Master's Thesis

Effects of high-fat diet and prebiotic fiber supplementation on hippocampal inflammation and adult neurogenesis

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The increase in high energy food as a part of regular nutrition in the last decades has been shown to cause negative health effects related to obesity and to alter the gut microbiota composition. In the past years, the amount of evidence pointing towards a connection between the gut microbiota and the brain, the gut-brain axis (GBA), has increased. Previous studies have shown a link between the abundance of a specific gut microbe, *Faecalibacterium prausnitzii*, and improvements in intestinal disorders as well as depression-like and anxiety-like behavior, suggesting that it influences both gut and brain health. *F. prausnitzii* is extremely oxygen sensitive, making its use as a probiotic difficult. Thus, its abundance was attempted to be manipulated by using a prebiotic fiber, xylo-oligosaccharide (XOS), in rats fed with high or low-fat feed. XOS has been shown to affect the abundance of *F. prausnitzii* in the gut. In this study the effects of a high-fat diet and/or dietary XOS on *F. prausnitzii*, and hippocampal inflammation and adult neurogenesis were examined. After a twelve-week dietary intervention, the rats were euthanized, and their hippocampi were sectioned and stained with immunohistological methods. The high-fat diet was found to increase weight gain and a low-fat diet with XOS was observed to have a positive influence on the abundance of *F. prausnitzii*. There were unexpected differences in the markers of activated microglia between the groups, as the low-fat diet seemed to associate with higher levels of markers. The number of newborn neurons did not differ between the groups, suggesting that the different diets did not affect adult neurogenesis.

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Paljon energiaa sisältävän ravinnon lisääntyminen osana tavallista ruokavaliota on viimeisten vuosikymmenien aikana todettu johtavan ylipainosta aiheutuviin terveyshaittoihin ja muuttavan suolistomikrobiston rakennetta. Viime vuosien aikana on tullut lisää näyttöä, joka viittaa suoliston ja aivojen väliseen yhteyteen, jota kutsutaan suoli-aivo-akseliksi. Suolistobakteeri *Faecalibacterium prausnitzii*n määrän on todettu olevan yhteydessä niin suoliston häiriöiden, kuin masennuksen ja ahdistuksen kaltaisten häiriöiden lieventymiseen, mikä viittaa sen vaikuttavan sekä suoliston että aivojen terveyteen. *F. prausnitzii* on erittäin anaerobinen, mikä tekee sen käytöstä probioottina vaikeaa. Tässä tutkimuksessa sen määrään pyrittiin vaikuttamaan prebioottisen xylo-oligosakkaridin (XOS) avulla sellaisten rottien suolistossa, joille syötettiin vähä- tai korkearasvaista ruokaa, joko XOS:n kanssa tai ilman. Pyrin selvittämään korkearasvaisen ruokavalion ja XOS:n vaikutusta *F. prausnitzii*n määrään sekä aikuisiän neurogeneesiin ja inflammaatioon aivotursossa. Kahdentoista viikon ravitsemusintervetion jälkeen rotat lopetettiin, niiden aivot viipaloitiin leikkeiksi ja värjättiin immunohistologisin menetelmin. Korkearasvaisen ruokavalion todettiin johtavan suurempaan painonnousuun ja vähärasvaisen ruokavalion havaittiin XOS:n kanssa johtavan korkeampaan *F. prausnitzii*n määrään. Ryhmien väliset erot mikrogliojen aktiivisuuden merkkiaineissa olivat yllättäviä: vähärasvainen ruoka vaikutti olevan yhteydessä korkeampaan aktiivisuustasoon. Uusien hermosolujen määrät olivat ryhmissä samalla tasolla, viitaten siihen, ettei ruokavalio vaikuttanut neurogeneesiin.

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ABBREVIATIONS

BBB = Blood-brain barrier

CA1-3 = *Cornu Ammonis* area 1-3

DCX = Doublecortin

DG = Dentate gyrus

F. prausnitzii = *Faecalibacterium prausnitzii*

GABA = Gamma-amino-butyric acid

GBA = Gut-brain axis

GCL = Granule cell layer

HFD = High-fat diet

Iba1 = Ionized calcium-binding adapter molecule 1

LFD = Low-fat diet

PB = Phosphate buffer

PBS = Phosphate buffered saline

PBS-T = Phosphate buffered saline with Triton-X

Prebiotics = Food components that are beneficial for probiotics

Probiotics = Microorganisms that are beneficial for their host's health

SCFA = Short-chained fatty acid

SUB = Subiculum

TBS-T = Tris buffered saline with Triton X-100

XOS = Xylo-oligosaccharide

1. INTRODUCTION

1.1 Background

The amount of overweight and obese people has increased drastically in the past few decades. The simplest explanation for excess body weight is the imbalance between energy intake and expenditure, which is largely prevalent due to increasingly sedentary lifestyle and consumption of energy-dense food (Popkin et al., 2012). Obesity is a condition with multiple negative health effects, such as musculoskeletal disorders, cardiovascular diseases, and diabetes (Herzog, 2020).

High-fat diet (HFD), which is a partial cause to the obesity epidemic, has been found to negatively impact the function of the intestinal barrier. In a healthy gut, this barrier facilitates transport of beneficial and necessary substances from the gut to the rest of the body, while keeping toxins and harmful microbes outside (Rohr et al., 2020). It consists of mucus, epithelial cells, and lamina propria, where there are a lot of immune cells. The mucus is habited by microbes, that can be beneficial or harmful, and it impedes dangerous molecules and the harmful microbes from reaching the underlying epithelial cells. The ratio of these microbes is regulated, among other things, by antimicrobial molecules in the mucus, which may be secreted by other microbes, or by the epithelial cells. The epithelial cells themselves are tightly connected to each other through tight junctions, to prevent molecules in the gut from leaking through paracellular spaces to the other side of the barrier and into the bloodstream. Finally, the immune cells in the lamina propria can secrete proteins, such as cytokines and immunoglobulin A, in response to antigens (Camilleri, 2019). When these defenses are compromised, by a HFD or otherwise, harmful substances in the gut can more easily spread throughout the body and cause inflammation, weaken the blood-brain barrier (BBB), and produce countless other problems (Obrenovich, 2018).

Inflammation is a response to injury, infection, or toxins, which exists to combat these detrimental factors by attracting plasma proteins and leukocytes to the site of harm. It is supposed to acutely deal with the insult causing the inflammation, and

continue to a resolution phase, where possible cellular debris is cleared, and tissues are repaired. But while it is an inherently protective process, failure to resolve the offending factors may lead the inflammation to become chronic (Medzhitov, 2008). This dysfunction can cause problems, as it instigates stress to the surrounding tissue, and can even lead to fibrosis, causing the tissue to lose its function, which can hinder the function of an organ (Ferrero-Miliani et al., 2007).

The blood-brain barrier refers to the attributes of blood vessels in the brain that highly regulate transport from the blood to the brain. Because of this barrier, the milieu in which the brain operates remains stable and is protected against pathogens and toxins. This is possible because of the endothelial cells of the capillaries that are tightly connected by tight junctions, similarly to the epithelial cells of the gut. Since molecules cannot use paracellular spaces to get to the brain, the endothelial cells can effectively regulate what goes through them. This enables the BBB to keep the potassium levels lower in the brain than in the plasma, which supports synaptic signaling. The BBB also restricts the access of neurotransmitters of the peripheral nervous system and macromolecules, such as many proteins, to the brain (Abbott et al., 2009). Still, the BBB does facilitate the transport of many molecules, for example nutrients, into the brain, and waste products out of the brain (Daneman & Prat, 2015).

In this study the possible effects of a HFD and the prebiotic xylo-oligosaccharide (XOS) on hippocampal inflammation and adult neurogenesis were examined. We also investigated whether possible changes in the gut microbiota composition were associated with these effects. Rats were fed with low-fat (LFD) or high-fat diet, with half of them receiving the prebiotic in the feed. Prebiotics are usually dietary fibers, that can be used for energy by probiotics, which are live microorganisms that are beneficial for the host's health. The goal of this experiment was to positively influence the abundance of a beneficial bacterium, *Faecalibacterium prausnitzii*, which was hypothesized to impact the hippocampus. After the dietary intervention, the hippocampi of the rats were sectioned and stained for analysis. The aim was to

determine the effect of the diets on adult neurogenesis and microglial activity in the hippocampus.

1.2 The Gut-Brain Axis

The gut-brain axis (GBA) is a bidirectional communication pathway between the intestine and the brain. Recently, the gut microbiota has been added to the concept, sometimes referred to as the microbiota-GBA. There are several disorders of the central nervous system, such as autism and anxiety-depressive behavior, that have been linked with dysbiosis, or a microbial imbalance, in the gut, pointing towards a connection between the two (Foster & McVey Neufeld, 2013). The communication can be neural or humoral, or it can work through endocrine or immune signaling (Figure 1). The GBA is usually studied on germ-free animals, or with probiotics, antibiotics, or infection models (Carabotti et al., 2015). There is also evidence from human studies that probiotics can influence the brain (Tillisch et al., 2013).

The autonomic nervous system works as a go-between, between the gut and the central nervous system. It consists of sympathetic, parasympathetic, and enteric nervous systems. The sympathetic nervous system mainly has an inhibiting effect on the intestine, as it slows down secretion and gastrointestinal transit. The parasympathetic nervous system is especially important for GBA due to the vagus nerve (Mayer, 2011), which is a long nerve between the medulla oblongata and the gut. It also innervates various other organs, such as some neck muscles and the heart (Breit et al., 2018). In the gut it mediates vago-vagal motor reflexes and the cephalic phase of gastric acid secretion. It signals to enteroendocrine cells, enterochromaffin-like cells and enterochromaffin cells, leading to secretion of histamine and serotonin in the gut (Mayer, 2011). The vagus nerve is also important for communication from the gut to the brain. Its role in the GBA has been studied with rats whose vagus nerve has been severed, by treating their depression- and anxiety-related symptoms with a probiotic. The vagotomized rats were shown not to exhibit the usual beneficial neurochemical and behavioral effects that were present in non-vagotomized rats treated with a probiotic (Bravo et al., 2011).

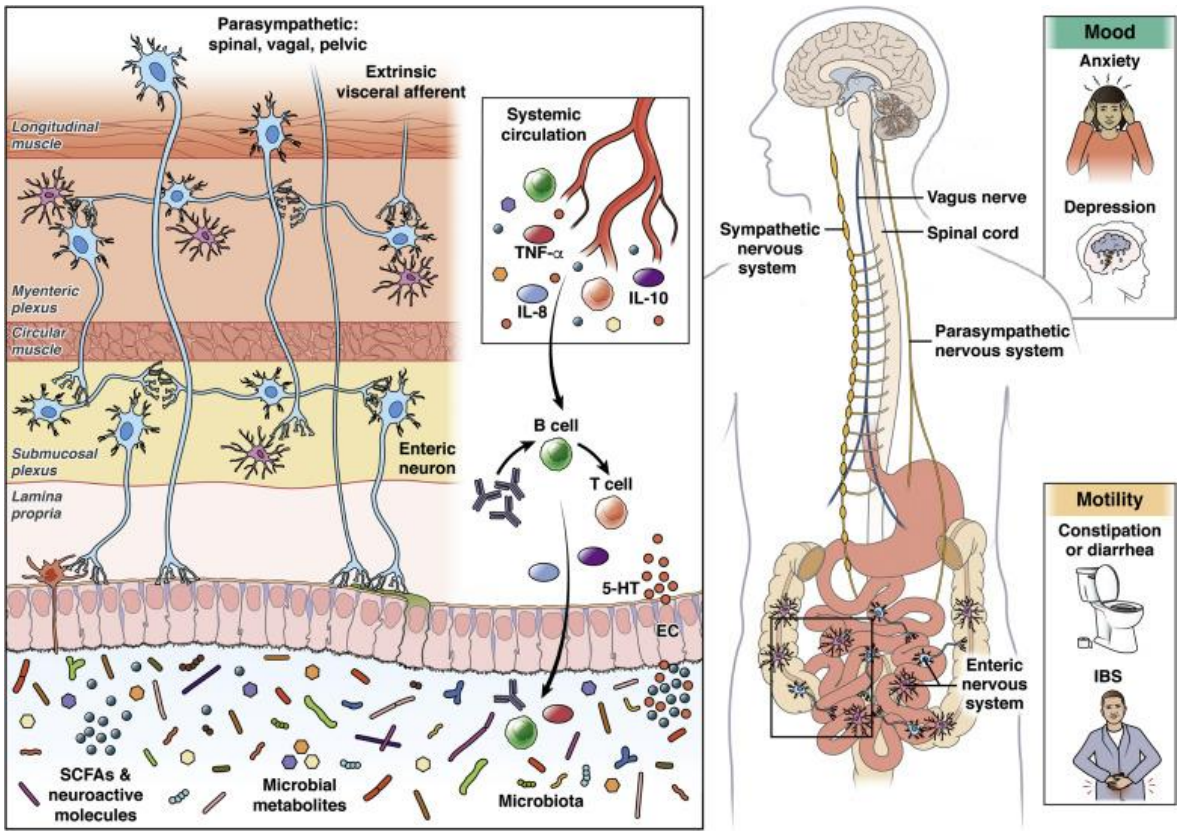

Figure 1. Major molecules and neural pathways of the autonomic nervous system that play a part in the gut-brain axis. Represented are the sympathetic and parasympathetic nervous systems, and the enteric nervous system. Microbiota in the gut can produce short-chain fatty acids, such as butyrate, which can reach the brain through circulation. They can also induce the production of inflammatory cytokines. Adapted from Margolis et al. (2021)*.

The ENS is the biggest part of the autonomic nervous system, with two plexuses, or neural networks, that are important for the GBA. The myenteric plexus controls the muscle functions of the intestinal wall, and the submucosal plexus monitors the environment of the lumen, controls blood flow of the digestive tract, and regulates the secretion and functions of the epithelial cells (Nezami & Srinivasan, 2010). The ENS is connected with the brain through the vagus nerve (Furness et al., 2014).

The hypothalamic-pituitary-adrenal (HPA) axis regulates responses to stressors. Through the hypothalamus it is connected to the limbic system (Carabotti et al., 2015), which consists of several brain structures, such as the hippocampal formation and amygdala. The functions of the limbic system include memory and emotional regulation (Rajmohan & Mohandas, 2007) and through that, the HPA axis is relevant for memory and emotional responses. Environmental factors, such as emotions and

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stress, and inflammatory agents can activate signaling from the hypothalamus, which through the pituitary gland causes the adrenal glands to secrete cortisol. Cortisol is a neuroendocrine hormone that is linked to the consolidation of fear-based memories of stressors (Hannibal & Bishop, 2014). Probiotics have been shown to reduce corticosterone secretion caused by stress (Bravo et al., 2011).

Gut microbes can affect the central nervous system in numerous ways, as they influence the neurochemistry of the brain, and by extension, memory. For example, brain-derived neurotrophic factor (BDNF) plays an important role in the function of the hippocampus. In mice infected with a pathogen, the decreased expression of BDNF, and subsequent memory deficits, were ameliorated with probiotics (Gareau et al., 2011). Gut microbiota has also been shown to affect the serotonergic circuitry in the limbic system, influencing anxiety and emotional behavior (Neufeld et al., 2011), as well as cause changes in the expression of the neurotransmitter gamma-amino-butyric acid (GABA) in multiple brain areas, such as the prefrontal cortex, amygdala, and the hippocampus (Bravo et al., 2011).

Chronic systemic inflammation that can be influenced by gut microbiota, can lead to inflammatory molecules being produced in the brain. It may also increase the activation of microglia and cause peripheral immune cells to cross into the brain (Perry et al., 2003). Bacteria can influence the quantity of molecules that are used to produce neurotransmitters in the gut. They can also impact the actual production of the neurotransmitters, or even produce them by themselves (Silva et al., 2020). These neurotransmitters can induce signaling from the gut to the brain through the vagus nerve (Breit et al., 2018), and they may be able to circulate into the brain directly (Sherwin et al., 2018; Strandwitz, 2018; Abdel-Haq et al., 2019). For example, about 90 % of all serotonin is produced in the gut, through tryptophan metabolism (Abdel-Haq et al., 2019). Gut microbes can influence tryptophan metabolism, which can cause tryptophan to be metabolized into kynurenine (O'Mahony et al., 2015) that can lead to numerous biologically active metabolites (Badawy, 2017). When this happens, the available tryptophan decreases, and less serotonin is produced (O'Mahony et al., 2015).

Short-chained fatty acids (SCFAs), that are the fermentation products of many gut microbes, are an important energy source for various tissues. The three most dominant SCFAs are butyrate, which is used for energy primarily by colonocytes, propionate that is metabolized by the liver, and acetate, which is used by the peripheral tissues (Wong et al., 2006; Silva et al., 2020). However, not all SCFAs are metabolized for energy and they can exert beneficial effects throughout the body. They improve the function of the intestinal barrier (Lewis et al., 2010), and butyrate for example regulates the differentiation of immunosuppressive regulatory T cells (Arpaia et al., 2013; Togashi et al., 2019). When receiving sufficient nutrition, SCFAs increase the activity of the sympathetic nervous system, while ketone bodies produced by ketogenic conditions reduce the activity. Thus, SCFAs are essential for maintaining metabolic homeostasis through the regulation of energy consumption (Kimura et al., 2011). They can cross and improve the function of the blood-brain barrier and upkeep the homeostasis of the brain. They have been shown to reduce inflammatory signaling in the brain (Silva et al., 2020) and impact levels of neurotransmitters and their precursors, at least in the hypothalamus (Frost et al., 2014). They have also been shown to influence intraneuronal potassium levels and could therefore affect neuronal signaling (Oleskin & Shenderov, 2016).

1.3 Memory and the hippocampal formation

1.3.1 Memory

Memory can be divided into two types: implicit and explicit memory. Implicit memory covers perceptual and motor skills, and it relies mainly on areas of the brain other than the hippocampus. Explicit memory on the other hand, is heavily hippocampus dependent and it includes semantic and episodic memory (Kandel et al., 2014). Semantic memory refers to overall knowledge that a person has acquired, and it is necessary for the ability to use language, for example (Tulving, 1972). Episodic memory contains object, temporal, and spatial memory, meaning that it is responsible for storing a collection of experiences in relation to time and space (Tulving, 1972; Babb & Johnson, 2011).

Memory is believed to require physiological changes in the brain that are possible through neural plasticity. This refers to microglial activity, adult neurogenesis, synaptogenesis, and synaptic plasticity (Bruel-Jungerman et al., 2007). Neurogenesis is the formation of new neurons, which mainly takes place in the dentate gyrus and the lateral ventricles. As this phenomenon is studied in this thesis, it is worth highlighting that adult neurogenesis is thought to be important for spatial memory (Bruel-Jungerman et al., 2007; Aimone et al., 2014). Synaptogenesis refers to the formation of new synapses, which increases signaling between neurons. Although there is evidence of learning-induced synaptogenesis, some studies have found no connection between the two, and it is possible that a remodeling of existing synapses plays a larger role. To support this, multiple morphological changes in synapses have been seen after learning (Bruel-Jungerman et al., 2007).

1.3.2 The hippocampal formation

The hippocampal formation is a part of the limbic system, located on both sides of the brain in the medial temporal lobes (Rajmohan & Mohandas, 2007), connecting to the lateral ventricles (Dekeyzer et al., 2017). It is essential for cognitive functions, such as memory and learning (Sutherland & Rudy, 1989), and it can influence emotional behavior (Strome et al., 2002). It also enables us to perceive the relationships between learned information (Rubin et al., 2014). The hippocampus is necessary for spatial memory, as it makes it possible to remember the locations of things and understand their relative locations with each other. This is achieved through the formation of a spatial and temporal context for information, referred to as a cognitive map (Lisman et al., 2017).

The hippocampal formation is a three-layered structure, that consists of the hippocampus proper (later 'the hippocampus'), the dentate gyrus (DG), and the subiculum (SUB). From the deepest to the most superficial, the layers have been named the polymorph layer, the cell layer, and the molecular layer. Notable for this thesis is that the polymorph layer of the DG is called hilus and the cell layer is a granule cell layer (GCL). In the hippocampus and the SUB the cell layers are

pyramidal cell layers (Figure 2). The hippocampal formation receives signaling mostly from the entorhinal cortex, which projects to all areas of the formation (van Strien et al., 2009).

The hippocampus is divided into three *Cornu Ammonis* (CA) areas: CA1, CA2, and CA3 (Bienkowski et al., 2018). The axons of the pyramidal cells in the CA3 area are connected to the CA1 region and have been given the name Schaffer collaterals. (Purves et al., 2001; Teixeira et al., 2018). The axons of the DG's granule cells are called mossy fibers and they are synaptically connected to the CA3 pyramidal cells of the hippocampus (Amaral et al., 2007). Both Schaffer collaterals and mossy fibers are part of a trisynaptic circuit, which is a major network in the hippocampal formation. The trisynaptic circuit has been found to be important for learning and memory, especially for spatial learning (Crusio & Schwegler, 2005; Stepan et al., 2015; Teixeira et al., 2018). The granule cells born through adult neurogenesis in the DG have the potential to become a part of this network (Aimone et al., 2014).

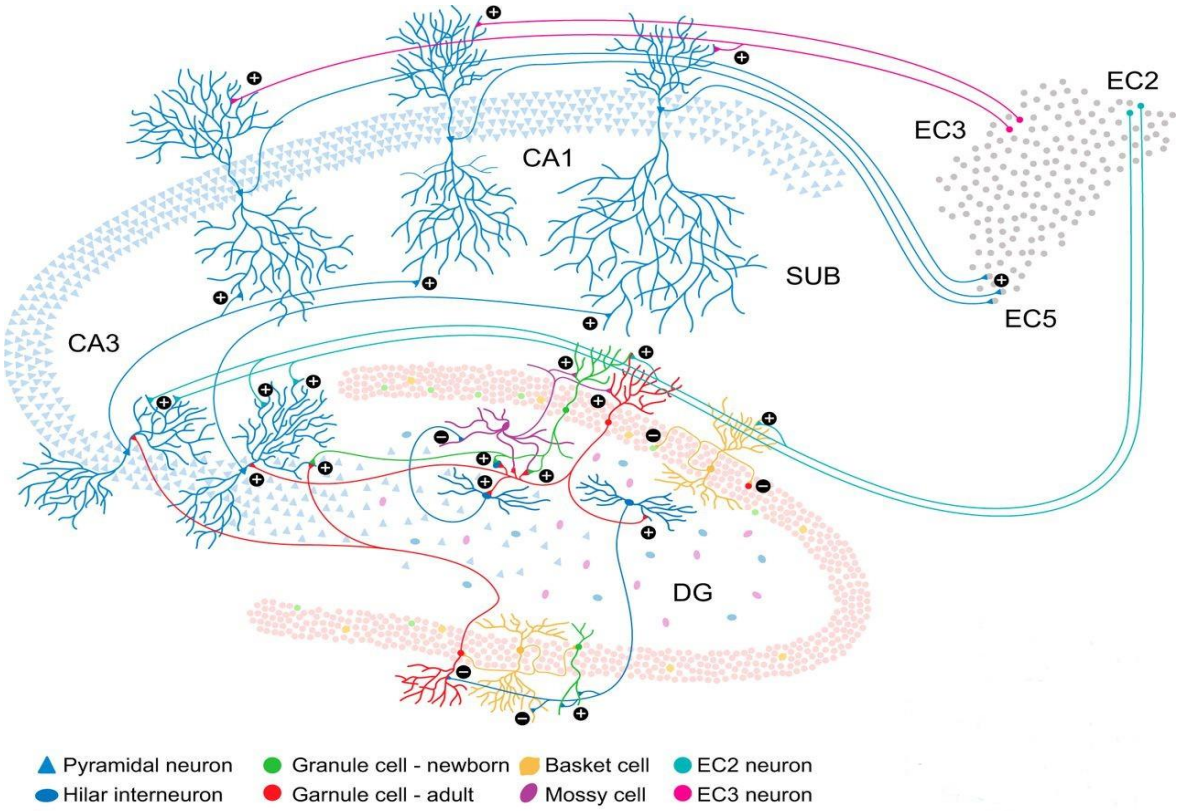

Figure 2. Schematic anatomy of rat hippocampal formation, showing the orientation of CA1 and CA3 regions of the hippocampus, the dentate gyrus (DG), subiculum (SUB) and the entorhinal cortex (EC). The locations of granule cells and hilar cells of the DG, and the grouping of pyramidal cells in the hippocampus can also be seen. Adapted from Aimone et al. (2014)*.

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The SUB is the region between the hippocampus and the entorhinal cortex. It has large pyramidal cells alongside smaller interneurons. The SUB is essential for the hippocampus' output signaling, as the CA1 projects primarily to the SUB, which then projects to various cortical and subcortical areas, especially back to the entorhinal cortex (O'Mara, 2005). However, the CA1 (van Strien et al., 2009) and CA2 regions project to the entorhinal cortex as well (Tzakis & Holahan, 2019). Signaling pathways between the entorhinal cortex and the hippocampal formation are visualized in Figure 3.

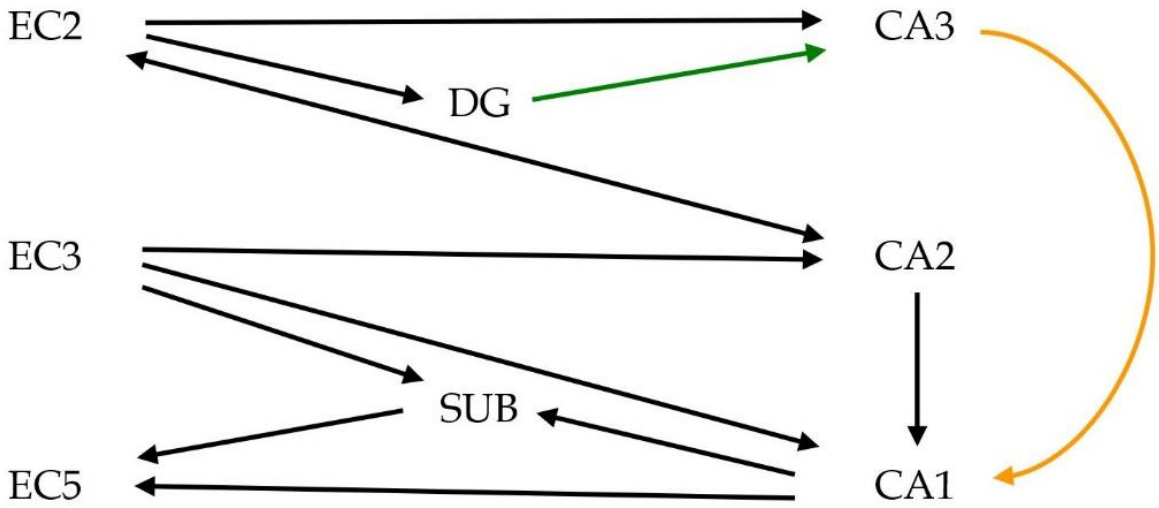

Figure 3. Neural circuit of the hippocampal formation. EC2, EC3, and EC5 are different layers in the entorhinal cortex. The green arrow represents mossy fibers and orange Schaffer collaterals. From the diagram, it can be seen that the entorhinal cortex projects to all areas of the hippocampal formation, and that the SUB, CA1 and CA2 regions project back to the entorhinal cortex.

1.4 Faecalibacterium prausnitzii

Faecalibacterium prausnitzii is one of the most abundant bacterial species in a healthy human gut, constituting about 5% of all gut microbes (Martín et al., 2017). *F. prausnitzii* is a non-motile and gram-positive bacterium, and is non-spore forming and extremely oxygen sensitive (Ferreira-Halder et al., 2017). It belongs to the *Firmicutes* phylum and has been reported to be beneficial for health. For instance, it has been found to be less abundant in the gut of humans with intestinal disorders,

such as Crohn's disease and irritable bowel syndrome (Martín et al., 2017). *F. prausnitzii* has also been observed to ease depression-like and anxiety-like behavior induced by chronic unpredictable mild stress in rats (Hao et al., 2019). These findings indicate that *F. prausnitzii* could be linked to health both in the periphery and in the brain.

F. prausnitzii possesses various anti-inflammatory properties. It has been found to increase the proportional production of anti-inflammatory interleukin-10 (IL-10) compared to pro-inflammatory interleukin-12 (IL-12), when measured from peripheral blood mononuclear cells (Sokol et al., 2008). Treatment of human intestinal epithelial cells with *F. prausnitzii* culture supernatant has been shown to reduce secretion of pro-inflammatory interleukin-8 and inhibit the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which among other molecules regulates the expression of pro-inflammatory genes (Sokol et al., 2008; Singh & Singh, 2020). On 2,4,6-trinitrobenzenesulphonic acid -induced colitis animal models, it has been found that treatment with *F. prausnitzii* ameliorates the symptoms of colitis and reduces the abnormally high amounts of the inflammatory cytokines tumor necrosis factor alpha and IL-12. At the same time the treatment increases the anti-inflammatory IL-10, and restores the microbial balance in the gut, normalizing the prevalence of *F. prausnitzii* (Sokol et al., 2008). Peripheral pro-inflammatory cytokines have also been found to activate microglia, which may reduce adult neurogenesis in the brain (Chesnokova et al., 2016). As *F. prausnitzii* has been found to cause a reduction in peripheral pro-inflammatory cytokines, this could imply possible benefits from increasing *F. prausnitzii* abundance, in terms of adult neurogenesis and neuroinflammation.

One way through which *F. prausnitzii* can influence the health of the brain is that it is an important producer of butyrate (Heinken et al., 2014), which is a SCFA. There is evidence that SCFAs can cross the blood-brain barrier (Silva et al., 2020), and butyrate has even been shown to be able to improve the BBB function (Bourassa et al., 2016). While butyrate produced in the gut is a vital energy source for colonocytes (Heinken et al., 2014), some of the butyrate is not metabolized there. Instead, it is transported

to the liver through portal circulation, and mostly used for energy. However, some of the butyrate continues to the systemic circulation and from there can reach the brain (Silva et al., 2020).

Butyrate has anti-inflammatory effects on the peripheral tissues and the brain, as it has been shown to protect primary microglia and the hippocampus against inflammation, caused by lipopolysaccharide (LPS) treatment of rats (Huuskonen et al., 2004). Butyrate is a histone deacetylase (HDAC) inhibitor (Berni Canani et al., 2012; Silva et al., 2020). HDACs can remove acetyl groups from histones, which allow tighter packaging of DNA, preventing gene expression. Therefore, by inhibiting this action, butyrate can influence gene expression (Waldecker et al., 2008), which has opened two possible paths for its protective effects against LPS. In the first path, butyrate can inhibit the activity of proteasomes (Yin et al., 2001), which are sizeable protein complexes, that degrade proteins found in cells (Tanaka, 2009). This inhibition leads to the up-regulation of proteins called inhibitor of nuclear factor kappa B, which inhibit the function of cytosolic NF-κB (Yin et al., 2001), preventing inflammation. In the second path, butyrate can downregulate the expression of toll-like receptor 4 (TLR4) (Böcker et al., 2003). TLR4 are receptors that can be activated by bacterial LPS. This activation initiates intracellular signaling, that leads to the activation of NF-κB and proinflammatory cytokine production (Pålsson-McDermott & O'Neill, 2004; Vaure & Liu, 2014). Therefore, downregulation of TLR4s can also reduce inflammation.

Since *F. prausnitzii* is extremely oxygen sensitive, its use as a probiotic would be difficult. Probiotics are live microorganisms that when consumed in adequate amounts, can provide health benefits by improving the gut flora. *F. prausnitzii* has not been accepted to be used as a probiotic supplement in humans. Therefore, it makes sense to try and influence its prevalence in the gut through other means, such as in the case of this study, prebiotics. Prebiotics are usually dietary fibers that are not digested by the host, but instead are fermented for energy by health beneficial bacteria in the gut. *F. prausnitzii* have been shown to be able to process for example inulin and apple pectin (Heinken et al., 2014). Here, XOS was chosen as the prebiotic

to be used, as it has been shown to increase the abundance of *F. prausnitzii* (Finegold et al., 2014). XOS has also been shown to increase the abundance of other beneficial species of bacteria and the production of butyrate, when paired with a HFD, while decreasing the inflammation caused by a HFD (Fei et al., 2019).

1.5 Microglia

Microglia are non-neuronal cells of the nervous system, constituting around 10 % of the cells in the central nervous system, where they function as macrophages and are a part of the innate immune system (Ji et al., 2013). During development they enhance the survival of neurons (Ueno et al., 2013) but also cause apoptosis and phagocytize apoptotic cells, without causing inflammation. They also guide the formation of vasculature and support the development of neuronal networks (Kierdorf & Prinz, 2017). This is achieved by monitoring and pruning synapses (Paolicelli et al., 2011), which means that they eliminate unused, extra synapses (Tierney & Nelson, 2009). Short-chained fatty acids produced by the gut microbiota are important for the development of microglia themselves (Erny et al., 2015).

During adulthood, microglia have two states. One state is often called resting state, which is a bit of a misnomer, as the cells are not resting, but instead they are surveilling the environment (Chen & Trapp, 2016). Microglia in this surveillance state have a small stationary soma, and protrusions, that can move and alter their size while scanning the environment, to upkeep homeostasis in the brain. In regions of the brain that support neurogenesis, microglia sustain the neurogenic niche that is needed for neurogenesis to occur. This is done by phagocytosis of not only apoptotic cells, but also some neuronal progenitor cells, so that the cellular density is contained (Kierdorf & Prinz, 2017). Microglia are constantly in contact with neurons, from which they can receive signaling through receptors that bind ligands such as fractalkine and CD200, which inhibit microglial cytotoxicity and inflammation. Disruptions in signaling from neurons, can cause microglia to become activated (Biber et al., 2007), which is their other state (Chen & Trapp, 2016).

Microglia can also be activated by brain injury or infection (Loane & Kumar, 2016) and they are the first cells that react to these offences (Gehrmann et al., 1995). Activated microglia have both neuroprotective and neurotoxic functions (Loane & Kumar, 2016) and are capable of presenting antigens (Gehrmann et al., 1995). Their activation results in changes in their morphology (Ji et al., 2013), migration to the site of insult, proliferation (Kettenmann et al., 2011), and the secretion of cytotoxic and inflammatory mediators (Gehrmann et al., 1995). Although they initially produce pro-inflammatory cytokines, this is usually downregulated after the offending condition has been resolved, and they can begin the production of anti-inflammatory molecules. However, if the pro-inflammatory signaling is not downregulated correctly, microglia may become neurotoxic and cause neurodegeneration (Loane & Kumar, 2016; Chitnis & Weiner, 2017). This neurotoxicity can also negatively affect neurogenesis. Nevertheless, when the neurotoxic stage is not reached, microglia may promote neurogenesis in the hippocampus and lateral ventricles, in response to injury (Chen & Trapp, 2016). There is some data suggesting that injury-related neurogenesis that is guided by microglia could also take place in other regions of the brain, but there is not enough supporting evidence for this (Ohira et al., 2010). Microglia can also perform synaptic stripping, in which they detach inhibitory axosomatic synapses, surround the neuronal cell bodies, and promote anti-apoptotic transcription. By doing this, microglia reduce the tissue damage that could otherwise be caused by injuries (Chen & Trapp, 2016). Finally, a high-fat diet has been shown to impact microglial activity and cause neuroinflammation (Thaler et al., 2012; Valdearcos et al., 2014; Gao et al., 2014; Cavaliere et al., 2019). However, there is evidence that a HFD increased pro-inflammatory signaling after three days, but the signaling was switched to anti-inflammatory after prolonged (four to eight weeks) exposure to such a diet (Baufeld et al., 2016).

Ionized calcium binding adaptor molecule 1 (Iba1) is a protein found in microglia and other macrophages. It is expressed in activated microglia for participation in membrane ruffling and phagocytosis (Ohsawa et al., 2004) and can therefore be used as a marker for neuroinflammation.

1.6 Adult neurogenesis

Adult neurogenesis has been determined to take place in at least two areas of the rodent brain: the subgranular zone (SGZ) of the dentate gyrus (Gould et al., 1998) and the subventricular zone (SVZ) of the lateral ventricles (Doetsch & Alvarez-Buylla, 1996). Newborn neurons migrate from the SGZ to the granule cell layer in the dentate gyrus, whereas neurons from the SVZ migrate to the olfactory bulb. There is also some controversial evidence for neurogenesis taking place in other regions of the brain (Aimone et al., 2014).

Hippocampal adult neurogenesis begins by neural stem cells in the SGZ dividing and producing neural progenitor cells (NPCs). There are three types (1-3) of progenitor cells, each representing a different stage in the development of a progenitor cell. Type 3 cells can transform into immature granule cells with the potential of becoming mature granule cells (Kempermann et al., 2004; Aimone et al., 2014). The newborn granule cells form connections with the pyramidal cells of the CA3 region of the hippocampus (Zhao et al., 2006). The type 2 and 3 progenitor cells, and the immature granule cells express a protein called doublecortin (DCX), which can be used as a marker for newly generated neurons in neurogenesis studies (Kempermann et al., 2004). As DCX does not have to be stained *in vivo*, it can also be used in human studies (Couillard-Despres et al., 2005).

NPCs can differentiate not only into neurons, but into glial cells as well. Because of this, NPCs can be found throughout the brain, and it has been found that these cells from regions that do not usually support neurogenesis, can generate neurons *in vitro* (Palmer et al., 1999). Therefore, it is likely that in the hippocampus, there are specific conditions which support neurogenesis. Such factors could be astrocytes, whose protein expression can increase or decrease neurogenesis depending on the protein, and vasculature, since NPCs are usually located near blood vessels, from which they may receive molecules that affect neurogenesis (Aimone et al., 2014). Microglia can also influence the process of neurogenesis, as newborn NPCs often undergo apoptosis in a few days after birth, after which microglia clear the dead cells, preserving homeostasis (Sierra et al., 2010). How microglia are activated and whether

the activation is acute or chronic, dictates whether they release pro- or anti-inflammatory cytokines. Microglia can influence neurogenesis through cytokines, for example, by steering the differentiation of NPCs into astrocytes. Different neurotransmitters have an impact on neurogenesis as well. GABA decreases proliferation in favor of differentiation. GABA and glutamate support survival, while serotonin possibly increases proliferation and acetylcholine is likely important for maturation and survival of the differentiated cell (Aimone et al., 2014).

Adult neurogenesis has been found to be affected by exercise and diet. For instance, caloric restriction and consumption of curcumin, blueberry polyphenols, resveratrol and polyunsaturated fatty acids have been shown to support neurogenesis, while a diet high in fat or sugar has been shown to decrease it. In addition, aging, oxidative stress, injuries, and neuroinflammation can decrease neurogenesis (Poulose et al., 2017). Hippocampal neurogenesis has been shown to benefit from probiotic treatment in mice. A two-week probiotic supplementation with *Lactobacillus helveticus* and *Bifidobacterium longum* in C57BL/6 mice prevented the decrease in neurogenesis and increase in intestinal permeability, caused by daily water avoidance stress sessions (Ait-Belgnaoui et al., 2014).

2 AIMS OF THE STUDY

The first objective of this study was to establish whether a high-fat diet increases weight gain. The second objective was to determine whether a high-fat diet affects microglial activation and neurogenesis in the hippocampal formation. The third objective was to find out whether dietary xylo-oligosaccharide influences the abundance of *F. prausnitzii*, and through that, whether it has a beneficial effect on neurogenesis and the activity of microglia. To achieve these objectives, experimental work was carried out with forty rats, divided into four groups, that were fed either with normal or high fat feed, with or without XOS supplementation. After the dietary intervention, the hippocampi of the rats were sectioned and immunohistologically stained with markers of activated microglia and newborn neurons. The number of immunopositive cells were calculated, and the results were statistically analyzed.

The first hypothesis was that a high-fat diet would lead to higher weight gain than a low-fat diet. The second hypothesis was that XOS fiber would increase the abundance of *F. prausnitzii*, prevalence of which would normally be diminished by the high-fat diet. The third hypothesis was that a high-fat diet would decrease hippocampal neurogenesis but adding XOS to the diet would ameliorate or remove the effect. Finally, it was hypothesized that the inflammation in the brain and consequently the heightened microglial activity caused by a HFD would be relieved by XOS.

3 MATERIALS AND METHODS

The first two sections of this chapter detail work done prior to this thesis, as a part of a larger research project. More details can be found from an article published by Lensu et al., 2020. The tasks performed by me begin from the paragraph titled 'sectioning'.

3.1 Rats

The brains used in this study came from four groups of ten rats. The first group was a low-fat control (LF control diet, 10 % energy from fat). The second group was fed the low-fat diet with xylo-oligosaccharide (concentration of 0.12 %, included in the LF control diet). The third group was fed with a high-fat diet (HFD, 60 % energy from fat), and the fourth group received the high-fat diet with XOS (concentration of 0.12 %, included in the HFD). The low-fat diets contained the normal amount of fat in the feed, but compared to the high fat counterparts, the diets are titled low fat for the purposes of this study. All diets were purchased from Labdiet/Testdiet, UK and were irradiated. All rats had the diets and normal tap water available *ad libitum*, the consumption of feed was measured every 24 h by weighing the feed.

The rats were male Wistar rats, and they were purchased from Charles River (Sulzfeld, Germany) at the age of 10-12 weeks. The basis for choosing Wistar rats was that pilot studies (Lensu et al., 2020) had shown that they harbor *F. prausnitzii* in their gut microbiota. The rats were habituated to their new environment for two weeks before any experiments begun. The diet intervention was started when the rats were about 15 weeks old, and it lasted for 12 weeks. Approval for the experiment had been acquired from the National Animal Experiment Board of Southern Finland (ESAVI/8805/4.10.07/2017). All experiments were performed in accordance with the Guidelines of the European Community Council directives 86/609/EEC and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123,

Strasbourg 1985). During the intervention, the rats were single-housed in standard Makrolon IV cages (Techniplast, Italy) in room temperature 21 ± 2 °C, humidity 55 ± 10 % and the light period was from 8.00 to 20.00 hr. All animals had aspen chips (Tapvei, Kaavi, Finland) as a bedding and nesting material.

Before and after the diet intervention, the rats went through several cognitive and behavioral tests which were recorded on video, for analysis in other research.

3.2 Sample collections

When the dietary intervention was completed, the rats were anesthetized with carbon dioxide-air mixture and their blood was drawn by cardiac puncture. Several tissues were collected and stored for other research purposes, while the cerebrum was cut in half in the midline. One half of the brain, including cerebellum, was fixed with 4 % paraformaldehyde for 48 h at +4 °C, washed twice with 0.01 M phosphate buffered saline [1xPBS, diluted from 10x PBS, 19.87 g disodium hydrogen phosphate (Na_2HPO_4), 2.57 g sodium dihydrogen phosphate (NaH_2PO_4) and 87.67 g sodium chloride (NaCl), V = 1 l, pH 7.2] and stored at +4 °C in PBS. To get information about the abundance of *F. prausnitzii* in the gut, DNA was extracted from the contents of the cecum and ran through real-time quantitative PCR.

3.3 Sectioning

The right side of each brain was cryoprotected in 30 % sucrose-solution (300 g sucrose in 0.1 M phosphate buffer [PB, 0.1 M disodium hydrogen phosphate (Na_2HPO_4), 0.1 M sodium dihydrogen phosphate (NaH_2PO_4), solutions were mixed until pH was 7.4] and the hippocampus was cryosectioned into 40 µm thick coronal sections using a sliding microtome (Leica SM 2010 R microtome, Leica Instruments GmBH, Germany). The tissues were attached to the microtome plate with the help of 0.1 M PB, Tissue-Tek (Sakura) and dry ice.

The sections were collected to 12 eppendorf tubes containing cryoprotective antifreeze solution (500 ml 0.05 M PB, 150 g sucrose, 300 ml ethylene glycol). Every

12th section went into one tube until the whole hippocampus had been sectioned, resulting in around 10 sections per tube. The sections were then stored in -20 °C.

3.4 Doublecortin staining

For DCX staining the sections were taken from the eppendorf tubes and put on a petri dish with 0.1 M Phosphate Buffer (PB) in it. On the petri dish the sections were counted, and their condition was inspected. For the staining, sections were put in labeled glass vials in which they were washed thrice in 2 ml of PB, with 5 min incubation in between, on a shaker plate.

PB was changed to 0.01 M tri-Na-citrate and the vials were boiled in 85 °C water bath for 15 minutes for the antigen retrieval. The citrate solution was washed with PB and the sections were left to cool for 15 minutes after which the endogenous peroxidase was blocked in 1% H_2O_2 for 30 minutes in room temperature. Then the sections were washed with 0.5 M Tris buffered saline with Triton X-100 (TBS-T, 6.06 g of Trizma hydrochloride and 1.39 g Trizma base with 29.2 g NaCl and 0.25 % Triton X-100) thrice and serum blocking was done for 1 h in room temperature. The serum blocking solution contained 0.5 M TBS-T and 2 % normal rabbit serum (S-5000, Vector; Lot. ZA0812). Finally, the sections were incubated with primary antibody solution [TBS-T + 1:2000 goat anti-DCX (sc-0866, Santa Cruz Biotechnology) + 2 % normal rabbit serum] overnight in +4 °C on a shaker plate.

Next day the sections were washed 3 x 5 min in TBS-T and incubated with secondary antibody solution 1:1000 Biotinylated Rabbit Anti-Goat IgG Antibody (BA-5000, Vector Labs/Mediq) in TBS-T for two hours in room temperature on a shaker plate.

After that, the sections were washed 3 x 5 min in TBS-T and put in tertiary antibody solution 1:1000 Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE Healthcare/VWR) in TBS-T for two hours in room temperature on a shaker plate.

The sections were washed 2 x 5 min in TBS-T and 1 x 5 min in 0.05 M Tris-buffer (6.08 g of Trizma hydrochloride and 1.4 g Trizma base, dissolved in distilled water,

pH 7.6, V = 1 l) and the staining was developed using 3,3'-Diaminobenzidine (DAB) (D4293, Sigma) as a chromogen. DAB was dissolved in 0.05 M Tris buffer, sonicated, and filtered. 30 % H_2O_2 was added to it, to catalyze the staining development. Solution was added into the vials in room temperature and color development was monitored for three and a half minutes. Reaction was ended by washing the sections 3 x 1 minute in 0.1 M PB and leaving them in PB overnight in +4 °C on a shaker.

A gelatin solution was prepared from water, gelatin (porcine skin, Type A, 300 Bloom) and potassium chrome sulphate. A petri dish was filled with the gelatin solution and the sections were transferred on it from the glass vial. After determining the correct anatomical order for the sections, they were mounted on a glass slide and left to dry in room temperature.

Sections were counterstained with Cresyl Violet and dehydrated with a rising alcohol series and xylene, and coverslipped with DEPEX mounting medium (VWR).

3.5 Ionized calcium-binding adapter molecule 1 staining

Iba1 staining was done very similarly to the DCX staining, except that on the first day a 3 % H_2O_2 solution was used during the peroxidase blocking and the serum was made of PBS-T + 2 % normal goat serum (Biowest, Cat# S200H-500, Lot# S114525200H). The primary antibody solution had Iba1 1:1500 (Host = rabbit, Thermo Fisher, Cat# PA5-27436, Lot# SI2446351E) in PBS-T + 2 % normal goat serum.

During the second day the TBS-T washes were done with PBS-T and the secondary staining was performed with goat anti-rabbit biotin 1:500 [Biotinylated goat anti-rabbit IgG (H+L), Vector Laboratories, Cat# BA-1000, Lot# 2A0324] in PBS-T. In the tertiary staining the antibody solution was 1:1000 Streptavidin-Horseradish Peroxidase Conjugate (RPN1231, GE Healthcare/VWR) in PBS-T. 1 ml of Nickel ammonium sulphate-solution was added to the DAB solution.

3.6 Microscopy and scanning

The number of DCX-positive newly generated cells were counted manually from the DCX stained samples, using an Olympus BX50 microscope (Olympus corporation, Japan), with a Colorview III camera, using 40x (Olympus UPIanFI, NA 0.75) and 10x (Olympus UPIanFI, NA 0.3) objectives. The DCX-positive cells were counted from the GCL and hilus regions of one hemisphere of the brain. The results were multiplied by two to account for the other hemisphere, multiplied by twelve to account for the sections between the stained sections, and divided by the number of stained sections. The results represent an estimate for the total number of DCX-positive cells in the DG, made proportional with the number of examined sections (sections n = 8-11).

The samples stained with Iba1 were scanned on Hamamatsu NanoZoomer-XR (Hamamatsu Photonics, Japan) with NDP.scan 3.2 software at the central hospital of Central Finland (Keski-Suomen keskussairaala). The scanned images were then analyzed using QuPath software (Quantitative Pathology & Bioimage Analysis, open source).

3.7 Image processing

In QuPath, images of the Iba1 stained samples were annotated for analysis. The CA1 and CA3 regions were annotated with 100 x 100 μm boxes. Hilus region was annotated with a 200 x 200 μm box. The granule cell layer was outlined by hand, manually (Figure 4). Four sections from the middle of the hippocampal formation of each rat were used for this analysis. To measure the number of Iba1-positive cells in the samples, the appropriate intensity thresholds were determined to distinguish them from the background. This was done by establishing the proper threshold for one sample by averaging the intensity across all the annotated areas (baseline sample). Then the true background intensities of all samples were measured from a small section of the samples, where there were no Iba1-positive cells. The threshold was then adjusted for the rest of the samples, by multiplying the baseline sample's average intensity by its true background and divided by the true background of

another sample. The resulting intensities were used in conjunction with a positive cell detection setting that made it so only cell shaped objects were detected. Finally, the results were made proportional to 1 mm^2, because of the varying sizes of the annotations.

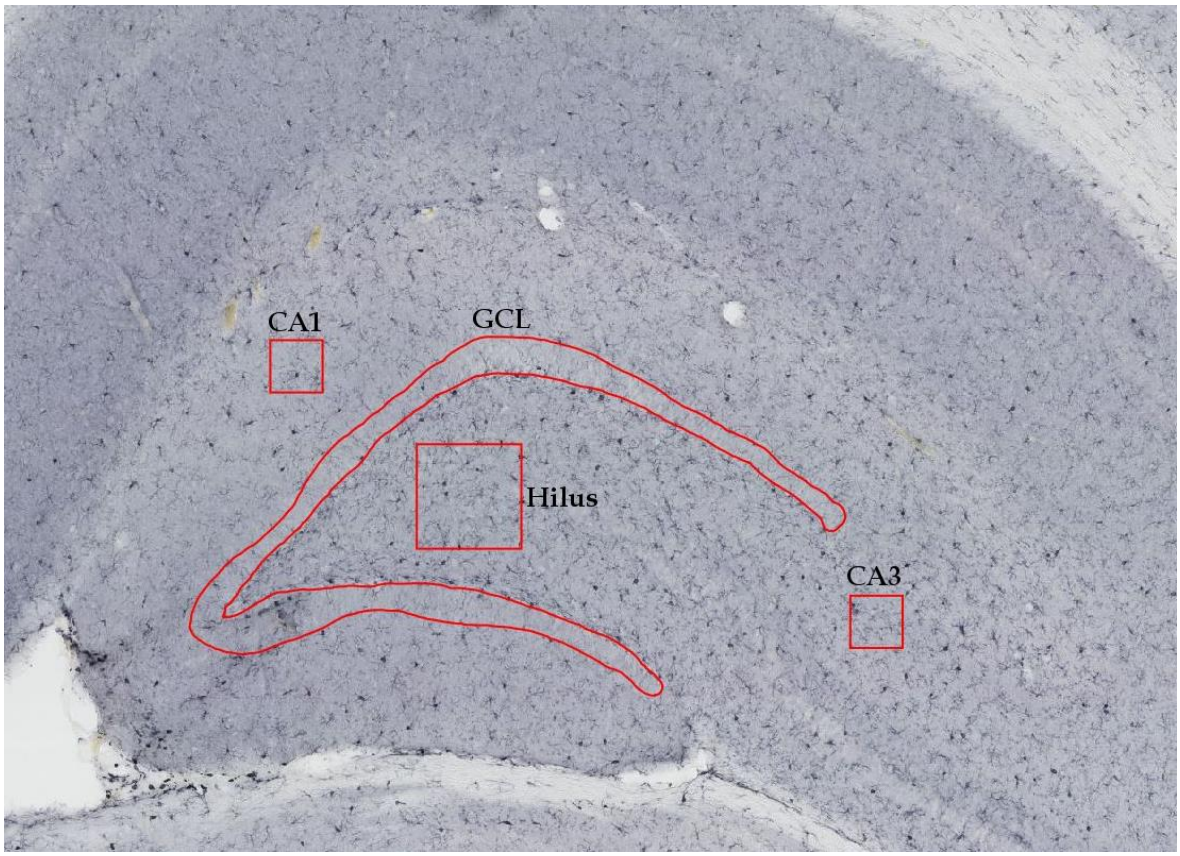

Figure 4. To analyze the number of ionized calcium-binding adapter molecule 1 positive cells in sections of the hippocampal formation, they were imaged and annotated in QuPath software. The annotations were placed in the *Cornu Ammonis* 1 (CA1), CA3, granulce cell layer (GCL), and hilus regions of the formation.

3.8 Statistical analysis

The statistical analyses for the acquired data were performed using IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA). To determine the significance of the different diets on neurogenesis, markers of microglial activity, and body weight, the parametric two-way analysis of variance (ANOVA) was chosen as the test to be used. The factors in the ANOVA tests were the amount of fat in the feed and whether XOS was supplemented. The abundance of *F. prausnitzii* was analyzed with Cohen's d test, because the deviations in microbiota compositions are large and

therefore parametric tests cannot be used. Thus, a test for effect size is more appropriate, with the effect sizes in Cohen's d being small (d = 0.2), medium (d = 0.5) and large (d = 0.8). Correlations between all variables were tested with Spearman's non-parametric correlation test.

4 RESULTS

4.1 Body weight, food consumption, and energy intake

The average body weight of the rats increased in all groups during the 12-week dietary intervention (Figure 5). The increase was higher with high-fat diets, than the low-fat diets ($p < 0.001$) (Table 1), indicating that HFD efficiently induced obesity. Xylo-oligosaccharide had no effect. The weight gain during the dietary intervention remained relatively steady in all groups (Figure 6).

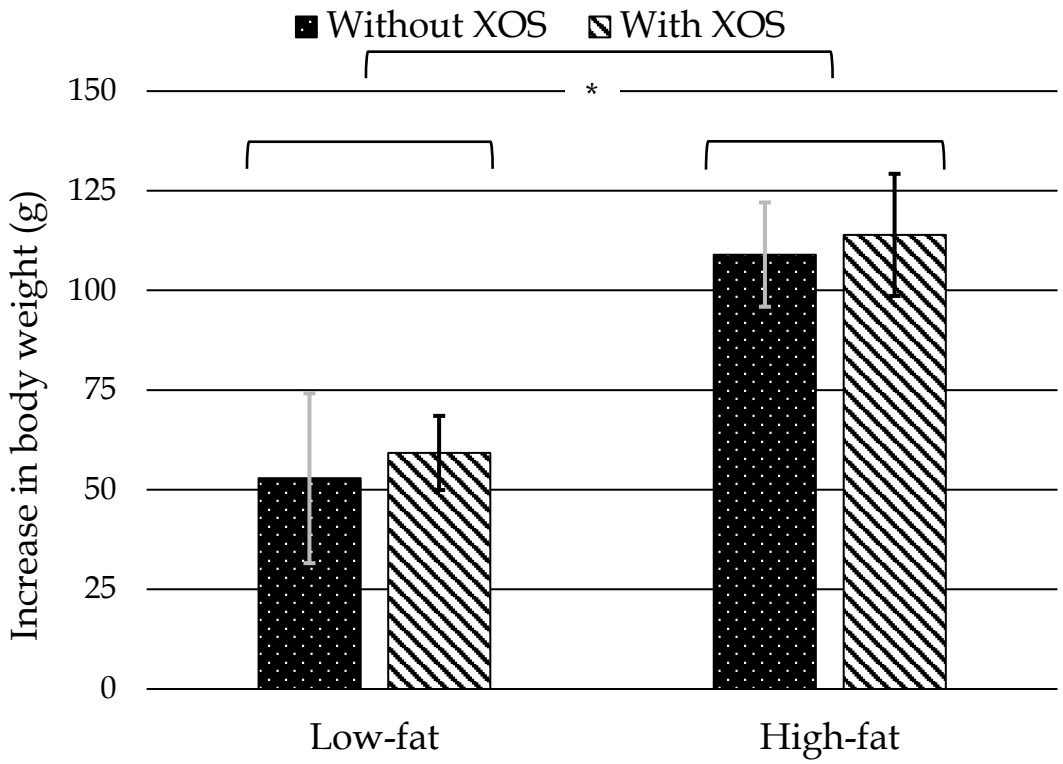

Figure 5. Four groups of rats were fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation for twelve weeks. During the diets, their body weights increased. Based on a factorial analysis of variance, with factors being the amount of fat in the diet and whether XOS was supplemented, the increase strongly associated with amount of fat in the diet ($p < 0.001$)*. The descriptors represent the mean increase of the groups' body weight during the diet and the 95 % confidence intervals, n = 10/group.

Table 1. The significance of the mean increase in body weight of four groups of rats, that were fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation for twelve weeks, were tested with a factorial analysis of variance. The factors were the amount of fat in the feed and whether the prebiotic XOS was supplemented or not. Significant values are in bold, n = 10/group.

	F-statistic	Error DF	DF	p-value
Amount of fat	66.436	36	1	**< 0.001**
Prebiotic	0.691	36	1	0.411
Amount of fat x prebiotic	0.011	36	1	0.918

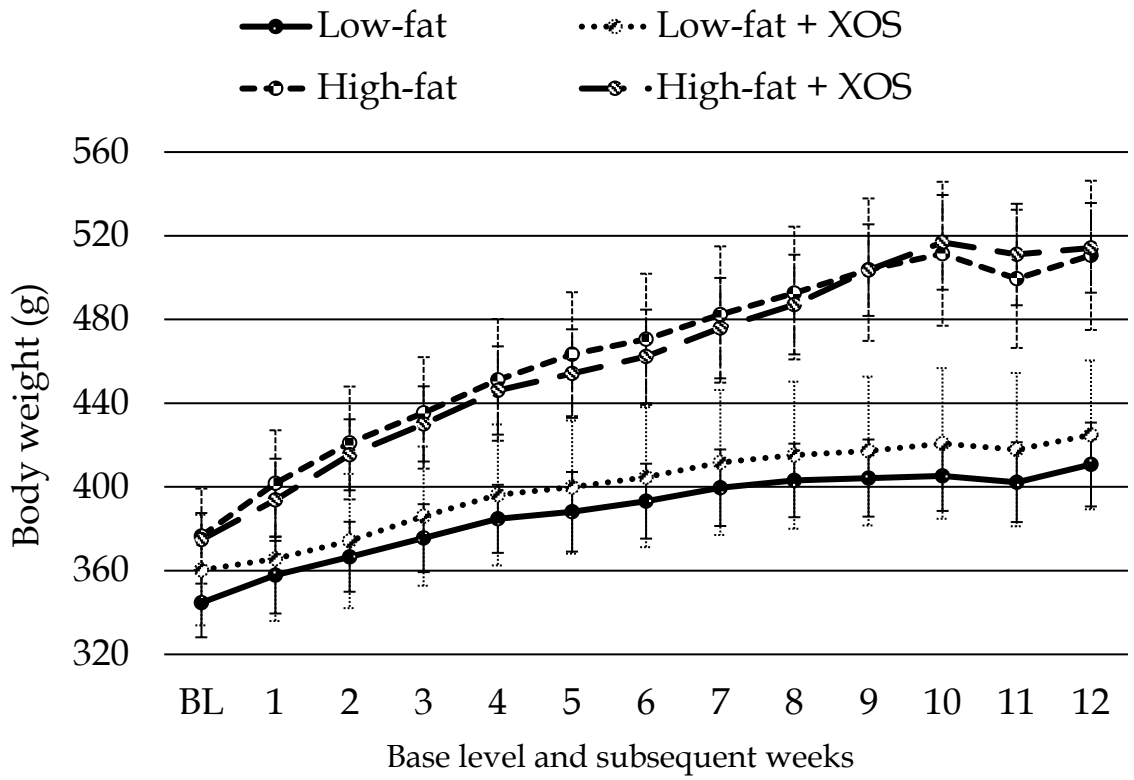

Figure 6. The body weights of four groups of rats, fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide supplementation were measured and the group means were calculated. The descriptors represent group mean body weights and standard deviations, n = 10/group.

During the dietary intervention, rats in the low-fat groups consumed more food than those in the high-fat groups ($p < 0.001$) (Table 2). The difference in consumption between the groups grew larger as the intervention continued further (Figure 7). However, when calculated as energy intake (Figure 8), it can be seen that the two low-fat groups initially consumed less energy than their high-fat counterparts. The difference narrowed as they begun eating more, and during the last four weeks the energy intake of all groups was more similar with each other. Still, on average, the higher amount of fat in the feed associated with a higher energy intake ($p < 0.001$) during the entire dietary intervention (Table 3).

Table 2. During a twelve-week dietary intervention, the daily food consumption of four groups of rats was weighed. The mean consumption was calculated from daily weighings that were a week apart and the results were used to calculate group means. The association of this group mean food consumption with different variables in the diets, low or high-fat (60 % of energy from fat), with or without xylo-oligosaccharide (XOS) supplementation, was tested with a factorial analysis of variance. The factors were the amount of fat in the feed and whether the prebiotic XOS was supplemented or not. Feeding the rats with a low-fat diet associated strongly with a higher food consumption. Significant values are in bold, n = 10/group.

	F-statistic	Error DF	DF	p-value
Amount of fat	41.894	36	1	**< 0.001**
Prebiotic	0.991	36	1	0.326
Amount of fat x prebiotic	2.562	36	1	0.118

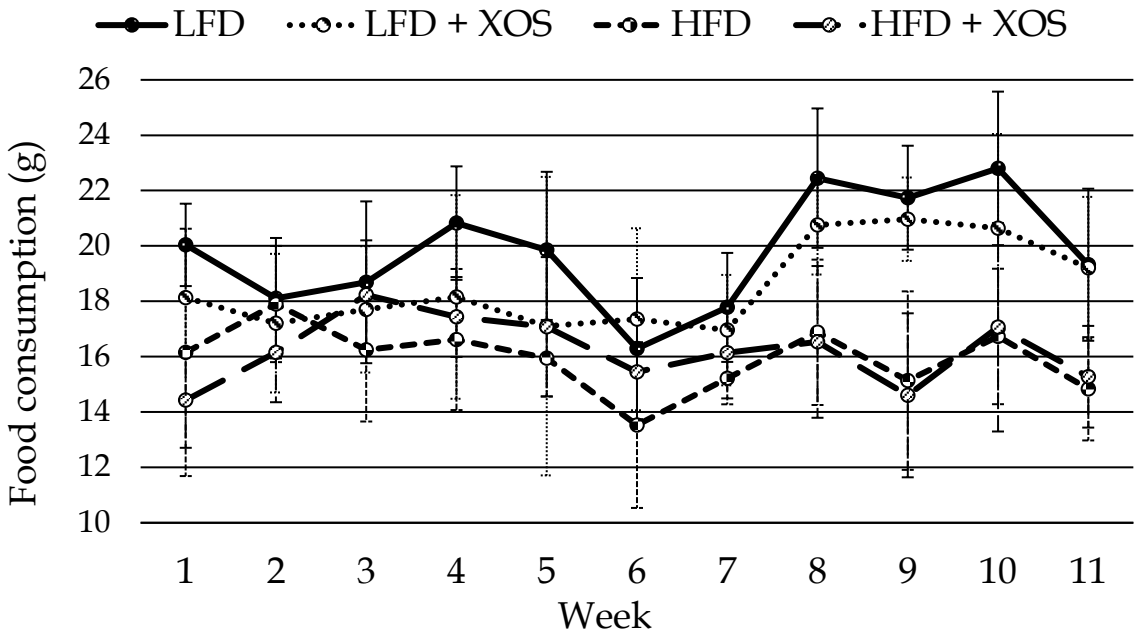

Figure 7. The food consumption of four groups of rats, fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide supplementation, was measured daily and the group means were calculated from weighings that were a week apart. The descriptors represent group mean food consumption and standard deviations, n = 10/group.

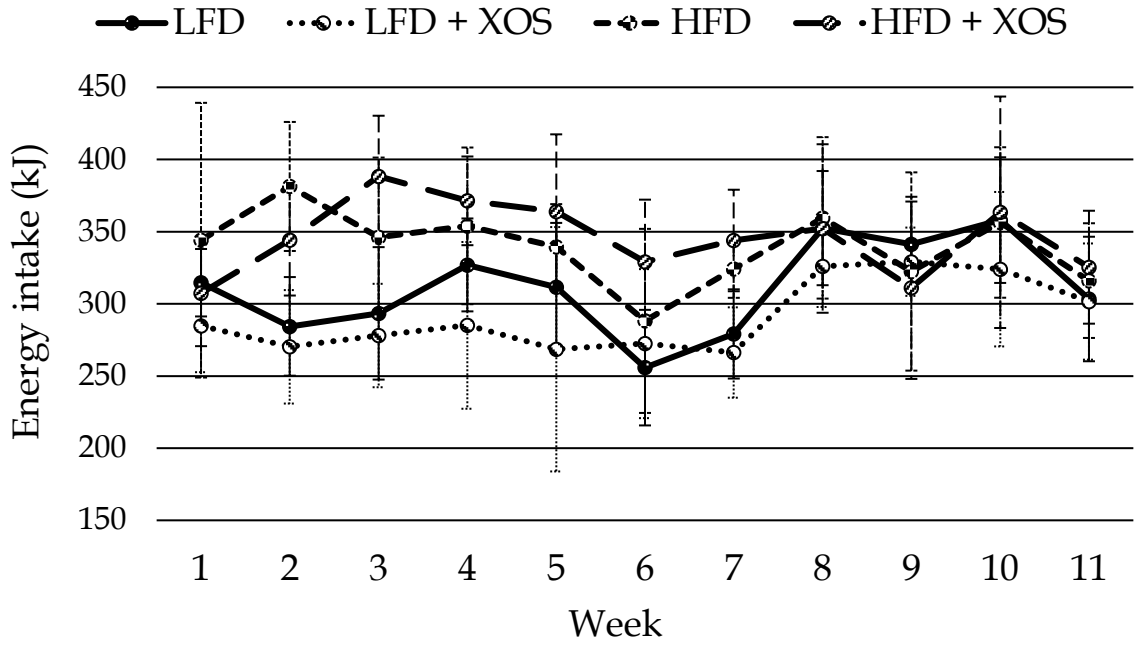

Figure 8. The energy intake of four groups of rats, fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide supplementation, was calculated based on food consumption, from which the group means were calculated. The descriptors represent group mean energy intake and standard deviations, n = 10/group.

Table 3. The significance of mean group average daily energy intake of four groups of rats, fed with low or high-fat (60 % of energy from fat), with or without xylo-oligosaccharide supplementation, were tested with a factorial analysis of variance. The factors were the amount of fat in the feed and whether the prebiotic XOS was supplemented or not. Significant values are in bold, n = 10/group.

	F-statistic	Error DF	DF	p-value
Amount of fat	21.473	36	1	**< 0.001**
Prebiotic	0.567	36	1	0.456
Amount of fat x prebiotic	2.106	36	1	0.155

4.2 Abundance of *F. prausnitzii*

After the dietary intervention, the abundance of *F. prausnitzii* in the low-fat groups tended to associate with XOS (d = 0.454) (Table 4). When comparing the effects of the different fat concentrations between the groups that received XOS, the low-fat diet tended to associate with a higher abundance of the bacterium (d = 0.613). After the diet intervention, the mean abundance of *F. prausnitzii* in the low-fat with XOS group was higher than in the rest of the groups (Figure 10).

Table 4. After a twelve-week dietary intervention, in which four groups of rats were fed with low (LFD) or high-fat (HFD) diets (60 % of energy from fat), with (LFD + XOS, HFD + XOS) or without xylo-oligosaccharide supplementation, the rats were euthanized and their cecal contents were gathered. The contents were processed with a qualitative PCR to measure the number of 16S rRNA gene copies in a gram of the cecal contents. These values were tested with a non-parametric Cohen's d test for pairwise comparison of the groups [values are significant when d ≥ 0.2 (low effect d = 0.2, medium effect d = 0.5, high effect d = 0.8)]. Significant values are in bold, n = 10 in HFD group, 9 in the rest.

	Cohen's d
LFD / LFD + XOS	**0.454**
HFD / HFD + XOS	0.162
LFD / HFD	0.014
LFD + XOS / HFD + XOS	**0.613**

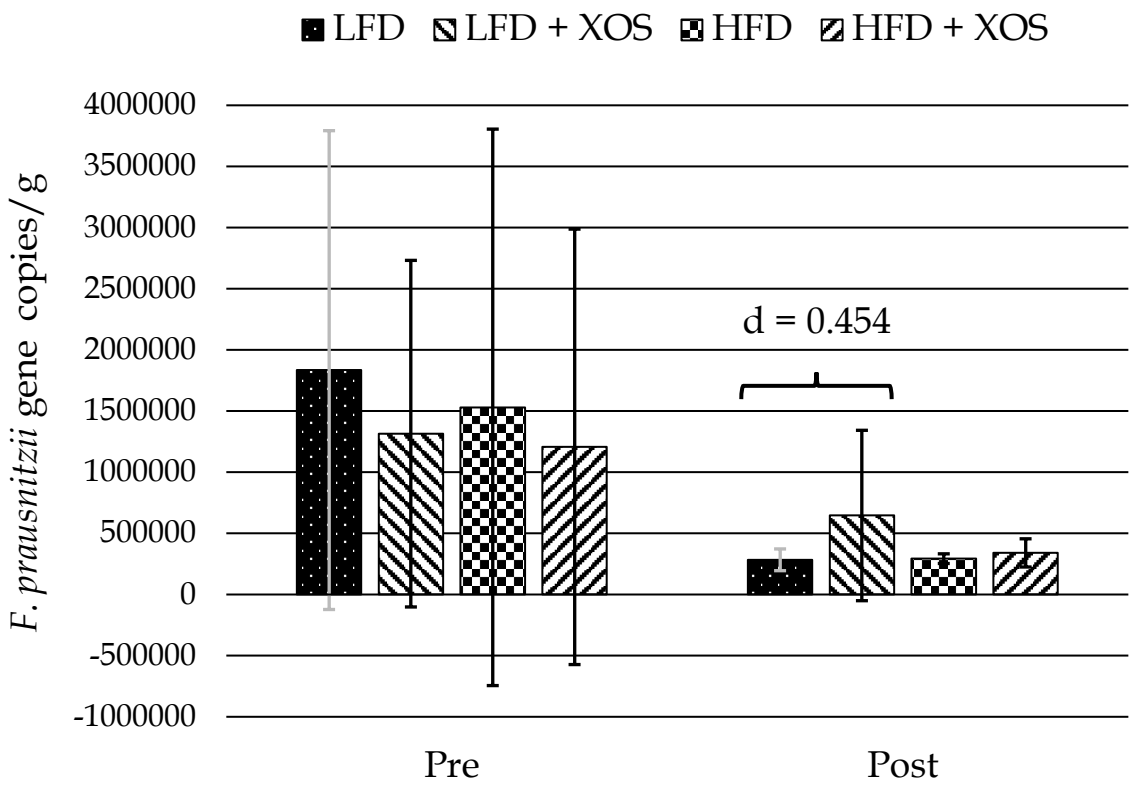

Figure 9. Four groups of rats were fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation for twelve weeks. Before the dietary intervention (pre), the abundance of *F. prausnitzii* was measured from feces, and after from the cecal contents (post) of the rats. This was done using qualitative PCR to determine the number of 16S rRNA gene copies per gram of feces or cecal contents. Finally, the group means were calculated. The descriptors represent group means and standard deviations. The resulting effect size from Cohen's d test (d = 0.454) between the low-fat and low-fat with XOS groups is highlighted with the bracket, n = 8-9/pre groups, 9-10/post groups.

4.3 Markers of microglial activity

The dietary intervention resulted in differences in the markers of microglial activity of the CA3, GCL, and hilus regions of the hippocampal formation (Figure 10). The high-fat diet resulted in less markers of microglial activity in the GCL region ($p = 0.028$) and tended to lessen the markers of microglial activity in the CA3 region ($p = 0.057$) (Table 5). In the hilus I found interaction between the amount of fat in the feed and the prebiotic in the markers of microglial activity ($p = 0.040$). A subsequent post-hoc test indicated a significant statistical difference between the low-fat group with XOS and the high-fat group with XOS ($p = 0.049$) (Table 6). The XOS

supplemented low-fat group had more markers of microglial activity than the XOS supplemented high-fat group.

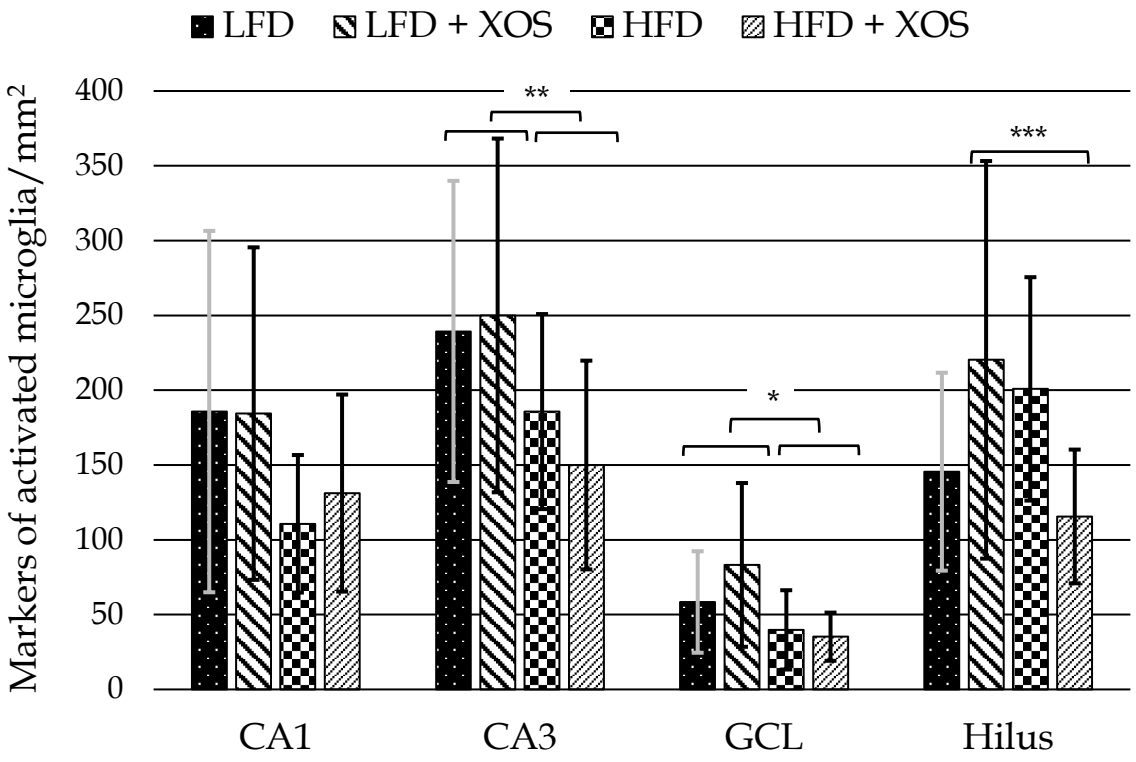

Figure 10. After the twelve-week dietary intervention, where four groups of rats were fed with low or high-fat (60 % of energy from fat) diets, with or without a prebiotic xylo-oligosaccharide (XOS) supplementation, sections of their hippocampi were stained with ionized calcium binding adaptor molecule 1, and images of these sections were analyzed. The analyses included 100 μm² areas from the CA1 and CA3 regions of the hippocampus and a 200 μm² area from dentate hilus. The granule cell layer (GCL) was outlined by hand. All counts were made proportional to mm² and analyzed. Based on a factorial analysis of variance, with the factors being the amount of fat in the feed and whether XOS was supplemented, the high-fat diets resulted in less markers of microglial activity in the GCL region ($p = 0.028$)* and tended to associate with less markers of microglial activity in the CA3 region ($p = 0.057$)**. Based on the same test, there was interaction between the amount of fat in the diet and the prebiotic supplementation ($p = 0.040$), which based on Fisher's least significant difference post-hoc test was the result of a significant statistical difference between the low and high-fat diets, that included the prebiotic ($p = 0.049$)***. The descriptors represent the group means of the markers of activated microglia in each of the four investigated regions (CA1, CA3, GCL, and hilus) and the 95 % confidence intervals, n = 7-8/group.

Table 5. After the twelve-week dietary intervention of four groups of rats with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation, their hippocampi were sectioned and stained with ionized calcium binding adaptor molecule 1 (Iba1). The number of Iba1 positive cells were counted from four regions of the hippocampal formation [CA1, CA3, granule cell layer (GCL), and hilus] and made proportional to mm^2. The results were tested with a factorial analysis of variance, the factors were the amount of fat in the feed and whether the prebiotic XOS was supplemented or not. Significant and close to significant values are in bold, n = 7-8/group.

Region	Independent variable	F-statistic	Error DF	DF	p-value
CA1	Amount of fat	2.803	26	1	0.106
	Prebiotic	0.063	26	1	0.804
	Amount of fat x prebiotic	0.082	26	1	0.777
CA3	Amount of fat	3.952	26	1	**0.057**
	Prebiotic	0.105	26	1	0.749
	Amount of fat x prebiotic	0.361	26	1	0.553
GCL	Amount of fat	5.473	25	1	**0.028**
	Prebiotic	0.504	25	1	0.484
	Amount of fat x prebiotic	1.062	25	1	0.313
Hilus	Amount of fat	0.443	26	1	0.512
	Prebiotic	0.020	26	1	0.889
	Amount of fat x prebiotic	4.662	26	1	**0.040**

Table 6. After the twelve-week dietary intervention of four groups of rats with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation, their hippocampi were sectioned and stained with ionized calcium binding adaptor molecule 1 (Iba1). The number of Iba1 positive cells were counted from four regions of the hippocampal formation [CA1, CA3, granule cell layer (GCL), and hilus] and made proportional to mm2. The results were tested with a factorial analysis of variance, the factors were the amount of fat in the feed and whether the prebiotic XOS was supplemented or not. The test revealed interaction between the amount of fat in the feed and the supplementation with XOS, which was analyzed further with the Fisher's least significant difference post-hoc test. Significant values are in bold.

		Mean difference	Std error	p-value
Low-fat	Low-fat with XOS	-74.777	52.411	0.166
	High-fat	-55.357	54.130	0.316
	High-fat with XOS	29.911	52.411	0.573
Low-fat with XOS	High-fat	19.420	52.411	0.714
	High-fat with XOS	104.688	50.634	**0.049**

4.4 Neurogenesis

The number of newborn neurons in the dentate gyrus was similar in all diet groups. (Figure 11). Neither the amount of fat in the diet, nor whether XOS was included in the feed seemed to impact the amount of neurogenesis (Table 7).

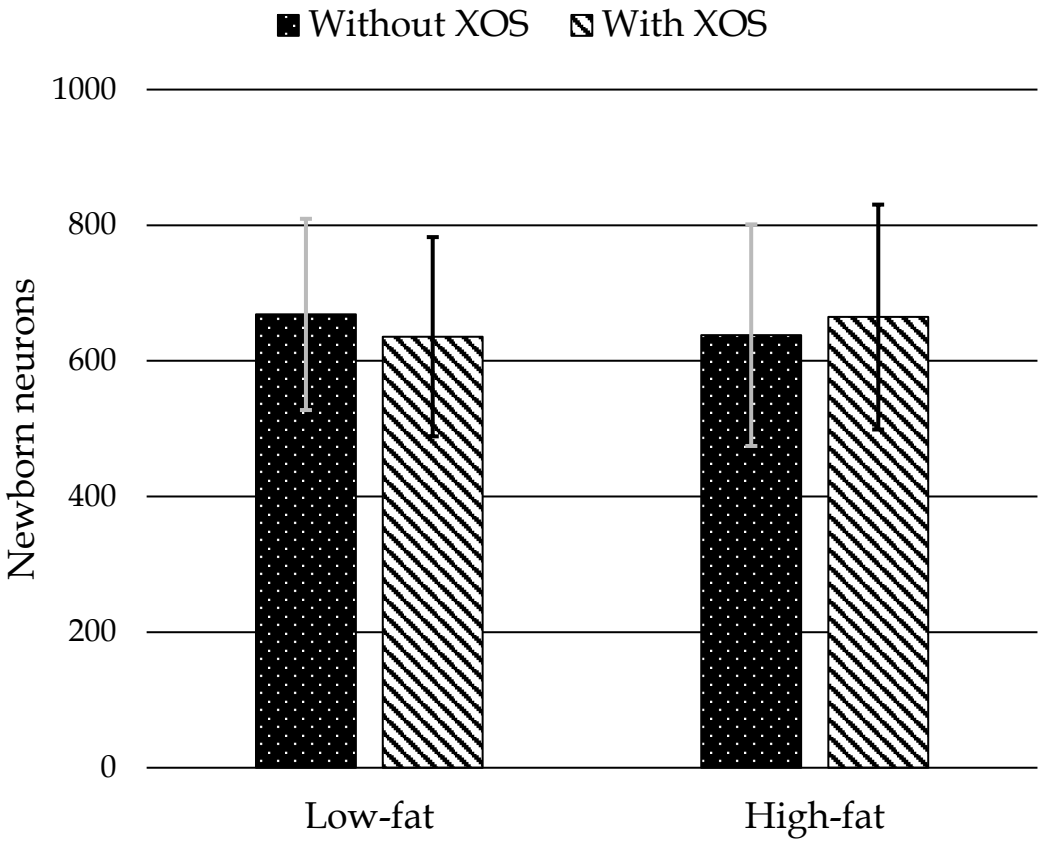

Figure 11. After the twelve-week dietary intervention of four groups of rats, fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide supplementation, their hippocampal formations were sectioned and stained with doublecortin (DCX). The number of DCX positive cells were counted from the granule cell layer and hilus of the dentate gyrus of one hemisphere of the brain. The results were adjusted for the other hemisphere and the missing sections and divided by the number of sections. Finally, the group means were calculated. The descriptors represent the group means for the newborn cells and the 95 % confidence intervals, n = 9-10/group.

Table 4. After the twelve-week dietary intervention of four groups of rats, fed with low or high-fat (60 % of energy from fat) diets, with or without xylo-oligosaccharide (XOS) supplementation, their hippocampal formations were sectioned and stained with doublecortin (DCX). The number of DCX positive cells were counted from the granule cell layer and hilus of the dentate gyrus of one hemisphere of the brain. The results were adjusted for the other hemisphere and the missing sections and divided by the number of sections. Finally, the group means were calculated and analyzed with a factorial analysis of variance, with the factors being the amount of fat in the feed and whether XOS was supplemented or not. Significant values are in bold, n = 9-10/group.

	F-statistic	Error DF	DF	p-value
Amount of fat	0.000	34	1	0.991
Prebiotic	0.002	34	1	0.964
Amount of fat x prebiotic	0.193	34	1	0.663

4.5 Correlation analyses

Based on Spearman's correlation (Table 8), the markers of microglial activation in the CA1, CA3, and GCL regions tended to correlate with food consumption ($p = 0.076$, 0.063, and 0.079 respectively). The increase in body weight associated with energy intake and food consumption ($p < 0.001$ and $p = 0.002$).

Table 5. After the twelve-week dietary intervention of four groups of rats that were fed with low or high-fat diets, with or without xylo-oligosaccharide supplementation, the markers of activated microglia in four regions of the hippocampal formation [CA1, CA3, granule cell layer (GCL), and hilus], the abundance of *F. prausnitzii* (FP), the number newborn cells (NG), the energy intake (EI), the food consumption (FC), and the body weight (BW), were measured. The results were tested with Spearman's non-parametric correlation test, to determine, whether there was correlation between any of the examined factors. There was strong correlation between the increase in body weight and the energy intake, and strong negative correlation between the increase in body weight and the food consumption. The markers of activated microglia in three regions of the hippocampal formation tended to correlate with the food consumption. Significant and close to significant values are in bold.

		FP	NG	EI	FC	BW
CA1	CC	-0.105	0.011	0.023	0.329	-0.095
	p-value	0.595	0.954	0.903	**0.076**	0.619
	N	28	30	30	30	30
CA3	CC	-0.231	-0.092	-0.150	0.343	-0.209
	p-value	0.238	0.627	0.429	**0.063**	0.267
	N	28	30	30	30	30
GCL	CC	-0.163	-0.031	-0.182	0.332	-0.096
	p-value	0.417	0.875	0.345	**0.079**	0.620
	N	27	29	29	29	29
Hilus	CC	-0.074	-0.121	-0.189	0.042	-0.107
	p-value	0.710	0.524	0.316	0.826	0.573
	N	28	30	30	30	30
Increase in body weight (BW)	CC	-0.092	0.036	0.652	-0.470	
	p-value	0.586	0.831	**< 0.001**	**0.002**	
	N	37	38	40	40	
Food consumption (FC)	CC	-0.168	0.065	0.059		
	p-value	0.320	0.698	0.719		
	N	37	40	40		
Energy intake (EI)	CC	-0.036	-0.007			
	p-value	0.833	0.966			
	N	37	38			
Neurogenesis (NG)	CC	0.009				
	p-value	0.959				
	N	35				

5 DISCUSSION

5.1 Recap of the hypotheses

I hypothesized that a high-fat diet would cause higher weight gain than a low-fat diet. Secondly, I hypothesized that a high-fat diet would decrease the abundance of *F. prausnitzii,* but supplementation with xylo-oligosaccharide would increase it. The third hypothesis was that a high-fat diet would decrease hippocampal neurogenesis, but XOS would alleviate or cancel the effect. My last hypothesis was that XOS would decrease the markers of microglial activity, which would be increased by a high-fat diet.

5.2 Energy leads to weight gain

The energy density of the diets was found to have a strong impact on body weight, as the HFD groups gained on average almost twice the weight of what the LFD groups gained, supporting previous findings (Cordner & Tamashiro, 2015).

The increase in body weight correlated strongly with the measured energy intake. This makes sense, as an increase in body weight requires an energy surplus. Interestingly however, the energy intake and food consumption fluctuated throughout the dietary intervention, whereas the increase in body weight was rather consistent. The fluctuations could be caused by fluctuations in activity, although that was not measured.

I found a significant negative correlation between the food consumption and increase in body weight: the low-fat groups consumed more food but gained less weight. This can be explained by the fact that low-fat feed is not as dense in energy and must therefore be eaten more. When the dietary intervention began, the food consumption was quite even between the four groups (Figure 7), whereas the energy intake was higher in the high-fat groups (Figure 8). However, as the intervention progressed, the food consumption increased in the low-fat groups, while (slightly) decreasing in the high-fat groups. That led to the difference in

energy intake being more moderate between all groups in the later weeks of the intervention. This could imply that satiety is driven more by energy than volume, which is supported by previous research (De Graaf & Hulshof, 1996). It could be interesting to study whether the rate of weight gain would even out in a longer intervention. If not, it could be possible that a HFD reduces activity, causing more weight gain. This could also have negative implications for neurogenesis and neuroinflammation, as exercise has been shown to improve them (Seo et al., 2019).

5.3 *F. prausnitzii* abundance increased with a healthy diet and XOS

The hypothesis was that a high-fat diet would decrease the abundance of *F. prausnitzii*, while supplementation with XOS would have an increasing effect on it. Based on the results, XOS did seem to have a positive influence on the abundance of the bacterium, when paired with a low-fat diet. Therefore, it is possible that XOS could be a beneficial supplementation to a healthy diet, causing an increase in the abundance of *F. prausnitzii* that might support health. However, in the high-fat groups, XOS did not significantly affect *F. prausnitzii* abundance. It could be that the negative effects of the HFD were too severe for XOS to overcome, but that would indicate that the HFD did indeed have negative effects. Yet, the abundance in the high-fat groups did not significantly differ from that in the control group, and therefore no such conclusions can be made.

When inspecting the measured abundance of *F. prausnitzii* before the dietary intervention and comparing it with the data gathered after the intervention (Figure 9), it would appear that the overall abundance across all groups decreased quite a lot. However, the earlier measurements were made from feces, while the latter were from cecal contents, which makes the data incomparable. Besides, the large deviations in the data gathered at the start of the intervention imply that the true means for the abundances could be closer to the values in the latter data.

5.4 The LFD with XOS -supplementation increased neuroinflammation

The dietary interventions had an impact on the markers of microglial activity in the hippocampus. The impact was strong in the GCL and hilus regions, and tentative in the two other regions investigated. The low-fat groups had more markers of microglial activity than the high-fat groups in the CA1, CA3 and GCL regions (Figure 10). These results contradict previous findings, where a HFD has been shown to increase inflammation in the brain (Valdearcos et al., 2014).

Despite the prebiotic was expected to decrease inflammation, we did not observe the anticipated role of it, as regardless of the XOS -supplementation, the markers of microglial activity were highest in three hippocampal regions of the low-fat group that received XOS (Figure 10). In the hilus, we found interaction between the amount of fat in the feed and the supplementation with XOS. Further analysis revealed that XOS -supplementation decreased the markers of microglial activity when paired with a high-fat diet but increased it with a low-fat diet. There is existing evidence that XOS -supplementation can reduce inflammation caused by a HFD (Fei et al., 2019), which is challenged by our results. Although the abundance of *F. prausnitzii* was not measured, the same study did examine the levels of butyrate, which is an important fermentation product of *F. prausnitzii*, and found that it was decreased by a HFD, but increased with the supplementation of XOS (Heinken et al., 2014; Fei et al., 2019).

F. prausnitzii has been shown to reduce inflammation (Huuskonen et al., 2004; Heinken et al., 2014), and XOS has been shown to increase the abundance of *F. prausnitzii* (Finegold et al., 2014). Therefore, the low-fat group that received XOS should have had the lowest number of the markers of activated microglia, especially as it was found to have the highest abundance of the bacteria. However, the opposite was observed in this study. It is unlikely that *F. prausnitzii* would be the culprit for the higher inflammation level. Instead, a more reasonable explanation could be that the higher abundance would be a result of compensation against the inflammation, similarly to what have been observed in the gut microbiota composition of patients suffering from bipolar depression (Lu et al., 2019).

Even though a high-fat diet did not increase the markers of microglial activity, eating habits could still have an impact on neuroinflammation. This notion comes from the finding that food consumption had tentative correlation with the markers of microglial activity in three regions of the hippocampus. Therefore, further research on the effects of high-volume consumption on inflammation might be warranted.

5.5 Neurogenesis was unaffected by the diets

We had set out to investigate the effects of a high-fat diet on neurogenesis, and whether a prebiotic supplementation would impact the effects. After the twelve-week dietary intervention, no significant differences could be observed in any of the four groups. A diet high in fat has been shown to negatively influence adult hippocampal neurogenesis (Lindqvist et al., 2006; Park et al., 2010), partially by causing neuroinflammation (Cavaliere et al., 2019). However, inflammation seemed to be lower in the high-fat groups, which contradicts this belief. Still, this does not explain why there were no differences in the occurrence of neurogenesis between the groups. Since inflammation was highest in the brain of the rats on the LFD with XOS, it would be expected that this group would have had the least newborn neurons. This could have been ameliorated by the fact that the same group had the highest abundance of *F. prausnitzii* in the gut. The issue is that the main way *F. prausnitzii* could positively influence neurogenesis is by lessening inflammation, which obviously was not observed in this group. In any case, the witnessed markers of microglial activity, or the differences in the abundance of *F. prausnitzii* cannot account for the absence of significant differences between the groups. This is because the abundance of the bacteria was significantly higher in only one group and the markers of microglial activity varied among multiple groups.

There are a few studies about the effects of a HFD on adult neurogenesis. One study has shown a decline in neurogenesis using male Sprague-Dawley rats (Lindqvist et al., 2006), while another study with similar results used male C57BL/6 mice (Park et al., 2010). A third study has found that a HFD led to a decrease in neurogenesis in

female C57BL/6 mice, but not male (Robison et al., 2020). None of these studies used Wistar rats, which were chosen for this thesis, because it had been previously demonstrated that they harbor *F. prausnitzii* in the gut, while, for instance Sprague-Dawley rats do not (Lensu et al., 2020). Therefore, it is possible that the mere presence of *F. prausnitzii* led to amelioration against the effects a HFD, and that its higher abundance in the low-fat with XOS group was unnecessary for this protection. This and the contribution of gender to deficits in neurogenesis caused by a HFD could warrant further research. Also, additional investigation of neurogenesis in different strains and species of animals could clarify these contradictory results. According to Snyder et al. (2009), rat neurogenesis appears to be more robust than that of mice, as granule cells mature around a week or two slower in mice than rats. While the proliferation of precursor cells is equal in both species, more cells survive in rat brains and the number of new and functioning granule neurons is higher in rats. There are differences in the rate of neurogenesis between different strains of mice (Snyder et al., 2009), and spatial learning -related neurogenesis has been shown to differ between Sprague-Dawley and Long-Evans rats with the latter showing less doublecortin-positive immature neurons (Epp et al., 2011). Therefore, it is possible that neurogenesis in Wistar rats could respond differently to a HFD than in Sprague-Dawley rats.

It is worth noting that if *F. prausnitzii* protected against the adverse effects of a HFD on neurogenesis, this protection was preventive. Similarly, if there had been differences between the groups that received XOS -supplementation and those that did not, the effect of XOS could have been preventive, since the supplementation was begun at the same time as the high-fat diets. Hence, if further research is going to be conducted, either with XOS -supplementation or directly with *F. prausnitzii*, it could be reasonable to also explore whether the possible beneficial effects could reverse impairments caused by a prior high-fat diet. This would also be interesting in the framework of the current obesity epidemic.

5.6 Conclusions

As was expected, the higher amount of fat in the feed led to a significantly higher increase in the body weight of the rats. This increase correlated with the measured energy intake as well. After the dietary intervention, the abundance of *F. prausnitzii* was higher in the group that had been fed low-fat feed with XOS. No meaningful difference could be observed between the two high-fat groups. Therefore, it is possible that XOS could be beneficial as a supplement in a healthy diet, as *F. prausnitzii* has been found to have health benefits, but it should not be relied on as a cure for the problems caused by a diet high in fat. The differences in the markers of microglial activity did not concur with my hypothesis, and there were no substantial disparities in neurogenesis between the four groups. As the number of newborn neurons was similar in the study groups to that of the control group, it would seem logical that the diets had no negative effects on the occurrence of neurogenesis.

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