Université catholique de Louvain – Louvain Drug Research Institute

Metabolism and Nutrition Research Group

Roles of gut microbes in the gut-to-brain axis controlling hedonic/reward responses to food intake in physiological condition and in the pathology of obesity

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Abstract

Overweight and obesity are now considered as multifactorial pathologies, affecting 39% of the world's population. The dysregulation of food intake in favor of hedonic food intake (based on the pleasure, and linked to the brain reward system) rather than food intake based on the energy needs of the body (homeostatic), has been identified as one of the main cause of body weight gain. Identifying factors contributing to this dysregulation of food intake would limit the obesity epidemic, the prevalence of metabolic disease associated with obesity, and healthcare costs.

Our team and others have broadly described the roles and mechanisms by which the gut microbiota influences hunger and satiety to regulate food intake in function of the energy needs. However, the roles of gut microbes in the hedonic food intake have been poorly investigated so far. The mechanisms behind the dysregulations of hedonic food intake associated with obesity are still unknown.

The aim of this PhD work is to investigate the roles of the gut microbiota as a potential therapeutic target to tackle dysregulations of the hedonic/reward system in the context of obesity.

We aim to know if the gut microbiota is involved in the hedonic responses to food intake in physiological and pathological conditions (obesity). Our objective is to prove the causal role of the gut microbiota in reward dysregulations associated with obesity. After that, we investigated the mediators of the gut-brain axis influencing the reward responses to food intake. Finally, we studied the underlying mechanisms of the roles of the gut microbiota in hedonic/reward responses to food intake.

We used the fecal material transplantation model in mice to prove the causal role of the gut microbiota in the reward system on a behavioral and neuronal points of view. Thanks to metabolomics studies, we were able to identify a gut-derived metabolite, the 3-(3-hydroxyphenyl)propionate as a potential mediator in the gut-brain axis influencing the reward system. Moreover, we have demonstrated that the modulation of the gut

microbiota following the administration of *Akkermansia muciniphila* allows to restore the reward alterations in obese rodents, potentially through a decrease in neuro-inflammation.

In conclusion, the therapeutic approach based on the modulation of the gut microbiota and in particular, the administration of beneficial bacteria would represent a promising strategy to treat the food intake alterations linked to the pleasure.

Our results demonstrate that the gut microbiota represents an important therapeutic target in the treatment of food intake dysregulations associated with obesity.

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I. Introduction

1. Obesity

1.1. Definition and epidemiology

The World Health Organization (WHO) defines overweight and obesity as an abnormal or excessive fat accumulation representing a risk for health. Body mass index (BMI) is a relevant measure of overweight and obesity as defined by the WHO and the most common tool used in clinical practice for adults in this context. It is based on the ratio between the weight (kg) and the height (m^2). Between 25 and 30 kg/m^2, the subject is considered as overweight, and above 30kg/m^2 as obese (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight[1]). Epidemiological studies showed that an increased BMI represents the major risk for the development of several non-communicable diseases such as cardiovascular diseases (the first cause of death in the world), musculoskeletal disorders, type 2 diabetes and some types of cancers [1]. Although difficult to measure in clinical practice, the repartition of fat should also be taken into consideration in the definition of obesity, as visceral fat is more associated with metabolic risk than subcutaneous fat [2]. The metabolic risk is often referred to as metabolic syndrome, a multifactor disease characterized by several cardiovascular and type II diabetes risk factors, including obesity (increased waist circumference), hypertension, glucose intolerance (increased blood glucose levels and fasting glycaemia) and dyslipidemia (increased fasting triglyceridemia and reduced levels of high-density lipoprotein cholesterol (HDL-C)) [3].

The worldwide prevalence of obesity has almost tripled these last 40 years. The latest report from the WHO is alarming: in 2016, 39% of adults were overweight and 13% were obese worldwide (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight[1]). If the trend continues, half of the world's population will be overweight by 2030. Pathological fat mass accumulation does not only occur in adults, since more than 124 million children and adolescents (6% of girls and 8% of boys) were obese in 2016 [4].

[1] Consulted on the 11th of April, 2022

The prevalence of overweight in Belgium is comparable to the European average in men (54% in Belgium versus 58% in average in Europe) and in women (44% versus 45%), but higher than global percentages cited above. Half of the Belgian population is overweight and 21% is obese (https://www.healthybelgium.be/en/health-status/determinants-of-health/weight-status[1]).

Prevention of obesity is key to reduce its prevalence worldwide. At global and national levels, significant expanses are invested in prevention campaigns, inducing considerable costs, in addition to the healthcare costs (2% and 7% of all health-care expenditures in developed countries) and expenditures linked to treatments of comorbidities (https://www.mckinsey.com/~/media/mckinsey/business%20functions/economic%20studies%20temp/our%20insights/how%20the%20world%20could%20better%20fight%20obesity/mgi_overcoming_obesity_full_report.ashx[1]).

Beyond the WHO definition, based on fat mass and risk factor, obesity is now considered as a complex physiopathological state itself. It has been broadly associated with number of metabolic disorders including insulin resistance, fatty liver disease, low-grade inflammation and high blood pressure [5]. More recently, psychological disorders such as depression, anxiety, low self-esteem and addiction have also been correlated with obese condition [6].

Given the epidemic curve, the economic impacts and health consequences, obesity prevention campaigns do not seem effective in this context. So far, the first intention treatment is lifestyle modifications including low calorie diet and increasing physical activity. However, given the altered biological processes associated with obesity, these interventions are not sufficient to induce considerable weight loss and stabilize it [7]. If obesity is associated with comorbidities or is categorized as severe (BMI > 40kg/m²) a surgical intervention could be suggested. Bariatric surgery is the most effective treatment to lose weight, and improve metabolic disorders (insulin sensitivity, dyslipidemia, blood pressure) [8]. As another complementary approach, in overweight patients with comorbidities or with severe obesity (BMI > 40kg/m²) a pharmacological treatment can be

[1] Consulted on the 11th of April, 2022

considered. There are currently six anti-obesity drugs approved on the market in Europe [7]. They can be categorized following their pharmacological site of action. Cathine is a sympathicomimetic (only for short-term use); orlistat is a pancreatic lipase inhibitor; naltrexone/bupropion are opioid receptor antagonist and dopamine/noradrenaline reuptake inhibitor; liraglutide is a glucagon-like peptide-1 receptor (GLP1R) agonist. Another GLP1R agonist, semaglutide has been approved in June 2021 by the Food and Drug Administration as anti-obesity drug in the United States [9]. The majority of anti-obesity drugs targets the central nervous system to impact food intake regulation, potentially inducing addiction and major side effects such as depression. For example, rimonabant, molecule with anorexigenic effect targeting the endocannabinoid receptor 1 (CB1), had to be retracted from the market due to increased suicide ideas and depression [10]. Besides side effects, anti-obesity medications show only moderate weight loss as compared to bariatric surgery: 3-7% after 6-12 months versus 28% over a year [7].

Therefore, focusing on the food intake regulation in the central nervous system by a more physiological approach, avoiding adverse effects, would be innovative and could be very helpful in the treatment of obesity. In this thesis work, we will address this question using the modulation of the gut microbiota.

1.2. Potential causes

The etiology of obesity is a complex interplay between internal and external environment factors contributing to a positive energy balance (Figure 1). In most cases, preclinical studies only take into account internal factors, from the inner environment, in opposition to the outer environment and the society (Figure 1). This probably explains why promising results about drug-therapies of obesity fail to induce substantial and stable body weight loss in clinical studies [7]. The most commonly evoked environmental factors responsible for the prevalence of global obesity is increased access to energy-dense food together with reduced physical activity [11]. A particular eating behavior promoting the intake palatable diet (rich in fat and sugar) independently of energy needs of the body, appear to be involved in the physiopathology of obesity and is often referred to as “food addiction” [12].

Questionnaire-based studies have shown that food addiction was highly associated with obesity since present in 25-37% of obese subjects and up to 60% in severe obese patients [13].

Besides food addiction, it exits a clear link between obesity and depression. Following meta-analysis data, obese adults have 23–36% increased odds of developing depressed mood as compared with non-obese controls [5]. The atypical subtype of depression associated with obesity is characterized by anhedonia (decreased experienced pleasure), hyperphagia, lethargy and hypersomnia. As anti-depressant drug can promote weight gain, the vicious circle of obesity is even more fed in these patients.

Among internal factors, genetic heritage also called heritability, contributes to 40-70% to the BMI [14]. In most common cases, several genes participate to body weight gain: this is polygenic obesity or common obesity [15]. Some monogenic mutations inducing severe and early fat mass gain due to chromosomal deletion or single-gene defects, also contribute to obesity but represent minor cases in the prevalence of obesity. For example, the mutation in the receptor of melanocortin 4 (MC4R) is only present in 1 to 2% of obese adults [16].

Importantly, the gut microbiota has emerged as a key player influencing several factors contributing to obesity: inflammation, adipose tissue expansion, insulin resistance, psychiatric disease, addiction, neurocircuits of appetite and satiety regulation, brown adipose tissue, and more recently the reward system [17; 18].

Of note, body weight gain could be promoted by using certain drugs such as anti-epileptic and psychotropic drugs [19].

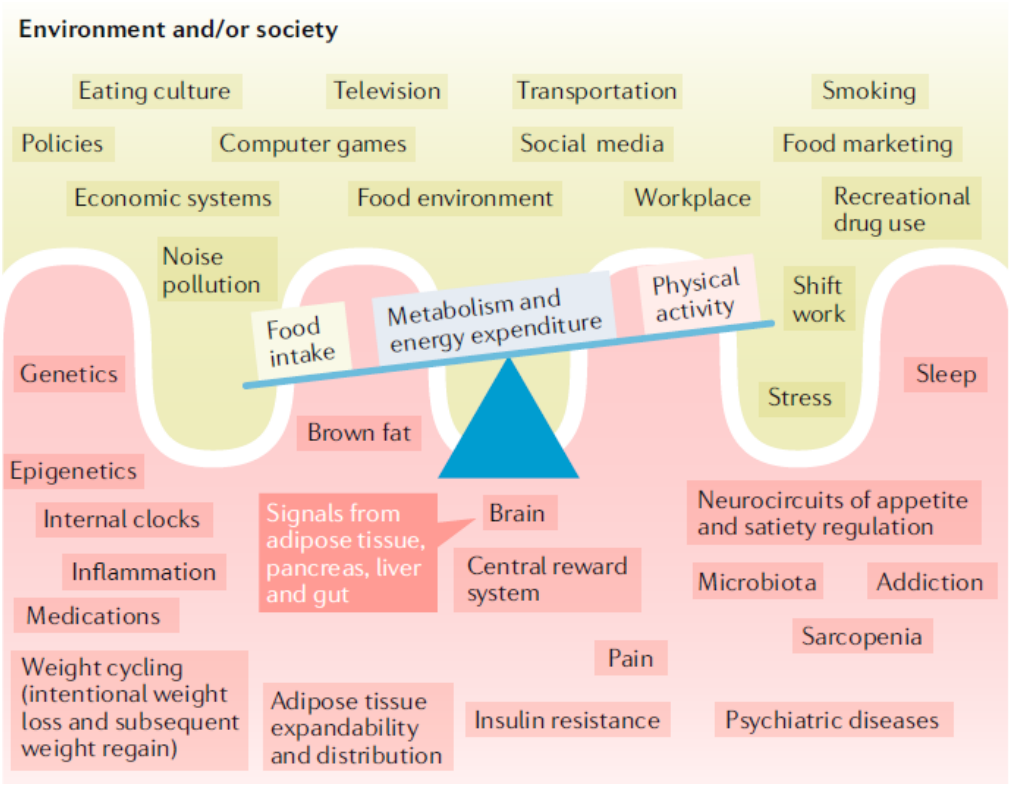

Figure 1 : Complex inner and outer environments contributing to obesity [20].

2. Food intake

[Adapted from the review de Wouters d'Oplinter et al., submitted in Frontiers in Neurosciences]

A fine-tune regulation of energy intake is crucial for a person's survival since undernutrition as well as overnutrition are deleterious for human health. Inappropriate food behavior is one of the main drivers for fat mass development leading to obesity, therefore it is of utmost importance to understand the mechanisms behind the regulation of food intake.

Food intake is regulated according to energy expenditure to maintain a stable body weight over time through a process called homeostatic regulation of food intake [21]. However, food intake is also regulated by a non-homeostatic system, namely the reward system. This system induces an eating behavior for pleasure rather than for energy needs and stimulates the intake of high nutritional value food, rich in sugar and/or fat, known as "palatable food" [22; 23]. Even if some dysregulations of the homeostatic system of food intake have been reported during obesity and are still an important field of research, there is now a very strong current of thought that a major cause of the increase in food intake associated with the rise of obesity resides in the hedonic rather than the homeostatic system. Indeed, in the present context of the increased availability, accessibility and affordability of energy-dense foods also referred as the obesogenic environment, homeostatic system can be overridden by the hedonic regulation of food intake [24]. That said, the investigation of the homeostatic regulation of food intake is still important to consider.

2.1. Homeostatic regulation (hypothalamic)

In physiology

The complex and dynamic processes involved in the homeostatic regulation of food intake are coordinated by the central nervous system (CNS) in accordance with information coming from peripheral organs - including the gut [25]. Based on these peripheral signals relayed to the brain, food intake, appetite and satiety are mainly integrated at the level of hypothalamic neuronal circuits [26]. Neurons within the hypothalamus play critical roles in

the homeostatic regulation of energy and body weight by adjusting energy intake to energy expenditure in response to biological and environmental cues.

The homeostatic feeding is mainly orchestrated in the arcuate nucleus of the hypothalamus (Arc) by different types of neuron populations: the co-expressing Agouti-related protein (AgRP) and neuropeptide Y (NPY) neurons and the co-expressing pro-opiomelanocortin (POMC) and cocaine-amphetamine-related transcript (CART) neurons, with orexigenic and anorexigenic functions, respectively (Figure 2) [27]. Importantly these populations of neurons interact together by inhibiting each other. Upon activation, POMC neurons release α-melanocyte-stimulating hormone (α-MSH) that binds to MC4R in the paraventricular nucleus (PVN) and leads to decreased food intake. On the opposite, AgRP/NPY activation in the Arc, stimulates the release of AgRP and NPY, inhibiting MC4R-expressing neurons in the PVN. Overall, the PVN is characterized by neurons expressing the single minded 1 transcription factor (SIM1) [21]. The Arc and PVN are located at a strategic position in the hypothalamus along the third ventricle, a brain region devoted to the blood-brain barrier and thereby accessible for peripheral signals. Arc neurons senses metabolic signals from the periphery since it express receptors for circulating molecules, produced by peripheral organs such as peptide YY (PYY), ghrelin, leptin and glucagon-like peptide-1 (GLP-1) [28]. The Arc communicates with the brainstem, which also collect information from the periphery. The vagal afferent fibers end in nodose ganglion, that transfers the information to the nucleus tractus solitarus (NTS), which also expresses receptors for peripheral signals (cholecystokinin (CCK), GLP-1).

The main peripheral actors described in the homeostatic regulation of food intake are insulin, leptin and ghrelin. Insulin is produced and secreted by the pancreas into the circulation in response to postprandial increase in blood glucose and it readily reaches tissues throughout the body, including the brain. Even if the main effects of insulin in the periphery is to induce glucose uptake by the tissues, in the brain, the majority of glucose transport into neurons occurred independently of insulin. In the brain, insulin inhibits AgRP/NPY neurons and activates POMC neurons in the Arc thereby inducing anorexigenic effects (Figure 2) [8; 29; 30].

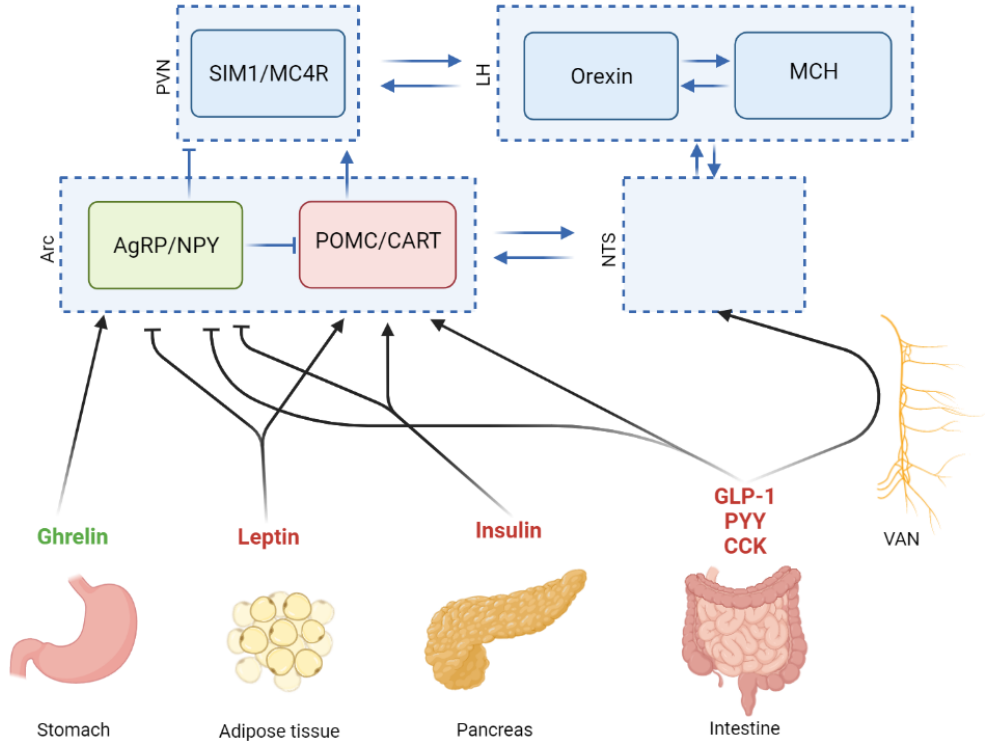

Figure 2: Homeostatic regulation of food intake according to peripheric signals. PVN: paraventricular nucleus; SIM1: single-minded family BHLH transcription factor 1; MC4R: receptor of melanocortin 4; LH: lateral hypothalamus; MCH: melanin-concentrating hormone; Arc: arcuate nucleus; AgRP: agouti-related protein; NPY: neuropeptide Y; POMC: pro-opiomelanocortin; CART: cocaine-amphetamine-related transcript; NTS: nucleus tractus solitarius; GLP-1: glucagon-like peptide-1; PYY: peptide YY; CCK: cholecystokinin; VAN: vagal afferent fibers.

The idea that fat mass signals are involved in the hypothalamic control of food intake has already been proposed in 1953 [31]. Later, this signal from the fat mass has been identified with the discovery of leptin in 1994 [32]. Leptin is produced by adipocytes in a proportional quantity to the fat mass [33] and is released in the circulation thereby reaching the brain. Like insulin, leptin in the brain inhibits AgRP/NPY neurons and activates POMC neurons in the Arc inducing anorexigenic effects (Figure 2) [34; 35; 36; 37].

On the opposite, the main hormone involved in orexigenic effects is ghrelin. Ghrelin is synthesized by the stomach during fasting and hypoglycaemia and increases the synthesis of AgRP and NPY in the Arc, inducing its orexigenic effects (Figure 2) [38; 39; 40]. Ghrelin

also inhibits POMC neuronal activity, however since ghrelin receptor expression has not been reported in POMC neurons, this inhibitory effect may be mediated by the activation of NPY/AgRP neurons interacting and inhibiting POMC neurons [41; 42].

Besides the stomach other parts of the intestine also synthesize peptides targeting the hypothalamus to regulate food intake. Enteroendocrine cells (EECs) located all along the intestine, from the duodenum to the colon, are involved in the synthesis and release of gastro-intestinal peptides some of which are involved in the homeostatic regulation of food intake.

CCK is produced by CCK cells, which are EECs located all along the intestine with the majority of them being in the duodenum [43; 44]. The CCK is produced in response to nutrients in the intestinal lumen, mainly lipids and protein and is inducing anorexigenic effect by the activation of POMC neurons through the vagus nerve [45; 46; 47; 48].

The glucose-dependent insulinotropic peptide (GIP) is synthesized within and released from intestine, mainly in the duodenum and proximal jejunum [49]. GIP is secreted in response to nutrient ingestion, especially glucose or fat and its effect on food regulation are mainly mediated by GLP-1 [50]. GLP-1 is another anorexigenic peptide produced by EECs (L-cells) mainly found in the ileum and distal colon, with fewer cells in the proximal gut [51]. GLP-1 production and secretion is induced in response to the presence of nutrients in the intestine either by a direct or indirect effect mediated by the GIP [50]. Even if the activation of central GLP1R plays a role in mediating food intake effect of GLP-1, since the short half-life of GLP-1, it is likely that endogenous gut-derived GLP-1 suppresses food intake by acting through the vagus nerve [52; 53]. Both central and peripheral GLP-1 agonists are able to inhibit NPY and AgRP neurons and activate POMC neurons thereby inducing its anorexigenic effects [54; 55; 56]. PYY is an additional anorexigenic peptide mainly secreted in ileum and large intestine EECs in response to fat ingestion [57; 58]. PYY induced its anorexigenic effects through the inhibition of NPY/AgRP neurons in the hypothalamus (Figure 2) [59].

Importantly, the different hormones and brain structures do not act separately but interact to influence food intake [60]. The next section (3.2) describes how reward-related structures influence food intake. The connections between the homeostatic and non-homeostatic regulations of food intake are essential to maintain a stable weight and mainly occur at the level of the lateral hypothalamus (LH) (Figure 2). The LH is composed of 2 neuronal populations: one expressing melanin-concentrating hormone (MCH) and one expressing orexins. MCH neurons project to the nucleus accumbens (Nac), whereas orexin neurons project to the ventral tegmental area (VTA), two brain areas involved in the reward system [61].

In physiopathology (obesity)

Over the past 25 years, sequencing studies have led to the discovery of several genes involved in homeostatic regulation of food intake whose disruption causes morbid obesity in humans. The detailed study of people with these monogenic obesity syndromes has provided unique insights into the role these neural circuits play in modulating human appetite and body weight [62]. To date, almost all of these genes encode proteins involved in the leptin-melanocortin pathway [63]. However, mutations that disrupt the production or bioactivity of leptin or in the gene encoding the leptin receptor are very rare and contribute to only 1% to 5% of severe obesity in children [64; 65; 66]. Therefore, other mechanisms are involved in food intake dysregulations during obesity.

In fact, hyperleptinemia and resistance to reducing body mass are two characteristics of typical obesity. Indeed, leptin is overexpressed at the gene level in the adipose tissue of individuals with obesity and strong positive associations exist between plasma leptin levels and body fat percentage [33; 67]. Therefore, several studies point towards leptin resistance instead of loss of leptin during obesity [68].

Similar observations are made for insulin since it is becoming increasingly apparent that neurons in the brain also become resistant to insulin; however, the relative contributions of central insulin resistance to the development of obesity and T2D remain poorly understood [29; 69]. In conclusion, any disruption in the production and or action of these

mediators involved in homeostatic regulation of food intake can lead to overconsumption and obesity.

2.2. Non-homeostatic regulation (mesocorticolimbic dopaminergic system)

In physiology

As mentioned earlier, food intake is also influenced by non-homeostatic signals, inducing food intake for pleasure. The hedonic intake of food depends mainly on taste, odor, texture and appearance [70; 71; 72]. The reward system was essential for our hunter-gatherer ancestors to favor a good energy storage in order to increase the survival rate. However, today in most western countries, humans are in omnipresence of palatable food and do not have to face a period of famine. Therefore, in these conditions, the reward system can lead to overeating and positive energy balance and induce obesity [73; 74].

In 1954, Olds and Milner were the first to identify rewarding sites in rat brains [75]. Few years after this discovery, the catecholaminergic system has been highlighted to be implicated in the processes of reward [76; 77; 78]. The mesocorticolimbic pathway includes dopaminergic neurons located in the VTA and their axons projects to the striatum, including the Nac and to the prefrontal cortex (PFC) (Figure 3) [79]. Other structures have been proved to be implicated in reward processes including the amygdala and the hippocampus [79; 80; 81].

Dopamine seems to be the major and common driver of the food and drug reward in the mesocorticolimbic pathway [81; 82; 83; 84]. It has already been demonstrated in 1989 that blocking the dopaminergic pathway inhibits the response for food during an operant task [85]. In response to rewarding stimuli, such as palatable food, dopamine is released in the mesocorticolimbic pathway promoting reward behaviors. In physiological state, dopamine is synthetized in two steps: first tyrosine is used by a rate-limiting enzyme, the tyrosine hydroxylase (TH) to produce 3,4-dihydroxyphenylalanine (DOPA), then the aromatic amino acid decarboxylase dopamine produces dopamine. Dopamine is stored in vesicles, before being released in the synaptic space. Eighty percent of the dopamine is recaptured by the

dopamine transporter (DAT), whereas the rest is degraded into 3-methoxytyramine (3-MT) and 3,4-dihydroxyphenylacetic acid (DOPAC). Both are transformed into homovanillic acid (HVA).

In addition to dopaminergic neurons, GABAergic and glutamatergic neurons are present in the VTA and involved in the reward processes - either by modulating the activity of dopamine neurons or independently of dopamine by sending projections to the brain structures innervated by VTA dopamine neurons [86]. Opioid signaling is also involved in the reward system since opioid receptors and their endogenous ligands are expressed in several area of the brain, including in the VTA, Nac and PFC [87]. Bioactive lipids such as endocannabinoids, are involved in both homeostatic and rewarding mechanisms of food intake. Endocannabinoids, endocannabinoid receptors including CB1 receptors, and synthesizing enzymes are all present in the mesolimbic system and, in particular, in the Nac and VTA [88]. Of note, the endocannabinoid system is further detailed later in this introduction.

The reward system encodes for the three components of food intake: the wanting, the liking and the learning. The dopaminergic system seems to control mostly the wanting component while GABAergic and opioid systems seem to be also implicated in the liking component [17; 89].

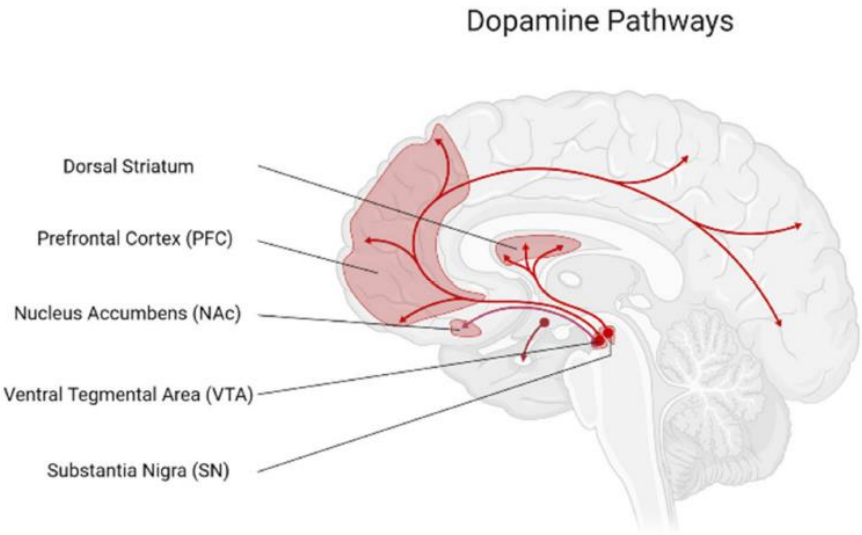

Figure 3: Dopaminergic projections in mesocorticolimbic areas of the brain.[90]

- ***Wanting***

The wanting component is related to the motivation to obtain a reward, and is the predominant behavior during the first phase of the food intake, the appetitive phase (Figure 4). This component induces an effort to acquire a specific nutrient in order to access to the rewarding value of it. A neuronal activation in the reward-related areas is observed in humans in response to visual cues of high caloric food compared to control food [91]. This component present dynamic fluctuations depending on the physiological state, when hungry, the "wanting" is significantly amplified [92].

In humans, the motivational component can be evaluated with analysis through a work to access rewarding food, for example through computer game [93]. While in rodents, the wanting component is often assessed with an operant conditioning test. The mouse must press on a lever to obtain a palatable food or other rewarding nutrients. The more the mouse is motivated, the more it will press on the lever. A principle of progressive ratio is often used to really assess the motivation with an incremental number of lever presses asked for each additional reward delivered, in contrast to the fixed ratio which delivers one reward after one lever press [94].

- ***Liking***

The liking component refers to the hedonic part of the reward system; it is related to the pleasure felt by eating a specific food, prominent during the consummatory phase (Figure 4). Hedonic word derives from the ancient Greek *hedonikós*, meaning "pleasurable". Before consumption of food, the hedonic component is neutral and become active only after ingestion [89; 95].

In human adults, a subjective self-report is used to report the rate how much they like (or dislike) a stimulus [96]. In animals and in young humans, orofacial reaction can be analyzed after an oral administration of substance to assess the liking component [96]. This hedonic component is essential to discriminate control (neutral) from palatable food and induces a preference for the intake of this latter: it helps to make a choice between tastes. The liking component can be assessed with a food preference test. The preference is analyzed using two or more food samples free of access presented to animals. In humans, lists of ranked food are often asked [73].

- ***Learning***

An attraction to certain tastes like sweet or fat exists thanks to a conditioning process. It is a learning process making the link between associated stimuli and the pleasure felt post-ingestion via a Pavlovian conditioning. This component is the last to occur in the food reward response (after the ingestion), and will strongly influence the wanting, depending on the type of liking experienced (Figure 4). In his experiment, Pavlov rang a bell every time he served food to his dog. Then one day, Pavlov rang the bell without giving food at its dog. However, this one was already drooling. It was the discovery of the learning process [97]. The brain anticipates the rewarding value of a specific food and encourages its absorption. This component is essential to make the link between stimuli and long-term effects on health. This component is stable upon physiological state (hungry or not) and also helps to make food choices [98]. For example, the gastro-intestinal consequences of a food intake will impact the future intake of this type of food.

With rodents, the learning component can be assessed with a conditioned place preference (CPP). The system is made of two different easily recognizable compartments characterized by different walls and grounds. To know the preferred compartment in baseline, the time spent by the mouse in each compartment is recorded during a pre-test with full access to each compartment. During trainings, the mouse is restrained in one compartment with the associated treatment. The less preferred compartment is used for rewarding trainings, the mouse can have access to palatable food or receive injection to certain drugs, whereas the most preferred compartment is used as control. After several days of trainings, the test ends with a session of free access to each compartment and no treatment. The aim of this test is to reverse the preference for one compartment thanks to rewards received during trainings [94]. This test is often used with pharmacological treatment but not yet well developed to assess the food reward learning.

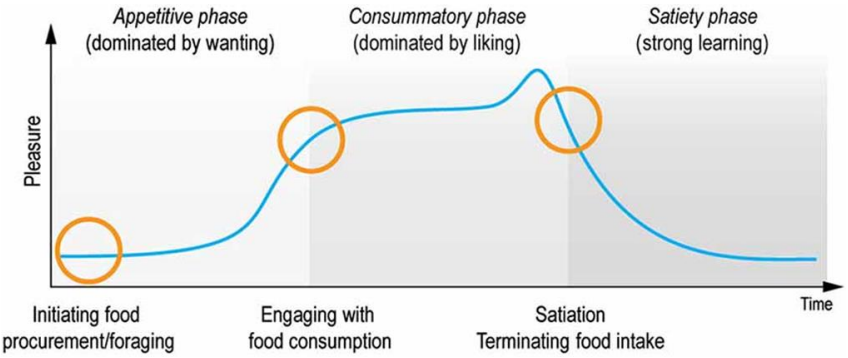

Figure 4: The three components of the food reward system over time : the wanting, the liking and the learning [99].

Even if each component seems to activate distinct circuits in the reward system, it is mostly impossible to really separate them during food behaviors analysis. In the literature, there is still controversy about this discrimination [100; 101; 102]. For example, the Leeds Food Preference Questionnaire permits an analysis of an explicit wanting and an implicit liking in humans. For the wanting component, participants are asking to answer to “which food do you most want to eat now?” between two food pictures [103; 104]. Studies using pharmacological manipulations of the dopaminergic system tried to dissect the effect on the different components of the reward system. The injection of amphetamines in the

systemic circulation or locally in the nucleus accumbens raises extracellular levels of dopamine and amplifies the wanting without modifying the liking component, suggesting a key role of the dopamine transmission through its receptors in the reward conditioning. To go further, dopamine receptors 1 and 2 (DRD1 and DRD2) seem to modulate different aspects of the wanting. Indeed, only specific D1 inhibition prevented the acquisition of a conditioned reinforcing value [105]. Optogenetic study also showed different effects of D1 and D2 striatal neuronal populations in the conditioning. The stimulation of D1 receptor-expressing neurons promotes place-based self-stimulation *i.e.* reinforcement, whereas the stimulation of D2 receptor expressing neurons promotes avoidance (punishment) [106].

In physiopathology (obesity)

In 2001, Wang et *al.* were the first to show a dysregulation of the dopaminergic reward system in obese humans [107]. Several studies have shown that long-term overeating in both rodents and humans induces a decrease in the dopamine released, in the dopamine turnover (ratio between DOPAC and dopamine), in the expression of dopamine receptors 1 and 2 (DRD1 and DRD2) and an increase in the expression DAT (Figure 5) [108; 109; 110; 111; 112] (for more information see Reviews [22; 113]).

In the context of obesity, altered food reward behaviors have been demonstrated in preclinical models. Studies have proven that rats under high-fat diet pressed less on the lever during the operant conditioning test to obtain a food reward compared to lean rats, suggesting an alteration of the wanting component. During the conditioned place preference, these obese rats spent less time in the compartment associated with palatable food than lean mice. Therefore, the learning component seems also to be dysregulated in the context of obesity [110; 114]. Furthermore, several studies demonstrated that obese mice present a decrease in the preference for palatable food compared to lean mice, suggesting an alteration of the liking component in the context of obesity [115; 116].

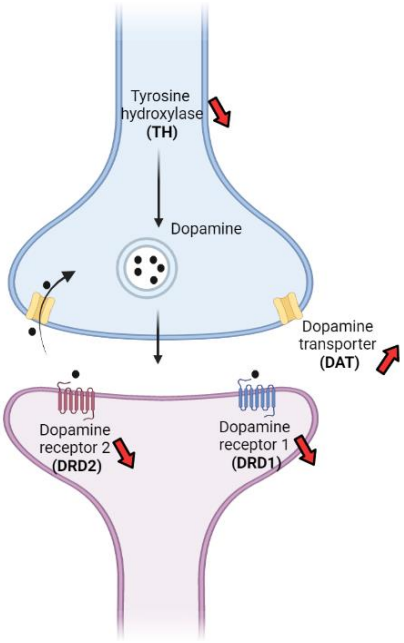

Figure 5: Dopaminergic synapse in obese condition. Red arrows represent the change in mRNA expressions observed after long-term energy dense diet [113].

Stice *et al*. have proved that obese humans show a decrease of the striatal activation in response to food reward consumption compared to lean humans [109; 117]. These altered food reward behaviors are often called food addiction, because of neuronal and behavioral similarities with drug addiction, as well as neuronal adaptations. After the stimulation with repeated palatable stimuli, the individuals need to increase the size of the portion of food ingested to obtain the same pleasure, for the same food reward stimulation, obese individuals will show less striatal activation. However, the scientific community is not yet unanimously agreed on the term food addiction [74; 81; 118].

One human condition associated with altered reward process is particularly interesting to study to understand the underlying biological mechanisms. The TaqIA A1 allele polymorphism affects 30-40% of the population and is associated with 30-40% decrease in the DRD2 abundance in the striatum [119]. These neuronal changes correlate with addiction and compulsive behavior [107; 120]. One study showed that this genetic susceptibility was present in 67% of overweight or obese subjects included in the study [121].

Importantly, even if the homeostatic and non-homeostatic systems are described separately, these systems work in concert to regulate food intake. For instance, Figlewicz et *al.* have demonstrated that dopaminergic neurons in the VTA expresses insulin and leptin receptors and several studies have shown their implications in the food reward [122]. Indeed, mice with a specific leptin receptor long-term RNA mediated knockdown in the VTA present an increase in the palatable food intake [123]. Therefore, it is now suggested that these systems need to be simultaneously investigated instead of separately.

3. Gut-brain axis mechanisms regulating food intake

[Adapted from the review de Wouters d'Oplinter et al., submitted in Frontiers in Neurosciences]

The gut-brain axis is a complex bi-directional communication system connecting the gastrointestinal (GI) tract and the CNS. This connection allows the brain to be informed among other components of the energy status in the periphery. The CNS sends then feedbacks to maintain energy homeostasis [124]. Two pathways are involved in this communication: the vagal and the systemic pathways (Figure 6).

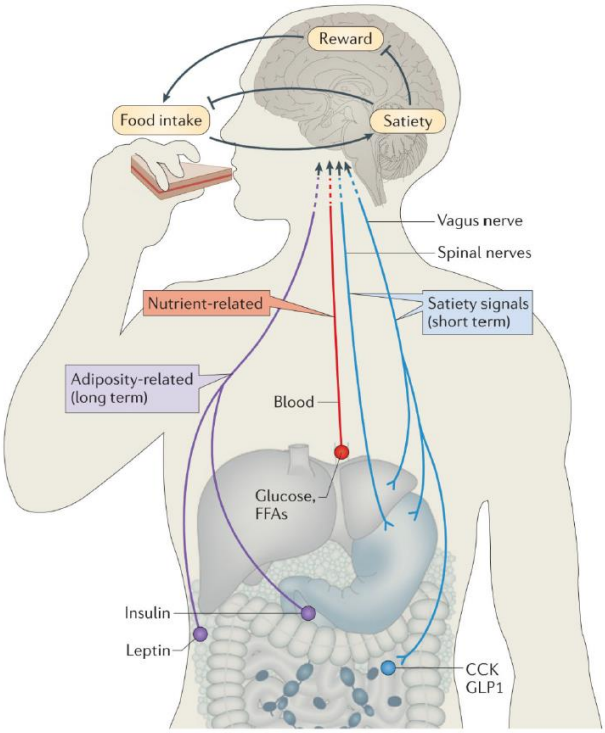

Figure 6: Homeostatic and non-homeostatic regulations of food intake [21]. FFA: free fatty acids; CCK: cholecystokinin; GLP: glucagon-like peptide-1.

3.1. Nervous pathway

The intestine (often seen as our second brain) is the largest place where intestinal neurons are following in number those from the CNS. The gastrointestinal tract is innervated by a lot

of vagal afferent nerves that connect to the CNS. These connections maintain the brain informed about the energy status from the periphery. The vagal afferences come from the intestine and have their cell bodies in the nodose ganglia and project to the NTS in the brainstem. The NTS contains projections to several regions of the brain including the hypothalamus [125; 126]. Vagal afferents detect mobility or distension and express a vast variety of gut hormones receptors such as GLP-1, CCK, PYY or ghrelin receptors [125; 127]. It has been proven that specific stimuli from the gut activate distinct population of vagal afferents [128; 129].

Few years ago, functional synapses between EECs and vagal afferents nerves have been discovered. Basolateral cytoplasmic elongations of EECs, called neuropods, connects directly to the vagal pathway permitting a fast and precise signal to the brain, through the nodose ganglia (Figure 7) [130; 131].

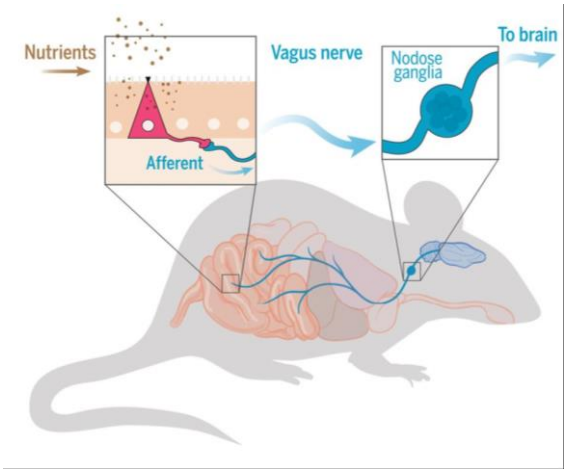

Figure 7: A gut-brain neuroepithelial circuit [131].

The key role of the vagus nerve in the gut-brain axis regulating the non-homeostatic food intake was shown by Han *et al.* [132]. For the first time, the authors demonstrated using optogenetics tools a functional pathway linking the gut to the brain reward system, involving the vagus nerve, the right nodose ganglion, the NTS and the dorsal striatum [132].

3.2. Systemic pathway

Besides neurons, the gut also contains various types of cells. The gut is one the largest compartment where the immune system is developed (*e.g.* the gut-associated lymphoid tissue (GALT) and immune infiltrating cells). Moreover, although the intestinal EECs are representing only 1% of the total intestinal epithelial cells, the gut constitutes the biggest endocrine organ [133]. Thirty hormones have been identified in the gastrointestinal (GI) tract and therefore represents an incredible reservoir of peptides acting at distance from the gut and on different organs. EECs are present all along the GI tract and form an important endocrine organ implicated in the regulation of food intake as described in the previous paragraph. In a postprandial state, EECs secrete GLP-1, CCK and PYY all of them acting as anorexigenic hormones [134]. In contrast, ghrelin is released in the stomach during fasting condition and act as an orexigenic hormone [135]. These hormones can be released in the blood or activates the vagal afferents nerves, thereby targeting the brain.

4. Gut microbiota

By influencing the production of signals of the systemic and vagal pathways in the gut-brain axis, the gut microbiota is a key mediator in that cross talk. This section will describe how the gut microbiota acts to communicate with the brain and influence both homeostatic and non-homeostatic regulations of food intake.

The microbiome includes microorganisms (bacteria, archaea, viruses, yeast and fungi) that colonize our body, their genomes and metabolites. The gut microbiota refers to the microbiome in the gut, where the highest colonization occurs as compared to other body parts, reaching a total abundance of 10^{14} bacteria in the human colon [136]. With 10 million microbial genes (150-fold higher than the human genome), it is not surprising that the metabolic capacity of the gut microbiota is revealed as so broad and powerful [137]. For several years, microbiota studies were based on culturing bacteria and measuring the relative abundance after 16S ribosomal RNA sequencing. Today, the development of molecular biology techniques and bioinformatics tools allows describing not only the composition, but also the functionality of the gut microbiota. Such tools confirmed the relevance to use the mouse model to study the gut microbiota on a translational approach for human, as its gut microbiota functionality is similar to the human one [138].

To characterize gut microbiota compositions, α- and β-diversity are often measured, giving a broader picture about the microbial ecology. α-diversity index describes the diversity within a sample, whereas β-diversity evaluates similarities or dissimilarities of two communities of samples [139].

4.1. In physiology and physiopathology (obesity)

4.1.1.The development of a microbiome

As an ecosystem, the gut microbiota composition varies over time and upon various factors, always in symbiosis with the host. Some key determinants in early life will shape the gut microbiota, such as the delivery mode (cesarean *vs* vaginal), breastfeeding, and early antibiotic exposure [140]. After 3 years of life, the global gut ecology is considered as stable,

led by three predominant phyla (Firmicutes, Bacteroidetes, Actinobacteria) and other less abundant (Tenericutes, Proteobacteria, Verrucomicrobia) [141]. The gut microbiota composition will still depends on environmental factors, mostly the diet and antibiotic exposure [142; 143].

Ingested food is in direct contact with the host gut microbiota, in this way, certain macronutrients directly influence its composition. For example, the consumption of a diet rich in fat and sugar alters rapidly the gut microbiota in a dose-response way [144]. Non-digested nutrients are utilized by some bacteria as metabolic substrates, mostly in the lower part of the intestine. Since some bacteria take advantage of this nutrient intake, it leads to correlations between their abundance and a specific diet intake [145]. It is thus not surprising that in many metabolic diseases associated with a dysregulated food behavior (but not only), including obesity, the gut microbiota composition is altered: the microbial diversity and the microbial richness (number of genes) are decreased [146; 147; 148]. At the phylum level, some studies have suggested a decrease in the ratio between Firmicutes and Bacteroidetes in obese condition [149; 150]. However, this biomarker of altered microbiota is not consistent between studies because the ratio varies upon methodological differences and the consideration of life-style associated factors [151].

4.1.2.Dysbiosis

An altered composition of the gut microbiota compromises its functionalities, that are essential for the host. This concept is often referred to as a dysbiosis. Dysbiosis is not only reported during metabolic disorders and obesity, but is also associated with other various pathologies (Figure 8). In the context of this thesis, it is important to note that the gut microbiota is also affected in neurological diseases associated with dopaminergic deficits (autism, Parkinson disease, depression, anxiety) [18].

Among the diseases listed in Figure 8, the gut microbiota has been causally linked only in a few, including obesity. Its causal role has been proven using models of mice depleted in gut microbiota: germ-free (axenic) or antibiotic-treated mice. Then, fecal material from donors harboring a gut microbiota of interest, is transferred into recipient mice without any gut

microbes, so that the causal link could be suggested. A pioneer study demonstrating a causal role of gut microbes in energy metabolism showed in 2004 that germ-free mice developed less adiposity than conventional mice despite higher food intake [152]. After colonization with gut microbes, these ex-germ-free mice reversed the trend and showed greater adiposity. The same observations were found in mice treated with antibiotics: they are protected against diet-induced obesity and inflammation [153].

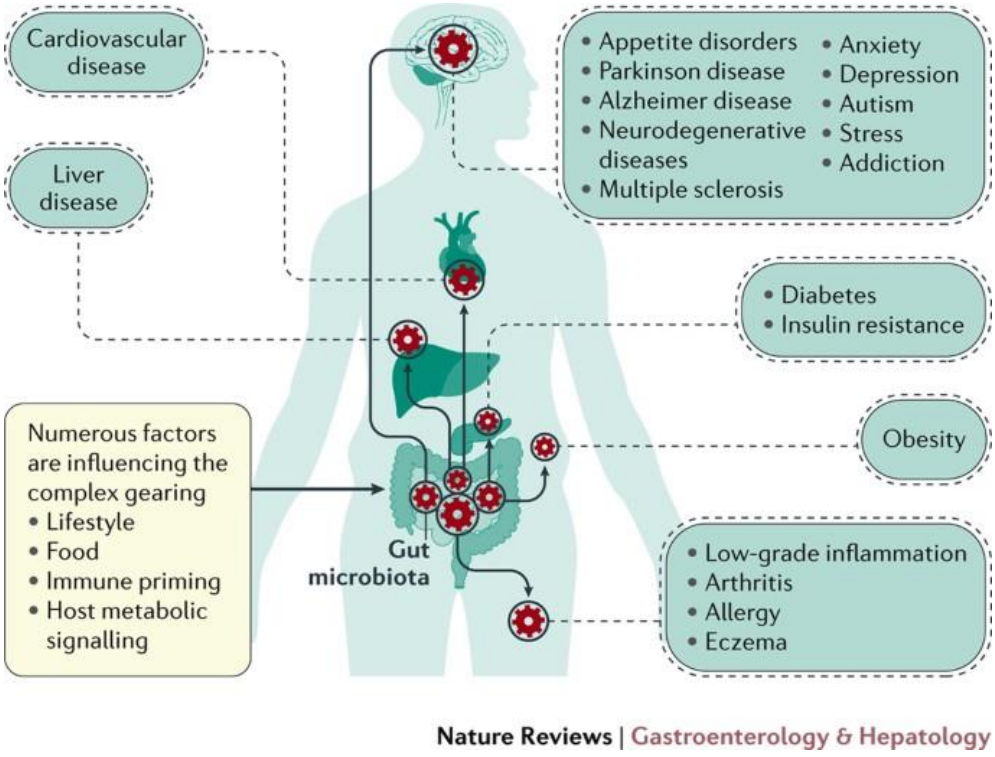

Figure 8 : Pathologies with disturbed gut microbiota composition and function [18].

4.1.3.Mechanisms of interaction with the host

Short chain fatty acids (SCFAs)

The gut microbiota not only uses fibers (*i.e.* non-digested ingredients) as energy source, but also produce metabolites able to interact with the host. By anaerobic fermentation, some bacteria produce SCFAs (butyrate, propionate and acetate) able to interact with the host to

influence its metabolism, including the regulation of food intake (further detailed in this thesis), glucose and lipid homeostasis, inflammation, immunity and cancer [136].

Several studies have pointed out associations between decreased abundance of butyrate-producing bacteria and the development of metabolic disorders [154]. These bacteria include *Roseburia*, *Faecalibacterium prausnitzii* and *Dysosmobacter welbionis* [155; 156]. On the opposite, the beneficial effects of prebiotics, *i.e.* substrates selectively utilized by host microorganisms conferring a health benefit to the host, are mostly due to an increased SCFA production [157]. SCFAs bind to intestinal G-protein-coupled receptor (GPR) 41 and 43 to impact the host health and have systemic effects after their absorption through the portal vein [158]. Labelled SCFAs are quantifiable in the cerebro-spinal fluid (CSF), therefore, it is suggested that SCFAs could pass the blood-brain-barrier through their transporter, the monocarboxylate transporter (MCT), to influence the brain and behavior [159; 160].

Dietary compounds with prebiotic properties are broad and include fermentable carbohydrates, human milk oligosaccharides, fructo-oligosaccharides (FOS), galacto-oligosaccharides and inulin [157]. The supplementations in polymers of fructose (FOS, inulin) have been described in rodents and humans to attenuate metabolic disturbance associated with obesity: decrease in fat mass gain, improvement of glucose and lipid homeostasis as well as inflammation [161; 162; 163; 164; 165; 166].

During carbohydrate fermentation, intermediate products of SCFAs metabolism such as succinate, fumarate and lactate can also affect the host, but their effects are more controversial than SCFAs benefits [158]. Some studies have associated succinate with beneficial effects on the host in the context of obesity showing reduced body weight, improved plasma glucose and fatty liver through intestinal neoglucogenesis [167]; while another study in overweight adults positively correlates succinate and fumarate with body weight [168].

Endocannabinoids

The gut microbiota can also influence the endocannabinoid tone, a broad family of bioactive lipids, involved in inflammation, energy homeostasis, gut barrier function and food intake [169; 170; 171; 172; 173]. The most characterized molecules of this system are anandamide (AEA), 2-arachidonoylglycerol (2-AG) binding to CB1 and cannabinoid receptor 2 (CB2). However, the activity of the endocannabinoid system is not limited to CB1 and CB2, as endocannabinoids can also activate the transcription factor peroxisome proliferator-activated receptor α (PPARα), GPR55 and transient receptor potential cation channel V1 (TRPV1) [174]. Other molecules structurally similar to endocannabinoids, are synthetized and degraded by the same enzymes but binds different receptors than CB1 and CB2. They include other *N*-acylethanolamines (*N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA), *N*-linoleylethanolamine (LEA)) and acylglycerols (2-palmitoylglycerol (2-PG) and 2-oleoylglycerol (2-OG)). The endocannabinoid system appears as a key mediator at the intersection between the gut microbiota and the host. Indeed, even if it does not represent the major production pathway, the gut microbiota is able to produce some precursors of endocannabinoids, and to influence the endocannabinoid production by the host [169; 176]. In turn, the host modifies the gut microbiota composition through ethanolamines production [169; 175]. Since an alteration in the endocannabinoid tone is associated with obesity and could participate to the physiopathology, the gut microbiota represents a key player in the crosstalk between the gut, peripheral organs and the brain through the endocannabinoid system.

Vitamins

The symbiosis between the gut microbiota and the host is essential as the gut microbes generates a broad catalogue of molecules, influencing different systems of the host. For example, it synthetizes vitamins that the host is not able to produce itself : vitamins K, B9, B12… [175].

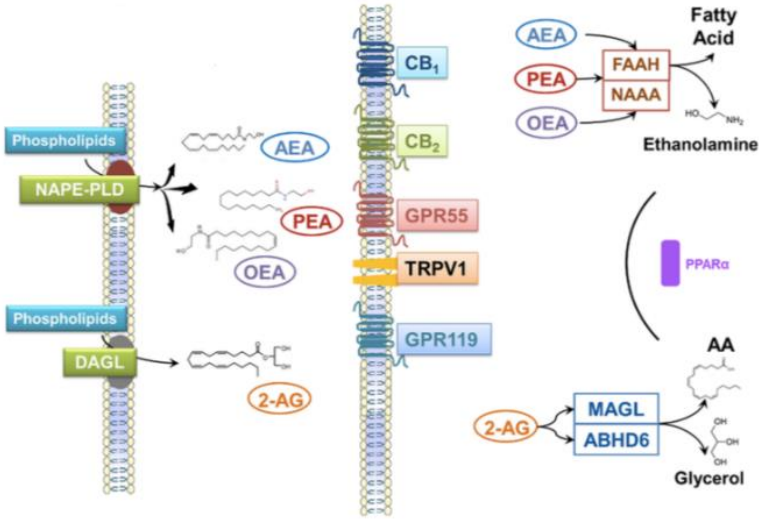

Figure 9 : The endocannabinoid system, adapted from Cani et al., 2014 [176]. AEA, PEA and OEA are synthetized on demand from membrane phospholipids by the N-acylphosphatidylethanolamine phospholipase D (NAPE-PLD). 2-AG is produced the same way by diacylglycerol lipase (DAGL). AEA, PEA and OEA are degraded by fatty acid amide hydrolase (FAAH) and N-acylethanolamine-hydrolyzing acid amidase (NAAA) into ethanolamine and fatty acids. 2-AG is degraded by two hydrolases: monoacylglycerol lipase (MAGL) and the α/β hydrolase domain 6 (ABHD6) into glycerol and arachidonic acid (AA).

Neurotransmitters

Some bacteria have adequate enzymes to release neurotransmitters (serotonin, γ-aminobutyric acid (GABA), glutamate, dopamine and noradrenaline) locally to the host [177]. For example, bacteria expressing β-glucuronidase produce dopamine and noradrenaline in the gut [178]. The impact of neurotransmitters produced in the gut on the brain is still not clear. Some evidence show that the administration of specific bacteria is able to modulate neurotransmitters concentrations in the brain. After 4 weeks of supplementation in *Lacticaseibacillus rhamnosus*, the concentrations in GABA and glutamine in the brain are significantly increased in mice [179]. The administration of *Bifidobacterium infantis* also modifies brain metabolites, but will be further discussed in paragraph 4.2.2. [180].

Secondary bile acids

Some gut microbes also use bile acids, essential to lipid absorption, as substrates to produce the secondary bile acids, including deoxycholic acid (DCA) and lithocholic acid (LCA).

Secondary bile acids bind farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) to influence thermogenesis, energy expenditure [143].

Lastly, the gut microbiota uses branched-chain amino acids as substrates [143]. Dysbiosis associated with obesity blunts the production of the metabolites described above and have deleterious effects on the host.

Gut barrier function

Besides the ability to produce various compounds, the gut microbiota exerts another essential function to the host: it participates to the gut barrier integrity. The gut barrier consists in several lines of protection between the intestinal lumen and the blood circulation, to prevent the entry of pathological compounds (allergens, pollutants, viruses, parasites) or bacteria. The mucus layer, anti-microbial peptides secreted by epithelial and goblet cells, epithelial cells expressing tight junction proteins (zonula occludens-1 (ZO-1), occludin and claudin), plus immune cells make the barrier effective (Figure 10). The gut microbiota plays a key role in the integrity of the gut barrier as it is able to regulate each factor involved in gut barrier function [181].

Obesity is characterized by an increased gut barrier permeability together with changes in gut microbiota composition. Animal and human studies have shown a decreased expression and function of tight-junction proteins, reduced mucus thickness and anti-microbial peptides production, together with modifications of intestinal immune cells (Figure 10) [153; 182; 183]. These alterations facilitate the translocation of bacterial components such as the lipopolysaccharide (LPS) into the blood. Indeed, obese subjects have 2- to 3-fold increased concentration of LPS in their blood compared to lean subjects; this phenomenon is defined as metabolic endotoxemia [153; 184].

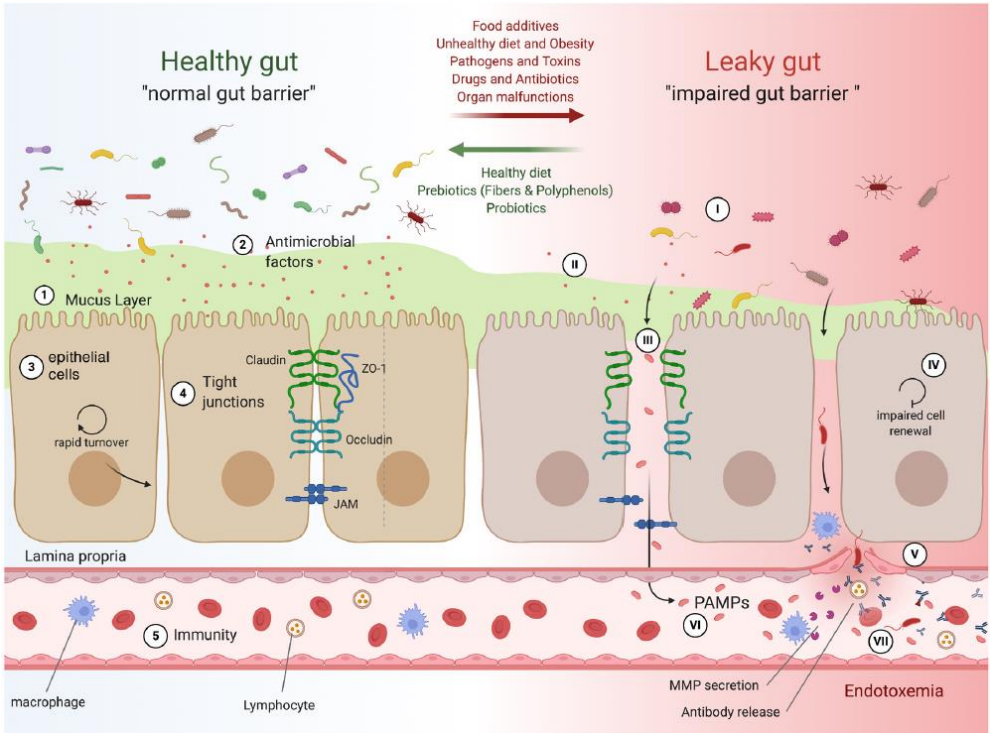

Figure 10: The gut barrier function in health and disease [181]. JAM, junctional adhesion molecule; MMP, matrix metalloproteinase.

LPS is a component of cell wall of gram-negative bacteria, inducing inflammation through the activation of innate immunity. LPS is a pathogen-associated molecular patterns (PAMPs), binding to toll-like receptors (TLR). This signaling pathway leads to the activation of the transcriptional factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), inducing the expression of pro-inflammatory cytokines (tumor necrosis factor-α (TNFα), interleukine-6 (IL-6), interleukine-1β (IL-1β)…) [153; 184]. In turn, inflammation weakens even more the gut barrier by altering the expression of tight junction proteins by pro-inflammatory cytokines TNFα and IL-1β [185].

LPS is a key inducer of inflammation and can reach host blood thanks to two pathways of blood circulation penetration. On the one hand, it could translocate through the intestinal epithelium due to an increased gut permeability; on the other hand, it could be integrated into chylomicrons during the digestion of dietary lipids [186]. In this last case, lipids induce

post-prandial inflammation on a more physiological way [187]. That said, in the context of obesity, low-grade inflammation is chronic, and present even at distance from a meal [153]. Importantly, some saturated lipids also bind TLR4, inducing the same signaling pathway as LPS [188].

It has been shown that high-fat diet-induced obesity is associated with inflammation in the brain, including brain areas involved in feeding behavior [189; 190; 191]. Pro-inflammatory cytokines and LPS pass the blood-brain barrier, and can activate the NF-κB pathway centrally (Figure 11). Moreover, inflammation in the brain is characterized by activation of the microglia and astrocytes, and a disruption of the blood-brain barrier, allowing immune cells infiltration [191; 192].

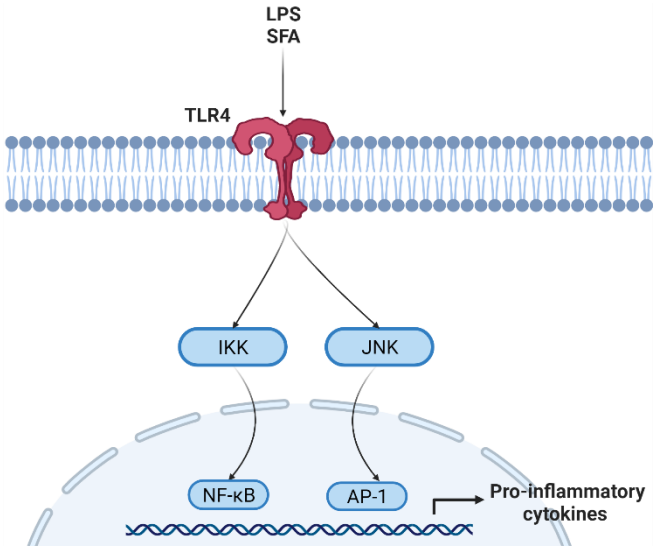

Figure 11: TLR4-mediated pro-inflammatory pathway. SFA: Saturated fatty acid; IKK : IκB kinase; JNK : c-Jun N-terminal kinases.

4.2. Gut microbiota and the regulation of food intake

[Adapted from the review de Wouters d'Oplinter et al., submitted in Frontiers in Neurosciences]

The gut microbiota has emerged as a key player into the gut-brain axis through the vagal and humoral pathways to modulate host metabolism and food intake [143; 193; 194]. Several tools have been used to modulate the gut microbiota composition such as the

supplementation in prebiotics or oral administration of beneficial bacteria, and some approaches have been shown to impact host food intake. Moreover, some gut microbiota components and metabolites have also been described to be involved in the homeostatic regulation of food intake.

4.2.1.Gut microbiota and homeostatic regulation of food intake

GLP-1 and PYY

Gut microbes are able to modulate GLP-1 and PYY levels in the circulation of the host thereby affecting food intake. To the best of our knowledge, the earliest demonstration describing specific changes in EEC physiology and linked to gut microbes came from experiments using germ-free (GF) animals. In a first study published in 1989, it was shown that the circulating levels of "enteroglucagon" and PYY were much higher in animals raised without microbes compared to conventionally raised animals [195]. Surprisingly, in the proximal small intestine, protein levels of key peptide the GLP-1 and PYY were found to be significantly lower in GF mice [196]. In the same study, the density of the EEC was lower in the ileum while higher in the colon of GF mice.

Wichmann *et al.* aimed at deciphering how the gut microbiota could influence the GLP-1 producing cells and confirmed the increased expression of proglucagon gene, increased GLP-1-positive cell number in the colon and higher circulating GLP-1 in GF mice versus conventionalized mice [197]. Interestingly, SCFAs are involved in the dialogue between gut microbes and EEC [197]. Indeed, monocolonization of the intestine of GF mice with *Escherichia coli* (which does not produce SCFAs) does not affect proglucagon expression or L cell number, while using a known SCFAs producer such as *Bacteroides thetaiotaomicron* is significantly affecting these parameters.

More than 20 years ago, our laboratory discovered in either genetically or diet-induced obesity rodents that treating them with a diet enriched with prebiotics such as inulin or oligofructose (*i.e.* inulin-type fructans) improves metabolic parameters together with decreasing food intake [198; 199; 200; 201; 202]. Seeking for the molecular mechanism that

explains the lower food intake and improved glucose tolerance, the improved phenotype was associated with higher GLP-1 content and proglucagon mRNA expression in the intestinal segments and higher levels of GLP-1 in the portal vein blood [199; 200; 201]. In the same line, deleting the production of PYY is linked with obesity and hyperphagia in mice [203], whereas prebiotic treatment is associated with increased level of PYY in the circulation [204].

Later, our laboratory discovered that these effects were accompanied by an increased number of EEC L-cells [205; 206]. Of note, these properties were not limited to inulin-type fructans since resistant starches and arabinoxylans also fermentable and modifying the gut microbiota, decreased food intake, fat mass and body weight gain, together with increased plasma GLP-1 and PYY levels [207; 208]. Similarly, the microbial fermentation of these other fiber compounds led to the production of SCFAs.

SCFAs

On a mechanistic point of view, there are several effectors linking SCFAs and the secretion of gut peptides from the EEC. GPR-41 and GPR-43 are expressed on EEC L-cells and are directly activated by the SCFAs produced by the gut microbiota and eventually promote the secretion of gut peptides such as GLP-1 and PYY [136]. Of note, mice lacking these receptors display altered secretion of GLP-1 and PYY after exposure to SCFAs or specific prebiotics [158; 209; 210].

There is a great therapeutic interest in modulating the production of SCFAs by using the gut microbiota. Indeed, targeting the endogenous production of GLP-1 and PYY is highly attractive since the levels of these peptides are decreased in obese and diabetic individuals [211; 212; 213; 214; 215; 216]. Indeed, in overweight adults, the supplementation in inulin-propionate ester (delivering specifically propionate in the colon) not only increased PYY and GLP-1 plasma concentrations, but decreased also significantly energy intake, leading to weight loss [217].

However, SCFAs themselves are also beneficial in the context of food intake regulation. It has been shown that gut-derived acetate is able to reach the brain and impact food behavior suppressing appetite [160].

Endocannabinoids

Endocannabinoids and related bioactive lipids are other mediators involved in this crosstalk between gut microbes and host food intake as previously discussed. Among those metabolites, some of them are strong agonists of GPR119. Importantly, activating GPR119 present on the L-cells triggers GLP-1 secretion and eventually may regulate food intake, glucose and energy metabolism [218; 219]. More recently, by using in GF mice, an extensive analysis of the endocannabinoidome (eCBome) which comprises numerous bioactive lipid families biochemically related to endocannabinoids, their receptors, and metabolic enzymes, our laboratory discovered that the gut microbiota plays an important role on the regulation of this system and this on numerous organs and targets including the EEC and their products [220].

The eCBome is widely distributed in various tissues and organs including the hypothalamus and different areas of the brain but also in the liver, the gut, and the adipose tissues. Therefore, the eCBome is now viewed as a key target to modulate numerous physiological functions such as the regulation of food intake but also glucose/lipid metabolism and inflammation among other pathways. Hence, understanding the link between the eCBome and more specifically the role of the gut microbiota on this system constitutes an important target.

LPS

As previously mentioned, when the gut barrier function is altered, it facilitates LPS translocation into the circulation, metabolic endotoxemia and the induction of pro-inflammatory pathways mainly through TLR4 binding. However, in a healthy context, implying an effective gut barrier, LPS has been demonstrated as a beneficial bacterial compound: it is able to stimulate the secretion of GLP-1 from EEC through TLR4 [90].

Caseinolytic peptidase B (ClpB)

Some bacterial components are also able to enter the blood circulation to reach the brain. It has been described for ClpB, a chaperone protein from *Escherichia coli*, that mimics anorexigenic hormone α-MSH and binds to its receptor MC4R [221]. This protein has been associated with eating disorders (anorexia nervosa, bulimia, binge-eating disorders) and obesity in human populations [221].

4.2.2.Gut microbiota and non-homeostatic regulation of food intake

As described above, the regulation of non-homeostatic food intake is mainly driven by dopaminergic neurons at the level of the mesocorticolimbic brain areas. Interestingly, animal models depleted in gut microbiota have demonstrated the key role of gut microbes in the dopaminergic transmission. In one study, GF mice showed increased synthetizing dopamine turnover (ratio DOPAC/dopamine) in the striatum as compared to conventional specific pathogen free (SPF) mice [222]. Another study showed that GF rats have lower dopamine degradation turnover rate (ratio HVA/dopamine) in the striatum as compared to SPF mice [223]. These results were further confirmed with another approach of gut microbiota depletion: mice treated with antibiotics [224]. These pioneering studies opened the field for the investigation of the role of gut microbes in many dopaminergic-related disease including Parkinson disease, addictions or hedonic dysregulations associated with obesity [225].

The impacts of the alterations of the dopaminergic system in absence of gut microbiota on a food intake and more precisely on behavior were then demonstrated. Indeed, GF mice show increased hedonic intake (increased preference for lipid solution and increased intake of highly concentrated sucrose solution) compared to control mice [196; 226]. However, the roles of gut microbes on other components of the food reward system (*i. e.* wanting and learning) still need to be demonstrated and in more physiologic model as well as the mechanisms involved in this crosstalk.

Amino acids metabolism

The gut microbiota modulates the production of neurotransmitters involved in the reward system including serotonin and dopamine from key amino acids precursors such as tryptophan, phenylalanine and tyrosine [177]. For instance, it has been shown that gut microbes increase colonic expression of tryptophan hydroxylase 1, the rate limiting enzyme synthetizing serotonin from tryptophan [227].

Moreover, amino acids themselves can influence the gut microbiota composition and the reward system through the gut-brain axis. Indeed, tryptophan has not only homeostatic anorexigenic properties (shown on the hypothalamus, in hungry conditions), but has also inhibiting effects on palatable food intake. In fact, intra gastric infusion of tryptophan reduced palatable intake in satiated mice and was associated with decreased cFOS expression in the Nac shell and core [228].

Recently, a large-scale clinical study strengthened the key associations between fecal amino acids, the gut microbiota and the reward system in the context of obesity [229]. After confirming the alterations of the reward system in obesity at the level of the Nac, the authors characterized the obese gut microbiota by a higher *Prevotella/Bacteroides* ratio. They further linked this ratio with Nac alterations and fecal tryptophan, with significant interactions. In this model, subjects with higher BMI, would have higher *Prevotella/Bacteroides* ratio and decreased fecal tryptophan. Whether if the link between reduced tryptophan and alterations of the reward system is mediated by modulations of neurotransmitters remains to be demonstrated. Another human study showed a negative association between bacterial gene functionality coding for the enzyme synthetizing phenylalanine, the precursor of dopamine, and the ventral striatal functional magnetic resonance imaging (MRI) response during reward anticipation [230]. These results suggest a potential role of gut microbes in neurotransmitters production in the intestine and their impacts on the brain. However so far, these were only correlations, the causal role of neurotransmitters modulated by the gut microbiota on the reward system remains to be demonstrated.

Vagus nerve

Reward-signaling pathways from the gut to the brain have been highlighted thanks to the development of optogenetic technologies and vagotomy protocols. In 2018, Han *et al.* demonstrated that one key mechanism by which the gut communicates with the brain and influence the food reward system is the vagus nerve, and uses glutamate as neurotransmitter [231]. So far, it is not clear if the gut microbiota uses this pathway to influence the food reward system. However, several evidence have been raised in favor of that hypothesis, since vagal afferent nerves (VAN) expressed receptors for gut-derived compounds such as SCFAs or gut peptides (GLP-1) influencing homeostatic and hedonic food intakes [128; 232].

GLP-1

Acetate, butyrate and propionate have been broadly described as beneficial gut-derived metabolites in the context of obesity for their impact on food intake through the release of satiety gut peptides GLP-1 and PYY [136]. Importantly, homeostatic and non-homeostatic systems can interact as GLP1R are also expressed in reward-related areas including the VTA and the Nac [233; 234]. Moreover, micro-injections of exendin-4, a GLP-1 analogue, in the Nac core and in the VTA reduces sucrose and fat intakes, whereas the antagonist of GLP1R, exendin-9 has opposite effects [233]. The same pharmacological approach demonstrated that GLP1R activation was able to reverse the CPP-induced behavior and operant conditioning for sucrose reward [235], potentially acting through an inhibition of dopaminergic firing and signaling in the Nac [236; 237]. Therefore, one may suggest that modulation of GLP-1 by gut microbes could also impact the reward system. Of note, GLP-1 is also produced locally in the brain, from preproglucagon neurons [238]. The implication of gut-derived GLP-1 from brain-derived GLP-1 on the reward system is not yet clearly established and the roles of gut microbes in the regulation of brain-derived GLP-1 remain to be investigated.

SCFAs

In the context of the reward system, effects of SCFAs can also be observed independently of GLP1 and PYY modulations. Indeed, a small clinical study involving healthy men showed that the administration of inulin-propionate ester, increasing selectively colonic propionate, was associated with reduces anticipatory reward responses in the human striatum to high-energy foods [239]. These results were not linked with changes in plasma PYY or GLP-1 suggesting an effect of propionate on the food reward system independent of GLP-1 and PYY. In another context of reward alterations, *i.e.* the chronic unpredictable mild stress (CUMS), the supplementation in propionate intra-rectally, restore the sucrose preference, together with the concentration of dopamine in the PFC of rats [240]. Conversely, another study investigating CUMS showed an increase in sucrose preference after inducing stress and a decrease in sucrose preference upon SCFAs administration [241]. It is worth noting that in this study, SCFAs administration did not change SCFAs levels in the feces.

Endocannabinoids

Endocannabinoids have been described as important neurotransmitters involved in the food reward system mainly through the activation of CB1 and CB2 in the brain and in the gut, and by mediating fat preference [242] [243]. The vagus nerve implication in the effects of endocannabinoids on the reward-related processes has been demonstrated in this context. Indeed, a recent study showed that blocking peripheral CB1 decreased dopaminergic neurons activation and palatable food intake though the vagus nerve [244]. The vagal pathway is also token by other bioactive lipids related to the endocannabinoid such as OEA. This a gut-derived endocannabinoid was identified as an intestinal lipid sensor, to increase striatal dopamine release, and decrease high-fat intake in favor of low-fat solution intake [245; 246].

More studies need to investigate respective effects of other endocannabinoids on hedonic food intake. One study in binge-eating subjects showed that plasma levels of AEA was significantly elevated after eating palatable food without changes in 2-AG levels [247]; while another one in rodents showed that 2-AG was elevated after binge-eating episode [244].

Interestingly since gut microbes are able to modulate endocannabinoids and related lipids they could represent potential mediators between gut microbes and food reward. Indeed, GF mice exhibited altered brain and intestinal eCBome (*i.e.* bioactive lipids and their receptors), while these effects are reversed after fecal material transplantation (FMT) [220]. It is clear that endocannabinoids signal through the vagus nerve to influence the reward system and that gut microbes are able to modulate endocannabinoids and related lipids both in the intestine and the gut [220; 248]. However, the direct role of gut microbes through eCBome on the food reward system need to be demonstrated.

Inflammation

Among the mechanisms underlying food reward alterations, inflammation has emerged as a promising pathway [249]. Activation of pro-inflammatory pathways in reward-related areas (Nac) has been described after high-fat feeding, together with impaired motivation for sucrose rewards during an operant conditioning task [250]. For example, silencing NF-κB pathway reverses the compulsive sucrose seeking [190]. Alterations in the gut microbiota composition has been identified to mediate inflammation through the systemic circulation and the brain, notably through the disruption of the gut and blood-brain barrier integrity [153; 183; 251; 252]. More precisely, in the Nac, the inflammation induced by a high-fat diet (HFD) is reversed after antibiotic treatment and transferable by FMT [253]. The role of the gut microbiota in inflammation-mediated behavioral alterations of the food reward system still needs to be answered.

In a mechanistic point of view, it has been shown that modulation of the gut microbiota by the administration of SCFAs restores microglia (immune cells of the brain) malformation and immaturity of the cortex of GF mice [252]. It has also already been demonstrated that another bacterial metabolite, indole, with anti-inflammatory potential, correlates positively with functional connectivity of the Nac in humans [254; 255]. Furthermore, a derivative from the indole metabolism, the indolepropionic acid was found to be inversely correlated with food addiction score in female [256]. However, the causal role of indole and derivatives on brain reward inflammation and food reward behavior still needs to be demonstrated.

Gut microbiota modulation as therapeutic target for food reward disorders

In the treatment of eating disorders such as binge eating, strongly associated with obesity, only few pharmacological approaches appear to be effective [257]. Considering the gut microbiota interactions with the food reward system previously described the modulation of the gut microbiota seems promising to treat eating disorders. However, so far, only few studies have investigated this approach.

Some interesting associations were found between the use of *Bifidobacterium infantis* and dopamine modulation in reward-related areas of the brain. Its supplementation revealed anti-inflammatory properties in rodents, coupled with increased plasma concentrations of tryptophan and decrease DOPAC in the cortical area of the brain, however its impacts on food reward remains to be demonstrated [180]. Another potential beneficial bacterium in this context is *Bifidobacterium pseudocatenulatum.* Indeed, this bacterium influences hedonic food behavior by reversing HFD-induced reduction in sucrose preference [258]. The same research group also investigated the administration of *Bacteroides uniformis* in a binge eating model of mice. Its supplementation decreases the total caloric intake of the binge episode, but with no difference in hedonic intake (sucrose solution). Besides, *Bacteroides uniformis* was associated with an increased expression of *Drd1* in the PFC [259]. However, the effects of gut microbes on other components of the food reward, as well as the effect of other beneficial microbes and the mechanisms related to these effects still need to be investigated.

The prebiotic approach modifies the gut microbiota composition with beneficial effects on the host. If their effects on homeostatic food intake are consistent, it is not the case in hedonic mechanisms. On the one hand, the administration of FOS to diet-induced obese (DIO) mice decreased their motivation for sucrose pellets as compared to DIO mice, after an overnight fasting, suggesting a worsening of their wanting component of the reward [260]. These behavioral results were not associated with changes in dopaminergic markers expressions in the Nac. On the other hand, another study using prebiotics, has shown that inulin (a long chain fructan) increases sucrose preference and ameliorates gut microbiota composition alterations induced by a HFD [261]. Finally, an oligosaccharide from the human

milk namely the 2-fucosyllactose, has been shown to be potentiates the motivation for food reward through the vagus nerve [262].

In conclusion, these studies support the interest of using gut microbiota-targeted approaches to modulate food reward behavior that is altered during obesity. However, the causal roles of gut microbes in food reward alterations during obesity and related mechanism remain to be addressed.

4.3. Parabacteroides

In the context of obesity, *Parabacteroides* appears as a potential beneficial microbe. From the Bacteroidetes phylum and Tannerellaceae family, *Parabacteroides* is a Gram-negative bacterium, anaerobic, commensal in rodents and humans [263; 264]. Importantly, *Parabacteroides* is a GABA-producer, the main inhibitory neurotransmitter in the brain [265]. Up to now, 15 different species of *Parabacteroides* have been described, including *P. distasonis, P. goldsteinii, P. johnsonii* and *P. merdae.*

Some studies in rodents and humans have found inverse associations between the abundance of *Parabacteroides* and the development of obesity [266; 267; 268; 269]. Importantly, a recent study showed that following a dietary intervention in overweight and obese subjects leading to weight loss, *Akkermansia muciniphila* abundance increased the most, followed by *P. distasonis* [270]. In obese rodent models, the administration of different genus of *Parabacteroides* shows beneficial effects on metabolism. The supplementation with *P. goldsteinii* reverses diet-induced weight gain, endotoxemia, glucose intolerance, intestinal permeability and inflammation [271]. *P. distasonis* also alleviates obesity, hyperglycemia, and hepatic steatosis in diet-induced obese mice, as well as in genetic obese mice (*ob/ob*) [167]. In this study, the mechanisms behind the beneficial effects of the supplementation have been investigated: they imply the production of secondary bile acids and succinate. On the one hand, *P. distasonis* is able to metabolize bile acids into LCA and ursodeoxycholic acid (UDCA), improving the gut barrier and decreasing dyslipidemia associated with obesity. On the other hand, *P. distasonis* increases the concentration in succinate in the gut, an intermediate metabolite of SCFAs, able to decrease

hyperglycemia. If *P. distasonis* appears to be a promising beneficial bacterium to tackle obesity, it is important to note that in other contexts, *Parabacteroides* is not systematically associated with beneficial effects [272].

In brain disorders, the beneficial effects of *Parabacteroides* seem to be dependent of the pathology context. During epilepsy, the beneficial effects of a ketogenic diet (rich in fat and low in carbohydrates) against seizure episodes correlated with the increased abundance of *P. merdae, P. distasonis* and *Akkermansia muciniphila* [273]. Further supplementation in *P. merdae, P. distasonis* and *A. muciniphila* recapitulate the beneficial effects on seizure and was associated with the normalization of GABA and glutamine levels in the hippocampus [273]. However, in the context of learning process, supplementation in *P. distasonis* and *P. johnsonii* in adolescent rats altered memory in a novel recognition object task [274]. These behavioral results were associated with significant changes in gene expressions in the hippocampus (the main brain area regulating memory), including some genes involved in Alzheimer and Parkinson diseases.

Studies describing *Parabacteroides* are emerging these lasts years and are not unanimous about its effects so far. Further studies on the specific role of *Parabacteroides* in the context of obesity, gut-brain axis and food intake are needed. This thesis will participate to answer to some of these questions.

4.4. Akkermansia muciniphila

Like *Parabacteroides, Akkermansia muciniphila* is a Gram-negative bacteria, anaerobic commensal found in rodents and humans, it represents 0.5 to 5% of the total gut microbiota in healthy humans [275; 276; 277]. *Akkermansia* is the only representative of the Verrucomicrobia phylum in the gut [277]. It lives inside the outer mucus layer of the intestine, degrades the mucus and thereby produces metabolites such as SCFAs propionate and acetate [278].

Strong literature in humans and animals supports that the abundance of *A. muciniphila* is inversely related to the severity of many metabolic disorders including obesity [279].

Indeed, this bacterium is almost lacking in obese/diabetic mice and humans, while in rodents the administration of prebiotics restored its abundance [206]. Based on these interesting associations, our team were the first to prove the beneficial effects of *A. muciniphila* in the context of obesity and metabolic disorders.

Our lab discovered that the administration of *A. muciniphila* alive and pasteurized decrease body weight, fat mass gain and metabolic disorders, the pasteurized form being even more effective (Table 1) [170; 183; 280; 281]. *A. muciniphila* supplementation reduced endotoxemia, hepatic lipid accumulation, and improves glucose tolerance and insulin sensitivity [170; 183; 280; 281]. The pasteurized form was further associated with increased whole body energy expenditure and increased fecal caloric content [280; 281]. While trying to understand how the pasteurized form could impact the host, our lab discovered that an outer membrane protein, Amuc_1100, was highly abundant and stable after pasteurization. The supplementation in this protein in DIO mice, recapitulates most of the beneficial effects of *A. muciniphila* pasteurized (Table 1) [280]. Next, our team translated the discoveries from rodents to humans in a small double-blind placebo-controlled clinical study demonstrating that *A. muciniphila* administration was safe and well tolerated in overweight volunteers [282]. Interestingly, this pilot study also provided preliminary data showing that pasteurized *A. muciniphila* slightly decreased body weight and fat mass, reduced inflammation and improved insulin sensitivity, and plasma total cholesterol [282]. Importantly, the supplementation did not modify the overall gut microbiota composition of subjects.

Akkermansia muciniphila alive 2*10^8 CFU	*Akkermansia muciniphila* pasteurized 2*10^8 bacterial cells	Amuc_1100 protein 3µg
Everard, 2013 [183]; Plovier, 2017 [280]	Plovier, 2017 [280]; Depommier, 2020 [281]	Plovier, 2017 [280]
↓ endotoxemia	↓ endotoxemia	↓ endotoxemia
↓ fat mass gain	↓↓ fat mass gain	↓↓ fat mass gain
↓ body weight gain	↓↓ body weight gain	↓↓ body weight gain
↑ glucose tolerance	↑↑ glucose tolerance	↑↑ glucose tolerance
↑ insulin sensitivity	↑↑ insulin sensitivity	↑↑ insulin sensitivity
↓ dyslipidemia	↓ dyslipidemia	↓ dyslipidemia
↓ gut barrier permeability	↓↓ gut barrier permeability	↓↓ gut barrier permeability
	↑ fecal energy content	
	↑ energy expenditure	

Table 1 : Non-exhaustive summary of the effects of Akkermansia muciniphila supplementation in preclinical model of obesity

Mechanistically, it exists several hypotheses linked to the beneficial effects of *A. muciniphila* during obesity and metabolic disorders. *A. muciniphila* can produce propionate and acetate, both acting as agonists for GPR-41, GPR-43, stimulating the release of GLP-1 [275]. The administration of *A. muciniphila* can also increase the levels of specific bioactive lipids related to endocannabinoids (*i.e.* OEA, 2-OG, 2-AG and 1-PG/2-PG) [183; 283]. Some of these lipids, including OEA and 2-OG have been shown to trigger the secretion of GLP-1 by activating GPR-119 [284; 285; 286; 287]. This suggests that *A. muciniphila* exerts beneficial effects on the metabolic health of its host by modulating the production of GLP-1 through different metabolic products. More recently the role of the protein P9 specifically produced by *A. muciniphila* has also been proposed and shown to promote the secretion of GLP-1 by stimulating the L-cells via a different mechanism than GPR-43/41 or GPR119-dependent pathways [288; 289].

In the gut-brain axis, the administration of *A. muciniphila* has already shown interesting effects. Indeed, after replicating the benefits of *A. muciniphila* on the gut barrier function, one study demonstrated that the administration of *A. muciniphila* improved hippocampal alterations induced by a HFD. It reduces hippocampal microgliosis, the expression of proinflammatory cytokines TNF-α, IL-1β and IL-6 and restores neuronal development and

synapse plasticity, thus ameliorates defects in learning and memory through a mechanism TLR4-dependent. The improvement in memory task by *A. muciniphila* was confirmed in mice in which high-fat high-cholesterol diet altered their learning process [290]. In chronic restraint-mice, inducing chronic stress, *A. muciniphila* restores the depressive-like behavior, and the serum corticosterone levels (an hormone upregulated during stress conditions), as well as hippocampal brain-derived neurotrophic factor (BDNF), a marker of brain impairments. The beneficial effects of *A. muciniphila* on depressive-like behaviors and cognitive impairments were further confirmed in a mouse-model of Alzheimer disease [291].

Surprisingly, none of the studies describing beneficial effects of *A. muciniphila* in the context of obesity were related to changes in the food intake. However, *A. muciniphila* could act on multiple pathways involved in the reward system including inflammation, the production of SCFAs, the endocannabinoid tone, making it a good candidate in the regulation of food reward alterations. Only one study found an inverse correlation between the abundance of *A. muciniphila* and the food addiction score in women [256]. Therefore, we will address this potential pathway in this thesis.

II. Objectives

In the last decades, overeating has been suggested as one of the main contributors to fat mass gain and obesity. Foods rich in fat and sugar, are more and more eaten for their hedonic properties (“liking”) related to the release of dopamine. The repetitive association between the intake of palatable food and the pleasure (“learning”) in certain conditions may contribute to increase the incentive salience for this kind of food (“wanting”). When the food intake is driven by hedonics instead of homeostatic signals, this leads to metabolic and psychologic alterations involved in the physiopathology of obesity. Neuronal adaptations in the reward system due to long-term overeating further feed this vicious circle of obesity including weight gain and inappropriate food intake. Caloric restriction is the first therapeutic approach to obesity, but could even worsen the condition, creating frustrations, impulsivity and cravings, leading to binge eating and further weight gain. Pharmacological approaches targeting the food intake regulation are not sufficient to induce sustainable and substantial weight loss. Other therapeutic approaches are needed to tackle the food intake dysregulations associated with obesity.

In this context, the modulation of the gut microbiota represents a promising therapeutic alternative to fight obesity. Indeed, the administration of specific bacteria or the supplementation with food ingredients stimulating the growth of beneficial bacteria, improve metabolic parameters and homeostatic signals regulating food intake. However, studies showing beneficial effects of the gut microbiota on food intake regulation mainly focus on the total energy intake mastered by the hypothalamus, and not the palatable intake involving the reward system. The role of the gut microbiota in the regulation of the reward system on neuronal and behavioral points of view remains poorly understood. Its causal implication in the reward alterations associated with obesity, has never been clearly established.

In this PhD work, we will try to uncover these unmet needs by addressing several research questions, illustrated in Figure : Is the gut microbiota involved in hedonic and reward process to food intake in lean conditions (physiology)? Is the gut microbiota contributing to the reward alterations associated with obesity (in physiopathology)? If yes, by which gut-

brain mechanisms microbes are implicated in such effects? And finally, is the modulation of the gut microbiota composition improving the reward alterations in obese conditions?

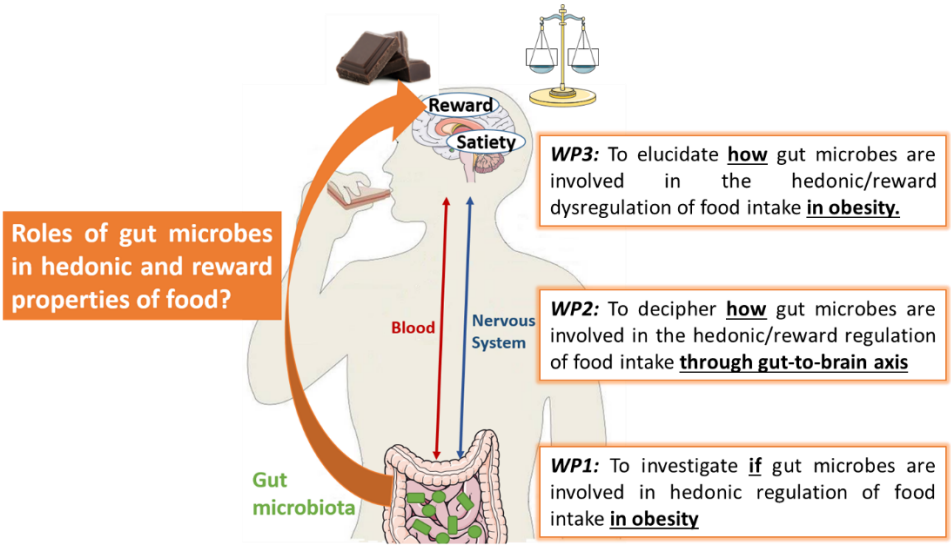

Figure 1: Thesis objectives. Adapted from Morton et al., 2014 [21].

To prove the causal role of the gut microbiota in the food reward system, we used the gold standard method, the FMT. Therefore, we sampled the feces of lean and DIO donor mice and inoculate them in lean recipient mice, previously depleted in gut microbes. We analyzed and compared the food behaviors and the specific markers in reward-related brain areas of donor and recipient mice. In the context of this thesis, we will focus on the three psychosocial components of the food reward proposed by Berridge in 1996: the liking, the wanting and the learning. We will also mostly focus on dopaminergic markers in reward-related brain areas as dopamine is the main neurotransmitter involved in hedonic food intake.

In the first chapter, we will first confirm the hedonic food intake alterations associated with obesity, then we will use FMT to prove the causal role of the gut microbiota in the liking component of the food reward. We will also use correlations to discover some bacteria involved in the hedonic food intake.

The second chapter will focus on the wanting and the learning components of the food reward. We will again confirm the alterations of these components of the food reward in obesity, then use the FMT to prove the causal role of the gut microbiota in these two components. We will go further in the understanding of gut-brain mechanisms by screening metabolites in the plasma and in the cecal contents of donor and recipient mice in order to pinpoint metabolite candidates involved in the gut-brain regulation of food reward.

In chapter three, we will use the approach modulating the gut microbiota composition by the administration of potential beneficial bacteria in DIO mice and assess the effects on the behavior and on reward markers in the brain.

Altogether, this PhD work provides new insights of the influence of the gut microbiota in physiological and pathological process associated with obesity, but also new potential mechanisms by which the gut microbiota communicates with the brain.

III. Results

1. CHAPTER 1 - *Gut microbes participate in food preference alterations during obesity*

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Gut microbes participate in food preference alterations during obesity

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1.1. Abstract

Hypothalamic regulations of food intake are altered during obesity. The dopaminergic mesocorticolimbic system, responsible for the hedonic response to food intake, is also affected. Gut microbes are other key players involved in obesity. Therefore, we investigated whether the gut microbiota plays a causal role in hedonic food intake alterations contributing to obesity. We transferred fecal material from lean or DIO mice into recipient mice and evaluated the hedonic food intake using a food preference test comparing the intake of control and palatable diets (HFHS, high-fat high-sucrose) in donor and recipient mice. Obese mice ate 58% less HFHS during the food preference test ($p < 0.0001$) than the lean donors, suggesting a dysregulation of the hedonic food intake during obesity. Strikingly, the reduction of the pleasure induced by eating in obesity was transferable through gut microbiota transplantation since obese gut microbiota recipient mice exhibited similar reduction in HFHS intake during the food preference test (40% reduction as compared to lean gut microbiota recipient mice, $p < 0.01$). This effect was associated with a consistent trend in modifications of dopaminergic markers expression in the striatum. We also pinpointed a highly positive correlation between HFHS intake and *Parabacteroides* ($p < 0.0001$), which could represent a potential actor involved in hedonic feeding probably through the gut-to-brain axis. We further demonstrated the key roles played by gut microbes in this paradigm since depletion of gut microbiota using broad-spectrum

antibiotics also altered HFHS intake during food preference test in lean mice. In conclusion, we discovered that gut microbes regulate hedonic aspects of food intake. Our data demonstrate that gut microbiota modifications associated with obesity participate in dysregulations of the reward and hedonic components of the food intake. These data provide evidence that gut microbes could be an interesting therapeutic target to tackle hedonic disorders related to obesity.

1.2. Introduction

In the physiopathology of obesity, overeating and consumption of calorie-dense food are major aspects contributing to a positive energy balance (energy input is greater than energy output) and the storage of fat [21]. In this context, the reward system, that drives eating behaviors associated with pleasure, is over-stimulated and becomes the major driver for food intake [21; 73; 111; 292]. Palatable food, rich in fat and sugar, can stimulate dopaminergic neurons and induce a release of dopamine mainly in the cortico-limbic areas of the brain (including the striatum, Nac and PFC) [79; 293; 294]. However, obesity, which is often the result of long-term overeating, is associated with a reduction of dopamine concentration in response to palatable food intake and a downregulation of dopaminergic markers. The expressions of dopamine receptors 1 (DRD1) and 2 (DRD2) are decreased, as well as the rate-limiting synthetizing enzyme (TH), whereas DAT is increased [107; 109; 295; 296; 297]. This hypofunctioning of the dopamine pathway has been suggested to feed the vicious circle of weight gain since it leads to an increase of the meal size of fatty and sweet food in an attempt to feel the same rewarding effect as before the development of obesity [298].

The microbiota plays a key role in the gut-to-brain axis influencing the food behavior. For instance, gut microbes have been shown to modulate the production of satiety hormones (GLP-1 and PYY), neurotransmitters (including GABA, dopamine and serotonin) and to interact with the vagus nerve [193; 299]. Mechanisms underlying the regulation of homeostatic food intake through the gut-to-brain axis are well-studied and supported by strong literature consistencies [21]. In contrast, mechanisms of hedonic food intake have

often been overlooked. It has only been a decade since de Araujo *et al.* demonstrated that post-ingestive signals, different from oral taste perception, were involved in triggering dopamine release in cortico-limbic areas of the brain [300]. These findings strengthened the gut as a key sensor in the regulation of food intake. However, so far, nothing has been shown about the direct role of the gut microbiota on the hedonic food intake. Therefore, we aimed to demonstrate the causal role of the gut microbiota in the hedonic responses to palatable food by using FMT and subsequent appropriate food behavioral tests.

1.3. Results

DIO donor mice show alterations in hedonic eating

First, we exposed 10 donor mice to a low-fat (control, CT) or HFD for 5 weeks to induce a lean or obese phenotype (DIO), respectively. As expected, mice fed with a HFD showed an increase of 12% in body weight (Figure 1a-b) and 230% in fat mass gain (Figure 1c-d) compared to CT-fed mice. Then, in order to study the hedonic component of food intake, we analyzed the pleasure associated with palatable food consumption in these mice.

To assess spontaneous hedonic food intake, the donor mice underwent a food preference test in which they were exposed for the first time to a palatable diet (HFHS). During this food preference test, donor mice were exposed to HFHS and CT for 3 hours during the light phase, and we recorded the consumption of each diet (Figure 1e and Fig. S1). Both lean and obese mice preferred the HFHS diet to CT as they ate more HFHS than CT during the food preference test. However, lean mice showed a faster tropism toward HFHS since they ate significantly more HFHS than CT from the beginning of the test, whereas DIO mice preferred a significantly palatable diet to control diet only after 90 min (Fig. S1). Overall, DIO mice were significantly less attracted to palatable diet, eating 58% less HFHS ($p < 0.0001$) than lean mice over the whole food preference test (Figure 1e).

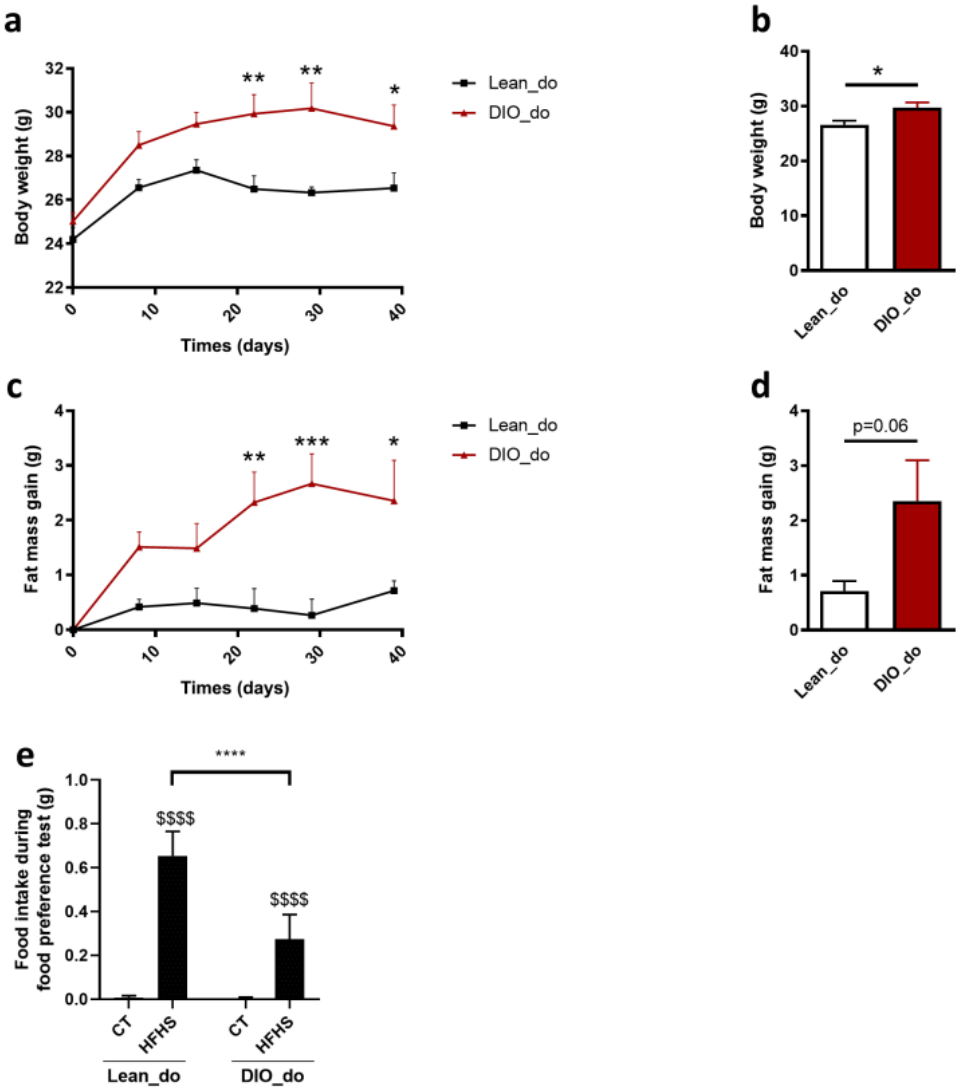


Figure 1. Obese mice present a reduced food preference for HFHS compared to lean mice. (**a,b**) Body weight evolution (g) (**a**) and final body weight (g) (**b**) of lean (Lean_do) and DIO donor mice (DIO_do) after a 5 weeks period. (**c,d**) Fat mass gain evolution (g) (**c**) and final fat mass gain (g) (**d**) of lean (Lean_do) and DIO donor mice (DIO_do). (**e**) Food preference test showing total HFHS and CT intake after 180 minutes of test by lean (Lean_do) and DIO donor mice (DIO_do) after 5 weeks of follow-up. Data are shown as mean ± SEM (n = 5/group). P-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test (**a, c, e**), unpaired Student's t-test (**b,d**). *: p-value ≤0,05 ; **: p-value ≤ 0,01; ***: p-value ≤ 0,001; ****: p-value ≤ 0,0001. : $$$$: p-value≤ 0.0001 between CT vs HFHS intake (**e**). $$$$: p-value ≤ 0.0001 between CT vs HFHS intake (**f**).

Obese gut microbiota transplantation transfers alterations in hedonic eating associated with obesity

To study the causal role of the gut microbiota in obesity-related hedonic eating disorders, we transplanted the gut microbiota from two lean and two obese donor mice into seven and eight recipient mice, respectively. All recipient mice were fed with the same CT diet during the whole experiment (Figure 2a).

Lean and obese gut microbiota recipient mice (Lean_rec and DIO_rec, respectively) did not show any difference in terms of body weight (Figure 2b-c) or fat mass gain (Figure 2d-e). However, DIO gut microbiota recipient mice tended to gain more fat mass over time, with a statistical significance at day 64 (Figure 2d). In order to investigate the energy metabolism of lean and obese gut microbiota recipient mice, we also performed precise measurements of O2 consumption and CO2 production in metabolic chambers. We did not observe any difference between mice receiving an obese or lean gut microbiota (Fig. S2). These results suggest that donor mice did not transfer their obese phenotype to recipient mice in terms of fat mass and body weight after fecal transplantation. It is worth noting that the transfer of the obese phenotype after FMT has been debated in the literature [301].

Interestingly, during the entire follow-up, lean and obese gut microbiota recipient mice had a similar intake of control diet (Fig. S3a-b). However, during their first exposure to palatable food (*i.e.* food preference test), we revealed differences in HFHS intake (Figure 2f and Fig. S3c).

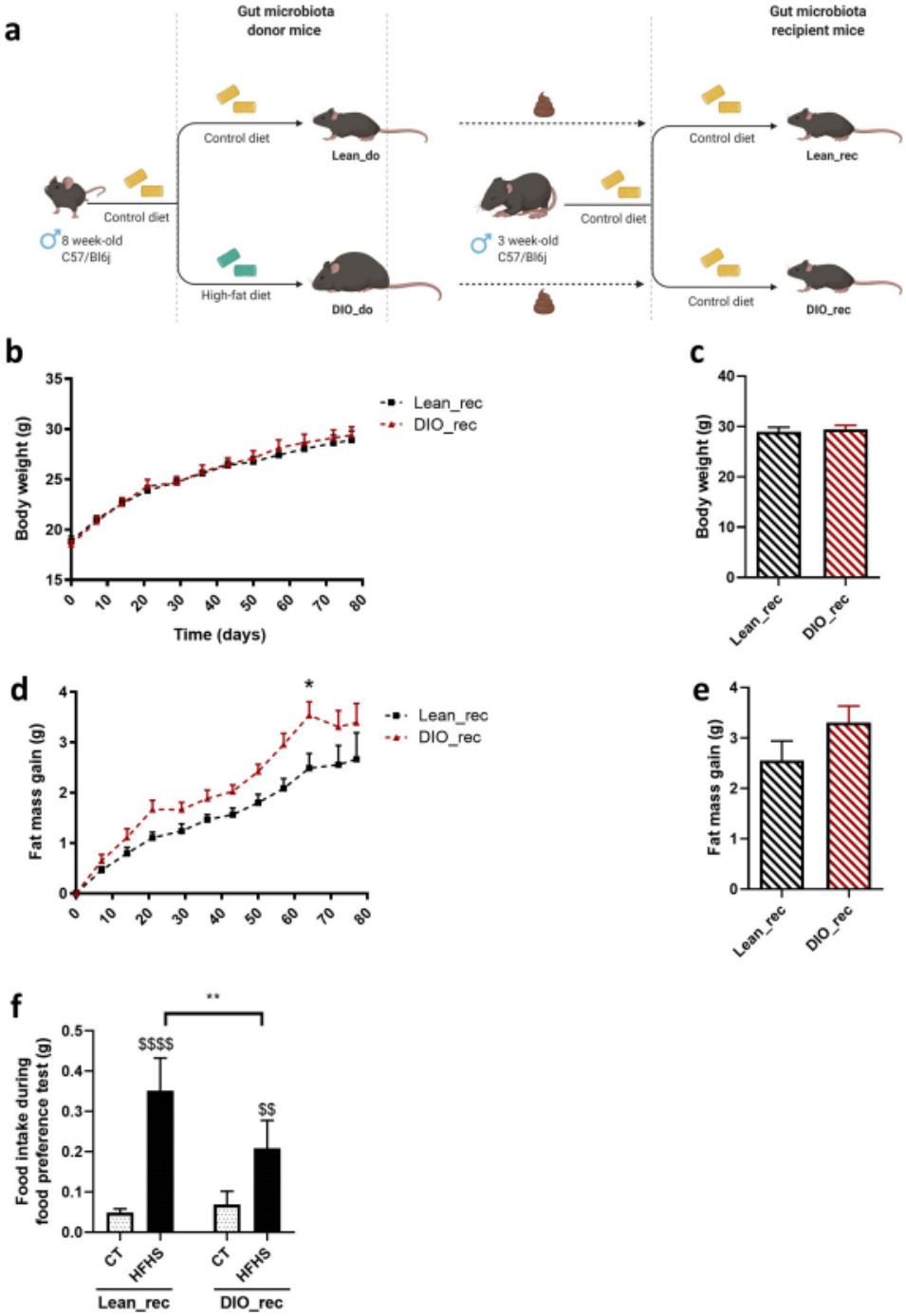

a
Gut microbiota donor mice
Gut microbiota recipient mice
Control diet
Lean_do
8 week-old C57/Bl6j
High-fat diet
DIO_do
3 week-old C57/Bl6j
Lean_rec
DIO_rec
b
Body weight (g)
Time (days)
Lean_rec
DIO_rec
c
Body weight (g)
d
Fat mass gain (g)
e
f
Food intake during food preference test (g)
$$$$
$$
**
CT
HFHS

Figure 2. Recipient mice show hedonic food behavior similar to donor mice after fecal transplantation. (**a**) Experimental plan of the FMT protocol. (**b,c**) Body weight evolution (g) (**b**) and final body weight (g) (**c**) of lean (Lean_rec) and DIO recipient mice (DIO_rec). (**d, e**) Fat mass gain evolution (g) (**d**) and final fat mass gain (g) (**e**) of lean (Lean_rec) and DIO recipient mice (DIO_rec). (**f**) Food preference test showing total HFHS and CT intake after 180 minutes of test by lean (Lean_rec) and DIO recipient mice (DIO_rec) after 12 weeks of follow-up. Data are shown as mean ± SEM (n = 7–8/group). P-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test (**b,d,f**) or unpaired Student's t-test (**c,e**). *: p-value ≤ 0,05 ; **: p-value ≤ 0,01. $$: p-value < 0.01; $$$$: p-value ≤ 0.0001 between CT vs HFHS intake (**f**)

Lean gut microbiota recipient mice displayed a faster preference for HFHS than DIO gut microbiota recipient mice. In fact, lean gut microbiota recipient mice ate significantly more HFHS than CT diet after 90 minutes of test, whereas the difference between HFHS and CT intake in DIO gut microbiota recipient mice was only significant after 150 and 180 min (Fig. S3c). Like the donor mice, the two recipient groups showed a preference for a palatable diet over a CT diet. Strikingly, the total HFHS intake was 40% less important in DIO gut microbiota recipient mice compared to lean gut microbiota recipient mice ($p < 0.01$, Figure 2f). These results demonstrate that lean and DIO gut microbiota recipient mice show similar patterns in terms of hedonic eating behavior as their respective microbiota donors and this effect is independent of obesity development or non-hedonic feeding behavior. Of note, ambulatory activity during the test was comparable between recipient mice, suggesting a similar exploratory behavior toward this novel food high in sugar and fat (Fig. S3d). Taken together, we uncovered a causal role of the gut microbiota in the hedonic food behavior alterations associated with obesity.

Dopaminergic markers in the striatum suggest a hypofunctional food reward system in DIO recipient mice

Pleasure associated with palatable food intake is mainly driven by dopaminergic pathways in the mesocorticolimbic system. Indeed, ingestion of diet rich in fat and sugar has been shown to be associated with the release of dopamine in the striatum in proportion to the self-reported level of pleasure derived from eating the food [302]. DRD1 and DRD2 are the most expressed dopamine receptors of the reward system [303] and the scientific literature describes a downregulation of these receptors in the context of obesity in humans and rodents, which is associated with a reduction of the pleasure related to palatable food

ingestion [107; 295]. Since transplantation of obese gut microbiota replicated food preference alterations associated with obesity (Fig. 2f), we wondered if this was associated with modifications in dopaminergic markers. Therefore, we investigated the expression of dopaminergic markers in the striatum of recipient mice by qPCR.

Our results show that after microbiota transplantation, DIO recipient mice express at least 60% less DRD1 and DRD2 in the striatum compared to lean recipient mice, although this failed to pass the statistical threshold due to high variability in the Lean_rec group ($p > 0.05$, Figure 3a-b). The expression of TH, the rate-limiting enzyme synthesizing dopamine, was also decreased (50%) in mice receiving obese microbiota compared to mice receiving lean microbiota ($p > 0.05$, Figure 3c). In line with these results, the DAT, responsible for the recapture of around 80% of the dopamine released, was two-fold more expressed in DIO_rec compared to Lean_rec ($p > 0.05$, Figure 3d), suggesting low function of the dopaminergic system in obese gut microbiota transplanted mice. Of note, the modifications of expression of dopaminergic markers are not associated with changes in the ambulatory activity (Fig. S2 a-b and Fig. S3d) suggesting that the qPCR results observed in the striatum are specific to the reward system rather than the motor function.

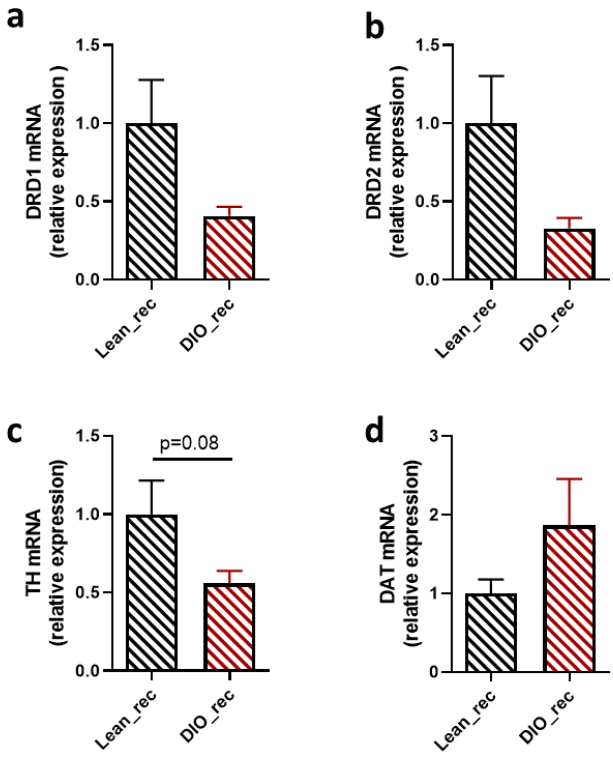


Figure 3. Alterations in dopaminergic signaling in recipient mice with obese gut microbiota. Striatal mRNA expressions of dopamine receptor 1 (DRD1) (**a**), dopamine receptor 2 (DRD2) (**b**), tyrosine hydroxylase (TH) (**c**) and dopamine transporter (DAT) (**d**) were measured by qPCR in lean (Lean_rec) and DIO recipient mice (DIO_rec) after 12 weeks of follow-up. Data are shown as mean ± SEM (n = 7–8/group). P-values were obtained after unpaired Student's t-test (**c**) or non-parametric Mann–Whitney test (**a,b,d**).

Besides the dopaminergic system in the striatum, other brain areas are involved in food reward such as caudate putamen, Nac, and PFC. Therefore, we further investigated and analyzed mRNA levels of the dopaminergic markers in these regions (Table S1). We did not observe any difference between lean and obese gut microbiota recipient mice in these brain areas, suggesting that modulations of the expression of dopaminergic markers between lean and obese gut microbiota recipient mice seem to be specific to the striatum.

FMT from obese donors into lean recipient mice is efficient

To validate the efficiency of the gut microbiota transplantation, bacterial composition of cecum contents from donor and recipient mice were analyzed using 16S rRNA sequencing. We compared common operational taxonomic units (OTUs) between donors and recipients at the end of each experiment, just after food preference tests (Figure 4a). Two mice from each donor group (CT-fed or HFD-fed) were donors for 7 Lean_rec and 8 DIO_rec recipient mice, respectively, with one donor mouse for 3 or 4 recipient mice. Venn diagram showed a high similarity of OTUs (more than 50%) between donors and recipients, confirming the colonization of antibiotic-treated recipients with gut microbiota from donors (Figure 4a).

Furthermore, as represented on the principal coordinate analysis (PCoA), obese donors, and obese gut recipient mice have gut microbiota profiles that differ from lean donors and lean gut microbiota recipient mice according to the principal component PC2 (Figure 4b).

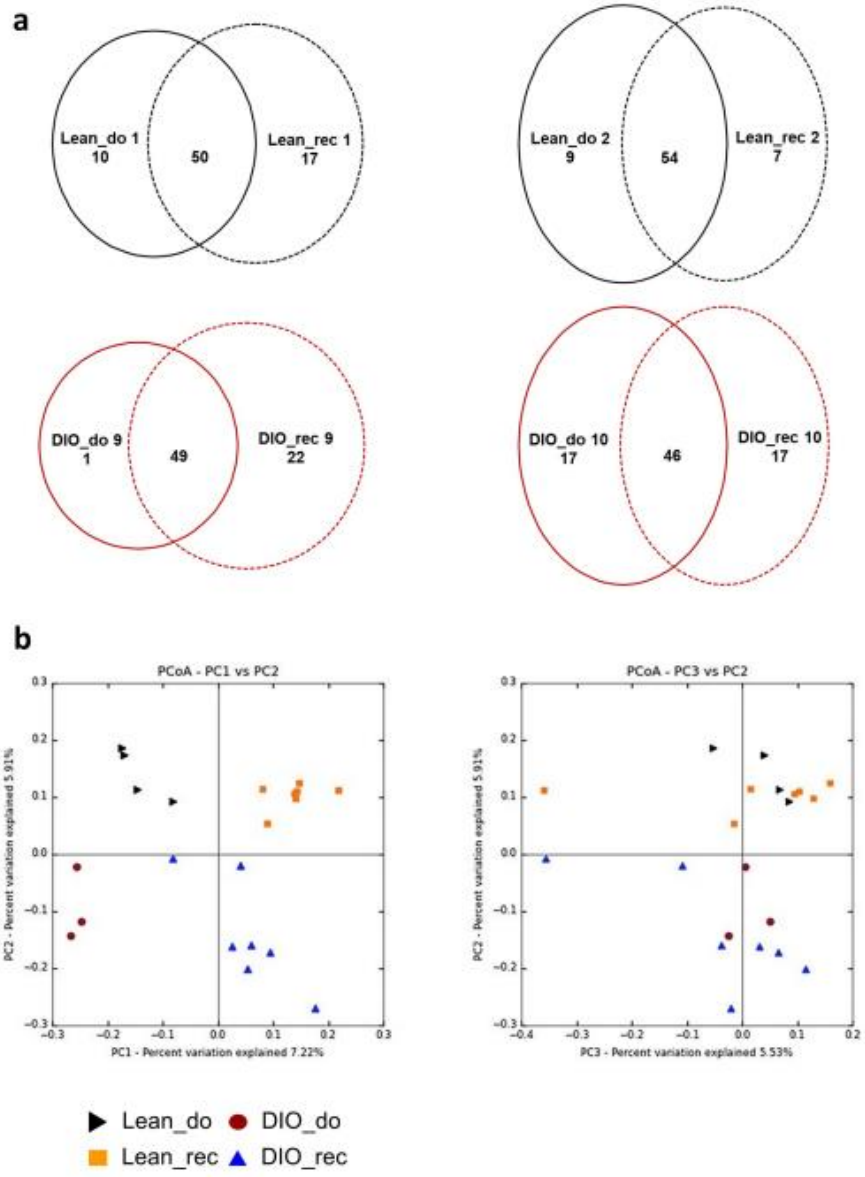


Figure 4. The gut microbiota of recipient mice is similar to the gut microbiota from donor mice. Venn diagram is based on OTUs similarity between donor (Lean_do and DIO_do) and recipient (Lean_rec and DIO_rec) mice , cecal contents were harvested after 5 weeks of follow-up in donor mice and 12 weeks of follow-up in recipient mice (**a**). Principal coordinates analysis (PCoA) is based on the unweighted UniFrac analysis on operational taxonomic units (OTUs). Each symbol representing a single sample is colored according to its group (**b**).

Parabacteroides represent a potential link in the gut-to-brain axis controlling hedonic food intake

As a preliminary approach to highlight a potential link between the gut microbiota and the food reward system in the context of obesity, we used Spearman's correlations to establish associations between several parameters of the food reward system and the gut microbiota. Data from donor and recipient mice were combined to create the correlation matrix. The heatmap showed that 18 OTUs correlated with the total HFHS intake measured during the food preference test (Figure 5a). In addition, positive correlations were found between the unidentified genus of the Peptococcaceae family and the mRNA expression of DRD1, DRD2, and TH (Figure 5a). However, after correcting for multiple comparisons using the FDR (false discovery rate) method, only *Parabacteroides* remained highly positively correlated with the HFHS intake (Figure 5b). This suggested that the more *Parabacteroides* the mice had, the more HFHS they ate during the food preference test. Based on this, *Parabacteroides* could represent a potential link between the gut microbiota and hedonic food behavior.

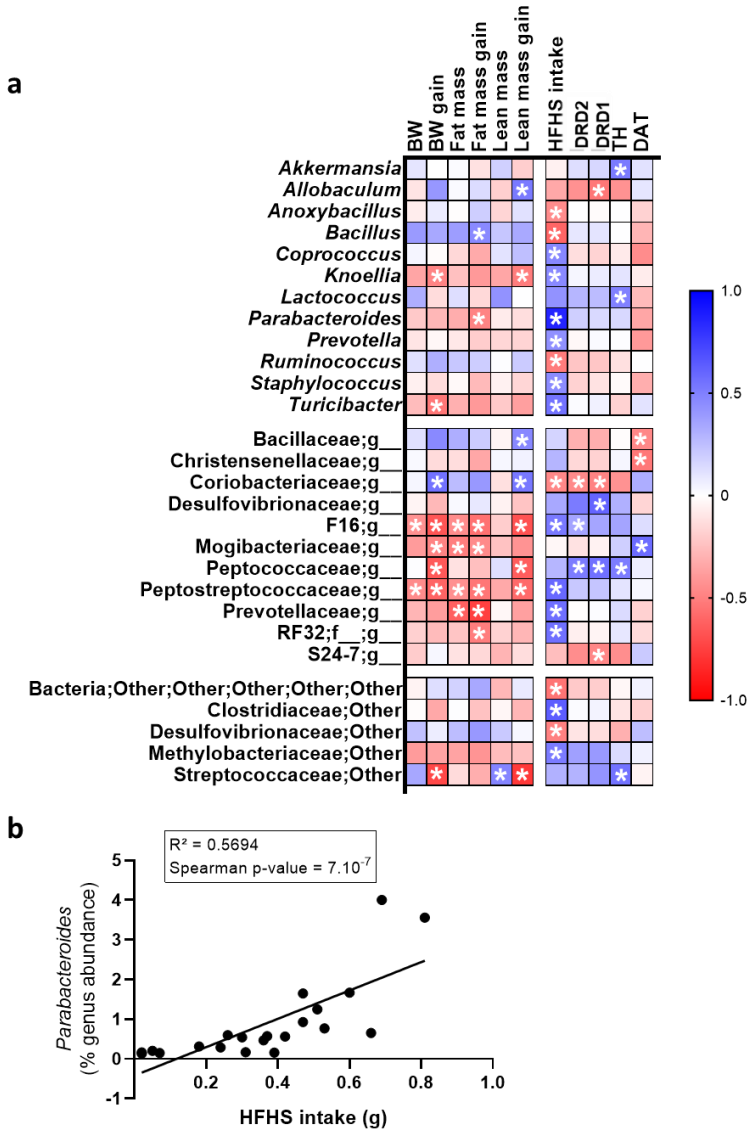


Figure 5. Correlations between gut microbes and dopaminergic markers. Heatmap of bacterial composition and food reward patterns. Spearman's correlations were calculated for each parameter for donor and recipient mice, cecal contents were harvested after 5 weeks of follow-up in donor mice and 12 weeks of follow-up in recipient mice (**a**). P-values were obtained after Spearman's correlation test. *: p ≤ 0,05; Spearman's correlation after FDR correction (**b**).

Depletion in gut microbiota over-stimulates hedonic eating in lean mice

To further investigate the causal role of the gut microbiota in the food reward system, we depleted the gut microbiota of mice by using a mixture of antibiotics. For this purpose, another cohort of mice, including mice under CT (Lean) and HFD (DIO) was used and followed during 5 weeks. Within each group, half of the mice received daily a mixture of antibiotics by oral gavage (Lean_AB and DIO_AB). A saline solution was administered daily by oral gavage to the other half (Lean_NaCl and DIO_NaCl). We first validated the model of depletion of the gut microbiota by quantifying the total bacterial load by quantitative polymerase chain reaction (qPCR) (Fig. S4a). After 5 days of daily gavage with antibiotics, 99% of the total bacterial load was suppressed in each group.

As predicted, mice under HFD gained significantly more weight (Figure 6a-b) and had a higher fat mass gain than the CT-fed mice (Figure 6c-d). Importantly, antibiotic treatment did not affect body weight (Figure 6a-b) or fat mass gain (Figure 6c-d).

To investigate the impact of the depletion of the gut microbiota on the "liking" component of the reward (pleasure), a food preference test was done in this cohort (Figure 6e and Fig. S4b). Obese mice (DIO_NaCl) ate 76% less HFHS than lean mice (Lean_NaCl) during the food preference test ($p < 0.0001$), as already observed in our first cohort of mice. These results demonstrated the reproducibility of our data and thereby their validity. We also observed that even after treatment with antibiotics, DIO mice (DIO_AB) displayed the same behavior and consumed 84% less HFHS compared to lean mice supplemented with antibiotics (Lean_AB) ($p < 0.0001$). Strikingly, this test showed that lean mice treated with antibiotics (Lean_AB) ate significantly more HFHS than lean mice with an intact gut microbiota (Lean_NaCl) from the first hour until the end of the test (Figure 6e and S4b). These results demonstrate for the first time that a healthy gut microbiota is involved in hedonic response to palatable food and that any type of alteration of the gut microbiota composition might affect the rewarding response to food.

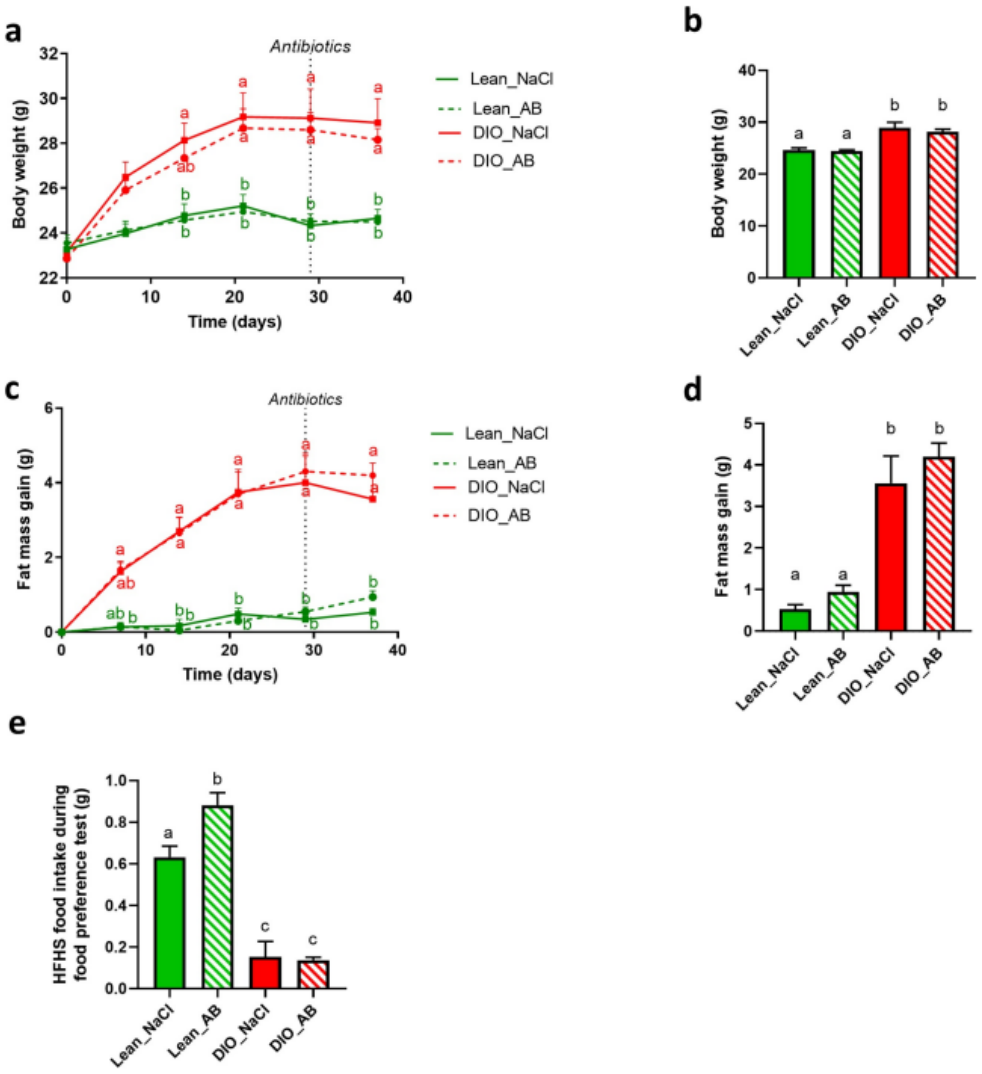


Figure 6. Antibiotic treatment affects food preference and HFHS intake independently of body weight and fat mass accumulation. Body weight evolution (**a**) and final body weight (**b**) of lean and DIO mice treated with antibiotics (Lean_AB and DIO_AB) or saline (Lean_NaCl and DIO_NaCl). Fat mass gain evolution (**c**) and final fat mass gain (**d**) of lean and DIO mice treated with antibiotics (Lean_AB and DIO_AB) or saline (Lean_NaCl and DIO_NaCl). (**e**) Food preference test showing total HFHS and CT intake after 180 minutes of test by lean and DIO mice treated with antibiotics (Lean_AB and DIO_AB) or saline (Lean_NaCl and DIO_NaCl) after 5 weeks of follow-up. Data are shown as mean ± SEM (n = 10/group). P-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test **(a,b,c,d,e)**. Different superscript letters represent significant p-values between groups and type of diet (CT or HFHS) (**e**).

1.4. Discussion

In this study, we provided data supporting the dysregulation of the reward system associated with obesity from a food behavioral point on view [116; 260]. Indeed, during the food preference test, obese mice showed a delayed preference for HFHS and ate significantly less palatable food compared to lean mice. These results suggest that eating a high-fat diet during a long period of time results in a hypofunctioning of the food reward system: palatable food is attributed less hedonic value in obese mice. In the mesocorticolimbic system, this implies a downregulation of the dopaminergic system associated with weight and fat mass gain as seen in the literature [295]. In this study, we focused on the "liking" component of the food reward, associated with the pleasure induced by palatable food. However, food reward also includes two other components, not investigated in the present study, such as the "wanting" (the motivation to obtain a reward) and the "learning" (the capacity to associate an environment with pleasure related to palatable food intake). These two other components can be evaluated with behavioral tests such as the operant wall and the CPP respectively.

Importantly, by using two different approaches we discovered that the gut microbiota is an active regulator of the hedonic component of the food intake. Indeed, we demonstrated that transplanting the gut microbiota from obese donor into lean recipient mice, replicated the obese altered hedonic behavior. In addition, we showed that the depletion of the gut microbiota using an antibiotic treatment increased the consumption of palatable food during a food preference test.

Altogether, we found out that alterations of the food intake related to the pleasure in obesity was at least partially explained by gut microbiota modifications associated with obesity but not due to obesity itself. Indeed, the altered hedonic behavior of obese mice was transferable into recipient mice through gut microbiota transplantation, but this was independent from obesity development and HFD consumption since obese gut microbiota recipient mice displayed the same body weight and similar energy metabolism as lean gut microbiota recipient mice. There was a minor increase in fat mass gain in obese gut

microbiota recipient mice as compared to lean gut microbiota recipient mice, but this had negligible effects on body weight evolution. Even if some data in the literature suggest that obese phenotype can be transferred by the gut microbiota, this hypothesis has been debated since the type of diet plays a key role in the development of fat mass gain and obesity [301]. Importantly, microbiota analysis revealed a high similarity between gut bacterial composition from donor and recipient mice, suggesting that the gut microbiota inoculum from donor efficiently engrafted the recipient cecum.

A key question that remains a matter of debate in the scientific literature as discussed by Berthoud *et al.* : it is not clear whether predisposing differences in reward functions cause overeating and weight gain, or whether repeated exposure or secondary effects of the obese state alter reward functions [304]. Using caloric restriction experiments in obese rodents, they demonstrate that weight loss resulted in a restoration of the sucrose concentration–response curve similar to the curve observed in lean rats. They concluded that most of the difference between lean and obese rats was due to secondary effects of the obese state, not to preexisting differences in reward processing. Using obese gut microbiota transplantation, our data demonstrate that an altered gut microbiota consequently to obesity contributes to alterations in hedonic food intake. Thereby, we provide new insight into the role of obesity state in hedonic alterations suggested by Berthoud *et al.*

In addition to behavioral evidence of a dysregulation of the food reward system in obese gut microbiota recipient mice, qPCR analysis also suggested a hypofunctioning of the dopaminergic pathways at the level of the striatum as typically described during obesity. All the dopaminergic markers investigated for synthesis and receptors are decreased, whereas DAT, responsible for dopamine recapture, is increased in DIO_rec compared to Lean_rec mice. Although the striatum is described as the main structure involved in the gut-induced reward [231], other structures are also involved in the food reward system. These include the Nac, the PFC, and the caudate putamen. However, we did not observe any difference in the expression of dopaminergic markers in these brain areas. Hence, our data suggest that modulations of the expression of dopaminergic markers between lean and obese gut

microbiota recipient mice are specific to the striatum. Besides the dopaminergic system, other systems are involved in the food reward. These include opioids such as dynorphin, that have been identified as key mediators involved in the pleasure induced by palatable food and could represent another interesting perspective to investigate in this context [115; 305].

As an early approach to investigate the mechanisms linking the gut microbes to the brain reward system, we showed a highly significant positive correlation between the abundance of *Parabacteroides* and the quantity of HFHS diet ingested during the food preference test. This suggests that *Parabacteroides* could play a potential role in driving the preference for HFHS rather than for CT diet, and stimulate the intake of food rich in fat and sugar, reflecting a functional food reward system. In genetic-obese mice and diet-induced obese mice, *Parabacteroides distasonis* has already been described as a protective bacterium against weight gain and participates to restore obesity-associated metabolic disorders such as hepatic steatosis and insulin resistance [167].

We used HFHS during the food preference test because sugar and fat follow different gut-to-brain pathways and have supra-additive effects on the striatal dopamine release [294]. On the one hand, Han *et al.* demonstrated that fat uses a PPARα-dependent pathway and the vagus nerve to inform the striatum and induce dopamine release [132]. On the other hand, sugar-induced striatal dopamine release is mainly due to the metabolism of the sugar in the gut, completed with the information from a portal vein sensor [306]. To go deeper into the mechanisms, Kaelberer *et al.* have demonstrated that intestinal EECs are able to communicate with the vagus nerve, in order to transduce an afferent message to the brain [131]. Since our team has already shown the key interplay between the gut microbiota and EECs to release peptides regulating the food intake (GLP-1, PYY), the investigation of the role of these hormones as key mediators in the crosstalk between the gut microbiota and the reward system, would be an interesting perspective [307].

In conclusion, these results demonstrate for the first time the implications of gut microbiota in the regulation of the reward pathway and hedonic aspects of food intake in mice (Figure

7). Our data also reveal the causal role of gut microbiota modifications associated with obesity into the dysregulations of the dopaminergic reward system and the hedonic food intake in obesity (Figure 7). Therefore, we provide here evidence that the gut microbiota could be an interesting therapeutic target to tackle hedonic food intake alterations related to obesity.

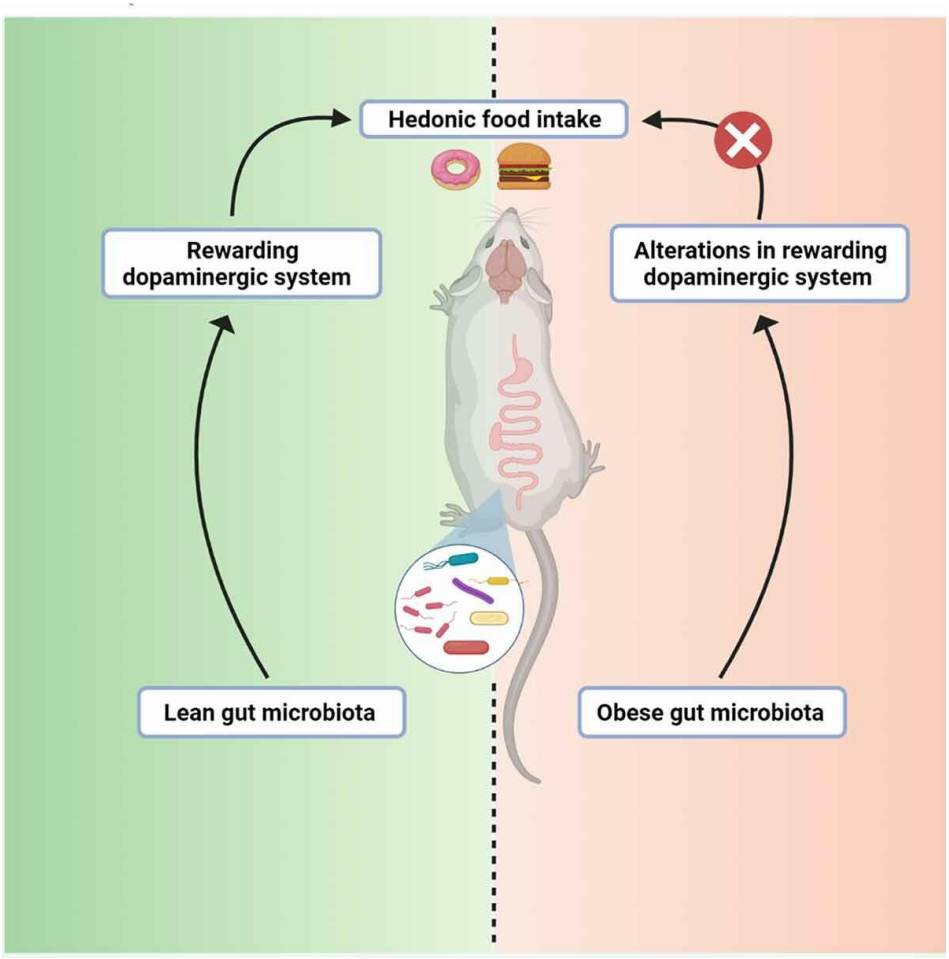
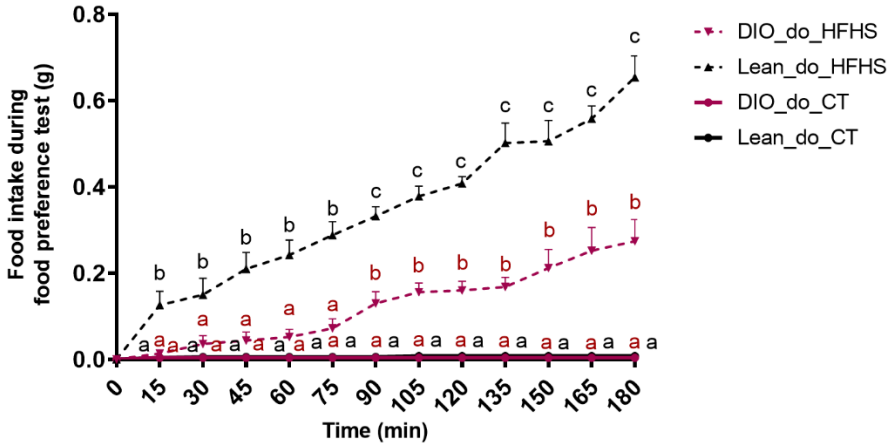
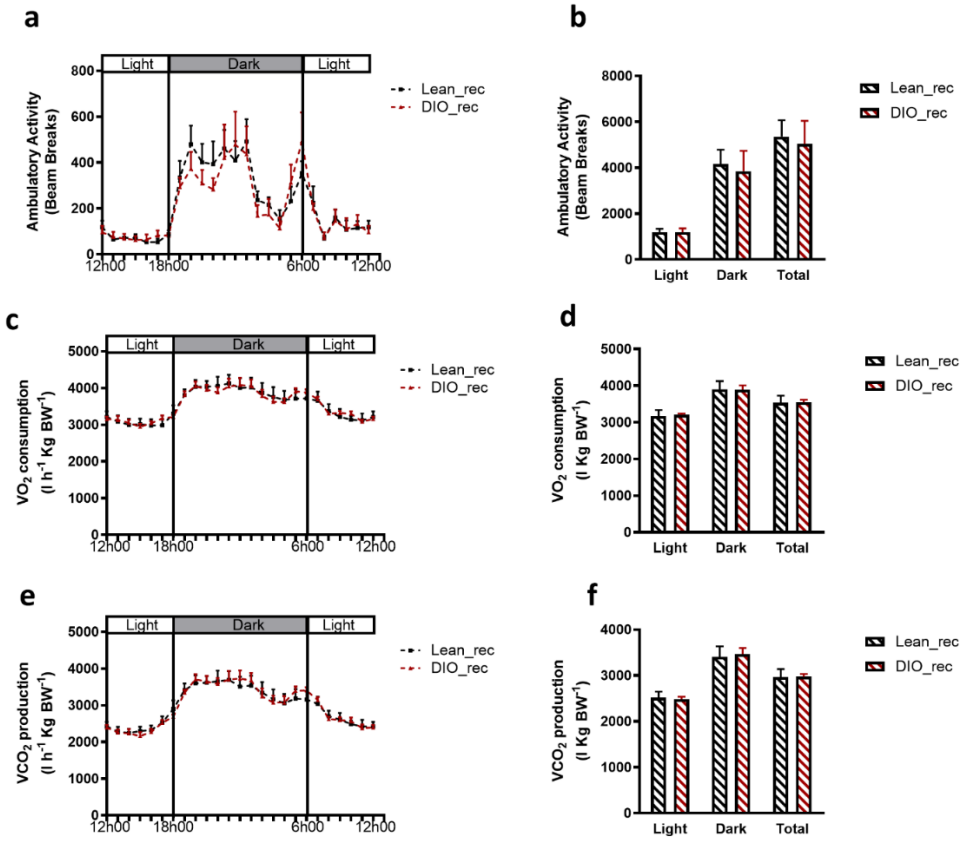


Figure 7: This study demonstrates for the first time the implication of the gut microbiota in the regulation of the reward pathway and hedonic aspects of food intake in mice. The data obtained also reveal the causal role of gut microbiota modifications associated with obesity into the dysregulations of the dopaminergic reward system and the hedonic food intake during obesity.

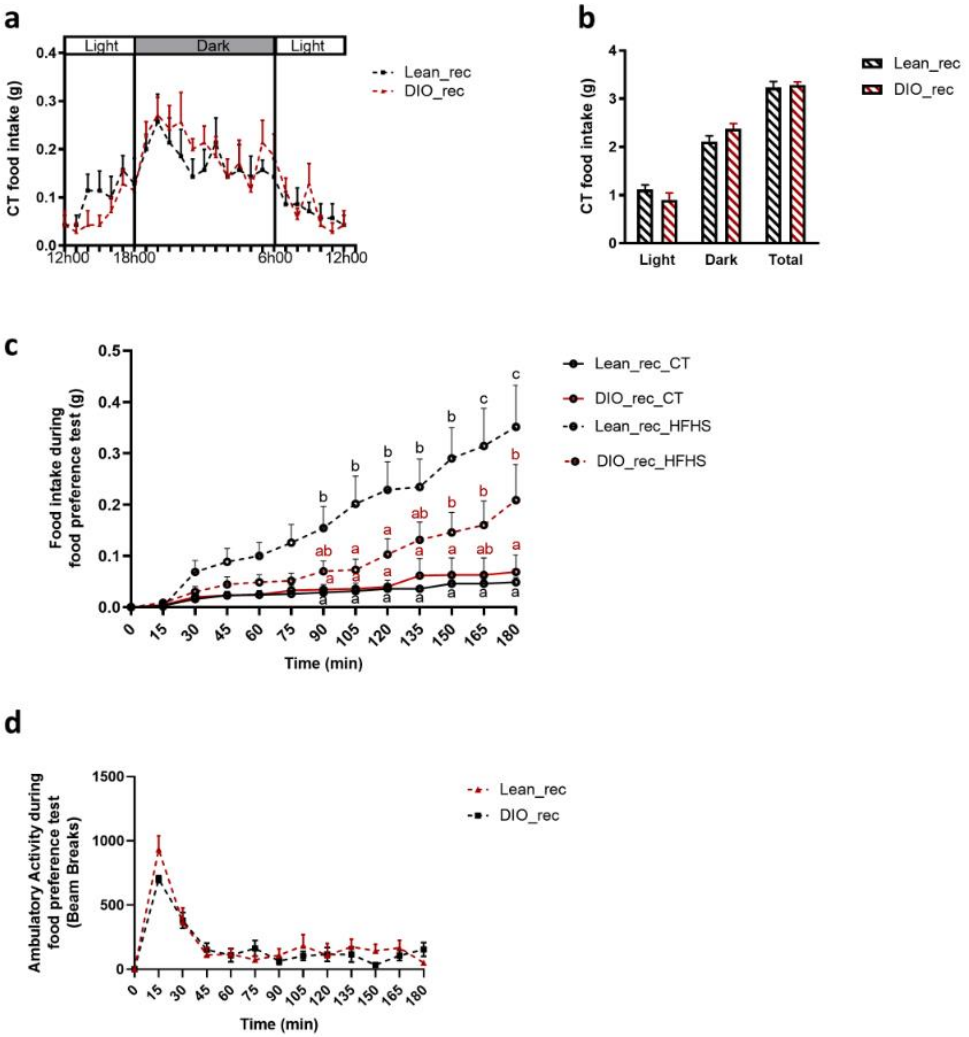
1.5. Supplemental information



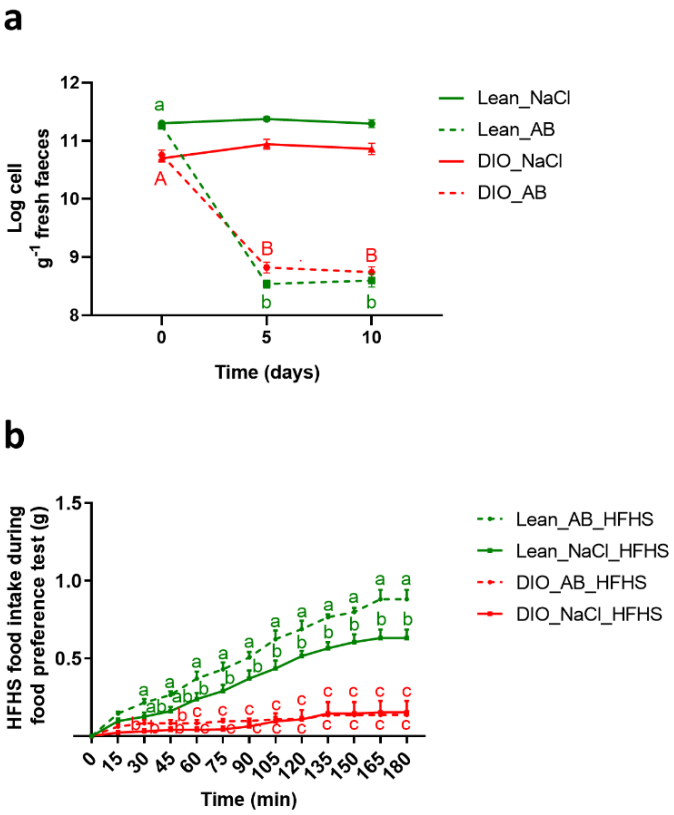
Supplemental figure 1: **Obese mice present a reduced food preference for HFHS compared to lean mice.** Food preference test showing HFHS and CT intake every 15 minutes during 180 minutes by lean (Lean_do) and DIO donor mice (DIO_do) after 5 weeks of follow-up. Data are shown as mean ± SEM (n=5/group). P-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test. Different superscript letters represent significant p-values ($p < 0.05$) between groups and type of diet (CT or HFHS) at each time-point.



Supplemental figure 2: Metabolic profiles do not differ between lean and obese gut microbiota recipient mice. Light, dark, and total ambulatory activity (Beam Breaks) (**a,b**). Light, dark and total O_2 consumption (l h-1 Kg body weight-1) (**c,d**). Light, dark and total CO_2 production (l h−1 Kg body weight−1) (**e,f**) during the 5th week of follow-up. Data are presented as the mean ± SEM (n=7/group). Data were analyzed by repeated measures two way ANOVA followed by Tukey post-hoc test (**a,c,e**) or unpaired Student's t-test within each phase (**b,d,f**).



Supplemental figure 3: Hedonic food intake alterations in obese gut microbiota recipient mice are specific to the reward system. Light, dark, and total control diet intake (g) (**a,b**). Food preference test showing HFHS and CT intake every 15 minutes during 180 minutes by lean (Lean_rec) and DIO gut microbiota recipient mice (DIO_rec) (**c**). Ambulatory activity (Beam Breaks) measured every 15 minutes during 180 minutes of the food preference test (**d**). Data were collected during the 5th week of follow-up are shown as mean ± SEM (n=7/group). P-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test (**a,c,d**) or unpaired Student's t-test within each phase (**b**). Different superscript letters represent significant p-values ($p < 0.05$) between groups and type of diet (CT or HFHS) at each time-point (**c**).



Supplemental figure 4: Depletion of gut microbial load by antibiotics induces hedonic intake alterations in control-fed mice. Total fecal bacterial load quantified by qPCR after 5 weeks follow-up (log cell g-1 fresh feces) in CT- or HFD-fed mice treated (Lean_AB, DIO_AB) or not with antibiotics (Lean_NaCl, AB_NaCl) (**a**). Food preference test showing HFHS and CT intake every 15 minutes during 180 minutes by lean and DIO mice treated with antibiotics (Lean_AB and DIO_AB) or saline (Lean_NaCl and DIO_NaCl) after 5 weeks follow-up (**b**). Data are shown as mean ± SEM (n=10/group). P-values were obtained after Two-way Anova, followed by Bonferroni post-hoc test. Different subscript letters represent significant p-values between time-point within a group (a) or between groups and type of diet (CT or HFHS) at each time-point (**b**).

	DIO_rec								
	Nucleus accumbens			Prefrontal cortex			Caudate putamen		
	Mean	SEM	p-value	Mean	SEM	p-value	Mean	SEM	p-value
DRD2	1,38	± 0,64	0,60	1,22	± 0,44	0,73	1,21	± 0,22	0,46
DRD1	1,70	± 0,69	0,41	1,21	± 0,27	0,56	1,15	± 0,24	0,59
TH	0,97	± 0,3	0,94	1,09	± 0,10	0,71	0,98	± 0,07	0,85
DAT				0,59	± 0,07	0,43			

Supplemental table 1: Dopaminergic markers in other reward-related regions in recipient mice. Nac, PFC and caudate putamen mRNA expression of dopamine receptor 2 (DRD2), dopamine receptor 1 (DRD1), tyrosine hydroxylase (TH) and dopamine transporter (DAT) measured by real-time qPCR in lean (Lean_rec) and DIO recipient mice (DIO_rec) after 11 weeks follow-up. As the relative control group, Lean_rec is not shown (means = 1 for each marker). Data are shown as mean ± SEM (n=4-7/group). P-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test.

1.6. Materials and methods

Mice and experimental design

All mouse experiments were approved by the ethical committee for animal care of the Health Sector of the UCLouvain, Université catholique de Louvain under the specific number 2017/UCL/ MD/005 and performed in accordance with the guidelines of the local ethics committee and in accordance with the Belgian Law of May 29, 2013 regarding the protection of laboratory animals (agreement number LA1230314).

Donor mice

A cohort of 8-week-old specific-opportunistic and pathogen-free (SOPF) male C57BL/6 J mice (10 mice, n = 5 per group) (Janvier laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2°C,12 h daylight cycle) in groups of two mice per cage, with free access to sterile food (irradiated) and sterile water. Upon delivery, the mice underwent an acclimatization period of 1 week, during which they were fed a control diet (CT, AIN93Mi, Research Diet, New Brunswick, NJ, USA). Then, mice were randomly divided in two groups, and were fed for 5 weeks with a controlled low-fat diet (CT, AIN93Mi) or a high-fat diet (HFD, 60% fat and 20% carbohydrates (kcal/100 g) D12492i, Research diet, New Brunswick, NJ, USA). Body weight, food, and water intake were recorded once a week. The body composition was assessed by using 7.5 MHz time domain-nuclear magnetic resonance (TD-NMR, LF50 Minispec, Bruker, Rheinstetten, Germany). After 4 weeks of follow- up, the mice entered the metabolic chambers to perform the food preference test.

Recipient mice

A cohort of 3-week-old specific-opportunistic and pathogen-free (SOPF) male C57BL/6 J mice (15 mice, n = 7–8 per group) (Janvier laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2°C,12 h daylight cycle) in groups of two mice per cage, with free access to sterile food (irradiated) and sterile water. Mice were fed a low-fat control diet (CT, AIN93Mi) during the entire transplantation protocol as well as after gut microbiota transplantation. Body weight, food, and water intake were recorded once a

week. The body composition was assessed by using 7.5 MHz time domain-nuclear magnetic resonance (TDNMR, LF50 Minispec, Bruker, Rheinstetten, Germany). After 12 weeks of follow-up, the mice entered the metabolic chambers to assess precisely their food intake and metabolism then perform the food preference test.

Fecal microbiota transplantation

At the end of the donor experiment, the cecal content was collected in sterile containers, filtered with 100µm filters and immediately diluted (1:50 w/vol) in sterile Ringer buffer (4,5g NaCl, 200 mg KCl, 125 mg CaCl2). This suspension was then diluted (1:1 v/v) in 20% (w/ v) skim milk (Nonfat dry milk, Biorad, 2005668 A) before storage at −80°C. Two CT-fed mice and two HFD-fed mice from the donor cohort were selected as fecal microbiota donors for seven or eight recipient mice per group, respectively, with one donor for three or four recipient mice. As previously described, prior to gut microbiota inoculation, 3-week-old SOPF recipient mice were depleted in intestinal microbiota by daily gavage of a broad spectrum, poorly absorbed mix of antibiotics during 5 days (100 mg/kg of ampicillin, neomycin, and metronidazole, and 50 mg/kg of vancomycin diluted in sterile water) added with antifungal (amphotericin B 1 mg/kg).31,32 Antibiotic treatment was then followed by a bowel cleansing with the administration of 600 µl of PEG solution (Macrogol 4000, Colofort, Ipsen, France) by oral gavage in two times at 30 min intervals after a 2-h fasting. Colonization was then achieved by intragastric gavage with 300 µl of inoculum three times a week for 1 week. During antibiotic treatment and inoculation, mice were transferred into clean cages 4 times a week. All the recipient mice were kept under CT diet (CT, AIN93Mi).

Antibiotic-treated mice

A cohort of 8-week-old SOPF male C57BL/6 J mice (40 mice, n = 10 per group) (Janvier Laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2°C,12 h daylight cycle) in groups of two mice per cage, with free access to sterile food (irradiated) and sterile water. Upon delivery, the mice underwent an acclimatization period of 1 week, during which they were fed a control diet (CT, AIN93Mi). Then, mice were fed with a low-fat control diet (CT, AIN93Mi) or a high-fat diet (HFD, D12492i). Treatment

continued for 5 weeks, after which the mice entered the metabolic chambers to perform the food preference test. Body weight, food, and water intake were recorded once a week. The body composition was assessed by using 7.5 MHz time domain-nuclear magnetic resonance (TD-NMR, LF50 Minispec, Bruker, Rheinstetten, Germany). The same antibiotic mixture as previously described was administered to half of the mice under the CT diet (Lean_AB) and to half of the mice under HFD diet (DIO_AB) from 7 days prior to the food preference test until the end of the experiment. Amphotericin B treatment ended after 5 days in order to avoid any toxicity.

Metabolic chambers

After 11 weeks of follow-up, recipient mice were separated and housed individually one week before entering metabolic chambers (Labmaster, TSE systems GmbH, Bad Homburg, Germany). Then, they underwent 4 days of metabolic assessment before the food preference test. The mice were analyzed for oxygen consumption, and carbon dioxide production using indirect calorimetry (Labmaster, TSE systems GmbH). These parameters were expressed as a function of the whole-body weight. Locomotor activity was recorded using an infrared light beam-based locomotion monitoring system (expressed as beam breaks count per hour). Sensors recorded the precise food intake of each diet every 15 minutes. Inside the chambers, measurements were taken every 15 minutes. The final data representation (total, day, or night) corresponds to all the values measured and summed (light phase or dark phase). The means (n = 7) were finally compared between groups.

Food preference test

During 3 hours in the daylight, mice were exposed to two kinds of diets: a low-fat, control diet (CT, AIN93Mi, Research diet, New Brunswick, NJ, USA) or a high-fat high-sucrose diet (HFHS, 45% fat and 27.8% sucrose (kcal/100 g) D17110301i, Research diet, New Brunswick, NJ, USA) in metabolic chambers (Labmaster/Phenomaster, TSE systems, Germany). Sensors recorded the precise food intake of each diet every 15 minutes.

Tissue sampling

At the end of each experiment, mice were fed and exposed for 1 h to HFHS before anesthesia with isoflurane (Forene, Abbott, England). This aims to mimic the conditions of the food preference test and stimulate the dopaminergic food reward system. Then the mice were euthanatized by exsanguination and cervical dislocation. Striatum, Nac, PFC, and caudate putamen were precisely dissected, the cecal content was harvested and immediately immersed into liquid nitrogen, then stored at −80°C for further analysis.

RNA preparation and qPCR analysis

Total RNA was prepared from the striatum, Nac, PFC and caudate putamen using TriPure reagent (Roche). Quantification and integrity analysis of the total RNA were performed by running 2 µl of each sample on an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent). If the RNA integrity number (RIN) obtained less than 6, the sample was excluded from further analyses. cDNA was prepared by reverse transcription of 1 µg total RNA using the GoScript Reverse Transcriptase kit (Promega, Madison, WI, USA). Real-time PCR was performed with the QuantStudio 3 real-time PCR system (Thermo Fisher, Waltham, MA, USA). Rpl19 RNA was chosen as the housekeeping gene. All samples were performed in duplicate, and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of amplification. Sequences of the primers used for qPCR are available in Table 1.

Table 1. qPCR primer sequences for the targeted mouse genes.

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
RPL19	GAA-GGT-CAA-AGG-GAA-TGT-GTT-CA	CCT-TGT-CTG-CCT-TCA-GCT-TGT
D2R	CCA-AGA-ACG-TGA-GGG-CTA-AG	TGA-GGA-TGC-GAA-AGG-AGA-AG
D1R	GAG-CCA-ACC-TGA-AGA-CAC-C	TGA-CAG-CAT-CTC-CAT-TTC-CAG
TH	GCC-AAG-GAC-AAG-CTC-AGG-AAC	ATC-AAT-GGC-CAG-GGT-GTA-CG
DAT	AAA-TGC-TCC-GTG-GGA-CCA-ATG	GTC-TCC-CGC-TCT-TGA-ACC-TC

DNA isolation from mouse cecal samples and sequencing

The cecal contents were collected and kept frozen at −80°C until use. Metagenomic DNA was extracted from the cecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications.33 The V1–V3 region of the 16S rRNA gene was amplified from the cecal microbiota of the mice using the following universal eubacterial primers: 27Fmod (5'AGRGTTTGATCMTGGCTCAG-3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG -3'). Purified amplicons were sequenced utilizing a MiSeq following the manufacturer's guidelines. Sequencing was performed at MR DNA (www. mrdnalab.com, Shallowater, TX, USA). Sequences were demultiplexed and processed using the QIIME pipeline (v1.9 using default options: Q25, minimum sequence length = 200 bp, maximum sequence length = 1000 bp, maximum number of ambiguous bases = 6, maximum number of homopolymers = 6, maximum number of primer mismatches = 0). For the 22 samples analyzed, 102 OTUs have been identified (97% similarity). The minimum number of sequences per sample was 48 170 and the maximum number of sequences per sample was 86 360. The median number of sequences per sample was 61 143 and the mean number of sequences per sample was 63 7392 ± 10 798 (standard deviation). The Q25 sequence data derived from the sequencing process were analyzed with the QIIME 1.9 pipeline. Briefly, the sequences were depleted of barcodes and primers. Sequences of 1000 bp were then removed; sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, and operational taxonomic units (OTUs) were generated. Chimeras were also removed. OTUs were defined by clustering at 3% divergence (97% similarity). The final OTUs were taxonomically classified using BLASTn against a curated Greengenes database. PCoA was generated with QIIME using the unweighted UniFrac distance matrix between the samples and as previously described [172; 308; 309; 310]. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB46582.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.1.2 for Windows (GraphPad Software, San Diego, CA, USA) except for microbiota analyses as described above. Data are expressed as mean ± SEM. Differences between the two groups were assessed using unpaired Student's t-test. In case variance differed significantly between groups according to the Fisher test, a nonparametric (Mann–Whitney) test was performed. Differences between more than two groups were assessed using one-way ANOVA or two-way ANOVA if repeated measurements, followed by Tuckey or Bonferroni, respectively, post-hoc test. If variance differed significantly between groups, a non-parametric Kruskal–Wallis test was performed, followed by the Dunnett post-hoc test.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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1.7. Complementary discussion, limitations and perspectives

With this first study, we proved that an alteration of the gut microbiota due to prolonged HFD, was causally involved in hedonic food intake alterations during a food preference test. After analyzing the composition of the gut microbiota by 16S rRNA sequencing, we suggested that low levels of *Parabacteroides* could be a risk factor for altered hedonic food intake, based on the highly positive correlation between the relative abundance of *Parabacteroides* and the palatable food intake. However, general conclusions based on comparisons of relative abundance between different samples from different groups, must be carefully taken into account. Together with the emergence of gut microbiota studies, the analysis of gut microbiota composition is evolving. The distinction between relative and absolute quantities of bacteria is a quite new concept in the literature [311]. We took this limitation into consideration for our second FMT experiment and subsequent gut microbiota analysis.

Here we supposed that the alterations in the gut microbiota would be the causal role into the brain reward alterations leading to inappropriate palatable food intake. However, it has also demonstrated that the chronic consumption of high-sugar diet leads to increased *Parabacteroides* abundance in the gut [274]. The high correlation that we observe between the abundance of *Parabacteroides* and palatable food intake during the food preference test may be to that, rather than potential beneficial effects of *Parabacteroides* on the reward system. That said, the food preference test only represents an acute exposure to HFHS diet and *Parabacteroides* is in most cases, a bacterium reduced in obese conditions, in humans and rodents [268; 269].

As a perspective of this study, we are currently trying to isolate *Parabacteroides* from inoculum solutions from this experiment, to administer this specific strain to lean and diet-induced obese mice and assess their behavior and reward system.

2. CHAPTER 2: *Obesity-associated microbiota intensifies the motivation for food reward: Identification of a new mediator in the gut-brain axis*

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[The results of this chapter have been included in a paper submitted to Nature Communications]

In this chapter, we will use the same model as the one described in the first chapter, *i.e.* the FMT, to pinpoint the role of the gut microbiota in the two other components of the reward system: the wanting and the learning. To assess these components, we previously validated appropriate protocols for behavioral tests: the operant wall test and the CPP test. Moreover, we went further into the mechanisms by screening the metabolites present in the cecal content and the plasma of donor and recipient mice, to highlight potential mediators involved in the reward system through the gut-brain axis.

2.1. Abstract

Obesity is a worldwide epidemic. Dysregulation of food intake, in the way of excessive hedonic consumption (linked to the pleasure) is considered as one of the main drivers for weight gain. Identifying the contributors of this dysregulation would help to tackle obesity and associated metabolic diseases. Meanwhile, the gut microbiome is altered during obesity and has been shown to regulate host metabolism including food intake. Here, by using FMT from lean or obese mice into lean recipient mice, we demonstrated that gut microbes play a role in the regulation of food reward (*i.e.* wanting and learning processes associated with palatable food intake) and could be responsible for compulsive behavior and excessive motivation to obtain sucrose pellets. Indeed, gut microbiota recipient mice from obese donors present an increased motivation to obtain food reward during operant wall task. Moreover, the transplantation of gut microbes from obese mice into lean mice

alters dopaminergic and opioid markers in reward-related brain areas. By using untargeted metabolomic approach in the cecal and plasma of donor and recipient mice we highlighted potential mediators in the gut-brain axis influencing the reward system. We identified a gut-derived metabolite, the 3-(3-hydroxyphenyl)propionate (33HPP) as highly positively correlated with motivation during operant wall test. By administrating 33HPP in mice we revealed its effects on food reward. Therefore, our data suggest that targeting the gut microbiota and its metabolites would be interesting as therapeutic strategy for compulsive eaters, preventing inappropriate hedonic food intake.

2.2. Introduction

Inappropriate food behavior combined with an increased availability of energy-dense foods is an important contributor to the emerging worldwide obesity epidemic [1]. To maintain a stable body weight, energy intake and energy expenditure should be balanced. This process is regulated at the level of the hypothalamus and is referred to as the homeostatic regulation of food intake [21]. By opposition, the reward system is referred as the non-homeostatic regulation of food intake and induces hedonic – or pleasure-related – consumption of foods enriched in fat and sugar. The pleasure is encoded by a mechanism linked to the stimulation of dopamine release from dopaminergic neurons in mesocorticolimbic area of the brain [312]. These dopaminergic neurons project from the VTA to the striatum, the NAc and the PFC. Although dopamine is the main neurotransmitter of the reward system, other mediators such as opioids and endocannabinoids have also been involved in hedonic feeding [244; 313]. The food reward can be divided into three components: the liking, the wanting, and the learning [314]. The liking refers to the hedonic value attributed to food, the wanting to the motivational process to seek out and consume certain foods, and the learning to the reinforcing of conditioning behavior, associated with food intake stimulus [99; 315]. Food reward alterations are considered to be major drivers for body weight gain, promoting obesity by overriding the homeostatic regulation of food intake. In fact, obesity itself is often characterized by inappropriate behavioral components of the food reward system, as well as by a hypofunctioning of the dopaminergic pathways

which then leads to overconsumption of food in an attempt to compensate for the lack of pleasure generated. This has been suggested in obese patients [107; 112; 113; 316].

Both neuronal (*i.e.* the vagus nerve) and humoral pathways link the gut and brain to modulate homeostatic and non-homeostatic food intakes [317]. Importantly, these two types of food intake regulations can overlap. For example, key hormones mediating homeostatic food intake (leptin, insulin, ghrelin and GLP-1) also influence reward responses [21; 234; 318; 319]. In obesity, the plasma concentrations of these mediators are altered, leading to a perturbation of the gut-brain axis and dysregulated food behavior[21].

The gut microbiota has emerged as a key player mediating the gut-brain axis by interfering with vagal and humoral pathways to modulate host metabolism and food intake [136; 143; 193; 194]. Gut microbes are able to stimulate the production of satiety hormones (GLP-1 and PYY) through the production of SCFAs, and to interact with the vagus nerve [193; 299]. In obesity, the gut microbiota composition is unbalanced and contributes to reinforcing the impairment of the gut-brain axis. Although, the role of the gut microbiota in homeostatic regulation of food intake is largely described and supported by strong literature, the causal role of the gut microbiota in non-homeostatic regulation of food intake is poorly understood. We previously demonstrated the proof-of-concept that gut microbes from obese mice are involved in alterations of the liking component of food intake, since FMT is able to reproduce hedonic alterations of obese donors mice in lean mice transplanted with the microbiota harvested from obese animals [320].

In this study, we aim to assess the role of gut microbes in the wanting and the learning component of food reward by means of the operant wall test and the CPP test respectively, both of which are validated tools for behavioral analysis in rodents. We evaluated these components in lean and obese mice (*i.e.* donor mice) as well as in lean mice transplanted with the gut microbiota harvested from these lean and obese donors (*i.e.* gut microbiota recipient mice). Our second objective is to point out how microbiota from obese donors could impact the food reward system, and to identify new mediators involved in the gut-brain axis by using untargeted metabolomics analyses.

2.3. Results

FMT does not transfer obese phenotype (fat mass and body weight) into lean recipient mice

We first validated the development of obesity in the cohort of donor mice: four weeks after starting the HFD treatment, obese donors (DIO_do) showed a higher body weight compared to lean donors (Lean_do, Fig. 1A). Fat mass difference was significant between lean and obese donors after 5 weeks of follow-up (Fig. 1B). For the food behavior tests, caloric restriction to reach 85% of initial body weight was required to potentiate the activation of the food reward system. The caloric restriction allowed to potentiate the reward response to the stimulus [321; 322]. However, the differences in body weight and fat mass were maintained all along the experiment and mice under HFD remained significantly heavier and fatter than mice under CT diet during the subsequent behavioral tests (Fig. 1A,B). We used the FMT protocol previously described to assess the role of the gut microbiota in the learning and motivational components of the food reward [320]. In brief, we sampled fecal samples from lean (Lean_do) or obese (DIO_do) mice and inoculated them by oral gavage into antibiotic-treated lean recipient mice (Lean_rec and DIO_rec respectively). All recipient mice were kept under the same low-fat control diet. In contrast to the donors, there was no difference in terms of body weight (Fig. 1C) or fat mass (Fig. 1D) between mice receiving a gut microbiota from obese donors (DIO_rec) compared to mice receiving a gut microbiota from lean donors (Lean_rec). As in our previous FMT experiment and many others in the literature, donor mice did not transfer their obese phenotype into recipient mice after FMT [301; 320].

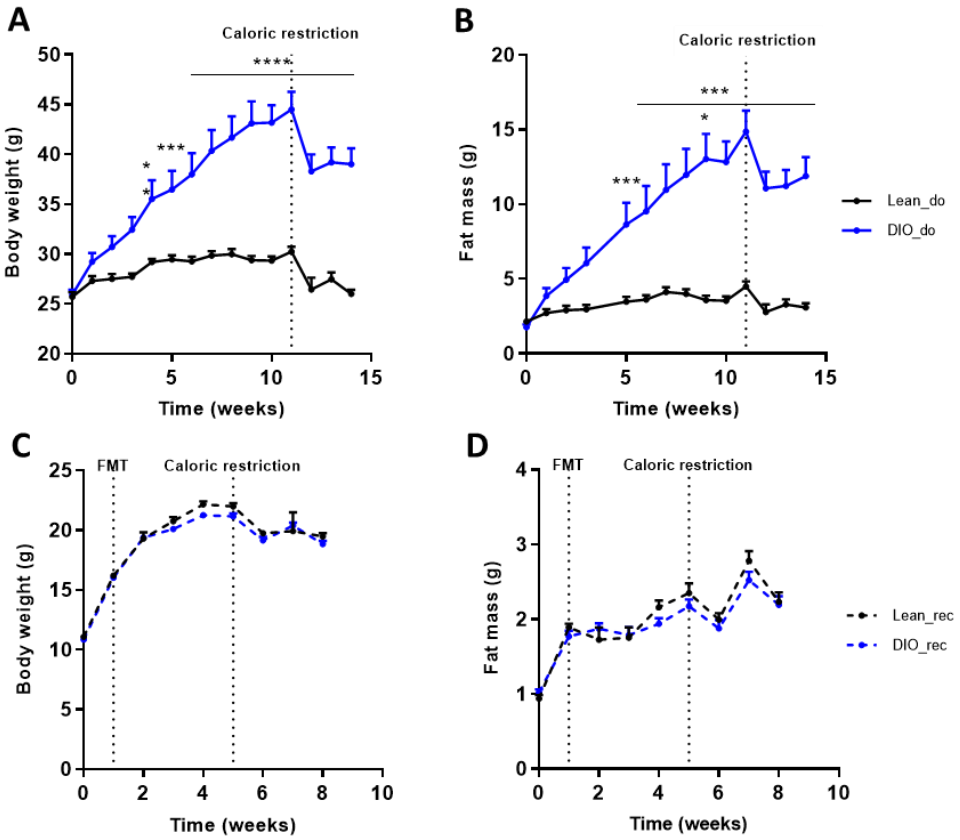


Figure 1: High-fat diet-induced obesity is not transferred through FMT in recipient mice. (**A**) Body weight and (**B**) fat mass evolutions (g) of lean (Lean_do) and DIO donor mice (DIO_do). (**C**) Body weight and (**D**) fat mass evolutions (g) of lean (Lean_rec) and DIO recipient mice (DIO_rec). Data are shown as mean ± SEM (n=7-8/group). p-values were obtained after Two-way ANOVA, followed by Bonferroni post-hoc test. **: p-value ≤ 0,01; *** : p-value ≤ 0,001; **** : p-value ≤ 0,0001.

Alterations in the learning component of the food reward associated with obesity are partially transferred through FMT

The learning component of the food reward is related to the cognitive process between the intake of specific food and the pleasure. To investigate the roles of gut microbes in the learning, we assessed if the obese-related alterations of learning component of the food reward were transferred through fecal transplantation, by testing donor and recipient mice for CPP (Fig. 2). The aim of this test is to evaluate to what extent mice could be conditioned to prefer a compartment with a food stimulus, even after the stimulus was removed. Here

we aimed to increase the time spent by the mouse in one side of the cage after being restrained in this side during the training sessions with a palatable food pellet stimulating the reward system (Reese's®). A pre-test is used to determine whether mice had a pre-existing preference for any of the compartments at baseline: the less preferred compartment would then be used for palatable food conditioning trainings, whereas the most preferred is used for the neutral conditioning trainings.

Both lean and obese donors spent more time in the compartment associated with palatable food (Reese's®) during the test than during the pre-test, suggesting that they are both able to reverse their initial preference for one side of the cage after the training sessions (Fig. 2A). However, the learning component of the food reward is more efficient in lean mice (Lean_do) than in obese mice (DIO_do). Indeed, the difference of time spent in the palatable food associated compartment compared to neutral compartment tends to be lower in obese mice compared to lean mice ($p=0.1$, Fig. 2A).

In order to evaluate the roles of gut microbes in the learning component of the food reward, we investigated the ability of gut microbiota recipient mice from lean and obese donors to associate an environment with food-induced pleasure during the CPP. Recipients of a lean gut microbiota (Lean_rec) reversed their initial preference for one compartment and significantly increased the time spent in the palatable side during the test as compared to the pre-test (Fig. 2B). In opposition, even if they spent more time in the palatable associated compartment during the test, gut microbiota recipient mice from obese donors (DIO_rec) showed no significant difference in the preference score for the palatable side during the test as compared to the pre-test (Fig. 2B). The DIO_rec group failed to reverse their initial preference for one side of the cage, reflecting their inability to effectively associate the side of the cage with palatable food-induced pleasure. These results suggest that recipient of gut microbiota from obese donors have a dysregulated learning component of the food reward. Altogether, our data demonstrate that the slight alterations of the learning component of the food reward associated with obesity are transferred through fecal material transplantation between donor and recipient mice.

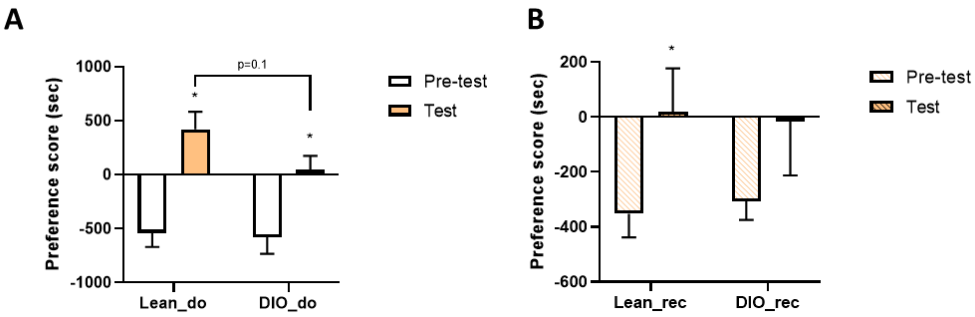


Figure 2: Obese mice show an alteration of the learning component of the food reward, transferred by gut microbes. (**A**) Preference score of conditioned place preference based on the difference of time spent (s) in the palatable food-associated side *vs* the time spent in the neutral-associated side of the cage during the pre-test and the test by lean (Lean_do) or DIO donor mice (DIO_do) after 11 weeks follow-up. (**B**) Preference score of conditioned place preference based on the difference of time spent (s) in the palatable food-associated side vs the time spent in the neutral-associated side of the cage during the pre-test and the test by lean (Lean_rec) or DIO recipient mice (DIO_rec) after 5 weeks follow-up. Data are shown as mean ± SEM (n=6/group). p-values were obtained after paired Student's t-test. *: p-value ≤ 0,05 between preference scores during test and pre-test.

Gut microbiota recipient mice from obese donors show excessive motivation for food reward

To assess the wanting component or the motivation to obtain food reward, donor and recipient mice underwent an operant wall test in which they had to press on a lever to receive a rewarding sucrose pellet (Fig. 3). The first three sessions of the test were based on a fixed ratio (FR) principle: one food reward required one lever press. In later sessions, mice had to press an incrementally increasing number of times (n+3) on the lever in order to obtain each new sucrose pellet. These progressive ratios sessions (PR) allowed us to assess their motivation to obtain a food reward.

Mice in obese condition (DIO_do) pressed significantly less on the lever during PR sessions as compared to lean mice (Lean_do) (Fig. 3A). The number of reward pellets obtained is also significantly less for obese donors (DIO_do) than for lean donors (Lean_do) during PR sessions (Fig. 3B). As PR sessions are the better reflection of the motivation to obtain a reward, our data show that obesity is associated with an alteration of the motivational

component of the food reward. These data are consistent with results previously described in literature [260; 323].

In order to decipher the roles of gut microbes in these alterations of wanting components associated with obesity, we investigated the motivation of mice transplanted with obese or lean gut microbiota to obtain sucrose pellets. Surprisingly, gut microbiota recipient mice from obese donors (DIO_rec) pressed more on the lever during PR sessions 2, 3 and 4 ($p=0.05$ during PR2, $p<0.05$ during PR3, $p=0.07$ during PR4), as compared to lean gut microbiota recipient mice (Lean_rec, Fig. 3C). This tendency was reflected by the higher number of rewards obtained by the mice inoculated with gut microbes from obese donors (DIO_rec) during the PR sessions 2, 3 and 4 (Fig. 3D).

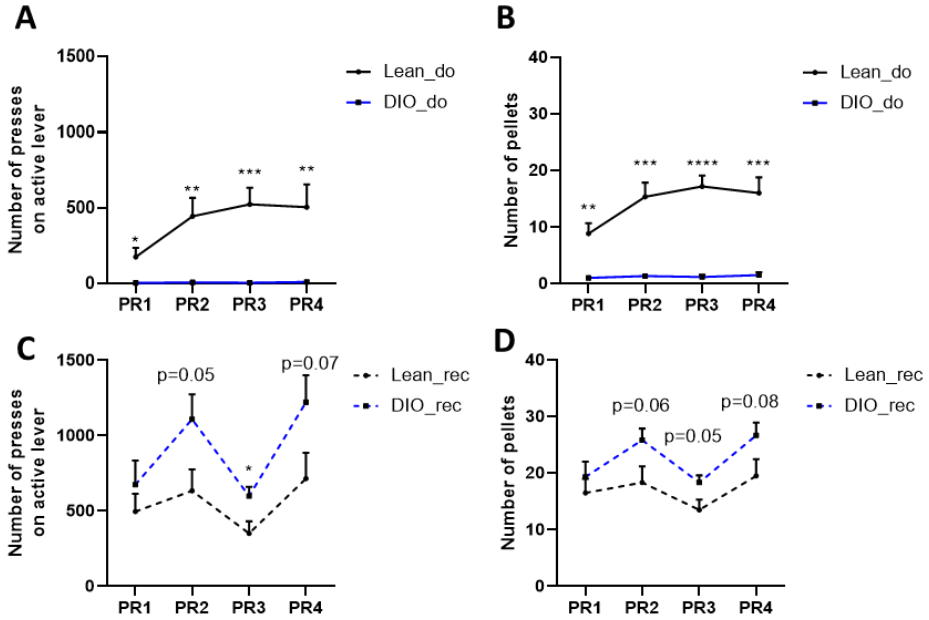


Figure 3: Gut microbes from obese donors lead to excessive motivation for food reward. Operant conditioning test showing (**A**) the number of active lever presses and (**B**) the number of pellets earned during the progressive ratio sessions (PR) by lean (Lean_do) and obese donor mice (DIO_do) after 11 weeks follow-up. Operant conditioning test showing (**C**) the number of active lever presses and (**D**) the number of pellets earned during the progressive ratio sessions (PR) by gut microbiota recipient mice from lean (Lean_rec) and obese donors (DIO_rec) after 5 weeks follow-up. Data are shown as mean ± SEM (n=6/group). p-values were obtained after unpaired Student's t-test. *: p-value ≤ 0,05 ; ** : p-value ≤ 0,01; *** : p-value ≤ 0,001; **** : p-value ≤ 0,0001.

Interestingly, these results suggest that gut microbiota recipient mice from obese donors (DIO_rec) behave in an opposite way as their obese donors (DIO_do) in this test assessing the motivation to obtain a reward, since the former pressed approximately 100 times more on the lever to obtain a food reward than the latter. It is worth noting that the absolute values of number of lever presses are similar between Lean_rec and Lean_do groups (Fig. 3A, C). Gut microbiota recipient mice from obese donors (DIO_rec) showed particularly higher values of active lever presses (Fig. 3D), suggesting an excessive motivation for a food reward, rather than a normal motived behavior observed in lean conditions.

Lean or obese microbiota signatures are maintained after FMT

In order to assess the correct engraftment of the donor gut microbiota into recipient mice, we compared gut microbiota compositions of donor and recipient mice by Illumina 16S sequencing. As expected and according to the scientific literature, β-diversity indices such as the Jaccard distance showed two clusters differentiating between the two diets along the PCoA1 axis (Fig. 4A). Indeed, Lean_do, Lean_rec and DIO_rec groups under CT diet stuck apart from the DIO_do group under HFD.

Importantly, Lean_do and Lean_rec samples overlapped on the PCoA plots, suggesting an appropriate transfer of gut microbiome from Lean_do to Lean_rec (Fig. 4A,B). Significant differences between these two groups shown by PERMANOVA ($p < 0.05$) in the case of Jaccard distances were most likely due to a significant difference in dispersion (Fig. 4A, PERMDISP, $p < 0.01$). DIO_do and DIO_rec samples formed separate clusters on the PCoA plots, but DIO_rec samples were also separate from the Lean_do and Lean_rec samples, suggesting that even if the composition of DIO_rec mice was affected by the CT diet, the difference from the lean gut microbiota persisted (Fig. 4A,B).

Consistently, the Venn diagram demonstrate that 76.9% of genera were shared between lean donors (Lean_do) and lean gut microbiota recipient mice (Lean_rec); and 74.7% of genera were shared between obese donors (DIO_do) and their recipient mice (DIO_rec) (Fig. 4C).

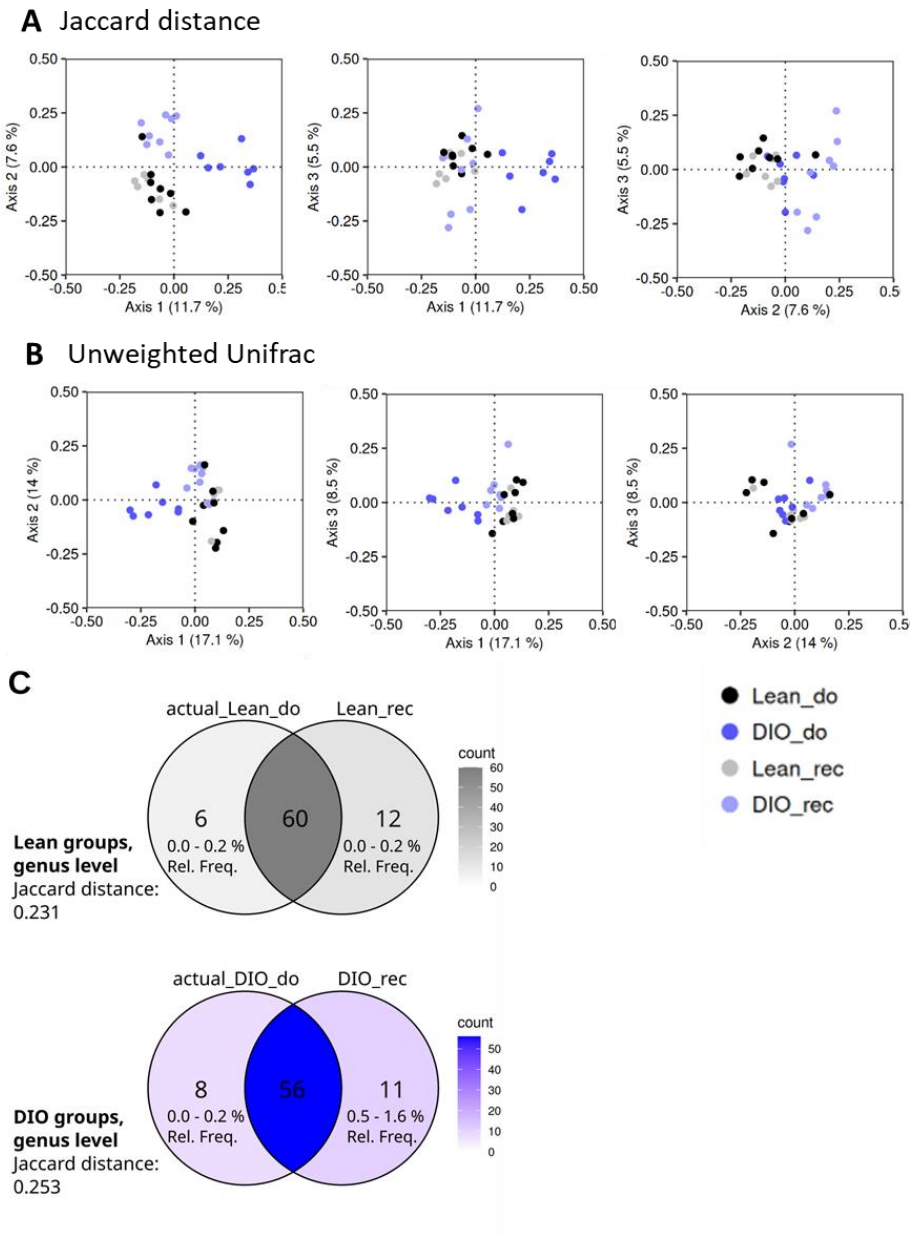


Figure 4: The majority of gut microbes from lean and obese donor mice engrafted lean gut microbiota recipient mice. Principal coordinates analysis (PCoA) for ASV-level data based on (**A**) the Jaccard distance and (**B**) the unweighted UniFrac. Each symbol representing a single sample is colored according to its group. (**C**) Venn diagrams depicting shared genera between donor (Lean_do and DIO_do) and recipient (Lean_rec and DIO_rec) mice. Cecal contents were collected after 5 weeks of follow-up in donor mice and after 11 weeks follow-up in recipient mice.

Gut microbes from obese mice alter the brain reward system of recipient mice

To better understand the excessive motivation of mice recipient of gut microbes from obese mice for a food reward, we investigated the expression of dopaminergic and opioid markers in the NAc of gut microbiota recipient mice from lean and obese donors (Fig. 5). We found a significant decrease in the expression of dopamine receptor 2 (*Drd2*) and the enzyme synthetizing dopamine, tyrosine hydroxylase (*Th*) in the NAc of mice recipient of a gut microbiota from obese donors compared to mice recipient of gut microbes from lean donors. Dopamine receptor 1 (*Drd1*) and the dopamine transporter (*Dat*) tended to be reduced in mice transplanted with gut microbiota from obese mice compared to mice transplanted with gut microbiota from lean mice, but this did not reach significance (DIO_rec *vs* Lean_rec, Fig. 5A).

Since the opioid system is also involved in food reward, and has been shown to be blunted in obese conditions [313; 324], we measured the expression of some key actors and found that DIO_rec had a significant reduction in the NAc expressions of μ-opioid receptor (*Oprm*), a similar trend for reduction in κ-opioid receptor (*Oprk*, p=0.05) and the precursor of the dynorphin (*Pdyn*, pre-prodynorphin, p=0.06, Fig. 5B). The expression of δ-opioid receptor (*Oprd*) did not differ between Lean_rec and DIO_rec (Fig. 5B).

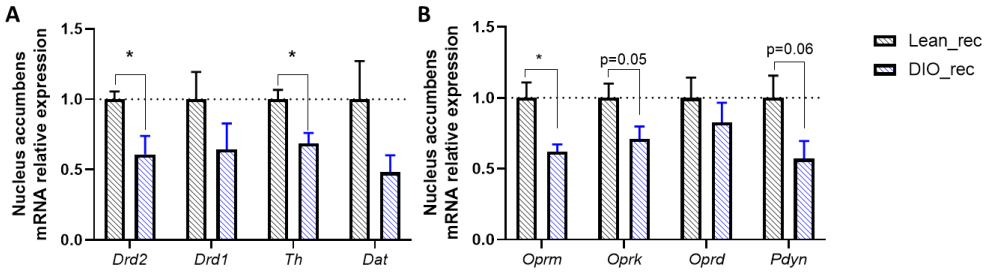


Figure 5: Dopaminergic and opioid systems of gut microbiota recipient mice from obese donors are downregulated. Nucleus accumbens mRNA expressions of (**A**) dopamine receptor 2 (*Drd2*), dopamine receptor 1 (*Drd1*), tyrosine hydroxylase (*Th*), dopamine transporter (*Dat*), (**B**) μ-opioid receptor (*Oprm*), κ-opioid receptor (*Oprk*), δ-opioid receptor (*Oprd*) and pre-prodynorphin (*Pdyn*) measured by qPCR in lean (Lean_rec) and DIO recipient mice (DIO_rec) after 11 weeks follow-up. Data are shown as mean ± SEM (n=7-8/group). p-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. * : p-value ≤ 0,05.

Excessive motivation for food reward is not associated with modulations of homeostatic regulators of food intake

To understand how gut microbes from obese mice could act on the behavioral and neuronal reward system in lean conditions (recipient mice), we analyzed several mediators of the gut-brain axis involved in the regulation of homeostatic food intake that are also able to influence the food reward system. Therefore, we measured ghrelin, insulin, leptin, GLP-1 and PYY in the plasma of recipient mice, as well as in donor mice. None of the homeostatic regulators analyzed in the plasma were different between gut microbiota recipient mice from lean and obese donors (Lean_rec vs DIO_rec, Fig. 6). In contrast, we observed typical hormonal changes associated with obesity in the plasma of obese donor mice such as a significant decrease in ghrelin (Fig. 6A), a significant increase in insulinemia (Fig. 6B) and leptinemia (Fig. 6C) compared to lean mice (DIO_do vs Lean_do). Plasma levels of GLP-1 and PYY were not significantly different between lean and obese donor mice (Lean_do vs DIO_do, Fig. 6D,E).

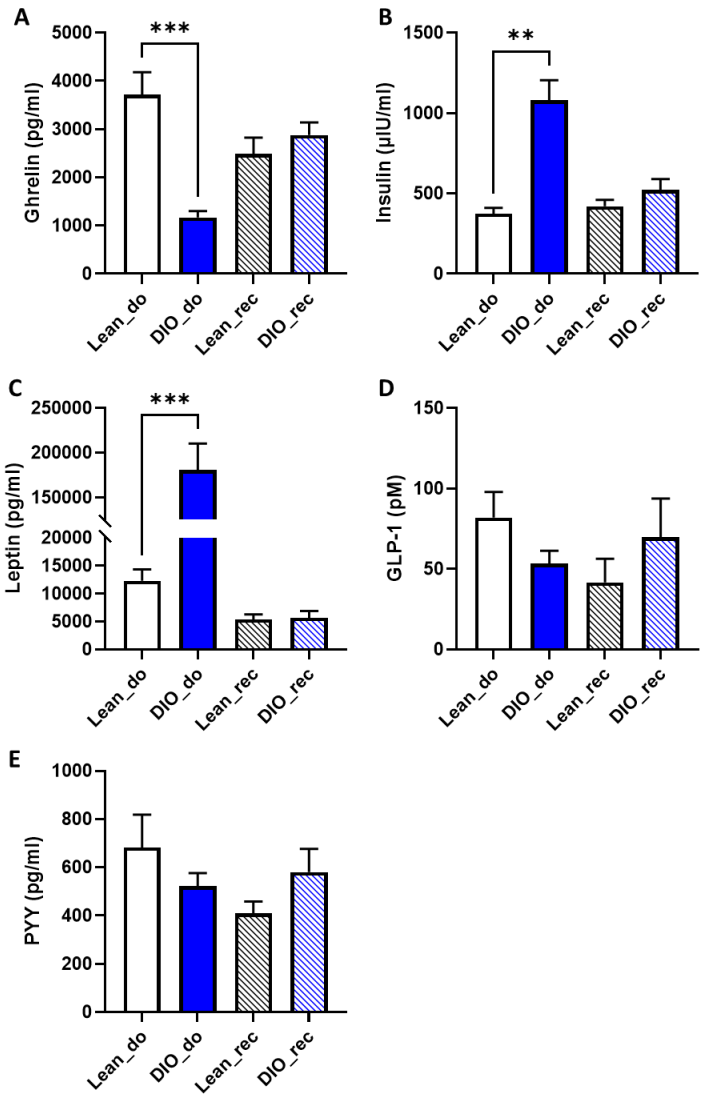


Figure 6: Homeostatic regulators of food intake are similar between recipient mice. Plasma concentrations of (A) ghrelin, (B) insulin, (C) leptin, (D) Glucagon-like peptide-1 (GLP-1) and (E) Peptide YY (PYY) in lean (Lean_do) and obese donor mice (DIO_do) after 11 weeks follow-up and gut microbiota recipient mice from lean and obese donors (Lean_do and DIO_rec) after 5 weeks follow-up. Data are shown as mean ± SEM (n=7-8/group). p-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test between lean and obese (DIO) donor and recipient mice separately. ** : p-value ≤ 0,01; *** : p-value ≤ 0,001.

Excessive motivation for food reward is associated with changes in metabolomic profile

Since none of the hormonal mediators of the gut-brain axis that we analyzed were discriminant between gut microbiota recipient groups, we used untargeted metabolomics to identify potential metabolites involved in the regulation of the reward system and the motivational behavior. We screened both plasma and cecal samples of mice recipient of gut microbiota from lean and obese donors and further univariate analyses such as volcano plots allowed us to illustrate metabolites affected (*i.e.* increased or decreased) by the gut microbiota from obese mice compared to lean mice in the plasma and the cecal content (Fig. 7A and B, and Table 1). Applying a p-value set at 0.05 for the Wilcoxon test and considering a minimal 2-fold change, we identified 14 metabolites significantly modified in the plasma of DIO_rec group compared to Lean_rec (Fig. 7A and Table 1). In the cecal content, 33 metabolites were significantly different between Lean_rec and DIO_rec (Fig. 7B and Table 1). Two metabolites, namely 3-(3-hydroxyphenyl)propionate (33HPP) and 3-(4-hydroxyphenyl)propionate (34HPP), were similarly affected in the plasma and the cecum of mice transplanted with the gut microbiota from obese mice. Since these compounds are specific gut microbiota metabolites, they represented interesting candidate molecules possibly involved in mediating the effects of gut microbes on the food reward. Indeed, we speculated that they would be absorbed in the intestine and then exert an action on the reward system through the blood circulation.

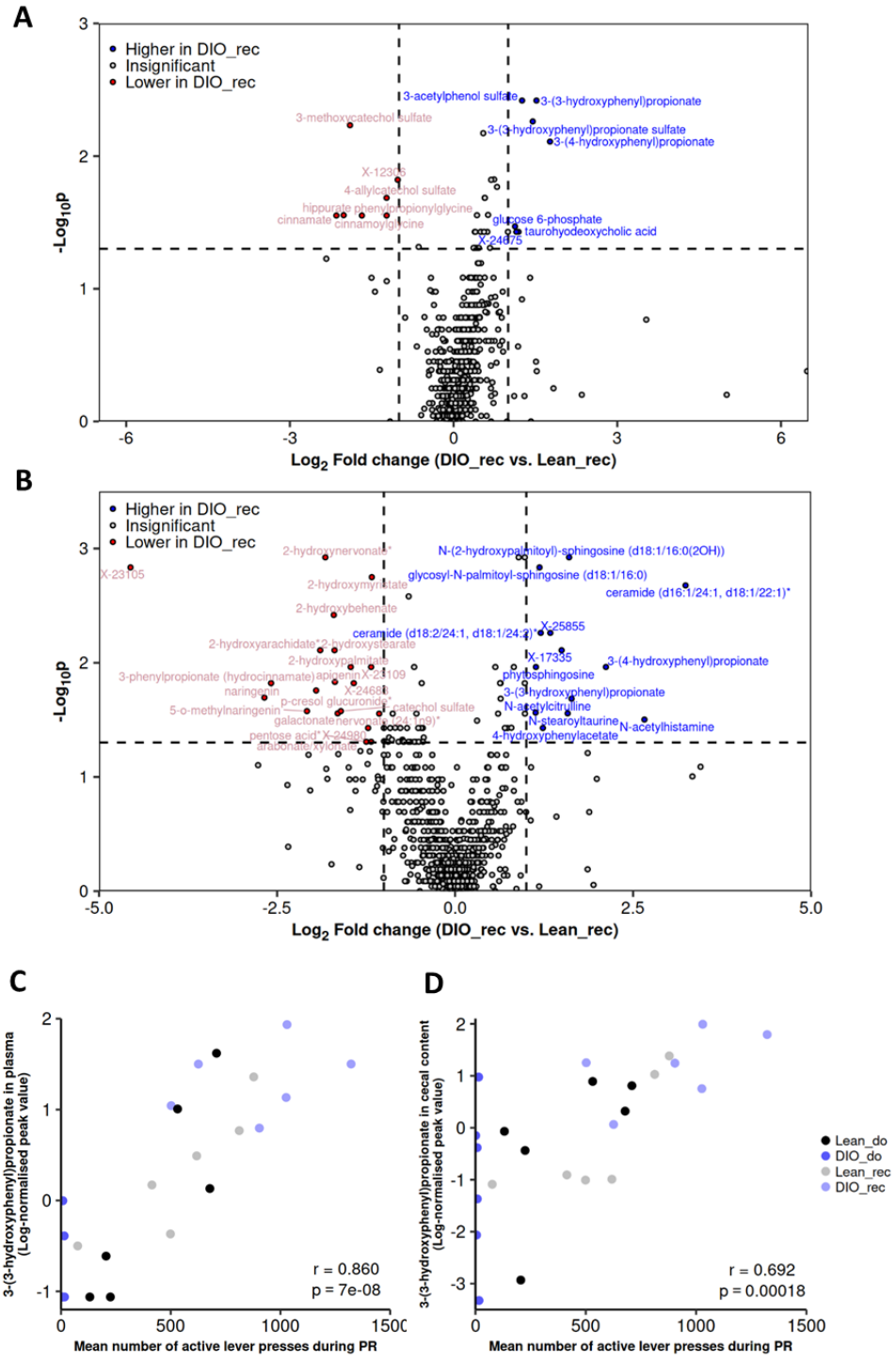

A
Higher in DIO_rec
Insignificant
Lower in DIO_rec
3-acetylphenol sulfate
3-(3-hydroxyphenyl)propionate
3-methoxycatechol sulfate
3-(3-hydroxyphenyl)propionate sulfate
3-(4-hydroxyphenyl)propionate
X-12306
4-allylcatechol sulfate
hippurate phenylpropionylglycine
cinnamate
cinnamoylglycine
glucose 6-phosphate
taurohyodeoxycholic acid
X-24675
-Log10p
Log2 Fold change (DIO_rec vs. Lean_rec)
B
Higher in DIO_rec
Insignificant
Lower in DIO_rec
2-hydroxynervonate*
N-(2-hydroxypalmitoyl)-sphingosine (d18:1/16:0(2OH))
X-23105
glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)
2-hydroxymyristate
ceramide (d16:1/24:1, d18:1/22:1)*
2-hydroxybehenate
X-25855
ceramide (d18:2/24:1, d18:1/24:2)*
2-hydroxyarachidate*
2-hydroxystearate
X-17335
3-(4-hydroxyphenyl)propionate
2-hydroxypalmitate
3-phenylpropionate (hydrocinnamate)
apigenin
X-23109
phytosphingosine
naringenin
X-24683
3-(3-hydroxyphenyl)propionate
p-cresol glucuronide*
N-acetylcitrulline
5-o-methylnaringenin
3-methylcatechol sulfate
galactonate
N-stearoyltaurine
N-acetylhistamine
pentose acid*
X-24980
4-hydroxyphenylacetate
arabonate/xylonate*
-Log10p
Log2 Fold change (DIO_rec vs. Lean_rec)
C
3-(3-hydroxyphenyl)propionate in plasma (Log-normalised peak value)
r = 0.860
p = 7e-08
Mean number of active lever presses during PR
D
3-(3-hydroxyphenyl)propionate in cecal content (Log-normalised peak value)
r = 0.692
p = 0.00018
Mean number of active lever presses during PR
Lean_do
DIO_do
Lean_rec
DIO_rec

Figure 7: Untargeted metabolomics analysis reveal a metabolic candidate onto the gut-brain axis regulating the motivation for food reward. Volcano plots showing metabolites discriminant between gut microbiota recipient mice from lean and obese donors in (**A**) the plasma and (**B**) in the cecal content after 5 weeks of follow-up. Metabolites with labeled point were significantly down (blue) or up (red) in gut microbiota recipient mice from obese donors (DIO_rec) after non-parametric Wilcoxon test (p-value < 0.05) and were more than 2-fold changed compared to lean gut microbiota recipient mice. Metabolites that did not satisfy the threshold of significance at Wilcoxon test and that were less than 2-fold changed compared to the lean gut microbiota recipient mice, appear in grey and are not labeled. The horizontal line corresponds to the p-value cutoff of 0.05 at non-parametric Wilcoxon test, the vertical lines correspond to the fold change cutoff of 2. See also **Table 1** for the detailed list of the significantly different metabolites between gut microbiota recipient mice from lean and obese donors. Metabolites with a star (*) are partially characterized, and. X metabolites are unidentified. Pearson's correlations between 3-(3-hydroxyphenyl)propionate (33HPP) concentrations in (**C**) the plasma or in (**D**) the cecal content of donor and gut microbiota recipient mice and the mean number of lever presses on the active lever during the progressive ratio (PR) sessions of the operant wall test.

To further explore this hypothesis, and to make the link between metabolites in the plasma and the motivational behavior, we integrated all metabolites from the untargeted metabolomics analysis of donor and recipient mice into a correlation matrix with the mean number of active lever presses during PR sessions of the operant wall test as a variable. After FDR correction, we discovered that the concentrations of 26 metabolites correlated significantly with the motivational parameter in both plasma and cecum (Suppl. Table 2, Fig. 7C,D). The 33HPP appeared as the highest correlated metabolite in the plasma, potentially acting through the gut-brain axis (Fig. 7C).

Metabolites in plasma	*Metabolites in cecal content*
3-(3-hydroxyphenyl)propionate	2-hydroxyarachidate*
3-(3-hydroxyphenyl)propionate sulfate	2-hydroxybehenate
3-(4-hydroxyphenyl)propionate	2-hydroxymyristate
3-acetylphenol sulfate	2-hydroxynervonate*
3-methoxycatechol sulfate	2-hydroxypalmitate
4-allylcatechol sulfate	2-hydroxystearate
cinnamate	**3-(3-hydroxyphenyl)propionate**
cinnamoylglycine	**3-(4-hydroxyphenyl)propionate**
glucose 6-phosphate	3-phenylpropionate (hydrocinnamate)
hippurate	4-hydroxyphenylacetate
phenylpropionylglycine	5-o-methylnaringenin
taurohyodeoxycholic acid	apigenin
X-12306	arabonate/xylonate
X-24675	catechol sulfate
	ceramide (d16:1/24:1, d18:1/22:1)*
	ceramide (d18:2/24:1, d18:1/24:2)*
	galactonate
	glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)
	N-(2-hydroxypalmitoyl)-sphingosine (d18:1/16:0(2OH))
	N-acetylcitrulline
	N-acetylhistamine
	N-stearoyltaurine
	naringenin
	nervonate (24:1n9)*
	p-cresol glucuronide*
	pentose acid*
	phytosphingosine
	X-17335
	X-23105
	X-23109
	X-24683
	X-24980
	X-25855

Table 1: List of the significantly different metabolites between gut microbiota recipient mice from lean and obese donors after untargeted metabolomics analysis. Metabolites significantly down (red) or up (blue) in gut microbiota recipient mice from obese donors (DIO_rec) after non-parametric Wilcoxon test (p-value < 0.05) and more than 2-fold changed compared to lean gut microbiota recipient mice after 5 weeks of follow-up. Metabolites with a star (*) are partially characterized, and. X metabolites are unidentified.

These strong correlations suggest that this microbial metabolite could be a potential candidate involved in the effect of gut microbes on the motivational component of the food reward in gut microbiota recipient mice and acting via the blood circulation. Of note, we found reduced 33HPP in the plasma of obese mice compared to lean donor mice (DIO_do vs Lean_do) and in contrast, particularly increased 33HPP in the plasma and in the cecal content of mice under control diet, receiving a gut microbiota from obese donors (DIO_rec, Suppl. Fig.1 A,B).

As 33HPP is a gut-derived metabolite, we sought to identify bacteria producing 33HPP in the gut of recipient mice from obese donors. For this purpose, we correlated gut microbiota sequencing data from donor and recipient mice with the levels of 33HPP in the plasma and in the cecum (Suppl. Table 3). We found that *Akkermansia* (Suppl. Fig. 2A)*, Muribaculum* (Suppl. Fig. 2B) and, considering the scatterplots, especially *Prevotellaceae* (Suppl. Fig. 2C), *Alloprevotella* (Suppl. Fig. 2D) and *Parabacteroides* (Suppl. Fig. 2E) were correlated with 33HPP levels in the plasma and in the cecal content.

The gut microbiota-derived metabolite 33HPP influence the expression of key markers associated with reward in the NAc

Since gut microbiota recipient mice from obese donors showed excessive motivation and expression of specific markers related to reward in the NAc (decrease of dopaminergic and opioid receptors), and that this was associated with a higher concentration of 33HPP in the cecum and the blood, we investigated whether 33HPP could have a direct impact on food reward in mice, independently of gut microbes. To this end, we administered 33HPP daily by intra-peritoneal injection to lean mice fed with a control diet. We evaluated the specific effect of 33HPP on dopaminergic and opioid receptors in the NAc and the impact on the operant conditioning.

Surprisingly, we found an increased expression of *Drd2* and *Drd1* in lean mice injected with 33HPP (33HPP) compared to lean mice injected with the vehicle (TBS) (Fig. 8A). The expression of *Dat* tended to be decreased after 33HPP supplementation (p=0.06, Fig. 8A), whereas the expression of the *Th* was not significantly affected (Fig. 8A). In the same line,

the expressions of *Oprm* and *Oprk* were increased in 33HPP-treated mice as compared to TBS-treated mice, as well as the expression of *Pdyn* (Fig. 8B). The expression of the *Oprd* was not significantly affected by 33HPP administration (Fig. 8B).

However, 33HPP did not influence the motivation of lean mice to obtain a food reward since the number of presses on the active lever (leading to the delivery of a food reward) was equivalent to that of mice injected with the vehicle (33HPP vs TBS) (Fig. 8C). Interestingly, the number of presses on the inactive lever (not leading to the delivery of a food reward) was slightly higher in 33HPP mice compared to TBS mice (Fig. 8D). This trend was even more apparent when calculating the ratio between inactive and active lever presses (Fig. 8E). Mice injected with 33HPP were unable to distinguish the active from the inactive lever.

Since these results suggest a dysfunction in memory and learning process, we investigated dopaminergic markers in the hippocampus, a brain area also implicated in the food reward system as the main regulator for memory and learning process. Lean mice receiving 33HPP had significantly lower expression of *Drd1* compared to placebo treated mice (TBS), whereas the other dopaminergic markers analyzed were not changed (*Drd2, Th, Dat*) (Fig. 8F). This suggests that 33HPP is inducing an alteration of the memory and learning process associated with the reward system.

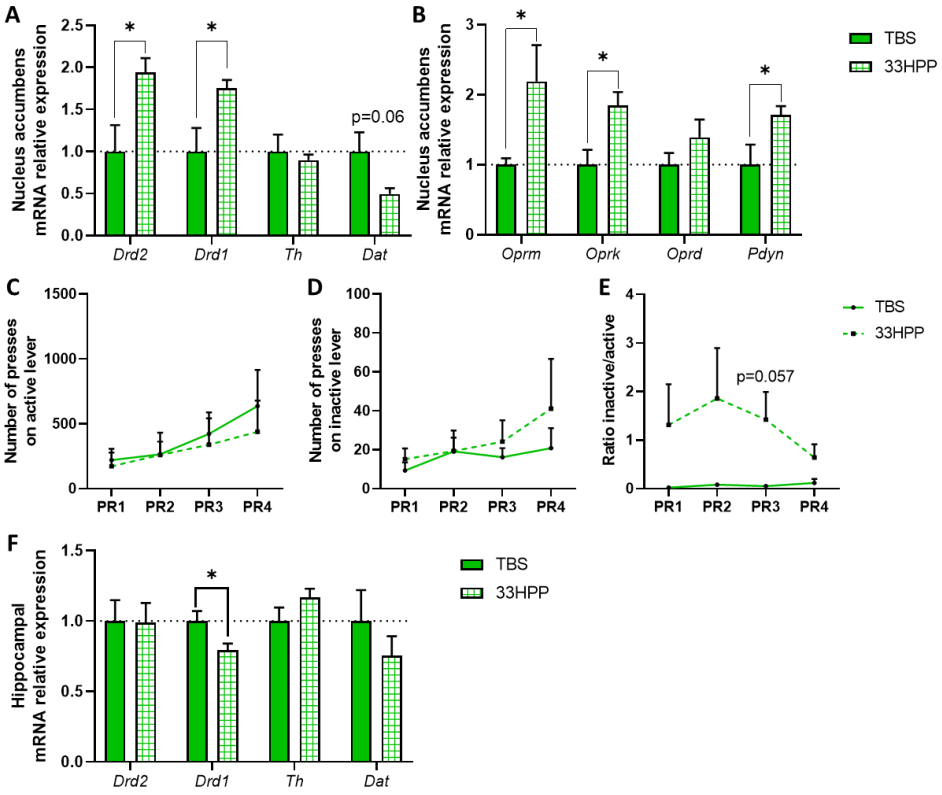


Figure 8: 33HPP modulates the food reward system and alters the memory. (**A**) Nucleus accumbens mRNA expressions of dopamine receptor 2 (*Drd2*), dopamine receptor 1 (*Drd1*), tyrosine hydroxylase (*Th*), dopamine transporter (*Dat*), (**B**) μ-opioid receptor (*Oprm*), κ-opioid receptor (*Oprk*), δ-opioid receptor (*Oprd*) and pre-prodynorphin (*Pdyn*) measured by qPCR in mice injected with TBS (TBS) and with 3-(3-hydroxyphenyl)propionate (33HPP) after 2 weeks of follow-up. Operant conditioning test showing (**C**) the number of active lever presses, (**D**) the number of inactive lever presses and (**E**) the ratio between the number of inactive lever presses on the number of inactive lever presses during the progressive ratio sessions (PR) by mice injected with TBS (TBS) and mice injected with 33HPP (33HPP) after 2 weeks of follow-up. (**F**) Hippocampal mRNA expressions of *Drd2*, *Drd1*, *Th*, *Dat* in mice injected with TBS (TBS) and mice injected with 33HPP (33HPP) after 2 weeks of follow-up. Data are shown as mean ± SEM (n=6/group). p-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test.

2.4. Discussion

In a previous proof-of-concept study using FMT from obese mice donors, we demonstrated that the gut microbiota played a causal role in the dysregulations of the liking component of the food reward system observed during obesity. In this study, we aimed to decipher the role of the gut microbiota in obese conditions in two other aspects of the reward system, *i.e.* the learning and motivational components, by using the CPP and the operant conditioning tests respectively. These behavioral approaches were applied in both lean and obese mice, as well as in lean recipient mice transplanted with gut microbiota from lean or obese donor mice. Interestingly, we discovered that gut microbes from obese mice affect the brain reward system of lean acceptor mice by inducing excessive motivation for food reward. Using untargeted metabolomics analysis on samples from gut microbiota recipient mice, we aimed to identify new metabolites potentially contributing to the altered food reward system during obesity and to the excessive impulse to obtain a food reward observed after inoculation with an obesity-associated gut microbiota. We then administered a newly identified metabolic candidate (33HPP) in lean mice and demonstrated that this metabolite could be implicated in the gut-brain axis, modulating the brain reward system and affecting behavior.

Since we aim to identify the specific roles of gut microbes on the reward system, the fecal transplantation model appears to be the more appropriate approach. In this study, the FMT from either lean or obese donor into lean recipient mice was effective to transfer bacterial genera from donors into recipient mice. Indeed, we have shown that a treatment with broad-spectrum, poorly absorbed, antibiotics and a laxative prior to the fecal material transplantation was efficient to deplete the cecal bacterial load of recipient mice with more than 99% [320]. Moreover, the use of young mice (3-weeks-old) for the transplantation of gut microbiota further improved the engraftment of donor gut microbes [325]. The high percentage of similar genera found in our study between donors and recipients confirms the efficient engraftment of gut microbes from donors into the recipient mice. Our FMT protocol also allows to cluster the gut microbiota composition of donor and recipient mice as illustrated by the PCoA, based on the presence or absence of some bacteria (Jaccard

distance). In this study, transfer of the gut microbiota from obese mice did not replicate the obese phenotype from HFD-induced obese donor into recipient mice (*i.e.* increased body weight gain and fat mass development). Since, mice recipient of gut microbes from obese mice were maintained under control, low-fat diet during the whole protocol, and it has been shown that the type of diet predominates to influence the phenotype rather than the fecal transplantation itself, this could explain our observations [326].

When investigating the reward system from a behavioral point of view, the training sessions with Reese's® were efficient to induce a conditioned behavior in lean donor and recipient mice, confirming the validity of our food-induced CPP protocol. Lean and obese donor mice reverse their initial preference for a particular test chamber. Interestingly, unlike lean donor mice, obese donor mice displayed an altered learning component of the food reward since they tend to spend less time in the palatable-associated compartment. Such cognitive impairments observed with CPP test and associated with obesity have already been described in the literature [114; 327; 328]. In gut microbiota recipient mice, both groups seemed able to associate one side of the cage with palatable food-induced pleasure since they both spent more time in the palatable food-associated chamber during the test as compared to the pre-test. However, only mice transplanted with a gut microbiota from lean mice were able to reverse their original preference after training sessions. Our data demonstrate that the altered learning component of the food reward associated with obesity was partially transferred through FMT.

We further characterized the reward behavior of obese mice and we found a reduced motivation for food reward, as already described in the literature [114; 260]. The altered learning component of the food reward and the decreased motivation in obese mice that we observed are a good reflection of their hypofunctional brain reward system [113; 116; 329]. Surprisingly, mice transplanted with fecal material from obese donors did not behave during the operant wall test like their obese donors. On the contrary, they pressed more often, and even excessively, on the lever to obtain sucrose pellets. This excessive motivation could induce an over-activation of the reward system, which afterwards downregulates as a negative feedback. This hypothesis could explain the downregulation of key mediators of

dopamine and endorphin signaling (*Drd2*, *Th* and *Oprm*, *Oprk*, *Pdyn* respectively) that we observed in mice transplanted with a gut microbiota from obese donors [329]**.** The exaggerated motivation of mice transplanted from obese donors associated with decreased *Drd2* expression could reflect the compulsive behavior that is described in the context of obesity. In 2010, Johnson and Kenny demonstrated that obese rats with decreased expression of *Drd2* showed an addiction-like behavior to eat palatable food [111]. Moreover, it has already been shown that mice fed with a HFD for 6 weeks then suddenly switched on a low-fat diet showed increased operant response for sucrose and fat reward pellets, suggesting craving for palatable food [330]. This was associated with modifications in dopaminergic transmission in the Nac including a decrease in the expression of TH, as we also observed in gut microbiota recipient mice from obese donors fed with a low fat diet and pressing excessively on the lever to obtain sucrose rewards [330].

Homeostatic regulators and their effects on behavioral and neuronal food reward system are well described in the literature [318; 331; 332]. It is well established that food restriction leads to increased reward response to palatable stimulus and that this is facilitated by the decrease in plasma leptin and insulin [333]. In fact, injections of insulin or leptin, reverses their respective effects and decreases the response in CPP and the operant wall tests [321; 334; 335]. Importantly, mice under a moderate HFD, with similar body weights and leptinemia as a control group, showed increased PR response during the operant wall test [336]. This demonstrates that body weight gain and adipose signals are major drivers to the decreased operant response to sucrose pellet under HFD. In our study, gut microbiota recipient mice from obese donors do not show any leptin or insulin elevation typical from obesity. This suggest that their behavior and changes in their brain reward system are mainly due to obese gut microbes rather than homeostatic mediators controlling the reward responses. These results assessing the learning and motivational components of the food reward complete our previous data showing that the liking component was transferable through FMT [320]. Since the liking, the wanting and the learning components of the food reward involve distinct brain areas, mediators and signaling, it could explain why we observe different results for these three components [314; 315].

In the second part of our study, we identified 33HPP as a metabolite being particularly increased in gut microbiota recipient mice from obese donors fed a control diet and correlating with the mean number of active lever presses during the operant conditioning. This metabolite is produced exclusively by bacteria (and not by the host) including by the genera *Clostridium*, *Eubacterium* and *Escherichia* and could be a degradation product from several polyphenols (chlorogenic acid, quercetin, naringenin, apigenin…) [337; 338; 339; 340; 341; 342]. Even if the source of 33HPP in our study remains unclear, some polyphenols compounds (naringenin, apigenin) appeared to be present in the plasma and in the cecal content of recipient mice after metabolomics analyses and could then represent a potential source of 33HPP. 34HPP, another potentially interesting metabolite that we identified in this context, can be produced through the degradation of DOPA by the gut microbiota, and some bacteria including *Enterococcus faecalis* and *Enterococcus faecium* can produce DOPA [225; 343]. This may represent a source of 34HPP in the cecum and in the blood of recipient mice. In the literature, one study showed that administration of polyphenols increases 33HPP levels in the blood and in the brain suggesting the capacity of 33HPP to pass the blood brain barrier [344]. Even if they have not been described so far as producers of 33HPP, unknown *Prevotellaceae*, *Alloprevotella* and *Parabacteroides* also correlated with 33HPP levels in the blood and in the cecal content. This strengthened our previous findings that proposed a role for *Parabacteroides* in the regulation of the food reward system [320].

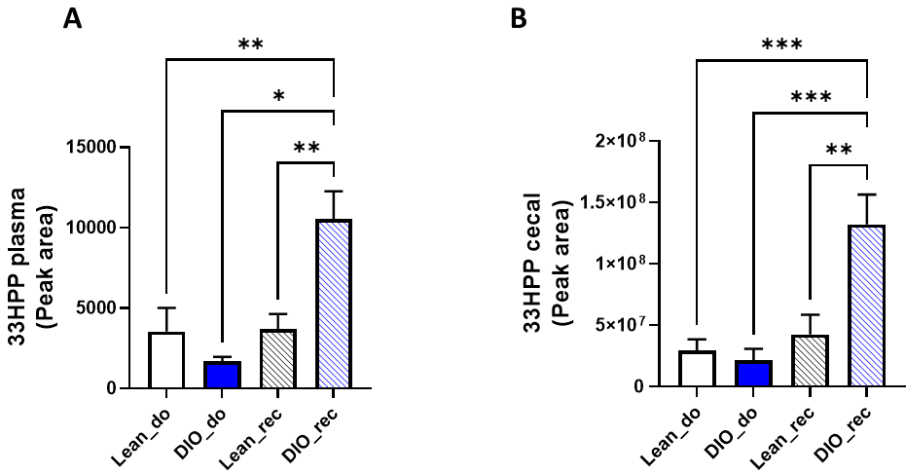
To decouple the effect of gut microbes from obese mice from the effect of 33HPP in mice recipient of gut microbes from obese mice, we injected 33HPP to lean mice under control diet. We found that 33HPP itself had no significant effect on the motivational component of the food reward (number of lever presses) despite an increase in the expression of dopamine and opioid markers. However, the learning process seemed altered in mice injected with 33HPP compared to placebo-treated mice. In fact, 33HPP-treated mice did not seem able to develop goal-directed behavior as they did not make the distinction between the active lever delivering a sucrose pellet and the inactive lever. Moreover, dopaminergic marker *Drd1* was decreased in the hippocampus of mice injected with 33HPP, suggesting an alteration of their reward memory and learning process. Interestingly, some studies

showed that phenolic acid compounds, among which 33HPP, were elevated in children with autism spectrum disorder (ASD) [345; 346; 347]. Among a panel of behavioral symptoms, ASD is characterized by stereotypical actions *i.e*. repetitive, invariant behavior pattern with no obvious goal or function. This pattern could be compared to lever pressing without aiming for the food reward. In the context of ASD, a recent study has shown that an oral drug adsorbing small aromatic or phenolic molecules, AB-2004, was associated with decreased levels of phenolic acids including 34HPP in the blood of mice [348]. Treatment with AB-2004 significantly improves compulsivity assessed with the marble burying test. This study confirms the alterations in the behavior such as anxiety or compulsivity driven by gut microbial production of phenolic compounds, and suggests a promising therapeutic approach through the adsorption of phenolic compounds [348].

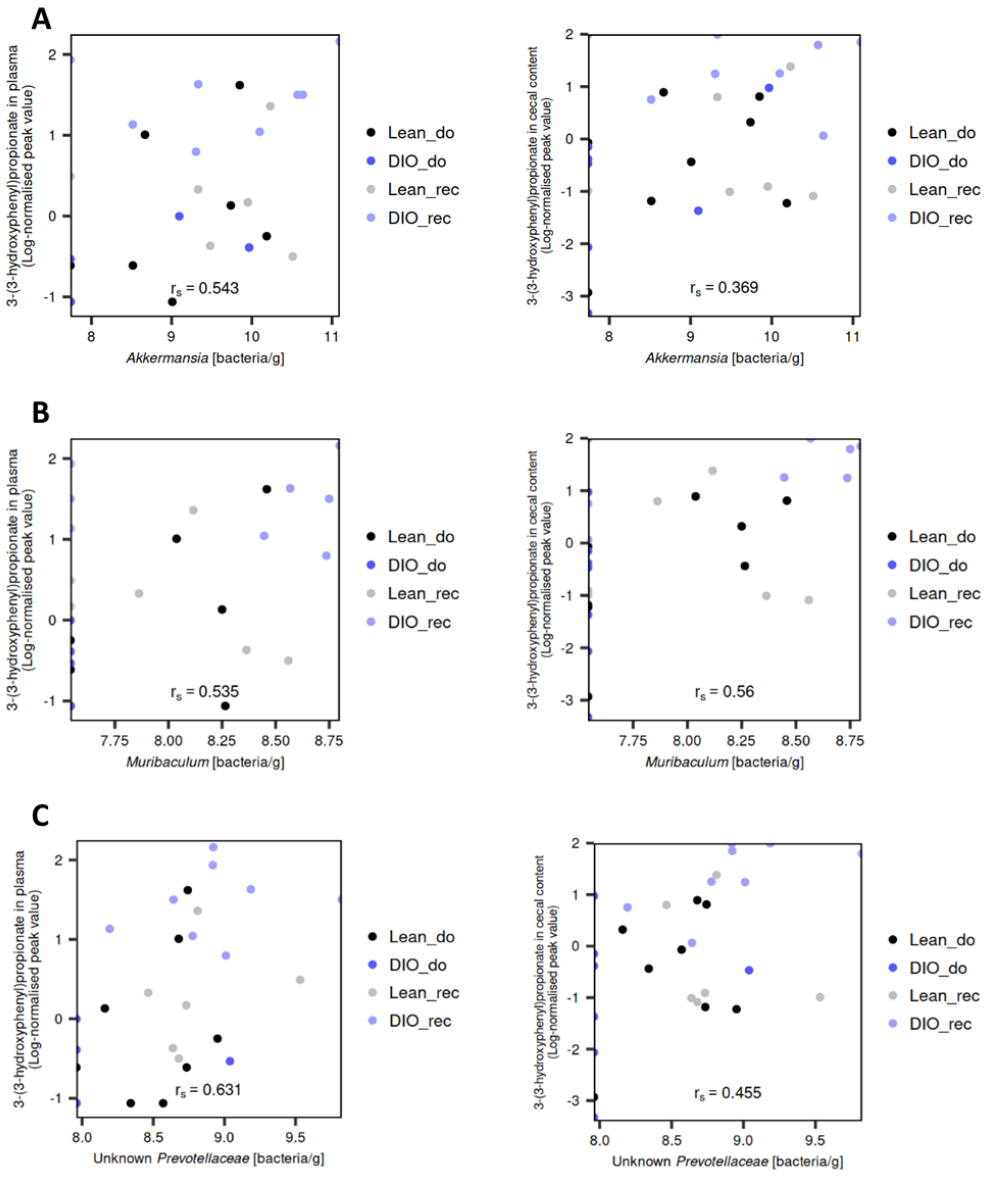
We show here that 33HPP stimulates the expression of key dopaminergic and opioid markers involved in the reward system (*Drd2*, *Drd1*, *Oprm*, *Oprk*, *Pdyn*) in control mice. By combining the results from the 33HPP experiment and the FMT experiment, we speculate that the elevated 33HPP in the blood and in the cecum of mice receiving a gut microbiota from obese donors made them particularly sensitive to a food reward, 33HPP being the main driver for the over-expression of dopaminergic and opioid markers. Over time and after repeated expositions to palatable food, obese recipient mice adapt and show a decreased expression of dopaminergic and opioid markers. These brain patterns are often associated with a compulsive behavior [111].

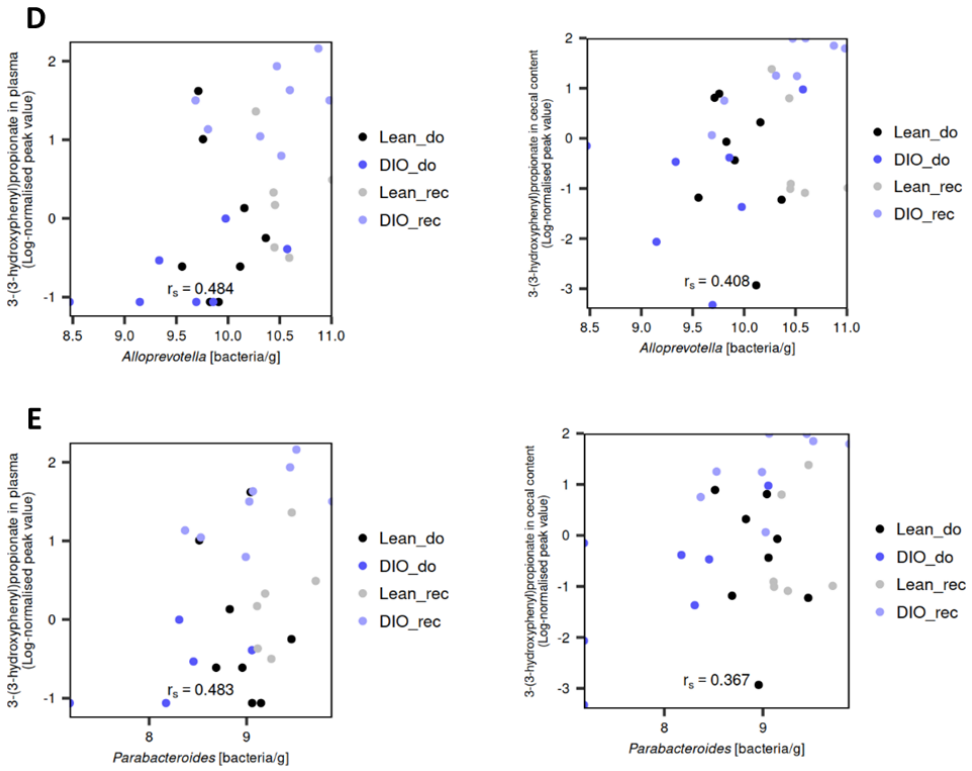
In conclusion, we confirmed that obese mice have a dysregulation of the learning and the wanting components of the food reward. We demonstrated that gut microbes play a role in the regulation of food reward and could be responsible for compulsive behavior and excessive motivation to obtain sucrose pellets. Moreover, obese gut microbes affected dopaminergic and opioid markers involved in reward system. We identified 33HPP as particularly increased in mice recipient of gut microbes from obese mice, and we were able to demonstrate its effects as key mediator of the gut-brain axis controlling the reward response to palatable food.

2.5. Supplemental information



Supplemental Figure 1: 33HPP concentrations in the plasma and in the cecal content of donor and recipient mice. (**A** Plasma concentrations of 3-(3-hydroxyphenyl)propionate (33HPP) in lean (Lean_do) and obese donors (DIO_do) after 11 weeks of follow-up and their gut microbiota recipient mice (Lean_rec and DIO_rec respectively) after 5 weeks of follow-up. (**B**) Cecal concentrations of 33HPP in lean (Lean_do) and obese donors (DIO_do) after 11 weeks of follow-up and their gut microbiota recipient mice (Lean_rec and DIO_rec respectively) after 5 weeks of follow-up. Data are shown as mean ± SEM (n=7-8/group). p-values were obtained after One-way ANOVA followed by Tuckey post-hoc test. * : p-value ≤ 0,05; ** : p-value ≤ 0,01; *** : p-value ≤ 0,001.


A
3-(3-hydroxyphenyl)propionate in plasma (Log-normalised peak value)
Akkermansia [bacteria/g]
rs = 0.543
3-(3-hydroxyphenyl)propionate in cecal content (Log-normalised peak value)
Akkermansia [bacteria/g]
rs = 0.369
B
Muribaculum [bacteria/g]
rs = 0.535
Muribaculum [bacteria/g]
rs = 0.56
C
Unknown Prevotellaceae [bacteria/g]
rs = 0.631
Unknown Prevotellaceae [bacteria/g]
rs = 0.455
Lean_do
DIO_do
Lean_rec
DIO_rec



Supplemental Figure 2: Correlations between 33HPP concentrations in the plasma and in the cecal content and abundance of bacteria in donor and recipient mice. Spearman's correlations between 3-(3-hydroxyphenyl)propionate (33HPP) concentrations in the plasma or in the cecal content of donor and gut microbiota recipient mice and the relative abundance of (A) *Akkermansia,* (B) *Muribaculum,* (C) Unknown *Prevotellaceae,* (D) *Alloprevotella*, and (E) *Parabacteroides* in lean (Lean_do) and obese donors (DIO_do) after 11 weeks of follow-up and their gut microbiota recipient mice (Lean_rec and DIO_rec respectively) after 11 weeks of follow-up.

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
RPL19	GAA-GGT-CAA-AGG-GAA-TGT-GTT-CA	CCT-TGT-CTG-CCT-TCA-GCT-TGT
D2R	CCA-AGA-ACG-TGA-GGG-CTA-AG	TGA-GGA-TGC-GAA-AGG-AGA-AG
D1R	GAG-CCA-ACC-TGA-AGA-CAC-C	TGA-CAG-CAT-CTC-CAT-TTC-CAG
TH	GCC-AAG-GAC-AAG-CTC-AGG-AAC	ATC-AAT-GGC-CAG-GGT-GTA-CG
DAT	AAA-TGC-TCC-GTG-GGA-CCA-ATG	GTC-TCC-CGC-TCT-TGA-ACC-TC
KOR	TGA-TCC-TGC-GCC-TGA-AGA-GT	ATG-CGG-CGG-AGA-TTT-CG
MOR	ACA-GGC-AGG-GGT-CCA-TAG-AT	GTG-ATG-ATG-AGG-ACC-GGC-AT
DOR	AAG-GCT-GTG-CTC-TCC-ATT-GAC	TGT-AGC-GGT-CCA-CGC-TCA-T
PPDyn	GCG-TGG-TCC-AGG-CTG-ATG	AGG-CAG-TCC-GCC-ATA-ACA-TT
Total bacteria	ACT-CCT-ACG-GGA-GGC-AGC-AG	ATT-ACC-GCG-GCT-GCT-GG

Supplemental Table 1: Sequences of primers for qPCR.

CHEMICAL_NAME	*r.plasma*	*P.plasma*	*q.plasma*	*r.cecum*	*P.cecum*	*q.cecum*
3-(3-hydroxyphenyl)propionate	0,8603	0	0	0,6916	0,0002	0,0044
3-(4-hydroxyphenyl)propionate	0,8569	0	0	0,8121	0	0,0001
X-21353	-0,7285	0,0001	0,0014	-0,7863	0	0,0003
gamma-tocopherol/beta-tocopherol	-0,7085	0,0001	0,0023	-0,7704	0	0,0006
docosadienoate (22:2n6)	-0,7061	0,0001	0,0024	-0,6138	0,0014	0,018
stearoyl ethanolamide	-0,6957	0,0002	0,003	-0,7532	0	0,0009
nonadecanoate (19:0)	-0,6939	0,0002	0,0031	-0,6951	0,0002	0,004
linoleate (18:2n6)	-0,6916	0,0002	0,0033	-0,6845	0,0002	0,0051
erucate (22:1n9)	-0,6865	0,0002	0,0037	-0,6799	0,0003	0,0056
arachidoylcarnitine (C20)*	-0,6676	0,0004	0,0054	-0,5589	0,0045	0,039
4-hydroxycinnamate	0,6604	0,0004	0,0061	0,6586	0,0005	0,0084
eicosenoate (20:1)	-0,6512	0,0006	0,0073	-0,6776	0,0003	0,0058
propionylglycine	-0,6506	0,0006	0,0074	-0,5489	0,0055	0,0442
3-hydroxylaurate	-0,6461	0,0006	0,008	-0,6326	0,0009	0,0133
1-methyl-5-imidazoleacetate	-0,6326	0,0009	0,0101	-0,5482	0,0055	0,0446
margarate (17:0)	-0,6123	0,0015	0,0141	-0,6043	0,0018	0,0208
xanthurenate	-0,6	0,0019	0,0171	-0,6663	0,0004	0,0073
arachidate (20:0)	-0,5765	0,0032	0,0241	-0,6883	0,0002	0,0047
dihomo-linoleate (20:2n6)	-0,5558	0,0048	0,0319	-0,6198	0,0012	0,0163
13-HODE + 9-HODE	-0,5489	0,0055	0,0349	-0,6952	0,0002	0,004
(16 or 17)-methylstearate (a19:0 or i19:0)	-0,5479	0,0056	0,0353	-0,6922	0,0002	0,0043
docosapentaenoate (n3 DPA; 22:5n3)	-0,5424	0,0062	0,0379	-0,5596	0,0045	0,0386
18-methylnonadecanoate (i20:0)	-0,5421	0,0062	0,038	-0,6562	0,0005	0,0088
thiamin (Vitamin B1)	-0,5392	0,0065	0,0395	-0,6309	0,0009	0,0136
hydroxystearate sulfate	-0,5292	0,0078	0,0447	-0,6755	0,0003	0,0061
X-24675	0,52	0,0092	0,0499	0,7158	0,0001	0,0025

Supplemental Table 2: Metabolites correlating significantly in the plasma and in the cecum with the mean active lever presses after Pearson's correlation and False Discovery Rate (FDR).

Taxon	*n*	*r.plasma*	*P.plasma*	*q.plasma*	*r.caecum*	*P.caecum*	*q.caecum*
f__Prevotellaceae	29	0,63	2,44E-04	5,02E-03	0,46	1,30E-02	9,23E-02
g__Akkermansia	29	0,54	2,32E-03	2,31E-02	0,37	4,89E-02	2,04E-01
g__Muribaculum	29	0,53	2,80E-03	2,63E-02	0,56	1,57E-03	2,43E-02
g__Alloprevotella	29	0,48	7,83E-03	5,23E-02	0,41	2,79E-02	1,46E-01
g__Parabacteroides	29	0,48	7,96E-03	5,29E-02	0,37	5,00E-02	2,07E-01

Supplemental Table 3: Genus correlating significantly with the concentration of 33HPP in the plasma and in the cecum after Spearman's correlation and False Discovery Rate (FDR).

2.6. Materials and methods

Mice and experimental design

All mouse experiments were approved by the ethical committee for animal care of the Health Sector of the UCLouvain, Université catholique de Louvain under the specific number 2017/UCL/MD/005 and performed in accordance with the guidelines of the local ethics committee and in accordance with the Belgian Law of May 29, 2013 regarding the protection of laboratory animals (agreement number LA1230314).

Donor mice

A cohort of 8-week-old SOPF male C57BL/6J mice (15 mice, n=7-8 per group) (Janvier laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2 °C, 12h daylight cycle) in groups of two mice per cage, with free access to sterile (irradiated) food and sterile (autoclaved) water. Upon delivery, mice underwent an acclimatization period of one week, during which they were fed a control diet (CT, AIN93Mi, Research Diet, New Brunswick, NJ, USA). Then, mice were randomly divided into two groups, and were fed for 14 weeks with control low-fat diet (CT, AIN93Mi) or a HFD (60% fat and 20% carbohydrates (kcal/100g) D12492i, Research diet, New Brunswick, NJ, USA). Body weight was recorded once a week. Body composition was assessed weekly by using 7.5 MHz time domain-nuclear magnetic resonance (TD-NMR, LF50 Minispec, Bruker, Rheinstetten, Germany). After 11 weeks of follow-up, the mice were transferred to behavioral cages to perform the conditioned place preference test and the operant wall test. During these tests, mice were food-restricted and body weight was maintained at 85%

of the initial body weight (before the behavioral tests). The caloric restriction allowed to potentiate the reward response to the stimulus [321; 322].

Recipient mice

A cohort of 3-week-old SOPF male C57BL/6J mice (15 mice, n=7-8 per group) (Janvier laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2 °C, 12h daylight cycle) in groups of two mice per cage, with free access to sterile (irradiated) food and sterile (autoclaved) water. Mice were fed a low-fat control diet (CT, AIN93Mi) during the entire procedure (before, during and after gut microbiota transplantation). Body weight was recorded once a week. Body composition was assessed weekly by using 7.5 MHz time domain-nuclear magnetic resonance (TD-NMR, LF50 Minispec, Bruker, Rheinstetten, Germany). After 5 weeks of follow-up, the mice were transferred to behavioral cages to perform the conditioned place preference test and the operant wall test. During these tests, mice were food-restricted and body weight was maintained at 85% of the initial body weight (before the behavioral tests). The caloric restriction allowed to potentiate the reward response to the stimulus [321; 322].

Fecal Microbiota Transplantation

During the experiment with donor mice, fecal samples were collected in sterile containers and immediately diluted (1:10 w/vol) in sterile PBS added with a mixture of antioxidants (0.5% ascorbic acid 0.5% L-cystein, 0.5% glutathione) and cryoprotectants (5% trehalose, 5% sorbitol). This suspension was filtered using 100µm filters. Then the filtrate was aliquoted in anaerobic conditions before storage at - 80°C. Three CT-fed mice and four HFD-fed mice from the donor cohort were selected as fecal microbiota donors for seven or eight recipient mice per group with 1 donor for 1, 2 or 3 recipient mice, according to quantity of feces from donors. As previously described, prior to gut microbiota inoculation, 3-week old SOPF recipient mice were depleted in intestinal microbiota by daily gavage of a broad-spectrum, poorly absorbed mix of antibiotics during 5 days (100 mg/kg of ampicillin, neomycin and metronidazole and 50 mg/kg of vancomycin diluted in sterile water) added with antifungal (amphotericin B 1mg/kg)[325; 349]. Antibiotic treatment was then followed

by a bowel cleansing with the administration of 800µl of PEG solution (PEG/Macrogol 4000, Colofort, Ipsen, France) by oral gavage in two times at 30 min intervals after a 2-hour fasting. Colonization was then achieved by intragastric gavage with 200 µl of inoculum three times a week for one week, then once a week until the end of the experiment. During inoculation, mice were transferred into clean cages 3 times a week. All recipient mice were kept under CT diet (CT, AIN93Mi).

33HPP experiment

A cohort of 7-week-old SOPF male C57BL/6J mice (20 mice, n=10 per group) (Janvier laboratories, France) were housed in a controlled environment (room temperature of 22 ± 2 °C,12h daylight cycle) in groups of two mice per cage, with free access to control sterile (irradiated) food (CT, AIN93Mi) and sterile water. Upon delivery, mice underwent an acclimatization period of one week. Then, mice were randomly divided in two groups, and were injected daily with 100µl of a vehicle solution (TBS) or a solution containing 33HPP (25mg/kg) during 4 weeks. After 2 weeks of follow-up, the mice were placed in behavioral cages to perform the operant wall test. During this test, mice were food-restricted and body weight was maintained at 85% of the initial body weight (before the behavioral tests). The caloric restriction allowed to potentiate the reward response to the stimulus [321; 322].

Conditioned place preference test

The learning component of the food reward is evaluated in donor and recipient mice by a CPP performed in the end of the light phase on a biased apparatus (Phenotyper chambers, Noldus, The Netherlands) as previously described with some modifications[350]. The behavioral cage is separated in two compartments characterized with smooth or rough floor and black or striped walls. All the compartments were completely cleaned before and after each session. Each session (pre-test, trainings, test) lasts exactly 30 minutes. Locomotor activity is recorded with infrared camera monitoring system and analyzed with the provided software (EthoVision XT 14). On day 1, a pre-test is used to determine the less preferred compartment in baseline (the one in which the mouse spent spontaneously less time) and is defined as the reward-associated compartment (biased CPP method). From day 2 to day

9, donor and recipient mice underwent eight trainings with or without a rewarding stimulus (Reese's®), in the less and in the most preferred compartment respectively (4 sessions in each compartment). During the test, the mouse is free to run in each compartment of the cage (in absence of rewarding stimulus), and the time spent in each compartment is recorded (analyzed with the provided software (EthoVision XT 14). Preference score is based on the difference of time spent (s) in the palatable food-associated side vs the time spent in the neutral-associated side of the cage during the pre-test and the test.

Operant wall test

The wanting component is linked to the motivation to obtain a reward and is evaluated by an operant wall test in donor and recipient mice as previously described with some modifications[260; 350]. Each session of the test was conducted during the end of the light phase, in operant conditioning chambers (Phenotyper chambers, Noldus, The Netherlands) and analyzed by the provided software (Ethovision XT 14). Briefly, the mice had intermittent access to an operant wall in their home cages. The operant wall system is composed of two levers and two lights and a pellet dispenser. One lever is arbitrarily designated as active, meaning that pressing on this lever initiates the delivery of a sucrose pellet (5-TUT peanut butter flavoured sucrose pellet, TestDiet, St. Louis, MO) and is associated with a light on. On the other side, another lever associated with a light off, is arbitrarily designated as inactive and will never deliver a reward. Mice were trained for the system twice overnight on a FR schedule (one lever press corresponds to one reward), then underwent 2 sessions of 1h30. Mice were then shifted to PR sessions (2h), the number of lever press to obtain a reward is incrementally increased (n+3) for every pellet.

Tissue sampling

At the end of each experiment, mice were maintained under caloric restriction before anesthesia with isoflurane (Forene, Abbott, England). This aims to mimic the conditions of the behavioral tests. Then the mice were euthanized by exsanguination and cervical dislocation. Blood was sampled from the portal and cava veins. Nac was precisely dissected,

the cecal content was harvested and immediately immersed into liquid nitrogen, then stored at −80°C for further analysis.

RNA preparation and quantitative PCR analysis

Total RNA was extracted from the Nac and the hippocampus using TriPure reagent (Roche). Complementary DNA was prepared by reverse transcription of 1µg total RNA using the GoScript Reverse Transcriptase kit (Promega, Madison, WI, USA). Real-time PCR was performed with the QuantStudio 3 real-time PCR system (Thermo Fisher, Waltham, MA, USA). *Rpl19* RNA was chosen as the housekeeping gene. All samples were performed in duplicate, and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of amplification. Sequences of the primers used for real-time PCR are available in Supplemental Table 1.

DNA isolation from mouse cecal samples, total bacteria quantification, and 16S amplicon sequencing

Cecal contents were collected and kept frozen at −80 °C until use. Metagenomic DNA was extracted from the cecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications [351]. The bacterial load in the cecal content was quantified using qPCR as described above, with universal bacterial primers (Supplemental Table 1). The V1–V3 region of the 16S rRNA gene was amplified from the cecal microbiota of the mice using the following universal eubacterial primers: 27Fmod (5'-AGRGTTTGATCMTGGCTCAG-3') and 519Rmodbio (5'-GTNTTACNGCGGCKGCTG-3'). Purified amplicons were sequenced using an Illumina MiSeq following the manufacturer's guidelines. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA). Sequence reads were demultiplexed and processed using the QIIME2 pipeline (q2cli 2020.11.1) [352], including primer removal using cutadapt [353] and denoising with DADA2 [354] using the following options in DADA2: maximum expected error = 2, truncation length = 280 nt. To ensure quality, only forward sequencing reads were used. For the 30 samples analyzed, 971 ASVs have been identified. These were decontaminated by mapping to the mouse genome GRCm39

(GCF_000001635.27) and, after taxonomic assignment, by aligning unassigned ASVs to the non-redundant nucleotide database of the National Center for Biotechnology Information using BLAST[355], resulting in 958 decontaminated ASVs. The taxonomic assignment of ASVs was performed using a classifier based on the SILVA 138.1 SSURef NR99 database [356] that was dereplicated and trimmed to the V1-V3 region using RESCRIPt. After this processing, samples contained between 21056 and 37447 sequence reads, with the median and mean number of sequence reads of 29687 and 29504, respectively. Diversity metrics were calculated using a sampling depth of 21056 reads and PCoA was performed using the Jaccard distances and unweighted UniFrac distances with QIIME2. The PCoA plots were visualized using the "tidyverse" collection of R packages "tidyverse". The sequencing data were submitted to the European Nucleotide Archive (ENA/EBI).

Plasma multiplex analysis

Plasma levels of total GLP-1, PYY, total ghrelin, insulin and leptin were measured by multiplex assay kits based on chemiluminescence detection and following manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). Analyses were done using a QuickPlex SQ 120 instrument (MSD) and DISCOVERY WORKBENCH®4.0 software.

Untargeted metabolomics analysis

Cecal and plasma samples were used for untargeted metabolomics analyses by UPLC-MS/MS (Metabolon, USA). To identify potential metabolites involved in the gut-to-brain axis and contributing to the reward system, the metabolomic data were then analyzed using R and R packages "stats" and "tidyverse". For each compound, the raw peak areas were median normalized and the minimum value was imputed for the missing values, followed by a log-transformation. Metabolites with both a fold-change > 2 and a p-value < 0.05 after the Kruskal-Wallis test were considered significant and the results were visualized with a volcano plot.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.1.2 for Windows (GraphPad Software, San Diego, CA, USA) except for microbiota analyses as described above. Data are expressed as mean ± SEM. Differences between two groups were assessed using unpaired Student's t-test. In case variance differed significantly between groups according to the Fisher test, a non-parametric (Mann-Whitney) test was performed. Two-way ANOVA was used if repeated measurements, followed by Bonferroni post-hoc test. Differences in microbial beta-diversity were assessed in QIIME2. Correlation of metabolite concentrations, either in plasma or in cecal content, and the wanting component of the reward system was performed using Pearson correlation on the log-transformed, imputed peak values from the metabolomic analysis, and the mean number of lever presses on the active lever during the PR sessions of the operant wall test. The metabolites that significantly correlated (FDR-adjusted p-value < 0.05) with the active lever presses in both plasma and cecal content were considered as relevant. Similarly, correlation of metabolite concentrations, either in plasma or in cecal content, and microbial genera in the cecal content was performed using Spearman correlation on the log-transformed, imputed peak values from the metabolomic analysis, and the genus abundances obtained by scaling the genus-level relative frequencies with the qPCR-based total bacterial abundances in the cecum. To ensure the relevance of correlations, only genera that appeared in at least 10 samples were considered. Two samples that were outliers for total bacterial abundances were omitted from this analysis. Correlations were visually inspected using scatterplots (Suppl. Fig. 2).

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Disclosure statement

PDC is cofounder of A-Mansia Biotech SA and AE, PDC and AdW are owners of patents on gut microbes and metabolic diseases.

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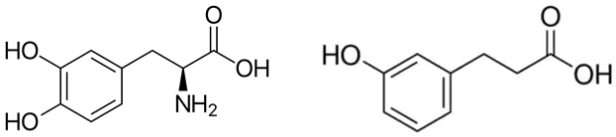
2.7. Complementary discussion, limitations and perspectives

After proving the role of gut microbes in the alterations of hedonic feeding associated with obesity (chapter 1), we aimed in this chapter, to prove its causal role in the motivation and the learning process of the food reward system, using FMT. We found that the gut microbiota recipient mice of obese donors behave as an opposite way as their donors at the operant wall test. Indeed, gut microbiota recipient mice from obese donors present an increased in motivation to obtain food reward during operant wall task suggesting that gut microbes could be responsible for compulsive behavior and excessive motivation to obtain sucrose pellets. We also described different metabolomic profiles in the cecum and in the plasma between gut microbiota recipient groups, potentially participating to the behavior.

Among the metabolites significantly different in the cecum and in the plasma of gut microbiota recipient mice from obese donors, we highlighted the 33HPP as a potential mediator into the gut-brain axis influencing the motivation for food reward. When administrating this metabolite to control mice, we found alterations of learning process linked to food reward, together with decreased *Drd1* expression in the hippocampus.

It is important to note how hydroxyphenylpropionic acids addressed in this chapter (33HPP and 34HPP) are structurally similar to L-DOPA, the precursor of dopamine (Figure 1). In fact, it has been shown that some bacteria of the gut microbiota are able to convert L-DOPA into hydroxyphenylpropionic acids *in vitro* [343]. L-DOPA represents the main treatment of Parkinson's disease. The link between benzoate metabolites and Parkinson's disease is relevant in the context of this thesis since Parkinson's patients show similarities with obese patients. In fact, they present an altered gut microbiota, together with decreased levels of dopamine in the reward-related brain areas and can show addiction-like behaviors. In a study investigating the blood metabolome of Parkinson's patients, blood levels of 33HPP were reduced in patients with Parkinson's disease [357], suggesting a link between 33HPP, Parkinson's disease and the gut microbiota.



L-DOPA 33HPP

Figure 1: Chemical structures of L-DOPA and 3-(3-hydroxyphenyl)propionic acid.

Based on the hypothesis that 33HPP could have a neuroactive potential, one perspective of this chapter is the absolute quantification of 33HPP and other structurally similar phenolic acids (including 34HPP) in the reward-related brain area of gut microbiota donor and recipient mice (FMT experiment), as well as 33HPP and TBS-injected lean mice (33HPP experiment).

3. CHAPTER 3: *Food reward alterations during obesity are associated with inflammation in the striatum: beneficial effects of Akkermansia muciniphila*

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[The results of this chapter have been included in a paper submitted to Science Advances]

In this chapter, in collaboration with Sabrina Huwart, PhD student in our team, we first confirmed the behavioral and neuronal alterations of the food reward system in the context of obesity, then we investigated the activation of pro-inflammatory pathways in reward related brain areas since inflammation has been suggested to be involved in several brain disorders. And finally, we used a gut microbiota-based approach, *i.e.* the supplementation in *Akkermansia muciniphila*, to tackle reward alterations associated with obesity.

3.1. Abstract

The reward system involved in hedonic regulation of food intake presents neuronal and behavioral alterations during obesity, leading to excess caloric ingestion. Obesity is also associated with low-grade inflammation in several organs, including the brain. Obese gut microbiota has been identified to promote this low-grade inflammation. The mechanisms underlying reward dysregulation during obesity are still not fully known. We investigated if inflammation affects reward-related brain areas during obesity using a cohort of control-fed or DIO male mice (n=20/group). We tested the potential effects of specific gut bacteria on the reward system during obesity by administrating *Akkermansia muciniphila* daily or a placebo to DIO male mice (n=10/group). We show that dysregulation of the liking and

wanting components of food reward is associated with inflammation and alterations in the blood–brain barrier in reward-related brain areas of obese mice. We identified *Akkermansia muciniphila* as a novel actor able to restore the dysregulated reward behaviors associated with obesity, potentially through a decrease in inflammatory pathways and lipid-sensing ability in reward-related brain areas as suggested by the reduced lipoprotein lipase expression (*Lpl*). This study allows to identify novel mechanisms and actors involved in reward dysregulation associated with obesity. Our results suggest that the supplementation of beneficial bacteria to attenuate reward alterations in obesity might be considered as an innovative therapeutic approach. This study provides an opportunity for further investigations of *Akkermansia muciniphila*-mediated behavioral improvement in other inflammatory neuropsychiatric disorders.

3.2. Introduction

The prevalence of obesity has steadily increased in recent decades. The last report of the World Health Organization (2016) announced that 13% of adults were obese [1]. Excess intake of energy-dense food is often considered as a major factor contributing to obesity [358]. Food intake is controlled by homeostatic regulation at the level of hypothalamic neuronal circuits, based on energy need, and by the food reward system, based primarily on hedonic value. Palatable food that is rich in fat and/or sugar stimulates hedonic, reinforcing and motivational processes of the reward system [99; 350]. Dopaminergic neurons in the mesocorticolimbic area of the brain comprise the reward system and are stimulated by palatable food to release dopamine from the VTA to the striatum, which includes the Nac. In 2001, Wang et *al.* first described a dysregulation of the reward system in obese individuals [107]. Since then, food reward alterations have been broadly proven in the literature in both human and rodent models [112] [113; 316]. Different studies have shown that long-term overeating is associated with a decrease in the release of dopamine, a decrease in the expression of dopamine receptors (DRD2 and DRD1) and an increase in the expression of DAT [107; 109; 296; 297; 316]. This hypofunctioning of the dopamine pathway leads to altered hedonic and motivational behavior feeding [107; 111; 359].

However, to date, the mechanism driving the desensitization of the reward system associated with obesity remains unknown.

Over the last 20 years, the gut microbiota has emerged as a key regulator of host metabolism, including the hypothalamic regulation of food intake through the gut-brain axis [143; 193; 194]. In obese individuals, the gut microbiota composition is altered, and gut permeability increases. These changes facilitate the translocation of bacterial components such as LPS across the gut barrier into the systemic circulation. The small increase in serum LPS levels observed in obesity is called metabolic endotoxemia [153; 184]. LPS activates NF-κB and c-Jun N-terminal kinase (JNK) inflammatory pathways through TLR4 and induces inflammation in several organs, including the brain [360]. Hypothalamic inflammation has been characterized by increased activation of inflammatory pathways, activation of microglia and astrocytes, and disruption of the blood–brain barrier (BBB) in both obese humans and rodents [191; 192]. Inflammation affects different regions of the brain and is associated with an increased prevalence of cognitive impairments [189; 361]. Therefore, we investigated the hypothesis that inflammation also affects reward-related brain areas during obesity. Our team recently determined the causal role of the gut microbiota in the dysregulation of the reward system in the context of obesity [320]. Using FMT from obese mice, we showed that the gut microbiota affects the hedonic components of food intake. One consistent gut microbiota disequilibrium associated with obesity was the decreased abundance of *Akkermansia muciniphila (A. muciniphila).* This bacterium was previously described as exerting beneficial effects on the prevention of body weight gain and metabolic disorders in rodents and humans [183; 280; 282]. In addition, *A. muciniphila* modulates inflammation and reduces metabolic endotoxemia, adipose tissue inflammation, insulin resistance and fat-mass gain in rodents fed a HFD [183; 280].

Our first aim in this study is to decipher the gut-brain mechanisms underlying the dysregulation of the reward system in obesity by measuring the levels of inflammatory markers in these brain areas. Second, we aim to investigate the potential beneficial effect of *A. muciniphila* on neuronal and behavioral alterations in the food reward system in an obesity model.

3.3. Results

DIO mice show behavioral and neuronal alterations in response to food reward

After 4 weeks of feeding a control low-fat diet (Lean group) or a HFD to establish obesity (DIO group), we assessed the liking and wanting components of the food reward system by performing behavioral tests. We first assessed the liking or hedonic component by analyzing the tropism towards palatable food (HFHS) over control food (CT) during a food preference test. An HFHS preference would suggest a rewarding response specifically to palatable food. As expected, and consistent with the literature [116; 260], lean mice showed a great preference for palatable food as shown by the higher intake of HFHS compared to CT ($p<0.0001$ CT vs. HFHS in the Lean group, Fig. 1A); in contrast, DIO mice had no preference for HFHS over the CT diet ($p=0.77$ CT vs. HFHS in the DIO group, Fig. 1A). Importantly, DIO mice ate more than 2-fold less HFHS than lean mice ($p<0.0001$ Lean and DIO mice, 1.16 g vs. 0.57 g, respectively).

We next assessed the motivational component of the food reward by subjecting the mice to an operant conditioning task in which their eagerness to obtain rewarding food (peanut butter-flavored sucrose pellets) was tested. Lean and DIO mice were evaluated for incentive motivation on PR sessions, which requires an increasing number of lever presses to obtain a new sucrose pellet [3 lever presses more for each subsequent reinforcer ($r=3n+3$; n= reinforcer number)]. The PR sessions thereby measure the amount of effort an animal was willing to exert to obtain food rewards and rely on the motivational aspect of the reward system. Compared to lean mice, DIO mice pressed significantly less on the lever to obtain a reward, suggesting a reduction of motivation (Figure 1B). Consistent with the number of lever presses, the breaking point or the number of sucrose pellets earned during a session was significantly lower in the DIO group than in the lean group (Fig. 1B).

To complete the behavioral analysis, we further investigated the reward system by analyzing the dopaminergic system in mesocorticolimbic structures of the brain (Fig. 1C). The expression of dopamine receptors (*Drd2* and *Drd1*) were decreased in the ventral striatum of obese mice ($p=0.028$ and $p=0.050$, respectively) compared to lean mice,

whereas the expression of *Dat*, which is responsible for the recapture of dopamine, tended to increase (p=0.068, Fig. 1C). The expression of *Th*, the rate-limiting enzyme synthetizing dopamine, was not affected in DIO mice (Fig. 1C).

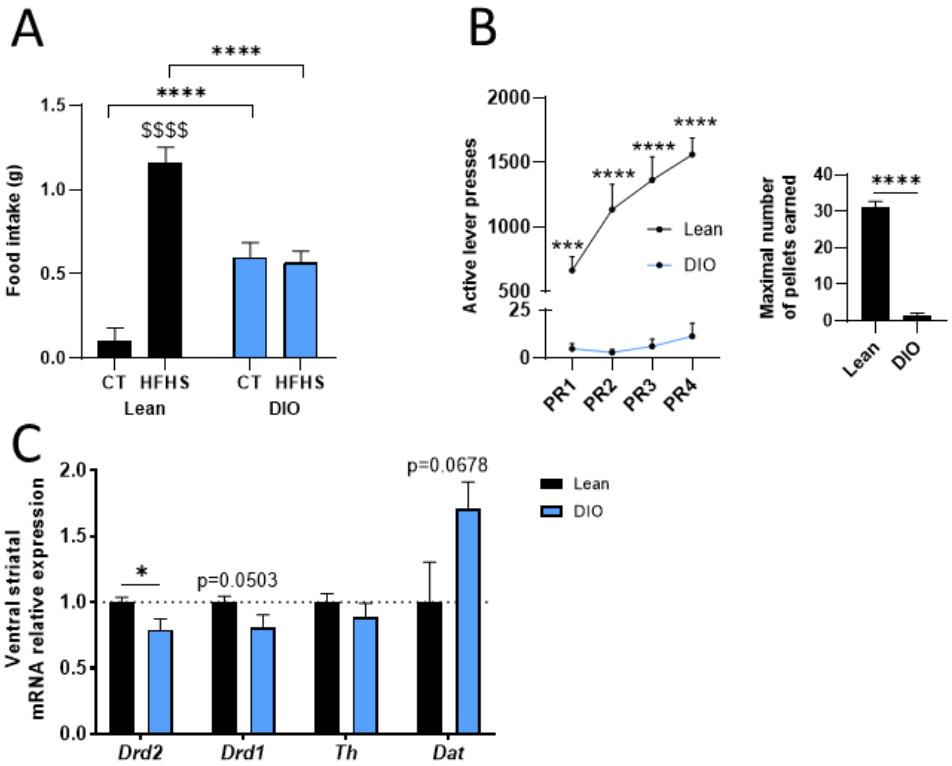
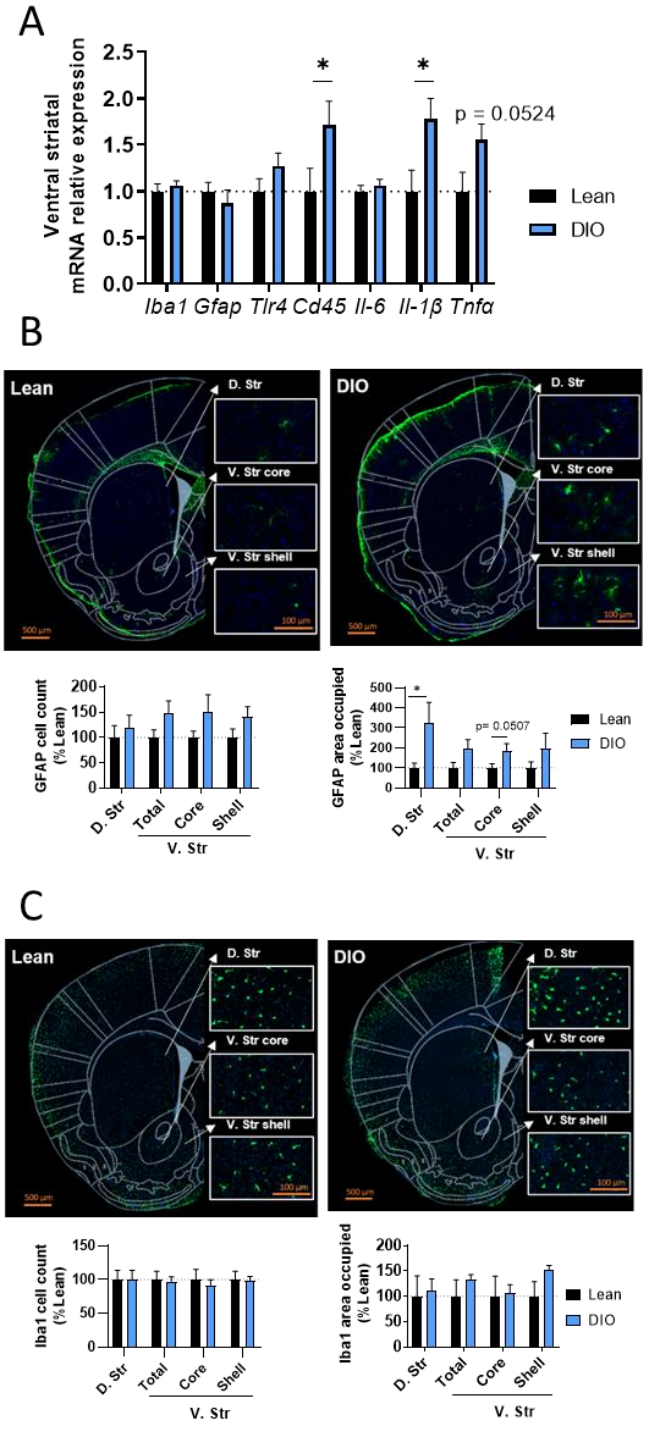


Figure 1. Obesity is associated with an alteration of the food reward system. (**A**) Food preference test showing HFHS and CT intake in grams after 3 hours of test by lean and DIO mice after 4 weeks of follow-up. (**B**) Operant conditioning test showing the number of active lever presses during the four progressive ratio sessions (PR) and the maximal number of pellets earned during the PR4 by lean and DIO mice after 4 weeks of follow-up. (**C**) Ventral striatal mRNA relative expression of dopamine receptor 2 (*Drd2*), dopamine receptor 1 (*Drd1*), tyrosine hydroxylase (*Th*) and dopamine transporter (*Dat*) measured by real-time PCR in lean and DIO mice after 4 weeks of follow-up. Data are shown as mean ± SEM. P-values were obtained after two-way ANOVA followed by Bonferroni post-hoc test. (n=10-12/group) (**A**) after two-way ANOVA followed by Bonferroni post-hoc test (n=12/group) (**B**) after unpaired Student's t-test or non-parametric Mann-Whitney test (n=9-12/group) (**B, C**) *: p-value <0,05; ***: p-value <0,001; ****: p-value <0,0001 between lean vs DIO. $$$$: p-value <0,0001 between CT vs HFHS food intake.

Obesity is associated with inflammation and blood–brain barrier alterations in reward-related brain areas

We investigated whether the dysregulation of food reward behaviors and dopaminergic pathways during obesity was linked to inflammation in reward-related brain areas. Since inflammation in the brain is associated with the activation of microglia and astrocytes, as reflected by an increase in the expression of ionized calcium-binding adaptor protein-1 (Iba1) and glial fibrillary acidic protein (GFAP), respectively, we analyzed the levels of these markers in the ventral striatum of lean and DIO mice using qPCR. We also analyzed the expression of the receptor for LPS (*Tlr4*), infiltrating immune cell markers (cluster of differentiation 45 (*Cd45*)) and proinflammatory cytokines (*Il-6*,*Il-1β* and *Tnfα*). We detected a significant increase in the expressions of *Cd45* and *Il-1β* and an increasing trend for *Tnfα* expression in obese mice compared to lean mice (p=0.044, 0.025 and 0.052, respectively, Lean vs DIO mice, Fig. 2A), suggesting the induction of inflammation in the mesocorticolimbic area by HFD-induced obesity.

We performed immunohistochemical staining for GFAP and Iba1 in the striatum to better visualize the potential activation states of astrocytes and microglia (Fig. 2B, C). We found that DIO induced astrocyte activation in the dorsal striatum and ventral striatum core, as shown by a significant increase in the GFAP-labelled area in the DIO group compared to the lean group (p=0.049 and 0.051, respectively, Lean vs DIO mice, Fig. 2B). Surprisingly, astrocytes were mainly activated in the right dorsal and the ventral striatum, while these regions in the left hemisphere did not present astrocyte activation (Supplementary Figure 1A, B). Iba1 immunostaining did not show a difference in microglial cell activation (Fig. 2C).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Ventral striatal mRNA relative expression
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2.0
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Lean
DIO
D. Str
V. Str core
V. Str shell
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GFAP cell count (%Lean)
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500
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DIO

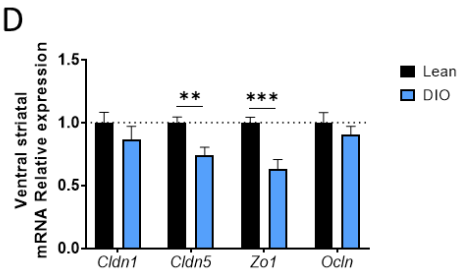


Figure 2. Obesity is associated with inflammation and blood-brain barrier alterations in reward-related brain areas. (**A**) Ventral striatal mRNA relative expression of ionized calcium-binding adapter (*Iba1*), glial fibrillary acidic protein (*Gfap*), toll-like receptor 4 (*Tlr4*), cluster of differentiation 45 (*Cd45*), interleukin 6 (*Il-6*), interleukin 1 beta (*Il-1β*) and tumor necrosis factor alpha (*Tnfα*) measured by qPCR in lean and DIO mice (n=10/group) after 4 weeks of follow-up. (**B**) Representative immunofluorescence of the dorsal striatum (D. Str), the ventral striatum (V. Str) total, core and shell and quantification of both the area occupied by astrocytes cells and the GFAP+ cells in these regions of lean and DIO mice (n=4-5/group) (**C**) Representative immunofluorescence of the dorsal striatum (D. Str), the ventral striatum (V. Str) total, core and shell and quantification of both the area occupied by microglial cells and the Iba1+ cells in these regions of lean and DIO mice. (n=5/group) (**D**) Ventral striatal mRNA relative expression of claudin-1 (*Cldn1*), claudin-5 (*Cldn5*), zonula occludens 1 (*Zo1*) and occludin (*Ocln*) measured by real-time qPCR in lean and DIO mice after 4 weeks of follow-up. (n=10/group) Data are shown as mean ± SEM. P-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. *: p-value <0,05; **: p-value <0,01; ***: p-value <0,001 between lean vs DIO.

The BBB is essential to protect brain structures from toxins, pathogens and excess immune cell infiltration and to maintain neuronal integrity [362; 363; 364]. Since inflammation in the CNS is associated with disruption of the BBB in several neurological disorders, including some associated with obesity [365; 366], we further analyzed the integrity of the BBB in reward-related brain areas by measuring the expression of key tight junction proteins. Interestingly, the expression levels of claudin-5 (*Cldn5*) and zonula occludens 1 *(Zo1)* were significantly decreased in obese mice compared to lean mice (p=0.0033 and 0.0004 Lean vs DIO mice, Fig. 2D), whereas claudin-1 *(Cldn1)* and occludin (*Ocln*) levels were not changed (Fig. 2D).

The administration of A. muciniphila restores the motivational component of food reward that is altered by DIO

A. muciniphila is a beneficial bacterium that counteracts DIO and metabolic disorders associated with low-grade inflammation [183; 280; 282]. Therefore, we evaluated the potential effect of *A. muciniphila* administration on the reward system in obese animals.

During the food preference test, obese mice treated with the placebo showed a preference for HFHS rather than for CT diet since they ate significantly more HFHS than CT (Fig. 3A). Nevertheless, DIO_placebo mice ate a lower quantity of HFHS than lean mice (mean=0.43 g and 1.16 g, respectively, Fig. 1A and 3A). This finding supports our previous results showing alterations in hedonic food intake in obese mice[320]. Obese mice treated with *A. muciniphila* did not show any preference for a specific diet (p=0.24 for the comparison between CT and HFHS in the DIO_Akk group, Fig. 3A), suggesting that the administration of *A. muciniphila* did not seem to improve the altered hedonic intake of obese animals.

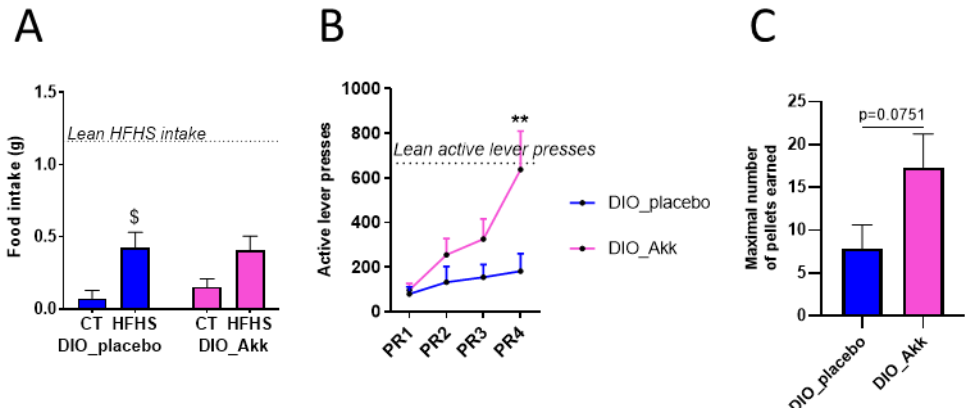


Figure 3. *Akkermansia muciniphila* administration improves the motivational component of food reward associated with obesity. (**A**) Food preference test showing HFHS and CT intake in grams after 3 hours of test by DIO mice treated with placebo (DIO_placebo) and *A. muciniphila* (DIO_Akk) after 4 weeks of follow-up. (**B**) Operant conditioning test showing the number of active lever presses during the four progressive ratio sessions (PR) and (**C**) the maximal number of pellets earned during the PR4 by DIO mice treated with placebo (DIO_placebo) and *A. muciniphila* (DIO_Akk) after 4 weeks of follow-up. Data are shown as mean ± SEM. P-values were obtained after two-way ANOVA followed by Bonferroni post-hoc test (n=7/group) (**A**), after two-way ANOVA repeated measure followed by Bonferroni post-hoc test (n=6/group) (**B**), after unpaired Student's t-test or non-parametric Mann-Whitney test (n=6-10/group) (**C**). **: p-value <0,01 between DIO_placebo vs DIO_Akk. $: p-value <0,05 between CT vs HFHS food intake.

Since reward-based food intake is attributed to several components, we decided to further investigate the motivation with an operant conditioning task. The PR sessions showed a statistically significant increase in the motivation of the obese mice treated with *A. muciniphila* at PR4 compared to obese mice treated with placebo (p=0.0022 DIO_placebo vs DIO_Akk groups, Fig. 3B). *A. muciniphila*-treated mice even reached a similar number of lever presses as lean mice during their first session of the PR test (mean number=666 in Lean mice, Fig. 1B, compared with mean number=638 in DIO_Akk mice). Consistent with the lever pressing parameter, the maximum number of reinforcers obtained tended to be increased in obese mice treated with *A. muciniphila* compared to placebo-treated mice (p=0.075, Fig. 3C).

Beneficial effects of Akkermansia muciniphila on the reward system is associated with a reduction in systemic and striatal inflammation, as well as a reduction in striatal Lpl expression

From a mechanistic perspective, the dysregulation of the reward system associated with obesity might be due to inflammation in reward-related brain areas (Fig 1 and 2). Based on the ability of *A. muciniphila* to reverse the motivational alterations induced by a HFD diet (Fig. 3B) and because systemic anti-inflammatory effects have previously been described for this bacterium, we analyzed several markers of inflammation in reward-related brain areas of obese mice treated with or without *A. muciniphila*.

First, we found that the administration of *A. muciniphila* decreased significantly plasma levels of TNFα (DIO_placebo vs DIO_Akk mice, Fig. 4A). Consistent with this decrease in the level of a systemic inflammatory marker, the expression of *Tlr4* was substantially decreased in the striatum of *A. muciniphila*-treated mice compared to placebo-treated mice (p=0.0015 DIO_placebo vs. DIO_Akk, Fig. 4B). The striatal expression of markers of infiltrating immune cells (*Cd45*) also tended to decrease in *A. muciniphila*-treated mice compared to placebo-treated mice (p=0.072 DIO_placebo vs. DIO_Akk, Fig. 4B). However, the striatal expression of markers of microglia (*Iba1*) and astrocytes (*Gfap*) was not changed between the DIO group compared to DIO_Akk group (Fig. 4B). Taken together, our results suggest that *A. muciniphila* supplementation reverses motivational alterations associated with obesity,

potentially through the modulation of *Tlr4* expression and the infiltration of immune cells in the mesocorticolimbic structures.

According to previous studies, a potential mechanism linking alterations in behavioral reward and dopaminergic transmission involves central lipid sensing through the lipid-processing LPL. A similar increase in motivational performance for food-seeking behavior has been assessed with viral-mediated knockdown of LPL in mice in a progressive ratio operant conditioning paradigm [350; 367]. Importantly, in our cohort, mice receiving *A. muciniphila* showed a highly significant decrease in *Lpl* expression in the striatum compared to placebo-treated obese mice (p=0.0058, Fig. 4C).

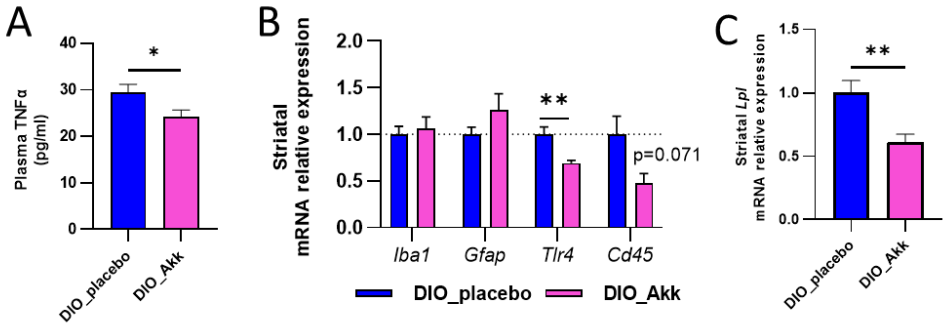


Figure 4. *Akkermansia muciniphila* administration reduces systemic and mesocorticolimbic markers of inflammation associated with obesity and lowers striatal *Lpl* expression. (**A**) Plasma concentration of the cytokines tumor necrosis factor alpha (Tnfα) in DIO mice treated with placebo (DIO_placebo) and *A. muciniphila* (DIO_Akk) after 4 weeks of follow-up. (**B**) Striatal mRNA relative expression of ionized calcium-binding adapter (*Iba1*), glial fibrillary acidic protein (*Gfap*), toll-like receptor 4 (*Tlr4*), cluster of differentiation 45 (*Cd45*) and (C) lipoprotein lipase (*Lpl*) measured by qPCR in DIO mice treated with placebo (DIO_placebo) and *A. muciniphila* (DIO_Akk) after 4 weeks of follow-up. (n=5-10/group) Data are shown as mean ± SEM. P-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. *: p-value <0,05; **: p-value <0,01 between DIO_placebo vs DIO_Akk.

3.4. Discussion

Alterations in reward processes are main contributors to excessive eating behaviors and play a crucial role in maintaining a healthy body weight. The identification of the mechanisms and factors involved in reward dysregulations associated with obesity is therefore of the utmost importance.

First, we aimed to validate behavioral and neuronal alterations in the reward system of obese animals. From a behavioral perspective, during the food preference test, mice were exposed for the first time to a palatable diet that stimulates the mesocorticolimbic system and induces pleasure. The absence of a preference for palatable food and the reduced intake of this diet in DIO mice suggests a dysregulation of the hedonic component of food intake associated with obesity. These results are consistent with other studies investigating the hedonic component of food intake through exposure to a palatable diet [116; 260; 320; 368] or using a variant of the food preference test, the two-bottle choice test, where obese rodents are less attracted to a sucrose solution [369]. Moreover, the results obtained using an operant conditioning paradigm confirmed the altered motivational reward system associated with obesity: DIO mice pressed less on the lever than lean mice, as previously reported in the literature [114]. In the brain, obese conditions are associated with modifications in the expression of key dopaminergic markers, such as dopaminergic receptors 1 and 2 and the dopamine transporter. These changes in the transcription of dopaminergic markers suggest hypofunctioning of the dopaminergic pathway and reflect the behavioral dysregulations of liking and wanting components of food reward observed during the food preference and operant conditioning tests.

The increased systemic low-grade inflammatory tone associated with obesity also suggests the presence of inflammation in the CNS. For many years, studies have focused on hypothalamic inflammation, which was shown to be involved in the development of the obese phenotype. However, recently, inflammation has been recognized as also affecting other brain regions, such as the cortex or hippocampus, in the context of obesity [189; 191; 363]. Furthermore, obesity-derived brain inflammation is associated with behavioral and

cognitive alterations [190; 361; 370]. We tested the inflammation hypothesis in reward-related brain areas to decipher the mechanisms underlying the altered reward system in obesity. qPCR and immunofluorescence staining revealed the activation of inflammatory mediators in the mesocorticolimbic area following HFD treatment. More specifically, we documented the upregulation of *Cd45*, which reflects the infiltration of immune cells, and increased expression of proinflammatory cytokines (*Il-1β* and *Tnfα*) in the ventral striatum of obese mice. Importantly, this study is the first to link inflammation in reward-related brain areas with altered hedonic and motivational reward behavior during obesity. We also showed astrocyte activation, which was detected using GFAP immunolabelling, in the dorsal striatum and in the core of the ventral striatum of obese mice. This activation seems to be specific to the right striatum, as the left striatum did not show any difference in the GFAP-positive area. In 2018, Han et *al.* reported that motivation and dopamine activity in the brain are mediated by right, but not left, vagal sensory ganglion activation [132]. Further investigations are needed, but the results suggest asymmetric brain pathways. GFAP immunolabelling results are consistent with studies suggesting the activation of astrocytes in the cerebellum and hypothalamus during obesity [189; 191]. Astrocytes adopt a reactive phenotype that alters their functions and impairs their abilities to maintain brain homeostasis. Moreover, astrocytes also contribute to the interaction between inflammation induction and the dopaminergic reward system. Indeed, astrocytes modify synaptic dopamine levels by presenting DAT on their own membrane to degrade dopamine [371]. Therefore, inflammation might be responsible for alterations in food reward and dopamine signaling during obesity.

Inflammation in the CNS may result from local activation of inflammatory pathways but might also be induced by increasing BBB permeability, allowing the translocation of proinflammatory mediators from the periphery into the brain. BBB function adapts to stimuli such as proinflammatory mediators and is, among others, regulated by astrocytes around microvessels [372]. In obese conditions, the BBB is chronically challenged by proinflammatory stimuli and presents increasing permeability, especially in the hypothalamus [362; 363; 364]. Here, we showed that the BBB was also affected in the

reward area in obese animals. We showed decreased mRNA expression of the tight junction proteins claudin 5 and zonula occludens 1. Claudin-5 is expressed at high levels in the brain, and its loss is associated with neurodegenerative, neuroinflammatory and neuropsychiatric disorders [373]. BBB leakage may result in cytokine and immune cell infiltration in the brain, activating inflammatory pathways [363; 364; 365; 373]. Consequently, high BBB permeability is considered a precursor of CNS inflammation and cognitive deficits. Based on our results, striatal inflammation in obese animals is potentially due to peripheral proinflammatory signals passing through the BBB.

Our team recently showed that the dysregulation of the hedonic component of food intake is transferable by gut microbiota transplantation from obese mice, indicating that the gut microbiota plays a major role in reward dysregulation associated with obesity through the gut-brain axis [320]. Here, we found that the administration of *A. muciniphila* reversed the altered motivation induced by a HFD. *A. muciniphila*-treated mice behaved as lean mice in terms of the number of lever presses during the operant conditioning test. Importantly, even if our study is the first to show that *A. muciniphila* is able to control food reward behavior, *A. muciniphila* has already been shown to be a beneficial bacterium in the gut-brain axis influencing behavior in other contexts such as in depression-like behavior induced by chronic stress [374], in cognitive impairments associated with obesity [290] as well as in spatial learning and memory in an animal model of Alzheimer's disease [291]. In our study, the administration of *A. muciniphila* did not seem to restore the altered hedonic intake typical of obesity. However, in a human cohort, *A. muciniphila* abundance was negatively correlated with the food addiction scale [256].

We investigated whether the positive effects of *A. muciniphila* administration on the reward system imply an improvement in inflammation. qPCR results showed that *A. muciniphila* supplementation in obese mice substantially decreased the expression of *Tlr4* and tended to decrease the expression of *Cd45* in reward-related brain areas. TLR4 is a key marker of inflammation in the context of obesity since it binds both LPS and fatty acids that are present at higher levels in the plasma of obese subjects. Then, TLR4 triggers inflammation via the NF-κB pathway. Sun, Luquet and Small hypothesized that NF-κB activation directly

or indirectly regulates the transcription of the dopamine DRD2 receptor [249]. The fatty acid - TLR4 pathway may be a potential mechanism by which *A. muciniphila* regulates food reward.

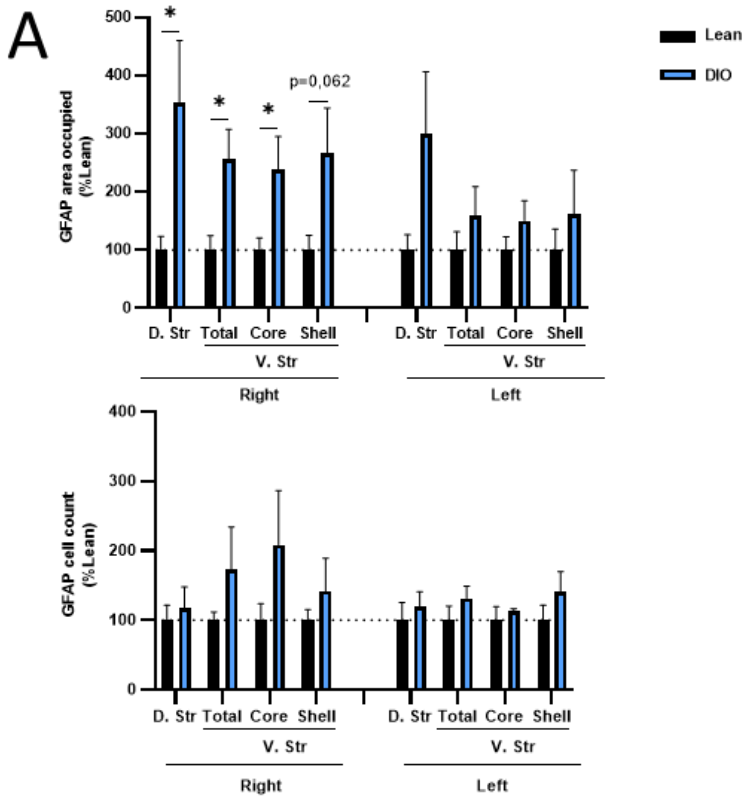
In addition, we observed that *A. muciniphila* supplementation in obese mice decreased the systemic inflammatory tone by reducing the plasma level of the proinflammatory cytokine TNFα. Circulating cytokines cross the BBB, especially when BBB permeability is increased, which we confirmed in reward-related brain areas of obese mice. Cytokines also activate the NF-κB proinflammatory pathway. In this context, the reduction of inflammation markers in the striatum by *A. muciniphila* observed in the present study might be linked to the systemic effect on TNFα levels. Together, our results suggest that *A. muciniphila* supplementation reverses the motivational alterations associated with obesity by modulating CNS inflammatory pathways. Consistent with our results, *A. muciniphila* administration has already been shown to be neuroprotective at the level of the hippocampus in a DIO model by modulating cytokine expression and improving learning and memory processes [375]. Moreover, *A. muciniphila* has been broadly described as an anti-inflammatory bacterium in several pathologies, including obesity (decreasing macrophage infiltration in the adipose tissue and serum LPS levels), alcoholic hepatitis, colitis and oral cavity infections [183; 376; 377; 378].

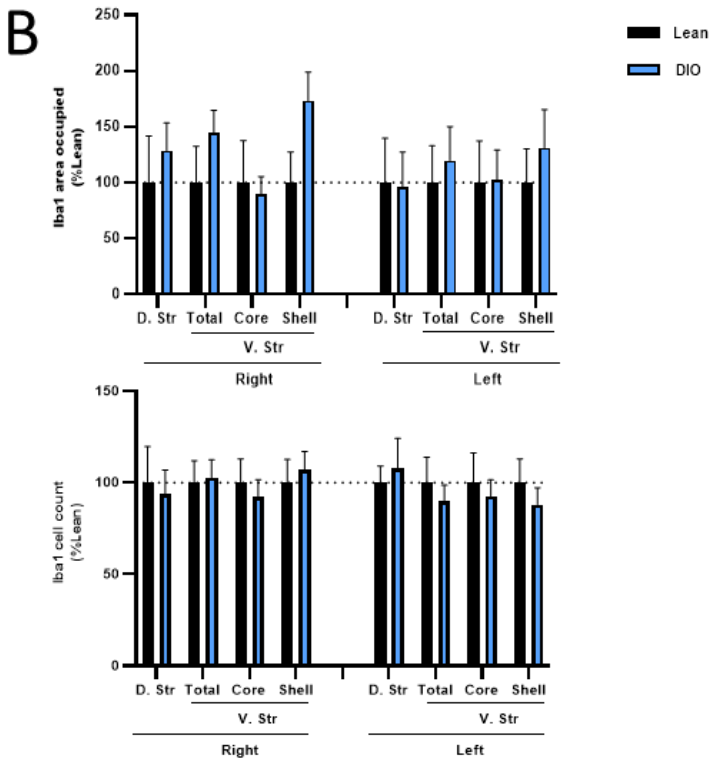
Finally, qPCR results showed a decrease in the expression of striatal enzyme *Lpl* in obese mice supplemented with *A. muciniphila*. In 2004, Cansell *et al.* identified this LPL, which is expressed at high levels in the striatum, as a lipid sensor modulating the motivational response to reward [367]. They reported that viral-mediated knockdown of *Lpl* in the ventral striatum to an extent that resembles the level observed with *A. muciniphila* supplementation leads to increased performance on operant conditioning test for a food response during the PR conditioning test and protects the reward system from deleterious effects of triglycerides in mice [367]. Furthermore, they recently proved that LPL expressed in the mesocorticolimbic pathway potentiates the action of circulating lipids and decreases the excitability of striatal DRD2 in medium spiny neurons [350]. Therefore, the reduction in *Lpl* expression and the subsequent reduction in the lipid sensing ability in the striatum

induced by *A. muciniphila* might participate in directly modulating dopaminoceptive signaling and behavioral output, restoring the motivational component observed during the operant conditioning test. Moreover, the potential reduction in fatty acids levels due to the reduction in LPL-mediated catabolism of triglycerides (TG) might also participate in the reduction in the expression and activation of the lipid-activated TLR4. Altogether, this potential pathway may be the mechanism by which *A. muciniphila* exerts its beneficial effects on the reward system. Furthermore, both the gut and the brain are fully equipped to detect TG and feedback to the reward system [379]. Hence, *A. muciniphila* supplementation may exert a positive effect on altering both gut TG sensing ability and vagal feedback to the brain and DA efflux, as reported by Tellez and co-authors [245]. Thus, *A. muciniphila* might exert beneficial effects by combining a brain-specific effects together with an indirect modulation of DA signaling by the gut-brain axis.

In conclusion, this study is the first to link the dysregulation of the food reward system with inflammation and alterations in the BBB in mesocorticolimbic areas of obese mice. Our data also identify beneficial effects of *A. muciniphila* administration on the brain reward system by reversing the motivational component of food reward altered by DIO. Moreover, we provide a potential mechanism to explain this effect through a decrease in inflammatory pathway activity and *Lpl* expression. Therefore, our results suggest that *A. muciniphila* supplementation might represent an additional and innovative approach to restore food reward behavior in obese individuals. Finally, this study provides an opportunity for further investigations of the effect of *A. muciniphila* on improving behavior in individuals with other neuropsychiatric disorders associated with CNS inflammation, such as Parkinson's disease or Alzheimer's disease.

3.5. Supplemental information





Supplemental Figure 1: Details of immunofluorescence quantification. (**A**) Immunofluorescence quantification of the area occupied by astrocytes cells and the Gfap + cells in the right and left dorsal striatum (D. Str), total ventral striatum (V. Str total), ventral striatum core (V. Str core) and ventral striatum shell (V. Str shell) of lean and DIO mice (n=4-5/group) after 5 weeks of follow-up. (**B**) Immunofluorescence quantification of the area occupied by microglial cells and the Iba1 + cells in the right and left dorsal striatum (D. Str), total ventral striatum (V. Str total), ventral striatum core (V. Str core) and ventral striatum shell (V. Str shell) of lean and DIO mice (n=5/group) after 5 weeks of follow-up. Data are shown as mean ± SEM. P-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. *: p-value <0,05; **: p-value <0,01 between lean vs DIO.

Target gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')
Rpl19	GAAGGTCAAAGGGAATGTGTTCA	CCTGTCTGCCTTCAGCTTGT
Drd2	CCAAGAACGTGAGGGCTAAG	TGAGGATGCGAAAGGAGAAG
Drd1	GAGCCAACCTGAAGACACC	TGACAGCATCTCCATTTCCAG
Th	GCCAAGGACAAGCTCAGGAAC	ATCAATGGCCAGGGTGTACG
Dat	AAATGCTCCGTGGGACCAATG	GTCTCCCGCTCTTGAACCTC
Iba1	TCCTCGATGATCCCAAATACA	GTTTCTCCAGCATTCGCTTC
Tlr4	CCCTCAGCACTCTTGATTGC	TGCTTCTGTTCCTTGACCCA
Gfap	GCTCCAAGATGAAACCAACC	CCAGCGATTCAACCTTTCTC
Cd45(Ptprc)	TGAGCACAACAGAGAATGCCC	AACACACCTGGATGATATGTGGT
Il-1β	TCGCTCAGGGTCACAAGAAA	CATCAGAGGCAAGGAGGAAAAC
Tnfα	TCGAGTGACAAGCCTGTAGCC	TTGAGATCCATGCCGTTGG
Il-6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
Cldn1	TTCGCAAAGCACCGGGCAGATACA	GCCACTAATGTCGCCAGACCTGAAA
Cldn5	TCATTGACCGGGAAGCTGAA	CCGGTGTCACAGAAGTACGA
Zo1	TTTTTGACAGGGGGAGTGG	TGCTGCAGAGGTCAAAGTTCAAG
Ocln	ATGTCCGGCCGATGCTCTC	TTTGGCTGCTCTTGGGTCTGTAT
Lpl	AGACTCGCTCTCAGATGCCCT	GCTTGCCATCCTCAGTCCC

Supplemental Table 1: Primer sequences

3.6. Materials and methods

Mice and experimental design

All mouse experiments were approved by the ethical committee for animal care of the Health Sector of the UCLouvain, Université catholique de Louvain under the specific number 2017/UCL/MD/005 and were performed in accordance with the guidelines of the local ethics committee and in accordance with the Belgian Law of May 29, 2013 regarding the protection of laboratory animals (agreement number LA1230314).

Experiment 1

A cohort of 9-week-old SOPF male C57BL/6J mice (Janvier laboratories, Le Genest-Saint-Isle, France) were housed in a controlled environment (room temperature of 22 ± 2 °C, 12h daylight cycle) in groups of two mice per cage, with free access to sterile food (irradiated) and sterile water. Upon delivery, mice were allowed to acclimatize during one week, during which they were fed a control diet (CT, AIN93Mi, Research Diet, New Brunswick, NJ, USA). Mice were then randomly divided in two groups (40 mice, n=20/group), and fed for 8 weeks with control low-fat diet (CT, AIN93Mi) or a HFD (or DIO, 60% fat and 20% carbohydrates (kcal/100g) D12492i, Research diet, New Brunswick, NJ, USA). After 4 weeks of follow-up, the mice were placed in behavioral cages to perform the food preference test and the operant wall test. During this last test, mice were food-restricted and body weights were maintained at 85% of the initial body weight (before the behavioral tests), as previously described [320]. The caloric restriction allowed to potentiate the reward response to the stimulus [334; 380].

Experiment 2

A cohort of 9-week-old SOPF male C57BL/6J mice (Janvier laboratories, France) were housed as described above. Upon delivery, mice underwent an acclimatization period of one week, during which they were fed a control diet (CT, AIN93Mi, Research Diet, New Brunswick, NJ, USA). Mice were then randomly divided in two groups (20 mice, n=10/group), and fed for 8 weeks with a HFD (60% fat and 20% carbohydrates (kcal/100g)

D12492i, Research diet, New Brunswick, NJ, USA). One group was treated with live *Akkermansia muciniphila* by oral gavage at a dose of 2.10^8 CFU suspended in sterile anaerobic PBS, produced and handled as previously described [280; 282]. The placebo group was orally administered an equivalent volume of sterile anaerobic PBS containing a similar end concentration of glycerol (2.5% vol/vol). Treatments continued until the end of the experiment (9 weeks in total). After 4 weeks of follow-up, the mice were placed in the behavioral cages to perform the food preference test and the operant wall test. During this last test, mice were food-restricted and body weights were maintained at 85% of the initial body weight (before the behavioral tests), as previously described [320]. The caloric restriction allowed to potentiate the reward response to the stimulus [334; 380].

Food preference test

During 3 hours in the daylight, mice were exposed to two diets: a low-fat, control diet (CT, AIN93Mi, Research diet, New Brunswick, NJ, USA) or HFHS (45% fat and 27.8% sucrose (kcal/100 g) D17110301i, Research diet, New Brunswick, NJ, USA) in Phenotyper chambers (Noldus, Wageningen, The Netherlands). The food intakes were recorded during a 3-hour session in the end of the light phase, in satiated state (access to food *ad libitum* before and after the test).

Operant wall test

The wanting component is linked to the motivation to obtain a reward and is evaluated by an operant wall test as previously described, with some adaptations [260; 350; 367]. Each session of the test was conducted during the end of the light phase, in operant conditioning chambers (Phenotyper chambers, Noldus, The Netherlands) and analyzed by the provided software (Ethovision XT 14). The mice had intermittent access to an operant wall in their home cages. The operant wall system is composed of two levers and two lights and a pellet dispenser. One lever is arbitrarily designated as active, meaning that pressing on this lever initiates the delivery of a sucrose pellet (5-TUT peanut butter flavoured sucrose pellet, TestDiet, St. Louis, MO, USA) and is associated with a light on. On the other side, another lever associated with a light off, is arbitrarily designated as inactive and will never deliver a

reward. Mice were trained for the system twice overnight on a fixed-ration schedule (one lever press corresponds to one reward), then underwent 2 sessions of 2 hours. Mice were then shifted to progressive ratio sessions (1h30), the number of lever presses to obtain a reward is incrementally increased (n+3) for every pellet.

Tissue sampling

At the end of each experiment, mice were maintained under caloric restriction and exposed for one hour to HFHS before anesthesia with isoflurane (Forene, Abbott, Maidenhead, England). This aims to mimic the conditions of the behavioral tests and stimulate the reward system. During anesthesia, blood was sampled from the portal and cava veins after which mice were euthanatized by decapitation. Striatum was precisely dissected and immediately immersed into liquid nitrogen, then stored at −80°C for further analysis.

RNA preparation and real-time PCR analysis

Total RNA was extracted from the striatum using TriPure reagent (Roche, Bale, Switzerland). cDNA was prepared by reverse transcription of 1µg total RNA using the GoScript Reverse Transcriptase kit (Promega, Madison, WI, USA). Real-time PCR was performed with the QuantStudio 3 real-time PCR system (Thermo Fisher, Waltham, MA, USA). *Rpl19* RNA was chosen as the housekeeping gene. All samples were performed in duplicate, and data were analyzed according to the $2^{-\Delta\Delta CT}$ method. The identity and purity of the amplified product were assessed by melting curve analysis at the end of amplification. Sequences of the primers used for real-time PCR are available in Supplementary Table 1.

Immunofluorescence

At the end of the experiment 1, mice were anesthetized by isoflurane and transcardially perfused using a solution of cold phosphate-buffered saline (PBS) followed by a solution of cold 4% (w/v) paraformaldehyde (PFA, Merck). The entire brain was carefully harvested, post-fixed in 4% PFA overnight at 4°C, cryoprotected overnight at 4°C in a solution of sucrose 30% (w/v), subsequently frozen in cold iso-pentane and stored at −80°C, as previously described[170]. Twenty micrometers thick serial coronal cryosection from fixed brain were

mounted on SuperFrost Plus slides (Menzel Gläser, Thermo Scientific, Waltham, MA, USA) and kept at −20 °C. For striatum, we harvested 8 serial sections per animal from bregma 0.61 mm to 1.41 mm according to 'The Mouse Brain in stereotaxic coordinates' (Paxinos, Franklin). Immunofluorescence was performed using Tyramide-signal amplification (TSA) technology, as previously described [170]. Briefly, after antigen retrieval (Dako S1699) by heating (2100 Antigen Retriever, Aptum, Southampton, UK) the endogenous peroxidases were inhibited in a solution of MeOH with H2O2 0.1% (v/v). Then the sections were incubated for 45 min in blocking solution (TBS, BSA 5%, Tween 20 0.1%) and then incubated overnight with a primary antibody (anti-GFAP 1/10 000, ab5804 from Merck or anti-Iba1 1/500, PA5-27436 from ThermoFischer). After washing, sections were incubated for 1h with Horse Radish Peroxidase-conjugated secondary antibody (DAKO K4003). The fluorescent signal was amplified using Alexa Fluor 488 Tyramide Reagent (B40953 ThermoFischer, USA). Finally, nuclei were stained with Hoechst 33342 (H1399 Invitrogen, USA). Slides were dehydrated and mounted with Dako Fluorescence Mounting Medium. Fluorescent GFAP scans were obtained using Oyster scanner (3DHistech Pannoramic P250 Flash III, Budapest, Hungary) and fluorescent Iba1 scans using Zeiss scanner (Axioscan.z1, Oberkochen, Germany). After blinding procedure, using Fiji software the region of interest (ROI) corresponding to the ventral striatum core, shell and striatum dorsal were delimited on each section using 'The Mouse Brain in stereotaxic coordinates' (Paxinos, Franklin) as reference and % of green area were measured. Positive neurons were manually counted within each ROI and a mean value was obtained for each animal. At least, three brain sections per animal were considered. Cell counting were double blinded. Quantifications corresponds to mean of two blinded independent experimenters.

Plasma multiplex analysis

Plasma levels of TNFα was measured by multiplex assay kits based on chemiluminescence detection and following manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD, USA). Analyses were done using a QuickPlex SQ 120 instrument (MSD, USA) and DISCOVERY WORKBENCH®4.0 software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.1.2 for Windows (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± SEM. Differences between two groups were assessed using unpaired Student's t-test. In case variance differed significantly between groups according to the Fisher test, a non-parametric (Mann-Whitney) test was performed. Difference between two groups and different time points was assessed using a two-way ANOVA repeated measurement, followed by Bonferroni post-hoc test. qPCR outliers have been excluded after Grubbs test.

Acknowledgements

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Disclosure statement

PDC, WMDV, AE and AdW are inventors on patent applications dealing with the use of *A. muciniphila* and its components in the treatment of metabolic disorders. PDC & WDV are cofounders of A-Mansia biotech SA.

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3.7. Complementary discussion, limitations and perspectives

With this paper, we demonstrated a new beneficial effect of the administration of *Akkermansia muciniphila* in the context of obesity: the restauration of a functional reward system and the attenuation of HFD-induced inflammation in reward-related brain areas.

In the submitted paper, we included the investigation of the liking and the wanting components of the food reward. However, we also assessed the learning component of the food reward with the CPP test (Fig. 1). Even if some data in the literature showed a beneficial effect of *Akkermansia muciniphila* in the learning and memory [375], in our study, the administration of *Akkermansia muciniphila* did not impact the conditioned behavior associated with a food reward stimulus (Fig. 1).

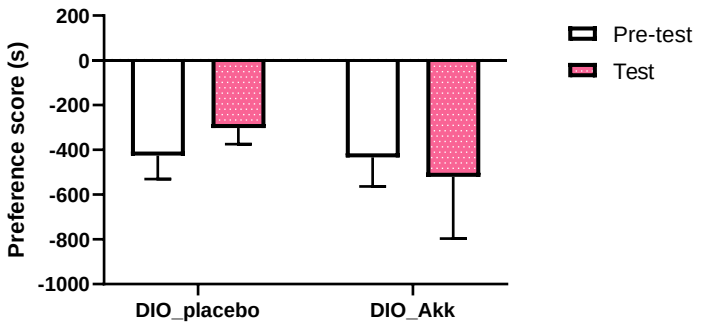

Figure 1 : **Preference score of conditioned place preference** based on the difference of time spent (s) in the palatable food-associated side vs the time spent in the neutral-associated side of the cage during the pre-test and the test by obese mice treated with the placebo (DIO_placebo) or *Akkermansia muciniphila* (DIO_Akk).

We evoked the increased expression of *Lpl* (involved in fatty acid uptake) as a potential mechanism linking the triggering of inflammation pathways and the alterations of the motivation for food reward in diet-induced obesity. We focused on the obese condition and did not include a control group as this study was purely exploratory. Hence, this is one main limitation of this study.

When examining the mechanisms behind the beneficial effects of *Akkermansia muciniphila* alive on the reward system, we suggested that inflammation and LPL are potential

mediators. However, there are many other potential pathways involved, the next paragraphs will discuss some that were analyzed or that needs further analyses. We found that the supplementation with alive *Akkermansia muciniphila* on the expression of the metabolic sensor *Lpl* seemed specific to the brain, as its expression is unchanged in the intestine (jejunum, data not shown). However, other lipases and lipid transporters could be involved in the brain lipid sensing, influencing inflammation and reward, including the adipose triglyceride lipase or the hormone-sensitive lipase. It has been shown that the hormone-sensitive lipase, hydrolyzing tri- and diglycerides plays a key role in the food intake and the energy homeostasis as the deficiency in this enzyme in the hypothalamus promotes food intake and body weight gain [381].

Metabolic sensors such as endocannabinoids could mediate the effect of alive *Akkermansia muciniphila* at the level of the intestine. Indeed, the supplementation with alive *Akkermansia muciniphila* changes the endocannabinoid tone in the gut, increasing OEA, 2-OG, 2-AG and 1-PG/2-PG) [170; 183; 283]. Since the vagal-endocannabinoid signaling plays a key role in the food reward and that OEA is described as a sensor influencing palatable food intake, this pathway deserves more investigation [244; 245].

In addition, it has already been shown that the administration of bacteria could impact neurotransmitters levels in the brain, influencing the behavior [180; 259]. Here we quantified neurotransmitters in the striatum of obese mice treated with the placebo or with alive *Akkermansia muciniphila.* Surprisingly, we did not find any difference in dopaminergic transmission (dopamine, DOPAC, HVA, 3-MT), but we found a significant decrease in epinephrine and a trend for the norepinephrine (Fig. 2A,B). The clear implication of noradrenaline and adrenaline in the reward processes is still unclear. However, noradrenaline and dopamine signaling are closely linked. Dopamine is the precursor of noradrenaline and adrenaline through dopamine β-hydroxylase in noradrenergic terminals and phenylethanolamine N-methyltransferase in adrenergic terminals [382]. At high concentrations, dopamine and noradrenaline can share their post-synaptic receptors and pre-synaptic transporters. Another interaction between catecholamines and dopaminergic systems, is the potentialization of dopamine release by noradrenaline. Psychostimulant

administration increases extracellular noradrenaline in mesocorticolimbic areas, which in turns induces burst firing of dopaminergic VTA neurons and increases dopamine release in the Nac [382]. We are the first to show that administration of *A. muciniphila* is associated with changes in adrenaline and noradrenaline in the striatum. It opens the way for future work to better understand how this could influence the reward system on a behavioral and neuronal approaches. Another team working on *Bacteroides uniformis* has also found changes in striatal levels of noradrenaline and adrenaline in the striatum after bacterial supplementation, and a reduction of binge eating and anxiety-like behavior [259].

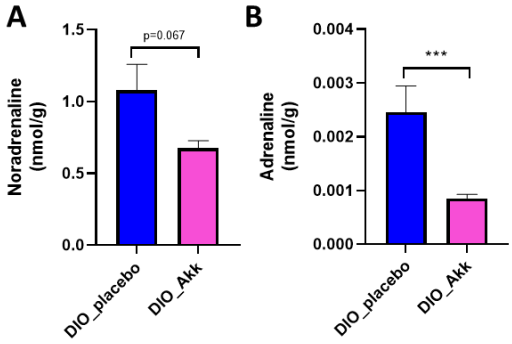

Figure 2: Striatal catecholamines modulation by *Akkermansia muciniphila*. (**A**) Noradrenaline and (**B**) adrenaline concentrations measured by UPLC-MS in HFD mice treated with placebo (DIO_placebo) or *Akkermansia muciniphila* (DIO_Akk) after 4 weeks of follow-up. P-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. ***: p-value <0,001.

IV. General discussion, conclusions and perspectives

Dysregulations of food intake and the susceptibility to overeat palatable food is thought to greatly contribute to the obesity pandemic. Targeting factors participating to the exaggerate hedonic food intake and associated neuronal alterations would help in the management of body weight loss, together with other therapeutic approaches like increasing physical activity and pharmacological treatments. Since the gut microbiota plays a key role on food intake regulation in the homeostatic brain areas, through different mechanisms, including the production of metabolites or vagus nerve activation, we hypothesized that the gut microbiota could also influence hedonic food intake and the food reward system.

We used FMT to prove the role of the gut microbiota in behavioral and neuronal alterations of the reward system associated with obesity. In addition, we tested other methods to modulate the gut microbiota such as antibiotics treatment and the oral administration of a potential beneficial bacteria, *Akkermansia muciniphila*.

The originality of this PhD work is to link the gut microbiota with the brain reward system, and thereby combine FMT with behavioral tests assessing the reward system, such as the food preference test, the operant wall test and the conditioned food preference test. The validation of these tests in the context of food-induced reward was an integrative part of my PhD work, and will represent new tools available in the field once published.

We validated the behavioral and neuronal alterations of the food reward in the context of obesity in chapter 1 and 2 (chapter 3 replicated the results). Chapter 1 focused on the liking component of the food reward, whereas the chapter 2 investigated the wanting and the learning components in diet-induced obese mice compared to lean mice. Obese mice eat less palatable food during the food preference test, suggesting an alteration of their liking, and press less on the lever to obtain a food reward, reflecting an alteration of their motivation. Importantly, even if the protocols were not exactly the same between experiments (for example, the duration of high-fat diet treatment was different in chapter 1 and 2), the behavioral alterations in the liking (chapters 1 and 2) and the wanting components (chapters 2 and 3) of the reward system are consistent in obese conditions.

The alteration of the learning component in DIO mice needs to be confirmed, because the effect was not significant in our study and not systematic among studies of our team. As every other preclinical study, our model of obesity and probably our behavioral tests do not perfectly reflect obesity development and the behavior of humans, because we work in a controlled environment, excluding many factors such as external cues. Indeed, human data showed that obese subjects do not rate decreased subjective liking but show increased wanting for palatable food [73]. This could be attributed to greater hedonic value due to past experience, and the associated conditioning. Indeed, obese individuals are hyper reactive to food cues, but have less activation of their reward system when actually eating palatable food, leading to overeating [73]. In our preclinical behavioral tests, we took care to use different palatable stimuli for each test to keep the novelty of the stimulus of the reward system. We hypothesized that obese mice already have a dysregulated reward system due to prolonged high-fat diet at the time of the behavioral tests, the liking, the wanting and the learning are thus already altered, as we have shown.

To investigate the roles of gut microbes in these alterations, the three behavioral components of the reward system were then assessed in lean mice receiving a gut microbiota from obese or lean donors. We proved that the transplantation of the gut microbiota from obese donors reproduced the hedonic alterations of food intake as seen in obese donors during the food preference test. The learning component was also transferred although the effect was less evident as the obese donors did not exhibit a clear alteration in their learning process during the CPP test. Surprisingly, concerning the wanting component, the recipient mice of a gut microbiota from obese donors were excessively motivated to obtain a food reward during the operant wall test.

By using metabolomic analyses, we discovered that recipient mice of a gut microbiota from obese donors had significantly higher levels of a gut-derived metabolite, the 33HPP, than mice receiving gut microbiota from lean donors. However, 33HPP was not elevated in obese donors. This difference might be due to the type of diet, since all recipient mice were kept under the same control diet. We hypothesized that the combination of a microbiota from obese donors and a control diet led to this high level of phenolic compound. In this context,

we suggested that 33HPP could be a potential inducer of a higher motivation for food reward.

As described in the thesis, to study the causal roles of gut microbes, we used the fecal material from lean or obese donors that we prepared and inoculate into lean mice previously depleted in gut microbiota through broad-spectrum antibiotics and laxative treatment. Then, to assess the efficiency of the gut microbiota engraftment from the donor into the recipient, we validated the efficacy of the transfer using Venn diagrams, showing common OTUs or genera shared in the cecal content of donor and recipient mice. However, this analysis does not take into consideration the proportion in relative abundance of the bacteria. It is not excluded that the engraftment was effective but that the overall gut microbiota composition and its subsequent metabolic functionality (as for the production of 33HPP) is not the same between donor and recipient mice.

However, and importantly, one innovative and consistent finding (replicated in chapters one and two) of this PhD work is the decrease in the expression of the dopaminergic markers in the reward-related brain areas (Nac and striatum) of mice receiving a gut microbiota from obese donors. Even if the gut microbiota compositions of obese donors and their recipient mice are not perfectly identical, the consequences on the brain reward system seem similar. Based on this, we can assume that the transplantation of obese-derived gut microbiota induces alterations in the dopaminergic reward system. Although FMT studies are considered as gold standard to prove causality between the gut microbiota and a physiopathology, and that we took care to use a validated FMT protocol (in the laboratory and in other studies), we never know exactly what we transfer. In other words, which bacteria and which metabolites are remaining after the preparation of the inoculum, the storage, the defreezing ? To answer that question, the analysis of the inoculum could be of great interest.

In this PhD work, we discovered a potential mechanism underlying the reward hypo-functioning in obesity: the triggering of inflammation pathways in reward-related brain area. This includes the activation of microglia, astrocytes and the increased expression of

pro-inflammatory cytokines in the striatum of DIO mice compared to lean mice. Some markers reflecting the integrity of the BBB were also affected in the reward-related brain areas of obese rodents, suggesting that the brain would not be isolated from potentially harmful molecules anymore (inflammatory cytokines, immune cells…).

We demonstrated the key role of the gut microbiota in reward behaviors with the supplementation with alive *Akkermansia muciniphila* in DIO mice. Alive *Akkermansia muciniphila*-supplemented mice showed an improvement of their motivation to obtain a food reward during the operant wall test. Even more, we showed that the administration of alive *Akkermansia muciniphila* decreased the expression of inflammatory markers in the striatum of HFD-fed mice. With this study, we discovered new beneficial effects of *Akkermansia muciniphila* on the behavior and inflammation in the brain.

In the context of obesity, our laboratory has demonstrated that the heat-killed formulation of the bacterium *Akkermansia muciniphila* was more efficient against diet-induced obesity and the development of metabolic disorders in rodents and humans [280; 281; 282]. Therefore, we launched a new experiment, including 3 groups (Fig. 1): mice under control diet, gavaged with a placebo (CT_placebo), mice under HFD supplemented with a placebo (HFD_placebo) and mice under HFD supplemented with pasteurized *Akkermansia muciniphila* (HFD_Akkpast). We took advantage of this new experiment to test the effects of the pasteurized form of *Akkermansia muciniphila* (heated at 70°C during 30min) on the food reward system, to compare it to the effects of the bacterium alive, at the same dose (2.10^8 bacteria per day). Surprisingly, in this study, we did not see any difference in term of body weight or fat mass between HFD mice treated with the placebo or with pasteurized *Akkermansia muciniphila* (Fig. 1A, B). That could be explained by the abnormal limited body weight gain during HFD in this experiment thereby limiting the effects of *Akkermansia muciniphila*. However, the fact that both HFD groups were perfectly body-weight and fat mass-matched allowed us to see effects on the food reward system independently from adipose signals.

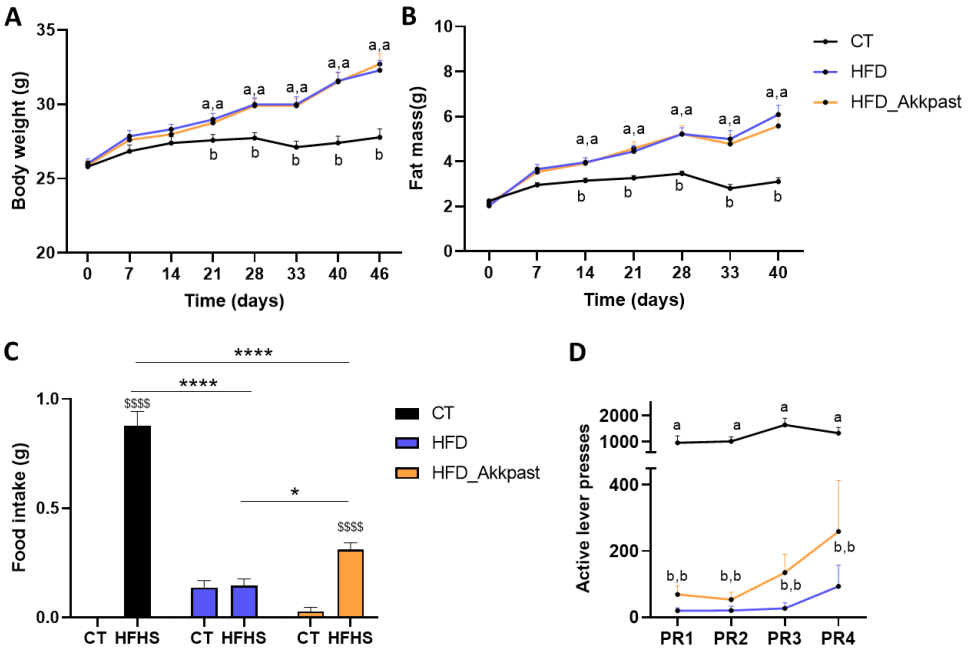

Figure 1: Effects of the supplementation of pasteurized *Akkermansia muciniphila* on the food reward. (A) Body weight and (**B**) fat mass evolutions (g) of control mice treated with a placebo (CT), high-fat-fed mice treated with a placebo (HFD) and high-fat-fed mice treated with pasteurized *Akkermansia muciniphila* (HFD_Akkpast). (**C**) Food preference test showing HFHS and CT intake in grams after 3 hours of test by control mice treated with a placebo (CT), high-fat-fed mice treated with a placebo (HFD) and high-fat-fed mice treated with pasteurized *Akkermansia muciniphila* (HFD_Akkpast) after 7 weeks of follow-up. (**D**) Operant conditioning test showing the number of active lever presses during the four progressive ratio sessions (PR) by control mice treated with a placebo (CT), high-fat-fed mice treated with a placebo (HFD) and high-fat-fed mice treated with pasteurized *Akkermansia muciniphila* (HFD_Akkpast) after 7 weeks of follow-up. Data are shown as mean ± SEM. P-values were obtained after (**A,B**) two-way ANOVA repeated measure followed by Bonferroni post-hoc test (n=14-16/group), (**C**) after two-way ANOVA followed by Bonferroni post-hoc test (n=6-8/group), (**D**) after two-way ANOVA repeated measure followed by Bonferroni post-hoc test (n=7-8/group). (**A,B,D**) Different letters mean significant differences at p-value < 0,05. (**C**) *: p-value < 0,05; ****: p-value < 0,0001 between CT *vs* HFD or HFD_Akkpast HFHS intake. $$$$: p-value < 0,0001 between CT and HFHS intake.

In this study, we first performed the food preference test. As expected, the two groups under HFD (HFD and HFD_Akkpast) eat less palatable food (HFHS) than lean mice under control diet (CT) (Fig. 1C). Importantly, we showed here that the administration of pasteurized *Akkermansia muciniphila* increased the hedonic food intake and induced a

preference for HFHS diet over CT diet. These results show that independently from adipose signals, the supplementation in *Akkermansia muciniphila* has beneficial effects on hedonic food intake in the context of obesity, as it reverses the high-fat diet-induced alterations.

Moreover, at the operant wall test assessing the motivation for a food reward, we repeated our previous findings describing the alteration of the motivational component of the reward system associated with obesity. Mice under HFD (HFD and HFD_Akkpast) pressed significantly less on the lever to obtain a reward than control mice (CT) during the PR sessions of the operant wall test (Fig. 1D). Interestingly, even if it did not reach statistical significance, it seems that mice treated with pasteurized *Akkermansia muciniphila* (HFD_Akkpast) pressed more on the active lever than placebo-treated mice (HFD). This trend follows the results of HFD mice treated with *Akkermansia muciniphila* alive (described in chapter three).

Altogether, the two studies involving the supplementation in *Akkermansia muciniphila* clearly show beneficial effects on the behavioral patterns of the food reward system in the context of obesity. If the pasteurized *Akkermansia muciniphila* also acts on inflammation and *Lpl* expression in reward-related brain areas still remains under investigation.

Of note, because we identified inflammation in the striatum as an underlying mechanism of reward alterations in obesity and a potential role for the gut microbiota, we investigated the inflammation hypothesis in the FMT experiment (from chapter 2). We quantified the expression of inflammation markers by qPCR to assess if the gut microbiota from obese donors was associated with increased inflammation in reward-related brain areas. We found an increased expression of *Tmem119*, and a trend for *Gfap* in the Nac of recipient mice of a gut microbiota from obese donors (Fig. 1), suggesting an activation of the microglia in DIO_rec group. Because of the limited quantity of cDNA and remaining brain tissues from the FMT studies, we were unable to make substantial analysis of the inflammation in the reward-related brain areas of recipient mice. Further investigations would be worth it, including the analysis of inflammatory markers by immunofluorescence after paraformaldéhyde-perfusion on animals.

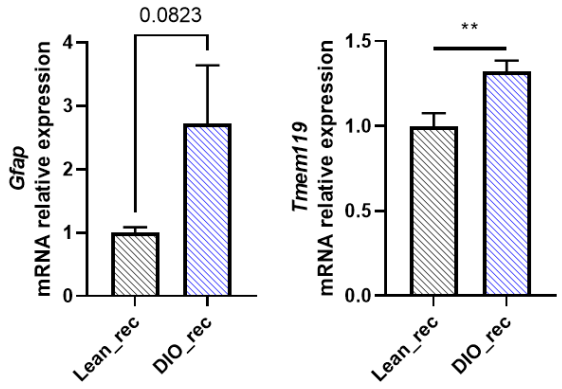

Figure 2: Inflammation markers in the nucleus accumbens of gut microbiota recipient mice. Nucleus accumbens mRNA expressions of glial fibrillary acidic protein (*Gfap*) and transmembrane protein 119 (*Tmem119*) measured by qPCR in lean (Lean_rec) and DIO recipient mice (DIO_rec) after 5 weeks of follow-up. Data are shown as mean ± SEM (n=7-8/group). p-values were obtained after unpaired Student's t-test or non-parametric Mann-Whitney test. **: p-value ≤ 0,01.

A general perspective to this PhD work would be the confirmation of our findings on the brain reward system, by other complementary techniques. In fact, we often use qPCR, as it a relevant and fast technique to assess metabolic changes. In the context of the reward system, we took the precaution to test the best conditions to evaluate food reward markers by qPCR after activation of the reward-related brain area. Therefore, we tested during a preliminary study the transcriptional effect in the striatum of the exposure to sucrose pellets 1 hour before the euthanasia. We showed that peanut-butter flavored sucrose pellets (the same used during the operant wall test) stimulate reward-related brain area as they induced an increased expression of *cFOS* in the striatum, as compared to fed mice and calorie restricted mice (Fig. 2).

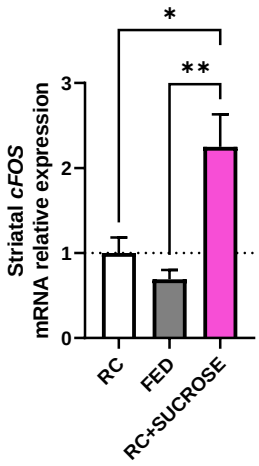

Figure 2: Neuronal activation of sucrose pellets under calorie restriction. Striatal mRNA expression of cFOS measured by qPCR in calorie restricted mice (RC), fed mice (fed) and calorie restricted mice exposed to sucrose pellets (RC+sucrose). Data are shown as mean ± SEM (n=4-7/group). p-values were obtained after unpaired One-way ANOVA followed by Tuckey post-hoc test. *: p-value ≤ 0,05; **: p-value ≤ 0,01.

On a practical point of view, to go further into the mechanisms, an analysis of DRD2 and DRD1 neurons activity in real-time during behavioral tests could provide additional information of the reward system. For this purpose, the use of fiber photometry technique would provide fluorescence emitted from a calcium sensor which can be expressed in targeted neurons specifically after genetic modifications. In the same idea, microdialysis technique give a real-time quantification of neurotransmitters thanks to the collection the dialysates from a semi-permeable membrane in the region of interest [383; 384; 385]. Both techniques require the implantation of a cannula in targeted regions [386; 387]. However, they could represent short-term perspectives as our team newly acquired stereotaxic material to implement cannula or to precisely make injection in the brain of awake mice. Replicating the experience with the administration of *Akkermansia muciniphila* alive (chapter 3) or with chronic treatment with 33HPP (chapter 2) and then evaluating the electrical activity in reward-related brain areas through electrophysiology technique would give a functional perspective of the reward system activity during the operant wall testing. To better understand the neuroactive potential of 33HPP, the stereotaxic injection of 33HPP

directly inside the nucleus accumbens and the assessing of the behavior could also represent an interesting perspective. In this PhD work, we often observe DRD1 and DRD2 mRNA variations after FMT or after 33HPP treatment. To decipher the specific roles of DRD1 and DRD2 activities in the behavior, the injection of specific agonists in the same conditions (*i.e.* after FMT or 33HPP treatment) would give substantial information about the pharmacological targets.

Finally and as mentioned in the introduction, one key mechanism linking the gut to the brain reward system is the vagus nerve [132]. Vagotomy studies would potentially clarify how gut microbiota interact with the brain reward system. Indeed, gut microbes such as *Akkermansia muciniphila* influence many mediators in direct contact with the vagus nerve. For example, *Akkermansia muciniphila* produces SCFAs that bind FFAR receptors on vagal afferent fibers to inform the brain [232]. *Akkermansia muciniphila* has also been shown to influence the number of EEC in the gut and it has been described that EEC act like a synapse with vagal afferents nerves, using glutamate as neurotransmitter, to transduce signals to the brain [131; 183]. Therefore, the investigation of the vagus nerve in this context could be a relevant perspective to uncover the mechanisms of crosstalk between gut microbe and the food reward.

Main conclusions

- Diet-induced obesity is associated with behavioral alterations of the food reward system: the liking, the wanting and the learning are decreased.
- The gut microbiota from obese donors participates to alterations of the liking and the learning components of the food reward whereas gut microbes could participate to compulsive behavior associated with obesity.
- The gut microbiota from obese donors induces brain reward dysregulations, including reduced expression of dopaminergic receptors.
- The 33HPP is a gut-derived metabolite positively correlated with the motivation to obtain a food reward.
- *Akkermansia muciniphila* restores the motivational component of the food reward and attenuates the inflammation in diet-induced obese mice.

V. Appendix

1. Abbreviations

2-AG	2-arachidonoylglycerol
2-OG	2-oleoylglycerol
2-PG	2-palmitoylglycerol
33HPP	3-(3-hydroxyphenyl)propionate
3-MT	3-methoxytyramine
AA	arachidonic acid
ABHD6	α/β hydrolase domain 6
AEA	anandamide
AgRP	agouti-related protein
AP-1	activator protein-1
Arc	arcuate nucleus
BBB	blood–brain barrier
BDNF	brain-derived neurotrophic factor
BMI	body mass index
CART	cocaine-amphetamine-related transcript
CB1	cannabinoid receptors 1
CB2	cannabinoid receptors 2
CCK	cholecystokinin
CD45	cluster of differentiation 45
ClpB	caseinolytic peptidase B
CNS	central nervous system
CPP	conditioned-place preference
CSF	cerebro-spinal fluid
CUMPS	chronic unpredictable mild stress
DAGL	diacylglycerol lipase
DAT	dopamine transporter
DCA	deoxycholic acid
DIO	diet-induced obese
DOPA	3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DRD1	dopamine receptor 1
DRD2	dopamine receptor 2
eCBome	endocannabinoidome
EECs	enteroendocrine cells

FAAH	fatty acid amide hydrolase
FDR	false discovery rate
FMT	fecal material transplantation
FOS	fructo-oligosaccharides
FR	fixed ratio
FXR	farnesoid X receptor
GABA	γ-aminobutyric acid
GF	germ-free
GFAP	glial fibrillary acidic protein
GI	gastrointestinal
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide-1
GLP1R	glucagon-like peptide 1 receptor
GPR	G-protein-coupled receptor
HDL-C	high-density lipoprotein cholesterol
HFD	high-fat diet
HFHS	high-fat high-sucrose
HVA	homovanillic acid
Iba-1	ionized calcium-binding adaptor protein-1
IBD	inflammatory bowel syndrome
IL-1β	interleukine-1β
IL-6	interleukine-6
JNK	c-Jun N-terminal kinase
LCA	lithocholic acid
LEA	*N*-linoleylethanolamine
LH	lateral hypothalamus
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAGL	monoacylglycérol lipase
MC4R	receptor of melanocortin 4
MCH	melanin-concentrating hormone
MCT	monocarboxylate transporter
MRI	magnetic resonance imaging
NAAA	N-acylethanolamine-hydrolyzing acid amidase
Nac	nucleus accumbens
NAPE-PLD	N-acylphosphatidylethanolamine phospholipase D

NF-κB	nuclear factor-κB
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OEA	*N*-oleoylethanolamine
Oprd	δ-opioid receptor
Oprk	κ-opioid receptor
Oprm	μ-opioid receptor
OTUs	operational taxonomic units
PAMPs	pathogen-associated molecular patterns
PCoA	principal coordinate analysis
Pdyn	pre-prodynorphin
PEA	*N*-palmitoylethanolamine
PFC	prefrontal cortex
POMC	pro-opiomelanocortin
PPARα	peroxisome proliferator-activated receptor α
PR	progressive ratio
PVN	paraventricular nucleus
PYY	peptide YY
qPCR	quantitative polymerase chain reaction
SCFAs	short-chain fatty acids
SIM1	single-minded family BHLH transcription factor 1
SOPF	specific-opportunistic and pathogen-free
SPF	specific pathogen free
TG	triglycerides
TGR5	takeda G protein-coupled receptor 5
TH	tyrosine hydroxylase
TLR	toll-like receptor
TNFα	tumor necrosis factor-α
TRPV1	transient receptor potential cation channel V1
VAN	vagal afferent nerves
VTA	ventral tegmental area
WHO	world health organization
ZO-1	zonula occludens-1
α-MSH	α-melanocyte-stimulating hormone

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