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Gut microbiota to counteract metabolic disorders and neuroinflammation

Impact of dietary factors and their potential to prevent Alzheimer's disease

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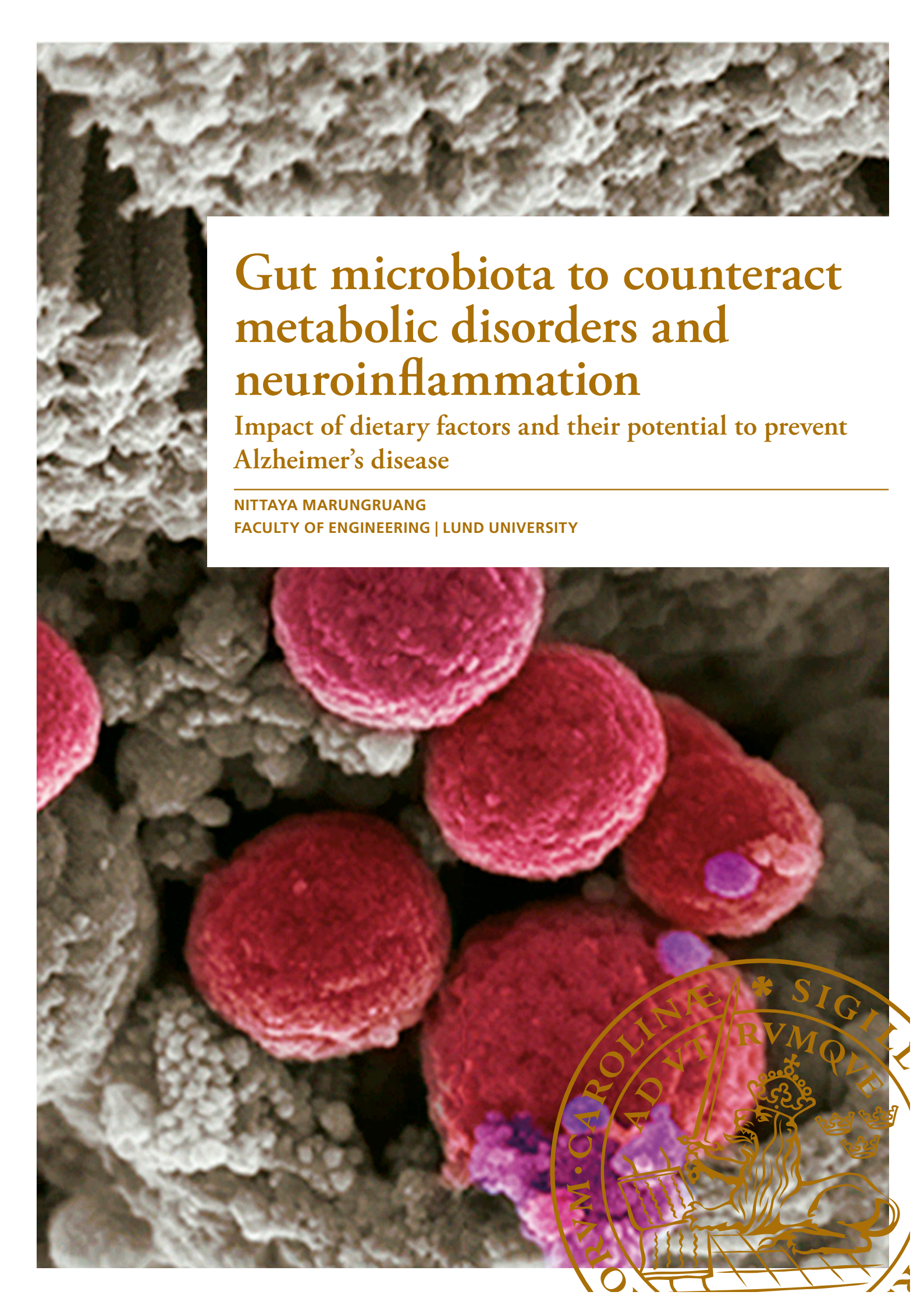
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Gut microbiota to counteract metabolic disorders and neuroinflammation

Impact of dietary factors and their potential to prevent Alzheimer's disease

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FACULTY OF ENGINEERING | LUND UNIVERSITY



Gut microbiota to counteract metabolic disorders and neuroinflammation

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Alzheimer's disease

Nittaya Marungruang



LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Engineering, Lund University, Sweden.

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Faculty opponent

Professor John F Cryan, Department of Anatomy & Neuroscience
University College Cork, Ireland

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<p>Abstract</p> <p>Alzheimer's disease (AD) is the most common form of dementia. Increased accumulations of senile plaques and tangles are the known neuropathological hallmarks of AD. However, the exact triggers for such protein accumulations, leading to cognitive impairment and important morphological alteration in AD, are still elusive. Ageing and genetic risk factors do not completely explain the global increase in the AD prevalence. Different environmental risk factors, including lifestyle and diet, as well as cluster risk factors of the metabolic syndrome (MetS) have been identified to influence the progression of the disease. Emerging evidence has suggested the important role of the commensal gut microbiota in brain development and function, affecting development of brain-related disorders. Hence, understanding the impact that gut microbiota may have on the risk factors for AD may provide possible preventive or therapeutic strategies for such neurodegenerative disease.</p> <p>Studies included in this thesis aimed to evaluate whether the gut microbiota may have influences on AD risk factors and how a healthy gut microbiota, amending gut-brain interaction, may be composed. The shifts of gut microbiota upon dietary changes, and their relation to neurodegenerative and metabolic disturbances in rodents and humans were investigated.</p> <p>The results showed that a mouse model of cerebral amyloidosis (APPPS1) displayed a distinct gut microbiota profile, as compared to that of healthy wild-type (WT) mice. Absence of the gut microbiota (germ-free, GF) in APPPS1 mice resulted in reduction of amyloid-beta (Aβ) deposition. Colonization of GF mice with the gut microbiota from APPPS1 mice resulted in increased Aβ pathology, relative to that seen with colonization of WT microbiota. Increased influx of inflammatory components seemed to be associated with Aβ pathology. A study in a neonatal rat model showed that dietary disturbances before weaning period affected the normal establishment of the gut microbiota, as well as gut barrier and blood-brain barrier (BBB) functions. Such disruption of a normal establishment of the gut microbiota early in life may have potential long-term adverse consequences. In human adults, whose gut microbial community has already established, a short-term dietary intervention with a multifunctional diet (MFD), including several active ingredients that may serve as substrates for the gut microbiota, seemed to be insufficient to produce a broad switch in the gut microbiota composition. Yet specific gut microbial genera that associated with the positive impact of the diet were identified. Studies in apolipoprotein E knockout (ApoE^{-/-}) mice showed that high-fat (HF) feedings with inclusion of dietary fibres from lingonberries (<i>Vaccinium vitis-idaea</i>) affect the gut microbiota, associated with the lingonberries' positive effects to counteract the impaired metabolic functions induced by HF diets. Inclusion of lingonberries also decreased levels of gut- and neuro-inflammation and increased synaptic density in the hippocampus of mice fed HF diets.</p> <p>The results of these studies suggest that the gut microbiota may be involved in the progression of AD by triggering systemic inflammatory responses. Chronic inflammation and ageing have been shown to increase vulnerability of the BBB. Inclusion of lingonberries in HF diets counteracted negative effects of the HF diets on metabolic and neuroinflammatory markers. Therefore, lingonberries may be included as a part of a functional diet to target specific gut microbiota associated with improved risk factors for developing AD or even other neurodegenerative diseases.</p>		
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To our companions in the gut

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Abstract

Alzheimer's disease (AD) is the most common form of dementia. Increased accumulations of senile plaques and tangles are the known neuropathological hallmarks of AD. However, the exact triggers for such protein accumulations, leading to cognitive impairment and important morphological alteration in AD, are still elusive. Ageing and genetic risk factors do not completely explain the global increase in the AD prevalence. Different environmental risk factors, including lifestyle and diet, as well as cluster risk factors of the metabolic syndrome (MetS) have been identified to influence the progression of the disease. Emerging evidence has suggested the important role of the commensal gut microbiota in brain development and function, affecting development of brain-related disorders. Hence, understanding the impact that gut microbiota may have on the risk factors for AD may provide possible preventive or therapeutic strategies for such neurodegenerative disease.

Studies included in this thesis aimed to evaluate whether the gut microbiota may have influences on AD risk factors and how a healthy gut microbiota, amending gut-brain interaction, may be composed. The shifts of gut microbiota upon dietary changes, and their relation to neurodegenerative and metabolic disturbances in rodents and humans were investigated.

The results showed that a mouse model of cerebral amyloidosis (APP^{PS1}) displayed a distinct gut microbiota profile, as compared to that of healthy wild-type (WT) mice. Absence of the gut microbiota (germ-free, GF) in APP^{PS1} mice resulted in reduction of amyloid-beta (A β) deposition. Colonization of GF mice with the gut microbiota from APP^{PS1} mice resulted in increased A β pathology, relative to that seen with colonization of WT microbiota. Increased influx of inflammatory components seemed to be associated with A β pathology. A study in a neonatal rat model showed that dietary disturbances before weaning period affected the normal establishment of the gut microbiota, as well as gut barrier and blood-brain barrier (BBB) functions. Such disruption of a normal establishment of the gut microbiota early in life may have potential long-term adverse consequences. In human adults, whose gut microbial community has already established, a short-term dietary intervention with a multifunctional diet (MFD), including several active ingredients that may serve as substrates for the gut microbiota, seemed to be insufficient to produce a broad switch in the gut microbiota composition. Yet specific gut microbial genera that associated with the positive impact of the diet were identified. Studies in apolipoprotein E knockout (ApoE^{-/-}) mice showed that high-fat (HF) feedings with inclusion of dietary fibres from lingonberries (*Vaccinium vitis-idaea*) affect the gut microbiota, associated with the lingonberries' positive effects to counteract the impaired metabolic functions induced by HF diets. Inclusion of

lingonberries also decreased levels of gut- and neuro-inflammation and increased synaptic density in the hippocampus of mice fed HF diets.

The results of these studies suggest that the gut microbiota may be involved in the progression of AD by triggering systemic inflammatory responses. Chronic inflammation and ageing have been shown to increase vulnerability of the BBB. Inclusion of lingonberries in HF diets counteracted negative effects of the HF diets on metabolic and neuroinflammatory markers. Therefore, lingonberries may be included as a part of a functional diet to target specific gut microbiota associated with improved risk factors for developing AD or even other neurodegenerative diseases.

Popular science summary

As we get older, we will eventually notice some slow thinking or experience trouble remembering things. However, serious mental decline is not a normal part of ageing, but a sign of dementia. Alzheimer's disease is the most common type of dementia. It is a progressive disease of nerve cell death and tissue loss throughout the brain, affecting both memory and behaviour. Most people with diagnosed Alzheimer's are 65 years or older, however, the disease could strike people already at younger age. Formation of protein plaques and tangles are suspected to block parts of the nerve cells to work properly and lead to nerve cell death. Amyloid-beta plaques are the most common protein plaques spreading in Alzheimer's brain. Ageing and genetic factors are the known risk factors for Alzheimer's. However, the exact cause of this protein deposition is currently unknown. There has been a growing number of people living with dementia around the world, partly due to global increase in the ageing population. However, it seems that environmental factors also contribute to the increasing number of Alzheimer's cases.

Evidence has suggested that people with the metabolic syndrome, such as obesity, type 2 diabetes and heart disease have increased risk for developing Alzheimer's disease. The most recent studies also suggested gut bacteria, a complex community of microorganisms living in the digestive tract, to be involved in metabolic syndrome and also some brain-related disorders.

The work done in this thesis revealed a possible role of the gut bacteria in the development of amyloid-beta plaques. Mice that are genetically modified to carry genes involved in the development of Alzheimer's disease, and therefore develop amyloid-beta plaques as they age, showed a unique gut bacterial community as compared to healthy mice. Also, mice that are completely lacking gut bacteria, so called germ-free mice showed lower levels of amyloid-beta plaques as compared to mice that are raised in a normal bacterial condition. The involvement of gut bacteria in the formation of the Alzheimer's plaques was further proven by the finding that germ-free mice receiving bacteria from Alzheimer's affected mice developed more plaques than mice receiving bacteria from healthy mice.

Gut bacteria is playing a crucial role in the immune system, and presence of some less beneficial groups of bacteria may trigger inflammation in the gut and the brain. Gut-brain interactions have been studied in several aspects and therefore it is plausible that gut bacteria could affect the formation of amyloid-beta plaques.

A study included this thesis also showed in rats that dietary disturbances before the weaning period can interfere with a normal establishment of the gut microbiota, which later in life may become more stable and less responsive to dietary changes. Dietary fibre is the component of vegetables or fruits that cannot be digested by

human enzymes in the small intestine and reaches the colon. It serves directly to the gut bacteria, making it the most important dietary factor for shaping the gut bacteria. The work included in this thesis revealed profound effects of dietary fibre in lingonberries when given to mice fed high-fat diet, increasing the abundance of bacteria associated with reduced levels of inflammation in the gut and in the brain, compared to mice fed high-fat diet without lingonberries. Mice consuming lingonberries also showed an increased number of synapses, a structure in the brain that is involved in communication between nerve cells. Including lingonberries as a part of a healthy diet may help to boost particular gut bacteria, potentially preventing or delaying the progression of metabolic disorders and brain-related diseases, such as Alzheimer's disease.

Populärvetenskaplig sammanfattning

När man blir äldre kan man så småningom märka av lite långsammare tänkande eller glömska. Allvarliga minnesproblem och nedsatt tankeförmåga tillhör dock inte en normal del av åldrandet, utan kan vara ett tecken på demenssjukdom. Alzheimers sjukdom är den vanligaste typen av demenssjukdom. Det är en kronisk sjukdom som orsakar nervcellsdöd och vävnadsförlust i hela hjärnan, vilket påverkar både minne och beteende. De flesta personer som får diagnosen är 65 år eller äldre, men sjukdomen kan även drabba människor i lägre ålder än så. Sjukdomen ger upphov till inlagring av proteinplack och nystan (tangles) som misstänks blockera delar av nervcellernas funktion och leda till nervcellsdöd. Amyloid-beta plack är de vanligaste proteinplacken som bildas i den Alzheimersdrabbade hjärnan. Åldrande och genetiska faktorer är kända riskfaktorer för Alzheimers sjukdom. Emellertid är den exakta orsaken till denna proteininlagring för närvarande okänd. Det har skett en ökning av antalet människor som lever med demens runt om i världen, delvis på grund av den åldrande befolkningen. Det verkar dock som att miljöfaktorer också bidrar till det ökande antalet av fall med Alzheimers sjukdom.

Forskning har visat att personer med det metabola syndromet, en sammansättning av rubbningar som kan inkludera fetma, typ 2 diabetes och hjärtsjukdomar, har ökad risk för att utveckla Alzheimers sjukdom. Nyligen har studier också visat att mikrobiotan, den komplexa uppsättningen av tarmbakterier som lever i matsmältningssystemet, kan vara involverad i det metabola syndromet och även vissa hjärnrelaterade störningar.

Arbetet i denna avhandling påvisar en möjlig roll hos tarmbakterierna för utvecklingen av amyloid-beta plack. Möss som är genetiskt modifierade för att bära gener som är involverade i utvecklingen av Alzheimers sjukdom, och som därmed utvecklar amyloid-beta plack när de åldras, uppvisade en annorlunda bakteriesammansättning i tarmen jämfört med friska möss. Möss som helt saknar tarmbakterier, så kallade bakteriefria möss, uppvisade också lägre nivåer av amyloid-beta plack jämfört med möss som är uppvuxna med bakterier i tarmen. Tarmbakteriernas inverkan på uppkomsten av amyloid-beta plack styrktes vidare av upptäckten att bakteriefria möss som mottog bakterier från Alzheimersdrabbade möss utvecklade mer plack än möss som mottog bakterier från friska möss.

Tarmbakterier spelar en avgörande roll för immunförsvaret, och närvaron av mindre fördelaktiga grupper av bakterier kan utlösa inflammation i tarmarna och hjärnan. Interaktioner mellan tarmfloran och hjärnan har setts i flera sjukdomssammanhang vilket gör det mer troligt att tarmbakterier även kan påverka bildningen av amyloid-beta plack.

En studie i denna avhandling gjord på råttor visade också att introducerade koststörningar före avvänjningsperioden från modersmjölken kan störa en normal etablering av tarmmikrobiotan, som senare i livet kan bli mer stabil och mindre mottaglig för kostförändringar. Kostfibrer är ingredienser i grönsaker eller frukter som inte kan smältas i tunntarmen och når därför tjocktarmen och utgör föda för tarmbakterierna. Detta gör kostfibrer till den viktigaste kostfaktorn för att ändra sammansättningen av tarmbakterierna. Arbetet i denna avhandling visade att kostfibrer i lingon som gavs till möss som åt en fet kost ökade mängden av vissa bakteriearter som är associerade med mindre inflammation i tarmarna och i hjärnan, jämfört med möss som enbart åt fet kost utan lingon. Möss som fick lingon hade också ett ökat antal synapser (sammankopplingar mellan nervceller i hjärnan). Att inkludera lingon som en del i en hälsosam kost kan således öka mängden av vissa gynnsamma tarmbakterier, som förhoppningsvis kan förebygga eller fördröja utvecklingen av Alzheimers sjukdom.

บทคัดย่อ

โรคอัลไซเมอร์ (Alzheimer's disease) เป็นโรคสมองเสื่อมชนิดที่พบได้บ่อยที่สุดในผู้สูงอายุ ส่วนใหญ่ผู้ที่ได้รับการวินิจฉัยว่าเป็นโรคนี้มีอายุตั้งแต่ 65 ปีขึ้นไป แต่โรคนี้ไม่ใช่โรคแห่งความชราและยังสามารถเกิดขึ้นได้กับผู้ที่อายุน้อยกว่า 65 ปีอีกด้วย การก่อตัวของคราบพลัก (plaque) ในสมองนับว่าเป็นหนึ่งในอาการสำคัญของโรคที่มีผลขวางการเชื่อมต่อและก่อให้เกิดการตายลงของเซลล์สมอง ซึ่งนำไปสู่การสูญเสียความทรงจำและการเปลี่ยนแปลงทางพฤติกรรม

อะไมลอยด์เบต้า (amyloid-beta) เป็นคราบพลักโปรตีนส่วนใหญ่ที่พบได้ในสมองของผู้ป่วยอัลไซเมอร์ จากปัจจัยที่ก่อให้เกิดโรคที่เป็นที่รู้จักคืออายุที่มากขึ้นและปัจจัยทางพันธุกรรม แต่อย่างไรก็ตามสาเหตุในการก่อตัวของคราบพลักยังไม่เป็นที่ทราบแน่ชัด จำนวนผู้ป่วยอัลไซเมอร์ที่เพิ่มมากขึ้นเรื่อยๆ ทั่วโลกอาจมาจากปัจจัยสิ่งแวดล้อมอื่นๆ ที่นอกเหนือจากการเพิ่มจำนวนของผู้สูงอายุทั่วโลก มีหลักฐานที่แสดงว่าผู้ที่มีภาวะผิดปกติเกี่ยวกับระบบเมแทบอลิซึมของร่างกาย (metabolic syndrome) อย่างเช่นภาวะโรคอ้วน โรคเบาหวาน หรือโรคหัวใจ มีความเสี่ยงมากขึ้นที่จะพัฒนาโรคอัลไซเมอร์ งานวิจัยล่าสุดได้มีการกล่าวถึงบทบาทของแบคทีเรียในลำไส้ต่อโรคเกี่ยวกับระบบเมแทบอลิซึมของร่างกาย และรวมไปถึงโรคที่เกี่ยวกับความผิดปกติทางสมองด้วย อย่างเช่น โรคออทิซึม เป็นต้น

งานวิจัยที่ศึกษาในเล่มนี้แสดงให้เห็นถึงบทบาทของแบคทีเรียในลำไส้ต่อการก่อตัวของคราบพลักอะไมลอยด์เบต้า หนูทดลองที่มีการตัดต่อพันธุกรรมให้พัฒนาคราบพลักอะไมลอยด์เบต้าเมื่ออายุมากขึ้นมีแบคทีเรียในลำไส้ชนิดที่ต่างจากหนูที่ไม่มีพันธุกรรมของโรคนี้ นอกจากนี้การพัฒนาตัวของคราบพลักอะไมลอยด์เบต้าในหนูที่โตในภาวะไร้ออกซิเจน (germ-free) นั้นช้าลง ถึงแม้ว่าหนูพวกนี้จะมีพันธุกรรมก่อโรค เมื่อหนูที่โตในภาวะไร้ออกซิเจนได้รับการถ่ายเทแบคทีเรียจากลำไส้ของหนูที่มีพันธุกรรมก่อโรค พบว่าการพัฒนาตัวของคราบพลักก็เพิ่มขึ้นและมากกว่าเมื่อหนูไร้ออกซิเจนได้รับแบคทีเรียจากหนูที่ไม่มีพันธุกรรมก่อโรค

แบคทีเรียในลำไส้มีบทบาทสำคัญในการพัฒนาของระบบภูมิคุ้มกัน การลดลงของแบคทีเรียที่มีประโยชน์และแทนที่ด้วยแบคทีเรียที่น้อยประโยชน์ในลำไส้จึงมีผลต่อระบบภูมิคุ้มกันทั้งร่างกายที่สามารถก่อให้เกิดการอักเสบระดับต่ำ (low-grade inflammation) ที่มีผลกระทบต่อทั้งร่างกาย งานวิจัยจำนวนมากในช่วงทศวรรษที่ผ่านมาได้แสดงให้เห็นถึงการเชื่อมต่อของระบบลำไส้และสมอง ดังนั้นจึงเป็นไปได้ที่แบคทีเรียในลำไส้จะมีผลกระทบต่อการทำงานของคราบพลักในโรคอัลไซเมอร์

กากใยอาหาร (dietary fibre) ที่พบได้ส่วนใหญ่ในผักและผลไม้เป็นส่วนของอาหารที่ไม่ถูกย่อยในกระเพาะอาหารและลำไส้เล็ก จึงถูกส่งผ่านไปถึงระบบลำไส้ใหญ่และเป็นอาหารหลักให้กับแบคทีเรียที่มีประโยชน์ ดังนั้นกากใยอาหารจึงเป็นส่วนอาหารสำคัญที่มีผลต่อชนิดของแบคทีเรียในลำไส้ งานวิจัยในผลไม้ที่เป็นที่นิยมในประเทศยุโรปตอนเหนือ ลิงกอนเบอร์รี่ (lingonberries) แสดงให้เห็นว่าผลไม้ชนิดนี้เพิ่มจำนวนแบคทีเรียที่มีประโยชน์ รวมถึงชนิดที่ไม่พบในหนูที่เป็นโรคอัลไซเมอร์ ซึ่งมีผลในการลดระดับของการอักเสบชั้นต่ำในลำไส้และในสมอง และเพิ่มจำนวนของเซลล์สื่อประสาท (synapses) ของหนูทดลองที่ได้รับอาหารที่มีไขมันสูง ดังนั้นลิงกอนเบอร์รี่จึงสามารถเป็นส่วนหนึ่งของอาหารเพื่อสุขภาพที่อาจจะมีผลในการต่อต้านการพัฒนาของโรคเกี่ยวกับระบบเมแทบอลิซึมของร่างกาย รวมถึงโรคอัลไซเมอร์ด้วย

List of scientific papers

- Paper I** **Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota**
Harach T, **Marungruang N**, Duthilleul N, Cheatham V, Mc Coy KD, Frisoni G, Neher JJ, Fåk F, Jucker M, Lasser T and Bolmont T
Scientific Reports. 2017 Feb 8; 7:41802. doi: 10.1038/srep41802
- Paper II** **Impact of dietary induced precocious gut maturation on cecal microbiota and its relation to the blood-brain barrier during the postnatal period in rats**
Marungruang N, Sureda EA, Lefrançoise A, Weström B, Nyman M, Prykhodko O and Fåk Hållenius F
Neurogastroenterology and Motility. 2018;e13285. doi: 10.1111/nmo.13285
- Paper III** **Improvement in cardiometabolic risk markers following a multifunctional diet is associated with gut microbial taxa in healthy overweight and obese subjects**
Marungruang N, Tovar J, Björck I and Fåk Hållenius F
European Journal of Nutrition. 2017 Nov 2. doi: 10.1007/s00394-017-1563-3
- Paper IV** **Lingonberries reduce atherosclerosis in Apoe(-/-) mice in association with altered gut microbiota composition and improved lipid profile**
Matziouridou C, **Marungruang N**, Nguyen TD, Nyman M, Fåk F
Molecular Nutrition and Food Research. 2016 May;60(5):1150-60. doi: 10.1002/mnfr.201500738
- Paper V** **Lingonberries and their two separated fractions differently alter the gut microbiota, improve metabolic functions, reduce gut inflammatory properties, and improve brain function in ApoE -/- mice**
Marungruang N, Kovalenko T, Osadchenko I, Voss U, Huang F, Burleigh S, Ushakova G, Skibo G, Nyman G, Prykhodko O and Fåk Hållenius F
Manuscript

The author's contributions

- Paper I** The author performed gut microbiota sequencing, sequence analysis using bioinformatic tool and took part in manuscript writing.
- Paper II** The author took part in sample collection in the animal experiment, performed gut microbiota sequencing, sequence analysis using bioinformatic tool, plasma LBP measurement, data analyses and wrote the manuscript.
- Paper III** The author performed gut microbiota sequencing, sequence analysis using bioinformatic tool, data analyses and wrote the manuscript.
- Paper IV** The author performed gut microbiota sequencing, sequence analysis using bioinformatic tool and took part in manuscript writing.
- Paper V** The author took part in study design, prepared raw materials (lingonberry fractions) for inclusion in animal diets, performed the animal experiment, oral glucose tolerance test, set up and took part in performing behaviour tests, performed gut microbiota sequencing, atherosclerosis plaque analysis, blood lipid measurement, data analyses and wrote the manuscript.

List of publications not included in the thesis

Heat-treated high-fat diet modifies gut microbiota and metabolic markers in ApoE^{-/-} mice

Marungruang N, Fåk F and Tareke E

Nutrition & Metabolism. 2016 13:22. doi:10.1186/s12986-016-0083-0

Evaluation of the microbiome in children's appendicitis

Salö M, Marungruang N, Roth B, Sundberg T, Stenström P, Arnbjörnsson E, Fåk F and Ohlsson B

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Molecular Properties of Guar Gum and Pectin Modify Cecal Bile Acids, Microbiota, and Plasma Lipopolysaccharide-Binding Protein in Rats

Ghaffarzagdegan T, Marungruang N, Fåk F, Nyman M

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Barley malt increases hindgut and portal butyric acid, modulates gene expression of gut tight junction proteins and Toll-like receptors in rats fed high-fat diets, but high advanced glycation end-products partially attenuate the effects

Zhong Y, Teixeira C, Marungruang N, Sae-Lim W, Tareke E, Andersson R, Fåk F and Nyman M

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Effects of two whole-grain barley varieties on caecal SCFA, gut microbiota and plasma inflammatory markers in rats consuming low- and high-fat diets

Zhong Y, Marungruang N, Fåk F, Nyman M

British Journal of Nutrition. Volume 113, Issue 10 28 May 2015, pp. 1558-1570. doi:10.1017/S0007114515000793

The physico-chemical properties of dietary fibre determine metabolic responses, short-chain fatty acid profiles and gut microbiota composition in rats fed low- and high-fat diets

Fåk F, Jakobsdottir G, Kulcinskaja E, **Marungruang N**, Matziouridou C, Nilsson U, Stålbrand H, Nyman M

PLoS One. 2015 May 14;10(5):e0127252. doi: 10.1371/journal.pone.0127252. eCollection 2015.

List of abbreviations

AD	Alzheimer's Disease
AMPs	Antimicrobial Peptides
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
APOE -/-	Apolipoprotein E knockout
APP	Amyloid Precursor Protein
ASD	Autism Spectrum Disorder
AUC	Area Under Curve
A β	Amyloid-beta
BBB	Blood-Brain Barrier
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD	Control Diet
CNS	Central Nervous System
COLOAD	Colonized with gut microbiota from APPPS1 mice
COLOWT	Colonized with gut microbiota from Wild-Type mice mice
CONVR	Conventionally-Raised
dwb	dry weight basis
EGCs	Enteric Glial Cells
ELISA	Enzyme-Linked Immunosorbent Assay
Ffar	Free-fatty acid receptor
GF	Germ-Free
GI tract	Gastrointestinal tract
GLP	Glucagon-like Peptide
HDL	High-Density Lipoprotein
HF	High-Fat
HSA	Human Serum Albumin

IFN	Interferon
IL	Interleukin
insLB	Insoluble fraction of Lingonberries
LB	Lingonberries
LBP	Lipopolysaccharide-Binding Protein
LDA	Linear Discriminant Analysis
LDL	Low-Density Lipoprotein
LEfSe	Linear Discriminant Analysis Effect Size
LF	Low-Fat
LPS	Lipopolysaccharide
MetS	Metabolic Syndrome
MFD	Multifunctional Diet
MS	Multiple Sclerosis
OGTT	Oral Glucose Tolerance Test
OPLS	Orthogonal Partial Least Squares
PAMPs	Pathogen-associated Molecular Patterns
PD	Parkinson's Disease
PHA	Phytohemagglutinin
PLS	Partial Least Squares
PRRs	Pathogen-recognition Receptors
PT	Protease
QIIME	Quantitative Insights Into Microbial Ecology
SCFAs	Short Chain Fatty Acids
solLB	Soluble fraction of Lingonberries
TLRs	Toll-Like Receptors
TNF	Tumour Necrosis Factor
wLB	Whole Lingonberries
WT	Wild-Type

Introduction

Alzheimer's disease (AD) is the most common form of dementia. The onset of this neurodegenerative disease affects life quality of both the individuals and their families, and large amounts of resources and money are spent in caring for people with AD (1). Approximately 35.6 million people of the world population are living with dementia and there has been a gradual increase each year, with estimated numbers doubling by 2030 and more than tripling by 2050. This has led the World Health Organization to raise awareness of dementia as a public health priority (2). Despite increasing research in this field, the exact cause of AD is still poorly understood and existing treatments are very limited to symptomatic treatments. Although AD usually affects people at the age older than 65 years, the disease is not a normal part of ageing. Despite being rare, the early-onset AD can also affect people at younger age (3). Genetic factors, such as inheritance of the *APOEε4* gene, *i.e.*, the most common susceptibility gene in human AD, are neither necessary nor sufficient for development of AD (4). Also, ageing and genetic risk factors do not completely explain the globally rapid increase in AD prevalence, which might instead be explained by additional environmental influences. Understanding the initial triggers for AD pathology is needed for the development of preventive or therapeutic approaches for AD.

Over the past decades research has revealed that the commensal gut microbiota and its metabolites play an important role in host health, including metabolism, physiology, nutrition, immune and barrier functions (5). More recently, its impact on brain functions and behaviour had also been revealed (6). This has emerged within the concept of gut microbiota-gut-brain axis, a bi-directional communication between the gut and the brain. A number of studies observed that specific groups of gut microbiota are associated with the development of brain-related disorders, such as autism spectrum disorder (ASD) (7-11), Parkinson's disease (PD) (12, 13) and multiple sclerosis (MS) (14). These further emphasize the possible involvement of the gut microbiota in neurodegenerative diseases, including AD. Yet the research regarding the role of the gut microbiota on AD is still in its early stages.

The gut microbiota and their hosts have long co-evolved and shared substrates for growth (15, 16). The establishment of the gut microbial community occurs during the first few years of life (17). Dietary disturbance during this period may affect the gut microbiota establishment and possess potential long-term effects on the host in

several aspects, especially since this period coincides with the key stage of brain development (18). Westernization of the diet *i.e.*, increased intake of refined foods rich in saturated fat and low in dietary fibre, is an important factor giving rise in the prevalence of metabolic syndrome (MetS), which is also associated with dementia (19-21). In human and pre-clinical models, unhealthy high-fat (HF) diets have been shown to cause an imbalanced community of the gut microbiota and be associated with the MetS (22). This may suggest the interactions between the gut microbiota, metabolic and neurodegenerative disorders.

Apart from understanding the exact mechanisms mediating the involvement of the gut microbiota in metabolic and neurodegenerative disorders, the current challenge is also to understand what a healthy gut microbiota is composed of and how to achieve a balanced gut-brain communication via gut microbiota manipulation. Dietary factors, especially dietary fibre, have been suggested as the most effective approach for optimizing and/or maintaining a balanced gut microbial community (23).

Studies included in this thesis expand existing knowledge regarding the role of the gut microbiota on host metabolism, brain function and behaviour, with special focuses on metabolic risk factors and AD. Dietary strategies for gut microbiota manipulation in human and rodent models were evaluated. The knowledge acquired here suggests possible strategies for optimizing the existing and future preventive, as well as therapeutic approaches toward metabolic and neurodegenerative diseases.

Background

Dementia and Alzheimer's disease

Dementia is defined as a set of neurodegenerative diseases which include severe impairment of cognitive function and deterioration in emotional control, social behaviour or motivation. The disturbance must be sufficient to interfere significantly with an individual's daily life, ranging from work, usual social activities, or relationships with others (24). The onset of dementia usually starts around the age of 65 and the prevalence doubles every five-year increment in age. The world population is ageing, and this clearly contributes to a negative impact on the number of people with dementia. In 2011, approximately 35.6 million people globally were living with dementia. Epidemiological studies have estimated that the number will double in 2030 and triple in 2050 in every part of the world (3). Although dementia mainly affects older people, it is not a normal part of ageing. Also, early-onset dementia can affect people already before the age of 65 (25). This is generally associated with a more rapid progression of dementia and accounts for approximately 1-5% of all dementia cases (3).

In Sweden, approximately 180,000 people were living with dementia in 2012, representing 1.8% of the total Swedish population, or 8% of those older than 65, or approximately half of those older than 90 years old (26). Also, the early-onset of dementia contributes up to approximately 9,000 dementia cases in Sweden (26).

AD is the most common form of dementia, accounting for approximately 60-80% of all dementia cases (27). Despite increasing awareness for the devastating effect of AD, and research trying to understand the exact pathogenic mechanisms of AD, there are currently no treatments to cure or alter the progression of the disease. The only prominent clinically effective drugs are those acting on enhancement of the central cholinergic function by the inhibition of acetylcholinesterase, which temporarily cure AD symptoms (28).

The neurofibrillary tangles and the senile plaques are the two most recognised neuropathological hallmarks of AD. The neurofibrillary tangles consist of abnormal accumulation of hyper-phosphorylated tau protein. The senile plaques consist of amyloid-beta ($A\beta$) peptide with abnormally configured neuronal processes (29).

The loss of synaptic components is the change that significantly leads to cognitive impairment and other important morphological alterations in AD (94).

Amyloid-beta ($A\beta$) and the amyloid hypothesis

There is evidence that $A\beta$ initiates the pathologies of AD, and deposition of $A\beta$ plaques commences decades before the development of clinical symptoms of AD (30). $A\beta$ is a sticky peptide firstly sequenced in the 1984 from the meningeal blood vessels of AD patients and individuals with Down's syndrome (31, 32). $A\beta$ is a product of amyloid precursor protein (APP) metabolism. The gene encoding APP is located on chromosome 21 (33). This couples to the previous recognition that Down's syndrome, which is trisomy 21, is associated with an increased risk for neuropathological development of AD (34). APP is produced in large quantities in neurons. The precise physiological function of APP is still elusive. However, mouse studies have shown positive effects of APP overexpression on neuron cell health and growth (35). Further, intracerebral injections of APP in adult animals resulted in improved cognitive functions and synaptic density (36, 37).

There are multiple alternate pathways for APP proteolysis, some of which lead to the generation of the $A\beta$ peptide and some of which do not. The enzymes that cleave APP usually are the alpha- (α -), beta- (β -), and gamma- (γ -) secretases. Proteolytic processing of APP by β -, and γ -secretases promotes generation of $A\beta$, while α -secretase cleavage on APP prevents $A\beta$ production (38). Several different $A\beta$ species exist. The slightly longer forms of $A\beta$, particularly $A\beta_{42}$, are more hydrophobic and fibrillogenic, and are the most abundant species deposited as senile plaques in the brain of AD patients. However, the $A\beta_{40}$ species, which is normally more abundantly produced by cells than $A\beta_{42}$, is usually colocalized with $A\beta_{42}$ in the plaques (39). Membrane disruption and ion dysregulation are the known components of $A\beta$ toxicity (40). Interestingly, individuals with short-term survival after head injury showed post-traumatic cerebral deposition of $A\beta_{42}$, similar to that seen in Down's syndrome and AD (41).

Development of drugs targeting $A\beta$ clearance has been shown to have no apparent clinical efficiency (42). The challenge could be that these drugs may have to be given at the earliest stage as possible for them to be successful, given that $A\beta$ pathologies develop long before the symptoms start. However, identifying the exact initial triggers for $A\beta$ deposition may be of more effective strategies to prevent or delay the progression of AD and other $A\beta$ -related brain disorders.

Risk factors of AD

Genetic risk factors

APP, *PSEN1* and *PSEN2* are genes encoding proteins that are involved in the breakdown of APP and in A β generation. Mutations in these genes have been identified to cause a proportion of early-onset AD (3). Yet a Finnish study has reported that mutation in these genes are rare in the Finnish population whom express familial early-onset AD, suggesting other risk factors to also be involved (43).

The apolipoprotein E (*APOE*) gene has been known as the most common genetic risk factor of AD in humans. APOE is a lipid-binding protein, which in humans, expresses as three common isoforms, including *APOE* ϵ 2, ϵ 3, and ϵ 4 (3). People with a single copy of *APOE* ϵ 4 have two- to three-fold increased risk, while those with two copies are associated with a five-fold or more increased risk for developing AD (44). The *APOE* ϵ 4 is also associated with mild cognitive impairment and with the progression to dementia (3). However, despite being a potent risk factor the presence of *APOE* ϵ 4 is neither necessary nor sufficient for developing AD (4). There are other unknown risk factors that can contribute to the disease.

Non-genetic risk factors

Genetic factors do not directly determine the prevalence of AD, and neither does ageing. Nevertheless, these factors are non-modifiable. Identification of other environmental risk factors that may be initial triggers of AD pathology is needed. Such environmental factors are modifiable and may be of an important strategy in a preventive approach for AD. Physical inactivity, mid-life hypertension, and cluster risk factors of the MetS, including diabetes mellitus, cardiovascular disease and obesity are among the known risk factors of AD (3). Recently, emerging research has suggested important roles of the commensal gut microbiota in the development of brain-related disorders and neurodegenerative diseases such as ASD (7-11), PD (12, 13) and MS (14). Thus, novel insights into the role of the gut microbiota in such neurodegenerative diseases, including AD are worth exploring.

The interest in the role of gut microbiota in AD has been growing exponentially during the past few years. Number of studies investigating the relation between the gut microbiota in AD that were published yearly from 2011 up until February 2018 is shown in **Figure 1**.

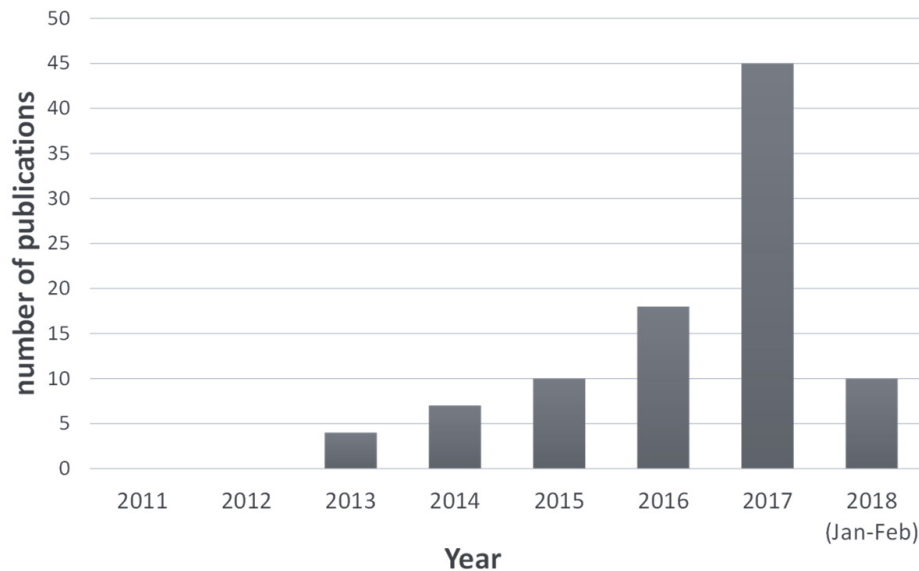


Figure 1 Number of yearly publications related to gut microbiota and Alzheimer’s disease from 2011 to February 2018. Data obtained by searching on PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) using the following keywords: gut microbiota, Alzheimer’s disease.

Gut microbiota

The mammalian gastrointestinal (GI) tract harbours trillions of microorganisms, known as the gut microbiota. It is predominated by bacteria, mainly anaerobes, with minor proportion of archaea, viruses, protozoa and fungi. Each individual has a unique gut microbiota, of which its composition is influenced by different host intrinsic and environmental factors. A collective number of over a thousand of bacterial species is estimated to inhabit the human gut (45, 46). The number of microbes vary along the length of the GI tract but is highest in the colon, the main site of gut fermentation of dietary components, such as dietary fibre (47). The gut microbial metabolites and their interactions with one another, as well as with the host, have a great impact on host health in several aspects. The majority of the gut microbiota is unculturable and thus its impact on the host has not previously been possible to be extensively studied. The advance in sequencing technology, however has now allowed us to characterize the complex community of the gut microbiota and brought about the subject of extensive research regarding the gut microbiota and its impact on host.

The gut microbiota is often referred to as a hidden ‘organ’ due to its great impact on host metabolism, physiology, nutrition and immune function (48). Changes in gut microbial population can result in both beneficial and harmful consequences beyond the GI tract. The ability of the gut microbiota and its metabolites to interact and

communicate with the brain has been emerging as an intriguing topic in human health and diseases (6).

Gut microbiota and host physiology, nutrition and metabolism

Microbes in the gut obtain nutrition from several substrates, including indigestible dietary components and host-derived components, such as shredded epithelium cells and mucus. The gut microbiota is crucial for regulatory effects on epithelial growth, differentiation and protection. Dysbiosis or durable alteration of the gut microbiota has been shown to be associated with impaired gut barrier functions, which subsequently leads to immune dysregulation. Studies have shown correlations between altered abundances of some specific gut bacterial taxa and certain disorders or conditions such as inflammatory bowel disease, irritable bowel syndrome, coeliac disease, as well as allergy, asthma and the MetS (49).

Substrate utilization by the gut microbiota produces several metabolites, generating energy for microbial cell processes and growth, as well as for a variety of metabolic functions of the hosts. This may be the primary evolutionary forces for the establishment of bacteria as human symbionts (50). Germ-free (GF) animals require up to 30% more caloric intake to maintain their body weight to the same as conventionally-raised animals, serving as a compelling example of the importance of the gut microbiota in host metabolism. Gut microbiota are capable to ferment dietary components, mostly indigestible carbohydrates, that cannot be digested by host enzymes. This results in production of short-chain fatty acids (SCFAs), mainly acetate, propionate and butyrate and gases such as CO₂, H₂ and methane, as well as vitamins, such as vitamin K and B₁₂. Colonic fermentation of protein by the gut microbiota, although less common due to high digestibility of dietary protein, often leads to production of potentially toxic metabolites for the colonocytes, such as ammonia, amines, and sulphides. Studies in animal models and *in vitro* have shown that these compounds have negative impacts on gut barrier function, leading to inflammation, and even colonic DNA damage and cancer progression (51). Also, as opposed to indigestible carbohydrates, protein fermentation leads to production of branched-chain fatty acids, such as iso-butyrate and iso-valerate (51).

SCFAs

SCFAs are important microbial metabolites for colonic health. It is well established that SCFAs, particularly butyrate, is the preferred substrate for the colonocytes (52). Butyrate has an important role in maintaining gut mucosal integrity, regulating colonocyte differentiation and apoptosis, and promoting clearance of dysfunctional cells (53, 54). Approximately 95% of the SCFAs produced in the colon, as a result of gut microbial fermentation, are rapidly absorbed by the colonocytes, and only

about 5% are secreted in the faeces (55). SCFAs produced by the gut microbiota can be found in hepatic, portal, and peripheral blood (56, 57), affecting lipid, glucose, and cholesterol metabolism in various tissues (58-61). Also, SCFAs, especially butyrate, play an important role in mediating communication between the microbes and immune responses (62). Unlike the known health effects of the three most abundant SCFAs, acetate, propionate and butyrate, little is known about other SCFAs. A number of bacteria has been shown to be capable of producing different SCFAs. A list of some examples of predominant SCFA producers, during growth on glucose is shown in **Table 1**. Presence of other carbohydrate substrates can as well induce SCFA productions by other different bacteria.

Table 1
Predominant SCFA producer (adapted from Ohira H *et al.*, (63))

SCFAs	Bacterial producer
Acetate	Most of the enteric bacteria <i>Bifidobacterium</i> (from Actinobacteria) <i>Bacteroides</i> , <i>Prevotella</i> (from Bacteroidetes) <i>Clostridium</i> , <i>Ruminococcus</i> , <i>Blautia hydrogenotrophica</i> , <i>Streptococcus</i> (from Firmicutes) <i>Akkermansia muciniphilia</i> (from Verrucomicrobia)
Propionate	<i>Bacteroides</i> (from Bacteroidetes) <i>Dialister</i> , <i>Veillonella</i> , <i>Coprococcus catus</i> , <i>Roseburia inulinivorans</i> , <i>Ruminococcus obeum</i> (from Firmicutes)
Butyrate	<i>Coprococcus comes</i> , <i>Coprococcus eutactus</i> , <i>Eubacterium rectale</i> , <i>Eubacterium hallii</i> , <i>Faecalibacterium prausnitzii</i> , <i>Roseburia</i> (from Firmicutes)

Gut microbiota and the SCFA metabolites have been shown to interact with L-cells, modulating the activity of gut peptides. These gut peptides include glucagon-like-peptide-1 (GLP-1) and GLP-2 that are involved in several biological functions of host physiology (64). GLP-1 has important roles in glucose homeostasis, appetite regulation and stomach emptying, while GLP-2 stimulates epithelial cell regeneration and thus play an important role in maintaining gut barrier functions (64).

Gut microbiota and immune system

The gut microbiota is segregated from the gut epithelial cells by a thick mucus layer. Despite this segregation, the gut microbiota is playing crucial roles in host immune development and homeostasis. The crosstalk between gut microbiota and the host immune system involves both innate and adaptive immunity that, in mammals, has developed a complex connection to maintain the homeostasis between the gut microbiota and immune regulation (65).

The mammalian immune system recognizes general pathogen-associated molecular patterns (PAMPs) by pathogen-recognition receptors (PRRs) (66). For example, lipopolysaccharide (LPS), the endotoxin from the outer membrane of Gram-negative bacteria, can be recognized by the toll-like receptors (TLRs), such as TLR-4, and their signalling lead to activation of the immune responses, under strong host regulation (67, 68). Dysbiosis of the gut microbiota is linked to aberrant immune responses, which are often accompanied by abnormal production of inflammatory cytokines (69). Changes in the abundances of specific gut bacteria have been shown to be associated with pro-inflammatory cytokine responses, such as interleukin-1 β (IL-1 β), IL-6, tumour necrosis factor- α (TNF- α) and interferon-gamma (IFN- γ), and may impact disease susceptibility (69).

Further, enteric glial cells (EGCs), representing the morphological and functional equivalent of astrocytes and microglia in the central nervous system (CNS), have recently been shown to be important in the regulation of inflammatory events in the gut (70). Similar to the CNS astrocytes, the EGCs express the S100B protein which exerts either trophic or toxic effects depending on its concentration (70-72). It has been shown that overexpression of glial S100B protein is observed in the early inflammatory processes in response to disruption of the intestinal barrier (73) and to particular bacteria (74).

Gut microbiota-brain interactions

Increasing evidence is pointing toward an important role of the gut microbiota in brain development, function and behaviour (5, 6). The complexity of these interactions is referred to as the gut-brain axis, which is a bidirectional signalling between the gut and the brain. The brain can influence the functioning of the gut, such as regional motility, acid secretion, bicarbonates and mucin production, epithelial fluid maintenance, gut permeability, and the mucosal immune response (75). Likewise, modulation in the gut microbial community has been reported to affect emotional behaviour, learning and memory, social interaction, eating behaviour, and different brain-signalling systems (75). Although the exact mechanisms mediating gut-brain interactions are not fully understood, these have been suggested to involve endocrine, immune and neural pathways (vagus nerve and enteric nervous system) (**Figure 2**) (76-78).

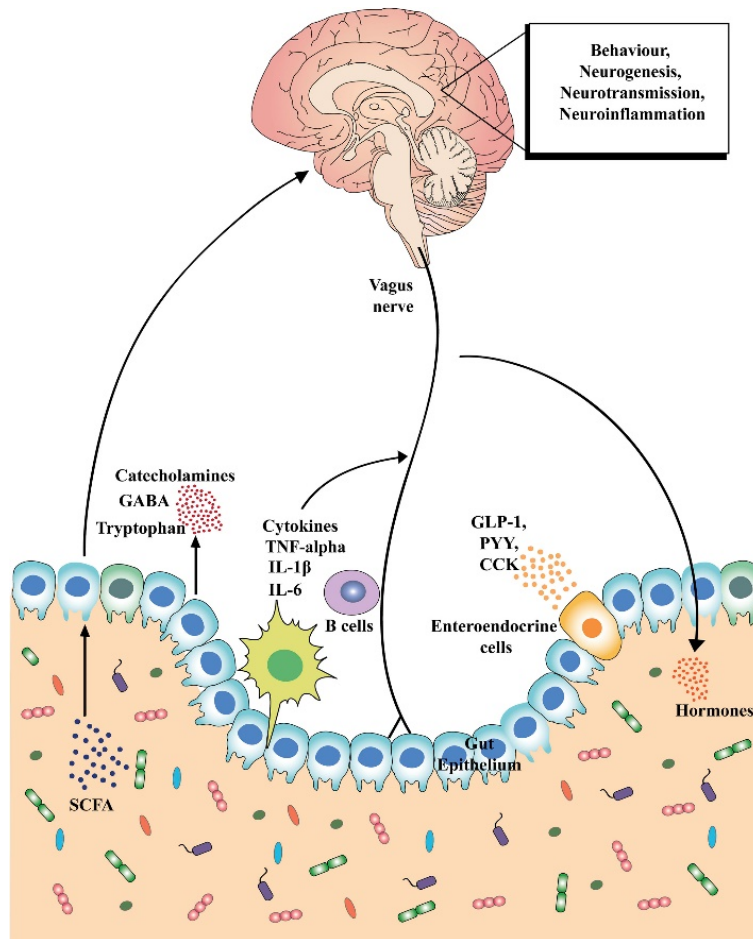


Figure 2 Potential pathways mediating the bi-directional gut-brain interaction include immune, endocrine, and neural pathways.

Activation of the vagus nerve, production of microbial antigens that recruit immune B cell responses, production of microbial metabolites (such as SCFAs), and enteroendocrine signaling from gut epithelial cells are the potential pathways of gut-brain axis communication. These signals control central physiological processes, such as neurotransmission, neurogenesis, neuroinflammation and neuroendocrine signaling. Received from © 2017 Foster Jane A, *et al.*, licensed under CC BY 4.0.

Gut microbiota and brain-related and neurodegenerative disorders

The revelation of the gut-brain interactions has brought about a paradigm shift in the traditional view regarding pathophysiology of brain-related disorders and neurodegenerative diseases (79). For example, in autism spectrum disorder (ASD), a range of chronic gastrointestinal (GI) symptoms including alterations in bowel habits, chronic abdominal pain and discomfort, and food intolerances have been observed as an important comorbidity of the disease (7, 11). Alteration in the gut microbiota composition and metabolites has also been implicated as a possible causative mechanism contributing to ASD pathophysiology. This hypothesis had been supported by a number of pre-clinical studies in rodent models (8). Ingestion of a *Bacteroides fragilis*, which restored gut barrier integrity, also ameliorated the ASD-like symptoms in a mouse model of ASD (9). Observational studies observed lower abundance of *Prevotella* and other carbohydrate-fermenters in the gut of ASD

children. Such gut microbial community assembles a hyper-westernization of the gut microbiota, and was suggested to play a role in the hyper-active adaptive immune system observed in ASD children (10).

In neurodegenerative diseases like Parkinson's disease (PD), the gut microbiota also showed to regulate motor deficits and neuroinflammation in a mouse model of PD (12). Colonization of the gut microbiota from PD patients in a GF mouse model of PD induced enhanced motor dysfunctions in the mice (12). In humans, the relative abundance of *Enterobacteriaceae* was shown to positively associate with the severity of PD symptoms (13). Further, observational studies in multiple sclerosis (MS) patients observed gut microbial dysbiosis, including lower abundance of *Faecalibacterium*, a butyrate producer whom is associated with a reduced inflammatory state (14).

Dynamic of gut microbiota development

It is still a topic of debate whether the initial gut microbial colonization may begin already before birth *in utero*, yet a huge inoculation occurs rapidly at birth. The first colonizers are facultative anaerobes, such as enterobacteria, coliforms and lactobacilli, followed by colonization of strict anaerobes such as *Bacteroides*, *Clostridium* and *Bifidobacterium* (17, 80). The gut microbiota of neonates is characterized by low diversity, with high relative abundance of Proteobacteria and Actinobacteria, while it becomes more diverse and with Firmicutes and Bacteroidetes being dominant at increased age, and fully developed toward an adult-like microbiota in terms of composition and diversity (81). The development of the gut microbiota during these first few years of life plays a key role in immunological and metabolic pathways and seems to be an important stage for long-term health status of the individual (17, 82). Infants born vaginally are exposed primarily to vaginal microbiota, which is mainly composed of lactobacilli. In contrast, those born via caesarean section are exposed primarily to the microbiota from the skins and the hospital environment, showing less abundance of lactobacilli, and increased risks of immune related disorders, such as allergies and asthma (80, 83).

Apart from mode of delivery, mode of feeding (breast milk or formula), introduction to solid food and antibiotics usage are other critical factors affecting the gut microbiota development in neonates (81). Interestingly, the period during the first few years of life when the gut microbiota is being developed coincides with the crucial period of brain development (18). Hence, a good establishment of the gut microbiota during this period may also profoundly ensure a normal development of the brain (84).

Once the community of an adult-like microbiota is established, the core gut microbiota is relatively stable throughout life and can be less responsive to dietary

changes (81). The human gut microbiota can be classified into three enterotypes based on variation of the levels of three robust cluster of bacterial genera, including *Bacteroides* (Enterotype 1), *Prevotella* (Enterotype 2) and *Ruminococcus* (Enterotype 3) (85). Yet gut microbiota alteration can be induced by a number of factors related to diet and lifestyle. A major shift in diets, such as from animal-based to plant-based, in human adults has been shown to induce changes in gut microbiota composition (86). With age, the core gut microbiota in an individual undergoes dynamic shifts, affecting diversity, stability and composition (87). Factors affecting gut microbiota development throughout life is shown in **Figure 3**.

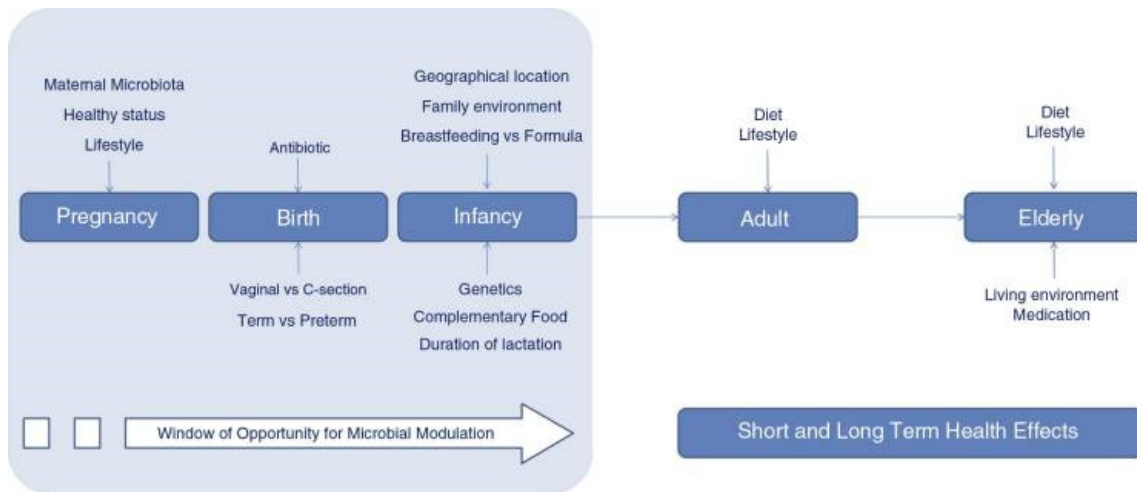


Figure 3 Factors affecting gut microbiota development throughout life. The first few years of life represent the most important period for gut microbiota establishment. Received from © 2015 Rodríguez Juan Miguel, *et al.*, licensed under CC by 3.0.

Dietary components

Dietary fat

Dietary fat refers to a class of lipid, classically defined as triglycerides (fats and oils), phospholipids, and sterols (cholesterol). Dietary fat is one of the main energy sources of the human body and plays a critical role in health and functions, including hormone production and fat-soluble vitamin transportation (88, 89). High consumption of dietary fat, especially saturated fat, *i.e.*, fatty acids with no double bonds, has been shown to be associated with increased plasma concentration of low-density lipoprotein (LDL-) cholesterol and risks for developing cardiovascular disease (90). Dairy products, butter, and red meats are examples of foods that contain high amount of saturated fatty acids (91). Hence, saturated fat intake, through the westernization of global diets, is now on the rise world-wide (92). Such

dietary pattern is associated with increased global prevalence of chronic diseases related to metabolic disorders, including cardiovascular disease, type 2 diabetes and obesity, collectively known as the metabolic syndrome (MetS).

Metabolic endotoxemia

HF diet has been shown to induce increased circulation of LPS, endotoxin from the outer membrane of Gram-negative bacteria (93), promoting metabolic endotoxemia and triggering low-grade systemic inflammatory responses in a range of tissues (47). The LPS translocation occurs mainly by its integration into chylomicrons during fat absorption, or is promoted by impaired gut barrier functions (94). LPS contains lipid A, a form of PAMPs, that triggers a signalling cascade of various pro-inflammatory activations, including the pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β . The endotoxic activity of LPS varies among different bacteria, depending on its chemical structure (95). Such chronic inflammatory tonus is a common pathophysiological element in the MetS, and both inflammatory and metabolic disorders have been known to also increase risk factor of dementia (96, 97).

Dietary fibre

Dietary fibre is a broad term for dietary carbohydrate polymers that are indigestible in the small intestine. When reaching the colon, the dietary fibre is served as a substrate for colonic fermentation by the gut microbiota (98). Dietary fibre contributes to faecal bulk through its physical presence and ability to adsorb water, promoting bowel health (99). Many of the health benefits associated to dietary fibres are mainly mediated by their fermentation by the gut microbiota, that leads to production of SCFAs, as described in the previous section. Dietary fibres can be divided into two primary classes on the basis of their water solubility, including soluble and insoluble dietary fibre (100). Soluble and insoluble dietary fibres exhibit unique structural components and, consequently, have different physiological effects. Soluble dietary fibres have been linked to metabolic effects such as lowering of blood cholesterol and the decrease in the intestinal absorption of glucose. Insoluble dietary fibres have been shown to reduce gut transit time, bind toxic and inflammatory compounds and transport them out of the body and in this way decrease the risk for constipation and perhaps also colon cancer (101).

Lingonberries

Lingonberries (*Vaccinium vitis-idaea*) are popular berries used in a variety of dishes in Nordic countries. Similar to other berries and fruits, lingonberries contain high amounts of dietary fibre, vitamins and minerals, as well as other bioactive compounds that provide health benefits beyond basic nutrition. Moreover, lingonberries are rich in different types polyphenols, a group of dietary components that have been shown to have anti-inflammatory and anti-oxidant properties (102, 103).

Objectives

The aim of this thesis was to evaluate whether the gut microbiota may have influences in AD development and how a healthy gut microbiota may be composed, in order to achieve a balanced gut-brain communication. The studies focus on an important aspect of preventive approach for reducing risk factors of metabolic and neurodegenerative diseases. The specific aims of the included papers are:

- To evaluate the involvement of the gut microbiota on A β pathology in the brain of a mouse model of AD. (**Paper I**)
- To investigate in a neonatal rat model, how dietary disturbances before weaning affect the establishment of the gut microbiota, as well as gut- and blood-brain barrier permeability. (**Paper II**)
- To assess whether a short-term human dietary intervention with a multifunctional diet (MFD) can alter the gut microbiota composition, and to identify bacterial candidates associated with the positive effects of the diet on human hosts. (**Paper III**)
- To test whether dietary fibre components in different fractions of lingonberries induce specific changes of the gut microbiota associated with improved metabolic profile, amending the gut-brain axis communication. (**Paper IV and V**)

Methodology

This section provides an overview and rationale for the methodologies used in the studies included in this thesis, which explored the gut microbiota shifts in relation to neurodegenerative and metabolic disturbances, as well as upon dietary changes in rodents and humans. Study design, dietary factors and specific biomarkers and statistical analyses mostly relevant for the discussion are summarized and reflected over below, while detailed methodologies are described in the corresponding original **paper I-V**, attached in the thesis.

Study design

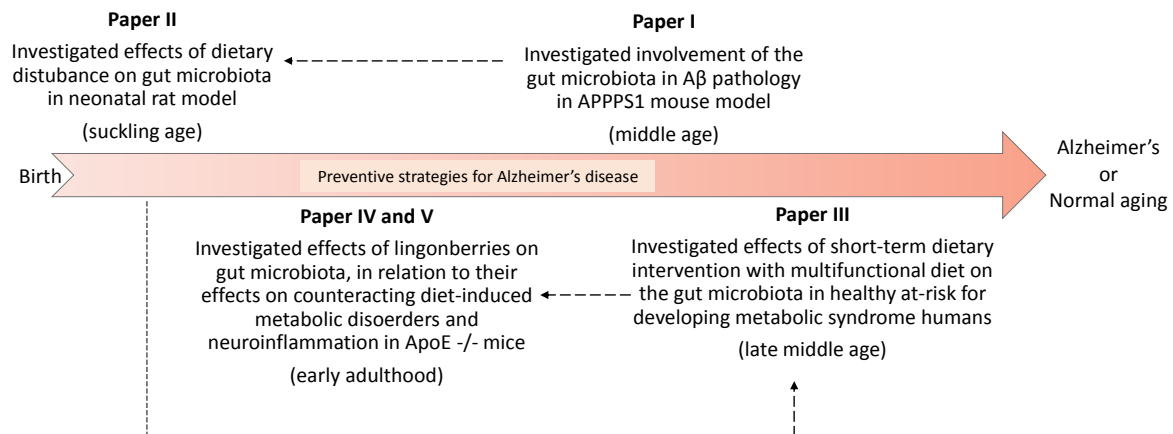


Figure 4 An overview of study design.

Studies included in this thesis explore the shifts in the gut microbiota composition in relation to neurodegenerative and metabolic disturbances, as well as upon dietary changes in rodents and human. The study in paper I was conducted in a transgenic mouse model of cerebral amyloidosis during middle age. Paper II was performed in rats during the suckling period. Paper III moved towards human cohorts at late middle age with increased risk for developing cardiometabolic syndrome whom underwent an 8-week dietary intervention with a multifunctional diet. Paper IV and V evaluated the effects of lingonberries and two isolated fractions of lingonberries, targeting specific changes of the gut microbiota composition, associated with improved diet-induced metabolic and cognitive disorders in ApoE^{-/-} mice.

An overview of the study design of the thesis is shown in **Figure 4**. **Paper I** was carried out in germ-free and conventionally raised APPPS1 mouse model of cerebral amyloidosis to evaluate whether the gut microbiota had a role in A β pathology, one of the most recognised pathologies in AD. Dietary and gut microbial alteration

during the first few years of life may have potential to profoundly affect host development and possess long-term consequences on host health (17, 18). Following this line of research, **paper II** investigated the effects of dietary disturbances early in life on the gut microbiota, as well as gut and blood-brain barrier (BBB). Further, MetS has been associated with cognitive dysfunctions and brain abnormalities (104), while improvements in cognitive functions have been observed with interventions targeting the MetS components (105, 106). Thus, **paper III** investigated the level of gut microbial shifts in healthy late middle-age human adults with increased cardiometabolic risk markers upon a short-term dietary intervention with a multifunctional diet (MFD). **Paper IV** investigated effects of lingonberries (LB) on the gut microbiota in relation to their positive metabolic effects. **Paper V** further evaluated the effects of LB and two separated fractions of LB on the gut microbiota associated with improved metabolic and inflammatory markers, as well as cognitive functions in ApoE $-/-$ on high-fat diets.

Gut microbiota studies in rodent models allow the complex host-microbiota interactions to be assessed, providing a better understanding of possible mechanisms in which different diseases may be triggered. Although absolute comparisons of the rodent and human gut microbiota might be difficult due to different confounding effects ranging from intrinsic differences between species to environmental influences, the processes responsible for the shifts in the gut microbiota upon disturbances are likely relevant (107).

Animal experiments

APP_{PS1} transgenic mouse model of cerebral amyloidosis

Paper I was carried out in an APP_{PS1} transgenic mouse model of cerebral amyloidosis. The study was divided into 3 sets of experiments. Firstly, the gut microbiota of APP_{PS1} transgenic mice was compared to that of wild-type (WT) mice at different ages to investigate the gut microbiota development in relation to the progression of cerebral amyloidosis. The APP_{PS1} mice co-express the KM670/671NL Swedish mutation of human amyloid precursor protein (APP) and the L166P mutation of human presenilin 1 (PS1) under the control of the Thy-1 promoter, showing age-dependent accumulation of parenchymal A β plaques (108). APP_{PS1} mice were generated on a C57BL/6 background. APP_{PS1} mice with both genders were included. Their age-matched WT littermates were used as a control group. The mice were fed rodent chow (Kliba-Nafag PN3437, Kaiseraugst, Switzerland) *ad libitum*. The APP_{PS1} and their WT littermates, referred to as conventionally-raised (CONVR)-APP_{PS1} and CONVR-WT, respectively, were cohoused in grouped cages (n=5 per cage) until analysed. The mice were sacrificed at the age of 1, 3.5 and 8 months (n=5 or 6), to follow the progression of A β

pathology in relation to their gut microbiota. The study was carried out at the Ecole Polytechnique Fédérale de Lausanne Animal Core Specific Pathogen-free Facility, with an approval from the Ethic Veterinary Committee of the Cantons of Vaud and Bern, Switzerland.

In the second set of experiments, germ-free (GF)- APPPS1 mice were generated at the Clean Mouse Facility at the University of Bern, Switzerland. The resulting litters were maintained in flexible film isolators and fed *ad libitum* with autoclaved diet (the same diet as given to conventionally-raised mice) and water. Both genders were included. The GF-APPPS1 mice and their control wild-type littermates (referred to as GF-WT) were cohoused (n=5 per cage).

The third set of experiments, GF mice were recolonized with the gut microbiota from conventionally-raised mice. Caecal contents were pooled from amyloid-depositing (12-month-old) CONVR-APPPS1 mice or CONVR-WT mice, then homogenized in sterile PBS (2 ml per cecum). A volume of 0.2 ml was immediately administered by oral gavage to 4-month-old GF-APPPS1 mice, at day 1 and day 4. The resulting recolonized mice were referred to as COLOAD-APPPS1 for those gavaged with CONVR-APPPS1 microbiota, and COLOWT-APPPS1 for those gavaged with CONVR-WT microbiota. The mice were housed in a conventional environment for 8 weeks under the same conditions and fed with the same diet as their CONVR-APPPS1 counterparts, prior to analysis.

For gut microbiota analysis, fresh faecal pellets were collected from each mouse at given time points and snap frozen in liquid nitrogen, prior to storage at -80 °C until analysed.

Neonatal rat model

Rodents are altricial species, *i.e.*, born with an immature gut barrier which is naturally highly permeable to macromolecules (109). Neonatal rats are relatively larger in size at birth as compared to mice, enabling the possibility to study the impact of dietary disturbance early in life on the gut microbiota development in **paper II**. The study was approved by Malmö-Lund's Ethical Committee of Animal Experiments, according to the European Parliament and Council Directive (2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:539) (Permit Number: M169-14). Sprague-Dawley rats (Taconic Biosciences Inc., Silkeborg, Denmark), of both genders at the age of 14 and 17 days (suckling), as well as 28 days (weaned) were included (n=10). Pups remained with their dam throughout the study. To ensure that the pups consumed only maternal milk, wall extenders were used to prevent access to solid chow. At the age of 14 days, rats were gavaged via stomach tube with one of the two investigated dietary gut provocative agents shown to induce changes in the gut barrier permeability in young animals (more details under dietary factors section). The dissection was performed at 17 days of age, 3

days after treatment. Two untreated additional groups were included in the study with dissection at 14 days (n=10) and 28 days of age (n=10), in order to assess the conditions before the treatments and after weaning. From day 21, the rats in the last group were separated from their dams (weaned) and had free access to water and standard rodent chow (RM1, SDS) until the day of dissection. The caecum was collected for gut microbiota analysis.

Atherosclerosis-prone apolipoprotein E-deficient (ApoE^{-/-}) mice

Apolipoprotein E (apoE) plays a central role in lipoprotein metabolism and is required for the efficient clearance of diet-derived chylomicrons by the liver (110). Hence, ApoE^{-/-} mice are prone to develop atherosclerosis. These mice are also more susceptible to endotoxemia and bacterial infection (111), serving as a useful model to investigate their metabolic disturbances and inflammatory states. The apoE has also received a great deal of attention as a risk factor for AD (112). Studies in **paper IV and V** were conducted in ApoE^{-/-} mice. The mice were fed high-fat diets to induce metabolic disorders during early adulthood. Experimental diets also included whole LB or one of two separated LB fractions (insoluble; insLB or soluble; solLB fraction, more details under dietary factors section) to investigate the effects of LB to counteract the detrimental effects of HF diets.

The ApoE^{-/-} mice (Scanbur AB, Karlslunde, Denmark) started the diets at 8-week-old and were fed the diets for 8 weeks (n=10) in the study of **paper IV**. At the end of the experiment, blood, liver, and epididymal fat pads and heart samples were collected and frozen at -40 °C until further analyses of lipid profiles and atherosclerotic plaque size. Also, caecum was collected for gut microbiota analysis. The study was approved by the local ethical review committee for animal experiments in Lund, Sweden (approval number M-295-12).

Similarly, in **paper V** the mice (Taconic Biosciences Inc., Silkeborg, Denmark) were fed HF diets with inclusion of whole LB (wLB) or insLB for 8 weeks. Since the mice fed solLB showed signs of intolerance to the diet, *i.e.*, rapid weight loss during a 1-week acclimatization, the mice in this group were only fed the diet for 2 weeks, starting at 14-week-old. Thus, the study included 2 sets of experiments. The effects of the experimental diets on lipid profiles and atherosclerosis were investigated. Spatial memory in a T-maze alternation test was conducted 1 week prior to the end of the experiment. Oral glucose tolerance test (OGTT) was conducted the day after the mice had performed the behaviour test. The levels of enteric glial S100B protein was also evaluated in different parts of the small intestine, as a marker for gut inflammatory states (113-115). The S100B was also evaluated in the brain. Brain histological samples were collected for evaluation of synaptic density under transmission electron microscope. Caecum was collected for gut microbiota analysis. The study was approved by the local ethical review committee for animal experiments in Lund, Sweden (approval number M 114-15).

Human dietary interventions

The study in **paper III** was carried out in a healthy cohort with increased risk for developing cardiometabolic complications. The subjects were between 50 and 73 years old of age, both genders, with body mass index (BMI) 25-33 kg/m² and normal fasting plasma glucose value, *i.e.*, ≤ 6.1 mmol/L. The subjects were also non-smokers and without any known medical condition. The volunteers came from towns and villages in Southern Sweden. A total of 52 subjects were recruited and 51 subjects were enrolled in the study. They were assigned to consume a MFD or a control diet (CD) for 8 weeks. Twenty-three subjects completed the multifunctional diet and 24 completed the control diet intervention. Thus, results from 47 completers (12 men and 35 women) were analysed. Cardiometabolic biomarkers were evaluated and reported in Tovar *et al.* (116). Faecal samples were collected on the day before starting the intervention (baseline), and after 8 weeks (endpoint), and were kept at -40 °C until analysed.

Dietary factors

Phytohemagglutinin (PHA) and microbial protease (PT)

Phytohemagglutinin (PHA), a lectin from red kidney beans, or microbial protease (PT), used in dairy and other types of foods was introduced during suckling period as a tool to study the effects of dietary disturbance early in life on the gut microbiota (**paper II**). These dietary gut provocative agents have been shown previously to affect gut barrier permeability to macromolecules in young animals (117-120).

Multifunctional diet (MFD)

A MFD in the study in **paper III** has been developed as a tool to decrease risk factors for cardiometabolic disease in healthy at-risk individuals. This diet has been shown to improve cardiometabolic biomarkers following an 8-week intervention (116). Both MFD and CD were designed in agreement with the Nordic Nutrition Recommendations (121) and supplied 2,500-2,600 kcal/day for men and 2,000-2,100 kcal/day for women, combining foods from plant and animal origins. Both diets incorporated commercial foods available in grocery stores, but the MFD also included prototype products, combining several functional concepts with potential ability to modulate different biomarkers related to the inflammatory tonus and cardiometabolic risk (122) (shown in **Table 2**).

Table 2

Major functional concepts and food ingredients included in the multifunctional diet (MFD)

Functional concept	Food ingredient
Natural antioxidant-rich foods	- Blueberries (<i>Vaccinium myrtillus</i>) - Cinnamon (<i>Cinnamomum cassia</i>)
Omega-3 fatty acids (triglyceride normalizing action)	- Salmon (<i>Salmo salar</i>), brisling (<i>Sprattus sprattus</i>) and mackerel (<i>Scomber scombrus</i>) - Cold-pressed rapeseed oil
Ingredients with prebiotic activity	- Intact barley kernels, whole rye flour and isolated barley fiber for baking a beta-glucan rich bread (2.3% beta-glucan, fresh basis) - Guar gum-containing bread (6.4 % guar gum, fresh basis) - Oat-based fiber drink (0.8% beta-glucan) - Rye/oat breakfast cereal - Oat-based muesli (12% beta-glucan)
Glycemic impact-modulating ingredients	- Whey protein - Vinegar
Blood cholesterol-normalizing ingredients	- Soybeans and soybean protein - Margarine enriched in stanol esters - Dry almonds

None of these active ingredients were included in the CD, except for minor amounts of ω -3 fatty acids. The CD contained dietary fibre from fruits, vegetables and wheat, with little contribution from whole grain products. The total dietary fibre content was 24 g/day for individuals consuming the CD and 62 g/day for the MFD.

Lingonberries (LB) and two separated lingonberry fractions

Freeze-dried lingonberries (*Vaccinium vitis-idaea*) used to prepare diets were provided by Orkla Foods Sverige AB (Eslöv, Sweden) (**paper IV**), or Skogsmat i Uddeholm AB (Karlstad, Sweden) (**Paper V**). The LB were milled into fine powder prior to shipping to Research Diets, Inc. (New Brunswick, USA) for preparation of rodent pellets with inclusion of essential macro- and micronutrients for rodents. Since pronounced effects of LB on the gut microbiota that seemed to be related to the improved diet-induced metabolic disorders was observed in **paper IV**, **paper V** aimed to investigate more specific components of LB that may contribute to the positive health impacts. Hence, insoluble and soluble fractions of the whole LB (wLB) were separated based on water solubility of the dietary fibre, following the protocol from Jakobsdottir *et al.* (123). Briefly, freeze-dried wLB powder was mixed with distilled water to a final volume of 5 L/kg, pH 2, and heated up to 80 °C for 10-15 minutes under constant stirring to solubilize the dietary fibre. The berry solution was then centrifuged and the supernatant (soluble fraction, solLB) was filtered through a sieve. The pellet was collected after centrifugation (insoluble fraction, insLB). Both fractions were frozen at -20 °C prior to freeze-drying. Dietary

fibre content in the wLB and each LB fraction was determined using a gravimetric, enzymatic method (124). The composition, *i.e.*, fat and dietary fibre contents of the HF control diets and whole LB diets in **paper IV and V** were slightly different.

Paper IV included 44% LB, giving 8% dry weight basis (dwb) dietary fibre in a high-fat (HF, 41% kcal) diet, whereas the control HF diet contained 8% (dwb) cellulose. A low-fat (LF, 10% kcal) diet was also included.

Since 8% dietary fibre from LB included in the HF diet in **paper IV** seemed to show pronounced effects on the investigated biomarkers, the dietary fibre contents from wLB and LB fractions in **paper V** were adjusted to minimize the LB content in the diets. **Paper V** included 26% whole LB or 20% insLB, each giving 6% (dwb) dietary fibre from the berries in a HF (38% kcal) diet. The initial formula for solLB diet contained 55% solLB fraction, also giving 6% (dwb) dietary fibre from solLB fraction. However, due to the intolerance as mentioned previously, the diet formula was adjusted to only contain 14% solLB fraction, giving 1.5% (dwb) dietary fibre from the fraction, with an extra 4.5% (dwb) dietary fibre from cellulose.

Biomarker analyses

Gut microbiota analyses

DNA extraction

Genomic DNA was extracted from 100-150 mg of faecal samples, or tissue and content of caecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The protocol from the manufacturer was followed with an addition of bead beating step using sterile glass beads (1 mm). Sterile glass beads (1 mm) were added together with stool lysis buffer to the samples and cell disruption was performed for 2×2 minutes at 25 Hz using a TissueLyser (Qiagen, Hilden, Germany), followed by a heating step at 95 °C for 5 minutes. After lysis, DNA-damaging substances and PCR inhibitors were removed using InhibitEX tablet (provided with the kit) and the DNA was purified on QIAamp Mini spin columns.

16S rRNA gene amplification and sequencing

The V3 and V4 region (**Paper I, II, III and IV**) or V4 region (**Paper V**) of the 16S rRNA gene were amplified using forward and reverse primers, containing Illumina overhang adaptor (**Table 3**) and unique dual indices, according to 16S sequencing library preparation protocol provided by Illumina. Briefly, PCR reactions were carried out in 25- μ L reactions with 0.2 μ M forward and reverse primers, with 12.5 ng template double-stranded DNA and 12.5 μ l of 2× KAPA HiFi HotStart Ready

Mix kit (KAPA Biosystems). Thermal cycling consisted of initial denaturation at 95° C for 3 minutes, followed by 25 cycles of denaturation at 95° C for 30 seconds, annealing at 55° C for 30 seconds, and extension at 72° C for 30 seconds, followed by a final step of 72° C for 5 minutes. The amplicons were purified with Agencourt AMPureXP Kit (Beckman Coulter, Inc.). A second PCR was thereafter performed to attach Illumina adapters and unique dual indexes to each sample (Nextera XT kit, Illumina), followed by a clean-up step with the AmPureXP Kit (Beckman Coulter, Inc.). Paired-end sequencing with a read length of 2×300 bp using Miseq V3 reagent kit (**Paper I, II, III, IV**) or with a read length of 2×250 bp using Miseq V2 reagent kit (**Paper V**) were carried out on a Miseq Instrument (Illumina Inc., San Diego, USA).

Table 3

Primer sequences for amplification of 16S rRNA genes

Primer name (the 16S gene region)	Sequence (5'-3') (The <u>Illumina overhang adaptor sequences</u> are underlined)
341f Forward (V3 - V4)	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> CCTACGGGNGGCWGCAG (125)
805r Reverse(V3 - V4)	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> GACTACHVGGGTATCTAATCC (125)
515f Forward(V4)	<u>TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG</u> GTGCCAGCMGCCGCGGTAA (126)
806r Reverse (V4)	<u>GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG</u> GGACTACHVGGGTWTCTAAT (126)

Sequence analysis

Sequences were analysed with the free software package Quantitative Insights into Microbial Ecology (QIIME) which allows analysis of high-throughput community sequencing data (127). Sequences with a shorter length than 200 nucleotides, contained ambiguous bases, primer mismatches or homo polymer runs in excess of six bases were removed. Forward and reverse reads were joined using Fastqjoin. Similar sequences were binned into operational taxonomic units (OTUs) using UCLUST, with a minimum pairwise identity of 97%, using the closed-reference OTU picking method in QIIME. The most abundant sequence in each OTU was chosen to represent its OTU. Representative OTUs were chosen based on abundance and aligned using PyNAST (a python-based implementation of NAST in QIIME) and taxonomy was assigned using the Greengenes database (version 13.8) (128) and the RDP classifier (129).

Permeability markers

Passage of albumins (approximately 70 kDa) was evaluated in **paper II**, to assess the permeability of gut-blod, and BBB to macromolecules in neonatal rats. On the day of sacrifice, the rats were separated from the dam one hour prior to gavage of human serum albumin (HSA, 1.25 mg per g body weight) as a marker for gut barrier permeability and intraperitoneal injection of bovine serum albumin (BSA, 0.5 mg per g body weight) as a marker for BBB permeability. Three hours after marker administration, the rats were anesthetized with Isoflurane (Abbott, Chicago, USA) and immediately exsanguinated by collecting blood samples via direct heart puncture before the decapitation. Brain samples were snap-frozen for further assessing the quantity of BSA that had passed from the bloodstream to the brain tissue.

LPS-binding protein (LBP) is increasingly being used as a relevant marker for leakage of LPS (130). Since quantification of LPS in biological samples is highly dependent on the sensitivity of the analytical method, plasma LPB levels in the rat pups were evaluated using enzyme-linked immunosorbent assay (ELISA) kit for a wide variety of species (Hycult Biotech, The Netherlands). The procedures were performed following the protocol from the manufacturer.

Behaviour test

T-maze spontaneous alternation

Spontaneous alternation reflects the operation of spatial working or short-term memory (131). The protocol from Deacon & Rawlins, 2006 (132) using a T-maze was followed with some modifications to investigate the cognitive functions of mice consuming LB and LB fractions in **paper V**. A T-maze with a start alley dimension and two goal arms of 30 × 10 cm (length × width) each was used (**Figure 5**). Guillotine doors cut to fit the maze were used to separate the start alley, and the two arms. Fresh woodchip bedding was put in the bottom of the maze and changed between each mouse. A mouse was put at the start alley for 10 seconds before the guillotine door was opened to allow the mouse to spontaneously select one of the two arms, with no rewarding system. Once the mouse had chosen the arm, the guillotine door to the chosen door was shut, keeping the mouse in the chosen arm for 30 seconds. The second trial was performed within 1 minute following the first. Alternation, *i.e.*, visiting the arm not chosen before was expected in the second trial. Three sets of trial in a day, during morning, afternoon and evening hours, were performed for each mouse. Percentage of alternation was calculated per mouse from

the three sets of trial. The trials were recorded, and the videos were analysed using a tracking software Ethovision XT 13.0 (Noldus Information Technology b.v., Wageningen, The Netherlands). Time taken for a mouse to move from the start alley, once the guillotine door was opened, until the middle part of the body had crossed the guillotine door to one of the arms, was analysed as time taken for making a decision.



Figure 5 T-maze spontaneous alternation system.

A T-maze with a start alley dimension and two goal arms of 30 × 10 cm (length × width) each. A mouse was put in a start alley before being allowed to choose one of the two arms. The mouse was kept in the chosen arm for 30 seconds before being put back to the start alley for 1 minute, followed by another session, where the mouse again was allowed to choose one of the arms.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6 or 7 (GraphPad Software, Inc., California, USA, www.graphpad.com). Data from biomarker analyses, and behavioural test were analysed using one-way analysis of variance (ANOVA), and subsequent Holm-Sidak's or Dunnett's test for multiple comparisons. Correlations between the investigated biomarkers and gut bacterial genera were analysed using SIMCA-14 software (Umetrics, Umeå, Sweden). Partial least squares (PLS) or Orthogonal PLS (OPLS) plot was used to illustrate correlations between different biomarkers and the gut microbiota. Pearson's correlation analysis was then used to analyse the significance of the correlations. The threshold for statistical significance was set at $p < 0.05$.

Results and Discussion

Overall findings

The results from **paper I** suggested an important role of gut microbiota in the development of cerebral β -amyloidosis, one of the most recognized pathologies in neurodegenerative disease, such as AD. The gut microbiota composition of APPPS1 transgenic mice, expressing increased cerebral A β pathology with age, differed from that seen in WT mice. Absence of the gut microbiota also resulted in reductions of cerebral A β pathology and neuroinflammation in the transgenic mice. Additionally, colonization of the GF transgenic mice with the gut microbiota from transgenic or WT mice restored A β deposition, to different levels, further indicating the involvement of specific gut microbial taxa in A β pathology. Increase or decrease of specific gut bacteria in transgenic mice may cause a disturbed gut barrier with increased influx of inflammatory components that also seemed to be positively associated with A β pathology. Controlling for a balanced gut microbial community that promotes proper gut and BBB functions, with reduced systemic inflammation, may be a new window for preventing the development of such neurodegenerative disorders. **Paper II** showed that dietary disturbances before weaning period affected the normal establishment of the gut microbiota, as well as gut barrier and BBB in neonatal rats. Although the investigated dietary components were found to cease gut permeability to macromolecules, they however increased plasma levels of LBP, reflecting leakage of LPS, bacterial endotoxin known to cause low-grade systemic inflammation.

Dietary interventions have been regarded as an effective way to modulate the gut microbiota. However, and surprisingly, the results from **paper III** showed that a short-term (8-week) dietary intervention with MFD in adults with already established gut microbial community seemed to be insufficient to produce a broad switch in the gut microbiota composition. Human dietary intervention studies carry several confounding factors that may contribute to the inter-individual variation, thus lowering the chance to observe diet-induced changes in the gut microbiota. Yet specific gut microbial genera associated with improved cardiometabolic biomarkers could be identified. **Paper IV** revealed that including LB in a HF diet reduced atherosclerosis in ApoE^{-/-} mice in association with changes in specific gut microbial taxa and improved lipid profile. **Paper V** further investigated that LB and two

separated fractions of LB included in HF diets induced gut microbiota alteration and improved different levels of diet-induced metabolic disturbances in ApoE^{-/-} mice. The mice also showed lowered levels of gut- and neuroinflammation, and increased hippocampal synaptic density.

Impact of gut microbiota on cerebral A β pathology in APPPS1 mice (Paper I)

Results from **paper I** (summarized in **Figure 6**) showed marked differences in the gut microbiota of CONVR-APPPS1 and CONVR-WT at 8-month-old, when remarkable differences in the levels of cerebral A β pathology were also observed. Absence of the gut microbiota was sufficient to significantly decrease the amount of cerebral A β plaques, reflecting the influences of the gut microbiota in A β pathology. In addition, recolonizing GF-APPPS1 mice with gut microbiota from CONVR-WT or CONVR-APPPS1 resulted in increased A β pathology to different levels, further indicating involvement of specific groups of bacteria in the A β pathology.

Gut microbiota composition of APPPS1 transgenic mice differed from WT mice

In the first set of the experiment, a minor gut microbiota shift was observed already in 1-month-old mice which gradually increased with age, following dynamics that showed increased Bacteroidetes and decreased Firmicutes phyla in CONVR-APPPS1 mice. The opposite dynamics in CONVR-WT mice were seen with age. At the age of 8 months, CONVR-APPPS1 mice displayed significant changes in most phyla, including reductions in Firmicutes, Verrucomicrobia, Proteobacteria and Actinobacteria, and increases in Bacteroidetes and Tenericutes, as compared to age-matched CONVR-WT mice.

At genus level, the 8-month-old CONVR-APPPS1 showed a significant decrease in relative abundance of *Akkermansia* and *Allobaculum*, and a significant increase in unclassified genera in the family *Rikenellaceae* and *S24-7*, as compared to CONVR-WT mice. *Akkermansia muciniphila* has been shown to play a crucial role in the mutualism between the host and gut microbiota, controlling gut barrier function and other physiological and homeostatic functions during obesity and type 2 diabetes (133). Similarly, *Allobaculum* has also been shown to increase with LF feeding in mice (134), and with prebiotics treatment in rats fed HF diet, associated with its capacity to prevent obesity and improve insulin resistance (135). On the other hand,

the family *Rikenellaceae* has been shown to increase in HF feeding (136), and enrichment of *Alistipes*, a member within the *Rikenellaceae*, has also been associated with type 2 diabetes in humans (137). The family *S24-7* have been linked to gut inflammation and gut immunity alterations, enriched in non-obese diabetic (NOD) mice, while probiotic administration counter-regulated autoimmunity and prevent type 1 diabetes, together with decreased *S24-7* abundance (138).

The WT and transgenic mice were cohoused (n=5 per cage) throughout the experiment to minimize cage effects and were given the same standard rodent chows. Despite this, the mice displayed distinct gut microbial profiles, reflecting that the genotype itself has a strong influence on the gut microbiota. Host genetics may have an impact on the availability and/or the level of host-derived glycans, which likely determine bacterial species capable of colonizing the mucosal surface.

Absence of the gut microbiota resulted in reduction of cerebral A β pathology and neuroinflammation

GF-APP_{PS1} mice showed significant reductions in both compact A β plaques and biochemical levels of A β , as compared to their age-matched CONVR-APP_{PS1} mice. Histopathological assessment of compact cerebral A β loads showed 77% and 57% reductions in A β loads in the brain of GF-APP_{PS1} mice at 3.5- and 8-month-old, respectively, as compared to their age-matched CONVR-APP_{PS1} mice. Interestingly, similar levels in cerebral APP levels were observed in 3.5-month-old GF-APP_{PS1} mice, and surprisingly, a 24% increase was observed in 8-month-old animals. This observation implies that the decrease of A β pathology in GF-APP_{PS1} mice is not due to lower APP expression, but rather results from a mechanism occurring downstream of APP cleavage or A β clearance. Significant reduction in plasma A β levels were observed in 3.5-month-old GF-APP_{PS1} mice, and no difference was observed in 8-month-old mice, suggesting no increased clearance of A β in blood. Thus, it is likely that although APP was produced at higher levels in GF animals, small amounts were cleaved into A β . Although the precise physiological function of APP is not known, in most studies APP overexpression showed a positive effect on neuron cell health and growth, while sequential APP proteolysis that generates the neurotoxic A β peptide is suggested to be the crucial step in the development of AD (139).

Further, the reduction of cerebral A β load in GF-APP_{PS1} mice was accompanied by an overall decrease in cortical neuroinflammation. As compared to their age-matched CONVR-APP_{PS1} mice, GF-APP_{PS1} animals exhibited a significant reduction in Iba-1a, a microglia marker at both 3.5 and 8 months. Significant reductions in the pro-inflammatory cytokines IFN- γ , IL-2 and IL-5 at 3.5 months,

and IL-1 β at 8 months were also observed in brain homogenates of GF-APP PS1 animals. These results are in line with previous findings that GF mice usually express immature microglia and reduced pro-inflammatory cytokine production (140). Yet the concurrent decreases in the levels of neuroinflammation and cerebral A β pathology, despite no reduction in APP production in GF-APP PS1 mice, suggest that the level of neuroinflammation may be a factor determining the level or pattern of APP proteolysis.

Colonization of GF-APP PS1 mice with gut microbiota restored A β deposition

To further assess the significant influence of the gut microbiota in the development of cerebral β -amyloidosis, GF-APP PS1 mice were colonized with the gut microbiota from CONVR-APP PS1 or CONVR-WT, referred to as COLOAD-APP PS1 and COLOWT-APP PS1 , respectively. After 8 weeks of colonization significant increases in cerebral A β levels were observed in both COLOAD-APP PS1 and COLOWT-APP PS1 mice, as compared to GF-APP PS1 mice. However, COLOWT-APP PS1 microbiota was surprisingly less effective in increasing cerebral A β levels. APP levels remained unaffected between the groups. These results suggested that pathogenicity of microbiota harvested from CONVR-WT mice was relatively less pronounced than from CONVR-APP PS1 mice, further underlying the involvement of specific gut microbial taxa in A β pathology.

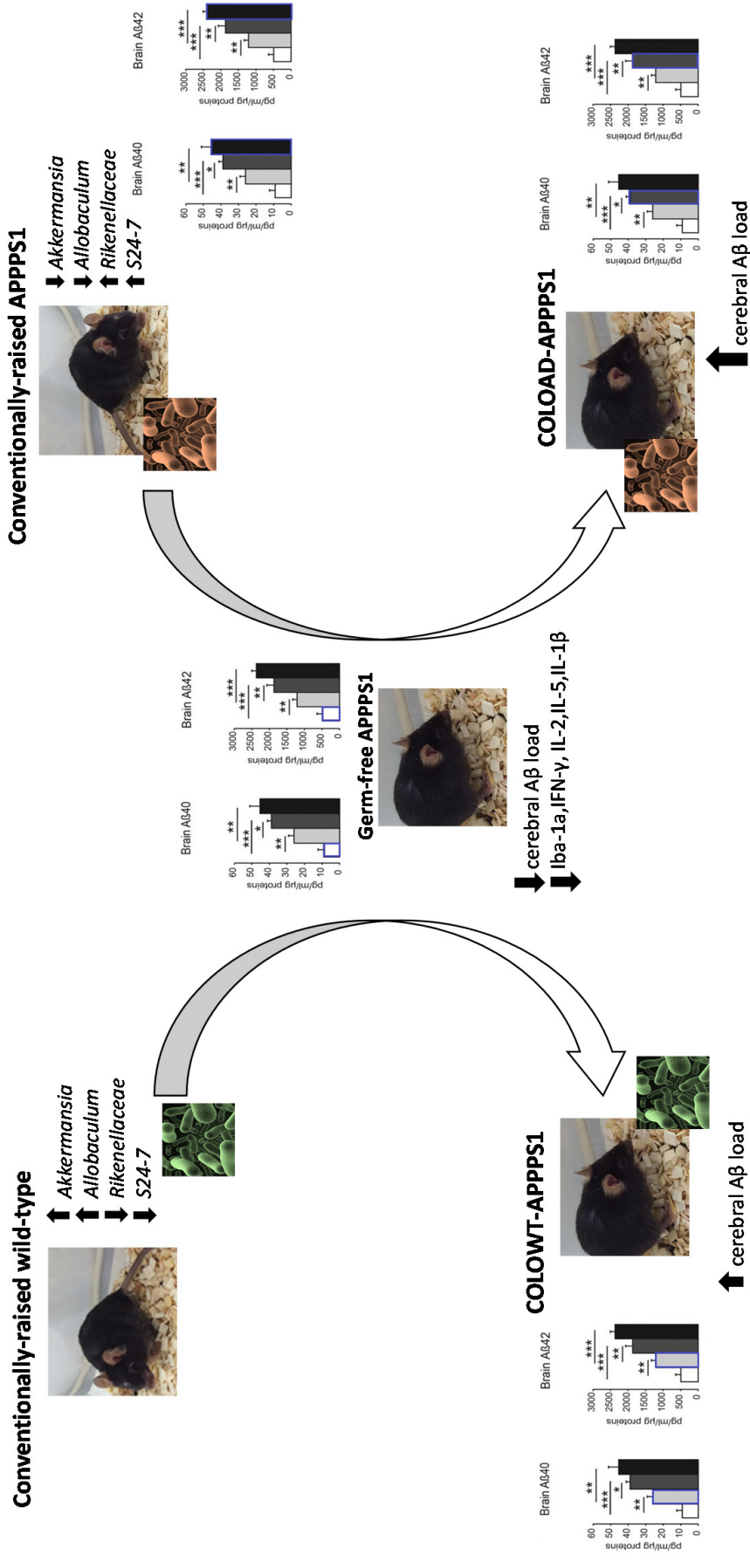
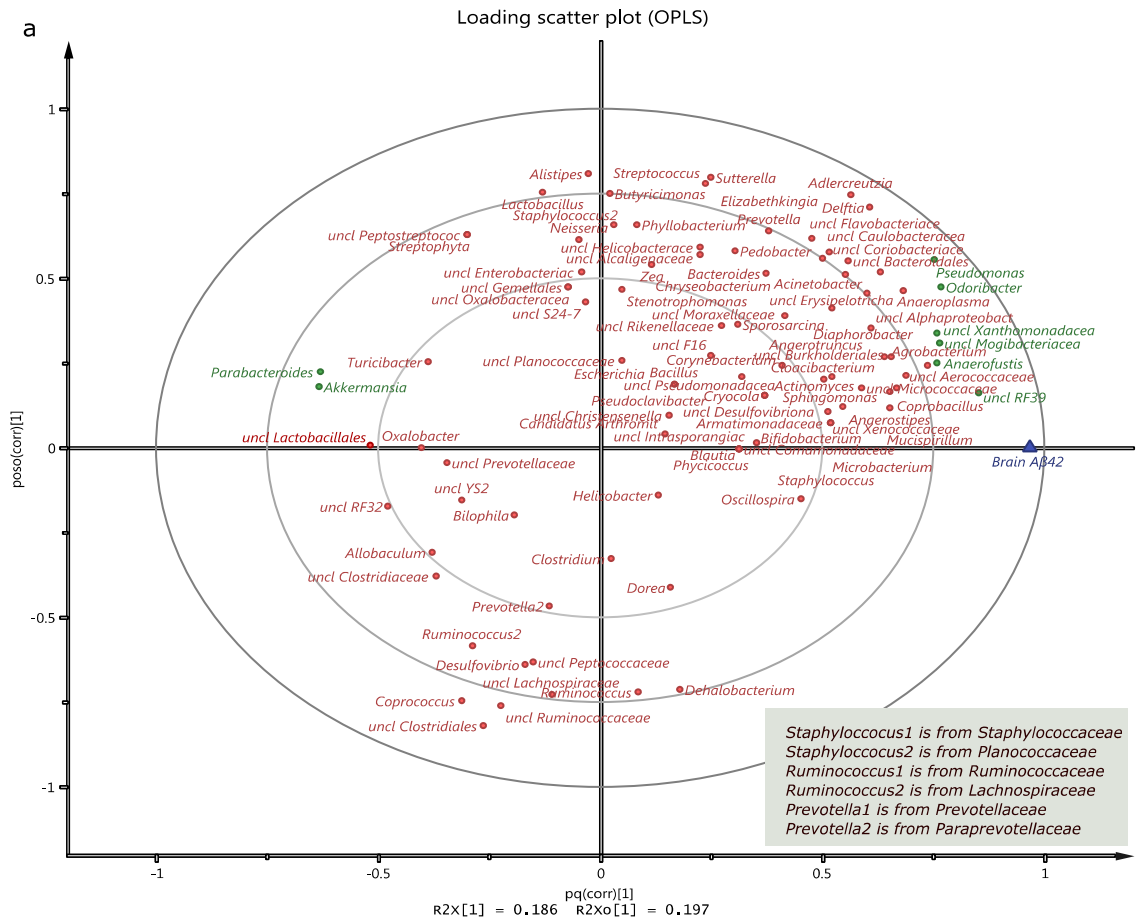


Figure 6 Overview of impact of gut microbiota on cerebral Aβ pathology and neuroinflammatory markers observed in paper I. Conventionally-raised transgenic APPS1 (CONVR-APPS1) mice showed different gut microbial profiles as compared to conventionally-raised wild-type (CONVR-WT) mice at the same age. APPS1 mice raised in germ-free conditions (GF-APPS1) displayed reductions in cerebral Aβ loads and neuroinflammatory cytokines. Colonization of GF-APPS1 mice with gut microbiota from CONVR-APPS1 or CONVR-WT, referred to as COLOAD-APPS1 and COLOWT-APPS1 mice, respectively, resulted in increased cerebral Aβ loads to a different level. Bacteria illustration was obtained and modified from <https://www.flickr.com/>, "Bacteria" by AJC1, licensed under CC BY 2.0.

Correlation between the gut microbiota and cerebral A β pathology

Eight bacterial genera (**Figure 7**) were identified to be associated positively or negatively with the amount of cerebral A β_{42} , the most abundant form of A β that seemed to represent the amount of compact A β plaques ($p < 0.05$, after correction for multiple comparisons). Most of the bacteria positively correlated with cerebral A β_{42} levels were Gram-negative bacteria, whose outer membranes contain LPS endotoxin, including *Odoribacter* from phylum Bacteroidetes, and *Pseudomonas* and *Xanthomonas*, members in Gammaproteobacteria. Further, an unclassified genus from *Mogiobacteriaceae* has been found to be associated with ulcerative proctitis, a chronic inflammatory bowel disease, in human subjects (141). On the other hand, *Akkermansia*, which showed negative correlation with A β pathology, has been shown to have an important role in gut barrier integrity as previously discussed, and thus control metabolic endotoxemia (133). *Parabacteroides* may also serve as another protective candidate, as it also showed negative correlation with A β pathology. A major species from this genus, *Parabacteroides distasonis*, has been shown to induce the anti-inflammatory cytokine IL-10 and suppressing the secretion of pro-inflammatory cytokines IL-17, IL-6, and IFN- γ , reducing the severity of intestinal inflammation in mouse models of acute and chronic colitis (142, 143). It is to be noted that these correlations do not imply causation. Whether or not these bacteria indeed have causative or protective roles in AD has to be investigated further.



b

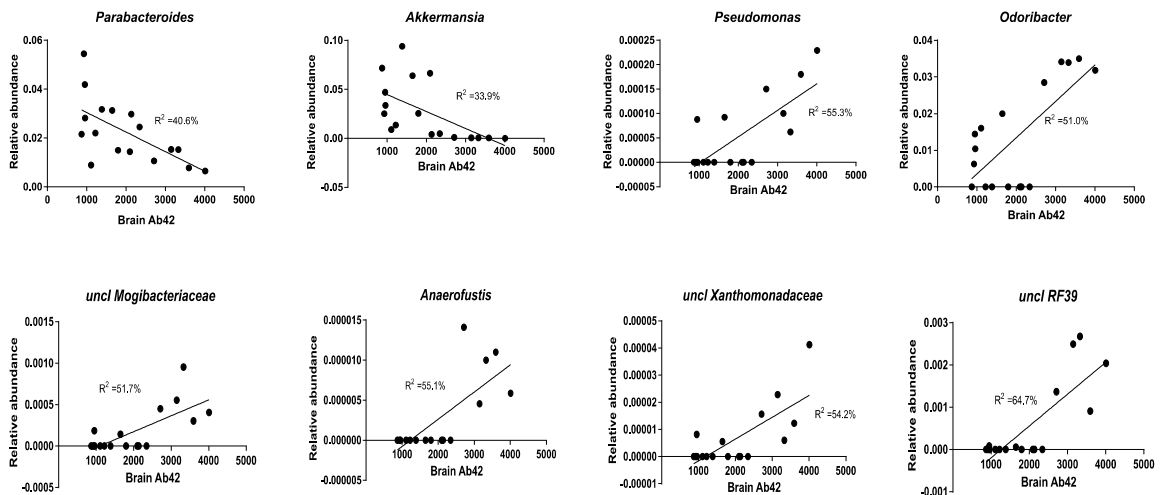


Figure 7 Correlations between the gut microbiota and cerebral $A\beta_{42}$ levels in 8 month-old CONVR-APPPS1 (n=6), COLOAD-APPPS1 (n=6) and COLOWT-APPPS1 (n=6) mice.

(a) Orthogonal partial least squares (OPLS) scatter plot giving an overview of the correlations. Variables situated closed to each other were positively correlated and variables situated opposite each other were negatively correlated. Eight bacterial genera (in green) were positively correlated with $A\beta_{42}$ levels ($p < 0.05$, Pearson's correlation with adjusted p-values for multiple comparisons using Benjamini-Hochberg procedure). (b) Scatter plot of the significant correlations with determination coefficient (R^2) indicating how well the data fits the model.

Possible mechanisms mediating the involvement of gut microbiota in A β pathology

Specific groups of gut microbiota seem to play an important role in the levels of neuroinflammation and cerebral A β pathology. It has been shown previously that A β not only has pathogenicity in AD, but also antimicrobial properties. The physiochemical and biological properties of A β has been shown to be similar to other antimicrobial peptides (AMPs), suggesting its possible function in the innate immune system (144). In addition, a recent study has shown that A β expression inhibits bacterial infection in the brain of 5XFAD transgenic mice, another mouse model of AD, as well as in the nematode *Caenorhabditis elegans*, and in cultured mammalian cell models (145). Furthermore, in the same study, APP knockout (APP-KO) mice, having low A β expression, also showed a trend toward attenuated survival after bacterial infection (145). One may hypothesize that a shift in the gut microbiota toward a community associated with disturbed gut barrier functions, such as reduction in *Akkermansia* seen in APPPS1 mice, may lead to increased influx of inflammatory components and systemic inflammation. Deteriorated function and structure of the BBB occurs as a part of ageing, or when the BBB is exposed to chronically elevated pro-inflammatory cytokines (146), rendering the brain more vulnerable to such inflammatory tonus. Although these findings suggest that increased accumulation of A β may serve as a mechanism to protect the body and the brain from bacterial infection or inflammation, over accumulation of these A β peptides can still be the cause of nerve cell toxicity in AD, due to the nature of A β to form plaques that penetrate the vesicular membrane (147). Therefore, the hypothesis that A β plaques are involved in AD development may not necessarily be rejected. Nevertheless, if it is true that A β production is a mechanism to protect the brain from inflammatory tonus, targeting A β clearance may not unravel the disease progression. Controlling for healthy gut barrier and BBB functions, by maintaining a balanced gut microbial community may be a way to minimize systemic inflammation and accumulation of A β peptides.

Dietary disturbances before weaning affect the establishment of the gut microbiota, as well as gut and blood-brain barriers in neonatal rats (Paper II)

Specific dietary components, including PHA and PT have been shown to affect the permeability to macromolecules through the gut barrier in young animals during the suckling period (117-119, 148-150). These dietary components were used as a tool to study the effects of dietary disturbances before weaning period on the regulation

of gut barrier and BBB, in relation to the gut microbiota. Neonatal rats, an altricial species born with an immature gut barrier to macromolecules (109), provide a suitable model for studying links between gut and BBB permeability. In **paper II**, single oral gavage with PHA or PT in suckling rats reduced gut barrier permeability to macromolecules. However, this led to significant shifts in the gut microbiota that seemed to be associated with increased plasma LBP levels, reflecting LPS leakages and low-grade systemic inflammation. Altered BBB permeability observed during brain development may be a response to endotoxemia and/or nutrient uptakes.

Single luminal exposure to PHA and PT decreased gut barrier permeability and altered gut microbiota, associated with low-grade systemic inflammation

High gut permeability to macromolecules (albumin, approximately 70 kDa) was observed in suckling rats at the age of 14 and 17 days, while ceased in weaned rats. The higher permeability to macromolecules of the gut barrier during the suckling period, in one way, allows optimal antigens and antibodies from breast milk to be transferred into infants. On the other hand, harmful antigens can also pass through and evoke adverse immune responses that could result in the development of medical conditions that newborns are especially susceptible to, such as necrotizing enterocolitis, toxigenic diarrhoea, and intestinal allergy (151). Accelerated epithelial growth has also been observed in human infants at weaning, when breast milk feeding is replaced with an increasing range of ingested nutrients (152). Such epithelial change is suggested to be intrinsically programmed since delayed weaning does not abrogate intestinal growth and maturation (153). Yet precocious weaning has been shown to be effective in modulating intestinal growth in young rats (154). Our study showed that a single luminal exposure to PHA or PT before weaning period was effective to cease the passage of albumin via gut-blood barrier. However, it also altered the gut microbiota toward a community that seemed to be associated with increased LPS translocation.

Although gut permeability to macromolecules of suckling rats treated with PHA or PT decreased to similar level as seen in weaned 28-day-old rats, their gut microbiota did not likewise the weaned rats. Proteobacteria was found to be the most dominant phylum in the young rats of 14-days with immature gut barrier. This has previously been observed in neonates along with a low diversity and high abundance of Actinobacteria (82). Milk oligosaccharides were the only substrates available for the gut microbiota during suckling period, which is consistent with the finding that the suckling rats at the age of 14 and 17 days showed similar SCFA profile, independently of the PHA or PT treatment. The availability of solid food after day 21 provided the microbiota with different substrates, causing important changes in

the gut microbiota composition. The Proteobacteria decreased, higher diversity was observed, and Firmicutes and Bacteroidetes became dominant in 28-day-old rats, establishing an adult-like microbiota. Also, a significant increase in caecal butyric acid was observed in 28-day-old rats. Butyric acid has been shown to improve gut barrier integrity (53) by being the most important substrate for gut epithelial cells. Instead of such gut microbiota changes, rats treated with PHA or PT showed a substantial increase in the relative abundance of Bacteroidetes to up to more than 75%. Particularly, *Bacteroides* and *Parabacteroides* were the most abundant genera, which mostly comprise Gram-negative bacteria. Members in *Bacteroidales* family can confer either positive or negative impacts on the host, depending upon their genetic content (155). However, the increase in Bacteroidetes in both PHA and PT treated rats was associated with increased plasma levels of LBP. Despite the decrease in gut barrier permeability to macromolecules, high LPS translocation seemed to occur, likely via paracellular leakage (156), causing endotoxemia that might induce bacteria-linked systemic inflammation (157). Stratification of gut epithelium at different states is shown in **Figure 8**.

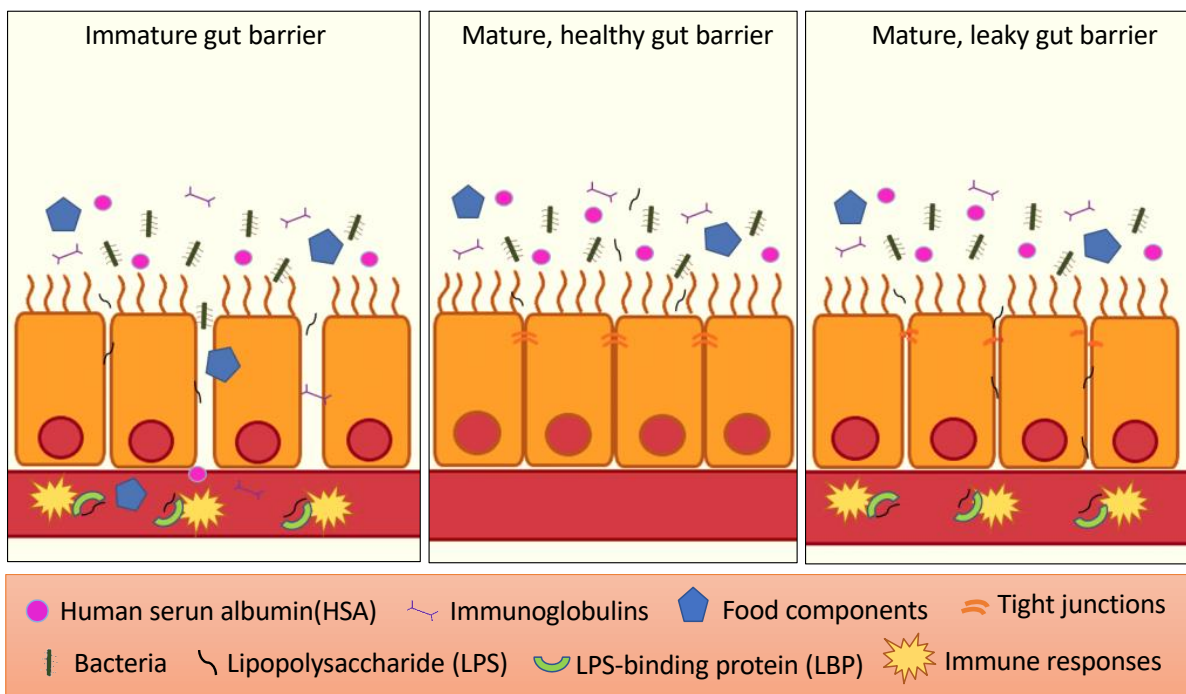


Figure 8 Stratification of gut epithelium with an immature (left), mature and healthy (middle), and mature but leaky (right) status.

Gavage of human serum albumin (approximately 70 kDa), as a marker for gut permeability to macromolecules, can be detected in the circulation of suckling rats, reflecting non-selective passage of luminal contents via both transcellular and paracellular pathways. Proper timing of solid feed at weaning results in a normal gut barrier maturation, where tight junctions are fully developed and passage of molecules becomes more selective. A single luminal exposure to phytohemagglutinin (PHA) or protease (PT) induced precocious gut maturation to macromolecules, yet leakages of LPS seemed to occur via paracellular pathway due to tight junction dysfunction. High plasma levels of LPS-binding protein (LBP) were detected in rats with immature gut barrier and those treated with PHA or PT.

Altered BBB permeability may be a response to bacterial-linked systemic inflammation and SCFA utilization

It has been documented that the BBB is well-established during early development, already in embryos and fetuses (158), but primed to respond to inflammatory stimuli (159). A previous pre-clinical study has revealed increased BBB permeability in GF mice, while recolonization with pathogen-free gut microbiota decreased BBB permeability and up-regulated expression of tight junction proteins, including zonula occludens-1, occludin, and claudin-5, suggesting the role of gut microbiota in modulation of BBB permeability (160). Negative association between LBP and passage of BSA, intraperitoneally injected as a marker of BBB change to macromolecules, from blood into the brain suggested a possible control between BBB and the inflammatory tonus. The increased BBB permeability in 28-day-old rats may suggest that BBB permeability is flexible during the brain development, for the sake of nutrient uptake, such as acetic acid which seemed to occur in a low systemic inflammation environment. The negative association between plasma LBP and BBB permeability did not seem to fully apply to the 14-day-old rats, which could be due to a not fully developed immune response to inflammatory stimuli in very young rats (161). The possibility that vascular spaces in the brain may have interfered with the BBB measurement cannot be completely excluded and our results obtained on the BBB will need further validation in future studies.

This study suggested links between gut barrier and BBB permeability, in response to changes in the gut microbiota. Dietary disturbances early in life, including early exposure to solid food, may deserve careful consideration since disruption of a normal establishment of the gut microbiota early in life may also have potential long-term adverse effects on the host.

An 8-week dietary intervention with MFD in healthy overweight adults only induced minor shifts in the gut microbiota (Paper III)

Low-grade systemic inflammatory tonus as a result of gut epithelial dysfunction has been observed in individuals with the MetS, and these are associated with increased risk of dementia (19). A MFD, targeting subclinical inflammation has been developed as a tool to decrease risk factors for cardiometabolic disease in healthy at-risk adult. An 8-week intervention with MFD has been shown to improve cardiometabolic biomarkers and systemic inflammation (116). The diet contains soybeans, oily fish and plant stanols, as well as several dietary fibre sources from rye, barley, oats and berries that could serve as substrates for the gut microbiota.

Thus, the effect of an 8-week intervention with MFD on the gut microbiota composition and its association with cardiometabolic biomarkers were evaluated in **paper III**. It was anticipated that the MFD would cause a distinct shift in the gut microbiota, given the inclusion of a variety of dietary fibre sources. However, the results showed that MFD did not significantly alter the gut microbiota composition at phylum or genus taxonomic levels. Only a minor shift at species level was observed. Yet positive or negative associations between specific bacterial genera and cardiometabolic biomarkers could be identified.

In a similar study in healthy at-risk adults there was also only minor shifts in the gut observed after an 8-week intervention with whole-grain diet (162). The period of 8-week intervention may have been insufficient to induce broad changes in the gut microbiota towards one direction in human adults whom already have an established community of the gut microbiota. Although a sudden switch from diet low in dietary fibre to one high in dietary fibre has been shown to elicit rapid changes in the gut microbiota within 24 hours, the change does not necessarily result in a permanent microbial shift (86). Short-term dietary changes tend to induce modest transient changes at best (163), or only as long as the substrates are available, before shifting back to the existing niches. Gut transit time and timing of gut microbiota sampling may also affect the level of the gut microbiota changes to be detected. Further, each single dietary component included in MFD may exert very different effects on the individual gut microbiota, resulting in less defined changes in overall gut microbiota, as those seen when the effect of only a single dietary component was investigated. Furthermore, the voluntary cohorts that participated in dietary interventions are usually of high nutrition awareness. A 32% reduction in breath hydrogen, *i.e.*, one of the products from bacterial fermentation was observed in participants consuming the control diet (CD). Although dietary fibre content was higher in the MFD (62 g/day), the dietary fibre content in CD (24 g/day) almost reaches the level of dietary fibre intake recommendation of 25-35 g/day in adults (121). Assigning the same low dietary fibre diet for 1-2 weeks prior to the dietary intervention may be of benefit to normalize for the baseline gut microbiota and increase the chance to detect bigger differences in the gut microbiota, as a result of the MFD diet.

Apart from the positive health outcomes of the MFD that may be derived from diet *per se*, the health benefits ascribed to dietary fibre are usually mediated by gut microbial fermentation and the metabolites that are produced (47). Although only subtle changes in the gut microbiota composition were observed after MFD, probably due to the multiple confounding factors that human dietary interventions carry as discussed above, the correlation analyses identified gut microbial genera that are associated with the improved cardiovascular risk markers.

Associations between gut microbial genera and cardiometabolic risk markers

In PLS correlation analysis, a number of bacterial genera associated with the investigated biomarkers of cardiometabolic risk was identified. Of note, *Treponema* correlated positively with blood pressure. This bacterium has been implicated in periodontal disease, a known risk factor for atherosclerosis, and its abundance in the oral cavity has, interestingly, also recently been associated with obesity in humans (164). Thus, the present study suggests that the gut abundance of *Treponema* may be linked to cardiometabolic risk factors such as increased blood pressure. In contrast, *Faecalibacterium* showed a negative association with blood pressure, which may reflect its proven anti-inflammatory capacities (165). Also, it has been reported that patients with MetS show a reduction in *Faecalibacterium prausnitzii*, a butyrate producer, compared to healthy individuals, which was restored upon a dietary intervention with a Mediterranean-type diet (166). In addition, *Ruminococcus* and *CF231* were associated with increased blood HDL-cholesterol levels, while *Bilophila* appeared to be associated with less favourable blood lipid profiles. *Bilophila wadsworthia* has been implicated in colitis in mice and it increases after high intake of saturated milk-fat through alterations in bile acid profiles (167).

This study suggests that although diet is one of the most influential factors modulating the composition of the gut microbiota, short-term dietary changes may be insufficient to induce broad and permanent microbial alteration in adults, whose gut microbial niches have already been established. Yet the health benefits of the dietary fibre are likely to be mediated by the gut microbiota and the metabolites that are produced. Identification of dietary components capable of targeting specific gut microbiota changes may be of benefit in improving future human dietary interventions and further highlight the positive health effects of the diets on human hosts.

Lingonberries and lingonberry fractions as potential functional diet targeting specific gut microbiota for prevention of diet-induced metabolic disorders and neuroinflammation (Paper IV and V)

Human dietary intervention studies have been showing inconclusive results regarding dietary impact on the gut microbiota, which is mainly due to the well-recognized inter-individual variations. Yet accumulating data has been pointing

towards the gut microbiota as a mediated factor of the impact of dietary fibre on host health (163). LB have previously been shown to prevent diet-induced obesity, improve insulin resistance and reduce inflammation in both animals and humans (168-170). LB are high in dietary fibre and several phenolic compounds (171). The results in **paper IV** showed that LB induced specific changes in the gut microbiota that seem to be related with improved lipid metabolism and reduced atherosclerosis in ApoE^{-/-} mice on a high fat diet. **Paper V** further investigated the specific effect of LB and two separated fractions of LB on the gut microbiota in relation to their potential effects on brain function and behaviour.

Lingonberries reduced atherosclerosis in ApoE^{-/-} mice in association with specific changes in the gut microbiota and improved lipid metabolism (Paper IV)

The results in **paper IV** showed that including LB in a HF diet reduced gut microbial diversity in mice, with specific increase in Bacteroidetes at phylum level. The linear discriminant analysis (LDA) effect size (LEfSe) method also detected an increase in *Akkermansia* and its Verrucomicrobia phylum. Although reduced microbial diversity has been shown to be a common feature of dysbiosis, and overgrowth of Gram-negative Bacteroidetes may play a role in chronic diseases such as inflammatory bowel disease, the increase in *Akkermansia* following consumption of LB seemed to be important in counteracting negative effects of HF feedings.

A recent study revealed the effect of LB in reducing plasma concentration of the inflammatory marker serum amyloid A (SAA) and the endotoxemia marker LBP in mice (170). **Paper IV** showed that LB significantly improved the lipid profiles, including significantly reduced plasma triglycerides and a tendency to reduced plasma total cholesterol levels. A concurrent reduction in atherosclerotic plaque size was also observed in mice fed HF diet with inclusion of LB as compared to HF control group. In fact, LB also showed a tendency ($p < 0.1$) to increase the relative gene expression of occludin in brain tissues of mice fed HF diet with inclusion of LB, to a level similar to that seen in mice fed LF diet (**Figure 9**, unpublished data), reflecting improved tight-junction functions of the BBB (172). Also, these mice had significantly increased level of free-fatty acid receptor-2 (Ffar2) gene expression in the brain (**Figure 9**, unpublished data). The Ffar2 is usually activated by SCFAs (173), thereby the increased level of Ffar2 gene expression may suggest an increased SCFA accumulation in the brain of mice fed LB. SCFAs, especially butyric acid, have been shown to be able to cross the BBB, protecting the brain and enhance plasticity in neurological disease models (174). The interesting effects of LB on the brain were further explored in **paper V**.

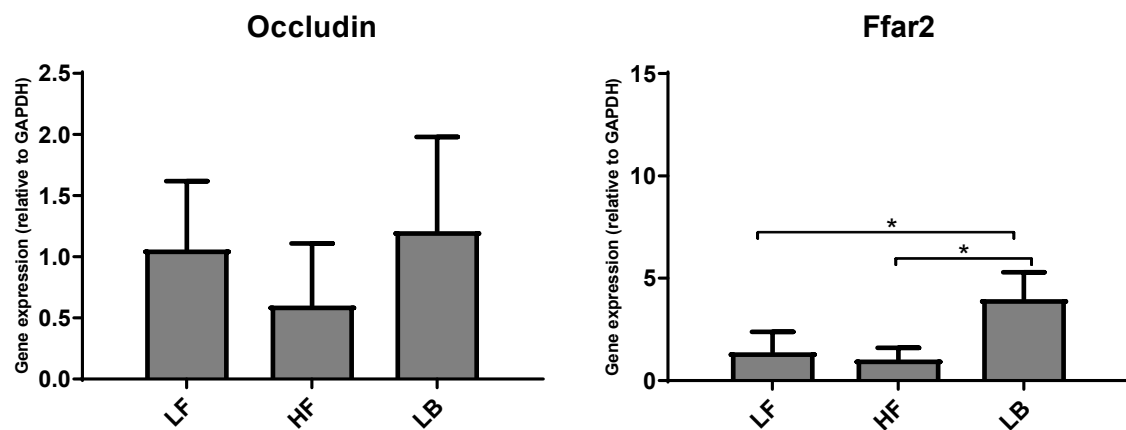


Figure 9 Gene expression (relative to GAPDH) of free fatty acid receptor 2 (Ffar2) and the tight junction protein occludin in the brain of mice

Mice were fed low-fat (LF), high-fat (HF) or HF diet with inclusion of lingonberries (LB) for weight weeks (n=10 per group). One-way ANOVA with Dunnett's post-hoc test was used to analyse significant differences between groups, using Graphpad Prism (*P<0.05).

LB contain high amount of dietary fibre and polyphenols, as well as other bioactive components with antioxidative properties (171, 175). In general, phenolic compounds are bound to dietary fibre and delivered to the lower part of the gut (176). Thus, the effect of LB on the gut microbiota may have been induced by the specific fibre content of the LB that reaches the lower part of the gut. Of total dietary fibre content, LB contain approximately 30% soluble and 70 % insoluble dietary fibre, as reported in **paper IV**. **Paper V** investigated whether dietary fibre of different solubility in LB may have an impact on the gut microbiota, in relation to inflammatory tonus, brain function and behaviour.

Lingonberries and their insoluble fraction modulate gut microbiota, reduce gut inflammation, improve glucose metabolism and brain functions (Paper V)

In this study, the effects of LB on brain function and behaviour were further evaluated. In order to try to identify components in LB that were responsible for their anti-metabolic disturbance and anti-inflammatory response, two fractions of LB were extracted based on their water solubility. All components in the lingonberries were collected in these fractions. Inclusion of wLB, or one of the two separated fractions of LB in a HF diet resulted in different levels of metabolic improvement. Since the aim of this study was to compare the effect of dietary fibre in each fraction of LB on the gut microbiota, the amounts of LBs and the two fractions added to the diet were calculated to give the same amount of dietary fibre. However, due to sign of intolerance in mice fed solLB diet, the study design had

adjusted for this group, as described in the methodology section. Instead, the mice in this group stayed on standard rodent chow during the first 6 weeks after arrival to the animal facility, prior to switching to the test diet only during the last 2 weeks. As mentioned previously, the solLB diet contained only 1.5% dietary fibre from solLB and 4.5% cellulose in the adjusted formula. As compared to its HF-control group, given the diet also for only 2 weeks, solLB showed effects on counteracting diet-induced obesity, but minor effects on other metabolic parameters and the gut microbiota. The composition of each fraction of LB needs further investigation. However, the weight loss effect of solLB fraction is suspected to be derived from benzoic acid (177) that is highly soluble in water (178). Resveratrol, another active component in LB (179), is a polyphenolic compound that have been proposed to have therapeutic potential in AD (180) is much less soluble in water (181) and would thus be present mostly in the insLB fraction. The effect of wLB and insLB seemed to also be related with the changes in the gut microbiota that may be involved in the improvement in brain function. Hence, the discussion will further focus on the effects of wLB and insLB fraction.

The wLB and insLB induced similar changes in the gut microbiota with mainly significant reductions in *Mucispirillum* and its Deferribacteres phylum, and significant increases in *Akkermansia* and its Verrucomicrobia phylum. *Mucispirillum* is usually found increased in mice on HF diets, and is suggested to play an important role in diet-induced obesity (182). Notably, this bacterium also showed positive correlation with atherosclerotic plaque size in **paper IV** (183). Prebiotics and diets rich in polyphenols have been shown to increase *Akkermansia* abundance, in association with improved metabolic profile (184-186). Phenolic components, especially flavonoids, are more enriched in the soluble components of plants, such as skin and seeds (187, 188) and this may be the reason that *Akkermansia* was increased in mice fed wLB and insLB, and not solLB.

There was evidence of increased levels of gut inflammation, as measured by the levels of enteric glial S100B protein, in mice fed HF-control diet. Including wLB or insLB in a HF diet significantly reduced levels of S100B protein in the small intestine, reflecting lowered EGC activation. Exposing human EGCs to pathogenic bacteria, like *Escherichia coli* has been shown to increase S100B expression, while exposure to *Lactobacillus paracasei* did not result in the same activation (74). Thus, the reduction in S100B protein in small intestine of mice fed wLB and insLB may be associated with the gut microbial profile. There was no significant difference in the S100B in the brain of the mice.

Mice fed wLB and insLB showed significant improvement in glucose responses as compared with their HF-controls after undergoing 4-hour fasting. The mice fed solLB showed similar area under curve (AUC) during 120 minutes following a glucose challenge, as those fed wLB and insLB, but the difference was not

significant when compared to their HF-controls that were on the diet also only for two weeks. Administration of viable *A. muciniphila* has been shown previously to improve glucose tolerance and decrease endogenous hepatic glucose production in HF-fed mice, together with improvements in gut barrier function (184). Thus, the increased *Akkermansia* abundance observed in mice fed wLB and insLB may be one of the factors involving in glucose and gut barrier homeostasis.

The finding that mice fed wLB and insLB showed increased hippocampal synaptic density is intriguing. This is also in line with, the slightly higher alternation rate and shorter time for making the decision in T-maze test, although these memory improvements were not significant. Alternation in a T-maze can also be used as a measure of spatial memory (132, 189). The ability of rodents to perform in T-maze task is sensitive to the function of the hippocampus (190). In fact, mitochondrial dysfunctions and synaptic damage are two main events that occur early in AD development (191). Study in older mice may increase the chance to detect more pronounced differences in the memory test.

Concluding remarks and future perspectives

Our pre-clinical results in **paper I**, although they need to be translated into humans, broaden our general conception that AD pathology in the brain may be triggered from the dysbiosis in the gut microbiota. The exact mechanisms mediating involvement of the gut microbiota in A β need to be studied further. Yet based on existing evidence, A β also function in the innate immune system, protecting the brain from inflammation (144, 145). BBB dysfunction developed during normal ageing has been suggested to be associated with inflammation (146). Increased influx of inflammatory components, including leakage of bacterial endotoxin from the gut into circulating system, that subsequently come into contact with the BBB, may be an important player in inducing APP cleavage into A β . Chronic inflammation, causing constant A β production, may lead to increased A β plaque formation overtime. Such chronic inflammatory tonus is a common pathophysiological element in the MetS, that has been known to also increase risk factor of dementia.

The bacterial candidates that seemed to be involved include:

- *Akkermansia* Decreased in APPPS1 mice which displayed increased levels of pro-inflammatory cytokines in brain homogenates, and also showed negative association with the amount of cerebral A β plaques (**Paper I**). Inclusion of wLB or insLB in a HF diet resulted in increased *Akkermansia* abundance, that may be associated with the lingonberries' effects in improving glucose tolerance, lowering inflammatory tonus, and increasing numbers of synapses in the hippocampus of ApoE^{-/-} mice (**Paper IV and V**).
- *Allobaculum* Decreased in APPPS1 mice (**Paper I**)
- **An unclassified genus from S24-7** Increased in APPPS1 mice (**Paper I**).
- *Parabacteroides* Correlated negatively with cerebral A β plaques (**Paper I**). It was also increased simultaneously with *Bacteroides* in suckling rats early exposed to dietary components that induced gut barrier maturation (reduced

permeability to macromolecules) but increased plasma LBP levels (**Paper II**).

- ***Bacteroides*** Increased simultaneously with *Parabacteroides* in suckling rats early exposed to dietary components that induced gut barrier maturation (reduced permeability to macromolecules) but increased plasma LBP levels (**Paper II**). This bacterium was increased with LB consumption but did not seem to induce inflammatory response (**Paper IV and V**). Specific responses to different species of *Bacteroides* should be further studied.

Interestingly, all these bacteria are Gram-negative and their cell wall component contains LPS, suggesting that the shift within the members of Gram-negative bacteria may play important role in host immune responses. It has been suggested that *Akkermansia*, although it contains LPS, increased the intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion, relating to glucose homeostasis (184). Moreover, pro-inflammatory stimulation of enterocytes by *Akkermansia* seems to keep the mucosa-associated immune system active at an appropriate level. On the other hand, LPS from some species of *Bacteroides* and some other Gram-negative bacteria can provoke a strong inflammatory cascade (192). Also, as mentioned previously, correlations do not prove causality. Colonization of germ-free mice with a single bacterium or a cocktail of those bacteria showing positive or negative correlations with the amount of cerebral A β plaques will further reveal whether they have causative or protective roles in AD.

Diet is an important modulator of the gut microbiota. Early exposure to some dietary components may disrupt a normal development of the gut microbiota in early life. Our human dietary intervention results (**Paper III**) showed only subtle microbiota changes following an 8-week consumption of MFD. This suggested that once a stable microbial niche has already established, short-term dietary changes may be less efficient to induce broad changes in the community. Yet the health benefits ascribed to dietary fibre are mediated by gut microbial fermentation and the metabolites that are produced (47). Identifying dietary components capable of promoting growth of the gut microbiota associated with positive health effects of the diet may further improve host health. LB induced increases in *Bacteroides* and *Akkermansia* that may be related with their positive effect to counteract diet-induced metabolic disorders and inflammation (**Paper IV**). The insoluble fraction of LB may contribute more to the increase in *Akkermansia* (**Paper V**), yet the effects were similar to those seen with intact whole LB. The observed effects of LB on the gut microbiota may further improve host metabolism, reduce systemic inflammation and have positive effects on brain functions, as seen by increased density of hippocampal synapses. Still, an increase in *Akkermansia* alone may not be taken as a cause of improved host health, but an indicator of a healthy gut barrier, e.g.,

increased mucus layer thickness. A lot more work is still needed to solve the complex puzzles of the gut microbiota-host interactions.

In conclusion, the studies included in this thesis add to the growing body of literature regarding the involvement of the gut microbiota in host metabolism, inflammatory responses, and the gut-brain axis communication. The progression of neurodegenerative disease, such as AD, may initiate from the dysbiosis of the gut microbiota, promoting systemic inflammation. Dietary disturbance early in life affected the establishment of the gut microbiota, associated with impaired gut barrier functions. Once the gut microbial community is established, short-term dietary changes may be insufficient to induce broad changes in the community. Specific dietary components, such as those from lingonberries, capable of modulating the gut microbiota towards a community that is associated with reduced systemic inflammation, may further promote positive health effects of the diets on the host, in terms of metabolism, and potentially brain functions. The important findings in this thesis are summarized and illustrated in **Figure 10**.

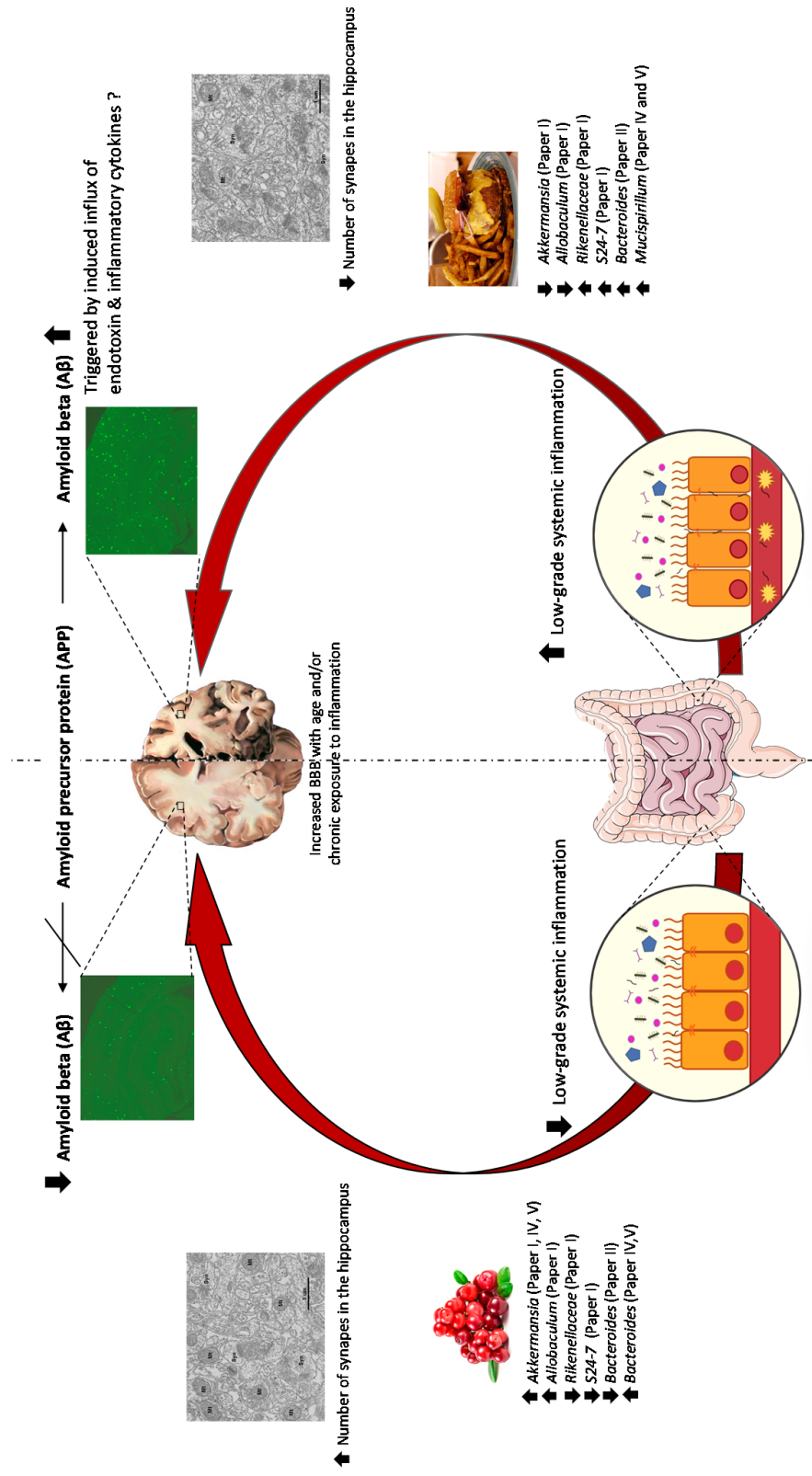


Figure 10 An overview of important findings in the thesis.

The progression of amyloid-beta ($A\beta$) pathology may initiate from the dysbiosis of the gut microbiota, associated with increased levels of systemic inflammation. Impaired gut barrier functions lead to increased influx of bacterial endotoxin and levels of low-grade systemic inflammation. The increased inflammation seemed to be associated with the levels of APP proteolysis into $A\beta$ peptides from $A\beta$ plaques, the common pathology seen in the brain of Alzheimer's disease. The diet plays an important role in shaping the gut microbiota. Dietary disturbance early in life affected the establishment of the gut microbiota, associated with impaired gut barrier functions. Once the gut microbial community is established, short-term dietary changes may be insufficient to induce broad changes in the community. Specific dietary components, such as lingonberries, capable of modulating the gut microbiota towards a community that is associated with reduced systemic inflammation, and improved brain function may further promote positive health effects of the diets on the host, in terms of metabolism, and potentially brain functions. Gastrointestinal illustration was obtained and modified from <https://smart.servier.com/>, licensed under CC BY 3.0. Alzheimer's brain illustration was reproduced with permission from ©2018 Alzheimer's Association. www.alz.org. All rights reserved, illustrations by Stacy Jannis.

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