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Direktor: Prof. Dr. Martin Dichgans

Ischemic Stroke induces a persisting perturbation of key bacterial populations and metabolites

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Philip William Melton

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der Universität München

Berichterstatter:	Prof. Dr. Arthur Johann Liesz
Mitberichterstatter:	Prof. Dr. Thomas Pfefferkorn
	Prof. Dr. Christian Opherk
	PD Dr. Tobias Schwerd
Mitbetreuung durch den promovierten Mitarbeiter:	
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	16.01.2025

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1. Affidavit

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Is my own work. I have only used the sourced indicated and have not made use of services of third party. Where the work of others has been quoted or reproduced, the source is always given.

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Place, Date

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Signature doctoral candidate

2. Table of Contents

1. Affidavit 4
2. Table of Contents 5
3. Summary 8
4. Zusammenfassung 10
5. Introduction 12
   5.1. Stroke 12
      5.1.1. Epidemiology 12
      5.1.2. Pathophysiology and clinical classification of ischemic stroke 13
      5.1.3. Current clinical management of ischemic stroke 15
   5.2. The gut microbiome 18
      5.2.1. Ischemic stroke affects the gut microbiome and its metabolites 19
      5.2.2. Probiotics as a modulator of the gut microbiome 20
   5.3. Aims of the Thesis 21
6. Materials and Methods 22
   6.1. Materials 22
      6.1.1. Equipment and Software 22
      6.1.2. Consumables 23
      6.1.3. FACS-Antibodies 25
   6.2. Methods 26
      6.2.1. StrokeMicroBiomics (SMB) 26
      6.2.2. Study design 26
      6.2.3. Ethical statement 26
      6.2.4. Study population 27
      6.2.5. Sample collection and preparation 28
      6.2.6. Outcome measures 29
      6.2.7. Population characteristics 31
      6.2.8. PRISE 32
      6.2.9. Study design 32
      6.2.10. Ethical statement 33

6.2.11. Study population 34
6.2.12. Sample collection and preparation 35
6.2.13. Study intervention 35
6.2.14. Outcome measures 36
6.2.15. Population characteristics 37
6.2.16. Animal experiments 38
6.2.17. Stroke model and severity assessment 39
6.2.18. Animal perfusion and tissue collection 39
6.2.19. Statistical Analysis 40
7. Results 41
7.1. A model of murine stroke induces a persisting state of gut dysbiosis 41
7.2. Key bacterial populations of the gut microbiome remain altered after ischemic stroke in a human cohort 44
7.3. Activation markers in the myeloid lineage and monocyte counts were relatively decreased 90 days after stroke 47
7.4. Ischemic stroke is associated with a reduction of key bacterial metabolites 48
7.5. Dysregulated bacterial populations associate with the production of short chain fatty acids 50
7.6. Probiotic intervention affects bacterial diversity of the gut microbiome and key bacterial populations in stroke patients 51
7.7. The gut microbiome may be colonized by probiotic intervention 53
8. Discussion 55
8.1. Summary of results 55
8.2. Gut dysbiosis primes a systemic immune response and drives secondary comorbidities 56
8.3. Stroke, intestinal immunity and the gut microbiome 58
8.4. Probiotics as a novel treatment option in ischemic stroke 59
8.5. Limitations 61
8.6. Outlook 62
9. List of Figures 63
10. List of Tables 64
11. References 65

12. List of Abbreviations 69
13. Acknowledgements.....71

3. Summary

Introduction: Stroke is a globally leading cause of death and disability. Previous studies in experimental, murine models of stroke have demonstrated, that acute cerebral ischemia induces a state of gut dysbiosis with a reduction in the abundance of gut resident bacteria. A concurrent dysregulation of key bacterial metabolites (such as short chain fatty acids) has also been described. Probiotics have previously been proposed to ameliorate the observed dysbiosis of the gut microbiome in other diseases. We aimed to observe the chronic condition of the gut microbiome and its associated metabolites in an observational cohort of mice, as well as stroke patients. Furthermore, we aimed to investigate the ability of a commercially available probiotic to alter the microbiome´s composition after stroke in a cohort of stroke patients.

Methods: Stool was collected at 3 time points (0, 3 and 14 days after surgery), while EDTA blood and ileum content were collected 14 days after surgery in a cohort of 5 sham operated vs. 5 fMCAo mice. Shotgun sequencing was performed on the stool samples and targeted mass spectrometry was used to analyse the SCFA concentrations in the gut. An exploratory cohort (SMB) of stroke and TIA patients was recruited from the stroke unit of the LMU clinic and EDTA blood as well as stool samples were collected within 7 days of acute symptom onset 90 days later. Faecal samples were split and used for both targeted/untargeted mass-spectrometry as well as metagenomics. EDTA blood was used for both mass-spectrometry on extracted plasma, as well as FACS analysis. Finally, an interventional cohort (PRISE) of stroke patients and household references was recruited from the stroke unit of the LMU hospital. Patients received either a probiotic compound or placebo for 90 days, after which stool was collected for metagenomic analysis.

Results: The metagenomics analysis of the murine cohort demonstrated a significant shift in the Bray-Curtis index when compared across all 3 time points, as well as a significant loss of the short chain fatty acid propionate in the ileum content 14 days after surgery. The SMB cohort showed statistically significant losses of key bacterial populations in the acute phase after

stroke, when compared to the control cohort, which did not recover after 90 days. Furthermore, key bacterial metabolites were dysregulated at both baseline and 90 days. Loss of key bacterial populations correlated with the loss of metabolites. The PRISE study showed significant changes in key bacterial populations between placebo and probiotics groups and possible colonization by the probiotic strains.

Discussion: To our knowledge, we demonstrate for the first time, that bacterial populations and potent bacterial metabolites that contribute to neuroprotection do not recover in humans 90 days after ischemic stroke, and this effect is robust across species. Furthermore, we demonstrate, that probiotics can affect the composition of the gut microbiome.

4. Zusammenfassung

Einleitung: Schlaganfall ist weltweit eine der führenden Ursachen für Tod und Behinderung. Frühere Studien an experimentellen, murinen Modellen des Schlaganfalls haben gezeigt, dass akute zerebrale Ischämie eine Dysbiose im Darm verursacht, die mit einer Verringerung der Menge an darmansässigen Bakterien einhergeht. Eine gleichzeitige Dysregulation wichtiger bakterieller Metaboliten (wie kurzkettige Fettsäuren) wurde ebenfalls beschrieben. Probiotika wurden bisher vorgeschlagen, um die beobachtete Dysbiose des Mikrobioms im Darm bei anderen Krankheiten zu verbessern. Unser Ziel war es, den chronischen Zustand des Darmmikrobioms und seiner assoziierten Metaboliten in einer Beobachtungskohorte von Mäusen sowie Schlaganfallpatienten zu beobachten. Darüber hinaus wollten wir die Fähigkeit eines kommerziell erhältlichen Probiotikums untersuchen, die Zusammensetzung des Mikrobioms nach einem Schlaganfall in einer Kohorte von Schlaganfallpatienten zu verändern.

Methoden: Stuhlproben wurden zu 3 Zeitpunkten (0, 3 und 14 Tage nach der Operation) gesammelt, während EDTA-Blut und Ileum Inhalt 14 Tage nach der Operation in einer Kohorte von 5 Scheinoperierten und 5 fMCAo-Mäusen entnommen wurden. Shotgun-Sequenzierung wurde an den Stuhlproben durchgeführt und gezielte Massenspektrometrie wurde verwendet, um die Konzentrationen von kurzkettigen Fettsäuren im Darm zu analysieren. Eine explorative Kohorte (SMB) von Schlaganfall- und TIA-Patienten wurde aus der Schlaganfallstation der LMU-Klinik rekrutiert, und EDTA-Blut sowie Stuhlproben wurden innerhalb von 7 Tagen nach akutem Symptombeginn, sowie 90 Tage später gesammelt. Stuhlproben wurden aufgeteilt und sowohl für gezielte/ungezielte Massenspektrometrie als auch für Metagenomik verwendet. EDTA-Blut wurde sowohl für Massenspektrometrie auf extrahiertem Plasma als auch für FACS-Analyse verwendet. Schließlich wurde eine interventionelle Kohorte (PRISE) von Schlaganfallpatienten und Haushaltsreferenzen aus der Schlaganfallstation der LMU-Klinik rekrutiert. Die Patienten erhielten entweder eine probiotische Verbindung oder ein Placebo für 90 Tage, wonach Stuhlproben für die metagenomische Analyse gesammelt wurden.

Ergebnisse: Die Metagenomanalyse der murinen Kohorte zeigte eine signifikante Verschiebung im Bray-Curtis-Index im Vergleich über alle 3 Zeitpunkte hinweg sowie einen signifikanten Verlust der kurzkettigen Fettsäure Propionat im Ileuminhalt 14 Tage nach der Operation. Die SMB-Kohorte zeigte statistisch signifikante Verluste wichtiger bakterieller Populationen in der akuten Phase nach Schlaganfall im Vergleich zur Kontrollkohorte, die sich nach 90 Tagen nicht erholte. Darüber hinaus waren wichtige bakterielle Metaboliten sowohl zu Beginn als auch nach 90 Tagen dysreguliert. Der Verlust wichtiger bakterieller Populationen korrelierte mit dem Verlust von Metaboliten. Die PRISE-Studie zeigte signifikante Veränderungen in wichtigen bakteriellen Populationen zwischen Placebo- und Probiotika-Gruppen und mögliche Kolonisation durch die probiotischen Stämme.

Diskussion: Wir zeigen zum ersten Mal, dass bakterielle Populationen und potente bakterielle Metaboliten, die zur Neuroprotektion beitragen, sich 90 Tage nach einem ischämischen Schlaganfall bei Menschen nicht erholen, und dieser Effekt ist artenübergreifend robust. Darüber hinaus zeigen wir, dass Probiotika einen geringen Einfluss auf die Darmzusammensetzung haben kann.

5. Introduction

5.1. Stroke

The term “stroke” is estimated to be around 400 years old, having first been used by the physician William Cole in a letter to a colleague in 1689 [1] and came to replace the ancient term “apoplexy”, the first documented use of which is credited to Hippocrates in the 5th century BC [2]. The modern definition of stroke is the onset of an acute neurological deficit with a duration of at least 24 hours and a cerebrovascular cause. This underlying cause can be typically attributed to the three major categories of stroke, either an infarction of an area of cerebral, spinal or retinal tissue, an intracerebral haemorrhage (ICH) or a subarachnoid haemorrhage (SAH) [3]. Stroke is often associated with symptoms such as unilateral motor impairment, facial paresis, unilateral sensory loss, impaired or slurred speaking, difficulty understanding speech, loss of vision, amongst others. In the mid-1960´s, clinicians introduced the term transient ischemic attack (TIA) to further distinguish acute neurological deficits with a duration of less than 24 hours. Newer definitions have emerged due to an improvement in imaging technology (in particular diffusion weighted magnetic resonance imaging (MRI)) that showed cerebral infarction in patients with neurological symptoms lasting less than 24 hours (classic TIA). The new definition of TIA distinguishes a tissue-damage based definition of stroke and TIA, as opposed to the previous time-based classification system [4]. In this dissertation, the time-based TIA classification was used.

5.1.1. Epidemiology

Stroke is the second-leading cause of death worldwide, with 12.2 million new cases, and almost 101 million prevalent cases of stroke reported in 2019. 62.4 % of all 12.2 million new strokes were reported to be ischemic strokes [5]. Furthermore, stroke was the third leading cause of disability adjusted life years (DALYs) in 2019, according to the WHO [6]. Of all strokes reported in the United States, 87% were ischemic, 10% ICH and 3 % SAH in origin. Projections indicate, that by 2030 an additional 3.4 million US citizens will have had a stroke, indicating a

20.5 % increase in prevalence since 2012 [7]. Internationally, there are considerable intercontinental differences in both the risk of stroke and access to stroke care. In Central Europe, the estimated lifetime risk of stroke is 31.7 %, while the global lifetime risk of stroke is around 25 % [8].

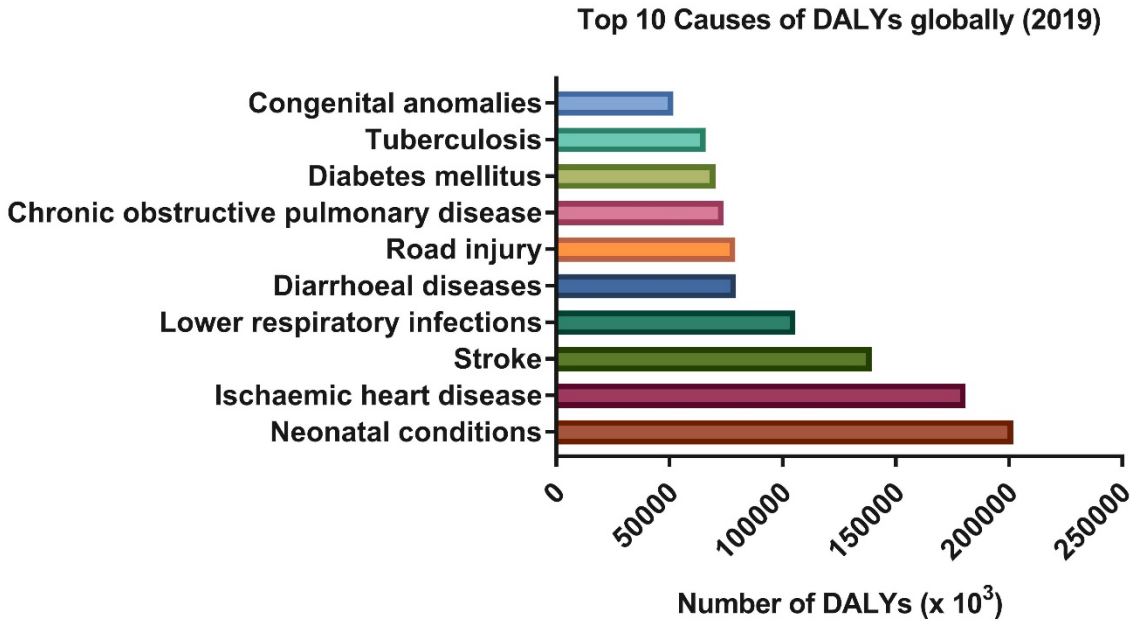

Figure 1: List of top 10 causes of DALYs globally, reported in 2019. (Data adapted from the WHO Global health estimates study 2020 [6])

In Germany the reported incidence of ischemic stroke in 2017 was 73.9, with a reported number of DALYs for the year 2016 of 543 per 100 000 citizens and a reported mortality in 2015 of 19.6 (Female) and 25 (Male) per 100 000 inhabitants [9].

5.1.2. Pathophysiology and clinical classification of ischemic stroke

Ischemic stroke results when an artery supplying the central nervous system becomes occluded and neuronal electrical function is impaired. In most patient a decrease in cerebral blood flow (CBF) of around 50% is not associated with the development of symptoms, however, a further loss of CBF induces reversible neuronal damage. If CBF is not restored within a reasonable time (this being wholly dependent on the severity of CBF loss), this

damage can become permanent, resulting in cerebral infarction or stroke with the concurrent development of symptoms consistent with the area of the brain affected [10]. In many patients, the viability of neuronal cells is maintained through collateral blood supply resulting in an ischemic penumbra, a halo of cells around the ischemic core of the stroke, which can still be salvaged, but are suffering from the effects of CBF loss [11]. How quickly the ischemic core expands and engulfs the penumbral tissue is a multifactorial process with many determinants, such as: age, localization of the infarct (i.e. grey and white matter have differing energy demands), previous vascular and cerebral conditions, degree of collateralization, genetic factors, and others. [12].

The oldest, and most commonly used system of stroke classification, is the TOAST criteria [13], developed in 1993 and containing 5 causes of ischemic stroke: Large artery atherosclerosis (LAA), Cardioembolism (CE), Small-vessel occlusion (SVO), Stroke of other determined aetiology, and stroke of undetermined aetiology. It has since been expanded upon and digitalized in the form of the Causative Classification of Stroke system (CCS), allowing for swift categorization in a clinical setting [14, 15]. To recognize a possible stroke outside of a hospital setting, the Canadian guidelines (amongst others) on acute stroke management, recommend the use by the general public of the FAST (Face, Arms, Speech, Time) screening system [16]. Other such screening tools have been implemented and are used internationally, such as the Shortened NIHSS for emergency medical services (sNIHSS-EMS) or the Austrian Prehospital Stroke Scale (APSS) [17]. The purpose of these scales is the fast identification of potential stroke victims, as the possibility and success of treatment is highly time dependent.

5.1.3. Current clinical management of ischemic stroke

In order to choose the adequate treatment algorithm, several diagnostic steps are first required. Upon entry to an emergency department with a potential stroke patient, the clinical assessment of a patient using standardised neurological scoring systems is customary. A typical and widely used example of a scoring system to gauge the impact of a stroke on clinical presentation is the National Institute for Health Stroke Scale (NIHSS), which consists of 11 items and measures focal neurological deficits [18]. The next essential step in the work-up of stroke patients presenting in an emergency department is the use of neuroimaging (typically conventional non-contrast CT) to primarily exclude the presence of a haemorrhagic stroke, such as an intracranial bleed or subarachnoid haemorrhage. This is often combined with CT-angiography and CT-perfusion imaging to allow for discrimination of stroke aetiology and a patients eligibility to receive treatment [19]. This imaging can be further augmented with imaging interpretation systems such as the ASPECTS score, to determine candidates for revascularisation therapy [20]. Newer strategies of acute stroke diagnosis include approaches utilizing artificial intelligence, which offer guidance on decision making and can act in a second reader capacity for stroke physicians [21].

The treatment of ischemic stroke has advanced considerably across the past 40 years, with the primary objective being the rapid restoration of blood flow to the brain to minimize cerebral damage and improve patient outcomes. Strategies for the treatment of ischemic stroke are generally categorized into two critical phases, acute management and secondary prevention.

In the immediate aftermath of an ischemic stroke, the focus is on promptly dissolving or removing the blood clot that is obstructing cerebral blood flow. The cornerstone of acute treatment is intravenous thrombolysis (IV tPA), specifically utilizing tissue plasminogen activator (tPA), a potent clot-dissolving agent [22]. When administered within 4.5 hours of symptom onset, tPA significantly enhances the likelihood of favourable outcomes, with the best results observed when it is administered as early as possible. However, the use of tPA is

limited by certain contraindications, including recent surgery, bleeding disorders, or if the stroke is haemorrhagic in nature rather than ischemic [23].

For patients experiencing a large vessel occlusion, especially in the anterior circulation, mechanical thrombectomy is the preferred intervention. This endovascular procedure involves the direct removal of the clot using devices such as stent retrievers or aspiration catheters. Mechanical thrombectomy has shown to be highly effective, particularly when performed within 6 (to possibly 24 hours) of stroke onset. The decision to proceed with thrombectomy is typically guided by advanced imaging techniques that assess the extent of brain tissue at risk. CT perfusion is instrumental in mapping the brain's blood flow dynamics, revealing areas with reduced perfusion where tissue is still viable but endangered. This data helps clinicians identify patients who are within the therapeutic window for thrombectomy, even if their symptoms have been present for an extended period (up to 24 hours in some cases) [23].

In cases where tPA is not administered, antiplatelet therapy, typically with aspirin or as a dual antiplatelet therapy (i.e., aspirin in combination with clopidogrel), is generally started within the first days after the onset of stroke symptoms, depending on the underlying aetiology. This early initiation aims to reduce the risk of early recurrent stroke by preventing further clot formation. However, when tPA has been used, the introduction of aspirin is carefully delayed to avoid the increased risk of haemorrhagic complications that can arise from combining the clot-busting effects of tPA with the blood-thinning properties of aspirin. The timing of aspirin administration in these cases is adjusted based on the patient's clinical condition and the judgment of the treating physician to balance the benefits of preventing recurrent stroke against the risk of bleeding [23, 24].

During the acute phase, supportive care is equally important. Managing blood pressure is critical, as both excessively high and low blood pressure can worsen outcomes. Blood sugar levels must be closely monitored and controlled, as hyperglycaemia can exacerbate brain injury. Body temperature management is also essential, as fever can increase metabolic demands on the brain and worsen outcomes [16]. Although still under investigation,

neuroprotective strategies aim to shield brain cells from further damage during the stroke and are an area of ongoing research [25].

Once the acute phase is managed, attention shifts to preventing future strokes. Antiplatelet or anticoagulant therapy plays a central role in this phase. Long-term antiplatelet agents, such as aspirin or clopidogrel, are commonly prescribed to reduce the risk of future thrombotic events. For patients with atrial fibrillation or other conditions that predispose them to embolic strokes, anticoagulants like phenprocoumon or direct oral anticoagulants (DOACs) are recommended to prevent clot formation [26].

Effective management of underlying risk factors is crucial for secondary prevention. Blood pressure control is paramount, as hypertension is one of the most significant modifiable risk factors for ischemic stroke. Lipid management, often involving the use of statins, is essential to lower cholesterol levels and reduce the risk of atherosclerotic plaque formation. For patients with diabetes, tight glycaemic control is necessary to prevent complications that can contribute to stroke risk. Lifestyle modifications are also a critical component of secondary prevention. Patients are encouraged to adopt a heart-healthy diet, rich in fruits, vegetables, and whole grains, and low in salt and saturated fats. Regular physical activity is recommended to improve cardiovascular health, and smoking cessation is strongly advised, as smoking significantly increases the risk of stroke [26, 27].

In some cases, surgical interventions may be necessary to reduce the risk of future strokes. For patients with significant carotid artery stenosis, carotid endarterectomy (the surgical removal of plaque from the carotid artery) or carotid artery stenting may be recommended to restore proper blood flow and prevent recurrent stroke from occurring [28].

5.2. The gut microbiome

The human gut is populated by bacteria, viruses and fungi (as well as bacteriophages), and the term gut microbiome refers to the entirety of the genetic material presented by the microbial residents of the gut. In this thesis however, it will refer only to the genetic material presented by the bacterial residents of the gut (also referred to as the bacteriome) [29]. The entire microbiome contains around 500 – 1000 different species of bacteria, with new methods of genotyping allowing for further division into subspecies, which represent over 99 % of all genes present in the human body [30]. The human gut is colonized at birth and progresses through a phase of chaotic bacterial communities until the stabilisation that arrives with the introduction of solid foods [31]. It has also been shown that although the composition of the gut microbiome fluctuates on a per day basis, largely due to factors stemming from inter-individual behaviour and health, the microbiome demonstrates long-term stability, that is, unless major events perturb the homeostasis of the gut microbiome [32, 33]. Characterized functions of the gut microbiota include the processing of dietary compounds, the de-novo synthesis of vitamins (such as B vitamins) [34, 35], the regulation of intestinal immunity and by extension the systemic immune system, as well as ensuring intestinal integrity [36]. To date, the precise definition of microbial dysbiosis remains elusive, primarily due to the vast variance of “normal” microbiome composition amongst healthy individuals. The key problem lies in the array of differing microbial profiles that persist in individuals that are healthy [37]. One suggested definition has been “a stable microbial community state that functionally contributes to the aetiology, diagnosis or treatment of a disease” [38]. For this reason, the key to understanding what even constitutes pathological change in the microbiome, versus natural responses to environmental stimuli by the gut microbiota, lies in the characterization of bacterial metabolites and key bacterial populations within different diseases [37, 39].

5.2.1. Ischemic stroke affects the gut microbiome and its metabolites

A study performed in patients with acute ischemic stroke demonstrated that there were acute dysregulations of key bacterial populations and faecal organic acids in patients with stroke versus control patients. Further studies performed in a patient cohort with large artery atherosclerosis based stroke, TIA and symptomatic atherosclerosis have shown that stroke leads to dysregulation of the gut metagenome, gut dysbiosis and downregulation of the gut metabolite trimethylamine-N-oxide (TMAO) [40, 41]. Another study demonstrated selective expansion of specific bacterial populations (such as various *Clostridium* species) and concurrent increases in intestinal gas pocket formation [42]. To our knowledge, very few studies have investigated the persistence of dysbiosis in stroke patients at chronic time points and they did not perform any metabolomics [43].

There are several mechanisms that have been proposed through which focal neuronal damage can cause gut dysbiosis. Brain injury models in mice have shown increased activation of the sympathetic system leading to intestinal paralysis and concurrent alterations of bacterial communities [44]. Another study in an experimental model of stroke in mice demonstrated that ischemic stroke can induce a loss of gut motility, leading to a decrease in the Shannon alpha diversity index [45]. The stroke induced dysbiosis and other forms of pre-existing, induced dysbiosis have been shown to worsen mortality, increase stroke volume, worsen functional outcomes and more in patients and experimental models of stroke [46]. However, other studies performed in experimental stroke models of mice have demonstrated that targeted modulation of the gut microbiome via intervention with antibiotics can reduce infarct volume, despite, or precisely because of the presence of prominent dysbiosis [47, 48].

5.2.2. Probiotics as a modulator of the gut microbiome

Originally, the concept of probiotic compounds was postulated by Elie Metchkinoff in 1900, a noble prize winner who first described associations between fermented products and gastro-intestinal health. This discovery gave birth to a market which was estimated to have a value of $36 billion dollars in 2013 [49]. Later, the term “probiotic” was defined in 1989 by Fuller as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” [50]. A typical example of a natural probiotic product is yogurt, which contains strains of *Lactobacilli*. Prebiotic compounds on the other hand are easily fermented fibres that can beneficially influence the expansion of select colonies of bacteria. Many commercially available compounds are a combination of the two, with inulin and fructo- or galacto-oligosaccharides added to the live microbial strains (“synbiotic”) [51].

Many commercial probiotics contain strains from the bacterial taxa *Bifidobacterium* and *Lactobacillus*. The functions of common probiotic factors (such as short-chain fatty acids or polysaccharides) have been demonstrated to range from the dampening of inflammatory processes to the upregulation of T_{reg} populations by stimulation of IL-10 production [52]. Across many years of research, many varying effects of probiotics have been proposed and investigated, however the research has been plagued by inaccuracy and a lack of standardisation, derived from a variety of factors, such as the use of a wide range of different bacterial species or combinations thereof combined with the use of self-reported outcomes or outcomes with very little manifest clinical value [53]. However, a large body of evidence has been gathered to describe the clinical implementation of probiotics from which at the very least the overwhelmingly positive safety profile of probiotic compounds can be deduced [54].

5.3. Aims of the Thesis

Ischemic stroke induces intestinal paralysis and a state of gut dysbiosis, which in turn affects the intestinal immune response and post-stroke neuro-inflammation [45]. Short chain fatty acids have been identified as effectors of the gut microbiome on the brain and are important neuroprotective metabolites that improve post-stroke recovery and modulate the host´s immune response [55].

On the basis of these previous findings, the objective of this dissertation was to identify the persistence of potential causes of chronic neuro-inflammation in the form of altering concentrations of key bacterial metabolites and differentially abundant bacterial populations. In order to substantiate this goal, the following specific objectives were addressed:

1. The characterization of bacterial metabolites and bacterial diversity in a cohort of mice
2. The identification of differentially abundant bacterial populations and dysregulated bacterial metabolites in a prospective cohort of stroke patients
3. The investigation of a commercially available probiotic and its efficacy in modulating post-stroke dysbiosis in patients

6. Materials and Methods

6.1. Materials

6.1.1. Equipment and Software

Product Name	Supplier
MVE YDH-1-127 Vapor Shipper	Chart Industries
BD FACSVerse™ Flow Cytometer	BD Biosciences
Mixer Uzusio VTX-3000L	LMS
Vortex-Genie 2	Scientific Industries
ND-1000 Nanodrop Spectrophotometer	Peqlab
TC20 Automated Cell Counter	Biorad
Centrifuge 5427R	Eppendorf
Centrifuge 5810R	Eppendorf
Research Plus Pipettes	Eppendorf
Accu-jet® *pro*	BRAND
Acculab ALC-80.4 Precision Analytical Balance	Acculab Sartorius Group
GraphPad Prism 6	Graphpad Software Inc.
FlowJo V10.6	Treestar Inc.

6.1.2. Consumables

Product Name	Supplier
General	
Cotton Swabs	MaiMed
PP-overalls	Falano
Medical Face Mask	Mölnlycke health care
Injekt 5ml	Braun
Kimtech Science Precision Wipes	Kimberly-Clark Professional
Microlance Needles 27G	BD
Vasco Nitril Gloves	Braun
Treatments	
Verum treatment (Contains: Corn Starch, Maltodextrin, Inulin P7, potassium chloride, hydrolysed rice protein, 9 probiotic bacterial strains (*B.bifidum W23, B. lactis W51, B. lactis W52, L. acidophilus W22, L. casei W56, L. paracasei W20, L. plantarum W62, L. salivarius W24, Lc. Lactis W19*), Magnesiumsulfate, Fructooligosaccharide P7, Amylase, Mangansulfate	Institute AllergoSan
Placebo Treatment (Contains: Corn Starch, Maltodextrin, Mangansulfate, Magnesiumsulfate)	Institute AllergoSan
Reagents	

Flow Cytometry Staining Buffer	Thermo Fisher Scientific, Germany
NaCl isotonic solution 0,9%	Fresenius Kabi, Germany
Isoflurane Iso-Vet 1000mg/g	Dechra, Germany
Ketamin 10%	Medistar, Germany
Xylanin 20mg/ml	WTD, Germany
Carprofen (Rimydyl 50ml/ml)	Zoetis, Germany
Octenisept	Schülke, Germany
Gibco Trypan blue solution	Thermo Fisher Scientific, Germany
Ficoll Paque Premium	Merck, Germany
Kits	
QIAmp Fast DNA Stool Mini Kit	Qiagen
Omnigene-Gut for microbiome OM-200	DNA genotek
Stool sampling tube with integrated spoon	Paracelsus Versand
Safety-Multifly^{R}-Needle	Sarstedt
7,5ml EDTA-Blood sampling tubes	Sarstedt

6.1.3. FACS-Antibodies

Marker/Fluorophore	Supplier
CD45 ef450	Invitrogen
CD11b APC Cy7	Invitrogen
CD14 PerCP Cy5.5	Invitrogen
CD16 FITC	Invitrogen
HLA PECy7	Invitrogen
CD163 APC	Invitrogen
CD36 PE	Invitrogen
CD3 FITC	Invitrogen
CD4 PerCP Cy5.5	Invitrogen
CD8a PE	Invitrogen
CD19 APC	Invitrogen
CD56 APC Cy7	Invitrogen

6.2. Methods

6.2.1. StrokeMicroBiomics (SMB)

6.2.2. Study design

SMB was a prospective, exploratory, observational clinical trial with a total of 17 patients included in the analysis. All patients who matched the inclusion/exclusion criteria and gave written informed consent were recruited from the stroke unit of the LMU university hospital Munich between the 16th of June 2019 and the 27th of April 2021. Patients were recruited on the basis of clinical presentation and imaging at the time point of admission into 2 distinct cohorts: stroke and transient ischemic attack (TIA). Two 7.5 ml EDTA blood vials and faecal samples were collected from all participants at baseline (within 7 days of onset of acute neurological deficits) and 90 days after cerebral event. Subsequent mass spectrometry (EDTA blood + stool sample), flow cytometry (EDTA blood) and shotgun sequencing (stool sample) was performed. Faecal metagenomic and mass spectrometry data from 7 additional, healthy volunteers recruited in an independent cohort in Kassel was included for the purposes of paired matching in the statistical analysis between stroke patients and healthy controls.

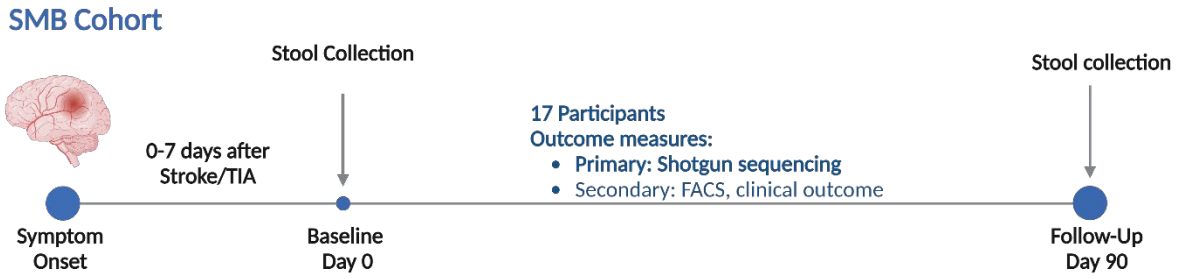

Figure 2: Study design of the SMB cohort

6.2.3. Ethical statement

The collection of EDTA blood had already been approved under the pre-existing ethics application (project number 121-09), which was approved by the independent ethics

commission (IEC) of the LMU medical faculty in 2009. In order to collect faecal samples, an amendment was written and approved by the IEC of the LMU medical faculty, expanding the collection of samples to include stool samples. After receiving approval for the collection of samples, a withdrawal application from the Biobank of the ISD (project number 121-09) was submitted to the IEC LMU medical faculty and subsequently approved.

Patients or their legal representatives were informed of the content and purposes of the sample collection. During the acquisition of written informed consent, patients and their legal representatives were also informed of their data protection rights and their ability to withdraw consent without any negative consequences for their further treatment at any point in time. This study was performed with adherence to the most current tenets of the declaration of Helsinki and following Good Clinical Practice (GCP).

6.2.4. Study population

The following inclusion and exclusion criteria were used to determine the patients' eligibility for participation in the study:

Inclusion criteria:

i. Admission to the Stroke Unit Großhadern of the Ludwig-Maximilians-University hospital
ii. Age ≥ 50 years
iii. Ability of patient or their legal participant to provide informed consent as detailed in the trial protocol of project number 121-09.
iv. NIHSS ≤ 3 at admission to hospital, acute neurological deficits may not persist for more than 24 hours (TIA group)

OR

NIHSS ≥ 4, radiologically confirmed cerebral infarction in the supply area of the anterior, medial or posterior cerebral artery (Stroke group). The stroke group was further divided into the following two subgroups:

a) Mild Stroke: NIHSS < 10 at time of admission
b) Severe Stroke: NIHSS ≥ 10 at time of admission

Exclusion Criteria:

i. Pregnancy
ii. Known, active cancer diagnosis
iii. Immunosuppression
iv. Infectious disease in the last 2 weeks
v. Gastrointestinal illness with diarrhoea or solitary diarrhoea within the last 2 weeks
vi. Operations within the last 4 weeks
vii. Active immune disease requiring treatment
viii. Chronic inflammatory disease
ix. Haemorrhagic stroke or intracranial bleed
x. Cerebellar stroke

Patients that withdrew consent, were lost to follow-up or could not participate in the 90 day follow-up visit for any other reason, were excluded from further participation in the trial.

6.2.5. Sample collection and preparation

This protocol was followed for both the baseline and day 90 visits. The patients were visited on the stroke unit of the LMU clinical for baseline and at home for day 90. A permit for house calls was applied for and then issued by the Bavarian regional government during the COVID-19 pandemic. All samples were assigned unique identifiers, which did not include any features allowing for the identification of a participant.

Stool samples:

After screening and recruitment of the patients were completed, patients were asked to call the ISD when they were ready to give a stool sample. Stool samples were collected via stool sampling tubes with integrated spoon (Paracelsus Versand). After acquisition of the stool, samples were immediately placed in a mobile liquid nitrogen container (MVE YDH-1-127 Vapor Shipper (Chart Industries)), to ensure minimal shifts in anaerobic and aerobic bacterial populations through exposure to air and a new environment. After collection and snap-freezing, the stool samples were stored at -80°C in a freezer at the ISD laboratory. To prepare the samples for shotgun sequencing, the stool samples were thawed before shipment and faecal DNA was extracted with the QIAmp Fast DNA Stool Mini Kit (Qiagen). DNA concentration and stability was subsequently measured via a Nano drop measurement for each sample. The extracted DNA samples were stored at -20 °C for a maximum of 2 weeks before shipment on dry ice.

EDTA blood:

Concurrently to the collection of stool, 2 vials of 7.5 ml EDTA blood were collected from the patients and placed on ice to cool them to 4°C. After transfer to the ISD laboratory, the first vial of EDTA blood was transferred to a 15 ml Falcon tube and spun in a centrifuge at 2000g relative centrifugal force (RCF) for 10 minutes. After the spin was completed, the resulting top layer of plasma was pipetted out of the falcon tube into multiple aliquots of 500 µl plasma in 1,5 mL Eppendorf Tubes, which were then stored at -80°C in a freezer at the ISD laboratory.

The second vial of 7.5 ml EDTA blood was used for immediate FACS analysis.

6.2.6. Outcome measures

The extracted faecal DNA aliquots were transported to the laboratory of Prof. Paul Wilmes at the University of Luxembourg, Luxembourg Centre for Systems Biomedicine. The subsequent shotgun sequencing was performed by the core facility team of the Luxembourg Centre for

Systems Biomedicine. Analysis was primarily performed by Dr. Velma Aho, a post-doctoral fellow in Prof. Wilmes laboratory.

Aliquots of at least 500 mg of snap frozen stool, as well as the matching plasma samples were also transported to the laboratory of Prof. Paul Wilmes at the University of Luxembourg, Luxembourg Centre for Systems Biomedicine. The core facility of this institute, under the direction of Dr. Christian Jäger then performed both targeted (for SCFAs and bile Acids) and non-targeted (polar and lipid liquid chromatography mass spectrometry (LCMS) and polar gas chromatography mass spectrometry (GCMS)), with lipid mass-spectrometry on the stool samples.

The second EDTA-blood vial was used for the characterization of PMBCs via flow cytometry using the following protocol:

i. PBMC isolation: For the isolation of peripheral blood mononuclear cells the 7.5 ml of EDTA-stabilised blood were diluted in 7.5 ml of cold (4°C), sterile PBS. The 1:1 dilution of blood was then gently layered on top of 20 ml of Ficoll Paque Premium using a pipette and a density gradient centrifugation was performed (Using the Eppendorf centrifuge 5810R) at room temperature, 400 g RCF for 30 minutes (With decreased acceleration and brakes). After centrifugation, the buffy coat was carefully removed via 1 ml Pipette and placed into a new 5 ml FACS tube and washed 3 times with 15ml of sterile PBS (Centrifuge settings: 4 °C, 300 g, 10 minutes), then resuspended in 200 µl of PBS.

ii. Cell counting: 10 µl PBMC cell suspension were diluted in 10 µl of trypan blue, a viability dye that accumulates in live cells. Cell counting was then performed in an automated fashion.

iii. Cell Staining: 100 µl of cell suspension were aliquoted into 2 separate FACS tubes. The first tube was stained with an Antibody cocktail consisting of 93 µl FACS Buffer, and 1 µl each of the cell dyes CD11b APC Cy7, CD14 PerCP Cy5.5, CD16 FITC, HLA PE Cy7, CD163 APC, CD36 PE and CD45 Ef450. The second tube was stained with

an antibody cocktail consisting of 94 µl FACS Buffer and 1 µl each of the cell dyes CD3 FITC, CD4 PerCP Cy5.5, CD8a PE, CD19 APC, CD56 APC Cy7 and CD45 Ef450. Both stainings were performed for 20 minutes at 4°C in a dark fridge. After staining was completed, 2 wash cycles (centrifuge settings: 5 minutes at 400g RCF, 20°C) were performed, the samples were resuspended in 100 µl, and flow cytometry on the BD FACSVerse Flow Cytometer initiated.

6.2.7. Population characteristics

More than 600 patients were screened for their participation in the observational, prospective SMB cohort. The majority of the screened patients did not meet the inclusion and exclusion criteria, or did not give their consent for participation. After excluding the patients who did not complete their inclusion in the trial due to loss-to-follow-up or death, 10 patients were included in the analysis with either a diagnosis of stroke or TIA upon admission to the stroke unit of the LMU university hospital (table 1). The stroke cohort consisted of 71% female participants (N = 5 out of a total 7 patients, with a median infarct volume of 14213 µl. 4 patients in the stroke cohort had a stroke of cardioembolic origin, 2 had a large artery atherosclerosis and 1 patient had an embolic stroke of unknown source. Patients in the stroke cohort had a median NIHSS of 3 at the time point of admission and 2 at the follow-up time point, with no change between the median baseline mRS of 2 and median follow-up mRS of 2. The TIA cohort consisted of 33% female participants (N = 1) out of a total 3 patients, presenting with a median infarct volume of 93.44 µl. 1 patient in the TIA cohort had a TIA of cardioembolic origin, 1 had a large artery atherosclerosis and 1 patient had a carotid artery dissection (Other). Patients in the TIA cohort had a median NIHSS of 0 at the time point of admission and 0 at the follow-up time point, with no change between the median baseline mRS of 0 and median follow-up mRS of 0. It was not possible to collect to EDTA-blood from one of the stroke patients at the chronic time point, thus this group is missing one data point

Table 1: SMB Cohort characteristics

Cohort	Stroke	Control	TIA
Number of patients	7	7	3
Age (Median, IQR)	71 (23)	71 (18)	60 (7)
Female, (%)	5 (71%)	5 (71%)	1 (33%)
Stroke characteristics			
Infarct volume in µl (median)	14213	-	93,44
Stroke Aetiology by TOAST			
LAA (n)	2	-	1
CE (n)	4	-	1
ESUS (n)	1	-	0
Other (n)	0	-	1
Antibiotic treatment prior to sample collection (n)	1	-	0
Clinical Presentation			
NIHSS (median)	3	-	0
NIHSS after 3 months (median)	2	-	0
mRS (median)	2	-	0
mRS after 3 months (median)	2	-	0

6.2.8. PRISE

6.2.9. Study design

The PRISE cohort was a prospective, randomized, double-blind, placebo-controlled interventional study. The study consisted of two intervention arms, named the probiotics and the placebo group. The probiotics group received a commercially available probiotic formulation containing 8 strains of bacteria and the prebiotic vehicle. The placebo group received only the vehicle without several of the prebiotic compounds (such as amylase, fructo-

oligosaccharides and inulin). A third group, termed the reference group, was also recruited. These participants were eligible to be recruited only if the lived in the same rooms as the study participants (e.g.., family members or spouses). This group provided only demographic data and a stool sample at the same time as the respective household member in the placebo or probiotic group. The recruitment of study participants was conducted between the 20.01.2021 and 28.11.2022, mono-centrically at the stroke unit of the LMU hospital. Double-blinding was carried out by an un-blinded member of the study team who was not involved in the screening, recruitment or examination of the patients.

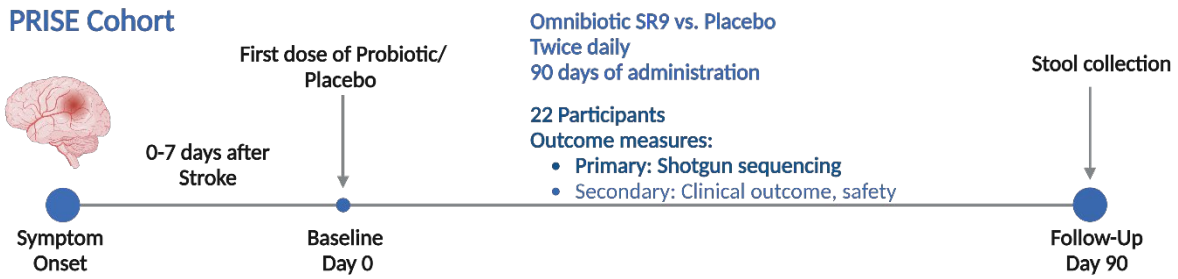

Figure 3: Study design of the PRISE cohort

6.2.10. Ethical statement

In order to initiate this project a new ethics application for a non-AMG/non-MPG interventional trial was submitted to the IEC of the LMU medical faculty. The IEC approved this project (labelled 20-0942) on the 11.12.2020. Due to the commercial licensing of the intervention as a food supplement, no additional approval was required for the study from the BfArM or other regulatory agencies.

Patients or their legal representatives were informed of the content and purposes of the trial. During the acquisition of written informed consent, patients and their legal representatives were also informed of their data protection rights and their ability to withdraw consent at any point in time without this having any negative repercussions for their further treatment. This study was performed with adherence to the most current tenets of the declaration of Helsinki and following Good Clinical Practice (GCP).

6.2.11. Study population

For the recruitment of patients into the probiotic and placebo arms of the PRISE study, the following eligibility criteria were used:

Inclusion criteria

i. Admission to the stroke unit of the LMU Hospital with a new diagnosis of stroke (Symptom onset ≤ 7 days)
ii. Age ≥ 18 years
iii. Written informed consent of the patient or their legal representative
iv. Patient must demonstrate sufficient compliance required for regular consumption of the study intervention

Exclusion criteria

i. Currently taking probiotics
ii. Pregnancy
iii. Participation in other interventional trials
iv. Known, active cancer diagnosis
v. Chronic immunological disease require treatment
vi. Immunosuppression
vii. Chronic inflammatory illness (i.e. pancreatitis)
viii. Haemorrhagic stroke or intracranial bleed
ix. Severely life-shortening prognosis
x. History of drug or alcohol abuse

During the 3-month participation of study patients, members of their respective household (both treatment groups) were recruited into the reference group during the telephone visits. This reference group underwent only stool sampling, using the same standardized stool sample collection kit. Participants in the reference group did not receive an intervention as part

of this study and were required to not have taken any other pre-/probiotic for at least 14 days before donating a sample.

6.2.12. Sample collection and preparation

The study participants of these 2 arms receive a standardized stool sample collection kit (OM-200.100-Microbiome Collection Kit) at the end of the 3-month intake period. Stool collection was done by the patient or with the assistance of supporting nursing staff/relatives.

6.2.13. Study intervention

The intervention group received the commercially available compound “OMNi-BiOTiC® SR-9” (a probiotic preparation consisting of corn starch, inulin, fructo-oligosaccharides and 9 live bacterial strains, distributed by Institut AllergoSan, Graz), while the placebo group received only corn starch as a placebo.

The study interventions (placebo/probiotics) were stored in sachets marked only with a number. The contents of the sachets were a white powder that was to be dissolved in water—thus, group allocation to either probiotics or placebo was not apparent to either the blinded study physician or the patient.

6.2.14. Outcome measures

Table 2: List of study specific measures

Group	Baseline Time point	Follow Up Time point (3 months)
Probiotics	NIHSS mRS	NIHSS mRS Stool collection BDI MoCA GSRS-IBS
Placebo	NIHSS mRS	NIHSS mRS Stool collection BDI MoCA GSRS-IBS
Reference	Stool collection	Not applicable

The primary endpoint of the study was the determination of gut microbiome composition, measured by phylogenetic diversity and the Shannon Diversity Index. Secondary endpoints of the study were the examination of the metabolome through blood samples and the collection of relevant clinical data through routine clinical examinations and questionnaires. The collected, pseudonymised clinical data include the infarct volume measured from CT/MRI routine examinations, the National Institute of Health Stroke Scale (NIHSS), the modified Rankin Score (mRS), Beck's Depression Inventory (BDI), Montreal Cognitive Assessment (MOCA), Gastrointestinal Symptom Rating Scale for Irritable Bowel Syndrome (GSRS-IBS), clinical laboratory chemistry from routine examinations, comorbidities, medication plans, and relevant data on stroke therapy and aetiology. The CT/MRI results are pseudonymised and evaluated at the ISD. The primary readout is the 16S RNA sequencing of the collected stool samples to determine the diversity and composition of the gut microbiome. Secondary

readouts are the National Institute of Health Stroke Scale (NIHSS) and modified Rankin Score (mRS) assessments at recruitment and sample collection. Additionally, bi-weekly telephone contact will be made to answer questions about preparation intake, changes in living circumstances (e.g., transfer to a care facility), and any complaints. Another secondary investigation involves the mass spectroscopic analysis of the collected blood samples. All described medical data will be pseudonymised from the medical records of the study participants.

6.2.15. Population characteristics

After excluding the patients who did not complete their inclusion in the trial due to loss-to-follow-up or death, 15 patients were included in the analysis with a diagnosis of stroke upon admission to the stroke unit of the LMU university hospital and randomly assigned to either the probiotic or placebo treatment group (table 3). The probiotic-treated cohort consisted of 16.7% female participants (N = 1) out of a total 6 patients, with a median infarct volume of 10472 µl. 2 patients in the probiotic-treated cohort had a stroke due to large artery atherosclerosis, 1 had a cryptogenic stroke, 1 patient had an embolic stroke of unknown source and 1 patient had a stroke from another cause. Patients in the probiotic-treated cohort had a median NIHSS of 4.5 at the time point of admission and 0.5 at the follow-up time point, with a change between the median baseline mRS of 2 and median follow-up mRS of 1. The placebo-treated cohort consisted of 22% female participants (N = 2) out of a total 9 patients, presenting with a median infarct volume of 637.75 µl. 2 patients had a stroke of cardioembolic origin, 6 patients had an embolic stroke of unknown source and 1 patient had a stroke from another cause. Patients in the placebo cohort had a median NIHSS of 2 at the time point of admission and 0 at the follow-up time point, with no change between the median baseline mRS of 1 and median follow-up mRS of 1. 7 household controls were recruited into the study as a reference cohort, which consisted of 85.7% female participants (N = 6) out of a total 7 participants.

Table 3: Characteristics of the PRISE Study population

Cohort	Probiotics	Placebo	Reference
Number of patients	6	9	7
Age (Median, IQR)	62 (11.25)	64 (22)	76 (30)
Female, (%)	1 (16.7%)	2 (22%)	6 (85.7%)
Stroke characteristic			
Infarct volume in µl (median)	10472	637.75	-
Stroke Aetiology by TOAST			
LAA (n)	2	0	-
CE (n)	0	2	-
Cryptogenic	1	0	-
ESUS (n)	2	6	-
Other (n)	1	1	-
Antibiotic treatment prior to sample collection (n)	0	1	-
Clinical Presentation			
NIHSS (median)	4.5	2	-
NIHSS after 3 months (median)	0.5	0	-
mRS (median)	2	1	-
mRS after 3 months (median)	1	1	-

6.2.16. Animal experiments

All procedures described here were subject to prior review and authorization by the responsible regulatory agency (Regierungspräsidium Oberbayern, Munich Germany). Male, WT, 8 week old C57BL6J mice were purchased from Charles River Laboratories (Germany, located in Sulzfeld) and housed at the animal core facility of the Centre for Stroke and Dementia Research (CSD). The holding facility for the mice was kept at regulated temperatures (22 ± 2 °C), with a 12-h light–dark cycle. All mice had access to food and water ad libitum. Any mice that died during surgery or before the completion of the designated trial period were excluded from further analysis. Furthermore, all animal experiments were reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines [56].

6.2.17. Stroke model and severity assessment

A previously published model of transient middle cerebral artery occlusion (MCAo) was used in this cohort [57]. Surgeries and clinical assessment were performed by Christina Bauer. Briefly, the mice were anesthetized with a mixture of isoflurane in 30% oxygen and 70% nitrous oxide. Body temperature was maintained at 37°C. After successful induction of anaesthesia, an incision exposed the temporal bone, and a laser Doppler probe was placed over the territory of the middle cerebral artery (MCA). The common and left external carotid arteries were ligated, and a 2-mm silicon-coated filament was inserted to block the MCA, confirmed by a > 80 % reduction in blood flow. After 45 minutes, the filament was removed. Mice were then returned to their cages with access to food and water. Sham-operated mice underwent similar procedures without prolonged filament insertion. Any mice that died during the surgery, had an inadequate drop in CBF (> 20 %) as measured by Doppler flow or had clinical presentation incongruous with a stroke at day 1 after surgery were excluded from further analysis. The drop-out rate was ~ 50 %, excluding sham-operated mice. Clinical assessment was performed daily for the first week after surgery.

6.2.18. Animal perfusion and tissue collection

Stool samples were acquired by collection of fresh droppings directly deposited in a 1 ml Eppendorf tube, these samples were then stored at -80 °C for later DNA extraction. In analogous fashion to the SMB cohort, DNA extraction was performed using the QIAmp Fast DNA Stool Mini Kit (Qiagen) with the help of Kerstin Thuß-Silczak, after which samples were kept at -20 °C for a maximum of 2 weeks until sequencing was performed. The DNA concentration and stability of each sample was subsequently measured via NanoDrop measurement for quality control purposes before sequencing. In preparation for internal tissue collection, the C57BL6J mice were anaesthetized using a drug cocktail consisting of ketamine (120 mg/kg) and xylazine (16 mg/kg). Then, venous blood was drawn directly from the right

cardiac ventricle and deposited in tubes (Sigma-Aldrich) containing 50 mM EDTA. This blood was subsequently centrifuged (settings: 2000g RCF, 10 minutes at 4°C) and the plasma stored at – 80 °C for later metabolomics analysis. Directly after the draw of EDTA blood from the heart, the mice were perfused via PBS injection to the left ventricle of the heart. After perfusion of the animals, the small intestines (from upper duodenum to lower ileum) were surgically removed and the contents of the ileum were placed in a 2 ml Eppendorf tube and stored at – 80 °C until ready for analysis via mass spectrometry.

6.2.19. Statistical Analysis

Data analysis was performed with the help of Adam Sorbie from the Institute for Stroke and Dementia Research (ISD), as well as Velma Aho from the Luxembourg Centre for Systems Biology. Sequencing data was analysed using the DESeq2 analysis package for the programming language “R”. All summary data is presented as the mean ± standard deviation (s.d.) unless indicated otherwise. The groups containing normally distributed dependent data were analysed using paired t tests (= 2 groups). Alpha diversity was compared using a Wilcoxon Signed Rank test. Beta diversity dissimilarity was compared on the basis of the Bray-Curtis index using adonis2 (PERMANOVA). P values < 0.05 were considered to be statistically significant, unless otherwise indicated.

7. Results

7.1. A model of murine stroke induces a persisting state of gut dysbiosis

In order to determine the persistence of gut microbiome dysbiosis past the initial acute phase of stroke, 5 male wild type C57BL6J mice received transient MCAo. Stool was collected manually from the mice directly before surgery, 3 days after fMCAo and 14 days after fMCAo. The mice were sacrificed 14 days post-fMCAo and EDTA-blood (for the subsequent collection of plasma) was extracted directly from the heart, while ileum content was collected from the small intestine (Figure 4).

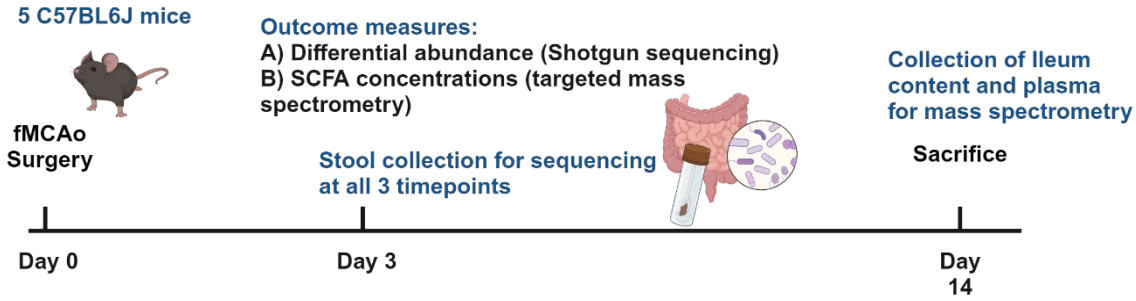

Figure 4: Study overview of murine pilot experiment. Mice were given fMCAo for 60 minutes and stool was collected at day 0 (before surgery), day 3 and day 14. The mice were sacrificed at day 14, EDTA-blood and ileum content were collected.

An additional cohort of 5 male C57Bl6J mice were sacrificed and EDTA-blood, as well as ileum content, was collected. This cohort served as the naïve reference control for the mass-spectrometry experiments. Shotgun sequencing was performed on the collected stool samples and an inter-time point analysis was performed. To analyse whether broad changes in the composition of the gut microbiome occurred after ischemic stroke, the alpha diversity measures of richness, Shannon index and inverted Simpson index were calculated. These analyses yielded no significant differences between any of the three time points (Figure 5).

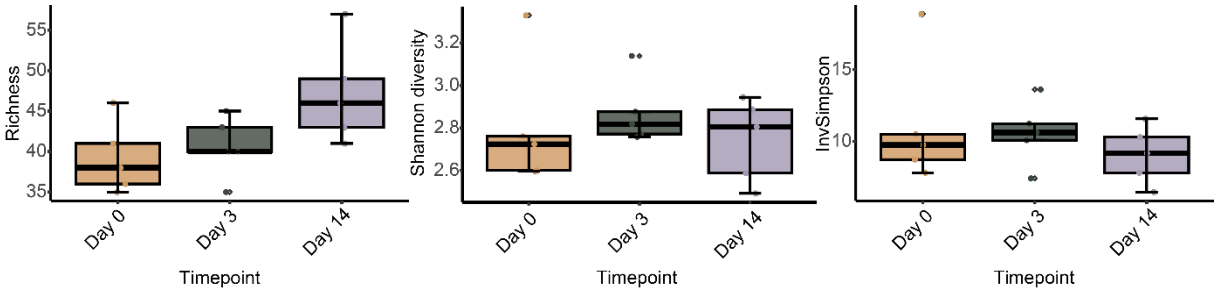

Figure 5: Alpha diversity measures of richness (left), Shannon index (centre) and inverted Simpson index (right) for each time point of stool collection.

In order to further investigate changes within the composition of the microbiome, the relative abundance of individual species comprising the genetic material of the microbiome were determined (Figure 6A), showing the expansion/reduction of the representation of individual species across 14 days (such as shows the expansion of *Bacteroides bacterium M10*). Finally, the shift in phylogenetic diversity on the basis of the Bray-Curtis index for each time point. In contrast to the diversity of bacterial species within the entire system remaining unchanged (alpha diversity measures), the beta diversity indices showed a significant ($p = 0.002$, $R^2 = 0.37$) change with a progression away from baseline across day 3 and day 14 (Figure 6B).

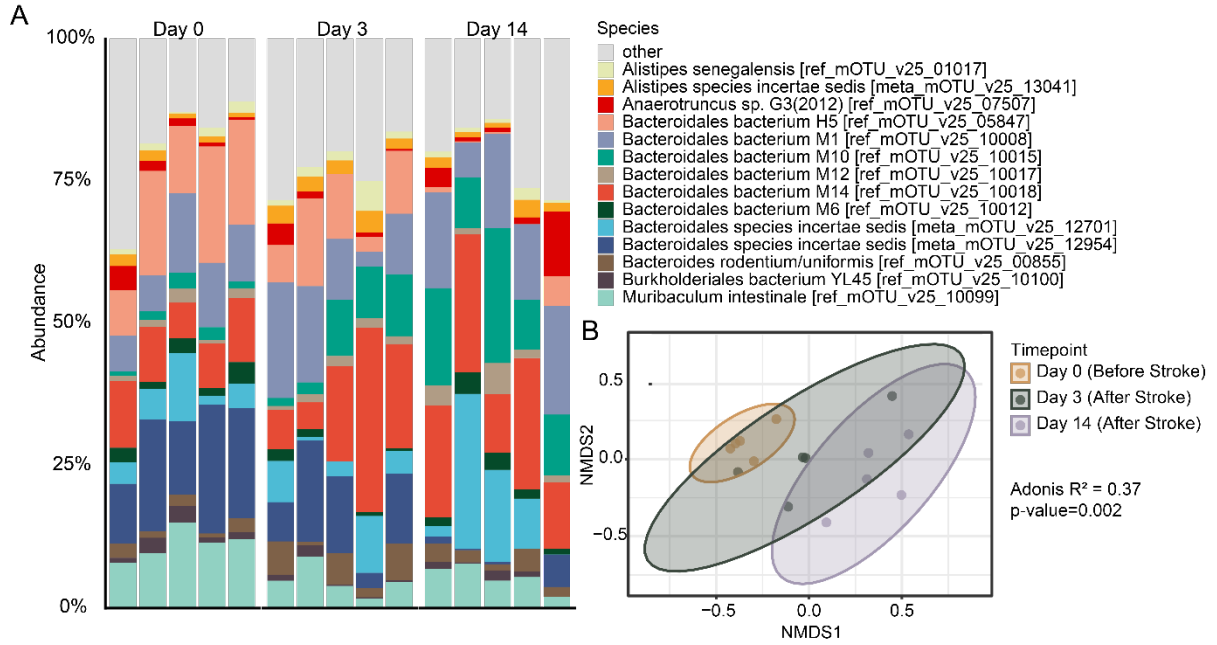

Figure 6: A) Relative abundance (in %) of bacterial species (see legend) at day 0, day 3 and day 14 after fMCAo surgery. B) PCA of all 3 time points (day 0, day 3 and day 14), plotted by using the Bray-Curtis index ($p = 0.002$, $R^2 = 0.37$)

Finally, the collected ileum content and plasma samples were analysed by targeted mass-spectrometry to measure the concentrations of the short chain fatty acids (propionate, isovalerate, formate, butyrate and acetate). The concentrations of the SCFAs did not significantly differ between the naïve and fMCAo cohorts in the plasma samples, nor were the isovalerate, formate, butyrate, acetate significantly different in the ileum content between the two cohorts. However, the concentration of propionate was significantly lower ($p = 0.032$) in the fMCAo cohort, than in the naïve cohort and the concentration of Acetate demonstrated a trend in that direction which may have been influenced by an outlier (Figure 7).

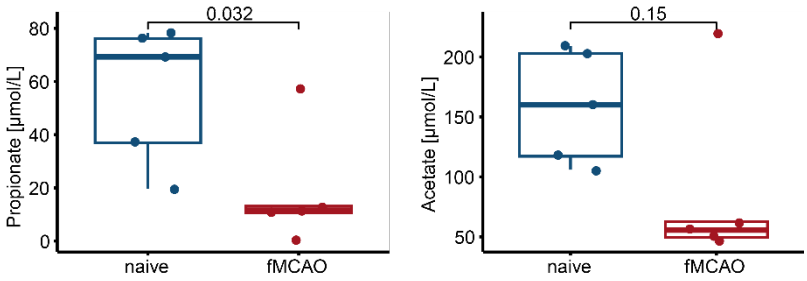

Figure 7: Concentrations (in µmol/L) of propionate and acetate as measured in the ileum content of a naïve and 14 days post-fMCAo cohort. The fMCAo cohort showed a significantly lower concentration of propionate than the naïve cohort, while a trend toward lower concentrations in the fMCAo cohort was observed for acetate.

7.2. Key bacterial populations of the gut microbiome remain altered after ischemic stroke in a human cohort

The relative abundance of measured bacterial genera in all 4 subpopulations captured in this study is shown in Figure 8 (Control, TIA, Mild Stroke and Severe Stroke). The TIA group was excluded from further analysis due to the small sample size and in all further analysis the mild and severe stroke groups were treated as a single group (stroke).

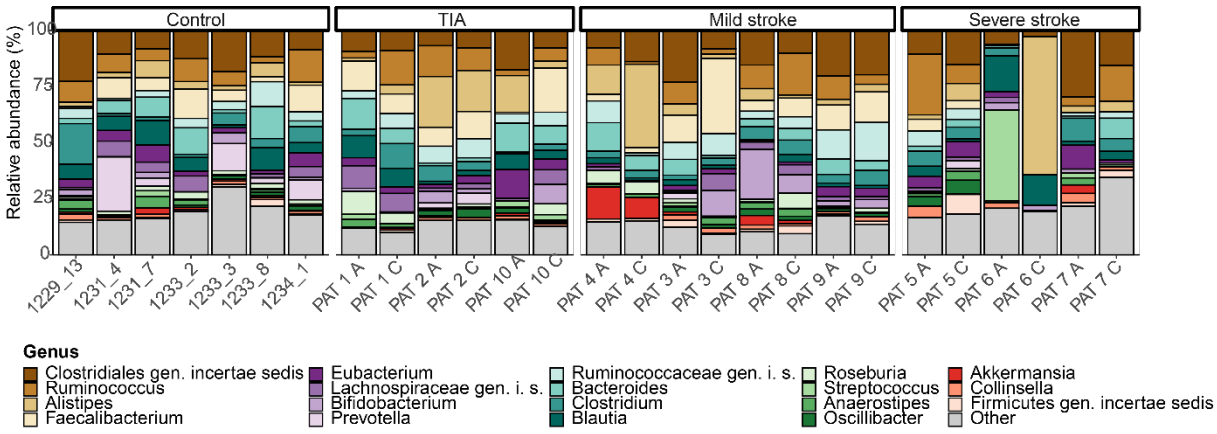

Figure 8: Relative abundance (in %) of measured bacterial genera (see legend) across all patient groups (from left to right): Control cohort from Kassel (one time point only), TIA group from LMU, mild stroke group from LMU, and severe stroke group from LMU. The x-axis show the unique patient identifiers used in the trial, as well as the time point of the measurement (A = acute; C= chronic).

After determining the relative abundance of individual bacterial genera in the individual samples, the groups were compared for shifts in broad bacterial composition using the alpha diversity measure of observed richness. There was a significant change ($p = 0.047$) between the acute stroke and chronic stroke cohorts (Figure 9, right). However, if the lowest value in the chronic stroke cohort (Data point PAT 6 C in Figure 8) is regarded as an outlier, the p value would only signify a statistical trend ($p = 0.094$). This finding shows a trend toward a loss of bacterial diversity that is not ameliorated across time. Furthermore, an NMDS analysis using the Bray-Curtis index revealed that there were no significant changes when comparing all 3 groups (Control, acute stroke and chronic stroke) (Figure 9, left).

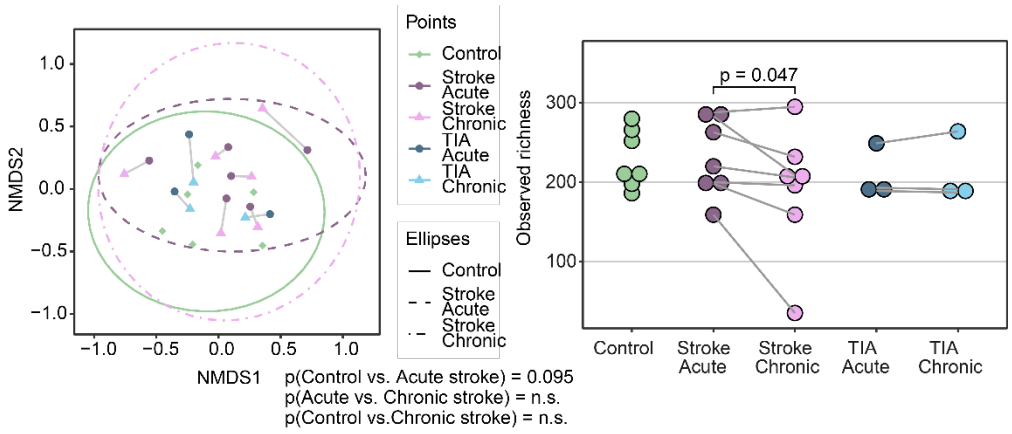

Figure 9: (Left) NMDS plot of the Bray-Curtis index showing 5 populations and 3 clusters, control vs acute stroke, acute vs. chronic stroke and control vs. chronic stroke. (Right) Observed richness (alpha diversity measure) of all 5 populations. Statistical measures were only compared between the control, acute stroke and chronic stroke groups.

When the Bray-Curtis indices were compared in a pairwise fashion, the acute vs. chronic stroke group analysis yielded no significance (not shown). However, the control vs acute/chronic stroke comparisons showed that the beta diversity of the gut microbiome is affected in the acute phase after stroke and had failed to recover 3 months later, indicating that stroke induces a persistent change to the microbiome, which is not ameliorated by the passage of time (Figure 10).

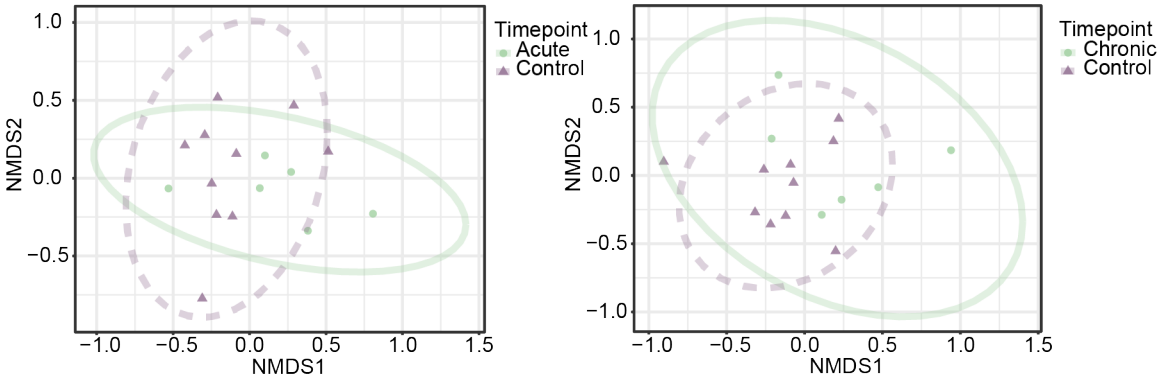

Figure 10: NMDS plots comparing the Bray-Curtis index between the acute stroke and control groups (left, p = 0.007, R^2 = 0.11), as well as the control and chronic stroke groups (right, p = 0.045, R^2 = 0.09)

To stratify the effect on the individual bacterial populations affected by stroke, the differential abundance of bacterial genera between the acute and chronic stroke groups were compared to the control population (Figure 11). A volcano plot was created to show the distribution of individual, significantly affected genera when comparing acute stroke with control, and chronic stroke with control. This analysis identified a significant loss of the bacterial genera *Prevotella*, *Eggertheliaceae genus incertae sedis, Proteobacteria*, and *Clostridiales* at both time points. These bacterial populations are heavily affected by the stroke in the acute phase and do not recover 3 months later.

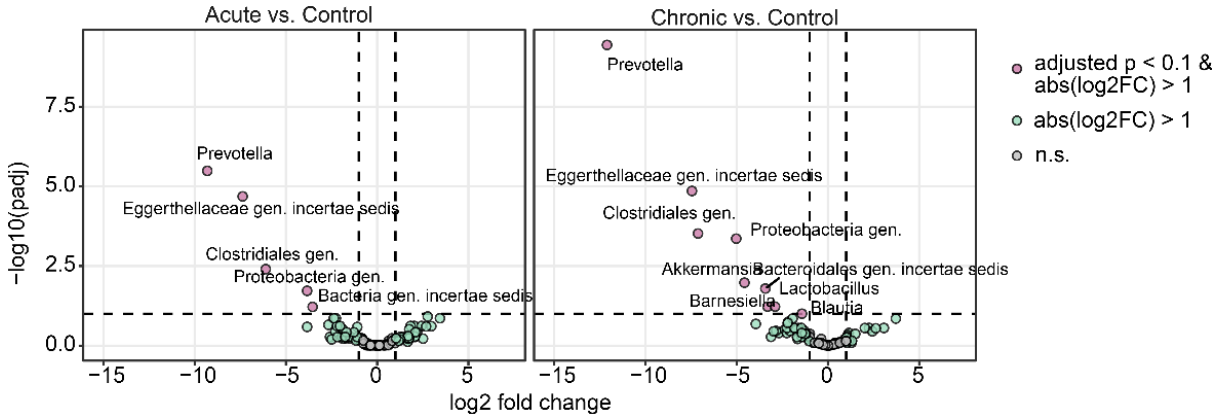

Figure 11: Volcano plot of differentially abundant genera between the acute and control groups (left) as well as the chronic and control groups (right).

7.3. Activation markers in the myeloid lineage and monocyte counts were relatively decreased 90 days after stroke

Flow cytometry analysis was performed on EDTA blood from patients at baseline and day 90. The fluorophores included in the analysis were designed to measure immune cells from both the lymphoid and myeloid lineages, such as B cells, CD8 and CD4 positive T cells, NK cells and monocytes. Additionally, several activation markers (including scavenger receptors CD163 and CD36) were included in the panels. The relative percentage of monocytes (CD45+ CD11b+ CD14+) and several activation markers within the myeloid lineage (CD45+ CD11b+ HLA+ cells, CD45+ CD11b+ CD36+ cells and CD45+ CD11b+ 163+ cells) was significantly decreased 3 months after stroke when compared to the acute phase (Figure 12).

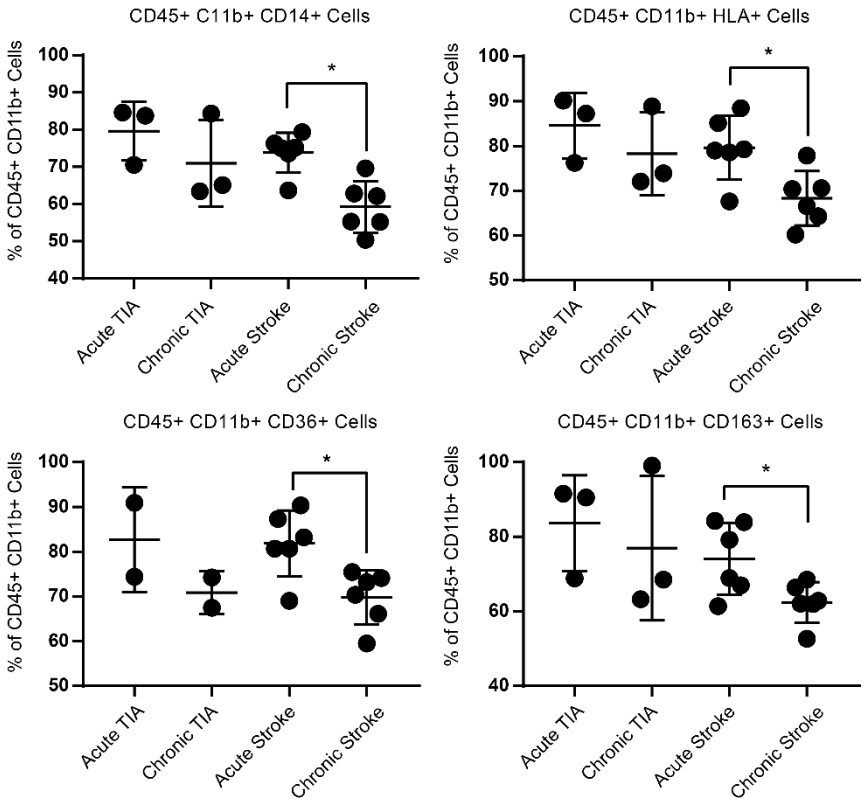

Figure 12: The dot plots show the relative percentage of myeloid CD45+ Cd11b+ cells in the that were signifantly different in acute stroke and chronic stroke groups between baseline and day 90. Acute TIA and chronic TIA were excluded from analysis for statistical reasons. Paired t-tests were used to compare the acute and chronic stroke cohorts.

7.4. Ischemic stroke is associated with a reduction of key bacterial metabolites

We further aimed to investigate the functional output of the gut microbiome by characterizing previously described gut metabolites via targeted and non-targeted mass-spectrometry. First, the results of the targeted mass-spectrometry were assessed, showing significant alteration in 3 key SCFAs. The concentration of faecal valeric acid (Figure 13, left) was significantly decreased in the acute phase after stroke ($p = 0.041$) and remained significantly decreased after 3 months ($p = 0.036$) when compared to the control group. In a similar fashion, propionic acid (Figure 13, right) showed a statistical trend towards reduction in the acute stroke group ($p = 0.069$), which became significant after 3 months ($p = 0.049$). Concentrations of butyric acid (Figure 13, centre) were significantly decreased ($p = 0.003$) in the acute phase after stroke but recovered after 3 months.

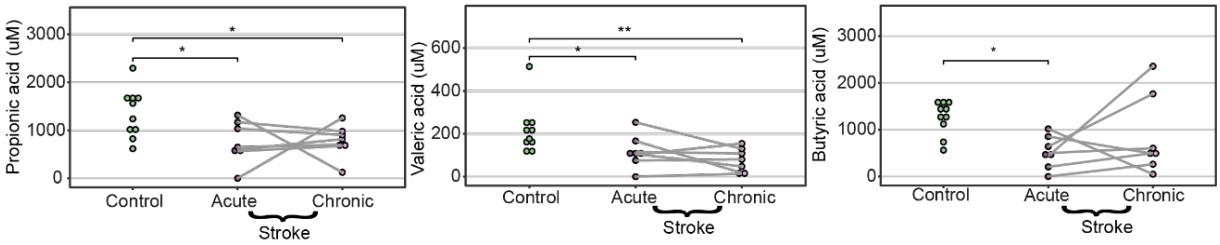

Figure 13: Concentrations in (µM) of valeric acid, butyric acid and propionic acid as measured in the ileum of the study participants. (Paired t test was performed, TIA was excluded)

Next, we assessed the key significantly different metabolites between the acute and chronic stroke groups in the untargeted mass-spectrometry. After excluding metabolites that showed a p value > 0.1 after correction for false discovery rate, two metabolites measured in the plasma remained (Figure 12). Indole-3-propionic acid was significantly increased ($p = 0.003$) between acute and chronic stroke conditions, while pyruvic acid, was significantly decreased ($p = 0.003$) between the two conditions.

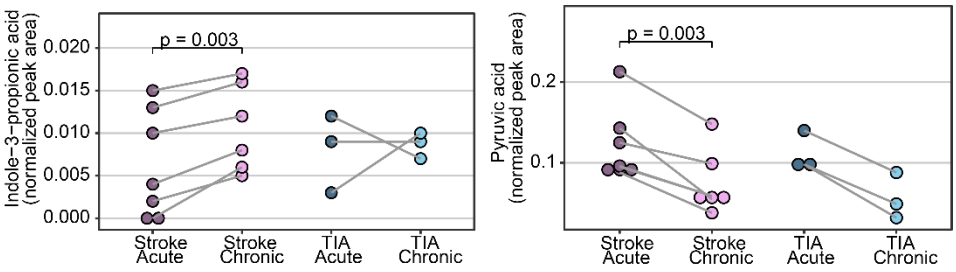

Figure 14: Normalized peak areas of the top 2 detected metabolites (Indole-3-propionic acid and pyruvic acid) in the plasma of study participants.

Table 4 presents the most prominently identified metabolites from the untargeted mass-spectrometry analysis. However, it is important to note that data from the control group are absent in this analysis. This gap occurred due to differing methods being employed for sample analysis (i.e., there was no LCMS performed on the control samples), resulting in a lack of comparable data for the control group in the untargeted mass-spectrometry analysis. Consequently, the identified metabolites in Table 4 reflect findings from only the stroke groups, hindering our ability to directly compare these metabolites with those in the control group.

Table 4: List of metabolites identified during untargeted mass-spectrometry that differed between the acute and chronic stroke time points. (Paired t-tests were performed, unknown compounds have been removed from this list)

Feature	p-value	FDR	Data source
Indole-3-propionic acid	0.003	0.088	Plasma LCMS
Pyruvic acid	0.003	0.088	Plasma LCMS
L-Cystine	0.019	0.250	Plasma LCMS
Indole-3-lactic acid	0.021	0.250	Plasma LCMS
Glutamic acid 3TMS	0.025	0.953	Plasma GCMS
L-Lactic acid	0.028	0.250	Plasma LCMS
Indole-3-acetic acid	0.028	0.250	Plasma LCMS
Betaine	0.029	0.250	Plasma LCMS
X5-Hydroxyindole-3-acetic acid 3TMS	0.032	0.766	Faecal GCMS
Fumaric acid 2TMS	0.043	0.766	Faecal GCMS
Thymine 2TMS	0.046	0.766	Faecal GCMS
Cytosine 2TMS	0.047	0.766	Faecal GCMS
Thymine T	0.048	0.708	Faecal LCMS

7.5. Dysregulated bacterial populations associate with the production of short chain fatty acids

Finally, we investigated whether the previously observed reduction in bacterial metabolites, particularly short-chain fatty acids (SCFAs), was associated with the bacterial genera (Figure 11) and mOTUs (not shown) that were significantly diminished under stroke conditions (Figure 15). Our analysis revealed a significant correlation between the genus *Prevotella* — which exhibited the most pronounced dysregulation — and the production of propionic acid and valeric acid across all samples. Similar correlations were observed for a genus of *Butyricicoccus*, a genus within the order *Bacteroidales*, and a genus of *Faecalibacterium*, all of which had been previously shown to decrease during both the acute and chronic phases following stroke. These findings suggest that the loss of these key bacterial populations may contribute to the observed reduction in SCFA production, potentially impacting gut-brain interactions in stroke pathology.

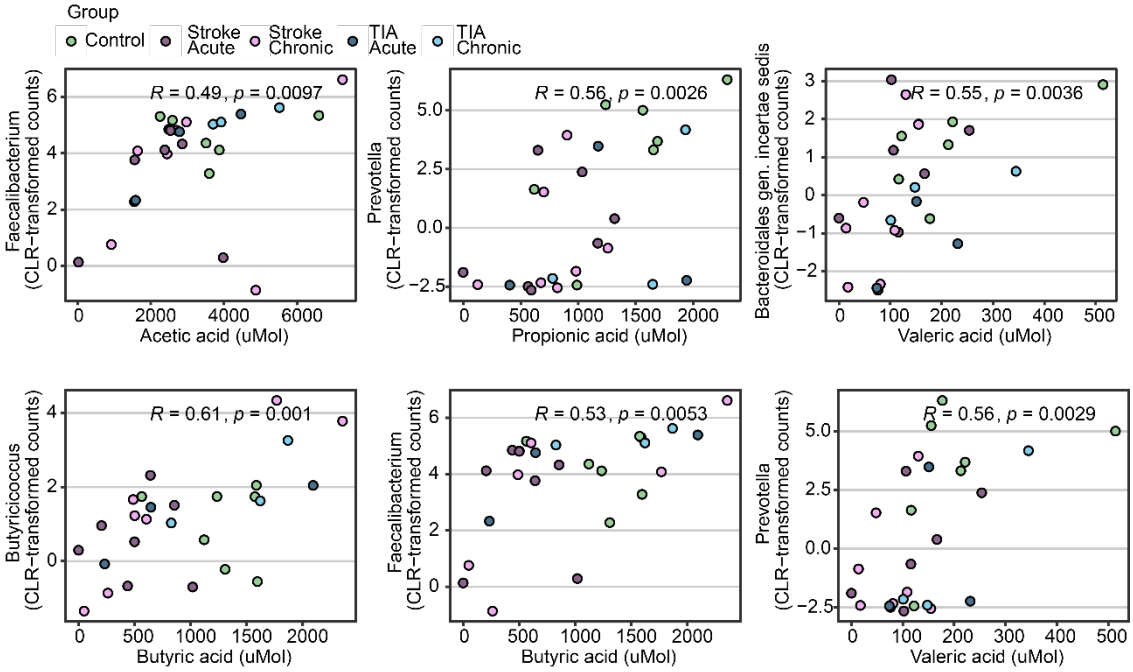

Figure 15: Plots show correlations identified between SCFAs and key bacterial populations in all samples. R = Spearman coefficient.

7.6. Probiotic intervention affects bacterial diversity of the gut microbiome and key bacterial populations in stroke patients

The primary outcome of the intervention study was to determine the effect of twice daily probiotic consumption on the gut microbiome of stroke patients. To characterise the changes in bacterial diversity, the alpha diversity index of observed richness, as well as the beta diversity index, Bray-Curtis, were compared between the treatment groups (reference, placebo, and probiotics). In both indices, when comparing across all 3 groups, no significant changes could be identified (Figure 16, left and right). Additionally, the observed richness between the control (reference) group and the pooled stroke group (from placebo and probiotics group) showed no significant differences (Figure 16, centre).

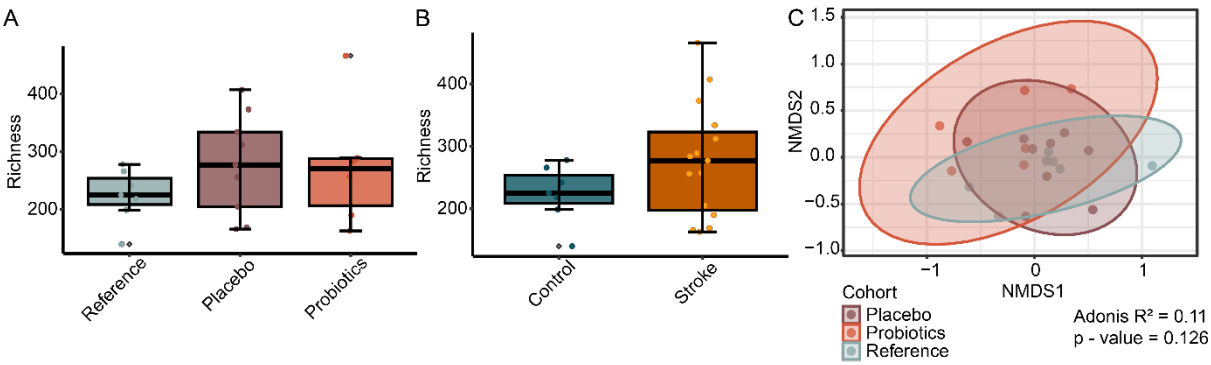

Figure 16: A) Observed richness across all 3 groups. B) Observed richness between reference and stroke group, as pooled from the placebo and probiotics groups. C) NMDS plot of the Bray-Curtis index comparing all 3 groups

However, when the Bray-Curtis indices were compared between the treatment groups in a pairwise comparison, the Euclidian distance from control between the reference and probiotics group was significantly increased (Figure 17), in contrast to the other pairwise comparisons, which were not significant (data not shown).

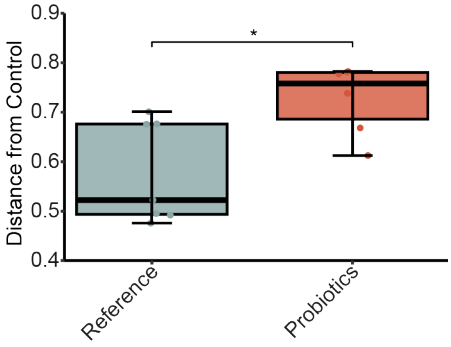

Figure 17: Euclidian distance of the placebo group from the reference group based on the Bray-Curtis index

After analysing shifts in the general populations of the gut microbiomes of the three treatment groups with alpha and beta diversity measures, the differential abundance of mOTUs (Figure 18) was compared between all three treatment groups. The mOTU level comparison of differential abundance yielded several species that were significantly ($p > 0.1$ and Log_2 FC > 1) increased or decreased between the probiotics and placebo groups. Decreased species included *Roseburia intestinales, Dialister invisus*, a species of the taxa *Firmicutes* and a species of the genus *Clostridiales*. Increased species included *Dorea longicatena*, a different species of the genus *Clostridiales* and a species of *Streptococcus*. The comparison of reference versus probiotics yielded a single significantly increased species (*Dialister invisus*). The analysis on a family and genus wide level did not yield any significant population shifts (likely due to sample size issues).

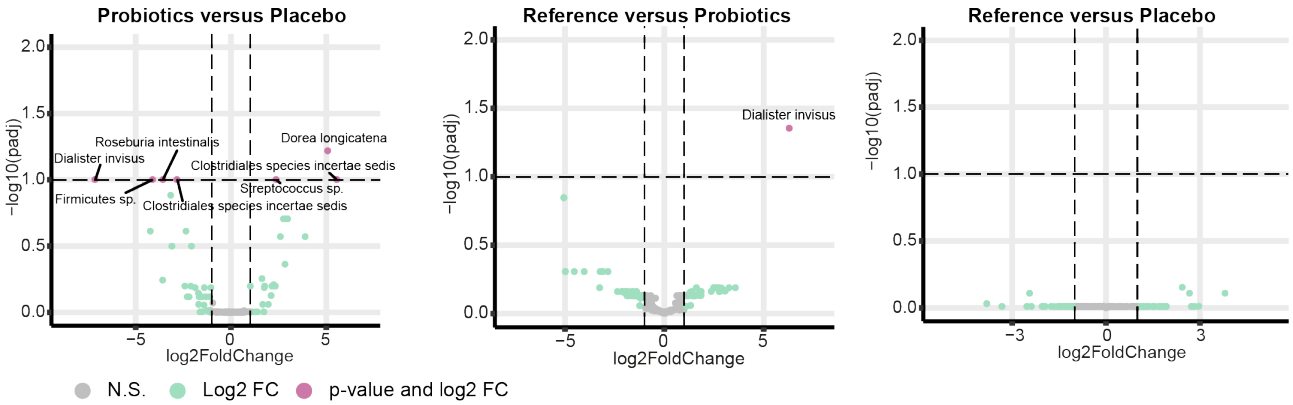

Figure 18: Volcano plot of differentially abundant bacterial mOTUs between the placebo and probiotics group. Significant p-values were defined as < 0.1.

After comparing all differential abundances across the treatment groups, a comparison of the relative abundances of the previously identified, stroke-affected, individual genera (in the SMB cohort) was performed (Figure 19). This analysis showed that a genus of *Eggerthellaceae* was significantly different between the probiotics treated group and the other groups. The genus *Prevotella* was also significantly increased in the placebo and probiotics groups. Finally, the genus *Barnesiella* was significantly decreased between reference and placebo.

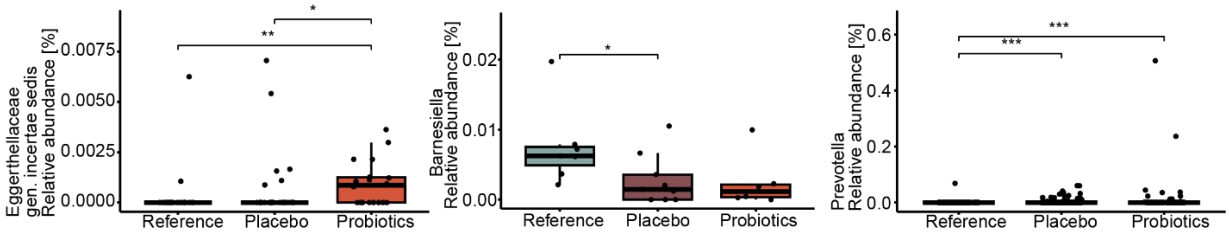

Figure 19: Relative abundances (in %) of key bacterial genera previously identified to be dysregulated after stroke in the observational cohort

7.7. The gut microbiome may be colonized by probiotic intervention

After analysing for differential abundance of individual genera, we investigated whether the probiotic strains could be identified in the faecal samples. Figure 20 shows a heat map of the relative abundance of all 8 bacterial species contained within the probiotic supplied by AllergoSan. *Bifidobacterium animalis* could be detected in 5/6 patients given probiotic intervention, with *Lactococcus lactis* and *Lactobacillus casei/paracasei* being detected in 4 of 6 patients. In 2 patients only a single strain of probiotic bacterium could be measured (*Bifidobacterium animalis* and *Bifidobacterium bifidum* respectively). Finally, 2 of the probiotic strains were not detected in any of the probiotic treated patient (*Lactobacillus salivarius* and *Lactobacillus acidophilus*). By contrast, the placebo and reference groups demonstrated far

fewer detectable probiotic strains. We deduce that, at least in part, the probiotic strains may be colonizing the gut microbiome of the probiotic group.

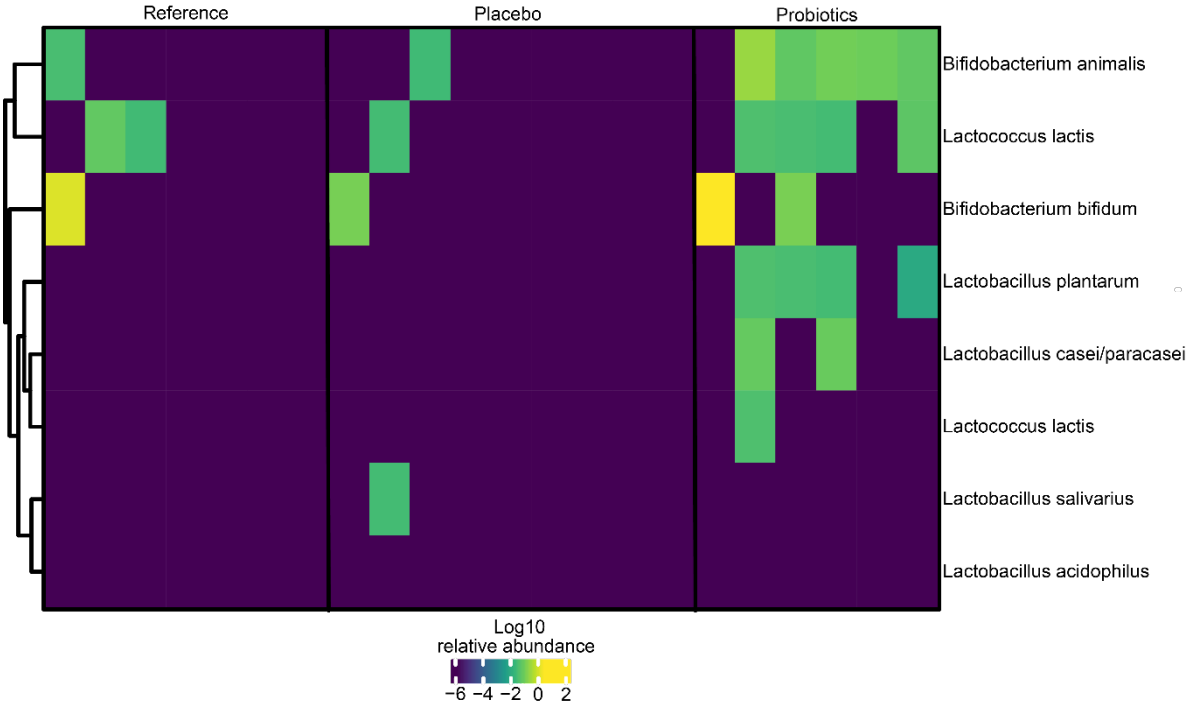

Figure 20: Heat map of the relative abundance of the 8 bacterial species of the probiotic as measured in the faecal samples of participants from all 3 treatment groups.

8. Discussion

8.1. Summary of results

The pilot experiments of this thesis demonstrated that the diversity of the gut microbiome in a cohort of C57BL6J mice was affected by a model of ischemic stroke. The trend in beta diversity 14 days after fMCAo was away from baseline. Concurrently, a significant loss of the concentration of a key SCFA, propionate, as compared to control mice and measured via targeted mass-spectrometry was demonstrated. In an observational cohort of stroke patients, a significant change in beta-diversity at both an acute time point, as well as at 3 months after stroke was measured, as compared with the control group. Furthermore, there was a significant decrease in alpha diversity of the microbiome between the acute and chronic time points of the stroke patient group. It was furthermore observed, in the targeted mass-spectrometry analysis, that the concentration of the SCFA butyrate was significantly decreased at the acute time point after stroke when compared to the control group, but recovered after 3 months. The concentrations of the SCFAs valerate and propionate were also significantly decreased after stroke as compared with control, but did not recover 3 months later. The untargeted mass-spectrometry revealed that further key bacterial metabolites were significantly different between acute and chronic stroke conditions, in particular Indole-3-propionic acid and pyruvic acid as measured in the plasma of stroke patients. Finally, the genera and mOTUs, that were significantly differentially abundant between baseline and follow up, were correlated with the main SCFAs (propionate, butyrate and acetate), as well as valerate, concentrations. This analysis yielded a significant positive correlation of the genus *Prevotella* with propionate and valerate, a genus of *Faecalibacterium* correlated positively with butyrate and acetate, a genus of the order *Bacteriodales* correlated positively with valerate and a genus of *Butyricicoccus* correlated positively with butyrate. The PRISE study revealed that the beta diversity of the gut microbiome of probiotic treated patients was significantly different when compared to the reference group, in contrast to the placebo group, however there were

no significant differences between placebo and probiotics. No further alpha or beta diversity measures were significantly different between any of the treatment groups. A comparison of the differential abundance of bacterial mOTUs between treatment groups revealed that individual species of bacteria were significantly different in abundance between probiotics and placebo treated groups, however no genera or families, as well as further comparison among all treatment arms revealed significantly differentially abundant bacterial populations (except the species *Dialister invisus* between probiotics and reference groups). Furthermore, the analysis of the specific genera that were significantly differentially abundant after stroke across either time points (acute and 3 months) in the observational cohort revealed that the genus of *Eggerthecellae* was significantly differentially abundant in the probiotic treated cohort. The genus of *Barnesiella* was also significantly differentially abundant between the reference and placebo group. The *Prevotella* genus was significantly differentially abundant in both comparisons between reference and either treatment arm. Finally, it could be shown that the strains of the probiotics could be identified in the stool of patient receiving probiotics to a higher degree versus reference and placebo. This indicates possible colonization of the gut microbiome by the probiotic strains.

8.2. Gut dysbiosis primes a systemic immune response and drives secondary comorbidities

The gut microbiome is capable of influencing the central nervous system directly via vagal impulses, through the blood stream via locally produced neurotransmitters (such as serotonin), hormones, metabolites, and circulating immune signals and by influencing the immune system via polarization of immune cells with metabolites and molecular patterns [58]. This means that signalling between the gut and the brain is bi-directional, while stroke induces dysbiosis, the gut microbiota are capable of acting upon the brain via the enteric nervous system, through direct connection with the brain through the vagus nerve, by sending chemical and humoral

signals, as well as differentiating immune cells that migrate to the brain or regulate pro- and anti-inflammatory cascades [59].

As demonstrated in previous literature and both the murine (Figure 6) and human observational cohorts (Figure 10), ischemic stroke leads to the acute dysbiosis of the gut microbiome. Here we have shown however, that this dysbiosis does not improve after 14 days in the murine cohort or 3 months in the human cohort, with beta diversity indices shown to clearly shift at the later time points of these cohorts. The persistence of gut microbiome dysbiosis can lead to the perturbation of intestinal immune homeostasis and thus the induction of chronic inflammation of the gut and by extension other organs. Gut dysbiosis has been characterized as a loss or weakening of host control over the gut microbiome and has been associated with numerous further diseases [60]. Chronic inflammation has been associated with development of numerous different types of cancer and intestinal inflammation, caused by dysbiosis, is no exception, as has previously been shown in mice models of colorectal cancer and colitis [61]. There is now also mounting evidence that dysbiosis is associated with the development of young-onset colorectal cancer in humans, with dysbiosis potentially becoming relevant for screening and diagnostics in the future [62]. Beyond the field of cancer research, gut dysbiosis has been shown to accelerate the development of diseases like non-alcoholic fatty liver disease by impairing the function of the intestinal barrier, thus allowing the pro-inflammatory molecules it produces (such as lipopolysaccharides and trimethylamine) to enter the liver and drive the existing inflammation forward [63]. In the field of auto-immune diseases, dysbiosis has been shown to be present in patients with systemic lupus erythematosus. Furthermore, interventions targeting dysbiosis were shown to ameliorate the disease progression of SLE in mouse models [64]. Gut dysbiosis has now been shown to be causally involved in the development and regulation of hypertension (a key risk factor for stroke) with protective molecules such as SCFAs and indole-3-lactic acid lowering blood pressure, while detrimental compounds like trimethylamine increase blood pressure [65]. Finally, there is now evidence from animal models, suggesting the microbiome and its metabolites may play a crucial role in

the formation of cognitive and behavioural functionality. Thus, in the presence of dysbiosis, the correct development of mental health (in the form of depression, anxiety, stress response and more) may be impaired [66].

8.3. Stroke, intestinal immunity and the gut microbiome

Our results here demonstrate that both in the murine and human observational cohort, the concentrations of key bacterial metabolites are reduced after stroke. Previous studies have shown, that ischemic stroke induces a dysregulation of the concentrations of bacterial metabolites in the acute phase after stroke [67] and our findings are in line with this. Short chain fatty acids have shown to be one of the main groups of bacterial metabolites, that act as mediators between the gut microbiome and immune system [68]. These SCFAs are produced by the gut microbiota from dietary fibres (the most common SCFAs being butyrate, propionate and acetate) and have been identified as playing a key role in numerous other diseases and cardiovascular diseases in particular [69, 70]. Amongst other functions, SCFAs affect the enteric nervous [71] and the intestinal immune system, leading to a differentiation of T regulatory and interleukin 10 producing T cells [72, 73]. SCFAs further affect immune cells (such as neutrophils, dendritic cells, macrophages, monocytes and T cells) by regulating the production of inflammatory cytokines (like Tumour Necrosis Factor and Interleukin 12) and chemoattractants (like CXCL1 and CXCL8), possibly through the inhibition of histone-deacetylases [74]. Furthermore, SCFAs have been shown to affect the brain through regulation of gut hormone release and production (e.g., upregulation of Peptide YY or suppression of Ghrelin production), as well as neural pathways, such as directly acting on vagal afferences within the gastrointestinal wall [74]. Additional studies have shown that SCFAs are capable of improving the integrity of the blood brain barrier and affecting expression of neurotransmitters in the hypothalamus [75]. We demonstrated that across 2 species, there is a reduction of these SCFAs at the acute and chronic time points and importantly the same major SCFA, propionate, is reduced across species.

The relevance of these findings lies in the previous publications that demonstrate how stroke induces a cascade of immunological responses both as an immediate and delayed response. Initially, disruption of the blood brain barrier allows for the infiltration of immune cells (in particular cell from the myeloid lineage, such as neutrophilic granulocytes and macrophages), that become activated by molecules released from dying neurons, leading to local inflammation and further death of penumbral neurons [76]. Furthermore, stroke induces the increased migration of immune cells to both peripheral immune organs and the brain from the small intestine [77]. The “healthy” gut microbiome primes an anti-inflammatory reaction to stroke, causing a shift in the Th_{17}-T_{reg} axis towards increased expression of neuroprotective T_{reg} cells [78]. A previous study performed on cynomolgus monkeys demonstrated that SCFAs (butyrate, acetate and propionate) were significantly decreased after experimental stroke surgery and did not recover 12 months later [79], but to our knowledge no such evidence has previously been reported in humans. In consideration of the protective effects that have been previously ascribed to SCFAs, our novel finding that the concentrations of valerate and propionate do not recover after three months in stroke patients has serious implications for the development of secondary comorbidities, as well as the recovery of stroke survivors.

8.4. Probiotics as a novel treatment option in ischemic stroke

We demonstrated that probiotics may successfully colonize the gut after a stroke, with specific genera and species of bacteria showing differential abundance in the probiotic-treated group. The administration of probiotics and synbiotics has been associated with a decrease in plasma levels of key pro-inflammatory signalling molecules, including high-sensitivity C-reactive protein (hsCRP), Interleukin-1 beta (IL-1ß), Tumour Necrosis Factor-alpha (TNF-α), and Interleukin-6 (IL-6) in previous studies (Table 5). These molecules play a critical role in the inflammatory response, and their elevated levels are linked to an increased risk of adverse cardiovascular events, such as heart attacks and strokes. By modulating the gut microbiota,

probiotics and synbiotics may help reduce systemic inflammation, which in turn could lower the risk of cardiovascular events [80]. However, the literature on this topic remains inconsistent due to several factors. As demonstrated in Table 5, the variability in probiotic strains, dosages, and durations of treatment, as well as differing reporting standards used in symbiotic intervention studies makes it challenging to draw definitive conclusions.

Table 5: Selection of synbiotic intervention trials that defined anti-inflammatory outcomes, CFU = Colony forming unit.

Trial	Population size (Synbiotic/ placebo)	Condition	Intervention, daily dosage (in CFUs), preparation	Duration	Significant change in inflammatory markers (synbiotic-treated group)
Federico et al. (2009) [81]	9/9	Colitis ulcerosa	*Lactobacillus paracasei* B21060 (5×10^9) Lyophilized powder	8 weeks	IL-6 and IL-8 ↓
Nova et al. (2011) [82]	18/18	Healthy subjects	*Lactobacillus acidophilus* La5, *Bifidobacterium animalis ssp. lactis* Bb-12, *Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus paracasei ssp. paracasei, Streptococcus thermophilus,* (2.4×10^9) Capsules	6 weeks	T Helper Cells, L-Selectin ↓
Kooshki et al. (2015) [83]	22/22	Type 2 Diabetes	Unknown dose/intervention Synbiotic capsule	8 weeks	IL-6, hsCRP, TNF-α ↓
Eslamparast et al. (2014) [84]	26/26	Non-alcoholic fatty liver disease	*Lactobacilluscasei, Lactobacillus rhamnosus, Streptococcus thermophilus, Bifidobacterium breve, Lactobacillusacidophilus, Bifidobacterium longum,* and *Lactobacillusbulgaricus* (2×10^8) Capsules	28 weeks	ALT, AST, GGT, hsCRP, TNF-α, NF-κB ↓
Rajkumar et al. (2014) [85]	15/15 (+15 treated with only probiotics)	Healthy subjects	*Lactobacillus salivarius* UBL S22 (2×10^9) Capsules	6 weeks	CRP, IL-6, IL-1β, TNF-α ↓

As a result, while some studies report significant reductions in inflammatory markers, others show minimal or no effects, highlighting the need for more standardized and large-scale clinical trials to better understand the potential benefits of probiotics and synbiotics in this context [86].

When it comes to experimental models of cerebral ischemia, research on the effects of probiotics is limited and the findings are inconsistent. This inconsistency is often attributed to

the lack of standardization in study designs and generally poor quality (see Table 5), including the absence of control groups. For example, in a study studying focal ischemia with the fMCAo model, mice pre-treated with a probiotic cocktail for several days exhibited a reduction in infarct volume and lower plasma levels of Tumour Necrosis Factor-alpha (TNF-α) compared to saline-treated controls [87]. Another study, which involved pre-treating ICR mice with 14 days of *Clostridium butyricum* oral gavage before inducing cerebral ischemia via bilateral common carotid artery occlusion, reported attenuation of apoptotic pathways, as evidenced by western blot and histological analysis, along with an increase in intracerebral butyrate concentrations [88].

Despite these promising findings, it is important to note the small sample sizes in these animal studies, which limits the generalizability of the results. Further research with more rigorous study designs and larger sample sizes is needed to better understand the role of probiotics in stroke recovery and their potential therapeutic applications.

8.5. Limitations

The murine observational cohort was hampered by a small sample size that allowed for few deductions about the metabolic data collected. Undoubtedly the analysis of the SCFA concentrations would have yielded more significant hits with a larger cohort of mice increasing the statistical power of the analysis. Furthermore, the origin microbiome of the breeding house may always have a potent influence on the outcomes measured and bias the generalizability of the results, however the congruency of results across species allows at least for a positive outlook on the comparability of these results.

The human cohorts were both affected primarily by a problem that affects many stroke cohorts. The bias in recruitment towards lower NIHSS and by extension lower infarct volumes is a well-documented problem [89]. This is in part due to the nature of consent and compliance required for the collections of samples and especially for the regular consumption of a probiotic. The

intervention cohort suffered severely from this effect and as a consequence the results are skewed towards participants that had few permanent effects following their strokes and thus may profit less from such a therapy. Furthermore, the small sample sizes of both the observational and probiotics intervention cohort means that inferences about clinical outcomes are statistically very difficult to draw and impedes the descriptive analysis due to the corrections necessary in multiple comparison analysis, which are unavoidable when analysing sequencing data. Nonetheless, the persistence of trends in dysbiosis and SCFA concentration loss across species indicated that these effects are robust and should be investigated in a larger cohort.

8.6. Outlook

On the basis of these findings, larger scale cohorts exploring the long-term effect of gut dysbiosis and associated metabolite alterations on intestinal immunity and disease specific immune response or prolongation should be considered. The characterisation of key bacterial metabolites in cohorts that have a higher power to detect sensitive changes could help understand the development and progression of secondary pathologies after stroke.

Newer publications have explored the potential of immune memory to influence disease progression after a stroke. If indeed the gut microbiome remains in a permanent state of dysbiosis, the continuous impact of the metabolic and by extension the immunological signalling on patient comorbidities may have serious clinical relevance which requires targeted therapy.

9. List of Figures

Figure 1: List of top 10 causes of DALYs globally, reported in 2019. (Data adapted from the WHO Global health estimates study 2020 [6]) 13

Figure 2: Study design of the SMB cohort 26

Figure 3: Study design of the PRISE cohort 33

Figure 4: Study overview of murine pilot experiment. Mice were given fMCAo for 60 minutes and stool was collected at day 0 (before surgery), day 3 and day 14. The mice were sacrificed at day 14, EDTA-blood and ileum content were collected. 41

Figure 5: Alpha diversity measures of richness (left), Shannon index (centre) and inverted Simpson index (right) for each time point of stool collection. 42

Figure 6: A) Relative abundance (in %) of bacterial species (see legend) at day 0, day 3 and day 14 after fMCAo surgery. B) PCA of all 3 time points (day 0, day 3 and day 14), plotted by using the Bray-Curtis index ($p = 0.002$, $R^2 = 0.37$) 42

Figure 7: Concentrations (in µmol/L) of propionate and acetate as measured in the ileum content of a naïve and 14 days post-fMCAo cohort. The fMCAo cohort showed a significantly lower concentration of propionate than the naïve cohort, while a trend toward lower concentrations in the fMCAo cohort was observed for acetate. 43

Figure 8: Relative abundance (in %) of measured bacterial genera (see legend) across all patient groups (from left to right): Control cohort from Kassel (one time point only), TIA group from LMU, mild stroke group from LMU, and severe stroke group from LMU. The x-axis show the unique patient identifiers used in the trial, as well as the time point of the measurement (A = acute; C= chronic). 44

Figure 9: (Left) NMDS plot of the Bray-Curtis index showing 5 populations and 3 clusters, control vs acute stroke, acute vs. chronic stroke and control vs. chronic stroke. (Right) Observed richness (alpha diversity measure) of all 5 populations. Statistical measures were only compared between the control, acute stroke and chronic stroke groups. 45

Figure 10: NMDS plots comparing the Bray-Curtis index between the acute stroke and control groups (left, $p = 0.007$, $R^2 = 0.11$), as well as the control and chronic stroke groups (right, $p = 0.045$, $R^2 = 0.09$) 45

Figure 11: Volcano plot of differentially abundant genera between the acute and control groups (left) as well as the chronic and control groups (right). 46

Figure 12: The dot plots show the relative percentage of myeloid CD45+ Cd11b+ cells in the that were signifantly different in acute stroke and chronic stroke groups between baseline and day 90. Acute TIA and chronic TIA were excluded from analysis for statistical reasons. Paired t-tests were used to compare the acute and chronic stroke cohorts. 47

Figure 13: Concentrations in (µM) of valeric acid, butyric acid and propionic acid as measured in the ileum of the study participants. (Paired t test was performed, TIA was excluded) 48

Figure 14: Normalized peak areas of the top 2 detected metabolites (Indole-3-propionic acid and pyruvic acid) in the plasma of study participants. 49

Figure 15: Plots show correlations identified between SCFAs and key bacterial populations in all samples. R = Spearman coefficient.....50

Figure 16: A) Observed richness across all 3 groups. B) Observed richness between reference and stroke group, as pooled from the placebo and probiotics groups. C) NMDS plot of the Bray-Curtis index comparing all 3 groups.....51

Figure 17: Euclidian distance of the placebo group from the reference group based on the Bray-Curtis index.....52

Figure 18: Volcano plot of differentially abundant bacterial mOTUs between the placebo and probiotics group. Significant p-values were defined as < 0.1. 52

Figure 19: Relative abundances (in %) of key bacterial genera previously identified to be dysregulated after stroke in the observational cohort.....53

Figure 20: Heat map of the relative abundance of the 8 bacterial species of the probiotic as measured in the faecal samples of participants from all 3 treatment groups. 54

10. List of Tables

Table 1: SMB Cohort characteristics 32

Table 2: List of study specific measures 36

Table 3: Characteristics of the PRISE Study population 38

Table 4: List of metabolites identified during untargeted mass-spectrometry (SMB) 49

Table 5: Selection of synbiotic intervention trials 60

11. References

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12. List of Abbreviations

AIS ..... Acute Ischemic Stroke

NIHSS ..... National Institute for Health Stroke Scale

mRS ..... modified Rankin Score

fMCAo ..... filamentary Middle Cerebral Artery occlusion

LAA ..... Large Artery Atherosclerosis

CE ..... Cardio-Embolism

IEC ..... Independent Ethics Commission

CBF ..... Cerebral Blood Flow

DALY ..... Disability Adjusted Life Year

CD ..... Cluster of Differentiation

HBP ..... High Blood Pressure (arterial)

RCF ..... Relative Centrifugal Force

ISD ..... Institute for Stroke and Dementia research

ICH ..... Intracerebral Hemmorrhage

SAH ..... Subarachnoid Hemmorrhage

PBMC.....Peripheral Blood Mononuclear Cell

SCFA ..... Short Chain Fatty Acid

FACS .....Fluorescence Associated Cell Sorting

MOCA .....Montreal Cognitive Assessment

PBS..... Phosphate Buffered Saline

ESUS .....Embolic Stroke of Unknown Source

TIA .....Transient Ischemic Attack

CE..... Cardiogenic Embolism

GCMS ..... Gas Chromatography Mass Spectrometry

LCMS .....Liquid Chromatography Mass Spectometry

MCA..... Middle Cerebral Artery

ACA..... Anterior Cerebral Artery

PCA..... Posterior Cerebral Artery

SAB.....Subarachnoid Bleed

ICH..... Intracerebral Hemmorhage

TMAO.....Trimethylamine-N-oxide

OTU .....Operational Taxonomic Unit

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