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Master thesis

Targeting microbiota-gut-brain axis in Alzheimer's disease in a diet-induced obesity model in mice

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AD - Alzheimer's disease

APP - Amyloid precursor Protein

Aβ - Amyloid-β

FOS - Fructooligosaccharides

GIT - Gastrointestinal tract

GOS - Galactooligosaccharides

GTT - Glucose Tolerance Test

HF - High-fat

NFT - Neurofibrillary tangles

OF - Open Field

SCFAs - Short chain fatty acids

SVM - Social V- Maze

T1D- Type 1 Diabetes

T2DM- Type 2 Diabetes mellitus

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. The onset of this neurodegenerative disease affects life quality of both the individuals and their families, and large amounts of resources and money are spent in caring for people with AD. Approximately 35.6 million people of the world population are living with dementia and there has been a gradual increase each year, with estimated numbers doubling by 2030 and more than tripling by 2050 (Jack et al. 2011). This has led the World Health Organization to raise awareness of dementia as a public health priority. Despite increasing research in this field, the exact cause of AD is still poorly understood and existing treatments are very limited to symptomatic treatments. Although AD usually affects people at the age older than 65 years, the disease is not a normal part of ageing. Despite being rare, the early-onset AD can also affect people at younger age. Genetic factors, such as inheritance of the apolipoprotein E gene, i.e., the most common susceptibility gene in human AD, are neither necessary nor sufficient for development of AD. Also, ageing and genetic risk factors do not completely explain the globally rapid increase in AD prevalence, which might instead be explained by additional environmental influences. Understanding the initial triggers for AD pathology is needed for the development of preventive or therapeutic approaches for AD (Veitch et al. 2019).

Over the past decades researchers have revealed that the commensal gut microbiota and its metabolites play a crucial role in host health, including metabolism, physiology, nutrition, immune and barrier functions (Bostancikliоğlu 2019). More recently, its impact on brain functions and behaviour had also been revealed (Quigley 2017). This has emerged within the concept of gut microbiota-gut-brain axis, a bidirectional communication between the gut and the brain. A number of studies observed that specific groups of gut microbiota are associated with the development of brain-related disorders, such as autism spectrum disorder, Parkinson's disease and multiple sclerosis (Westfall et al. 2017) . These further emphasize the possible involvement of the gut microbiota in neurodegenerative diseases, including AD (Ling Zhang et al. 2017). Yet the research regarding the role of the gut microbiota on AD is still in its early stages.

The gut microbiota and their hosts have long co-evolved and shared substrates for growth. The establishment of the gut microbial community occurs during the first few years of life (R. Liu et al. 2017).. Westernization of the diet i.e., increased intake of refined foods rich in saturated

fat and low in dietary fibre, is an important factor giving rise in the prevalence of metabolic syndrome, which is also associated with dementia (Holm-Hansen et al. 2016). In human and pre-clinical models, unhealthy high-fat (HF) diets have been shown to cause an imbalanced community of the gut microbiota (Veniaminova et al. 2020). This may suggest the interactions between the gut microbiota, metabolic and neurodegenerative disorders.

Apart from understanding the exact mechanisms mediating the involvement of the gut microbiota in metabolic and neurodegenerative disorders, the current challenge is also to understand what a healthy gut microbiota is composed of and how to achieve a balanced gut-brain communication via gut microbiota manipulation.

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

Aim of the thesis - To find bacterial strains of microbiota that can reduce inflammation and the development of AD under the diabetic conditions**.**

Objectives:

- To validate a diet-induced diabetes leading to AD model in mice.
- To analyse the anxiety like behaviour, cognition, social behaviour and physical activity in the diet-induced obesity in mice administrated with prebiotics.
- To assess whether a long-term mice dietary intervention altering the gut microbiota, is responsible for memory impairment.

1. LITERATURE REVIEW

1.1. AD and dementia association

Dementia is studied to be a syndrome that affects memory and other cognitive functions to the extent that it interferes with daily function. There are many circumstances that can provoke this syndrome, including neurodegenerative disorders cerebrovascular disease, brain injury, and certain infections (Alkasir et al. 2017).

Dementia systems mainly includes decline in memory and decline in at least one of the following cognitive abilities:

- Ability to generate proper speech or to understand spoken or written languages;
- To identify objects, assuming intact sensory function;
- To perform motor activities, sensory function, and comprehension of the required task
- Ability to think clearly, to make sound judgments, and execute a complex task.

The decline in cognitive abilities must be severe enough to interfere with daily life to confirm the patient's condition (Gaugler et al. 2016a).

The prevalence of dementia is rapidly increasing in developed countries because of social and demographic changes. This trend is expected to worsen in the coming decades, with the number of cases possibly even tripling in the next 25 years (Rodríguez-Gómez et al. 2019). AD is the most common cause of dementia (Gaugler et al. 2016b).

AD described the long-term study of the female patient Auguste D., whom Alois Alzheimer had observed and investigated at the Frankfurt Psychiatric Hospital in November 1901. On November 3, 1906, a clinical psychiatrist and neuroanatomist, Alois Alzheimer, reported "A peculiar severe disease process of the cerebral cortex" (Stelzmann, Norman Schnitzlein, and Reed Murtagh 1995)to the 37th Meeting of South-West German Psychiatrists in Tübingen (Dage et al. 2017). Also, it is proved that females are much more prone to get AD as compared to males (Evans et al. 2011).

AD has become one of the major diseases that threatening the health of elder people in modern society, affecting at least 27 million people and corresponding from 60% to 70% of all dementias cases (Frigerio et al. 2019; Silva et al. 2019). The age is the main associated risk factor and the age of 65 years is used to distinguished AD patients with an early-onset or late-onset form. It is likely that diverse factors contribute to the pathogenesis of late-onset AD (Spires-Jones and Hyman 2014).

AD can affect vast number of people in different ways, but the most common symptoms pattern starts with a gradual worsening difficulty in remembering new information. This is because disruption of neuron usually takes place in regions which are involved in forming new memories. As damage spreads, individuals experience various other difficulties. The following are alarming signs of AD:

- Memory loss that makes daily life more difficult.
- Challenges faced during planning a task or solving problems.
- Difficulty in completing familiar tasks at home.
- Confusion with time or place.
- Trouble understanding visual images and relationships.
- Problems with words in speaking or writing in native.
- Misplacing things and losing the ability to retrace them back.
- Poor judgment in difficult conditions.
- Withdrawal from social activities.
- Changes personality and stability.

Dementia due to AD is characterized by memory, thinking, and behavioural symptoms that impair a person's ability to function in daily life and that are caused by AD-related brain changes (Reitz and Mayeux 2014; Ding et al. 2019; J. Yang et al. 2019).

1.2. Development of AD

Numerous factors contribute to one's likelihood of developing AD (Terry 2006). The biggest risk factor for AD is aging. Most people diagnosed by AD are from the range of 65 or older. However, it is studies that some people younger than 65 age can also develop the disease, although this a rare case. Aging is not the only known risk factor for AD, there are many more

factors responsible as well (Mathay et al. 2017; R. Liu et al. 2017). It has been implied that AD is a complex multifactorial disorder, with many proposed theories as to the cause of AD, including:

- exacerbation of aging (Crous-Bou et al. 2017),
- degeneration of anatomical pathways, which includes the cholinergic and cortico-cortical pathways (A Armstrong 2018),
- an environmental factor such as exposure to aluminium, severe head injury (Colomina and Peris-Sampedro 2017),
- genetic factors including mutations of amyloid precursor protein (APP) and presenilin genes (Fenoglio et al. 2018),
- mitochondrial dysfunction in neurons (Wilkins and Swerdlow 2016),
- a compromised blood brain barrier (Cai et al. 2018),
- a dysfunction in immune system (Calsolaro and Edison 2016; Albensi 2019),
- infectious agents (Sochocka, Zwolińska, and Leszek 2017).

There is no cure yet found for AD and new therapeutic agents need to be discovered to cure this particular disease (Dage et al. 2017).

1.3. Pathology of AD

Characterization of AD is done by loss of neurons and synapses in the cerebral cortex and certain subcortical regions, also widening of cerebral sulci and ventricular dilatation is observed (Fjell et al. 2014). These atrophy is also marked as the medial temporal lobe with initial changes in the entorhinal cortex (functions as a hub in a widespread network for memory, navigation and the perception of time) (Moodley and Chan 2014) and subsequently the hippocampus; as hippocampus plays an important role in memory formation, learning and remembering the events (String et al. 2010). The neuropathological hallmarks of AD include extracellular β-amyloid plaques and intracellular neurofibrillary tangles (NFT) when tested histochemically (X. Hu, Wang, and Jin 2016).

In maximum cases of early-onset AD derive from a combination of genetic mutations in genes encoding APP and presenilins 1 and 2 (Janelidze et al. 2016). APP which is cleavage by β and

γ secretase complexes leads to the formation of amyloid-β (Aβ) peptides that can aggregate and form amyloid plaques outside the cell (Serý et al. 2013) (Figure 1).

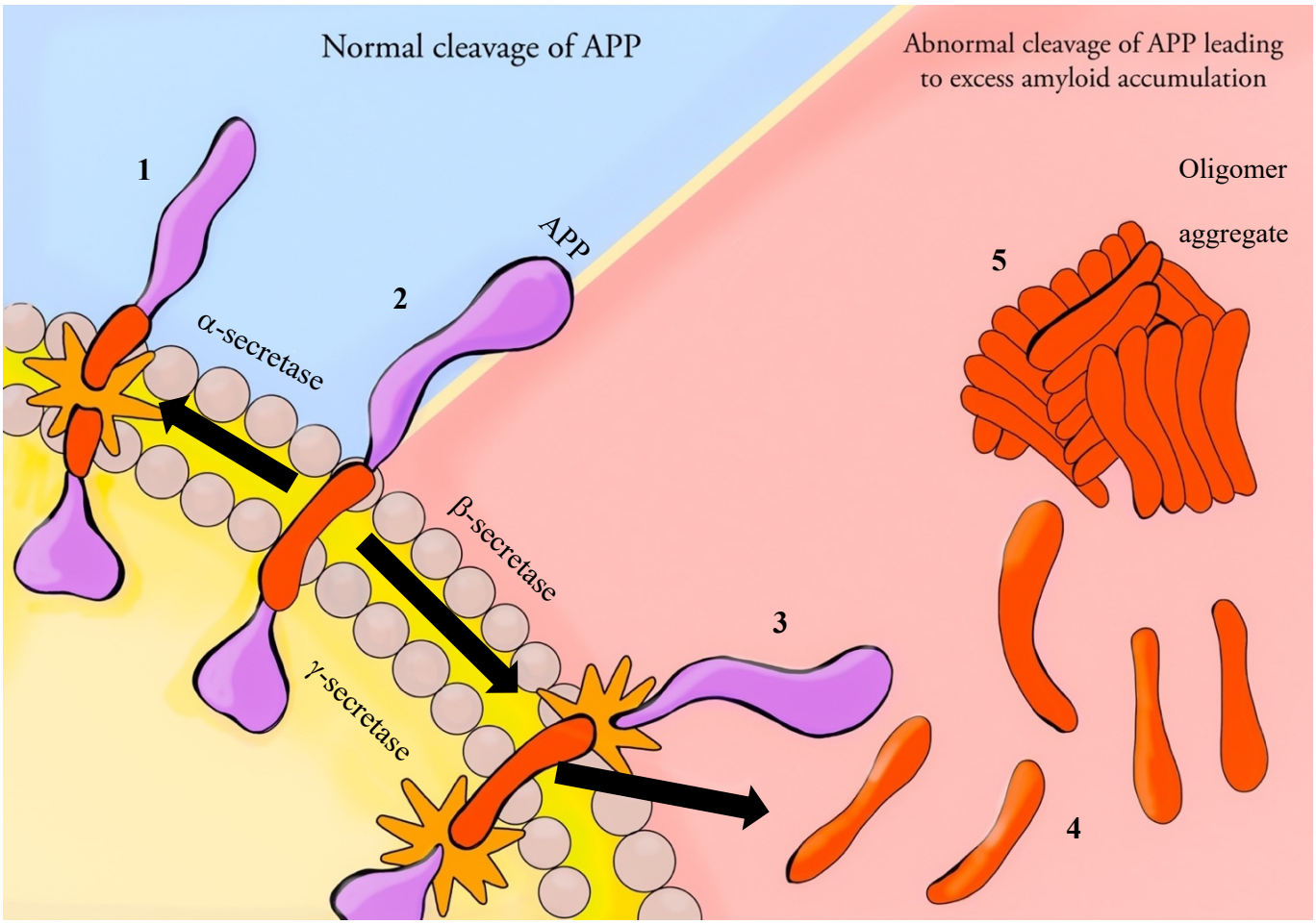


Figure 1. Aβ Formation

1. The APP present on the cell membrane of the neuron cleave for α-secretase which results in the formation of two soluble aggregate which can be digested by the microglial cells
2. When APP is cleaved by β-secretase and γ-secretase due to APP mutations and PSEN1/PSEN2 mutation it leads to formation of Aβ, insoluble aggregates.
3. These Aβ aggregates are insoluble and starts to aggregate outside the neuron
4. These aggregates are sticky in nature and can cause plaque in the brain which can be examined histochemical techniques.
5. These plaques are the confirmation of AD and are one of the major hallmarks of AD. These aggregates intrust in neuron signalling and also triggers the formation of another hallmark of AD – tau protein aggregation forming NFT.

These Aβ peptides are mainly composed of the 42 amino acid peptides, which is less abundant but more are prone to aggregation than the Aβ1–40 peptides (Weller and Budson 2018; Hane, Lee, and Leonenko 2017). Amyloid deposits and NFT, which comprises hyper phosphorylated tau (τ) protein (Figure 2), are the most important pathologic hallmarks of AD (Saha and Sen 2019). Plaques formed are very dense, and mostly insoluble beta amyloid peptide and cellular material outside and around the neurons (Mroczko et al. 2018). NFT are aggregates of the microtubule-associated protein tau, which has become hyperphosphorylated and accumulate inside the cells (A Armstrong 2018).

Hence, AD is a progressive neurodegenerative syndrome associated with the accumulation of proteinaceous misfolded Aβ fibrils and oligomers, together with neurofibrillary tangles consisting of hyper-phosphorylated tau protein, in the cerebral cortex and other brain regions (Bonfili et al. 2017) (Figure 3). Tau is the most important microtubule associated protein of a normal mature neuron, alternative splicing of its pre-mRNA generates six molecular τ isoforms in human brain (Ding et al. 2019).

Preclinical studies for AD have been crucial in establishing a causal role of the commensal gut microbiota in shaping brain function and behaviour (Fröhlich et al. 2016). Comprising trillions of symbiotic microorganisms, the gut microbiota is an essential element for the maintenance of the host's health (Giau et al. 2018). The gut microbiota residing organisms can form amyloid polymers which can trigger to AD progression (X. Hu, Wang, and Jin 2016).

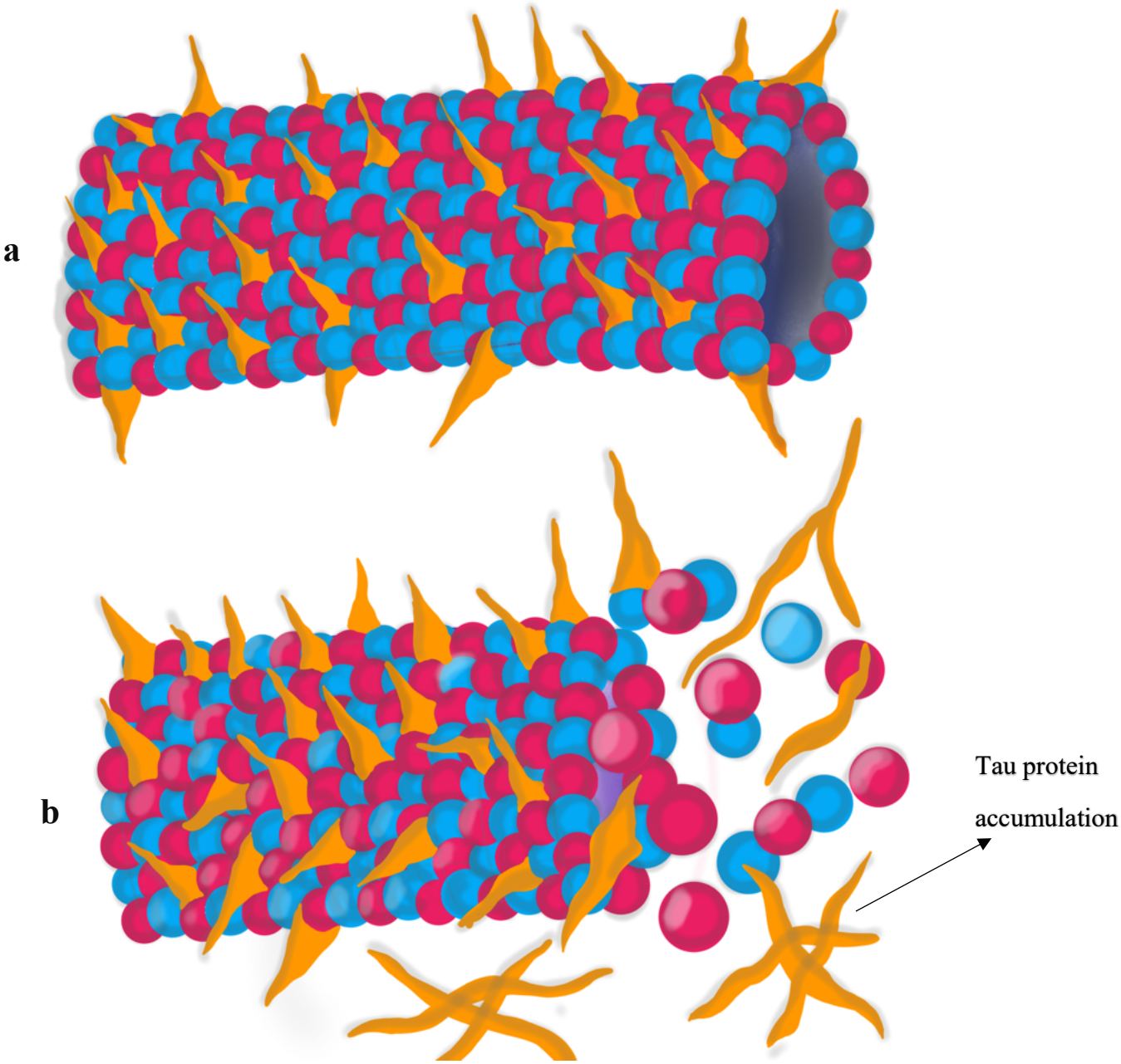


Figure 2. Comparison of microtubule in healthy neuron and diseased neuron.

a) In healthy neurons the tau protein binds to the microtubules and helps in stabilization.

b) In healthy neurons the tau protein binds to the microtubules and helps in stabilization. In AD patients, the tau protein which helps in stabilization of microtubules gets hyperphosphorylated and accumulates inside the neurons.

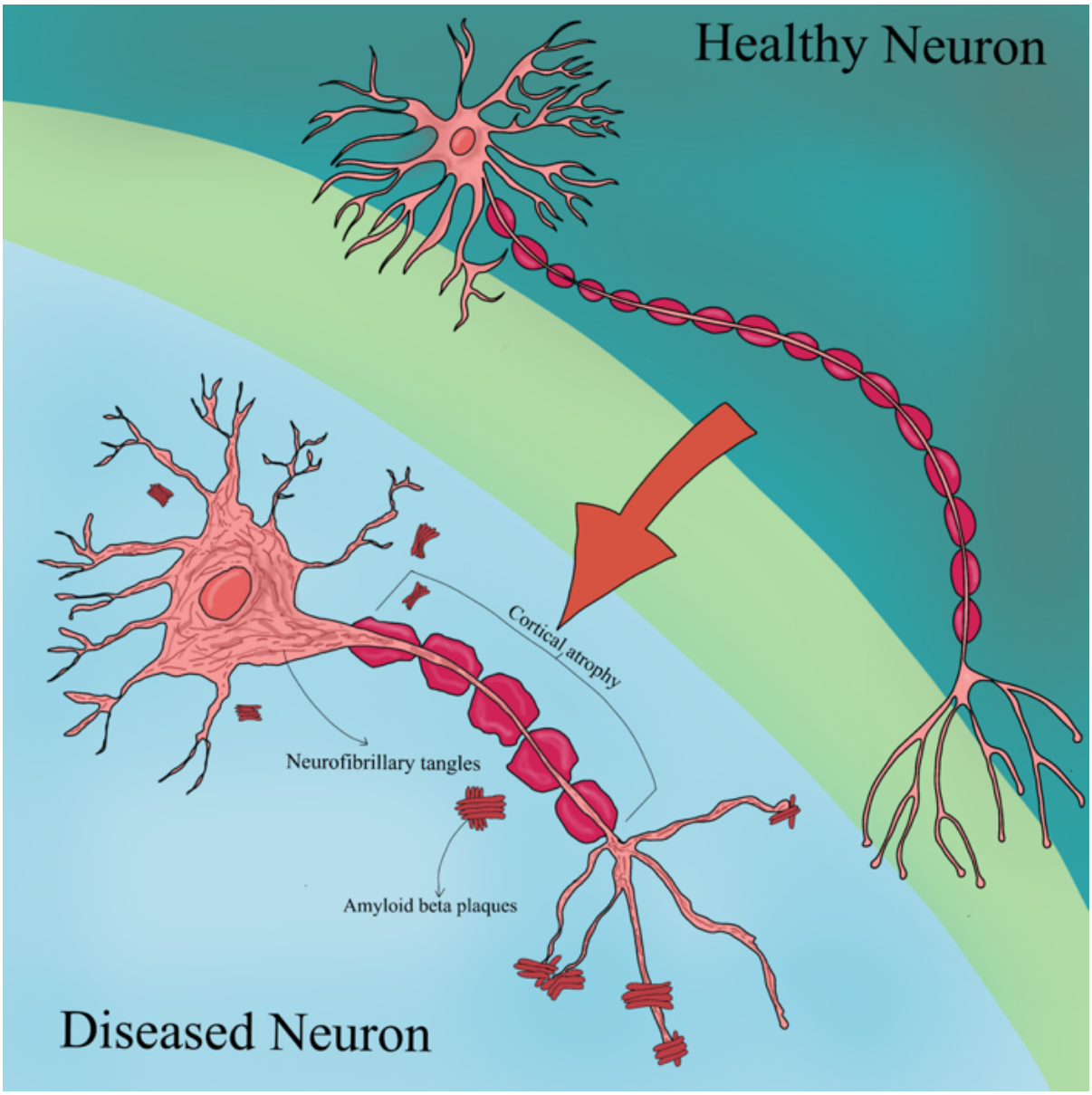


Figure 3. Comparison of healthy neuron with diseased neuron. Diseased neurons have Aβ plaques aggregated outside where they interfere in the signal responses in the brain. Neurofibrillary tangles disturbs the neuron structure where the neuron gets weak and cannot be active as a healthy neuron hence resulting in neuron death.

1.4. The effects of the gut microbiota on the brain

Mammals live in a coevolutionary association with huge quantities of commensal microorganisms resident on the exposed and internal surfaces of their bodies (Burokas et al. 2015). Microbiota, the ecological community of commensal, symbiotic and pathogenic

microorganisms in our body, has important regulatory functions in health and disease (de J.R. De-Paula, Forlenza, and Forlenza 2018). Comprising trillions of symbiotic microorganisms, the gut microbiota is an essential element for the maintenance of the host's health (Giau et al. 2018). Accumulating evidence from both clinical and basic medical research is driving our increased awareness of the significance of the human microbiota in maintaining a healthy CNS (Mayer et al. 2014) (Table 1). The Gastrointestinal Tract (GIT) is inhabited with numerous microorganisms, a figure thought to be 1.5 times that of the number of human cells in our bodies and 150 times as many genes as our genome (Martin et al. 2018). The gut microbiota plays a fundamental role in the modulation of the bidirectional signalling underlying the gut–brain axis (Giau et al. 2018). The brain-gut axis reflects the bidirectional, constant communication between the CNS and the GIT. There is also a growing body of evidence that the intestinal microbiota influences the brain-gut interactions in different points of time (from early life to neurodegeneration), as well as at different levels (from the gut lumen to the CNS) (Kowalski, Mulak, and Words 2019).

The gut microbiota is defined as the microbial community found within the gut. This microbial ecosystem is an essential endocrine "organ" involved in critical functions such as influencing metabolism and the absorption of food, providing trophic and protective functions, instructing innate immunity, and acting as a dynamic filter of environmental exposure in the host (Ratto et al. 2018). The initial scepticism about reports suggesting a profound role of an intact gut microbiota in shaping brain neurochemistry and emotional behaviour has given way to an unprecedented paradigm shift in the conceptualization of many psychiatric and neurological diseases.(Mayer et al. 2014) A bidirectional communication between the brain and gut exists that is referred to as the microbiota-gut-brain axis (Burokas et al. 2015). Gut microbiota is a complex system of microorganisms showing an unequal distribution from the mouth to the anus and covering several biological functions (Franceschi et al. 2019).

Humans have long considered the contents of the bowels as mere waste products, rather than a diverse and vital community whose intimate interactions with the body impact us on multiple levels (Tillisch, 2014). The microbiota-gut-brain axis reflects the bidirectional, constant communication between the CNS and the GIT. The human GIT, containing 95% of the human microbiota, harbours a genetically diverse microbial population that plays major roles in nutrition, digestion, neurotrophism, inflammation, growth, immunity and protection against foreign pathogens (Martin et al. 2018).

Table 1. The role of microorganisms residing in the gut and key players in gut-brain axis.

Sr. No	Organism	Increase/ Decrease	Role	Reference
1.	*Bacteroides fragilis*	↑	• Protect against Central nerves system demyelinating disease • In pregnant mice showed an immediate significant diminished autistic behaviour. •	(Cerovic, Forloni, and Balducci 2019) (Ochoa-Repáraz and Kasper 2018) (Colpitts et al. 2017)
2.	*Lactobacillus casei*	↑	Decrease in anxiety symptoms	(Rao et al. 2009)
4.	*Lactobacillus rhamnosus*	↑	• Increases anxiolytic behaviour • Ameliorate the inflammation level in the brain.	(Bravo et al. 2012) (Bravo et al. 2011) O'Mahony et al., 2005)
5.	*Streptococcus thermophilus*	↑	• Robust effect on activity in the brain regions that control central processing of emotion and sensation. • Degradation of Aβ 42 load.	(Bonfili et al. 2017)
6.	*Campylobacter jejuni*	↑	Induces anxiety-like behaviour	(Lyte et al. 2006)
7.	*Campylobacter rodentium*	↑	Leads to stress and contribute to behavioural abnormalities	(Gareau et al. 2011)
8.	*Porphyromonas gingivalis*	↑	Causes inflammatory response in liver, which subsequently leads to	(Bonfili et al. 2017)

			neuroinflammation and cause neurodegenerative disease.	
9.	*Eubacterium rectale*	↓	leads to amyloidosis	(Cattaneo et al. 2017)
10.	*Lactobacillus acidophilus*	↑	Improves the impairment in neural proteolysis	(Bonfili et al. 2017)
11	*Lactobacillus johnsonii*	↑	Improves gastric vagus nerve activity	(Tanida et al. 2005)
12	*Bacillus subtilis*	↑	Produce extracellular amyloid fibres	(Vogt et al. 2017)

More recently, the importance of gut microbiota has raised attention of researchers in the fields of neurochemistry, neurophysiology and neuropsychiatry, based on consistent pieces of evidence of the relationship between gut microbiota and brain homeostasis and subsequent implications of the disruption of gut microbiota to the pathogenesis of brain diseases. The composition of gut microbiota in humans has been found to be related to several medical diseases, including obesity (Ley et al. 2006) diabetes (Qin et al. 2012), asthma (Mennini et al. 2017), inflammatory GIT (Z. Liu, Cao, and Cong 2013) and other autoimmune diseases (Wu and Wu 2012).

1.5. The microbiota-gut-brain axis

The recognition of the human microbiota as a substantial contributor to nutrition, health and disease is a relatively recent one, and currently, peer- reviewed studies linking alterations in microbiota to the etiopathology of human disease are few (Hill et al. 2014). The term "microbiota-gut-brain axis" refers to a crosstalk between the brain and the gut involving multiple overlapping pathways, including the autonomic, neuroendocrine, vagus nerve, the immune system, or the metabolic processes of gut microorganisms and immune systems as well as bacterial metabolites and neuromodulatory molecules (Luca et al. 2019; R. Liu et al. 2017). The bidirectional signalling between the GIT and the brain is vital for maintaining homeostasis and is regulated at the neural (both central and enteric nervous systems), hormonal and immunological levels (Figure 4). Interest in the potential involvement of gut microbiota in brain function emerged, in part, due to the well-described pathways of communication between

the brain and the GIT which has been heavily studied in the area of food intake, satiety and the regulation of the digestive tract.

The gut microbiota assists a number of everyday functions in the brain, including the regulation of the hypothalamic-pituitary-adrenal axis activation state. The release of cortisol as a result of the hypothalamic-pituitary-adrenal axis activation can in turn govern the activation state of brain microglia, and effect cytokine release as well as attracting of monocytes from the periphery to the brain (H. X. Wang and Wang 2016). They also can rule actions in the periphery and CNS by various means of communication including vagal nerve and adrenergic nerve activation as well as producing several molecular candidates such as neurotransmitters, neuropeptides, endocrine hormones and immune-modulators (Ferrari 2019).

Host stress hormones such as noradrenaline, which might affect bacterial activities or signalling between bacteria, may change the microbial diversity and actions of the gut microbiota. However, these bacteria are capable of synthesizing and releasing many neurotransmitters and neuromodulators themselves, or evoke the synthesis and release of neuropeptides from enter endocrine cells.

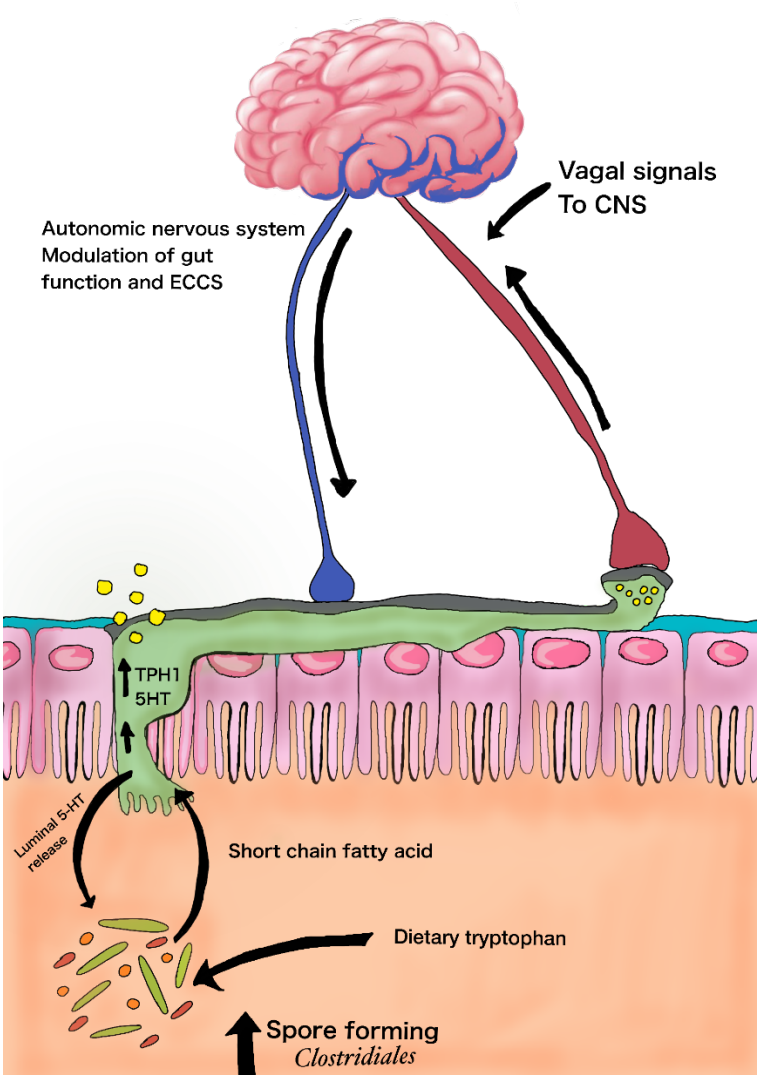


Figure 4. –Bidirectional brain-gut-microbiome interactions related to serotonin signalling. Enterochromaffin cells (shown in green) contain more than 90% of the body's serotonin. Serotonin syn-thesis in ECCs is modulated by SCFAs produced by spore forming. Clostridiales, which increase their stimulatory actions on ECCs with increased dietary tryptophan availability. ECCs communicate with afferent nerve fibers through synapse like connections between neuropod like extensions and afferent nerve terminals. The autonomic nervous system can activate ECCs to release serotonin into the gut lumen, where it can interact with gut microbes. TPH1, tryptophan hydroxylase type 1; 5-HT, serotonin.

Some microorganisms from the microbiota were associated with AD (Table 2). Moreover, microbiota of aged individuals with AD was found to have a lower level of bacteria producing butyrate (Rieder et al. 2017). With decreased butyrate levels, individuals with AD could then suffer from increased inflammation in the brain and the progression of cognitive loss. Butyrate production also is believed to enhance sleep and as a result may be beneficial in this capacity on cognition (Maiese 2019).

Dysfunction in the microbiota-gut-brain axis was investigated in irritable bowel syndrome, inflammatory bowel disease, depression, and anxiety, as well as neurodevelopmental disorders such as autism, Parkinson's disease, and AD (Schwabe et al. 2013).

Table 2. Case study of microorganisms mainly found in AD patients and transgenic mice.

Sr.no	Microorganisms		Organism	Location	Reference
1.	*Firmicutes /Actinobacteria*	↓	CONVR-APP/PS1 mice	GIT	(Westfall et al. 2017)(Salminen et al. 1998)
2.	*Bacteroides/ tenericutes*	↑			
3.	*E.coli / B. subtilis*	↑	AD patient with irritable bowel syndrome	Stool	(Alkasir et al. 2017)(Salazar et al. 2014)(Asti and Gioglio 2014)
4.	*E. rectale*	↓	AD patient	Stool	(Cattaneo et al. 2017)
5.	*Escherichia/ shigella*	↑			
6.	*B.fragilis*	↓			
7.	*Lactobacilli / Bifidobacteria*	↑	SAMP-8 mice	GIT	(Ma, Forsythe, and Bienenstock 2004)
8.	*Fusobacteriaceae*	↓	AD patients	Stool	(Rajilić-Stojanović, Smidt, and De Vos 2007)
9.	*Prevotellaceae*	↑		Stool	

Prebiotics are nondigestible food ingredients that selectively stimulate the growth of probiotic bacteria such as *Lactobacilli* and *Bifidobacteria* in the gut (Saulnier et al., 2013). Increasing the proportion of these bacteria with prebiotics such as galactooligosaccharides (GOS) or fructooligosaccharides (FOS) may have many beneficial effects on the gut, the immune system, and on brain function, providing initial support for further investigations of the utility of prebiotics in mental health and potential treatment of psychiatric disorders (Drakoularakou, Tzortzis, Rastall, & Gibson, 2010; Savignac et al., 2013; van Vlies et al., 2012; Vulevic, Drakoularakou, Yaqoob, Tzortzis, & Gibson, 2008). Recently, a study conducted on human has showed that subjects supplemented with GOS displayed a suppression of the neuroendocrine stress response and an increase in the processing of positive versus negative attentional vigilance, showing an early anxiolytic-like profile (Schmidt et al., 2014). Inulin-type fructans and lactulose modulate gut transit, decrease putrefactive activity within the gut

lumen, prevent GI infections, and mitigate microbiota regulation of the microbiota-gut- brain axis inflammatory responses (Casellas et al., 2007; Lewis, Burmeister, & Brazier, 2005; de Preter et al., 2008).

1.6. Neuro-inflammatory processes

Although each of the aforementioned neurodegenerative diseases presents with unique cellular pathologies and clinical symptoms, there is one feature common to all these diseases: chronic neuroinflammation (R. Liu et al. 2017). The hallmark features of chronic neuroinflammation include over activation and dysregulation of the immune cells of the brain, the microglia. In a suitable normal homeostatic conditions, microglia remain in a resting state. In this state, microglia take on a ramified shape and continuously extend and retract their cellular processes in search of foreign invaders and pathological stimuli in their nearby environment. When microglia encounter pathological agents, or detect injury, they become activated and take on an amoeboid morphology (Lee et al. 2019). This change in activation status is associated with modifications in the expression pattern of many cell membrane receptors, such as complement receptors, major histocompatibility complex, CD11a, CD40, and CD80 molecules, all of which have important immune functions. Once activated, microglia also alter their secretory profile, by upregulating the release of several chemokines and cytokines, such as interferon -γ, tumour necrosis factor- α, interleukin-1 β, and interleukin IN-6 , interleukin -8, Monocyte Chemoattractant Protein-1, as well as increase secretion of reactive oxygen species and reactive nitrogen species, all of which are secreted in aims of eliminating the noxious agents (Lin, Zheng, and Zhang 2018).

In AD, astrocyte and microglia activation has been observed, particularly in the brain regions containing higher concentration of Aβ plaques and NFTs (primarily the frontal cortex and hippocampus) (Cowan and Petri 2018). As compared to healthy controls, the brains of AD patients exhibit a high increase in interleukin -1β and Tumour necrosis factor-α concentration, both cyclooxygenase -1 and -2 expression, and also an increase in 12-lipoxygenase, and 15-lipoxygenase expression (Roy Sarkar and Banerjee 2019). These findings indicate that AD patients exhibit prominent neuroinflammation in their CNS. In addition, concentrations of C-reactive protein, an inflammatory marker, are elevated approximately eight fold in the serum of AD patients (Erny, Hrabě de Angelis, and Prinz 2017). Its levels have been positively correlated with severity of cognitive decline in elderly individuals, indicating that the chronic

neuroinflammation observed in AD may represent a significant contributing factor to the defining symptoms of this disease (X. Hu, Wang, and Jin 2016).

Although it is likely the neuroinflammation that occurs in these diseases is initially triggered by the pathological formations in the brain, other aspects of the human immune system may also influence the neuroinflammation observed in these neurodegenerative diseases (Hill et al. 2014). Emerging evidence suggests that the composition of, and communication with, the gut microbiota may have a notable impact on modifying the neuroinflammation in these particular neurodegenerative disease states.

The microbiota has been linked to the CNS via different proposed communication pathways:
(1) bacterial metabolic products such as tryptophan and short chain fatty acids (SCFAs) and others (Table 3),
(2) innervation of the gut by the vagus nerve, and
(3) microbe-associated molecular patterns that drive inflammation (Erny, Hrabě de Angelis, and Prinz 2017).
The mechanism by which bacterial products or these microbes associated molecular patterns trigger an inflammatory response in the brain may include entry to the circumventricular organs, areas in the brain where the blood brain barrier is less tightly regulated. While these molecules are limited in their entry throughout the blood brain barrier (Johnston and Webster 2009), cells present at these "leaky" locations (i.e., microglia), may be poised to respond to blood-borne molecular signals, and in turn propagate inflammation in a wave across the parenchyma (L.M. and H.L. 2018). Alternatively, microglia may respond to cytokine-induced reactivation of cells forming the blood brain barrier–endothelial cells, astrocytes, pericytes, etc (Varatharaj and Galea 2017); or via selective transport systems across these cells. A neural route for the microbiota-gut-brain axis suggests that a basal level of immune activation in the gut acts on the vagal afferents innervating the viscera and transduces inflammatory information to the nucleus of the solitary tract, at the level of the brainstem (Z. Liu, Cao, and Cong 2013; Mayer et al. 2014). Low levels of cytokines may activate this route, even when circulating pro-inflammatory cytokines are not detectable. This vagal-centric model for understanding the microbiota-gut-brain-axis has not yet been approached from the angle of microglial development, activation, or function, and remains of interest to the field. Researchers classified activated microglia as a classic, proinflammatory M1 phenotype and others as a noninflammatory, alternative M2 phenotype.

Table 3. Different microorganisms playing an important role in formation of metabolites in the human body.

Sr.no	Gut microorganisms	Metabolites	References
1	Lactobacillus	SCFAs, Acetylcholine	(Alkasir et al. 2017) (Ma, Forsythe, and Bienenstock 2004)
2	Bifidobacterium	Gamma-aminobutyric acid	(Roy Sarkar and Banerjee 2019; Alkasir et al. 2017)
3	Escherichia	Dopamine, Norepinephrine, Endotoxin and Serotonin	(Lopes and Sourjik 2018)(Asano et al. 2012)
4	Bacillus	Dopamine, Acetylcholine and Noradrenaline	(Johnson and Foster 2018)
5	Saccharomyces	Norepinephrine	(Asano et al. 2012)
7	Enterococcus	Histamine, Serotonin	(de J.R. De-Paula, Forlenza, and Forlenza 2018)

Microglia express a complex sensome, and are capable of integrating and responding to signals in their environment, whether pathogen associated molecular patter, damage associated molecular patterns, or other secreted factors, by secreting pro-inflammatory cytokines including tumour necrosis factor- α, interleukin -1β, and interleukin -6 hence showing the picture of M1 state (Ferreira et al. 2014). Secretion of the anti-inflammatory cytokines is the character of the M2 state, including IN 4, IN 10, IN 13, and transforming growth factor-β, with phagocytic capacity increased, where- as toxic nitric oxide produced not. These signalling features, their macrophage identity, and status as resident-CNS cells make microglia intriguing candidates for mediating the interactions between the host's microbiota and developing brain. Indeed, metabolic products from gut microbiota, as well as microbiota-induced peripheral immune products are capable of influencing microglial activity (Hanisch 2002; Cowan and Petri 2018; Grenham et al. 2011).

1.7. The role of gut microbiota in inflammation and diabetes

In the brain, astrocytes are the main energy reservoirs, accumulate glycogen, and help to sustain high-energy demands associated with neuronal activity. Astrocytes, present in the brain, play an essential role in long-term memory formation by converting glycogen into lactate and transporting it to the neurons (Salas and De Strooper 2018). Astrocytes express an insulin-independent glucose transporter, glucose transporter 1 protein (also known as solute carrier family 2); thus it has been believed that astrocytes take up glucose through the blood-brain barrier in an insulin-independent manner and convert the intracellular glucose to glucose-6-phosphate and then store as glycogen (W. Li et al. 2018). The brain expresses mainly insulin-independent glucose transporter 1 (endothelial cells of brain blood barrier, astrocytes), (Salas and De Strooper 2018; W. Li et al. 2018; R. Li et al. 2019). Insulin controls not only whole-body energy and glucose homeostasis in the periphery of the human body but also exerts specific effects in the brain through insulin receptor and the closely related insulin-like growth factor-1 receptor (R. Li et al. 2019). Epidemiological studies have indicated that diabetic patients have an elevated risk of developing AD (Diehl, Mullins, and Kapogiannis 2017). It has been studied over the wide range of patients with diabetes (males as well as females) which confirms the risk of AD is more higher in females (Chatterjee et al. 2016).

Diabetes is a very complex metabolic disorder characterized by a rise in blood glucose levels due to an altered insulin production by pancreatic cells (Type 1) of the human body or an impaired insulin response (Type 2) (Salas and De Strooper 2018). It is becoming increasingly evident that the gut microbiota is contributing to many human diseases including diabetes both type 1 and type 2. The chronic hyperglycemia and insulin resistance found in diabetic patients is commonly associated with vascular complications which eventually lead to alterations in the kidney (nephropathy), retina (retinopathy) and peripheral nerves (neuropathy), among other problems (W. Li et al. 2018). Not surprisingly, besides the abnormalities in peripheral organs, diabetic patients also show structural and functional changes in the CNS.

Type 1 diabetes (T1D) is an autoimmune disease that is caused by the destruction of pancreatic β-cells by the immune system and T1D is a T cell-mediated autoimmune metabolic disease which is commonly seen in children and young adults although it can also be present in older adults (Y. Hu et al. 2018). The insulin-producing beta cells of the pancreatic islets are damaged

and destroyed by activated autoreactive T cells resulting in disordered blood glucose regulation. Even though T1D is mainly caused by genetic defect, epigenetic and environmental factors have been shown to play an important role in this disease. Higher rates of T1D incidence have been reported in recent years that are not explained by genetic factors and have been attributed to changes in our lifestyle such diet, hygiene, and antibiotic usage that can directly affect microbiota (Luca et al. 2019). It has been shown that diabetes incidence in the non-obese diabetic subjects or patients was significantly increased which is in line with the observation that the rates of T1D is higher in countries with stringent hygiene practices (Salas and De Strooper 2018). A common trait between theories investigating the causes of neurodegenerative diseases is the presence of neuroinflammation that has been associated with activation of microglia and peripheral monocytes that cross the blood brain barrier (Townsend et al. 2005)(Ratto et al. 2018). Type 2 diabetes mellitus (T2DM) is the more common type of diabetes where peripheral insulin resistance and compensatory increased insulin secretion may accelerate the decrease in pancreatic islet secretory function, eventually leading to insulin deficiency (R. Li et al. 2019).

Obesity is a major global public health problem and is associated with the metabolic disorder, T2DM that is characterized by the progressive loss of glucose homeostasis, development of insulin resistance and pancreatic β-cell degeneration, leading to insufficient insulin secretion from β-cells to meet the demands of increased peripheral insulin resistance in the late stages of the disease (Le Zhang et al. 2019).

Different population-based cohorts studies have also suggested an increased risk to develop dementia in T2DM patients (Ott et al. 1999). T2DM and AD share many characteristics, including chronic inflammation, oxidative stress, impaired insulin signalling, insulin resistance, glucose intolerance, and cognitive impairment (R. Li et al. 2019). Increasing evidence has shown that insulin deficiency and resistance, the markers of T2DM, are also important in AD pathology (Salas and De Strooper 2018).

Evidence in animal and human models supports the hypothesis that obesity and T2DM are associated with a deep gut dysbiosis. Overeating could represent one of the main starting points to alter gut microbiota locally and to initiate systemic inflammatory processes through the mucosal barrier (Gaofeng et al. 2018). A decline in butyrate-producing *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, which may be metabolically beneficial, and an increase in

several opportunistic pathogen levels were observed from the stool samples of the T2DM patients. T2DM patients showed higher levels of *Lactobacillus* species in comparison to nondiabetics (Ferrell and Chiang 2019). Normal subjects conducted differed from patients with prediabetes with higher levels of *Faecalibacterium prausnitzii* and *Haemophilus parainfluenzae T3T1*, whereas *Verrucomicrobiaceae, Akkermansia muciniphila*, and *Clostridiales sp. SS3/4* were less abundant (X.-Y. Li, Shen, and Ji 2019). SCFAs, such as acetate, butyrate, and propionate, as well as the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, might constitute a link between the microbiota and systemic inflammatory diseases. In particular, butyrate seems to have a direct role in the development of extrathymic anti-inflammatory regulatory T cells (X.-Y. Li, Shen, and Ji 2019). The alteration on the production of SCFAs, especially butyrate, observed in T2DM patients, might have a key role in the development of low-grade inflammation (Gaofeng et al. 2018).

Another important role in the development of a metabolic syndrome has been demonstrated for the pattern recognition receptor such as the toll-like receptor 5, a component of the innate immune system expressed in the gut mucosa and one that helps defend against infection (Nakamura et al. 2019). Mice deficient of the toll-like receptor 5 exhibited hyperphagia and developed hyperlipidemia, hypertension, insulin resistance, and obesity, as well as an altered microbiota. Interestingly, the transfer of intestinal microbiota from TLR5-deficient mice to germ-free mice led to metabolic syndrome (W. Li et al. 2018). These data support the crosstalk of gut microbiota with the innate immune system and suggest that the alteration of this link is critical in the development of the metabolic syndrome. In addition, studies show that gut-derived endotoxin lipopolysaccharide might be involved in the chronic inflammation observed in T2DM (Naito, Uchiyama, & Takagi, 2018). HF diet increased the lipopolysaccharide content of the gut microbiota and resulted in metabolic endotoxemia (Biscetti et al. 2019). They observed that subcutaneous infusions of lipopolysaccharide into mice determined insulin resistance and obesity similar to that after feeding HF diet. Gut dysbiosis might increase lipopolysaccharide production by gram-negative bacteria and lead to metabolic endotoxemia and low-level inflammation that could contribute to the development of insulin resistance and T2DM (Biscetti et al. 2019; X.-Y. Li, Shen, and Ji 2019).

In AD the degradation of brain cells is observed at higher rate due to neuroinflammation. Microglia being responsible for this action, encounters the AD prone cells and triggers the inflammation process. Moreover, the gust microbiota playing a crucial role in inflammation

and microglia activation also has a big impact on various chronic disease, like T2DM or AD pathology. Therefore, it is necessary to understand the mechanisms of the microbiota-gut-brain axis evolvement in the onset of AD and its pathology.

1.8. AD and obesity models in mice

Transgenic mouse models that are modified now mimic a range of AD-related pathologies which has helped the researchers to know more about the disease (Puzzo et al. 2015). These models have coined new insights in the pathophysiology as well as novel therapeutic approaches (Ameen-Ali et al. 2017). The models, however, raise a number of issues of its own as well. It is clear that the success of transgenic mouse models has to depend on the overexpression of APP transgenes containing FAD-associated mutations at levels that are not physiological (Tai et al. 2017).

The experimental models of AD are critical to gain a better understanding of pathogenesis and to assess the potential of novel therapeutic approaches in research. The most commonly used experimental animal models are transgenic mice. These transgenic mice have a overexpress human genes associated with familial AD that result in the formation of amyloid plaques (Webster et al. 2014).

- The first example of such models was the PDAPP mouse, which expressed human APP with the Indiana mutation (APP^{V717F}) driven by the PDGF-β promoter (Castellano et al. 2011).
- Tg2576 mice expressed human APP with the double Swedish mutation ($APP^{K670N/M671L}$). These mice developed plaques in the frontal, temporal and entorhinal cortices, hippocampus and cerebellum (Wolf et al. 2016). APP23 mice have more pronounced CAA, immediately formed form compact plaques in comparison to the predominantly diffuse plaques found in Tg2576 mice, and have localized neurodegeneration that is not seen in the Tg2576 mice (Yin et al. 2016)(Kim et al. 2019).
- The most extreme APP mouse model that is widely used is the 5xFAD model; these mice express the Swedish ($APP^{K670N/M671L}$), London (APP^{V717I}) and Florida (APP^{I716V}) APP mutations (Tible et al. 2019).

Some of the rodent models potentially useful for diet-induced obesity, insulin resistance and T2DM research are C57BL/6J , SWR/J, A/J, C3H/HeJ, DBA/2J, NZO , and TALLYHO/Jng mice, DDR Sprague Dawley Rats. Monogenic group of models includes C57BL/6J–ob/ob and C57BLKS/J–db/dbmice, Otsuka Long–Evans Tokushima Fatty Rat, fa/fa Rat , Zucker Diabetic Fatty Rat (Kleinert et al. 2018).

It is not yet clear that the temporal appearance of plaque and cognitive deficits in brain of the mouse models mirrors exactly that of the human disease. Many of the mouse models, indeed, exhibit behavioural deficits before significant plaque pathology (Ling Zhang et al. 2017). Substantial plaques in the brain is probably present for some period of time before cognitive symptoms first appear in human AD (Saito et al. 2014). Interpretations of behavioural studies in the mouse are also complicated by the fact that it is difficult to know how precisely the behavioural tests used in mice model the cognitive deficits in humans (Moy et al. 2004).

2. MATERIALS AND METHODS

2.1. Animals

In this study C57BL/6JRj mice from Janvier Labs, France (n=80; 40 males and 40 females) were used. The weight of the mice was 16–22 g at the beginning of the experiment. Mice were housed four per cage in controlled laboratory conditions with the temperature maintained at 21 ± 1°C and humidity at 55 ± 10 percent and food and water provided *ad libitum*.. Mice were tested during the dark phase of a reverse light cycle (lights off at 7.00 a.m. and on at 7.00 p.m.). All experimental protocols were performed in accordance with recommendations for the proper care and use of laboratory animals [local regulations by director of Lithuanian State Food and Veterinary Service (law n° B1-866/2012); European (EU directive n° 2010/63/EU) regulations and were approved by the ethical committee. Body weight was registered twice a week during the experiment (Monday and Friday).

2.2. Study design

The study with a basal period lasted 10 months (Figure 5). Two independent cohorts participated in the study. The cohort 1 with 48 mice (24 males, 24 females) and the cohort 2 having 32 mice (16 males, 16 females).

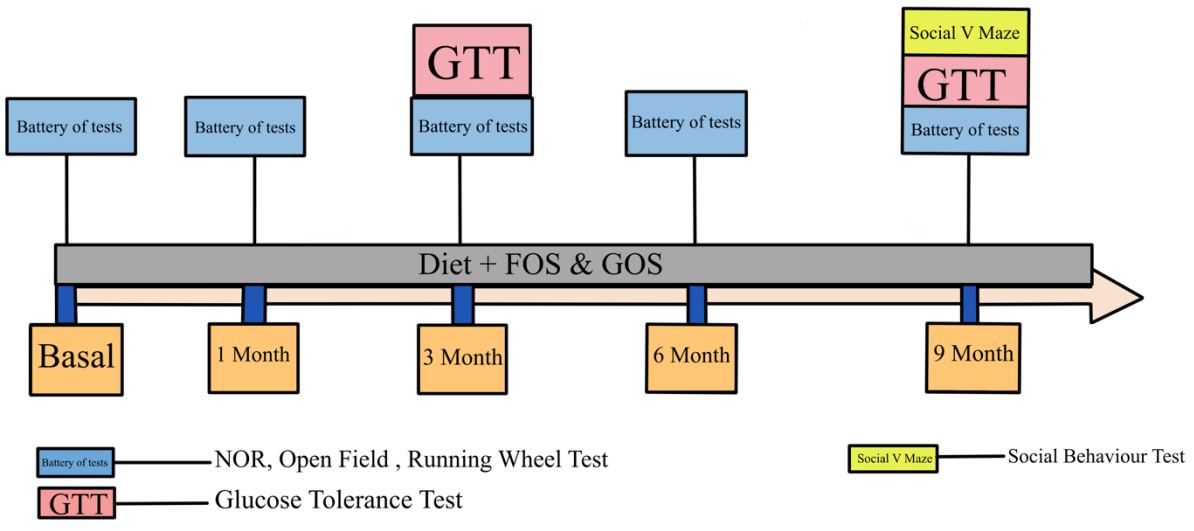


Figure 5. Experimental schedule during the 9 months of diet-induced obesity protocol.

2.3. Food and prebiotic administration

Mice were fed standard laboratory chow for one month to facilitate habituation to the new environment and then randomized into dietary treatment groups. After basal tests the first group of mice (n=20) was fed an low-fat diet [Altromin, Germany] (protein-18%, fat-10.2%, carbohydrate-71.8% and energy from fat 10 %) providing 3.76 kcal/g, as a control, while the second group (n=20) was fed a low-fat diet with prebiotics (FOS+GOS), the third group (n=20) was fed with HF diet [Altromin, Germany] (protein-18.1%, fat-61.6%, carbohydrate-20.3% and energy from fat 60%) providing 5.10 kcal/g and the fourth group (n=20) with HF diet with FOS+GOS. Mice were administered with prebiotics, a combination of FOS and GOS (dissolved in drinking water for 30 g/L), or water during all of the study.

2.4. Glucose tolerance test

For the glucose tolerance test, animals exposed to 3 and 9 months of obesity development were fasted for 14 hours prior to the beginning of the blood sample collection. Blood was sampled from the tail using a glucose monitoring system. This method is based on a slip-in sampling test strip technology, which requires a tiny amount of blood (5 µl). The glucose detection range and the relative standard variation stated by manufacturer for 20–600 mg/dl and less than 3.1 percent, respectively. After measuring basal levels of glucose (0 time point), animals were injected glucose 2 g/kg intraperitoneally, and blood glucose levels were measured at 15, 30, 45, 60 and 120 minutes.

2.5. Open field test for locomotor activity

The open field (OF) apparatus size was 40 cm x 40cm. The four square size apparatus were placed next to each other forming a 80cm x 80 cm which had four chamber for four individual mice. The walls of the open-field were smooth and 30cm high. The animals were introduced to the OF for 10 minutes. The walking pattern of the animals was recorded using a Bandicam (Bandicam company) camera connected to the Viewer 3 (Bioserve) software for analyses. The animals were introduced to the OF five times after as specified in Figure 5. After each OF session, the following parameters of the animals' walking patterns were analysed: total activity; percentages of time spent in each zone, Latency; and the percentage of time that the animals spent walking with their operated side pointing against the wall (Castel et al. 2018; Rico,

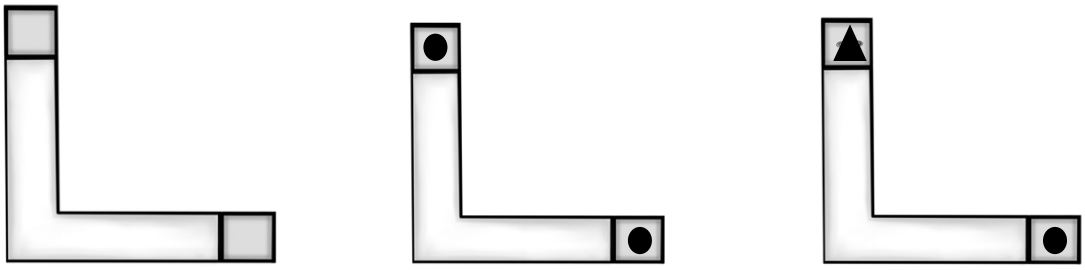
Bonuti, and Morato 2019). The OF test was conducted in room with illumination of 490-500 lux.

2.6. Running wheel test

For running-wheel conditions, activity wheels (12 cm diameter) were attached to the mice new cages. Each full wheel revolution was equivalent to 1.12 meters and each quarter-rotation of the wheel was recorded. Each chamber was enclosed in a sound-attenuating cubicle and equipped with 2 response levers, 2 stimulus lights, a pellet hopper, and a food holder (Seward et al. 2018). Equipment was controlled by Lab Chart Reader 7 Pro (AD instruments) for recording and analyses was done by Lab Chart 8 Reader software (Manzanares, Brito-da-Silva, and Gandra 2018).

2.7. Cognition

Cognitive function was assessed using the novel object recognition test. A cognitive task depending on hippocampal function. V shaped maze build with plexiglass with height 15 cm arm length 30 cm and the 4.5 cm wide. On the first day, mice were habituated for 9 min to the V-maze in which the task was performed, as previously described (Burokas et al. 2014) (Figure 6). On the second day, mice were put back in the maze for 9 min, two identical objects were presented and the time that the mice spent exploring each object was recorded. The mice were again placed in the maze 24 h later for 9 min, one of the familiar objects was replaced with a novel object and the total time spent exploring each of the two objects (novel and familiar) was computed. Object exploration was defined as the orientation of the nose to the object at a distance of less than 2 cm. A discrimination index was calculated as the difference between the time spent exploring either the novel or familiar object divided by the total time exploring the two objects. An elevated discrimination index is considered to reflect improved memory retention for the familiar object (Puighermanal et al. 2009). The novel object recognition test was conducted in a sound-attenuated room with dim illumination 14-15 lux.



Day 1-Habituation
9 Minutes

Day 2- training
9 Minutes

Day 3- Memory
9 Minutes

Figure 6. Demonstration of the V maze for Nobel Object Recognition Test. This test is divided into three days series. Day 1-habituation, Day 2- training and Day 3 preference for memory. Each test lasts 10 min. During this time the exploration of the experimental mouse towards the chambers at the end of the corridors was recorded with digital camera.

2.8. Social V- Maze

The Social V- Maze (SVM) was used in a sound-attenuated room with dim illumination 10-15 lux. A digital camera on top of the maze was used to record the sessions. The maze is built with black opaqueness plexiglass glass with dimensions of each arm's length 30 cm and 4.5 cm wide; the wall of the maze is 15 cm. The experiment was conducted for 15 minutes which was divided into three phases (Moy et al. 2004). Habituation session (Phase I) allows mice to freely explore and familiarize with the V-Maze. Sociability session (Phase II) is performed just after the habituation session. Preference for social novelty session (Phase III) is performed just after the sociability session. All three phases of the social test (habituation, sociability and preference for social novelty) were performed consecutively (Figure 7). SVM phases lasted 5 min as it has been described that during the first five minutes of interaction the majority of the social behaviours take place (Nadler et al. 2004). All the experiments were performed during the light phase of the dark/light cycle (lights on at 7 a.m. and off at 7 p.m.).

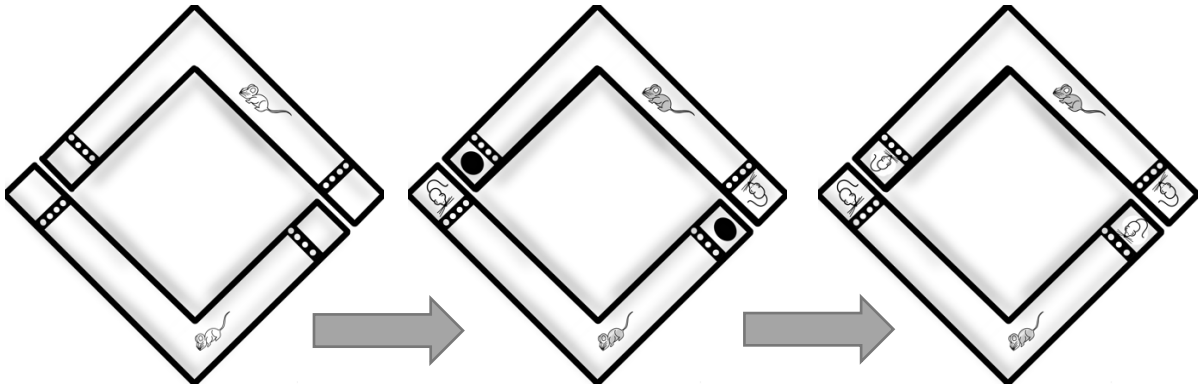


Figure 7. Scheme of the sociability and preference for social novelty test. There are three different phases: Phase I (habituation), Phase II (sociability) and Phase III (preference for social novelty). Each phase lasts 5 min. During this time the exploration of the experimental mouse towards the chambers at the end of the corridors was recorded on the three phases: both chambers empty (Phase I), Stranger mice vs. Object (Phase II) and Stranger mice vs. Familiar mice (Phase III).

3. RESULTS

3.1. Body weight

Body weight progressively increased by the continue exposure to HF diet (Figure 8 and 9). Body weight was progressively increased by the continued exposure HF diet in both males and females. In males two-way repeated measures ANOVA revealed significant main effects of diet ($F_{(3,36)}$ = 26.33; P<0.001), time ($F_{(9,54)}$ = 218.4; P<0.001) and interaction between both factors ($F_{(27,324)}$ = 23.30; P<0.001). Subsequent Dunnett's analysis showed a significant increase of body weight in obese and obese mice with FOS&GOS compared to lean controls from month 2 till month 9 (from P<0.05 to P<0.001) (Figure 8). Similarly, body weight was increased in female mice exposed to HF diet. Two-way repeated measures ANOVA revealed significant main effects of diet ($F_{(3,33)}$ = 25.82; P<0.001), time point ($F_{(9,48)}$ = 187.9; P<0.001) and interaction between both factors ($F_{(27,297)}$ = 27.784; P<0.001). Subsequent Dunnett's analysis showed a significant increase of body weight in obese mice from month 2 and obese mice with FOS&GOS from month 3 till month 9 compared to lean controls (from P<0.05 to P<0.001) (Figure 9).

Figure 8.

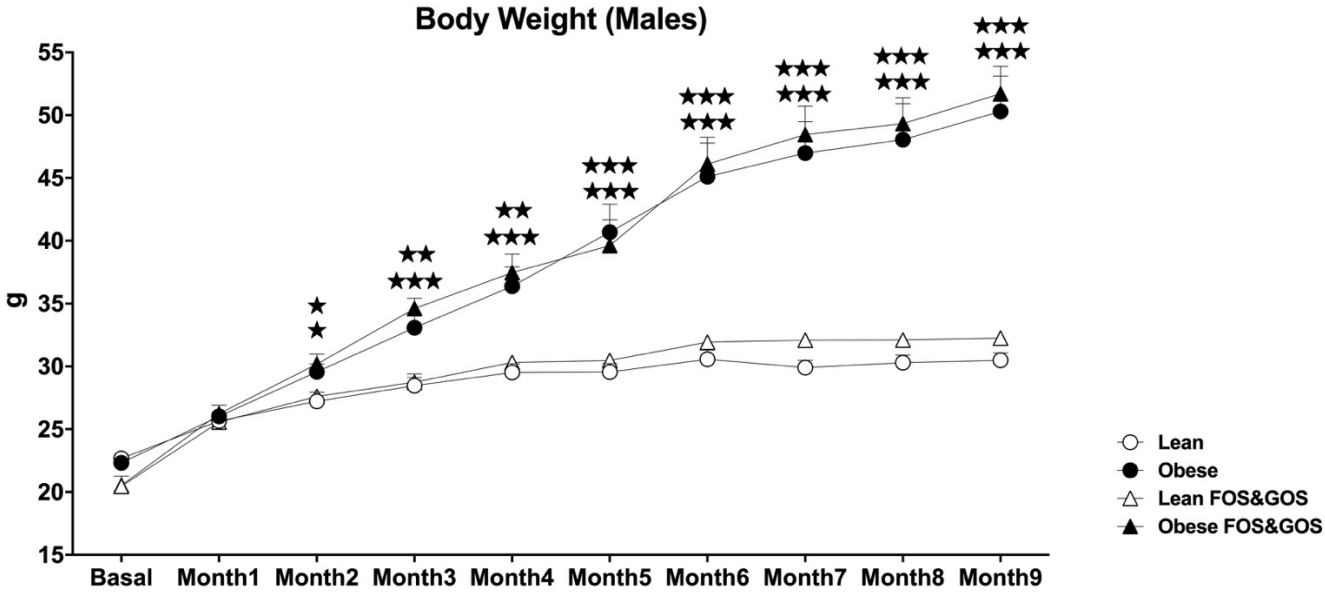
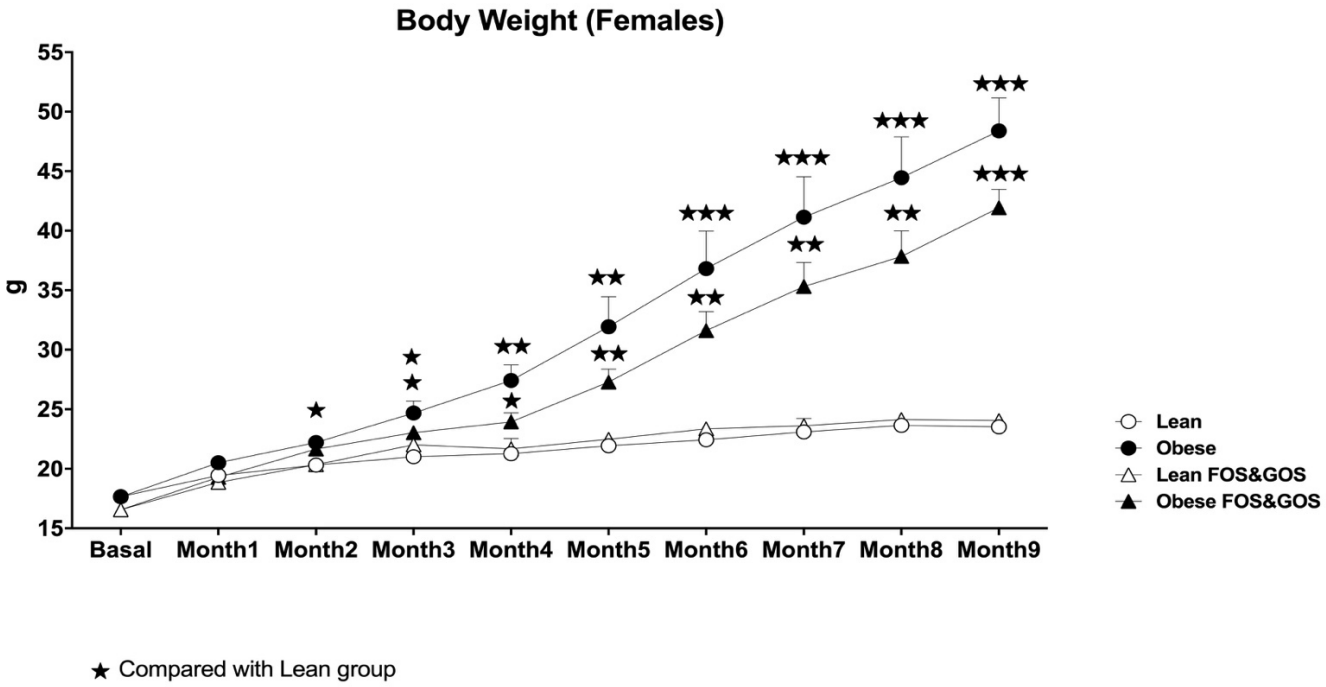


Figure 9.

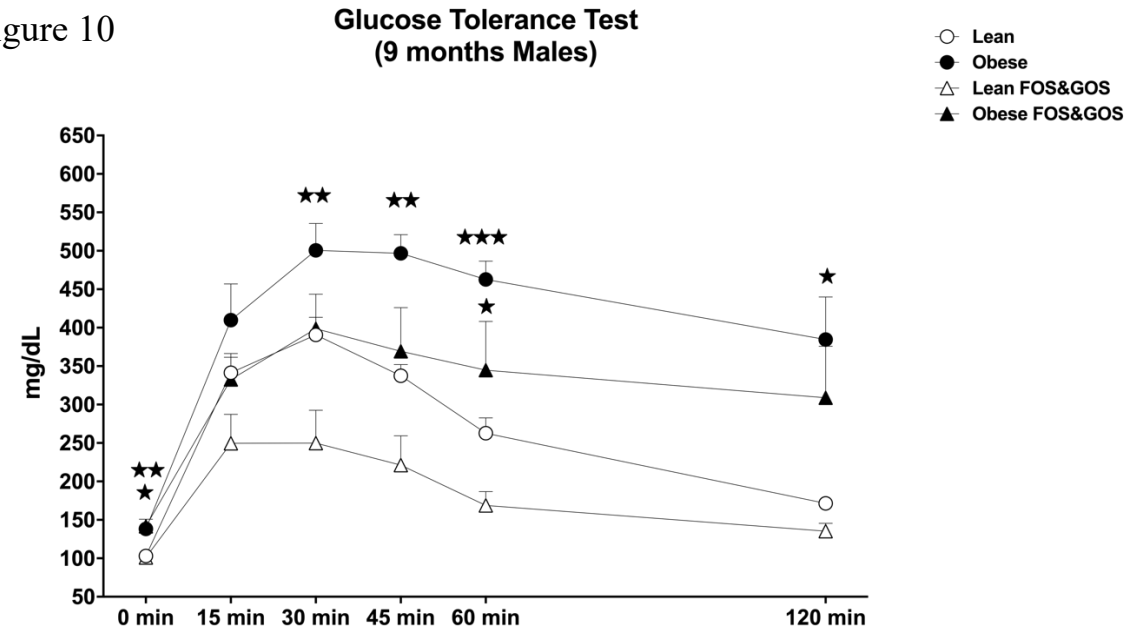


3.2. GTT

Fasted glucose levels in blood were increased in male mice exposed to HF diet for 9 months. Glucose tolerance was impaired in obese male mice. Two-way repeated measures ANOVA revealed significant main effects of diet ($F_{(3,19)} = 11.94$; $P<0.001$), time point ($F_{(3,60)} = 69.59$; $P<0.001$) and interaction between both factors ($F_{(15,95)} = 4.156$; $P<0.001$). Subsequent Dunnett's analysis showed a significant increase of glucose levels in obese and obese mice with FOS&GOS compared to corresponding lean controls at time point 0 and with obese mice at time point 30, 45, 60 and 120 min after glucose administration (from $P<0.05$ to $P<0.001$) (Figure 10). Similarly, fasted glucose levels in blood were increased in female mice exposed to HF diet for 9 months. Glucose tolerance was impaired in obese female mice too. Two-way repeated measures ANOVA revealed significant main effects of diet ($F_{(3,18)} = 19.88$; $P<0.001$), time point ($F_{(3,60)} = 50.44$; $P<0.001$) and interaction between both factors ($F_{(15,90)} = 5.774$;

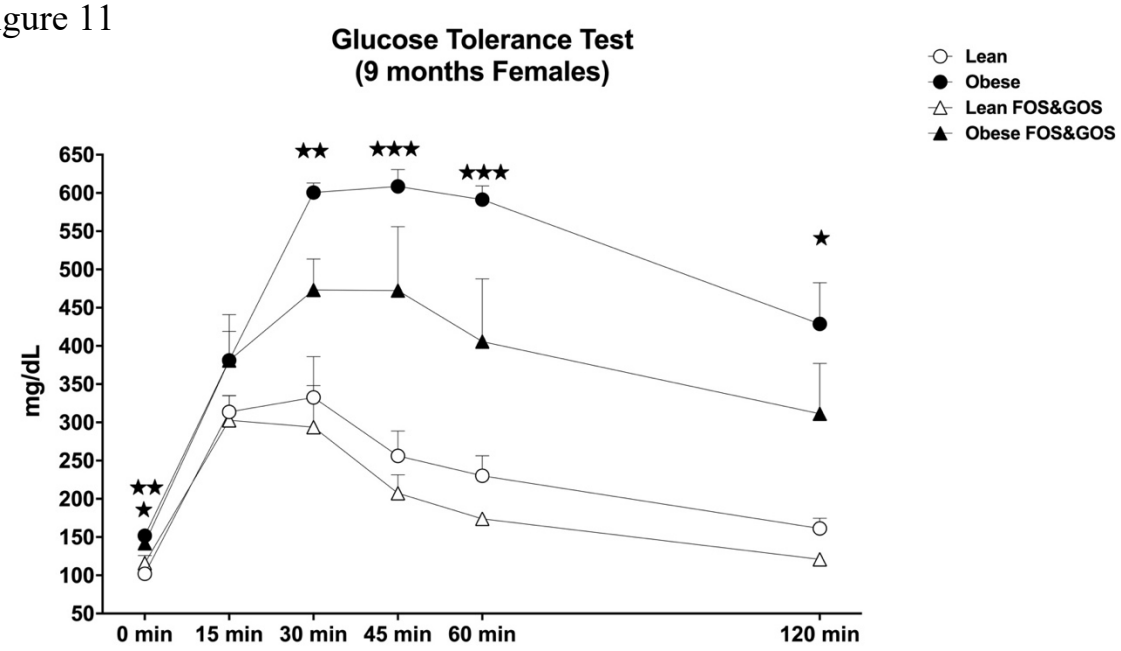
P<0.001). Subsequent Dunnett's analysis showed a significant increase of glucose levels in obese and obese mice with FOS&GOS compared to corresponding lean controls at time point 0 and with obese mice at time point 30, 45, 60 and 120 min after glucose administration (from *P*<0.05 to *P*<0.001) (Figure 11).

Figure 10



★ Compared with Lean group

Figure 11



★ Compared with Lean group

3.3. OF test

3.3.1. Latency to enter into the center

Latency to enter center during the OF test to check anxiety like behaviour in the mice was analysed in males as well as females. One-way ANOVA revealed significance difference for males, during the basal condition ($F_{(3,33)}$= 3.422; P<0.05 , Figure 12A), 3 months ($F_{(3,30)}$= 5.276; P <0.05, Figure 12B) but didn't show difference for 1 month ($F_{(3,34)}$= 0.9118; n.s., Figure 12D), 6 months ($F_{(3,18)}$= 0.7588; n.s., Figure 12E), 9 months ($F_{(3,17)}$= 0.9044; n.s., Figure 12C).Subsequent Dunnett's multiple comparison test showed an increase in anxiety like behavior in obese mice with FOS &GOS (Figure 12B). One-way ANOVA revealed no significant difference in females during basal condition ($F_{(3,31)}$= 0.5782; n.s., Figure 13A) 1 month ($F_{(3,29)}$= 2.363; n.s., Figure 13D), 3 months ($F_{(3,32)}$= 0.9054; n.s., Figure 13B), 6 months ($F_{(3,19)}$= 0.4725; n.s., Figure 13E), 9 months ($F_{(3,18)}$= 1.526; n.s., Figure 13C).

3.3.2. Percentage of time spent in the center

Percentage of activity time in the center zone was analysed to confirm the anxiety like behaviour in the mice (Figure 14, 15). One-way ANOVA did not revealed any significant difference in males during basal conditions ($F_{(3,36)}$= 0.6852; n.s.; Figure 14A), after 1 month ($F_{(3,36)}$= 0.05782; n.s.; Figure 14D), 3 months ($F_{(3,35)}$= 2.474; n.s.; Figure 14B), 6 month ($F_{(3,34)}$= 1.494; n.s.; Figure 14E), 9 months ($F_{(3,16)}$= 3.104; n.s.; Figure 14C). One-way ANOVA did not reveal any significant difference in female during basal conditions ($F_{(3,34)}$= 0.9868; n.s.; Figure 15A) 1 month ($F_{(3,34)}$= 1.498; n.s.; Figure 15D), 3 month ($F_{(3,31)}$= 1.954; n.s.; Figure 15B), 6 month females ($F_{(3,32)}$= 1.239; n.s.; Figure 15E) , 9 month females ($F_{(3,19)}$= 0.1163; n.s.; Figure 15C).

Figure 12

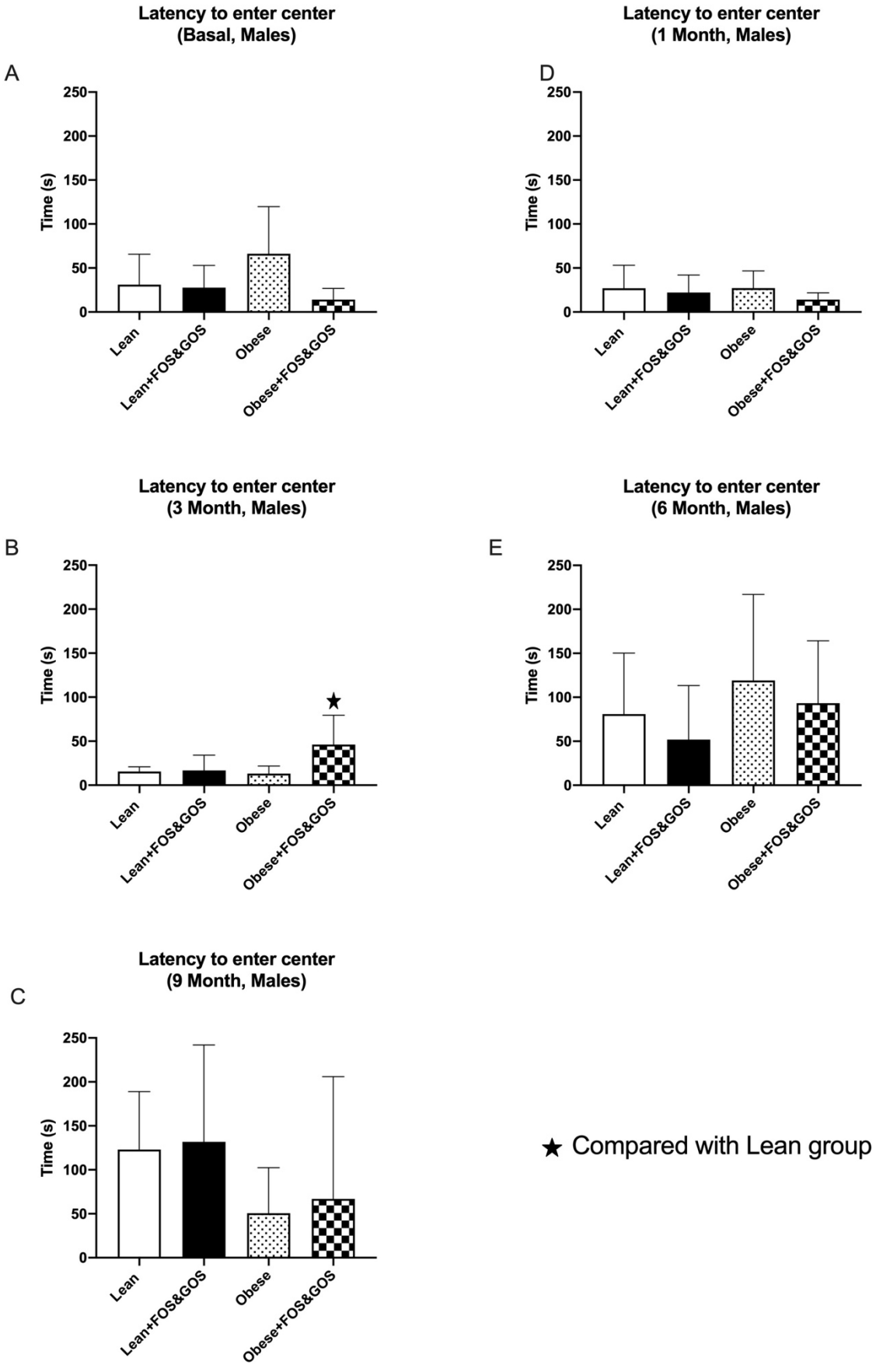

Latency to enter center
(Basal, Males)
A
Time (s)
250
200
150
100
50
0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
Latency to enter center
(1 Month, Males)
D
Latency to enter center
(3 Month, Males)
B
Latency to enter center
(6 Month, Males)
E
Latency to enter center
(9 Month, Males)
C
★ Compared with Lean group

Figure 13

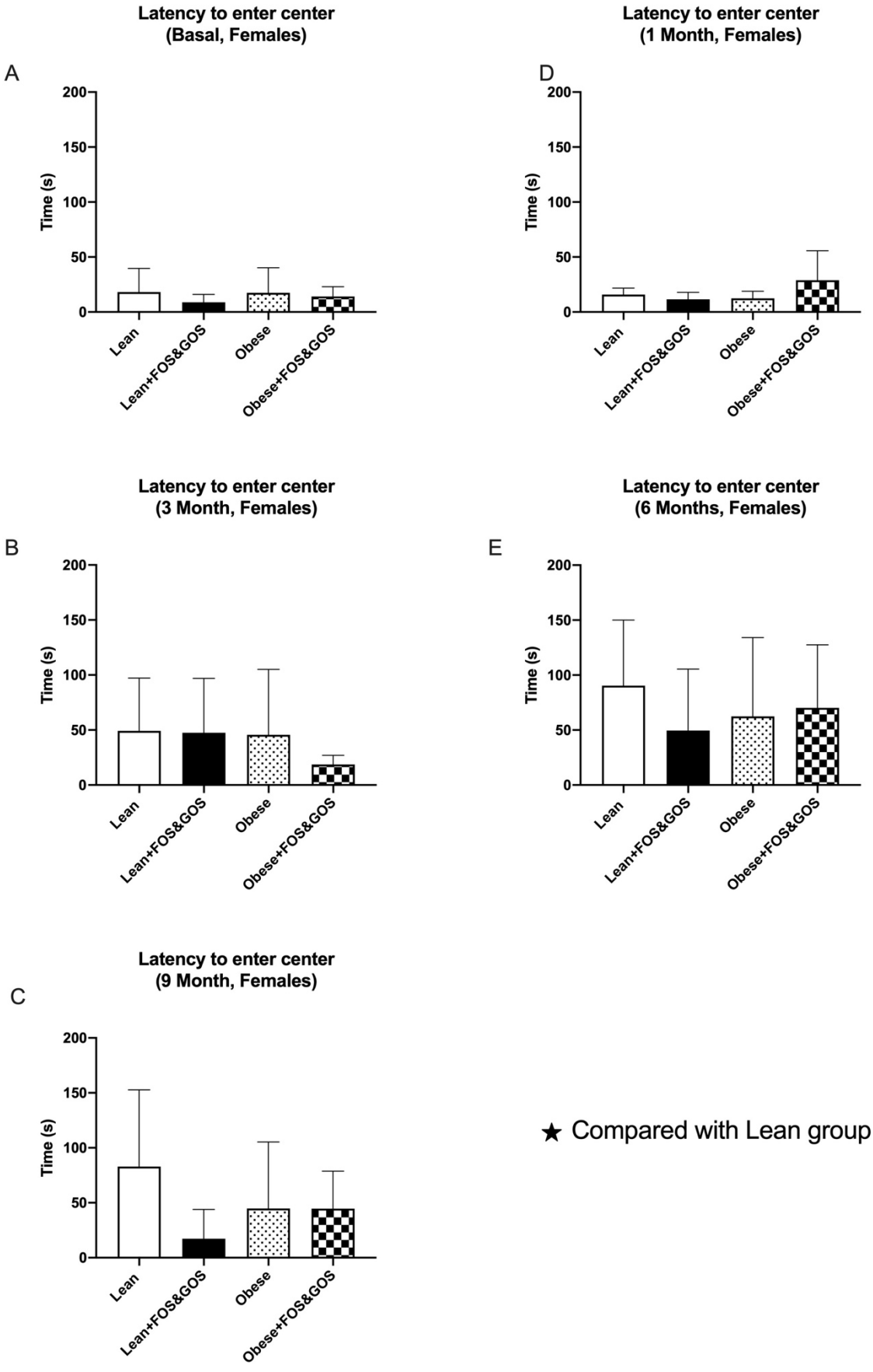

Latency to enter center
(Basal, Females)
A
Time (s)
200
150
100
50
0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
Latency to enter center
(1 Month, Females)
D
Latency to enter center
(3 Month, Females)
B
Latency to enter center
(6 Months, Females)
E
Latency to enter center
(9 Month, Females)
C
★ Compared with Lean group

Figure 14

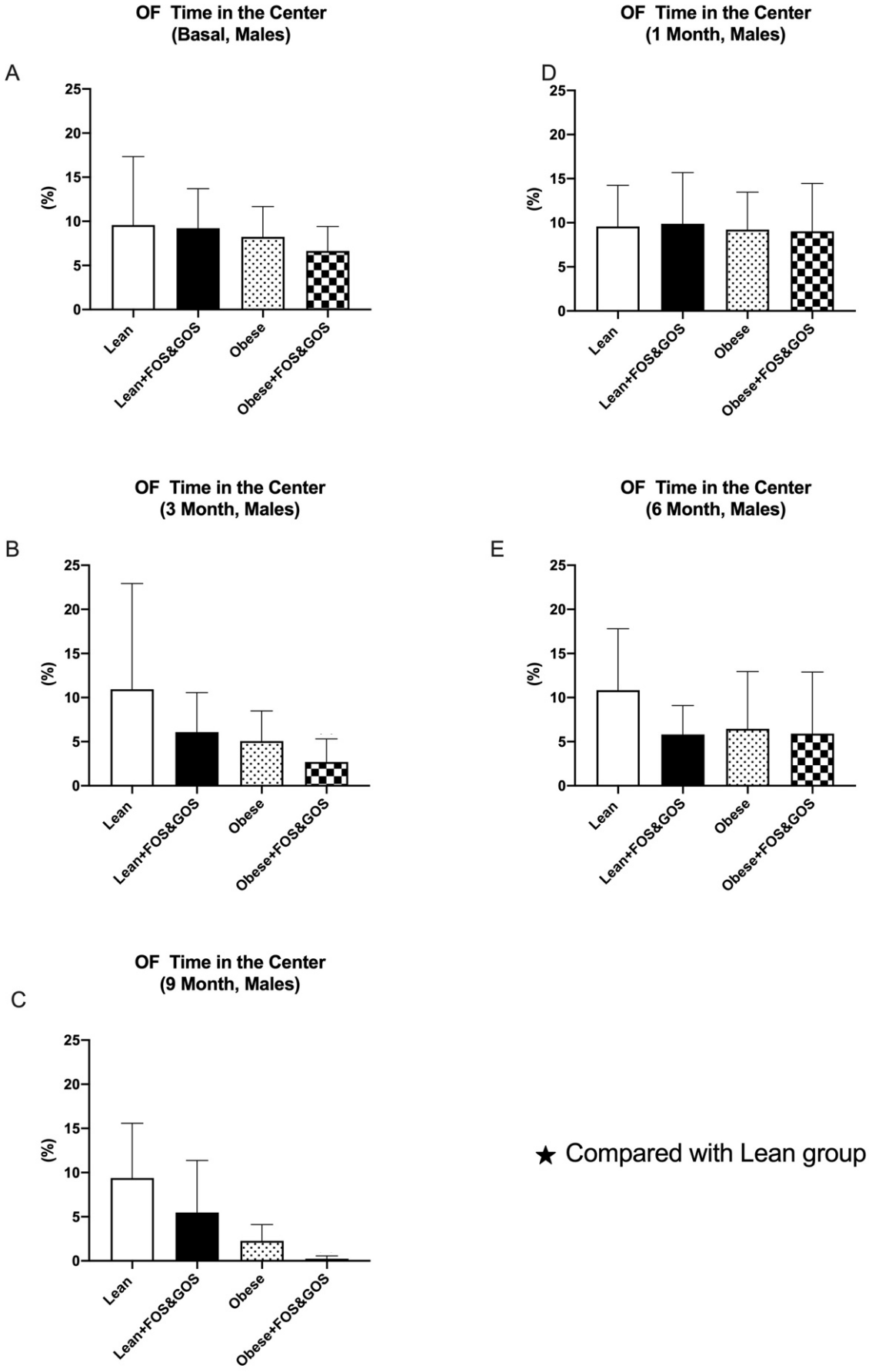
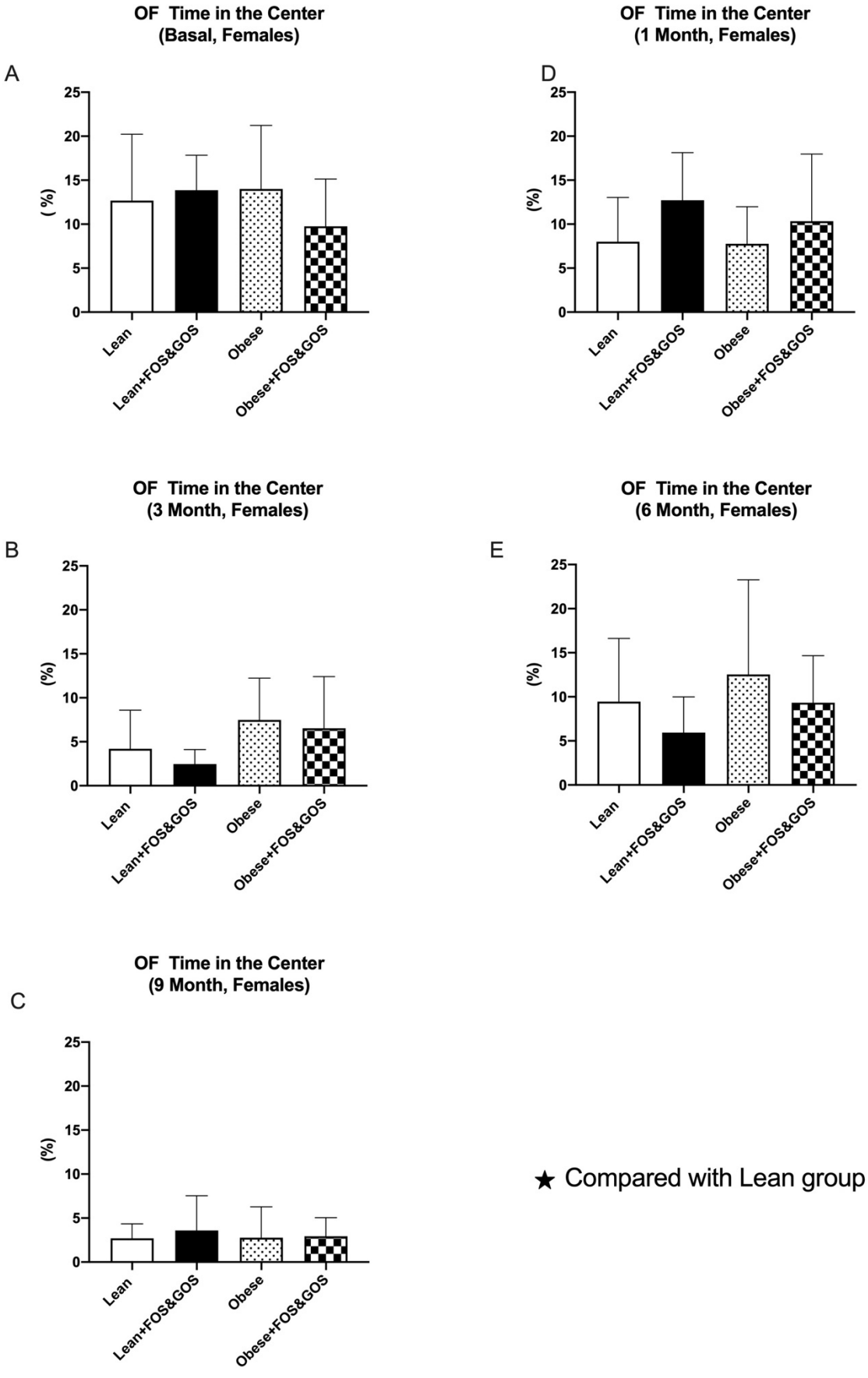

OF Time in the Center
(Basal, Males)
A
(%)
25
20
15
10
5
0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
OF Time in the Center
(1 Month, Males)
D
OF Time in the Center
(3 Month, Males)
B
OF Time in the Center
(6 Month, Males)
E
OF Time in the Center
(9 Month, Males)
C
★ Compared with Lean group

Figure 15


OF Time in the Center
(Basal, Females)
A
(%)
25
20
15
10
5
0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
OF Time in the Center
(1 Month, Females)
D
(%)
OF Time in the Center
(3 Month, Females)
B
(%)
OF Time in the Center
(6 Month, Females)
E
(%)
OF Time in the Center
(9 Month, Females)
C
(%)
★ Compared with Lean group

3.4. Novel object recognition test

Novel object recognition test was conducted successfully. Long term memory was observed in different time intervals (basal, 1 month, 3 months, 6 months and 9 months). One-way ANOVA did not revealed any significance difference in males at basal conditions ($F_{(3,20)}$= 0.4377; n.s., Figure 16A), 1 month ($F_{(3,20)}$= 0.1274; n.s., Figure 16D), 3 months ($F_{(3,19)}$= 0.4355; n.s., Figure 16B), 6 months ($F_{(3,20)}$= 0.09233; n.s., Figure16E), but it revealed significance difference at 9 months ($F_{(3,20)}$= 3.998; $P<0.05$, Figure 16C). Subsequent Dunnett's analysis showed that obese mice with prebiotics administration showed lower DI for long term memory after 9 months (Figure 16C). One-way ANOVA reveal no significant difference in long term memory impairment in female mice at basal conditions ($F_{(3,26)}$= 1.033; n.s., Figure 17A), after 1 month ($F_{(3,34)}$= 1.202; n.s., Figure 17D), 3 months ($F_{(3,33)}$= 1.096; n.s., Figure 17B), 6 months ($F_{(3,33)}$= 0.2872; n.s., Figure 17E), 9 months ($F_{(3,19)}$= 0.3644; n.s., Figure 17C).

Figure 16

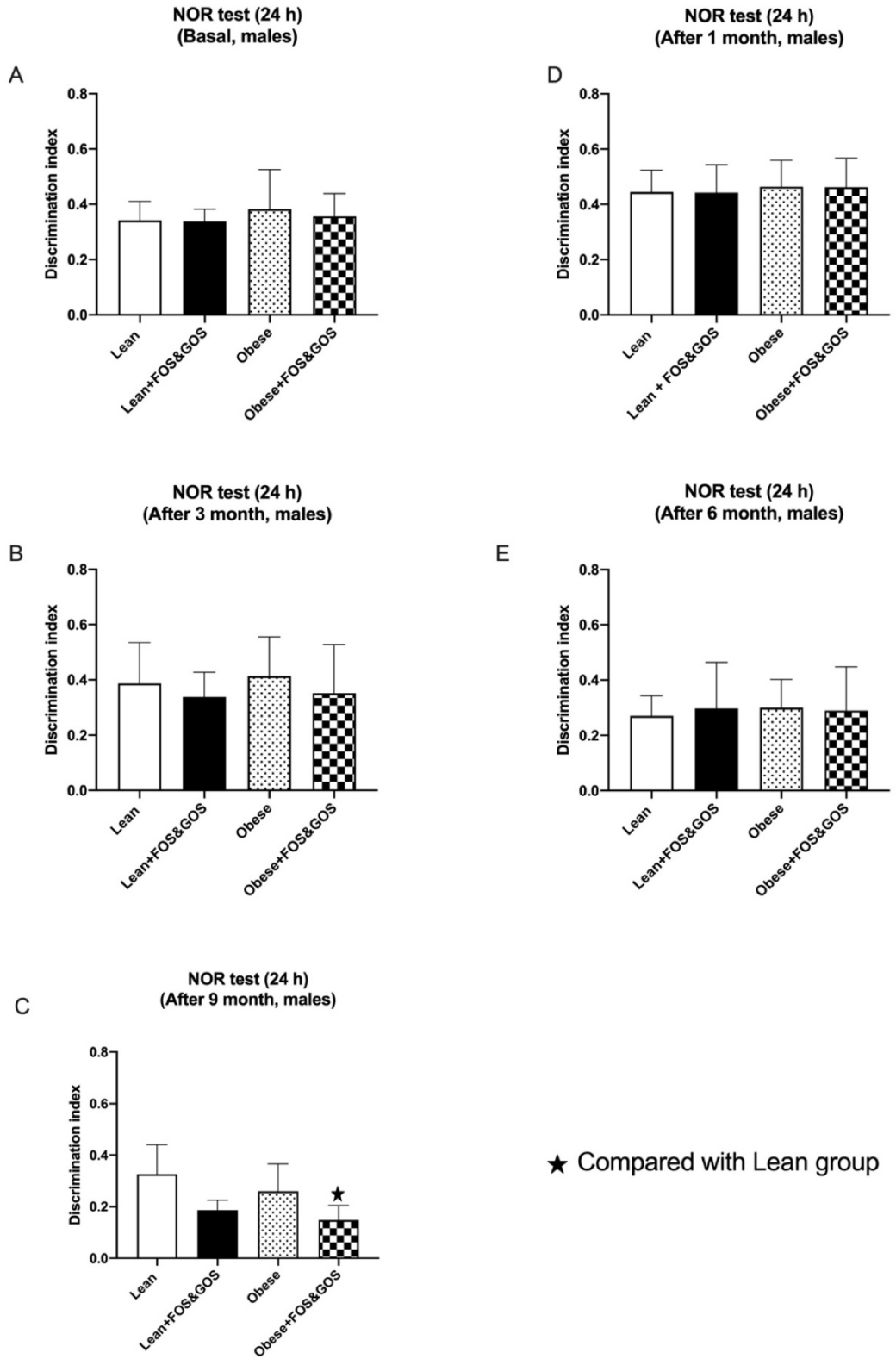
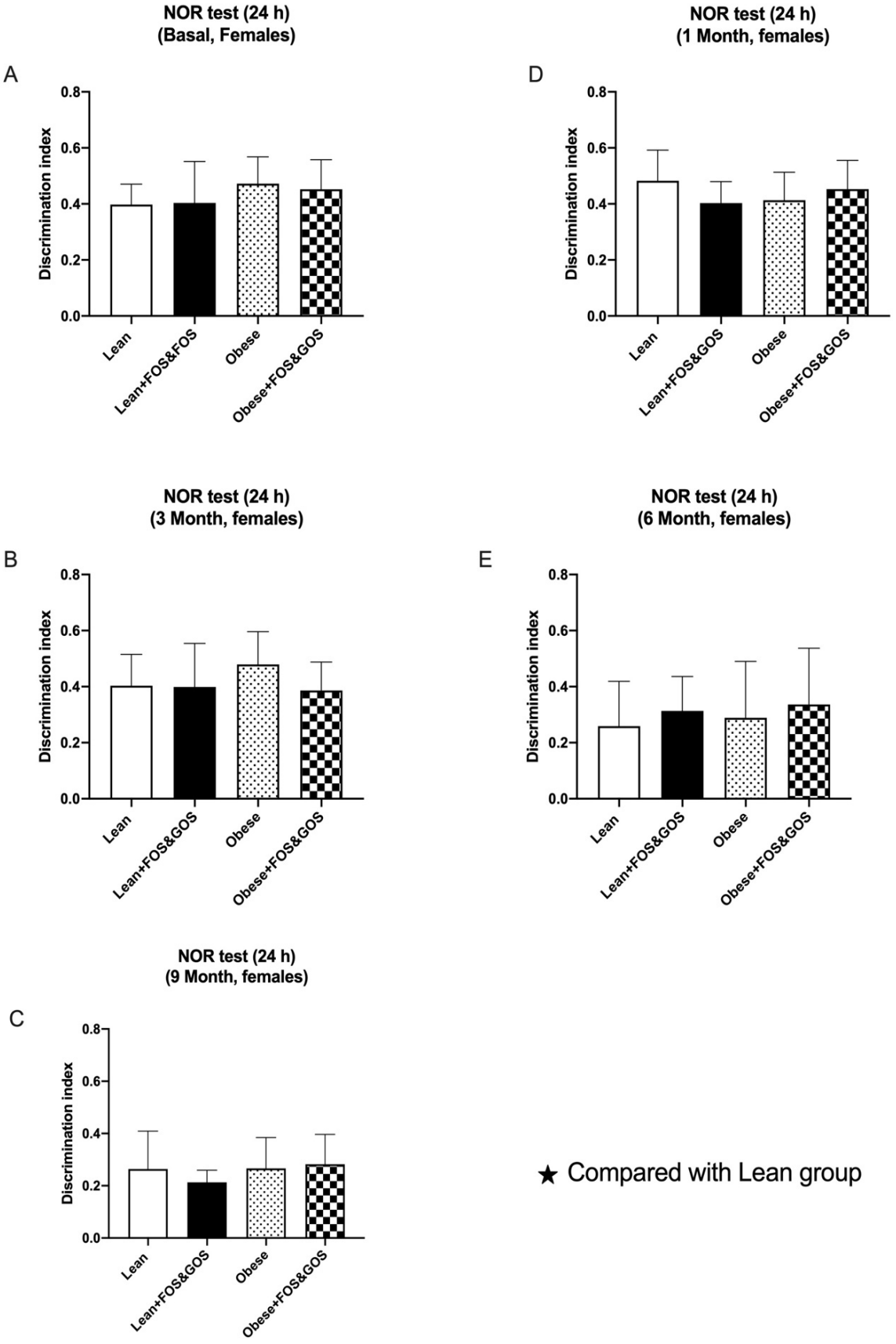

NOR test (24 h)
(Basal, males)
A
Discrimination index
0.8
0.6
0.4
0.2
0.0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(After 1 month, males)
D
Discrimination index
0.8
0.6
0.4
0.2
0.0
Lean
Lean + FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(After 3 month, males)
B
Discrimination index
0.8
0.6
0.4
0.2
0.0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(After 6 month, males)
E
Discrimination index
0.8
0.6
0.4
0.2
0.0
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(After 9 month, males)
C
Discrimination index
0.8
0.6
0.4
0.2
0.0
★
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
★ Compared with Lean group

Figure 17


NOR test (24 h)
(Basal, Females)
A
Discrimination index
0.0
0.2
0.4
0.6
0.8
Lean
Lean+FOS&FOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(1 Month, females)
D
Discrimination index
0.0
0.2
0.4
0.6
0.8
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(3 Month, females)
B
Discrimination index
0.0
0.2
0.4
0.6
0.8
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(6 Month, females)
E
Discrimination index
0.0
0.2
0.4
0.6
0.8
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
NOR test (24 h)
(9 Month, females)
C
Discrimination index
0.0
0.2
0.4
0.6
0.8
Lean
Lean+FOS&GOS
Obese
Obese+FOS&GOS
★ Compared with Lean group

3.5. Running Wheel Test

Running wheel test showed a decrease in the physical activity of the mice. HF induced mice has least physical activity as compared to other mice. Day time physical activity and the total physical activity (24 hours) was analysed in diet induced mice.

3.5.1 Total physical activity

One-way ANOVA did not reveal significant difference of diet on physical activity of the mice for males in basal condition ($F_{(3,33)}$= 0.2245; n.s.; Figure 18A), 1 month ($F_{(3,34)}$= 2.148; n.s. ; Figure 18D), but it revealed the significant difference in 3 months ($F_{(3,32)}$= 3.157; P<0.05; Figure 18B), 6 months ($F_{(3,35)}$= 6.898; P<0.0001; Figure 18E), 9 months ($F_{(3,18)}$= 8.993; *P* < 0.0001; Figure 18C). Subsequent Dunnett's multiple comparison test showed a significant decrease in physical activity in obese male mice during the day and night cycle after 6 and 9 months (from *P*<0.05 to *P*<0.0001) (Figure 18E and C). One-way ANOVA did not revealed significance difference for females during basal condition ($F_{(3,32)}$= 0.3982; n.s.; Figure 19A), and 3 months ($F_{(3,30)}$= 2.278; n.s.; Figure 19B), but it revealed significance difference in 1 month ($F_{(3,34)}$= 4.391; P<0.05 ; Figure 19D), 6 months ($F_{(3,32)}$= 6.276; P<0.001; Figure 19E), 9 months ($F_{(3,18)}$= 11.29 ; *P* < 0.0001; Figure 19C). Subsequent Dunnett's multiple comparison test showed a significant decrease in physical activity in obese female mice after 6 and 9 months and lean animals after 6 months administration with FOS&GOS (from *P*<0.05 to *P*<0.0001) (Figure. 19).

3.5.2 Physical activity during day time

One-way ANOVA did not revealed the significance difference for the effect of diet on physical activity of the mice during day time in males for basal condition ($F_{(3,34)}$= 0.2245; n.s.; Figure 20A), after 1 month ($F_{(3,34)}$= 1.622; n.s.; Figure 20D), but significant difference was revealed in 3 months ($F_{(3,36)}$= 3.157; P<0.05; Figure 20B), 6 months ($F_{(3,36)}$= 6.898; P<0.001; Figure 20E), 9 months ($F_{(3,20)}$= 8.993; P<0.001; Figure 20C). Subsequent Dunnett's multiple comparison test showed a significant decrease in physical activity in obese male mice after 6 and 9 months (Fig. 20). One-way ANOVA did not reveal the significant difference during day time in females for basal condition ($F_{(3,31)}$= 0.2194; n.s.; Figure 21A), 3 months ($F_{(3,30)}$= 2.278; n.s.; Figure 21B), but it revealed the significance difference for 1 month ($F_{(3,31)}$=3.474; P<0.05; Figure 21D), 6 months ($F_{(3,32)}$= 6.276; P<0.05; Figure 21E), 9 month ($F_{(3,17)}$= 25.42, *P* < 0.0001; Figure 21C). Subsequent Dunnett's multiple comparison test showed a significant decrease in physical activity in obese female mice after 6 and 9 months (Figure 21).

Figure 18

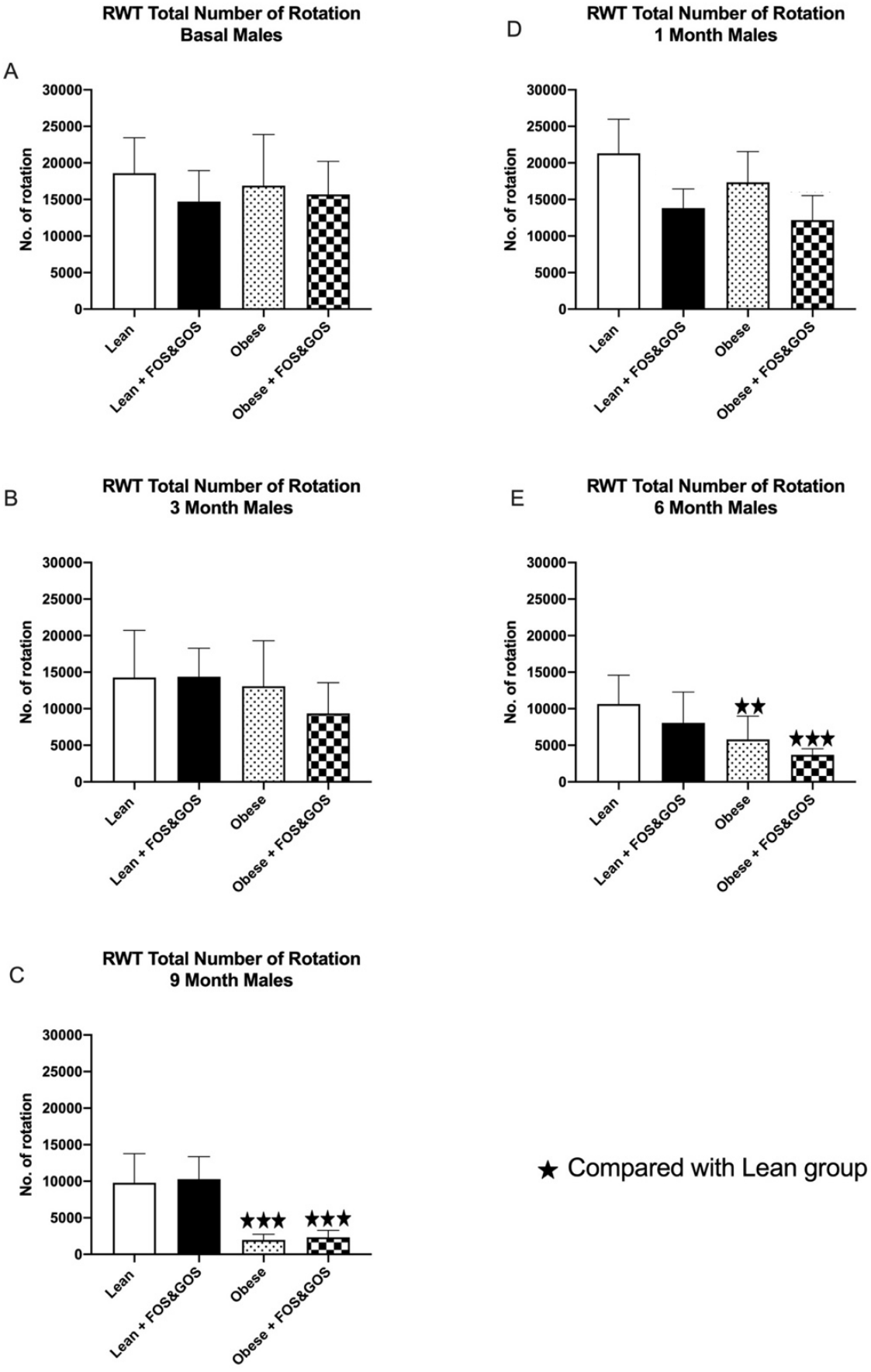

A
RWT Total Number of Rotation
Basal Males
No. of rotation
30000
25000
20000
15000
10000
5000
0
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
D
RWT Total Number of Rotation
1 Month Males
B
RWT Total Number of Rotation
3 Month Males
E
RWT Total Number of Rotation
6 Month Males
★★
★★★
C
RWT Total Number of Rotation
9 Month Males
★★★
★★★
★ Compared with Lean group

Figure 19

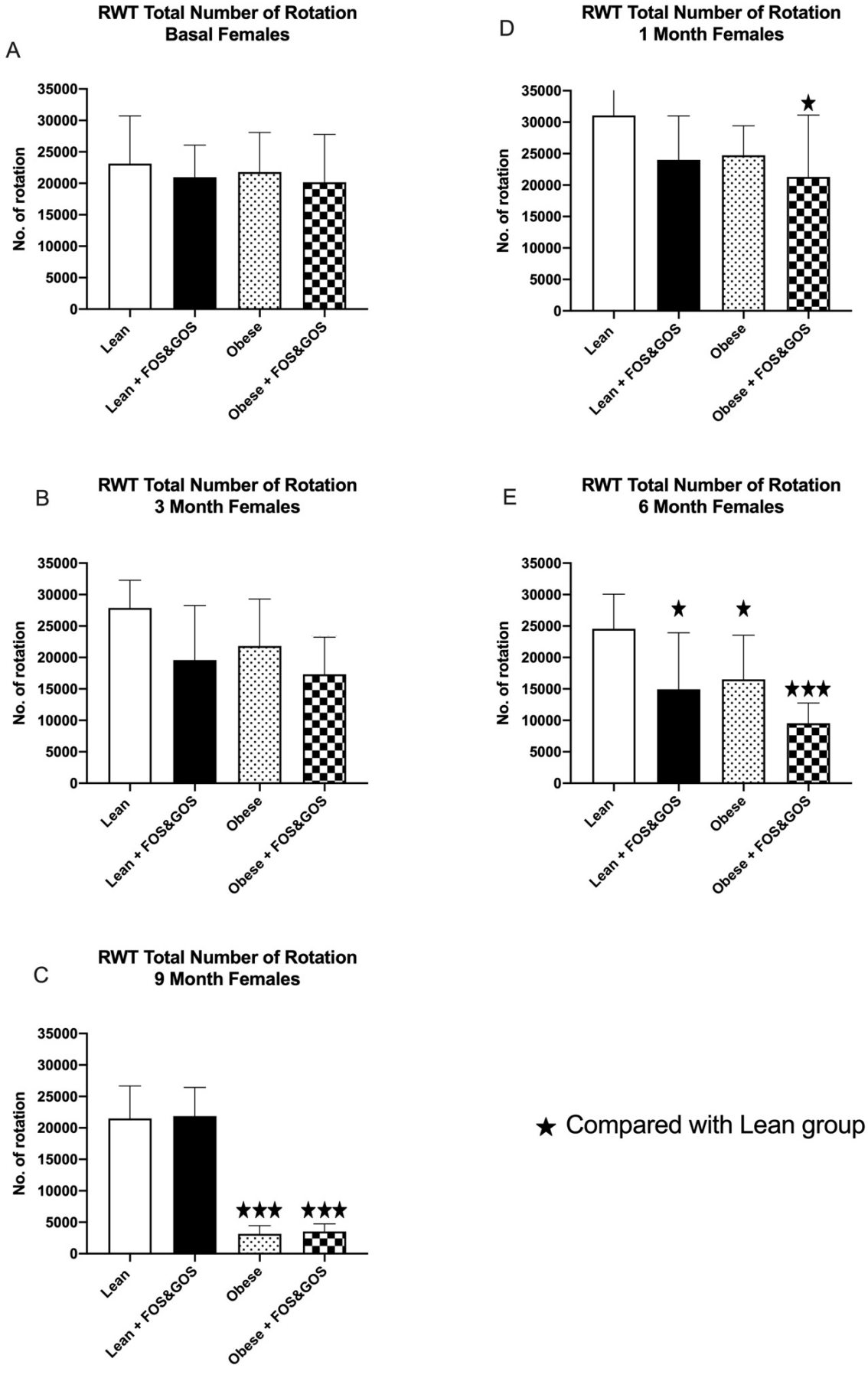

A
RWT Total Number of Rotation
Basal Females
No. of rotation
35000
30000
25000
20000
15000
10000
5000
0
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
D
RWT Total Number of Rotation
1 Month Females
★
B
RWT Total Number of Rotation
3 Month Females
E
RWT Total Number of Rotation
6 Month Females
★
★
★★★
C
RWT Total Number of Rotation
9 Month Females
★★★
★★★
★ Compared with Lean group

Figure 20

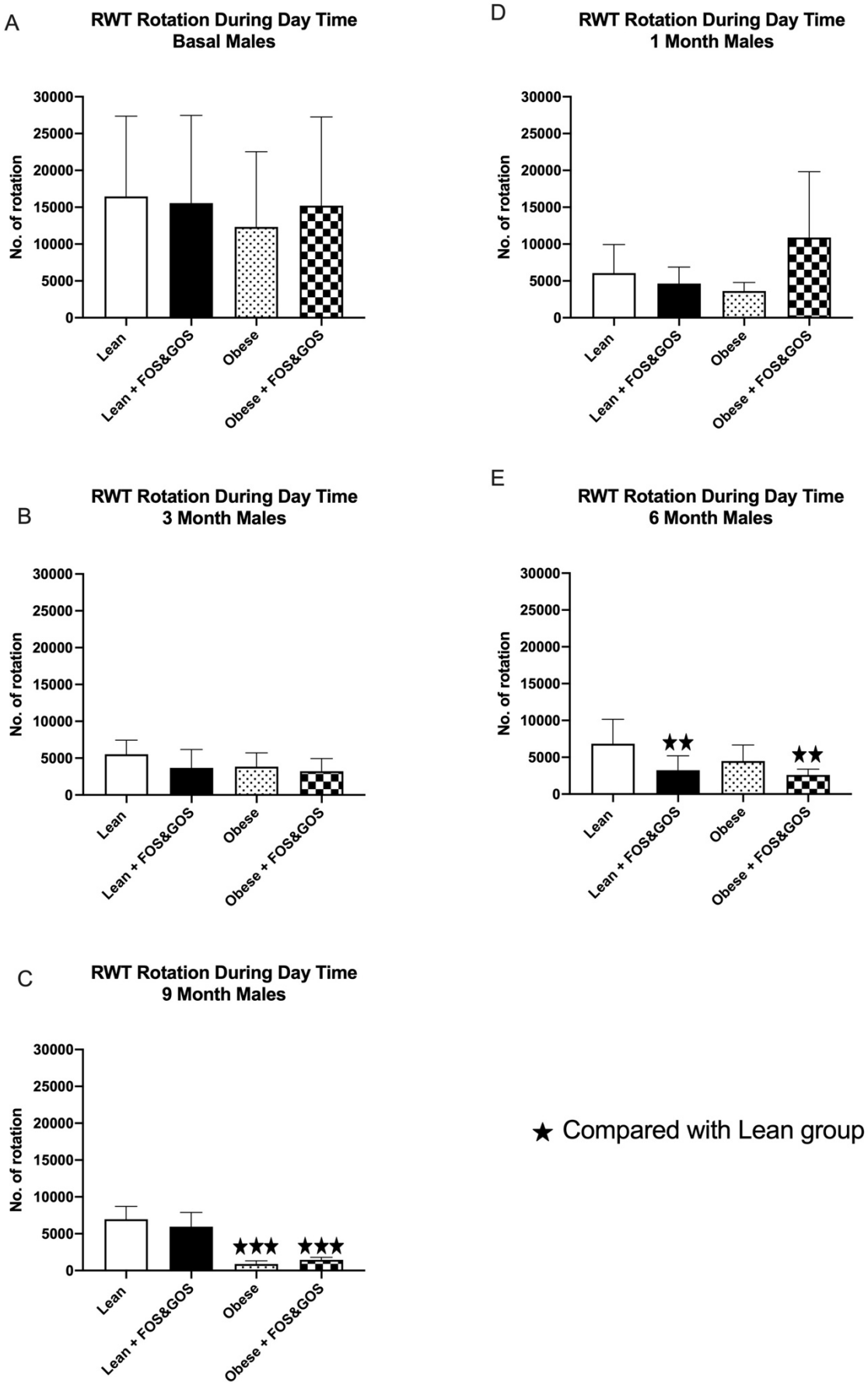
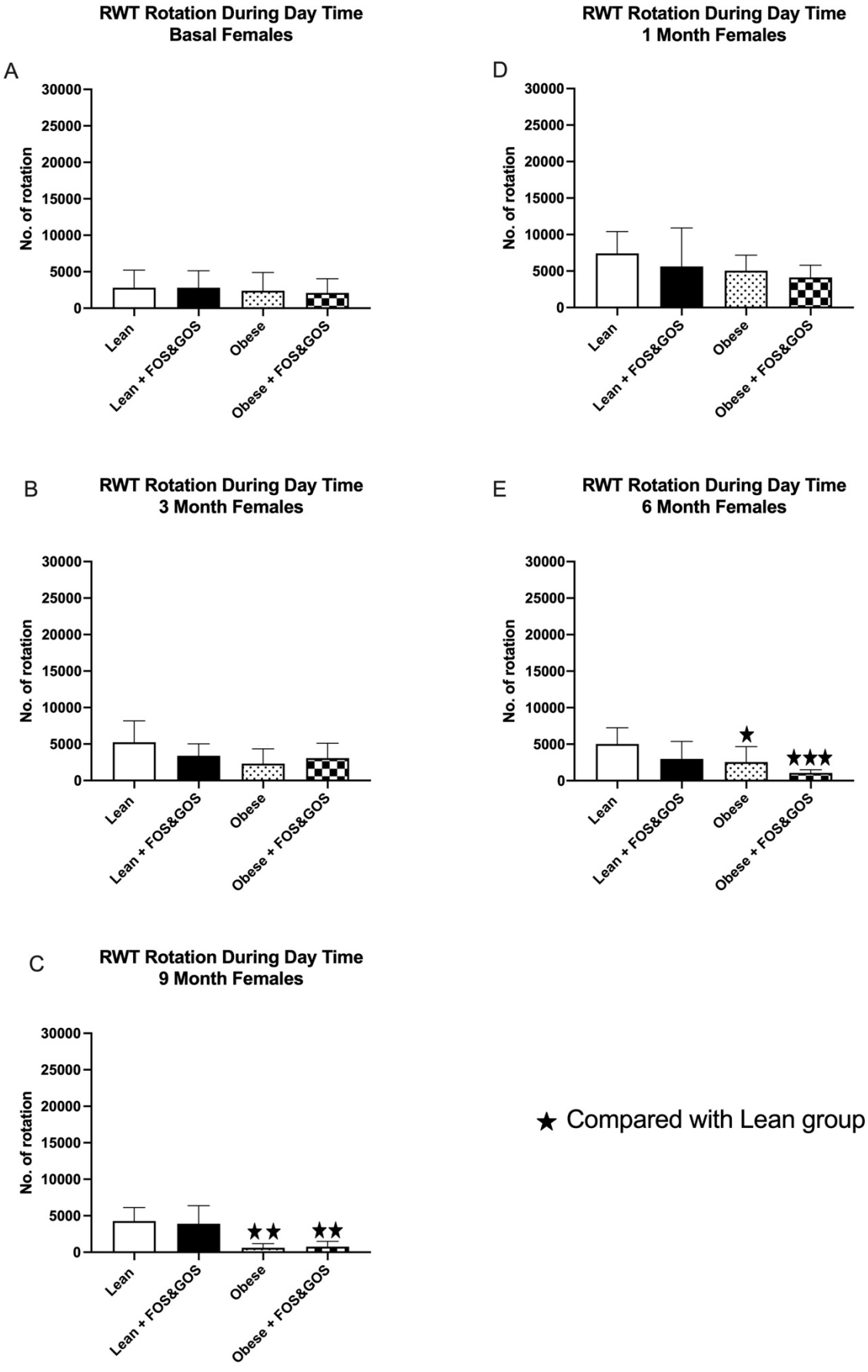

A
RWT Rotation During Day Time
Basal Males
No. of rotation
30000
25000
20000
15000
10000
5000
0
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
D
RWT Rotation During Day Time
1 Month Males
B
RWT Rotation During Day Time
3 Month Males
E
RWT Rotation During Day Time
6 Month Males
★★
C
RWT Rotation During Day Time
9 Month Males
★★★
★ Compared with Lean group

Figure 21


A
RWT Rotation During Day Time
Basal Females
No. of rotation
30000
25000
20000
15000
10000
5000
0
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
D
RWT Rotation During Day Time
1 Month Females
B
RWT Rotation During Day Time
3 Month Females
E
RWT Rotation During Day Time
6 Month Females
C
RWT Rotation During Day Time
9 Month Females
★ Compared with Lean group

3.6. SVM

Decrease in social behaviour was observed in obese mice. The SVM which was conducted in two categories mouse verses object and stranger mouse verses familiar mouse showed a strong decrease in sociability in mice after the HF diet induced obesity. One-way ANOVA revealed significant difference in both males and females giving stranger mouse verses object in males ($F_{(3,19)}$= 8.608; P< 0.001; Figure 22A), stranger mouse verses object in females ($F_{(3,19)}$= 10.28; P< 0.001; Figure 22B), stranger mouse verses familiar mouse males ($F_{(3,19)}$= 7.762; P< 0.05; Figure 23A), and did not show any significant difference in stranger mouse verses familiar mouse females ($F_{(3,18)}$= 1.913; n.s.; Figure 23B). Subsequent Dunnett's multiple comparison test showed a significant decrease in sociability in obese mice (from P<0.05 to P<0.001) (Figure 22 and 23).

Figure 22

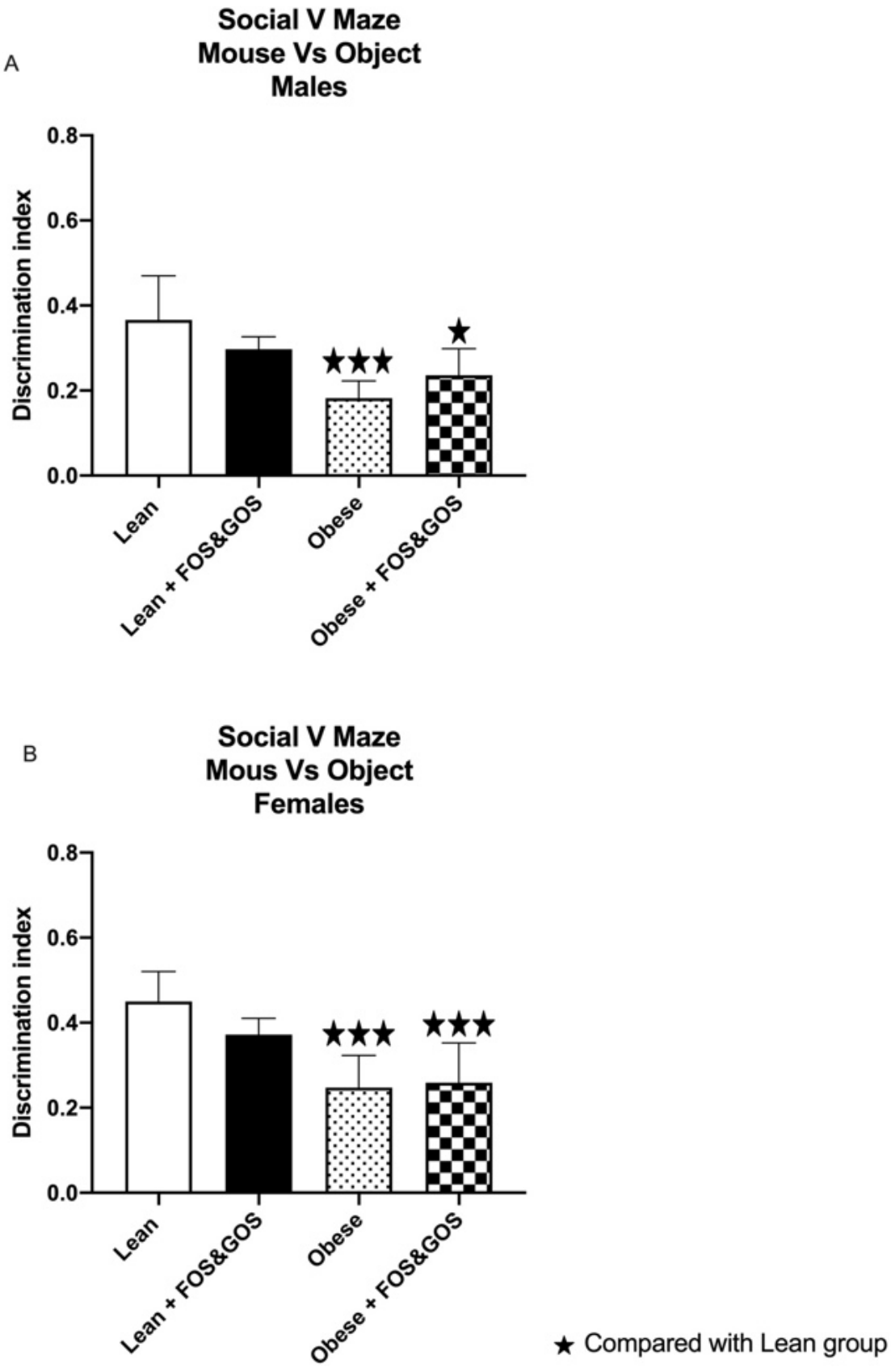
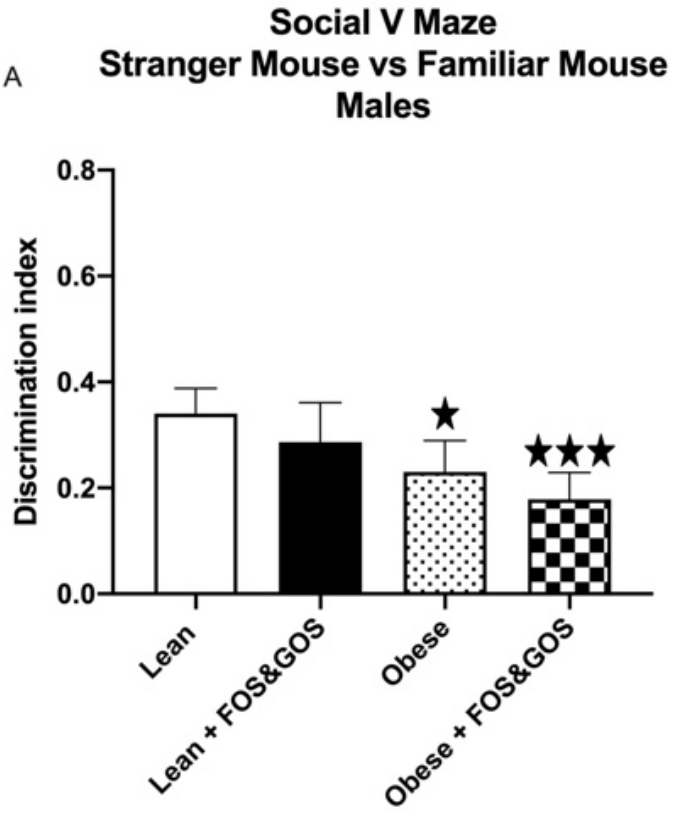
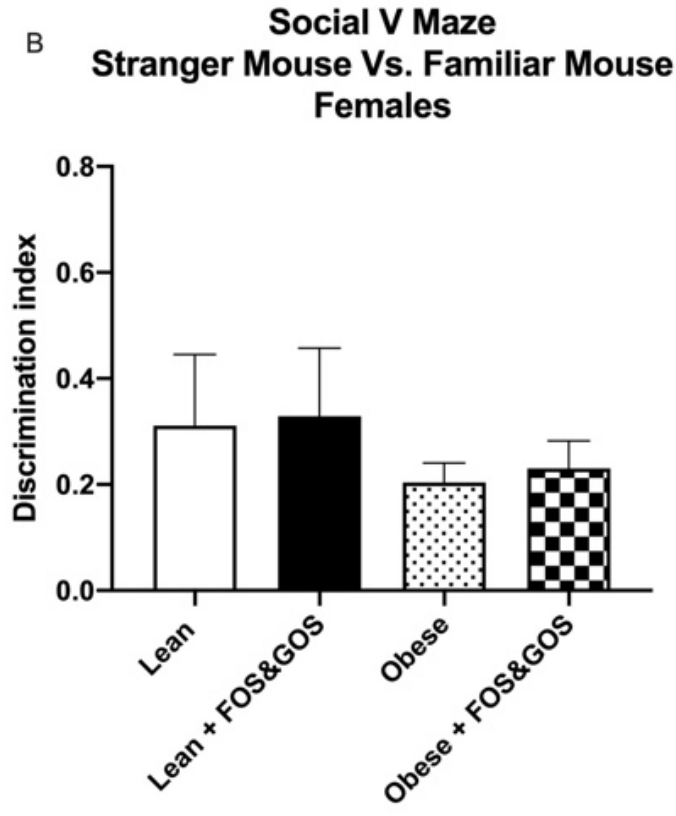

A
Social V Maze
Mouse Vs Object
Males
Discrimination index
0.8
0.6
0.4
0.2
0.0
★★★
★
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
B
Social V Maze
Mous Vs Object
Females
Discrimination index
0.8
0.6
0.4
0.2
0.0
★★★
★★★
Lean
Lean + FOS&GOS
Obese
Obese + FOS&GOS
★ Compared with Lean group

Figure 23

A



B



★ Compared with Lean group

4. DISCUSSION

4.1. HF diet effect on body weight and GTT

In our study conditions, mice (males and females) introduced with HF diet had significant weight gain from the second month with diet. HF diet led to significant overweight and glucose intolerance. It was found that some mice developed obesity while others remained lean when fed with the control diet. In this experiment, HF diet induced significant weight gain, as the effects were more pronounced in the HF than in the low-fat diet exposed mice. Similar observation was previously described (Espinosa-Carrasco et al. 2018; Burokas et al. 2018). Glucose tolerance was impaired in obese mice, as revealed by an upward shifted glucose curve. However, lean mice showed lower values than obese animals at almost all time points (except 15 min) suggesting better recovery. As expected, HF diet obese mice also showed the highest fasted glucose levels, which could be related to their highest increase of body weight. These results validate the obesity model leading to diabetes state in animals used in the present study because raised fasting glucose levels have been demonstrated in diet-induced, genetic, and metabolic syndrome mouse models similarly to humans.

Prebiotics are widely used as modulators of the intestinal and immune systems and are an important component of infant milk formulas (Chang et al. 2019). However, limited studies have focused on the effects of prebiotics on the CNS and behaviour (Chang et al. 2019; Whisner and Castillo 2018; Sarao and Arora 2017; Shokryazdan et al. 2017; Burokas et al. 2017). In this study, we report that prebiotics (i.e. FOS+GOS) were able to modify behaviour relevant to anxiety and memory in mice to some extent. The effects of prebiotics were observed on cognition, physical activity, and sociability. When compared the body weight of the mice, the obese animals with prebiotics didn't have a major weight gain as the only with HF diet animals.

4.2. Long term memory impairment and anxiety

Obesity and the consequent metabolic dysfunctions are strongly linked to metabolic syndrome-associated cognitive impairment and dementia (Bruce-Keller et al. 2017). Contrary, as expected, these HF diet mice did not exhibit cognitive function decline (only males after 9 months of diet). Both glucose and insulin intolerance impairments are demonstrated to be

essential and important pathogenic factors in the development of metabolic dysfunction-associated cognitive impairment (Bruce-Keller et al. 2015); here we show that HF diet treatment for 9 months increases glucose intolerance in mice (Figures 10, 11). Importantly, insulin resistance is one major risk factor of cognitive impairment in patients with obesity/diabetes or metabolic syndrome (McCrimmon, Ryan, and Frier 2012). Here we show as expected that HF diet-induced obesity in mice cognition was decreased over the 9 months duration (, similarly that previously was confirmed that strong metabolic regulation for insulin dependence in mice with HF diet-induced obesity was associated with dramatic cognition decline (Q. Wang et al. 2018). The object recognition task used in this study evaluates a hippocampal-dependent long-term memory. Hippocampal-dependent memory is not impaired in mice during the basal conditions. In this study, HF food intake impairs hippocampal long-term memory in mice. It is well-known that HF food modifies hippocampal-dependent long-term memory and decreases dendritic complexity within the hippocampus (Cope et al. 2018). When compared with control group obese mice characterized by deficient learning, do neither behave homogeneously in terms of learning impairment/inflexibility nor reinforcement, impulsivity and compulsivity (Burokas et al. 2018).

As obesity is a complex disorder and a major risk factor for many diseases and health problems, including heart disease, diabetes, stroke, high blood pressure, cancer, osteoarthritis, and cognitive impairment (Amiri and Behnezhad 2019). Additionally, decreased spatial memory, anxiety, and depression have been linked to obesity-associated brain inflammation (Rajan and Menon 2017). Previous studies have reported that HF diet-induced obesity in mice results in anxiety-like behaviour (Seravalle and Grassi 2017). However, similar to other obesity-driven phenotypes such as physical inactivity, anxiety-like behaviour is not simply caused by increased body mass (Singh et al. 2018). In our study we have not observed a profound effect of HF diet on open filed results for anxiety (only males after 9 months of diet). Interestingly, loss of body weight alone does not always correspond to improvements in mental health (Inglis et al. 2019). Thus, the mechanistic basis of obesity-induced anxiety-like behaviour is not yet fully understood. HF diet induces senescence in multiple organs, predominantly in perigonadal adipose tissue but also in other organs including the liver (Ogrodnik et al. 2019). Until now, the role of HF diet in the induction of senescence in the brain is largely understudied. Recently, several studies have uncovered a role for senescent glial cells in neurodegenerative diseases such as τ-dependent pathology (Rajan and Menon 2017). In the case of our study mice didn't show a strong effect on anxiety like behaviour.

4.3. Obese mice response to physical activity

It has been showed that obese mice are poor in physical activity (Carvalho et al. 2018; Manzanares, Brito-da-Silva, and Gandra 2018). This study presents the evidence that voluntary running wheel exercise can result in less activity in HF diet fed mice but also to some extent in mice low-fat fed mice over the time. The present study demonstrated that aged obese mice, significantly reduced physical endurance capacity by >70% compared with aged lean mice. As obese mice have gained weight and are less physically active the results showed decline in monitored running wheel test. Prebiotics administration didn't have any major effect on the physical activity of the mice.

4.4. Sociability of obese mice

The analysis of social interactions both in a familiar mouse and stranger mouse demonstrated that, obese mice (males and females) were less sociable, spending less time observing the mouse and spending more time exploring the object in the social V- maze compared to the lean mice. Moreover male obese mice showed lower preference for social novelty. These data can be interpreted as a sign of social avoidance of both familiar and unfamiliar mice displayed by animals fed with the HF food. It has been reported that obese mice present a factor of neophobia that could explain lower social interactions in cases where only the interactions with unfamiliar mouse would be reduced in mice exposed to the HF diet (Q. Wang et al. 2018). Our results are generally in line with several studies that reported diminished sociability in rodents after exposures to diets containing high amounts of fat/cholesterol (Holm-Hansen et al. 2016). In one of the most recent studies, Buffington and colleagues found that the even off springs of dams fed with a HF diet containing high amounts of cholesterol displayed markedly dropped number, frequency, and duration of social contacts both with familiar and unfamiliar mouse, as well as impaired long-term potentiation in the ventral tegmental area, a sign of a deficient synaptic plasticity (Buffington et al. 2016). These deficits were rescued by supplementary oxytocin and a restoration of normal microbiota parameters, which were affected in the offspring of dietary-challenged mice and can potentially mediate reported effects here. Moreover, another study with a HF diet containing high amounts of cholesterol showed that it can exacerbate social deficiency and cognitive rigidity in BTBR T+tf/J inbreed mouse line, a

model of autism (M. Yang et al. 2012). BTBR mice, after housing on a HF diet containing high amounts of cholesterol starting at weaning, demonstrated greater deficits in social memory, lowered preference for social novelty, and impaired learning of the T-maze than these mice fed with a regular diet (Newell et al. 2017).

In summary our results suggest that modelling a diet-induced obesity and diabetes leading to AD nine months are not sufficient at least for behavioural symptoms such as memory impairment. The rapid developed obesity and diabetes by HF diet may not be straight forward for AD behavioural symptoms. On the other hand, this does not mean that there is not already an early stage of development of AD.

CONCLUSIONS

1. HF diet already after two months significantly increased body weight of mice, both males and females and impaired glucose tolerance after 9 months of diet. Prebiotic administration mitigated the HF diet negative impact.

2. The mice showed no anxiety like behaviour or memory impairment after 9 months of HF diet or prebiotics administration. The obese mice were less physically active when they had a running-wheel test.

3. The sociability test revealed that obese mice were less social compared to the lean mice. They also had less preference for social novelty.

4. Our results suggest that nine months of HF diet is not sufficient that a diet-induced diabetes leads to an AD model in mice.

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VILNIUS UNIVERSITY

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Master thesis

Targeting microbiota-gut-brain axis in Alzheimer's disease in a diet-induced obesity model in mice

SUMMARY

The concept of the microbiota-gut-brain axis, the bidirectional communication between gut and brain, has been supported by many experimental and clinical studies. Clinical observations showed that the intestinal microbiota profile is altered in many neurologic and psychiatric disorders and diseases including Parkinson's disease and AD have given rise to the hypothesis that a disordered microbiota (dysbiosis) is a pathogenetic factor in these pathologies.

To shed more light on the communication between the intestinal microbiota and the brain function, and to find bacterial strains of microbiota that can reduce inflammation and the development of AD in diabetic conditions a diet-induced obesity dysbiosis model was established in C57BL/6JRj mice. To validate diet-induced diabetes leading to AD models in mice we have characterized by assessment of emotional, affective, physical and cognitive behaviour. In addition, the administration of prebiotics was used for positive microbiota modulation. Male and female mice were fed for 9 months with HF food and prebiotics (FOS+GOS) dissolved in drinking water. Animal body weight was recorded and glucose tolerance test was conducted at the 9 month time for the confirmation of diabetes in mice. Also anxiety-like behaviour, cognition, social behaviour and physical activity were analysed.

HF diet already after two months significantly increased body weight of the mice, both males and females and impaired glucose tolerance after 9 months of diet. Prebiotic administration mitigated the HF diet negative impact. Whereas anxiety-like behaviour and long term memory weren't altered, but had a major impact on physical activity and sociability after 9 month with HF diet. In summary, these results suggest that nine months of HF diet is not sufficient that a diet-induced diabetes leads to an AD model in mice.

VILNIAUS UNIVERSITETAS

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Magistrinis darbas

Mikrobiotos-žarnyno-smegenų ryšio vaidmens Alzheimerio ligos eigoje tyrimas panaudojant mitybos indukuotą pelių nutukimo modelį

SANTRAUKA

Mikrobiotos-žarnyno-smegenų ryšys, tai dvikryptė sąveika tarp žarnyno ir smegenų, kuri buvo parodyta daugeliu eksperimentų ir klinikinių tyrimų. Klinikiniai stebėjimai atskleidė, kad žarnyno mikrobiotos sudėties profilis yra pakitęs daugelio neurologinių ir psichinių sutrikimų bei ligų, įskaitant Parkinsono ir Alzheimerio ligas, ir iškėlė hipotezę, kad sutrikdyta mikrobiota (disbiozė) yra šių patologijų patogenezinis veiksnys.

Norėdami sužinoti daugiau apie žarnyno mikrobiotos ir smegenų ryšį ir surasti mikrobiotos bakterijų kamienus, kurie galėtų sumažinti neuro-uždegiminius procesus ir Alzheimerio ligos vystymąsi esant diabetui, buvo panaudotas mitybos indukuotas C57BL/6JRj pelių nutukimo/diabeto modelis kaip kartu ir nutukimo sukelta disbiozė pelėms. Norėdami patvirtinti mitybos indukuotą diabetą, kuris vėliau sukeltų Alzheimerio ligą pelėms, buvo vertinama gyvūnų nerimo būsena, fizinis aktyvumas, atmintis ir socialumas. Be to, teigiamam mikrobiotos moduliavimui buvo naudojami prebiotikai. Pelių patinai ir patelės 9 mėnesius buvo maitinami daug riebalų turinčiu pašaru ir prebiotikais (FOS+GOS), ištirpintais geriamajame vandenyje. Reguliariai buvo registruojamas gyvūnų svoris ir atliktas gliukozės tolerancijos tyrimas, siekiant patvirtinti pelių diabetą, bei atlikti minėti gyvūnų elgesio testai.

Daug riebalų turintis pašaras jau po dviejų mėnesių žymiai padidino pelių, tiek patinų, tiek patelių, kūno svorį ir pablogino gliukozės toleranciją po 9 mėnesių dietos. Prebiotikų vartojimas sušvelnino neigiamą šio riebaus pašaro poveikį. Pelių nerimas ir ilgalaikė atmintis nepakito, tačiau turėjo didelę įtaką fiziniam jų aktyvumui ir socialumui po 9 mėnesių maitinimo su pašarais, turinčiais daug riebalų. Apibendrinant, šie rezultatai rodo, kad devynių mėnesių neužtenka, kad daug riebalų turinčio pašaro indukuotas diabetas pelėms taip pat skatintų ir Alzheimerio ligos vystymąsi.