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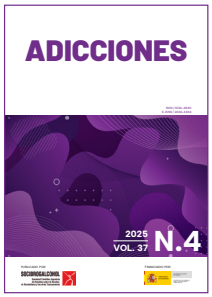

ORIGINAL

Loss of eating control and cognitive flexibility: Involvement of gut microbiota

Pérdida del control de la ingesta alimentaria y flexibilidad cognitiva: Participación de la microbiota intestinal

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Abstract

Loss of eating control is a crucial factor in developing obesity, which has become a global health concern, causing important cardiovascular, metabolic, emotional, and cognitive co-morbidities. A major cognitive alteration associated with loss of eating control and obesity is the impairment of cognitive flexibility and inhibitory control. An increasing number of studies confirm that gut microbiota is a significant contributor to loss of eating control, obesity, and cognitive function. Therefore, we have investigated whether gut microbiota transfer from humans with impaired/not impaired cognitive flexibility could substantially affect this behavioral response in mice in the context of obesogenic versus standard diet. Mice were pretreated with an antibiotic cocktail and later received a gut microbiota transplant from human subjects. The transferred microbiota was maintained in mice for seven weeks. Afterward, behavioral tests were performed to evaluate different cognitive responses, locomotor activity, anxiety-like, and depression-like behaviors. Antibiotic treatment significantly impaired short-term memory in mice, as previously reported. Furthermore, mice that received microbiota from high and low cognitive flexibility subjects modified their short-term and long-term memory performance depending on the diet exposure. Slight changes were observed in the locomotor activity, primarily in the high-fat diet-fed antibiotic-treated mice, and no significant alterations were observed in anxiety-like or depressive-like behaviors. In summary, this study shows that gut microbiota is a major contributor to cognitive flexibility, which may open novel therapeutic strategies for combating loss of eating control and related metabolic co-morbidities.

Keywords: Loss of eating control, obesity, food addiction, gut microbiota, cognitive flexibility, fecal microbiota transplant

Resumen

La pérdida del control de la ingesta alimentaria es un factor crucial en el desarrollo de la obesidad. Una alteración cognitiva importante asociada con la pérdida del control de la ingesta alimentaria y la obesidad es el deterioro de la flexibilidad cognitiva y el control inhibitorio. Un número cada vez mayor de estudios confirman que la microbiota intestinal contribuye significativamente a la pérdida del control de la ingesta alimentaria, la obesidad y la función cognitiva. En base a estos datos, hemos investigado si la transferencia de microbiota intestinal de humanos con obesidad/normopeso y flexibilidad cognitiva deteriorada/no deteriorada podría afectar sustancialmente esta respuesta conductual en ratones expuestos a una dieta obesogénica frente a una dieta estándar. Los ratones fueron tratados previamente con un cóctel de antibióticos y luego recibieron un trasplante de microbiota intestinal de humanos. Posteriormente, se realizaron diferentes test de comportamiento para evaluar la flexibilidad cognitiva como una medida del control inhibitorio. El tratamiento con antibióticos deterioró significativamente la memoria a corto plazo en ratones, como se ha informado previamente. Es importante destacar que los ratones que recibieron microbiota de sujetos con alta y baja flexibilidad cognitiva modificaron su rendimiento de memoria a corto y largo plazo en función de la exposición a la dieta. En resumen, este estudio muestra que la microbiota intestinal es un factor que contribuye de manera importante a la flexibilidad cognitiva, lo que puede abrir nuevas estrategias terapéuticas para combatir la pérdida de control de la ingesta alimentaria y las comorbilidades metabólicas asociadas.

Palabras clave: Adicción a la comida, obesidad, microbiota intestinal, deterioro cognitivo, trasplante de microbiota fecal

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Loss of eating control is a behavioral alteration closely related to the development of overweight and obesity, metabolic disorders that have reached epidemic levels, with over 890 million people being diagnosed with obesity (WHO. Obesity and Overweight, 2022). Overweight and obesity are serious health concerns associated with an increased risk of cardiovascular disease, type 2 diabetes, and cognitive alterations (Arnoriaga-Rodríguez et al., 2020a; Kloock et al., 2023). Current obesity-related treatments have limited efficacy, and 35 to 50% of obese individuals relapse during the first year, mainly due to a loss of eating control that several authors have even defined as a food addiction behavior with close similarities to the behavioral alterations that define substance use disorders (Hussain & Bloom, 2013).

Food addiction is a compulsive eating disorder arising from a disbalance between homeostatic and hedonic food intake control systems. Individuals with food addiction demonstrate a lack of cognitive flexibility and inhibitory control, leading to high compulsivity, motivation, and impulsivity toward highly palatable food (Domingo-Rodriguez et al., 2020). Despite not being included in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), food addiction can be diagnosed by the Yale Food Addiction Scale-2 (YFAS-2). YFAS-2 is a 35-item questionnaire based on the DSM-5 criteria for substance use disorder (Penzenstadler et al., 2019). Interestingly, according to YFAS-2, several studies have demonstrated that not all individuals with obesity have food addiction since around 25-37% of obese and 60% of morbidly obese individuals are diagnosed with food addiction (Gupta et al., 2020), pointing to a rather similar etiology of these eating disorders.

The Western diet is a modern diet high in refined carbohydrates and saturated fats, and is the major factor leading to loss of eating control and obesity (López-Taboada et al., 2020). Indeed, the high palatability of food can induce maladaptive changes in the brain, contributing to the development of these behavioral and metabolic alterations (Moore et al., 2017). Furthermore, cognitive flexibility and inhibitory control play a major role in the regulation of food intake and determining food choices. Several authors have demonstrated that people with obesity have memory alterations (Cheke et al., 2016) and impaired cognitive flexibility (Bocarsly et al., 2015; Song et al., 2022). In addition, high-fat diet consumption has major negative consequences on cognitive performance (Underwood & Thompson, 2016) and cognitive flexibility (Magnusson et al., 2015). On the other hand, memories about past eating experiences determine future eating habits and food choices and guide attention toward food cues, thus shaping our cognitive and inhibitory responses related to food intake control. It is clear that impairments in cognitive flexibility and cognitive performance can exacerbate overeating and contribute to the development of both obesity and food addiction (Higgs, 2016). Indeed, the engagement in secondary sedentary and attention-required activities disrupts memory formation and flexibility, resulting in enhanced food intake (Higgs, 2015). Furthermore, patients with memory impairment show overeating due to the inability to recall recent eating events (Higgs et al., 2008). Thus, a vicious cycle is formed in the case of obesity, where obesity induces cognitive alterations, which in turn further exacerbate the loss of eating control and overeating.

Several studies have highlighted the involvement of gut microbiota in various health conditions, pointing to a new possible tool for disease prevention and treatment. Gut microbiota is involved in gut homeostasis by maintaining the integrity of the gut epithelium and participating in metabolism and immunity. Apart from local effects in the gut, the microbiota is known to play a role in metabolic processes and affect brain functioning through the gut-brain axis (Hou et al., 2022; Megur et al., 2022). Conversely, microbiota alterations due to the consumption of a Western diet or the use of antibiotics often lead to microbial dysbiosis. Gut dysbiosis is associated with an increase in gut permeability and systemic low-grade inflammation, which subsequently contributes to increased blood-brain-barrier permeability and eventual neuroinflammation (Hrncir, 2022; Kearns, 2024). This neuroinflammation can further alter feeding behavior and impair cognitive processes and has been related to various psychiatric disorders (Alboni et al., 2017; DiSabato et al., 2016). Obese individuals display gut dysbiosis represented by a lower gut microbiome diversity and abundance, with a significant decrease in beneficial bacteria that may be related to their impairment in eating control, cognitive performance, and flexibility (Chen et al., 2021). Several of these previous studies have demonstrated that the cognitive alterations revealed in obese individuals can be transferred to mice by the use of fecal microbiota transplantation (FMT) (Arnoriaga-Rodríguez et al., 2020a; Arnoriaga-Rodríguez et al., 2021), further supporting the relationship between gut microbiota and behavioral responses. Indeed, FMT from obese individuals led to a decrease in short-term and working memory (Arnoriaga-Rodríguez et al., 2020a), and cognitive flexibility and inhibitory control impairment in mice (Arnoriaga-Rodríguez et al., 2021), indicating that microbiota modulations can potentially modify cognitive functions. FMT was also sufficient to transfer the depression phenotype from obese humans to mice (Mayneris-Perxachs et al., 2022a). Similarly, a recent study has demonstrated that a specific gut microbiota composition is necessary for the development of food addiction. Thus, food-addicted humans and mice demonstrated a decrease in *Blautia* sp., and the addictive phenotype was prevented by an oral administration of *Blautia wexlerae* in mice (Samulėnaitė et al., 2024). Based on these previous findings, we have now investigated the involvement of gut microbiota in

cognitive flexibility and inhibitory control by evaluating the consequences of FMT from humans with these behavioral disorders in mice exposed to obesogenic or standard diet.

Methods

Human participants

Twenty healthy human adult donors, women and men, were selected based on their cognitive flexibility scores, assessed by the Stroop Color-Word Test (SCWT) as described before (Castells-Nobau et al., 2024). The body mass index (BMI) and socioeducational status among the groups were investigated. SCWT is used to investigate inhibitory control and cognitive flexibility in humans. Cognitive flexibility/inhibitory control was evaluated in three subsequent steps. During the first part, a patient is introduced to 100 cards with printed words (color names) in black ink. During the second part, 100 cards of words are displayed in different colors of ink (green, red, blue). Ultimately, during the test, a patient is given 100 cards of color names, which are printed in a non-matching ink. Each part takes 45 seconds, after which a patient is evaluated. Cognitive flexibility/inhibitory control is evaluated using a formula: WC − WC′ (WC′= W × C/W + C). W (word) accounts for part one of the experiment, C (color) for part two, and WC (word-color) for the ultimate test. Those patients who obtained a final score lower than four were considered to have impaired cognitive flexibility/inhibitory control. In contrast, those who had more than six were considered to have optimal inhibitory control/cognitive flexibility.

Donor feces were prepared for oral administration to mice in sterile conditions with L-cysteine to reduce oxygen in an environment to protect anaerobic bacteria and 10% glycerol to protect bacteria from freezing-related damage. Samples were aliquoted and frozen at -80°C.

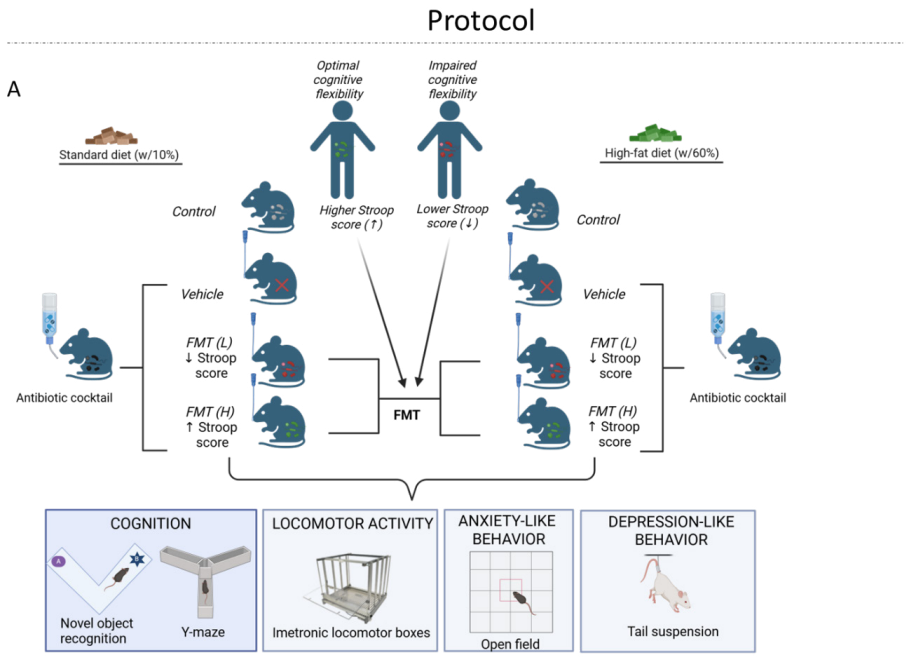
Animals and Experimental Design

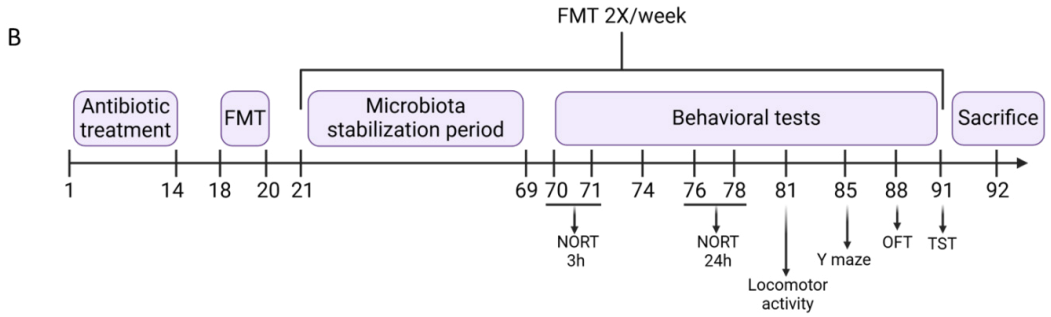
Seventy-seven male C57BL/6J mice (4 weeks of age, 17-21 g weight) were housed individually in controlled laboratory conditions with a temperature of 21 ± 1 ºC and humidity of 55% ± 10%, in an inverted cycle (lights off 7:30 a.m.-7:30 p.m.). Conventional mice, with no initial alterations in their gut microbiota composition, were used. Food and water were available *ad libitum*, and animals` body weight, food, and water intake were monitored during the experimental protocol. All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal – Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive (2010/63/EU).

The information regarding the experimental group assignment and the timeline is represented in Figure 1A and Figure 1B, respectively. Mice were randomly divided into eight groups, four of which were fed on a standard diet (w/10%) (~3.514 kcal/kg) and the other four on a high-fat diet (w/60%) (~5.228 kcal/kg). Irradiated, vacuum-packed diets were bought from Altromin (Germany). Both groups were further divided into 1) control, 2) vehicle, 3) FMT from individuals with low Stroop scores (FMT (L), 4) FMT from individuals with high Stroop scores (FMT (H) (Figure 1A). Each mouse received microbiota from a specific human donor, allowing for individual evaluation of the microbiota's effect on brain function, in the context of high-fat and standard diets.

Figure 1

Experimental protocol. A. Experimental protocol and group assignment. B. Experimental timeline (days)





Note. **A.** Fecal material from humans with impaired (low Stroop scores; FMT (L) or optimal cognitive flexibility (high Stroop scores; FMT (H) was processed for oral administration to mice. All mice, except for the control group, received antibiotic treatment, followed by an oral administration of vehicle or fecal microbiota transplantation (FMT) twice per week for the whole experimental protocol. Mice were fed on a standard or high-fat diet. At the end of the protocol, behavioral tests were performed: novel object recognition test (NORT) for short- and long-term memory, Y-maze for working memory, open field test (OFT), and elevated plus maze test (EPM) for anxiety-like behavior, and tail suspension test (TST) for depression-like behavior. Furthermore, locomotor activity was evaluated. **B.** Mice received antibiotic treatment for two weeks; after a 72-hour window, oral administration of vehicle or fecal microbiota transplantation was performed for three consecutive days and then twice per week for the whole experimental protocol. After seven weeks of stabilization, behavioral tests were performed to assess short-term (days 70-71) and long-term memory (days 76-78) by novel object recognition test, anxiety-like behavior by the elevated plus maze test (day 74), and open field test (day 88), and depression-like behavior (day 91) by tail suspension test.

All groups, except for both controls, received an antibiotic cocktail (500 mg/L ampicillin, 500 mg/L metronidazole, 500 mg/L vancomycin, 250 mg/L imipenem, 1g/l neomycin) daily for two weeks in drinking water, as previously described (Kelly et al., 2016). Seventy-two hours later, mice were orally gavaged with saline (1x PBS+1g/l L-cysteine+10% glycerol) (vehicle group) or fecal material from the donors (FMT groups) via oral gavage (200 mg/ml, 200 µL) for three consecutive days as previously described (Arnoriaga-Rodríguez et al., 2020a), later twice per week throughout the experimental protocol.

After seven weeks, mice were exposed to a series of behavioral tests, including a novel object recognition test (NORT 3h and NORT 24h, on days 70-71 and 76-78, respectively), locomotor activity (on day 81), Y maze (on day 85), open field test (OFT, on day 88) and tail suspension test (TST, on day 91). On the last day of the study (day 92), mice were sacrificed, and several brain areas and blood plasma were collected for further analysis (Figure 1B).

Cognition

Novel object recognition

A novel object recognition test (NORT) was performed to assess long-term and short-term memory as previously described (Burokas et al., 2014). Shortly, it is performed in a V-shaped maze (30 cm long × 4.5 cm wide × 15 cm height of each corridor), illuminated with 2.5 lux on both corners. On the first day, mice were habituated to a maze for 9 minutes. The following day, mice were introduced to a maze with two identical objects on opposite sides of the maze and left to explore for 9 minutes. After 3 hours or 24 hours, the test was performed to assess short-term or long-term memory, respectively. During the test, one of the familiar objects was replaced with a novel one, and mice were left to explore both figures for 9 minutes, and the total time spent exploring the two objects was measured. Mice were verified to ensure that there was no initial preference for the objects used. A discrimination index (DI) was calculated as the difference between the time the animal spent exploring either the novel (Tn) or familiar (Tf) object, divided by the total time exploring both objects: (DI = (Tn − Tf)/(Tn + Tf)). A higher discrimination index reflects high memory retention for the familiar object. Mice that explored for less than 10 seconds were excluded from the analysis. The analysis of the behavioral data was performed manually by the use of calibrated stopwatches.

Y maze

The Y maze test is based on the innate curiosity of rodents to explore previously unvisited areas. This test was performed to assess working memory as previously described (Vijaya et al., 2024). The maze is comprised of three identical arms intersecting at 120° (Y-shaped; 6.5 cm width × 30 cm length × 15 cm height), all three entries were illuminated with 10 lux. Entries into the arms of the maze were counted for 10 minutes (traversing the head and two front paws is considered a valid entry), and the percentage of spontaneous alternation was calculated by the sequential entries in all three arms divided by the total number of possible alternations. The analysis of the behavioral data was performed manually.

Locomotor activity

Locomotor activity was evaluated in the individual locomotor activity boxes (10.8 cm width × 20.3 cm length × 18.6 cm height, Imetronic, Pessac, France). The total activity (number of beam breaks) and the total number of rearings were detected during 1 hour by infrared sensors.

Anxiety-like responses

The open-field test (OFT) was used to assess anxiety-like behavior. This test is based on the conflict of innate fear and avoidance of bright, open areas (that mimic a

situation of predator risk) and an instinct to explore novel environments. Mice were placed in the middle of the box (90 cm x 70 cm, 500 lux), and their movements were recorded over the course of 5 minutes. The preference for being in the periphery of the box indicates higher anxiety-like behavior. The analysis of the behavioral data was performed with Smart 3.0 video tracking software.

Depressive-like responses

The tail suspension test (TST) evaluates depressive-like behavior. During the test, mice were suspended by their tails with tape in a position that prevented them from escaping or holding onto nearby surfaces for 6 minutes. The time of immobility (defined as the time during which the animal is hanging passively and motionless) was measured, and the higher the immobility time, the higher the depressive-like behavior. The analysis of the behavioral data was performed manually by the use of calibrated stopwatches.

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Figures were prepared using GraphPad Prism software (GraphPad Software), illustrations were done with Biorender, and statistical analysis was performed using IBM SPSS 28.0.

The normal distribution of the data was evaluated for all the datasets using the Shapiro-Wilk test to select appropriate statistical tests. Two-way ANOVA or Kruskal-Wallis comparisons were used when appropriate, followed by a Fisher`s post hoc analysis or Mann-Whitney U test, respectively, when necessary. The effect size for the two-way ANOVA was calculated as Eta squared (η^2), using SPSS, while the effect size for Kruskal-Wallis comparisons (η^2) was calculated manually. The values above 0.14 indicate a large effect. The effect size for the Mann-Whitney U test was calculated manually, as the rank biserial correlation (r), with r values above 0.5 indicating a strong effect. In Fisher`s post hoc test, the effect size was evaluated by Cohen`s d, which was calculated manually, and a large effect was considered when d values were above 0.8.

Results

Twenty healthy human donors, men and women with an average age of 48 ± 9, were selected. All individuals were obese, with an average BMI of 32.4 ± 9.8, of similar socioeducational status, with an average of 13 ± 4 years of education. The Stroop scores, evaluated by the SCWT, were lower than 4 in donors with impaired cognitive flexibility and higher than 6 in optimal cognitive flexibility donors. No significant differences between the age, sex, BMI, and socioeducational status were observed between the groups with different cognitive flexibility scores (Table 1).

Table 1
The information on human donors

Characteristic	Overall N = 20[1]	high STROOPI N = 10[1]	low STROOPI N = 10[1]	p-value[2]
age	48 ± 9	47 ± 7	49 ± 11	0.57
sex				0.63
1	6 (30%)	4 (40%)	2 (20%)	
2	14 (70%)	6 (60%)	8 (80%)	
BMI	32.4 ± 9.8	32.5 ± 10.8	32.3 ± 9.4	0.97
education_years	13 ± 4	13 ± 4	13 ± 3	0.81

[1] Mean ± SD; n (%)

[2] One-way analysis of means; Fisher's exact test

Note. The table demonstrates the age, sex, BMI, and years of education in the overall population and between donors with high or low Stroop scores.

A total of 77 male C57BL/6J mice, 4 weeks old, were used in the study. Mice were divided into 8 groups, with 9-10 mice per group (Figure 1A). Half of the mice were fed standard, and the other half were exposed to a high-fat diet. All mice, except for the control group, received an antibiotic cocktail for microbiota depletion and were orally administered a vehicle, as an additional control, or the gut microbiota from a human donor. Each mouse received microbiota from a specific human donor, allowing for individual evaluation of the microbiota's effect on brain function.

The impact of the gut microbiota on metabolic parameters

We monitored the body weight, food, caloric, and water intake in mice during the experimental protocol to examine the impact of diet and microbiota changes on these parameters. Although diet did not primarily affect mice`s body weight (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 585, n.s.; Figure 2A), microbiota modulations had a significant effect. First, standard diet-fed mice that received microbiota from impaired cognitive flexibility donors showed a significant decrease in body weight (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 14, r = 0.58, $p < 0.05$; Figure 2A, left), compared to the control group, with no significant changes in other standard diet-fed groups. However, high-fat diet-fed mice that received an antibiotic cocktail, and oral administration of vehicle had significantly decreased body weight compared both to the control group (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 0, r = 0.84, $p<0.001$; Figure 2A, right) and experimental groups that received FMT from low (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 11, r = 0.61, $p<0.001$; Figure 2A, right) or high (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 12, r = 0.62, $p<0.001$; Figure 2A, right) cognitive flexibility donors. In

addition, the experimental high-fat diet-fed FMT groups also demonstrated a decrease in body weight, compared to the control group (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, p < 0.001; FMT (L) post-hoc U = 2, r = 0.81, p < 0.001, FMT (H) post-hoc U = 0, r = 0.84, p < 0.001; Figure 2A, right).

Despite changes in body weight, food intake was not affected by diet or microbiota modulations (Kruskal-Wallis H Test; H (7) = 13.230, n.s.; Figure 2B). Due to a higher caloric content of the high-fat diet, mice consumed significantly more calories compared to the standard diet group (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 13, r = 0.84, p < 0.001; Figure 2C, right). Furthermore, several changes between the groups of different microbiota compositions were observed. First, standard-diet fed mice that received oral administration of vehicle (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 19, r = 0.52, p < 0.05; Figure 2C, left), or microbiota transplantation from impaired (Kruskal-Wallis H; Test H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 13, r = 0.84, p <0.01; Figure 2C, left), or optimal cognitive flexibility donors (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 22, r = 0.47, p < 0.05; Figure 2C, left) had significantly decreased calorie intake, compared to the control group. Less pronounced effects were observed in the high-fat diet-fed mice, since only the mice that received FMT from donors with high Stroop score values significantly decreased the calorie intake, compared to the control (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 21, r = 0.49, p < 0.05; Figure 2C, right),suggesting a potential protective role in the development of obesity.

Interestingly, high-fat diet-fed mice significantly decreased water intake compared to the standard diet group (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.414, p < 0.001; post-hoc U = 188, r = 0.64, p < 0.001; Figure 2D). Although global analysis has indicated microbiota-related alterations in water intake, it was not confirmed by the subsequent post hoc comparisons neither in the standard diet-fed mice (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.4143, p < 0.001; control x vehicle, U = 48, n.s., control x FMT (L), U = 28, n.s., control x FMT (H), U = 32, n.s., vehicle x FMT (L) U = 26, n.s.; vehicle x FMT (H) U = 33.5, n.s., Figure 2D, left) nor in the high-fat diet-fed mice (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.4143, p<0.001; control x vehicle, U = 31, n.s., control x FMT (L), U = 33, n.s., control x FMT (H), U = 47, n.s., vehicle x FMT (L) U = 40, n.s.; vehicle x FMT (H) U = 30, n.s., Figure 2D, right).

Diet also had an impact on locomotor activity, as observed by a significantly reduced activity (two-way ANOVA; diet, F (1, 69) = 26.712, η^2 = 0.279, p < 0.001; Figure 2E, right) and the number of rearings (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p<0.001; post-hoc U = 217.5, r = 0.61, p < 0.001; Figure 2F, right) in high-fat diet-fed mice. These alterations in locomotor activity were modulated by microbiota composition. Thus, mice of the vehicle group that had depleted gut microbiota with antibiotics showed a significant increase in total activity (two-way ANOVA; diet, F (1, 69) = 26.712, η^2 = 0.279, p < 0.001; post-hoc I-J = -978.933, d = 7.49, p < 0.001; Figure 2E, right) and number of rearings (Kruskal-Wallis H Test H (7) = 35.963, η^2 = 0.419, p < 0.001; post-hoc U = 17, r = 0.51, p < 0.05; Figure 2F, right), compared to the high-fat diet control mice. The high-fat diet group that received FMT from impaired cognitive flexibility donors demonstrated a decreased total locomotor activity compared to the vehicle (two-way ANOVA; groups, F (3, 69) = 3.393, η^2 = 0.129, p<0.05; post-hoc I-J = -673.389, d = 1.66, p<0.05; Figure 2E, right), but not the control group (two-way ANOVA; groups, F (3, 69) = 3.393, η^2 = 0.129, p<0.05; post-hoc I-J = -303.544, n.s.; Figure 2E, right). Ultimately, standard diet-fed mice that received a microbiota transplant from optimal cognitive flexibility donors demonstrated a decrease in the number of rearings compared to the vehicle (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p<0.001; post-hoc U = 23, r = 0.46, p < 0.05; Figure 2F, left), but not the control group (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p < 0.001; post-hoc U = 29, n.s.; Figure 2F, left).

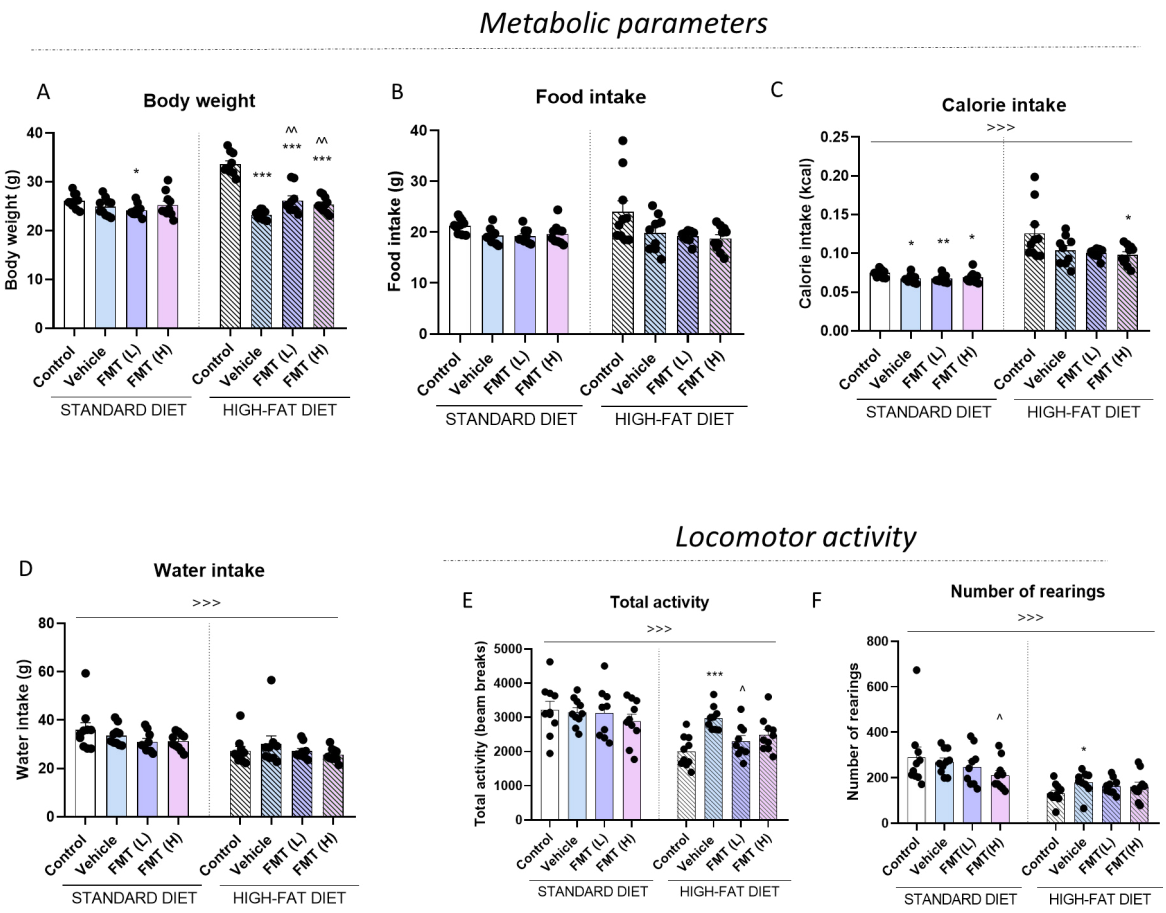
Fecal microbiota transplantation alters memory performance

Short-term, long-term, and working memory were assessed to better understand the relationships between gut microbiota, cognitive flexibility, and cognitive decline. First, no effect of diet was observed on short-term (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144; post-hoc U = 683, n.s.; Figure 3A), long-term (two-way ANOVA; diet F (1, 61) = 0.139, n.s.; microbiota F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; interaction F (3, 61) = 0.319, n.s.; Figure 3B) and working memory (two-way ANOVA; diet F (1, 69) = 1.644, n.s.; microbiota F (3, 69) = 1.509, n.s.; interaction F (3, 69) = 0.183, n.s.; Figure 3C).

The standard diet-fed vehicle group with antibiotic-depleted gut microbiota demonstrated a severely impaired short-term memory with a strong effect size (Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 6, r = 0.74, p < 0.001; Figure 3A). However, the same effect was not observed in the high-fat diet-fed mice (Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 23, n.s.; Figure 3A). This antibiotic-induced memory impairment was reversed by the microbiota transplantation from the impaired cognitive flexibility donors (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 14, r = 0.58, p < 0.05). Meanwhile, those who received microbiota from donors with optimal cognitive flexibility showed a trend toward improved short-term memory (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 22, p=0.065, n.s., Figure 3A, left). No significant

Figure 2

Metabolic parameters and locomotor activity of mice. **A.** *Average body weight (g).* **B.** *Average food intake (g).* **C.** *Average calorie intake (kcal).* **D.** *Water intake (g).* **E.** *Locomotor activity – total locomotor activity (beam breaks).* **F.** *Locomotor activity – number of rearings*



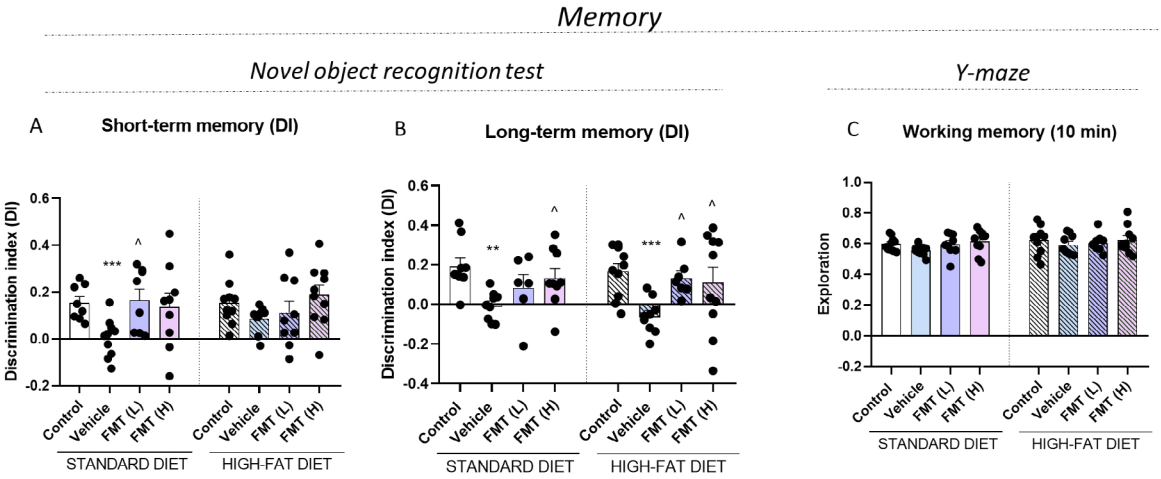
Note. A. Data were analysed using Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, p<0.001; *sig. STD control x STD FMT (L), post-hoc U = 14, r = 0.58, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc U = 0, r = 0.84, p < 0.001; ***sig. HFD control x HFD FMT (L), post-hoc U = 2, r = 0.81, p < 0.001; ***sig. HFD control x HFD FMT (H), post-hoc U = 0, r = 0.84, p < 0.001; ^^sig. HFD vehicle x HFD FMT (L), post-hoc U = 11, r = 0.61, p < 0.01; ^^sig. HFD vehicle x HFD FMT (H), post-hoc U = 12, r = 0.62, p < 0.01. C. Data were analysed using Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; >>> sig. diet, post-hoc U = 13; r = 0.84, p < 0.001; *sig. STD Control x STD vehicle, post-hoc U = 19, r = 0.52, p < 0.05; **sig. STD Control x STD FMT (L), post-hoc U = 13, r = 0.59, p < 0.01; *sig. STD Control x STD FMT (H), post-hoc U test U = 22, r = 0.47, p < 0.05; *sig. HFD Control x HFD FMT (H), post-hoc U = 21, r = 0.49, p < 0.05). D. Data were analysed using Kruskal-Wallis H Test; H (7) = 35.586, η^2 = 0.414, p < 0.001; >>> sig. diet, post-hoc U = 188, r = 0.64, p < 0.001. E. Data were analysed using two-way ANOVA groups F (3, 69) = 3.393, η^2 = 0.129, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc I-J = -978.933, d = 7.49, p < 0.001; ^sig. HFD vehicle x HFD FMT (L); post-hoc I-J = -673.389, d = 1.66, p < 0.05); >>> sig. diet, two-way ANOVA diet; F (1, 69) = 26.712, η^2 = 0.279, p < 0.001. F. Data were analysed Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p < 0.001; ^sig. STD vehicle x STD FMT (H), post-hoc U = 23, r = 0.46, p < 0.05; *sig. HFD control x HFD vehicle, post-hoc U = 17, r = 0.51, p < 0.05, >>> sig. diet, post-hoc U = 217.5, r = 0.61, p < 0.001. Abbreviations: STD – standard diet; HFD – high-fat diet; FMT – fecal microbiota transplantation; FMT (L) – FMT from low cognitive flexibility donors; FMT (H) – FMT from high cognitive flexibility donors.

differences were observed between the groups fed on a high-fat diet (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc control x vehicle U = 23; control x FMT (L) U = 35; control x FMT (H) U = 38; vehicle x FMT (L) U = 39, n.s.; vehicle x FMT (H) U = 23, n.s, Figure 3A, right).

The effects in long-term memory were more pronounced than in short-term memory. The vehicle group demonstrated a significant impairment in long-term memory independent of diet (STD control x STD vehicle two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.199, d = 5.82, p < 0.01); HFD control x HFD vehicle post-hoc I-J = -0.226, d = 6.37, p = 0.001; Figure 3B), with a large effect size. This cognitive impairment was alleviated by FMT from humans with high Stroop scores to standard diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.139, d = 3.63, p < 0.05; Figure 3B, left) and high-fat diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.169, d = 2.78, p < 0.05; Figure 3B, right). Furthermore, microbiota transplantation from impaired cognitive flexibility donors also improved long-term memory, compared to the vehicle group, but only in high-fat diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p<0.001; post-hoc I-J = -0.192, d = 5.97, p < 0.05; Figure 3B, right). These changes in short- and long-term memory reveal the involvement of gut microbiota in cognitive performance.

No significant differences between groups were observed on working memory in the context of different diets and microbiota compositions (two-way ANOVA diet F (1, 69) = 1.644, n.s.; microbiota F (3, 69) = 1.509, n.s.; interaction F (3, 69) = 0.183, n.s., Figure 3C).

Figure 3
Effect of fecal microbiota transplantation on short-, long-, and working memory in mice. **A.** *Discrimination index (DI) during a novel object recognition test for short-term memory (3h).* **B.** *Discrimination index (DI) during a novel object recognition test for long-term memory (24h). C. Exploration of the Y-maze (10 min) for working memory*

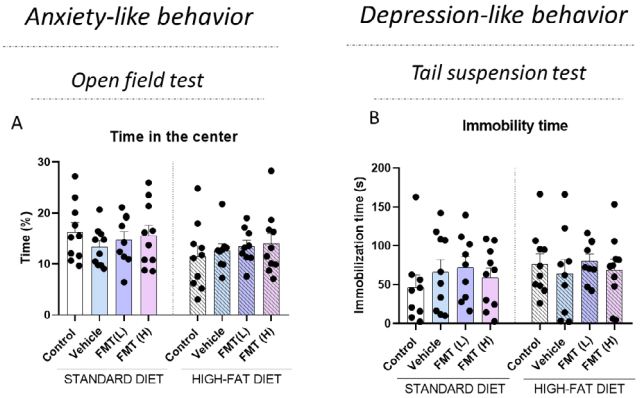


Note. A. Data were analysed using Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; ***sig. STD control x STD vehicle, post-hoc U = 6, r = 0.74, p < 0.001; ^sig. STD vehicle x STD FMT (L), post-hoc U = 14, r = 0.58, p < 0.05. B. Data were analysed using two-way ANOVA groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; **sig. STD control x STD vehicle, post-hoc I-J = -0.199, d = 5.82, p < 0.01; ^sig. STD vehicle x STD FMT (H), post-hoc I-J = -0.139, d = 3.63, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc I-J = -0.226, d = 6.37, p = 0.001; ^sig. HFD vehicle x FMT (L), post-hoc I-J = -0.192, d = 5.97, p < 0.05; ^sig. HFD vehicle x FMT (H), post-hoc I-J = -0.169, d = 2.78, p < 0.05.
Abbreviations: STD – standard diet; HFD – high-fat diet; FMT – fecal microbiota transplantation; FMT (L) – FMT from low cognitive flexibility donors; FMT (H) – FMT from high cognitive flexibility donors.

Fecal microbiota transplantation did not affect anxiety and depression

High-fat diet did not have any effect on anxiety-like (Two-way ANOVA; diet F (1, 69) = 3.020, n.s.; microbiota F (3, 69) = 0.403, n.s.; interaction F (3, 69) = 0.586, n.s.; Figure 4A) and depressive-like behaviors (Kruskal-Wallis H Test; H (7) = 5.237, n.s.; Figure 4B). Furthermore, no significant differences between the groups of different microbiota composition were observed in the open-field test, which represents a model of anxiety-like behavior (Two-way ANOVA; diet F (1, 69) = 3.020, n.s.; microbiota F (3, 69) = 0.403, n.s.; interaction F (3, 69) = 0.586, n.s.; Figure 4A), and the tail suspension test, representing depressive-like behavior (Kruskal-Wallis H Test; H (7) = 5.237, n.s.; Figure 4B).

Figure 4
Effect of fecal microbiota transplantation on anxiety-like and depression-like behavior in mice. **A.** *Time (%) in the center of an open field test.* **B.** *Immobility time (s) during the tail suspension test*



Discussion

In this study, we have revealed the relationship between cognitive flexibility and different parameters of cognitive performance, providing new insights to understand the mechanisms involved in the loss of eating control, which has close behavioral similarities with the behavioral alterations underlying substance use disorders. First, we demonstrated antibiotic-induced cognitive impairments, which were alleviated by FMT. Indeed, FMT from individuals with high cognitive flexibility/inhibitory control scores improved long-term memory independent of diet. However, no significant effect was observed in short-term memory. Remarkably, the microbiota transplantation from impaired cognitive flexibility donors improved short-term memory in the standard diet-fed mice and long-term memory in mice fed on a high-fat diet. Overall, our results indicate that diet and microbiota composition influence brain function. No effect of FMT or diet exposure was observed in anxiety- and depression-like behavior was revealed in our experimental conditions.

FMT has been widely investigated as a promising tool to investigate the role of gut microbiota in various pathophysiological conditions. Indeed, taking into account the involvement of gut microbiota in behavioral responses and metabolic functions, it has been observed that FMT can strongly metabolic alterations in mice, including an improvement in fasting blood glucose levels (Qiu et al., 2023), type 2 diabetes (Chen et al., 2023), insulin sensitivity (Vrieze et al., 2012; Wu et al., 2023), lipid profile (Liang et al., 2022), body weight (Arnoriaga-Rodríguez et al., 2020b; Mayneris-Perxachs et al., 2020)

and several centrally mediated behavioral impairments (Arnoriaga-Rodríguez et al., 2021; Arnoriaga-Rodríguez et al., 2020a). Apart from the peripheral effects, the gut microbiota also has a significant impact on brain-related behavioral responses. Several studies have shown that FMT from healthy donors has improved symptoms of various neurological and psychiatric conditions, including autism spectrum disorder, neuropathic pain, epilepsy, multiple sclerosis, Parkinson's disease, Alzheimer's disease, and depression, among others (Vendrik et al., 2020). Some of these studies have also highlighted the impact of gut microbiota on cognitive performance. Thus, humans with poor memory had low microbial diversity, underlying this bidirectional communication between the gut and the brain in cognitive control (El-Sayed et al., 2021). In mice, antibiotic-induced cognitive impairment was reversed by the use of two different bacteria strains through the production of neurotransmitters and antioxidant activity (Yarullina et al., 2024). Exercise, one of the major microbiota modulators, shows the potential to alleviate microbiota-induced hippocampal deficits. Indeed, antibiotic-treated mice display low-grade inflammation and deficits in pattern separation, which were mitigated by exercise (Nicolas et al., 2024). In another study, antibiotic-induced cognitive impairment was reversed by the use of two different *Lactobacillus* strains through the production of neurotransmitters and antioxidant activity (Yarullina et al., 2024). Similarly, in our study, we demonstrated that antibiotic treatment induced cognitive deficits that were improved by microbiota transplantation, independent of the human donors' cognitive flexibility scores, confirming both the detrimental effect of antibiotics and the crucial effect of microbiota recolonization on cognitive performance.

Interestingly, we previously found that gut microbiota from obese donors led to impairments in working and short-term memory. This cognitive impairment was associated with microbiota-related tryptophan metabolism (Arnoriaga-Rodríguez et al., 2020a). Other studies have demonstrated that methanogen archaea were correlated with better cognitive performance, while FMT from humans containing higher amounts of the archaea *M. smithii* to mice led to better inhibitory control (Fumagalli et al., 2025). Furthermore, viruses also play a crucial role in the gut microbiota function since higher levels of Caudovirales in humans were associated with improved executive functions and verbal memory. Interestingly, the FMT from human donors with high levels of Caudovirales improved short-term and emotional memories in mice (Mayneris-Perxachs, et al., 2022b), whereas FMT from *Blastocystis* carriers impaired cognitive function in mice (Mayneris-Perxachs et al., 2022c). Conversely, another study has shown that an eukaryote parasite found in the gut, namely *Blastocystis*, induced executive function deficits together with further alterations in gut microbiota. Obesity-related cognitive decline was linked to adipose tissue gene expression responsible for the alteration of cognitive function (Oliveras-Cañellas et al., 2023). In our study, we have demonstrated that microbiota transplantation from obese subjects (BMI above 30) with high and low cognitive flexibility differentially improved short- and long-term memory depending on the diet. Specifically, FMT from impaired cognitive flexibility donors improved short-term memory only in standard diet-fed mice and long-term memory in high-fat diet-fed mice. In contrast, FMT from high Stroop score donors significantly improved long-term memory independently of diet. No significant effects were observed on working memory, which strongly reflects cognitive flexibility, indicating that memory performance, rather than cognitive flexibility, might be modulated by microbiota modifications. Contrary to our findings, a previous study demonstrates a coherence in the results when FMT from obese humans with impaired inhibitory control led to similar alterations in the reversal test (Arnoriaga-Rodríguez et al., 2021), suggesting that more extensive behavioral tests to assess cognitive flexibility and inhibitory control could be performed in future experiments.

The Western diet is an unhealthy eating pattern with the consumption of highly palatable foods containing sugars and fats. Due to its high palatability, it is the leading cause of loss of eating control, obesity, and the associated microbial imbalance (Burokas et al., 2018; Espinosa-Carrasco et al., 2018; Mancino et al., 2015; Martín-García et al., 2010; Samulėnaitė et al., 2024). In this study, the exposure to a high-fat diet did not induce an obese phenotype in mice. Conversely, a combination of antibiotic treatment and a high-fat diet led to a significant decrease in body weight. Meanwhile, the FMT groups, independently of microbiota content, demonstrated an intermediate body weight, which was significantly higher than in antibiotic-treated mice, but lower when compared to the high-fat diet control. Such an antibiotic-related effect was previously described (Luo et al., 2023), suggesting an explanation to why the obese phenotype was not obtained, despite high-fat diet intake. The length of the protocol and continuous FMT procedures might also have contributed to the lean phenotype. On the other hand, a high-fat diet significantly decreased water intake, number of rearings, and total locomotor activity, which is in agreement with previous studies (Bjursell et al., 2008; Volcko et al., 2020; Yokoyama et al., 2020). Microbiota composition also altered locomotor activity. Indeed, antibiotic-treated, high-fat diet-fed mice demonstrated a substantial increase in total locomotor activity and rearings, which is in agreement with a lower body weight of these mice. This increase in locomotor activity was alleviated by FMT from low cognitive flexibility donors, but not from high cognitive flexibility donors, although a trend towards lower activity was observed. Despite no significant differences observed

in food intake, high-fat diet-fed mice significantly increased caloric intake, indicating obesity-associated changes in eating behavior. However, it was not strong enough to alter body weight. Palatable food exposure can also induce alterations in the brain reward system, contributing to the loss of eating control (Berding et al., 2021; Martín-García et al., 2011; Requena et al., 2018). Indeed, food palatability is a crucial component for the development of food addiction since mice maintained reliable operant conditioning mainly when the response was reinforced by highly palatable chocolate-flavored pellets (García-Blanco et al., 2022). However, despite the overlap between obesity and food addiction, a distinct microbiota profile between obese women with or without food addiction was revealed (Dong et al., 2020). Furthermore, executive functions are frequently impaired in food addiction, which contributes to further overeating. Interestingly, obese individuals also have poor inhibitory control, which positively correlated with gut potentially harmful microbiota changes, while the negative correlations were revealed with beneficial microbiota in both humans and mice (Arnoriaga-Rodríguez et al., 2021; Castells-Nobau et al., 2024; Samulėnaitė et al., 2024).

In summary, this study highlights the interaction between cognitive flexibility impairment related to the loss of eating control and gut microbiota alterations. We have demonstrated that microbiota plays a crucial role in cognitive performance since antibiotic-depleted mice had substantially impaired short-term and long-term memory. Furthermore, microbiota related to the cognitive flexibility phenotype in humans produces important cognitive changes through FMT in mice that depend on their diet exposure. These cognitive responses are closely related to the loss of eating control, which leads to overweight and obesity. Considering the lack of effective therapeutic strategies to fight against loss of eating control, our results could open a new therapeutic perspective for future microbiota-based strategies targeting this behavioral disorder and related comorbidities.

Despite these results, our study has certain limitations. First, the control groups do not fully explain the observed effects. Another control group with antibiotic-pretreated mice that receive autotransplant and an additional experimental group that receives FMT from an intermediate inhibitory control donor could potentially give more explanation for the results obtained. Furthermore, we used only male mice in our study, although human donors of both sexes were used. Ultimately, more behavioral tests to investigate cognitive flexibility in mice could be implemented for a better understanding of how gut microbiota affects cognitive flexibility.

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Conflict of interest

The authors declare no conflicts of interest

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