterpretations that resulted from off-target amplifications and false positive assignment due to primers competition in samples [51]. In order to obtain an optimal sequence for later analysis, a clear and sharp band visualized from the gel electrophoresis, by increasing the sample concentration for initial amplification, should be observed before sequencing.

Although the brain has traditionally been considered sterile, the sterility of brain by BBB protecting against foreign microbe's invasion has not been rigorously tested [52]. Metabolic diseases such as diabetes can increase permeability of the BBB via a loss of tight junction proteins [53], neurodegenerative diseases can also compromise BBB and further progression of Alzheimer's disease with amyloid-β deposition [54, 55]. Since the mice conducted in this study were healthy and very unlikely to have BBB impairments, the sequencing without low detection of microbiota was expected. The animals with Alzheimer's disease will be included in later study to compare with the healthy littermates once the current method was well established.

Overall, the findings from this experimental study provides a valuable insight into the bacterial composition in the murine brain tissue using different DNA extraction methods with microbial DNA enrichment. The bacterial DNA extraction and enrichment could be used on its own, or in conjunction with different PCR based techniques such as nested PCR to allow for a more accurate binding to the target DNA region. The significant number of bacteria exists in the brain despite the amount was too little to be identified. Studies have demonstrated that the bacteria in the brain can be detected, and the rooting cause can be identified such as the bacteria is easy to travel from the oral cavity to the brain for people with periodontitis, which can exacerbate Alzheimer's [56, 57]. However, it is not like the gut, fecal or saliva bacteria being well profiled. The neuroinflammation and mitochondrial impairment induced by high fat diet [32] mitigated by black currant supplemented to protect the integrity of BBB was also suggested from the findings of little amount of brain bacteria.

Once the methodology established, the profile of bacterial community could be used as an indicator to predict and prevent the occurrence of neurodegenerative disease like Alzheimer's. In addition, to better understand and evaluate the bacterial composition in the brain of mice, the gender should also be taken into consider as at the genus level, the gut microbiota of male and female mice are similar but the relative abundance is slightly different among the sex [58].

Diet is a key modifiable factor to induce a shift in gut microbiota [59]. Long term consumption of diets with various vegetables, fruits, cereals, and beverages not only provide vital nutrients to maintain body health but also rich in antioxidants such as polyphenols that reduce the oxidative stress in molecular level and offer protection against diseases development [60]. Ultimately, the modulated gut microbiome and enriched beneficial bacteria and its metabolites short-chain fatty acids (derived from fiber fermentation), for instance [61] may ameliorate, prevent or slow neurodegeneration by diet interventions.

Conclusion and Future Perspective

This study sought to explore a methodology for extracting bacterial DNA from host DNA in mice to provide a tool to explore the gut-brain microbiome in mice. The separation was achieved by using the methylation property in mammalian DNA and MBD2-Fc bound magnetic beads. The lower enrichment efficiency of Promega kit than Qiagen kit suggested Qiagen kit performed better in enrichment. It also resulted in Promega kit having higher concentration of DNA extraction from both host and microbiota which more host DNA needed to be eliminated. This study highlights that the enrichment step is essential for this method development to obtain the optimal bacterial DNA. Additionally, this highlights the need for future research on microbial DNA extraction kits targeted for brain tissue with increased amplification. Given the small sample sizes, microfluidic systems can potentially provide a solution to this ensuring repeatability and high sensitivity.

It is exhilarating and reasonable to hypothesize that different microbiome community in the CNS could contribute to neurodegenerative disorders such as AD caused by immune responses. While the DNA reading was low when sequencing, there was no valid taxonomic signals and no concluding evidence to support the idea of there could reside bacteria in the healthy mice brain. However, given the challenges in amplification, more research is needed. The existing of brain microbiome or even novel microbes not yet identified or recovered by standard approaches should not be excluded as a striking finding of novel microbial sequences were obtained in the course of sequencing human cell-free blood DNA [62].

This study concludes that DNA extraction with depletion of human and mice DNA before sequencing increase yield of bacterial DNA is critical to increase the signal intensity. Due to time constraints, future research should investigate how DNA extraction and separation influence the sequencing process for brain tissue. Additional studies and analysis will be required to determine the sequences in the brain of Alzheimer's sick animals. Identification of possible microbiota in the brain, especially in animal models with a damaged blood-brain barrier, can provide much needed into the evolution of this disease.

References

1. Costello, E.K., et al., *Bacterial community variation in human body habitats across space and time.* Science, 2009. **326**(5960): p. 1694-7.

2. Heather, J.M. and B. Chain, *The sequence of sequencers: The history of sequencing DNA.* Genomics, 2016. **107**(1): p. 1-8.

3. Luo, C., et al., *Direct Comparisons of Illumina vs. Roche 454 Sequencing Technologies on the Same Microbial Community DNA Sample.* PLOS ONE, 2012. **7**(2): p. e30087.

4. Behjati, S. and P.S. Tarpey, *What is next generation sequencing?* Arch Dis Child Educ Pract Ed, 2013. **98**(6): p. 236-8.

5. Hamilton, J.A., et al., *Brain Uptake and Utilization of Fatty Acids, Lipids and Lipoproteins: Application to Neurological Disorders.* Journal of Molecular Neuroscience, 2007. **33**(1): p. 2-11.

6. Ojo-Okunola, A., et al., *The Influence of DNA Extraction and Lipid Removal on Human Milk Bacterial Profiles.* Methods Protoc, 2020. **3**(2).

7. Helmersen, K. and H.V. Aamot, *DNA extraction of microbial DNA directly from infected tissue: an optimized protocol for use in nanopore sequencing.* Scientific Reports, 2020. **10**(1): p. 2985.

8. Heravi, F.S., et al., *Host DNA depletion efficiency of microbiome DNA enrichment methods in infected tissue samples.* Journal of Microbiological Methods, 2020. **170**: p. 105856.

9. Salter, S.J., et al., *Reagent and laboratory contamination can critically impact sequence-based microbiome analyses.* BMC Biol, 2014. **12**: p. 87.

10. Tran, S.M.-S. and M.H. Mohajeri, *The Role of Gut Bacterial Metabolites in Brain Development, Aging and Disease.* Nutrients, 2021. **13**(3): p. 732.

11. Bulgart, H.R., et al., *Microbial involvement in Alzheimer disease development and progression.* Molecular Neurodegeneration, 2020. **15**(1): p. 42.

12. Sureda, A., et al., *Oral microbiota and Alzheimer's disease: Do all roads lead to Rome?* Pharmacological Research, 2020. **151**: p. 104582.

13. Narengaowa, et al., *The Oral-Gut-Brain AXIS: The Influence of Microbes in Alzheimer's Disease.* Frontiers in Cellular Neuroscience, 2021. **15**.

14. Emery, D.C., et al., *16S rRNA Next Generation Sequencing Analysis Shows Bacteria in Alzheimer's Post-Mortem Brain.* Frontiers in Aging Neuroscience, 2017. **9**.

15. Singh, R.K., et al., *Influence of diet on the gut microbiome and implications for human health.* J Transl Med, 2017. **15**(1): p. 73.

16. David, L.A., et al., *Diet rapidly and reproducibly alters the human gut microbiome.* Nature, 2014. **505**(7484): p. 559-63.

17. Srour, B., et al., *Ultra-processed foods and human health: from epidemiological evidence to mechanistic insights.* The Lancet Gastroenterology & Hepatology.

18. Canfora, E.E., et al., *Gut microbial metabolites in obesity, NAFLD and T2DM.* Nature Reviews Endocrinology, 2019. **15**(5): p. 261-273.

19. Jardon, K.M., et al., *Dietary macronutrients and the gut microbiome: a precision nutrition approach to improve cardiometabolic health.* Gut, 2022. **71**(6): p. 1214-1226.

20. Gopalan, A., et al., *The health benefits of blackcurrants.* Food & Function, 2012. **3**(8): p. 795-809.

21. Cao, L., et al., *Long-Term Blackcurrant Supplementation Modified Gut Microbiome Profiles in Mice in an Age-Dependent Manner: An Exploratory Study.* Nutrients, 2020. **12**(2).

22. Oxford Martin School, Global Change Data Lab, and Our World in Data. *Cause of deaths for people who were 70 years and older, World, 2019*. 2019 [cited 2022 28 February]; Available from: https://ourworldindata.org/grapher/causes-of-death-in-70-year-olds?time=2019..latest.

23. World Health Organization. *Dementia*. 2021 [cited 2022 28 February]; Available from: https://www.who.int/news-room/fact-sheets/detail/dementia.

24. Jönsson, L., et al., *Determinants of costs of care for patients with Alzheimer's disease.* Int J Geriatr Psychiatry, 2006. **21**(5): p. 449-59.

25. Deshpande, P., N. Gogia, and A. Singh, *Exploring the efficacy of natural products in alleviating Alzheimer's disease.* Neural Regen Res, 2019. **14**(8): p. 1321-1329.

26. Alzheimer's association. *What is Alzheimer's Disease?* 2022 [cited 2022 28 February]; Available from: https://www.alz.org/alzheimers-dementia/what-is-alzheimers.

27. Kumar, D.K., et al., *Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease.* Sci Transl Med, 2016. **8**(340): p. 340ra72.

28. Tetz, G., et al., *Bacterial DNA promotes Tau aggregation.* Sci Rep, 2020. **10**(1): p. 2369.

29. Alonso, R., et al., *Infection of Fungi and Bacteria in Brain Tissue From Elderly Persons and Patients With Alzheimer's Disease.* Frontiers in Aging Neuroscience, 2018. **10**.

30. Vogt, N.M., et al., *Gut microbiome alterations in Alzheimer's disease.* Scientific Reports, 2017. **7**(1): p. 13537.

31. Marizzoni, M., et al., *Short-Chain Fatty Acids and Lipopolysaccharide as Mediators Between Gut Dysbiosis and Amyloid Pathology in Alzheimer's Disease.* Journal of Alzheimer's Disease, 2020. **78**: p. 683-697.

32. Cavaliere, G., et al., *High-Fat Diet Induces Neuroinflammation and Mitochondrial Impairment in Mice Cerebral Cortex and Synaptic Fraction.* Frontiers in Cellular Neuroscience, 2019. **13**.

33. Liang, Y., et al., *Gut Microbial Metabolites in Parkinson's Disease: Implications of Mitochondrial Dysfunction in the Pathogenesis and Treatment.* Molecular Neurobiology, 2021. **58**(8): p. 3745-3758.

34. Jackson, D.N. and A.L. Theiss, *Gut bacteria signaling to mitochondria in intestinal inflammation and cancer.* Gut Microbes, 2020. **11**(3): p. 285-304.

35. Houghton, D., et al., *Impact of Age-Related Mitochondrial Dysfunction and Exercise on Intestinal Microbiota Composition.* The Journals of Gerontology: Series A, 2017. **73**(5): p. 571-578.

36. Lee, H.J., et al., *Diabetes and Alzheimer's Disease: Mechanisms and Nutritional Aspects.* Clin Nutr Res, 2018. **7**(4): p. 229-240.

37. Carvalho, C. and S. Cardoso, *Diabetes–Alzheimer's Disease Link: Targeting Mitochondrial Dysfunction and Redox Imbalance.* Antioxidants & Redox Signaling, 2021. **34**(8): p. 631-649.

38. Kozich, J.J., et al., *Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform.* Appl Environ Microbiol, 2013. **79**(17): p. 5112-20.

39. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nature Biotechnology, 2019. **37**(8): p. 852-857.

40. Lister, R., et al., *Human DNA methylomes at base resolution show widespread epigenomic differences.* Nature, 2009. **462**(7271): p. 315-322.

41. Thoendel, M., et al., *Comparison of microbial DNA enrichment tools for metagenomic whole genome sequencing.* J Microbiol Methods, 2016. **127**: p. 141-145.

42. Green, M.R. and J. Sambrook, *Nested Polymerase Chain Reaction (PCR).* Cold Spring Harb Protoc, 2019. **2019**(2).

43. Deepachandi, B., et al., *A highly sensitive modified nested PCR to enhance case detection in leishmaniasis.* BMC Infectious Diseases, 2019. **19**(1): p. 623.

44. Alonso, R., et al., *Evidence for Fungal Infection in Cerebrospinal Fluid and Brain Tissue from Patients with Amyotrophic Lateral Sclerosis.* International Journal of Biological Sciences, 2015. **11**(5): p. 546-558.

45. Fuks, G., et al., *Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling.* Microbiome, 2018. **6**(1): p. 17.

46. Bukin, Y.S., et al., *The effect of 16S rRNA region choice on bacterial community metabarcoding results.* Scientific Data, 2019. **6**(1): p. 190007.

47. Allen, H.K., et al., *Pipeline for amplifying and analyzing amplicons of the V1-V3 region of the 16S rRNA gene.* BMC Res Notes, 2016. **9**: p. 380.

48. Wang, J., et al., *Diversity and Biogeography of Human Oral Saliva Microbial Communities Revealed by the Earth Microbiome Project.* Frontiers in Microbiology, 2022. **13**.

49. Rinninella, E., et al., *What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases.* Microorganisms, 2019. **7**(1).

50. King, C.H., et al., *Baseline human gut microbiota profile in healthy people and standard reporting template.* PLOS ONE, 2019. **14**(9): p. e0206484.

51. Bedarf, J.R., et al., *Much ado about nothing? Off-target amplification can lead to false-positive bacterial brain microbiome detection in healthy and Parkinson's disease individuals.* Microbiome, 2021. **9**(1): p. 75.

52. Link, C.D., *Is There a Brain Microbiome?* Neurosci Insights, 2021. **16**: p. 26331055211018709.

53. Hawkins, B.T., et al., *Increased blood–brain barrier permeability and altered tight junctions in experimental diabetes in the rat: contribution of hyperglycaemia and matrix metalloproteinases.* Diabetologia, 2007. **50**(1): p. 202-211.

54. Acharya, N.K., et al., *Diabetes and hypercholesterolemia increase blood-brain barrier permeability and brain amyloid deposition: beneficial effects of the LpPLA2 inhibitor darapladib.* J Alzheimers Dis, 2013. **35**(1): p. 179-98.

55. Sweeney, M.D. and B.V. Zlokovic, *A lymphatic waste-disposal system implicated in Alzheimer's disease.* Nature, 2018. **560**(7717): p. 172-174.

56. Mao, S., et al., *Association of periodontitis and oral microbiomes with Alzheimer's disease: A narrative systematic review.* Journal of Dental Sciences, 2022. **17**(4): p. 1762-1779.

57. Gurav, A.N., *Alzheimer's disease and periodontitis--an elusive link.* Rev Assoc Med Bras (1992), 2014. **60**(2): p. 173-80.

58. Chu, M. and X. Zhang, *Bacterial Atlas of Mouse Gut Microbiota.* Cellular Microbiology, 2022. **2022**: p. 5968814.

59. Leeming, E.R., et al., *Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration.* Nutrients, 2019. **11**(12).

60. Pandey, K.B. and S.I. Rizvi, *Plant polyphenols as dietary antioxidants in human health and disease.* Oxid Med Cell Longev, 2009. **2**(5): p. 270-8.

61. Silva, Y.P., A. Bernardi, and R.L. Frozza, *The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication.* Frontiers in Endocrinology, 2020. **11**.

62. Kowarsky, M., et al., *Numerous uncharacterized and highly divergent microbes which colonize humans are revealed by circulating cell-free DNA.* Proc Natl Acad Sci U S A, 2017. **114**(36): p. 9623-9628.

Supplementary Material

Table 1 Concentration of extracted and enriched DNA.

Sample type	Concentration		Enrichment efficiency	Extraction Kit
	Before enrichment	Before PCR		
DNA Saliva enriched	1.05	1.13	107.62 %	Qiagen
DNA Saliva enriched	13.1	3.38	25.80 %	Promega
DNA Brain enriched (BC 80, B2)	4.75	6.10	128.42 %	Qiagen
DNA Brain enriched (BC 80, B2)	56	3.52	6.29 %	Promega

Too low: < 0.5 ng/mL (unit: ng/µL)
Too high: > 600 ng/mL

Table 2 Taxonomic table. Contaminant microbes from laboratory reagents in bold.

Group	Phylum	Class	Order	Family	Genus	Percentage
PPE	n/a	n/a	n/a	n/a		n/a
PP	Unassigned	-	-	-		100%
PQE	Actinobacteria	Actinobacteria	Actinomycetales	-		100 %
SQ	-	-	-	-		95.0%
	Unassigned	-	-	-		5.0%
SP	-	-	-	-		93.3 %
	Firmicutes	Clostridia	Clostridiales	Ruminococaceae	*Gemmiger*	3.3 %
	Fusobacteria	Fusobacteria	Fusobacteriales	Leptotrichiaceae	*Sebaidella*	3.3 %
PQ	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Lautropia*	66.7 %
	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	*Veillonella*	12.5 %
	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Rothia*	12.5 %
	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	*Neisseria*	8.3 %
N	Proteobacteria	Gammaproteobacteria	Enterobacteriales	**Enterobacteriaceae**	-	71.4 %
	Firmicutes	Clostridia	Clostriales	Peptostreptococcaceae	*Clostridium*	28.5 %
NC	Proteobacteria	Alphaproterobacteria	Rhizobiales	Methylobacteriaceae	***Methylobacterium***	38.7 %
	Bacteroidetes	Bacteroidia	Bacteroidales	-		25.8%
	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae		19.4 %
	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Lysobacter*	9.7 %
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Actinetobacter*	6.4 %
PC	Proteobacteria	Gammaproteobacteria	Enterobacteriales	**Enterobacteriaceae**	-	45.8 %
	Firmicutes	Bacilli	Bacillales	Bacillaceae		23.5 %
	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae		16.3 %
	Firmicutes	Bacilli	Bacillales	Planococcaceae	*Staphylococcus*	7.1 %
	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	5.6 %
	Firmicutes	Bacilli	Lactobacillates	-		1 %
	Firmicutes	Bacilli	Bacillales	Listeriaceae	*Listeria*	0.6 %

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I would like to thank everyone who helped me along this journey with unforgettable memories. I want to thank both of my supervisors Frida and Olena for your support and guidance, the freedom and encouragement you gave me and willing to let me try and believe in me. A special thanks to our bioinformatician Stephan, for not only helping me analyze and interpret the data, but always having a valuable discussion with me to make my thoughts clear and logical.

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Last but not the least, to every beautiful soul that I have spoken with but have not mentioned, and you the reader, thank you.

Diet cures more than a doctor. The biggest milestone of this study was being able to establish a robust way to investigate the mighty little guys from the diet coexisting with us in the brain. Below, is a mice brain hemisphere from one of DNA extraction, imaged at Animal facility, 20 May 2022. May it also serve as a reminder that animals sacrifice their lives to make all this possible, we are grateful for their contribution.

LUND UNIVERSITY

LTH

FACULTY OF ENGINEERING

Department of Food Technology, Engineering and Nutrition

Lund University, Faculty of Engineering

Master Thesis