Exploring the Diet-Microbiota-Brain Axis in Childhood and Adolescence: An Integrated Approach

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THESIS SUMMARY

In recent years, there has been a significant increase in behavioral problems, anxiety symptoms, and learning disorders among children and adolescents. This growing impact on mental health underscores the urgent need to orient research and public policies toward preventive strategies, aiming to avoid the progression of these alterations into more severe clinical conditions and to strengthen resilience from early developmental stages.

Epidemiological evidence has shown that multiple environmental factors—including family and social interactions, physical activity levels, and notably dietary habits—can exert protective or adverse effects on mental health during childhood and adolescence, periods particularly sensitive for neurodevelopment. Among these factors, diet has gained special relevance, as specific dietary patterns and nutrients have been shown to induce metabolic, immune, and epigenetic changes, in addition to modulating the composition and function of the gut microbiota, potentially influencing brain development. In this context, the diet–gut–brain axis emerges as a key bidirectional pathway connecting the central nervous system with the gastrointestinal tract, capable of directly impacting neuropsychological functioning in children and adolescents.

However, to rigorously study these complex interactions between diet, microbiota, and mental health, it is essential to have valid, reproducible, and population-adapted dietary assessment tools that allow for reliable and comparable nutritional data. Among these instruments, Food Frequency Questionnaires (FFQs) are particularly suitable due to their ease of administration and interpretation. Nonetheless, in the absence of validated tools to accurately assess dietary intake in Spanish children and adolescents, as well as the limited availability of studies focusing on diet–microbiota interactions associated with mental health, this thesis aimed to address these knowledge gaps.

In the first chapter of this thesis, we aimed to adapt and validate the EPIC-FFQ in a sample of 150 Spanish children and adolescents aged 10 to 17 years, using the average of nine 24-hour recalls (24H-DR) as the reference. The results showed optimal reproducibility for food groups and energy-adjusted nutrients, with coefficients of 0.51 and 0.47, respectively, surpassing the 0.40 threshold considered indicative of moderate-to-adequate reproducibility. Regarding validity, values were somewhat low for unadjusted nutrients, but moderate and acceptable for energy-adjusted

and de-attenuated values (0.32, 0.50, and 0.50). Overall, these findings indicate that the EPIC-FFQ is a valid and reliable tool for assessing dietary intake in Spanish children and adolescents, providing a fundamental instrument to advance the study of diet–microbiota–mental health relationships.

The second and third chapters of this thesis report a cross-sectional study with 335–407 children and adolescents aged 5 to 17 years, aiming to investigate how diet, gut microbiota, and various lifestyle factors relate to behavioral problems and subclinical alterations in cognitive function, including learning and executive functions, which may precede clinical mental health disorders. Key dietary determinants were identified using logistic models. Microbiota differences were assessed through diversity metrics and differential taxon abundance analyses using DESeq2. Diet–microbiota interactions were explored through machine learning and mediation analyses.

Results from Chapter 2, focused on behavioral difficulties, showed a strong association between a Western-style diet and behavioral problems across all age groups. Participants with behavioral problems exhibited altered microbial profiles, with reduced levels of short-chain fatty acid (SCFA) producing bacteria and increased levels of potentially pathogenic bacteria such as *Campylobacter coli* and *Lautropia mirabilis*, frequently associated with low-quality diets.

In adolescents, *Lautropia mirabilis* mediated the association between Western diet consumption and behavioral problems, while *Anaerostipes rhamnosivorans* mediated the effects of fish intake. Including microbiota data in logistic regression models significantly improved the ability to identify individuals with behavioral problems compared to models based solely on nutrients or food groups.

Chapter 3, focused on executive function and learning difficulties, revealed distinct dietary patterns among subgroups with cognitive alterations compared to controls. Children with learning difficulties showed greater adherence to a pro-inflammatory diet, with lower intake of antioxidant nutrients and higher consumption of fats and dairy products. The executive function deficit group was characterized by low adherence to patterns rich in B vitamins and iron. Although global microbiota diversity did not differ from controls, relevant gender- and species-level differences were observed. Notable variations included species from the genera *Bifidobacterium* and *Lactobacillus*, SCFA producers such as *Coprococcus* and *Prevotella,* and potentially pathogenic or oral bacteria including Salmonella enterica and Staphylococcus aureus. The largest differences,

both in microbial diversity and dietary patterns, were recorded among subclinical groups, with key genera including *Akkermansia muciniphila*, *Segatella copri*, as well as, Lactic Acid Bacteria (LAB).

Integration analyses of diet and microbiota data revealed associations between zinc, lauric acid, and dairy consumption with *Bifidobacterium spp*. in participants with learning difficulties, whereas consumption of pastries and processed meats correlated with oral-type bacteria in the executive function deficit group. Mediation analyses identified *Segatella copri* and *Methanobrevibacter smithii* as mediators of the associations between oat and leafy green vegetable intake and learning difficulties, and *Lacticaseibacillus paracasei* as a mediator of associations with lauric acid (highly correlated with dairy intake).

Finally, Chapter 4 consisted of a comprehensive literature review, evidencing robust associations between diet, gut microbiota, and neurodevelopment. An increasing number of nutritional interventions and microbiome-targeted strategies show promising results for managing specific neuropsychiatric disorders such as depression, autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), social phobia, and epilepsy. However, significant knowledge gaps remain, including the scarcity of studies focusing on subclinical manifestations, a critical area for preventive interventions. This limitation underscores the need for dietary- and microbiota-based research and clinical trials to explore early mental health markers—not just clinical outcomes—related to the diet–microbiota–brain axis.

Overall, this doctoral thesis contributes to addressing methodological limitations through the validation of a dietary questionnaire and highlights the complex interaction between diet, gut microbiota, and behavioral and cognitive problems in childhood and adolescence. It reinforces the importance of continued research in subclinical populations to advance the development of preventive strategies based on diet–microbiota interactions, enabling interventions prior to the onset of clinical disorders.

RESUMEN DE LA TESIS

En los últimos años se ha observado un aumento significativo de los problemas de conducta, los síntomas de ansiedad y los trastornos del aprendizaje en la población infantil y adolescente. Este creciente impacto sobre la salud mental subraya la necesidad urgente de orientar la investigación y las políticas públicas hacia estrategias de prevención, con el fin de evitar la progresión estas alteraciones hacia cuadros clínicos más graves y fortalecer la resiliencia desde las primeras etapas del desarrollo.

La evidencia epidemiológica ha mostrado que múltiples factores ambientales —entre ellos las interacciones familiares y sociales, los niveles de actividad física y, de forma destacada, los hábitos alimentarios— pueden ejercer efectos protectores o adversos sobre la salud mental durante la infancia y la adolescencia, etapas especialmente sensibles para el neurodesarrollo. Entre estos factores, la dieta ha cobrado especial relevancia al demostrarse que determinados patrones alimentarios, así como, nutrientes específicos pueden inducir cambios metabólicos, inmunitarios y epigenéticos, además de modular la composición y función de la microbiota intestinal, influyendo potencialmente en el desarrollo cerebral. En este contexto, el eje dieta–intestino–cerebro se plantea como una vía clave y bidireccional que conecta el sistema nervioso central con el tracto gastrointestinal, pudiendo ejercer un impacto directo sobre el funcionamiento neuropsicológico infantil y adolescente.

No obstante, para estudiar con rigor estas complejas interacciones entre dieta, microbiota y salud mental es imprescindible disponer de herramientas de evaluación dietética válida, reproducible y adaptada a la población juvenil, que permitan obtener datos nutricionales fiables y comparables. Entre estos instrumentos, los cuestionarios de frecuencia de consumo (FFQ) resultan especialmente adecuados por su facilidad de administración e interpretación. Sin embargo, ante la ausencia de herramientas validadas para evaluar con precisión la ingesta dietética en población infantil y adolescente española, así como la limitada disponibilidad de estudios centrados en la identificación de interacciones dieta-microbiota asociadas a la salud mental, esta tesis se ha orientado a abordar estos vacíos de conocimiento.

En el primer capítulo de esta tesis hemos tenido como objetivo adaptar y validar el FFQ-EPIC en una muestra de 150 niños y adolescentes españoles de 10 a 17 años, utilizando como referencia la media de nueve

días de recordatorios de 24 horas (24H-DR). Los resultados mostraron una reproducibilidad óptima para los grupos de alimentos y para los nutrientes ajustados por energía, con coeficientes de 0.51 y 0.47, respectivamente, superando el umbral de 0.40 considerado indicativo de reproducibilidad moderada-adecuada. En cuanto a la validez, los valores fueron algo bajos para los nutrientes sin ajustar, pero moderados y aceptables para los nutrientes ajustados por energía y para los valores des-atenuados (0.32, 0.50 y 0.50). En conjunto, estos hallazgos indican que el FFQ-EPIC es un instrumento válido y fiable para evaluar la ingesta dietética en población infantil y adolescente española, proporcionando una herramienta fundamental para avanzar en el estudio de las relaciones entre dieta, microbiota y salud mental.

El segundo y tercer capítulo de esta tesis se realizó un estudio transversal con 335-407 niños y adolescentes de 5 a 17 años con el objetivo de investigar cómo la dieta, la microbiota intestinal y diversos factores de estilo de vida se relacionan con problemas conductuales y alteraciones subclínicas de la función cognitiva, relacionadas con el aprendizaje y las funciones ejecutivas, que pueden preceder a trastornos clínicos de salud mental. Los principales determinantes dietéticos se identificaron mediante modelos logísticos. Las diferencias en microbiota se detectaron mediante comparaciones de métricas de diversidad y abundancia diferencial de taxones con DESeq2. Las interacciones dieta-microbiota se exploraron mediante técnicas de machine learning y análisis de mediación.

Los resultados del capítulo 2, centrado en dificultades conductuales, mostraron una fuerte asociación entre una dieta de estilo occidental y la presencia de problemas conductuales en todos los grupos de edad. Los participantes con problemas conductuales presentaron perfiles microbianos alterados, con niveles reducidos de bacterias productoras de ácidos grasos de cadena corta (SCFA) y niveles elevados de bacterias potencialmente patógenas como *Campylobacter coli* y *Lautropia mirabilis*, frecuentemente relacionadas con dietas de baja calidad.

En adolescentes, *Lautropia mirabilis* actuó como mediador entre el consumo de una dieta occidental y la aparición de problemas conductuales, mientras que *Anaerostipes rhamnosivorans* medió los efectos del consumo de pescado. La inclusión de datos de microbiota en los modelos de regresión logística mejoró significativamente la capacidad para identificar individuos con problemas conductuales, en comparación con los modelos basados únicamente en nutrientes o grupos de alimentos.

Los resultados del capítulo 3, centrados en dificultades en las funciones ejecutivas y el aprendizaje, mostraron patrones dietéticos diferenciados

entre subgrupos con alteraciones cognitivas y controles. Los niños con dificultades de aprendizaje presentaron mayor adherencia a una dieta proinflamatoria, con menor ingesta de nutrientes antioxidantes y mayor consumo de grasas y productos lácteos. El grupo con problemas de función ejecutiva se caracterizó por una baja adherencia a patrones ricos en vitaminas del grupo B y hierro. Aunque la diversidad global de la microbiota no difirió respecto a los controles, sí se observaron diferencias relevantes a nivel de género y especie. Entre estas, destacaron variaciones en la abundancia de especies de los géneros *Bifidobacterium* y *Lactobacillus*, bacterias productoras de ácidos grasos de cadena corta como *Coprococcus* y *Prevotella*, así como de bacterias potencialmente patógenas y orales, incluyendo Salmonella enterica y Staphylococcus aureus. Cabe señalar que las mayores diferencias, tanto en diversidad microbiana como en patrones dietéticos, se registraron entre los grupos subclínicos. Entre los géneros más relevantes, se identificaron variaciones en *Akkermansia muciniphila*, *Segatella copri* y en la abundancia de bacterias acido lácticas (BAL)

El análisis de integración de datos de dieta y microbiota reveló asociaciones entre la ingesta de zinc, ácido láurico y los productos lácteos con *Bifidobacterium spp*. en participantes con dificultades de aprendizaje, mientras que el consumo de bollería y carnes procesadas se correlacionó con bacterias de tipo oral en el grupo con alteraciones ejecutivas. Los análisis de mediación identificaron a *Segatella copri* y *Methanobrevibacter smithii* como mediadores de las asociaciones entre el consumo de avena y verduras de hoja verde y las dificultades en el aprendizaje, y a *Lacticaseibacillus paracasei* como mediador de las asociaciones con el ácido láurico (altamente correlacionado con el consumo de lácteos).

Finalmente, el cuarto y último capítulo consistió en una revisión exhaustiva de la literatura científica, que evidenció asociaciones sólidas entre dieta, microbiota intestinal y neurodesarrollo. Asimismo, se identificó un número creciente de intervenciones nutricionales y estrategias dirigidas al microbioma con resultados prometedores para el manejo de trastornos neuropsiquiátricos específicos, como depresión, trastorno del espectro autista (ASD), trastorno por déficit de atención e hiperactividad (ADHD), fobia social o epilepsia. Sin embargo, también se evidenció una importante laguna de conocimiento, como la escasez de estudios centrados en manifestaciones subclínicas, un ámbito esencial para el desarrollo de intervenciones preventivas. Esta limitación resalta la necesidad de investigaciones y ensayos clínicos basados en la dieta y microbiota que exploren marcadores tempranos de salud mental, no solo clínicos, relacionados con el eje dieta–microbiota–cerebro.

Globalmente, esta tesis doctoral contribuye a subsanar limitaciones metodológicas a través de la validación de un cuestionario dietético y pone de manifiesto la compleja interacción entre la dieta, la microbiota intestinal y los problemas conductuales y cognitivos en la infancia y la adolescencia, reforzando la relevancia de seguir investigando en poblaciones subclínicas con el fin de avanzar en el desarrollo de estrategias preventivas, basadas en la relación entre la dieta y la microbiota, que permitan intervenir antes de la aparición de trastornos clínicos.

RESUM DE LA TESI

En els últims anys s'ha observat un augment significatiu dels problemes de conducta, dels símptomes d'ansietat i dels trastorns de l'aprenentatge en la població infantil i adolescent. Aquest impacte creixent sobre la salut mental subratlla la necessitat urgent d'orientar la investigació i les polítiques públiques cap a estratègies de prevenció, amb l'objectiu d'evitar la progressió d'aquestes alteracions cap a quadres clínics més greus i de reforçar la resiliència des de les primeres etapes del desenvolupament.

L'evidència epidemiològica ha mostrat que múltiples factors ambientals —entre ells les interaccions familiars i socials, els nivells d'activitat física i, de manera destacada, els hàbits alimentaris— poden exercir efectes protectors o adversos sobre la salut mental durant la infància i l'adolescència, etapes especialment sensibles per al neurodesenvolupament. Entre aquests factors, la dieta ha adquirit especial rellevància, demostrant-se que determinats patrons alimentaris i nutrients específics poden induir canvis metabòlics, immunitaris i epigenètics, a més de modular la composició i la funció de la microbiota intestinal, influint potencialment en el desenvolupament cerebral. En aquest context, l'eix dieta–intestí–cervell es planteja com una via clau i bidireccional que connecta el sistema nerviós central amb el tracte gastrointestinal, podent exercir un impacte directe sobre el funcionament neuropsicològic infantil i adolescent.

No obstant això, per estudiar amb rigor aquestes complexes interaccions entre dieta, microbiota i salut mental, és imprescindible disposar d'eines d'avaluació dietètica vàlides, reproduïbles i adaptades a la població juvenil, que permeten obtenir dades nutricionals fiables i comparables. Entre aquests instruments, els qüestionaris de freqüència de consum (FFQ) resulten especialment adequats per la seva facilitat d'administració i interpretació. Tanmateix, davant l'absència d'eines validades per avaluar amb precisió la ingesta dietètica en la població infantil i adolescent espanyola, així com la limitada disponibilitat d'estudis centrats en la identificació d'interaccions dieta–microbiota associades a la salut mental, aquesta tesi s'ha orientat a abordar aquests buits de coneixement.

En el primer capítol d'aquesta tesi, l'objectiu va ser adaptar i validar el FFQ-EPIC en una mostra de 150 nens i adolescents espanyols de 10 a 17 anys, utilitzant com a referència la mitjana de nou dies de recordatoris de 24 hores (24H-DR). Els resultats van mostrar una reproducibilitat òptima per als grups d'aliments i per als nutrients ajustats per energia, amb coeficients de 0,51 i 0,47, respectivament, superant el llindar de 0,40

considerat indicatiu de reproducibilitat moderada-adequada. Pel que fa a la validesa, els valors van ser una mica baixos per als nutrients sense ajustar, però moderats i acceptables per als nutrients ajustats per energia i per als valors desatenuats (0,32, 0,50 i 0,50). En conjunt, aquests resultats indiquen que el FFQ-EPIC és un instrument vàlid i fiable per avaluar la ingesta dietètica en població infantil i adolescent espanyola, proporcionant una eina fonamental per avançar en l'estudi de les relacions entre dieta, microbiota i salut mental.

En el segon i tercer capítol d'aquesta tesi es va dur a terme un estudi transversal amb 335–407 nens i adolescents de 5 a 17 anys, amb l'objectiu d'investigar com la dieta, la microbiota intestinal i diversos factors d'estil de vida es relacionen amb problemes conductuals i alteracions subclíniques de la funció cognitiva, relacionades amb l'aprenentatge i les funcions executives, que poden precedir trastorns clínics de salut mental. Els principals determinants dietètics es van identificar mitjançant models logístics. Les diferències en microbiota es van detectar mitjançant comparacions de mètriques de diversitat i abundància diferencial de taxons amb DESeq2. Les interaccions dieta–microbiota es van explorar mitjançant tècniques de machine learning i anàlisi de mediació.

Els resultats del capítol 2, centrat en dificultats conductuals, van mostrar una forta associació entre una dieta d'estil occidental i la presència de problemes conductuals en tots els grups d'edat. Els participants amb problemes conductuals presentaven perfils microbians alterats, amb nivells reduïts de bacteris productors d'àcids grassos de cadena curta (SCFA) i nivells elevats de bacteris potencialment patògens com *Campylobacter coli* i *Lautropia mirabilis*, sovint associats a dietes de baixa qualitat.

En adolescents, *Lautropia mirabilis* va actuar com a mediador entre el consum d'una dieta occidental i l'aparició de problemes conductuals, mentre que *Anaerostipes rhamnosivorans* va mediar els efectes del consum de peix. La inclusió de dades de microbiota en els models de regressió logística va millorar significativament la capacitat per identificar individus amb problemes conductuals, en comparació amb els models basats només en nutrients o grups d'aliments.

Els resultats del capítol 3, centrats en dificultats en les funcions executives i l'aprenentatge, van mostrar patrons dietètics diferenciats entre subgrups amb alteracions cognitives i controls. Els nens amb dificultats d'aprenentatge presentaven major adherència a una dieta proinflamatòria, amb menor ingesta de nutrients antioxidants i major consum de greixos i productes lactis. El grup amb problemes de funció executiva es

caracteritzava per una baixa adherència a patrons rics en vitamines del grup B i ferro. Tot i que la diversitat global de la microbiota no va diferir respecte als controls, es van observar diferències rellevants a nivell de gènere i espècie. Entre aquestes, destacaven variacions en l'abundància d'espècies dels gèneres *Bifidobacterium* i *Lactobacillus*, bacteris productors d'àcids grassos de cadena curta com *Coprococcus* i *Prevotella* així com bacteris potencialment patògens i orals, incloent-hi Salmonella enterica i Staphylococcus aureus. Cal assenyalar que les majors diferències, tant en diversitat microbiana com en patrons dietètics, es van registrar entre els grups subclínics. Entre els gèneres més rellevants, es van identificar variacions en *Akkermansia muciniphila* i *Segatella copri*, així com en l'abundància de bacteris làctics.

L'anàlisi d'integració de dades de dieta i microbiota va revelar associacions entre la ingesta de zinc, àcid làuric i productes lactis amb *Bifidobacterium spp.* en participants amb dificultats d'aprenentatge, mentre que el consum de bolleria i carns processades es va correlacionar amb bacteris de tipus oral en el grup amb alteracions executives. Els anàlisis de mediació van identificar *Segatella copri* i *Methanobrevibacter smithii* com a mediadors de les associacions entre el consum d'avena i verdures de fulla verda i les dificultats d'aprenentatge, i Lacticaseibacillus paracasei com a mediador de les associacions amb l'àcid làuric (altament correlacionat amb el consum de lactis).

Finalment, el quart i últim capítol consistí en una revisió exhaustiva de la literatura científica, que evidencià associacions sòlides entre dieta, microbiota intestinal i neurodesenvolupament. Així mateix, es va identificar un nombre creixent d'intervencions nutricionals i estratègies dirigides al microbioma amb resultats prometedors per al maneig de trastorns neuropsiquiàtrics específics, com depressió, trastorn de l'espectre autista (ASD), trastorn per dèficit d'atenció i hiperactivitat (ADHD), fòbia social o epilepsia. No obstant això, també es va evidenciar una important bretxa de coneixement, com l'escassetat d'estudis centrats en manifestacions subclíniques, un àmbit essencial per al desenvolupament d'intervencions preventives. Aquesta limitació subratlla la necessitat d'investigacions i assaigs clínics basats en la dieta i la microbiota que explorin marcadors primerencs de salut mental, no només clínics, relacionats amb l'eix dieta–microbiota–cervell.

Globalment, aquesta tesi doctoral contribueix a subsanar limitacions metodològiques a través de la validació d'un qüestionari dietètic i posa de manifest la complexa interacció entre dieta, microbiota intestinal i problemes conductuals i cognitius en la infància i l'adolescència, reforçant

la rellevància de continuar investigant en poblacions subclíniques per avançar en el desenvolupament d'estratègies preventives basades en la relació entre la dieta i la microbiota, que permeten intervenir abans de l'aparició de trastorns clínics.

INTRODUCTION

1 CHILD AND ADOLESCENT'S MENTAL HEALTH

MENTAL HEALTH FROM A PUBLIC HEALTH PERSPECTIVE

According to the World Health Organization (WHO) mental health is "a state of mental well-being that enables people to cope with the stresses of life, realize their abilities, learn and work well, and contribute to their community"[1]. By contrast, mental disorders are "characterized by a clinically significant disturbance in an individual's cognition, emotional regulation, and/or behavior"[2].A mental Health Atlas was released by WHO in 2020 in order to show a global view of the mental health situation in the world. This document reported that, although now there is greater awareness of the importance of mental health in the population, there is still a need for more efficient prevention and treatment services in health care, as well as more multidisciplinary research to translated findings into more holistic care measuresfor people with mental disorders[3].

The latest data provided by the Annual Report of our National Health System of 2023, reported that 34% of the Spanish population has a mental health problem and that about 50% of mental problems appear before the age of 14.In addition, 20% of population under 25 years is diagnosed with a mental disorders, with anxiety/stress, attention deficit hyperactivity disorder and learning difficulties being the most among young population[4] (Figure 1). Overall, the figures above, reveal the necessity for more research in causal factors and mechanisms and their translation into effective strategies for early detection of mental health risks and disease management especially in children and adolescents.

In fact, goal 3 of the 17 Sustainable Development Goals (SDGs) adopted by the United Nations in 2015 as part of the 2030 Agenda recognizes the promotion of mental health and well-being as an important health priority through the **prevention, early detection** and **treatment** of behavioral, developmental and neurological disorders[5]. In this context, tackling socio-emotional learning, is one of the most cost-effective ways to promote mental health and well-being in children and adolescents, according to major public international agencies, including the WHO, UNICEF and the European Commission[6–8]

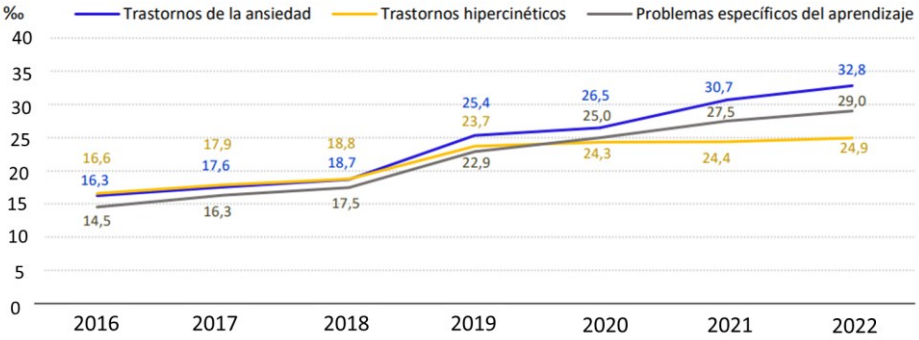

Figure 1. Most recent data on the prevalence of mental health problems among individuals under 25 years of age in Spain. Anxiety disorders, hyperkinetic disorders, and specific learning disorders are shown. *Source: National Health System of Spain, 2023* (original figure in Spanish; legend translated by the author)

Additionally, it is essential to consider that non-clinical or subclinical mental health conditions can substantially contribute to the onset or worsening of health problems [9]. Unhealthy lifestyle habits, such as those acquired or intensified during the COVID-19 pandemic (e.g. increased digital media use, reduced social interactions and isolation, etc.) may further exacerbate vulnerabilities for youth mental health[10,11]. Therefore, mental health research in subclinical youth populations is critical to detect and identify early risk and protective factors and, thus, contribute to developing preventive interventions and strengthening **resilience** before the onset of clinical disorders[12].

SENSITIVE PERIODS IN NEURODEVELOPMENT

Human brain development is a highly complex process that begins within a few weeks after conception and extends up the third decade of life. This process includes key stages such as, neurogenesis, neuronal migration, neuronal maturation, apoptosis, synaptogenesis, pruning, and myelination[13]

Preclinical and clinical studies using neuroimaging techniques have revealed new insights into brain structure and function and into the understanding of the multiple determinants throughout life[14,15]. These studies show that the brain is particularly sensitive to external influences during prenatal life and early childhood since it is a period when brain morphology and function are undergoing intense development and refinement[13]. For instance, findings indicate that when facing stressful situations during early life such as maltreatment and family, community or food instability, major brain circuits are altered[16,17].Notwithstanding, these studies have also revealed that both subcortical and cortical structures including hippocampus,amygdala and cerebral cortex, do not seem to be completely developed in early childhood, and that continue to exhibit maturational shifts into adolescence[18].

In this context, both preclinical and clinical studies have identified several brain structures and functions as key mediators of adaptive responses to adversity or stress during childhood and adolescence. These include microglial activation (the brain's immune cells), callosal connectivity, amygdala and hippocampal volume and activity, reward system sensitivity, and gray matter volume[19–26]. In addition, the serotonin (5-HT) system and the hypothalamus-pituitary-adrenal (HPA) also contribute play a central role in modulating stress responses and supporting resilience during childhood and adolescence[27,28]. These findings have highlighted that, although the first years of life (< 5 years old) constitute critical stages of neurodevelopment[17], youth stages, including those in middle childhood and adolescence, are periods susceptible to changes characterized by heightened synaptic pruning and plasticity[29–32]. In this regard, a recent study found that brain regions that support executive, social, and emotional functions seem to be quite responsive and repairable to the environment during childhood and adolescents[33]. As a consequence, the brain is more strongly influenced by positive or negative environmental events leading to structural and functional changes more easily[31,32].

Notably, epidemiological studies have found that there are many environmental factors, including the family and social interactions, socioeconomic conditions, dietary habits, and levels of physical activity, that can be adverse or protective for mental health during childhood and

adolescence when brain structure and function are still developing and adapting to the adult life[34–39].In light of this, childhood and adolescence represent as **sensitive periods for neural development when** the brain is susceptible to the impact of a number of risk or protective environmental factors [40–42]

A risk factor is understood to be any individual, family or contextual condition that increases the probability of presenting some health problem or disorder in the future. The risk factor must always be prior to the disorder. To illustrate, a mother with depression is a risk factor, since her son/daughter will be more likely to suffer from depression than any other child born to a non-depressed mother[43]. In contrast, protective factors is understood to be any individual, family or contextual condition that reduce the probability of presenting some health problem by mitigating the impact of risk factors[44].

Several risk or protective factors can act as determinants for the onset and progression of health problems.These mental health determinants can operate at multiple levels throughout life including environmental or psychosocial determinants such as community, economic, familial, school, demographic, lifestyle, and social and cultural factors[45]; biological determinants such as age, sex, genetic, diseases, temperament or intellectual quotient (IQ) factors; as well as individual determinants such as poor emotional coping skills or low self-esteem which in turn can be shaped by both environmental and biological determinants[46].Additionally, factors related to prenatal period such as prematurity, birth weight, pregnancy and delivery complications, parental age, maternal nutrition, and exposure to alcohol, drugs or other toxic substances during pregnancy together with postnatal and infancy factors related to healthy habits like physical activity, use of screens, sleep habits and nutrition have emerged as factors that could influence proper neural development in later stage of the life, resulting in adverse short- and long-term effects on mental health[47,48].

It is important to note that although these factors can appear throughout life, in the most sensitive periods of development, can be particularly detrimental[49]. Several research involving twins and gen-environment interaction have revealed that multifactorial and polygenetic etiology can affect mental disorders[50–52]. This studies found that genetic vulnerability coupled with shared and non-shared environmental factors can predict behavioral, mood and learning disorders[53,54].Furthermore, research on gen-environment interaction have found that negative psychosocial factor in early life may lead to greater impairment learning disorders as well as depressive and anxiety problems in childhood and adolescents due to genetic vulnerability to the environment[55,56]. This is explained by the

diathesis-stress concept that informs us how mental disorders develop from the interaction between a biological or genetic predisposition (diathesis) and environmental stressors (stress)[57]. For this reason, ambiental factors can enhance the risk of mental disorders in genetically vulnerable children and adolescents. In short, those studies show that environmental factors may be as important as genetic constitution in the onset and progression of mental illness [51,58]

Conversely, positive environmental or psychosocial factors have been shown to attenuate mental outcomes[59]. In this regards, recent studies have unveiled that modifiable lifestyle factors can attenuate mental illness in children and adolescents[60,61]. To put it simply, healthy protective factors may confer psychological resilience which could help to decrease the burden of mental disorders[59]. In this sense, recent longitudinal studies conducted in children found that environmental factors such as screen time, physical activity, diet or close friendship may alter the course of mental health conditions by affecting children's resilience in the face of multidimensional adversity situations[62–65]. In light of these studies, early intervention approaches that focus on protective and promotional factors are essential for improving and preventing mental health issues in children and adolescents[59,66]

Up until now, studies have focused on the relationship between specific lifestyle behaviors and typical clinical mental disorders. Conversely, a new wide-exposome perspective identifying modifiable lifestyle factors and their joint effect on different neurodevelopmental comorbidities comprising executive function, academic performance or mood and behavioral impairments as well as on other shared biological or social processes could contribute to acquire effective, preventive and holistic approach for promote or protect brain health, especially in contexts where multiple adversity factors coexist [59,67–71]. Studies like the ones performed within this Doctoral Thesis aim to expand our knowledge by detecting several psychosocial, anthropometric, prenatal, clinical, and lifestyle factors, highlighting dietary habits and intestinal microbiome, which could contribute to the psychological deterioration or improvement of non-clinical children and adolescents. This thesis could help lay the groundwork for preventive, management, and treatment strategies for mental health issues in youth populations

2 NUTRITIONAL INFLUENCE ON MENTAL HEALTH IN CHILDREN AND ADOLESCENTS

NUTRITIONAL EPIDEMIOLOGY AND PSYCHIATRY

Hippocrates, the 'father of medicine' (460-377 BC) stated that 'If we were able to provide everyone with the right amount of nutrition and physical exercise, neither in deficiency nor in excess, we would have found the way to health'. He emphasized the fundamental role of lifestyle factors, including physical activity and nutrition, in both disease prevention and therapy with the well-known phrase, "Let food be the medicine and let thy medicine be food."" [72,73].

Nutritional epidemiology studies the relationship between dietary habits and health in the population, identifying risk and protective factors and contributing to promote disease prevention through diet. This field has established the importance of dietary habits in public health and the global burden of diet-related non-communicable diseases like malnutrition, including obesity, diabetes or mental disorders.[74].However, research in this area faces important methodological challenges, specially related to the reliability of dietary assessments[75]. In children and adolescents, it is also crucial to adapt and validate the dietary assessment tools to ensure the accuracy and usefulness of the data[76]. Of the tools available, food-frequency questionnaires (FFQs) appear to be the most optimal methods for the child and adolescent population due to their ease of administration, coding and processing. Furthermore, the efficacy of these questionnaires will also depend on their careful validation and reproducibility [75,77,78]. To address those requirements, the first objective of this thesis was to adapt and validate the FFQ- EPIC questionnaire previously validated in other countries but not in Spanish children and adolescentsfor, for use in epidemiological studies of diet-health relationships in this population, ensuring reliable data collection.

The raise in mental disorders prevalence has been associated with profound sociodemographic changes, such as urbanization and globalization of food production systems and people's lifestyle and eating habits[79]. Although research on the effects of nutrition on mental health has steadily increased in recent years, further efforts in understanding how

food influences psychological well-being in different life stage could be crucial from a scientific and public health perspective [79].

The role of nutrition in mental health is relatively well-established in sensitive and critical periods for brain development[80]. In particular, maternal nutritional deficiencies in essential nutrients such as iron, folic acid, iodine, polyunsaturated fatty acids (PUFAs), polyphenols or vitamin D as well as inadequate dietary pattern and quality can affect learning, skills motor, behavior and socio-emotional development in prenatal stage by means of epigenetic modification with consequences in childhood and adolescence brain function [81–86].Subsequently, poor nutritional practices in early life, such as inadequate breastfeeding, inappropriate introduction to solid food, nutritional deficiencies (e.g. iodine deficiency), or chronic undernutrition may lead to improper brain development and long-term consequences for mental health [87–89]. In this respect, pre-clinical and clinical studies have found that essential micro- and macronutrients in the diet, like methionine, protein or PUFAs, as well as the type of dietary pattern in early life, can protect the brain, improve resilience to stressful events and lead to changes in brain morphology and function, such as enhance executive function and intelligence in childhood and adolescence [37,90,91]. In other words, nutrition in early life can lay the foundation for lifetime brain function.

Middle childhood and adolescents are periods recognized as critical windows of vulnerability where nutritional factors profoundly influence both physical and mental well-being. During these stages, growing independence in food choices, exposure to social and environmental pressures, and increased autonomy can lead to significant changes in dietary patterns-such as skipping meals or adopting diets high in ultra-processed foods, saturated fats, salt, and refined sugars. These behaviors often result in malnutrition, characterized by macronutrient imbalances and micronutrient deficiencies (e.g., iron-deficiency anemia, vitamin A and iodine deficiencies), as well as the risk of overweight and obesity[92–96].In fact, obesity is highly comorbid with mental disorders such as depression, anxiety, sleep disorders or academic achievement, showing a bidirectional relationship[97,98].Moreover, both obesity and mental disorders seem to share some pathological processes, like chronic inflammation, as well as similar behavioral and psychosocial risk factors[99,100].In this sense, understanding the mechanisms underlying this relationship can assist to design tailored intervention that address both metabolic and mental problems[101].Ultimately, it is during adolescence that eating disorders such as anorexia nervosa, bulimia and binge eating disorder tend to appear promoting unbalanced nutrition that can have serious consequences for physical and mental health[102].

Observational studies have focused on analyzing the effect of diet quality, such as adherence to the Mediterranean diet or specific nutrient deficiencies on psychological distress, socio-affective, behavioral problems or achieve/learning in children and adolescents[103–108]. In this context, preclinical and clinical studies have showed that synaptic architecture and brain structural development can be alter by diet,make nutrition a key tool for optimizing brain development, resilience and psychosocial well-being[37,109].Thus, this experimental studies show that poor-quality diets are associated with reduced dendritic arborization, widened synaptic clefts, and narrowed post-synaptic densities,structural alterations that compromise the speed, efficiency, and stability of neuronal signaling, ultimately impairing synaptic plasticity[37,109]. Meanwhile research in this area has unraveled some of the biological pathways and systems by which dietary intake may impact on synaptic function, brain morphology and mental health[37]. These included inflammation, oxidative stress, the hypothalamus–pituitary adrenal axis (HPA) regulating stress, the tryptophan pathways, the neurotransmitter, neuroplasticity, mitochondrial dysfunction, epigenetics and gut-brain axis, all of which are hereinafter described in the next section.

This evidence has set the groundwork for a new discipline of study defined as 'nutritional psychiatry', which incorporates nutrition as an essential pillar and modifiable factor alongside genetic, psychological and social factors to treat or prevent mental health problems.

MOLECULAR AND CELLULAR MECHANISMS UNDERLYING THE EFFECTS OF DIET ON MENTAL HEALTH

The understanding of the pathways through which diet can interact with the central nervous system (CNS) has substantially progressed through the use of animal models of mental disorders—highlighting autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), depression, or Alzheimer's disease—which have been instrumental[110–113]. These models allow the manipulation of diet and environmental conditions in a controlled way, revealing how dietary patterns (especially high-fat, high-sugar diets), involving both direct and indirect effects, can impact cognitive and behavioral functions. At the same time, clinical studies have helped translate preclinical findings to real-world contexts, establishing and confirming associations between dietary patterns, foods (constituents) and mental health outcomes[114]. Interventional studies have also reported how

dietary changes and nutrients supplements could help as adjuvant therapeutics and, above all, as preventive approaches for mental health. In this sense, most clinical trials have focused on evaluating the effects of dietary and nutrient-based interventions on the most common neurodevelopmental and mood disorders, with the aim of reducing associated clinical symptoms[115–118]

Dietary patterns and nutrients can modulates CNS by acting on pathways implicated in the pathophysiology of mental disorders [119] **(Figure 2).** For instance, scientific evidence confirms that diet plays a critical role in modulating low-grade inflammatory processes, which can affect brain development and function, especially during early life stages[120,121]. Previous studies demonstrated that Western dietary patterns rich in ultra-processed foods and pro-inflammatory nutrients such as saturated fats, sugars or cholesterol can elevate pro-inflammatory cytokines (e.g., IL-6, TNF-α, CRP), leading to chronic low-grade inflammation[122–124]. Moreover, preclinical studies have revealed that high fat and sugar diets can also lead to brain structural changes in the hippocampus, blood-brain barrier (BBB) disruption, microglial activation, mitochondrial dysfunction and the hypothalamic–pituitary–adrenal (HPA) dysregulation, and increased neuroinflammation in adolescents rodents[113,125–130]. Alongside all of this, mitochondrial dysfunction coupled with chronic inflammation leads to an overproduction of reactive oxygen species (ROS), which contributes to oxidative stress and further cellular damage, affecting proteins, lipids, and DNA.[131]. This excessive production of ROS can overwhelm antioxidant defenses resulting in structural and functional damage, particularly in hippocampal regions [132].

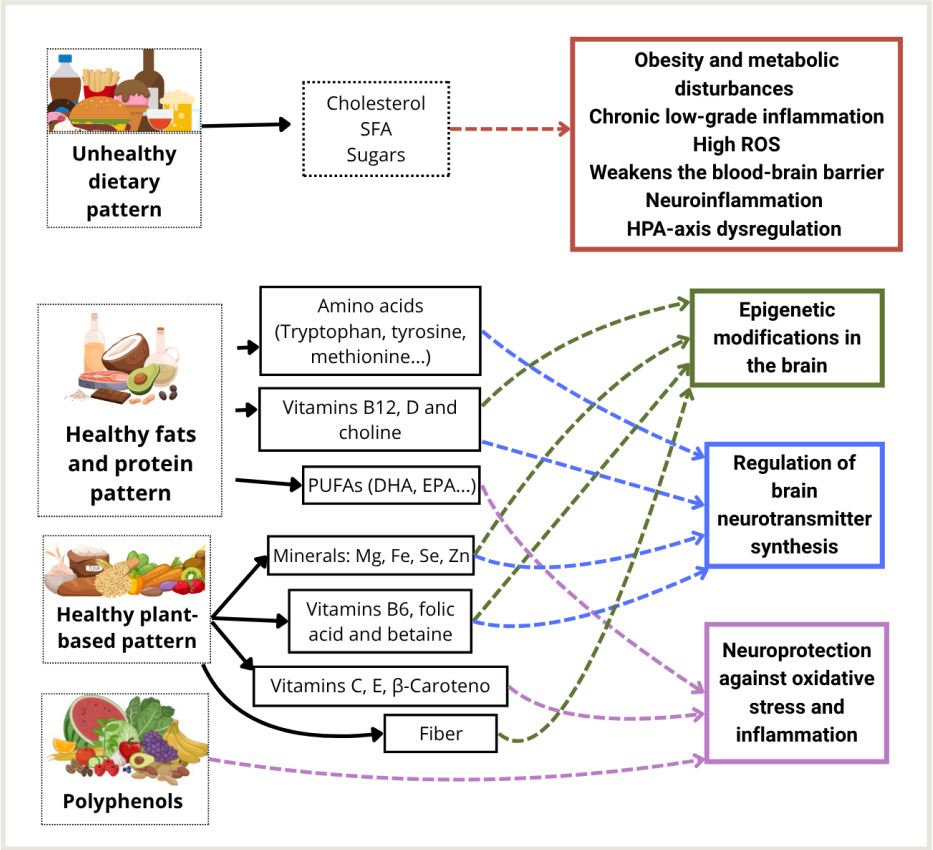

Figure 2. Schematic representation of dietary patterns and nutrients influencing pathways involved in the pathophysiology of mental disorders

Moreover, sustained consumption of these unhealthy dietary patterns can lead to excessive adiposity, obesity, impaired glucose metabolism and dyslipidemic processes, disrupted insulin signaling as well as altered brain neurotransmitter levels[133–136]. These metabolic disturbances can indirectly exacerbate systemic inflammation and oxidative stress, which may extend to the central nervous system and promote neuroinflammation[97,137,138]. As a consequence, the risk of mood or cognitive psychiatric disorders is elevated.

In summary, all of these diet-induced processes can impair synaptic plasticity and neurogenesis, which limits the brain's ability to adapt, learn, and repair connections. This ultimately impacts cognitive and mood

function, contributing to the onset and progression of mental disorders in children and adolescents[97,139,140]

Conversely, the adherence to of **healthy dietary patterns** like the Mediterranean pattern rich in vegetables, whole grains, legumes, fruits, fish and nuts as well as anti-inflammatory macro- and micro nutrients can act neuroprotectively[120,141–143]. For instance, a observational study conducted in ASD children reported that greater Mediterranean diet adherence was associated with reduced levels of pro-inflammatory cytokine IL-6, thereby potentially supporting cognitive function and enhancing quality of life in this population[144]. In addition, plant bioactive compound such as polyphenols can regulate inflammatory processes in the CNS by maintaining structural integrity or by suppressing microglia activation[145] while antioxidant nutrients such as vitamin C, E, β-Carotene, magnesium or selenium protecting neurons from oxidative stress-induced damage by antioxidant defense and anti-inflammatory mechanisms[146–149].

Several in vitro, preclinical and clinical studies have found that PUFAs can mitigate inflammation-induced impairments in neurogenesis, by promoting phospholipid and improving membrane fluidity and function—thereby facilitating synaptic signaling and plasticity—and alleviate oxidative stress by reducing reactive oxygen species (ROS)[150–152]. For instance, a recent intervention in adolescents with depression demonstrated that supplementation with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) —the primary PUFAs— improved lipid profiles, reduced oxidative stress, and alleviated depressive symptoms. The study highlighted distinct mechanisms of action: EPA exerted more pronounced anti-inflammatory effects, whereas DHA contributed to structural integrity and neuroprotection. Importantly, these benefits were observed only in patients who exhibited baseline lipid or inflammatory imbalances[152].

Animal and human research in effect of dietary variables and patterns on emotional, mood disorders and depression treatment or prevention has consistently identified the HPA axis as a central mediator in this complex interplay[153,154]. In this context, the Mediterranean dietary pattern has shown potential in attenuating HPA-axis dysregulation by lowering cortisol levels and reducing stress-induced activation of this system[118,155–157,158(p3),159]. This approach could represent a promising strategy for controlling HPA-axis hyperactivity and its contribution to mental health conditions. However, the current body of evidence remains inconclusive, and well-designed clinical trials are needed to substantiate these findings.

Conversely, synthesis of neurotransmitter represents another key pathway through which diet may influence mood disorders or emotional behavior. In this sense, high intake of saturated fat consumption has been shown to impair serotonin synthesis by insulin dysregulation, alteration of amino acid metabolism, increased competition among large neutral amino acids (LNAAs) for transport across the BBB, and activation of the kynurenine pathway, thereby contributing to affective disturbances[153,160–162]. Regarding the protective effects of diet mediated by neurotransmitter metabolism, several dietary components are capable of modulating the synthesis, release and function of this brain's chemical messengers related to reward circuitry and mood regulation including catecholamine (dopamine, norepinephrine) or monoamines (serotonin)[163,164].

In this matter, LNAAs — notably tryptophan and tyrosine— serve as critical **precursors** for serotonin and dopamine synthesis, respectively[143,164,165]. Thus, clinical and preclinical interventions have revealed that enhanced serotonin synthesis from tryptophan-diet has direct implications for the regulation of sleep alterations, mood and the prevention of depressive and anxious symptoms in young populations [166,167]. Additionally, deficiency or dysregulation of the major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) and the excitatory glutamatergic system has also been strongly associated with anxiety and depression in adolescents, although it remains unclear whether GABA levels increase or decrease in these conditions[168,169]. In this sense ketogenic diet has been studied for its ability to modulate both the GABAergic and glutamatergic systems, potentially offering therapeutic benefits in the management of mood disorders and epilepsy in children and adolescents. Meanwhile, vitamin B6 deficiency—an essential cofactor in the synthesis of GABA from glutamate—has been associated with compromised inhibitory activity of the GABAergic system[170,171].

Furthermore, PUFAs can modulate the serotonergic and dopaminergic systems while micronutrients such as vitamin D, zinc, iron, folate, and vitamin B12 have also been implicated as **cofactors** in the metabolism of monoaminergic neurotransmitters[172,173(p3),174–178]. Taken together, these dietary components underscore suggest that nutritional status plays a broader role in the etiology and modulation of emotional dysregulation, depression, impulsivity, and aggressive behaviors in children and adolescents.

Concerning the diet-induced metabolic changes that influence cognitive and function brain , micronutrients such as zinc and iron, Vitamin D, magnesium and group B vitamin (B12, B6 and folate) are the most

extensively studied by acting as **essential nutrients** in the treatment, prevention and promotion of mental health in children and adolescents[179,180]. In this context, these micronutrients appear to play a crucial role in key neurophysiological processes such as myelination and synaptogenesis, which are fundamental for the development of executive functions, brain connectivity, and cognitive processes[181,182].In this sense, preclinical studies have found that unhealthy dietary pattern rich in fat and poor in PUFAs maybe can significantly influence the noradrenergic and glutamatergic systems—key pathways involved in synaptic plasticity, neural excitability, and learning processes[183,184].Notwithstanding, combined administration of biotin, magnesium and arginine counteracted metabolic disorders induced by HFD in obese rats suggest supplementation with essential nutrients could be a promising strategy to address the connection between metabolic dysfunction and cognitive deficits in obesity[185].Complementing this, a series of randomized controlled trials conducted in adolescents have provided strong evidence that iron supplementation can improve not only iron biomarkers but also cognitive outcomes and brain electrophysiological activity[186,187]

Beyond the contribution of micronutrients, macronutrients intake, especially protein, have also been shown to modulate brain connectivity and neural development, influence neurotransmitter synthesis and contribute to better cognitive function in adults[143,165].Nonetheless, the existing literature on intellectual and academic performance in middle childhood and adolescence appears to be characterized by inconsistent and limited. Nonetheless, available evidence suggest that ultra-processed or high-fat diets, as well as low-protein and carbohydrates diets, may adversely affect learning and academic performance in these populations[107,188]. Although the mechanisms underlying this relationship remain unclear, it has been proposed that dysfunctions in energy metabolism and alterations in hippocampal and prefrontal cortex structure and function might mediate the impact of these dietary pattern on learning disabilities[130,189].Additionally, an observational neuroimaging study identified distinct associations between dietary patterns and intelligence in males and female's adolescents. These differences may be influenced by sex-specific metabolic and hormonal sensitivities during adolescence, highlighting how macronutrient roles in neurotransmitter synthesis and brain metabolism can vary between sexes during this critical developmental period[189].

Despite limited clinical investigation, the role of certain dietary compounds in regulating the expression of brain-derived neurotrophic factor (BDNF) a key protein involved in neurogenesis, brain morphological development

and variation of the hippocampus and prefrontal cortex, neuroinflammation, neurotransmitter synthesis, synaptic plasticity, and neuronal survival, also stands out as particularly relevant in cognitive and executive function in in both healthy children and adolescents and those with neurodevelopmental disorders[190–192]. Among these dietary compounds, zinc, flavonoids, vitamin E and low-carbohydrate and high-protein dietary pattern have demonstrated beneficial effects on enhance and regulate BDNF levels, synaptic mechanisms and cognitive processes [193–199]. In addition omega-3 supplementation have been linked to improvements both depressive symptoms and cognitive function in adolescents[200–202]. Nevertheless, compared to dietary factors, the effect of physical activity on BDNF levels in children and adolescents has been more extensively studied[203]. Therefore, despite these promising findings, more rigorous and long-term research is urgently needed to clarify the causal relationships and underlying mechanisms by which specific dietary components influence BDNF expression and, consequently, cognitive and neurodevelopmental outcomes in children and adolescents.

It is important to note that the effectiveness of dietary therapies for regulating emotional, cognitive, and stress-related processes in children and adolescents with mental health conditions—such as ASD, ADHD, learning disorders and depression— highlighted the critical role of baseline metabolic and nutritional status in shaping the treatment response. Specifically, individuals with pre-existing imbalances in lipids, inflammation, or micronutrients (e.g., low levels of PUFAs, zinc, or vitamin D) are more likely to benefit from dietary interventions[204]. Additionally, a recent review of clinical trials emphasizes the preventive effects of healthy dietary patterns and the adverse role of essential nutrients deficiencies in subclinical mental health symptoms[205]. In this regard, a clinical trial in healthy adolescents found that consuming 30 grams of walnuts per day increased ALA levels and improved sustained attention, fluid intelligence, and ADHD-related symptoms[206]

Not only metabolic pathways, but epigenetic modifications also have been extensively investigated to understand how diet may impact neurological diseases. On this regard, nutrigenomics and nutriepigenetic are emerging sciences that allows us to understand what nutrients and how can modulate gene expression and cause reversible epigenetic modifications that can impact brain development and function[207]. It is important to note that the link between nutrition and psychopathology is well-established in prenatal and postnatal stages where evidence support like poor early life diet influence epigenetics mechanism leading to later cognitive, behavior and emotional changes in children and adolescents[208–211]. In addition,

malnutrition in elderly may also provoke epigenetics changes with implications in neurodegenerative disorders[212,213].However research in children and adolescents remains limited[214.]

Nutrigenetics is the branch of nutritional genomics that examines how individual genetic variations, such as single nucleotide polymorphisms (SNPs), influence individual responses to dietary components and affect health outcomes.

In this regard, genome-wide association studies (GWASs) have identified genetic risk factors for ADHD, depression, and ASD. In addition, micronutrients GWASs data informed us about SNPs correlated to micronutrients concentrations [215–218]. On the basis, a recent study used data from mental health disorders and micronutrients GWASs to identify specific SNPs associated with micronutrient levels that show genetic correlations with the aforementioned mental disorders in children and adolescents. Particularly, elevated vitamin B12 levels correlated with heightened risk of ASD but reduced depression risk. In addition, iron-binding capacity levels was associated with raised risk of ASD while vitamin C levels with narrowed ADHD risk[219]. To sump up, these findings help elucidate how certain individual genetic variants may influence individual response to nutrients, ultimately modulating the risk of mental disorders in children and adolescents. This once again underscores the critical role of proper nutrition in promoting early mental health.

Concerning nutriepigenetics , the impact of methyl donors—including choline, betaine, folic acid, methionine, and vitamins B6 and B12—which are essential nutrients involved in one-carbon metabolism—are the dietary compound most studied in relation to epigenetic modifications[220]. It appears that the availability of methyl donors influences neurotransmitter synthesis, the maintenance of cellular membrane integrity, nucleotide synthesis, DNA and histone methylation reactions, and the trans-sulfuration pathways that, in turn, affect gene expression linked to brain development and function[221]. Among the small number of observational and interventions performed in middle-children and puberty, the most extensively investigated diet-related epigenetic modifications have been those mediated by methyl-donors and dietary fat and fiber in DNA methylation, histone modifications and non-coding ribonucleic acids, as they modulate metabolic pathways contributing to diseases such as asthma and obesity [222–226]. Regarding mental health, the only clinical intervention was conducted in children aged 7-12 with ADHD which were supplemented with a mixture of vitamins, minerals, amino acids, and antioxidants. The results demonstrated improvements in overall

functioning, reduced impairment, and enhanced attention, emotional regulation, and aggression control in the supplemented group. These behavioral benefits were accompanied by general improvements in methylation profiles, influenced in part by MTHFR genotype, although no specific genes were identified [227].

Finally, it is increasingly recognized that the gut microbiota plays also an important role in regulating the relationship between diet and mental health through the gut-brain axis. This bidirectional axis connects the central nervous system with the gastrointestinal tract through neuroendocrine, immune, and metabolic mechanisms, offering new insights into how dietary factors can influence neuropsychological functioning and mental disorders[228]. In children and adolescents, the gut microbiota has been implicated in several neurobiological pathways relevant to mental illness, including the modulation of the expression of the brain-derived neurotrophic factor (BDNF), serotonin neurotransmission, immune responses, and the hypothalamic–pituitary–adrenal (HPA) axis-mediated stress response, thereby with impacts on cognitive processes[229]. Notably, studies in children and adolescents with ADHD and ASD have shown that alterations in gut microbiota composition ,called intestinal dysbiosis, can exacerbate oxidative stress and neuroinflammation, impact blood–brain barrier permeability, and disrupt microglial function, ultimately worsening neuropsychiatric symptoms[230]. These findings highlight the gut-brain axis as a crucial and modifiable link between diet and mental health, suggesting that targeting the microbiota within this axis may offer novel strategies for the prevention and management of diet-related mental disorders. In the following section of the introduction of this thesis, we will detail the aspects that link the microbiota with mental health in young populations and the contribution of diet to the gut-brain axis

3 THE GUT MICROBIOTA-BRAIN AXIS ACROSS CHILDHOOD AND ADOLESCENTS

EARLY-LIFE FACTORS AND MICROBIOTA DISRUPTION

Humans live in a co-evolutionary and symbiotic relationship with the microbes that inhabit all their body systems. The gastrointestinal tract is the most densely populated ecosystem of human body, which is so called gut microbiota. Gut microbiota contains around 10^{11} to 10^{12} per milliliter

microbial cells, including more than 2,000 different species, known at the time[231]. The microbiota encodes a total of approximately 3.3 billions of genes, more than 100 times the amount of genes present in the human genome. The collective genome of all the bacteria residing in our gut microbiota is named microbiome. Although a variety of microorganisms such as archaea, eukaryotes, and viruses inhabit the human gut, the predominant members of the microbiota are bacteria, which account for approximately 99% of its total composition[232].

The initial colonization of the human body was traditionally believed to occur at birth. Some studies identified bacterial products in the placenta, amniotic fluid, and umbilical cord during pregnancy, suggesting that the exposure to microbes may begin before birth[233]. Nonetheless, the sterility of the intrauterine environment is still under discussion. Concerning the microbiotastructure and composition, the exact timing of stable adult-like gut microbiota formation remains unclear, but it typically occurs around 2.5 to 3 years of age. The microbiota also experience a maturation process during childhood and adolescence, where the use of antibiotics, hormonal shifts, pubertal changes and environmental exposures are key aspects for its modulation [234–236].

It is well-established that the prenatal and early infancy and to a less extent childhood and adolescence are periods where gut microbiota is more unstable and, therefore, at greater risk of suffering imbalances, generally known as dysbiosis, due to exposure to environmental stressors. However, these periods can also be considered as windows of opportunity to positively influence health through gut microbiota modulation[237]. Thus, the intestinal microbiome acts as a “translator” of environmental signals influencing the host's immune system, metabolism and neurological functions, which may determine the onset of several non-communicable diseases [238].

The maternal intestinal, vaginal, oral, and mammary microbiota constitute the primary source of microorganisms for the newborn. Concerning this, firstly **maternal conditions during pregnancy** including antibiotic exposure, gestational age, genetic, geography, environment, ethnic, lifestyle-related aspects such as stress or diet, and pathological conditions like obesity or gestational diabetes, can significantly influence the maternal microbial composition and subsequently modify the microbiome transmitted to the newborn[81,239,240]. These alterations may promote the proliferation of pathogenic bacteria while reducing the presence of beneficial ones, potentially influencing and shaping the initial

establishment of the fetal and neonatal microbiota. Secondly**, birth factors** such as the mode of delivery, prematurity, breastfeeding and perinatal antibiotics have also been shown to influence initial microbial colonization and microbiota maturation[241,242]. For instance, several studied have shown that neonates born by cesarean section exhibit a distinct hormonal, physical, bacterial, and medical conditions compared to those delivered vaginally. Cesarean-born neonates are typically characterized by a lower abundance of beneficial bacterial species, including members of the genera *Bacteroides*, *Bifidobacterium*, *Parabacteroides*, wheareas aerobic members including *Escherichia/Shigella and streptococcus* are enriched[243]. This microbial imbalance has been associated with an increased risk of immunological, metabolic, and developmental disorders[244–246]. In this sense, cesarean-born infants can partially recover a vaginal-like microbial profile through exposure to maternal vaginal microbiota, and similarly, breastfeeding has been shown to help restore gut microbiota in these infants[247,248]. Moreover, exposure to vaginal microbiota seem to accelerate healthy gut microbiota maturation, increase the presence of beneficial maternal bacteria and improve early indicators of neurodevelopment[249]. Thirdly, **postnatal care** also plays a critical role in shaping the developing gut microbiota throughout early and mid-infancy, and extending into toddlerhood (3 years old) [242,250] . In this early stage of the life birth factors continue to influence gut microbiota along with antibiotic use, family structure, environment, geography and introduction of solid foods.[251,252]

Additionally, childhood and adolescents also represent key periods in which environmental factors continue to modulate and significantly modify the composition and function of the microbiome as shown more recently. During **early childhood** (3-5 years old) a “mature microbiota” is considered to be established potentially carrying the imprint of perinatal and early postnatal factors[253]. In the **middle childhood** (6-11 years old) gut microbiota is significantly influenced by obesity and lifestyle factors, including stress, physical activity, screen time, outdoor exposure and dietary patterns and nutrient intake, being dietary factors the primary determinants during this stage of life[254]. In this context, a study that compared the gut microbiota composition of two adolescent populations adhering to distinct dietary patterns concluded that both the compositional and functional profiles of the microbiota reflected the nutritional differences between the Mediterranean and Western diets[255]. Apart from these, in **adolescents,** hormonal changes, puberty, academic stress, social pressures, and alcohol and tobacco use also appear as important factors in transforming the gut microbiota[256,257].

These key modifying factors and environmental influences can induce functional and structural changes in the CNS — as saw in the previous section— potentially contributing its impairment and enhancement of disease risk. Moreover, intestinal dysbiosis can affect the development and functioning of the central nervous system through the gut-brain axis, as explained below.

EXPLORING THE BRAIN–GUT AXIS

Parallel to the development and maturation of the intestinal microbiota, the CNS also undergoes significant development in early life and in latter stages, during childhood and adolescence. The gut microbiota not only play a crucial role in various physiological processes related to human health, including nutrition and digestion, maintenance of homeostasis, metabolism, immunomodulation, but also in the functioning of both the CNS and the enteric nervous system (ENS) **(Figure 3)**[258]

Gut bacteria maintain a bidirectional communication with our brain via the called 'gut–brain axis through neural, neuro-endocrine, metabolic and neuroimmune pathways[259,260].The CNS affect gut microbiota through neural pathways mediated by vagal nerve and ENS afferent neurons[261]. In turn, gut microbiota can directly or indirectly modulate processes associated with neural functioning, developmental, learning, behavioral, memory and mood [262]. The most evidence related to implication commensal microbiota in normal developmental brain comes from studies in germ-free mice (GF). Studies have shown that GF mice as well as mice treated with antibiotics to induce gut microbiota depletion, exhibit a reduced abundance and diversity of gut microbiota [263].This microbial imbalance affects neural circuitry through gut-brain axis pathways and produce alterations in anxiety-like behavior, cognition, and overall behavioral patterns[264]. Regarding these communication paths, several studies have observed that GF mice presented abnormal microglia and hippocampal morphology, dysregulation of the response of HPA system, increases in the permeability of the blood–brain barrier and impairs endocrine and immune pathways compared to specific-pathogen-free (SPF) mice[265–267].For instance, some studies carried out in mice found that persistent intestinal dysbiosis caused by repeated administration of antibiotics during the sensitive period of adolescence increases vulnerability to emotional and anxiety-behaviors disturbances in adulthood which may be due to dysregulation of HPA, changes in BDNF levels and absence of bacterial metabolites[268–271]. Concerning metabolic pathways,

preclinical studies have demonstrated that the intestinal microbiota affect brain metabolism, supporting the idea that the cerebrum of SPF animals is metabolically more active than that of GF mice[272]. In this regard, gut bacteria has been considered to acts as a "second brain" due to its ability to synthesize neuroactive compounds, such as neurotransmitters (serotonin, dopamine and GABA) and their amino acid precursors (phenylalanine, tryptophan) and to regulate tryptophan metabolism which can modulate neurophysicology and immunity[273,274].In addition, functional products of the intestinal microbiota such as bioactive lipids, vitamins, amino acids, trimethylamine-n-oxides, lipopolysaccharides (LPS) and bioactive peptides, secondary bile acid and SCFA, also seem to play a role in neuroimmuno, endocrine and inflammatory functions as they can cross BBB affecting brain structure and function[262,275,276]. In fact, these microbiota-derived metabolites can influence gene expression by inhibiting the activity of deacetylases (HDACs)[277].To illustrate this, animal studies have revealed that a microbiota composition low in SCFA-producing bacteria contribute to cognitive deterioration[278]. Moreover, increasing the abundance of SCFA-producing bacteria through dietary interventions, such as fiber supplementation, has been associated with reduced hippocampal neuroinflammation and may help prevent mental health disorders[279].This processes can influence intestinal mucosal immunity and barrier function , as well as contribute to the biosynthesis of neuroactive compounds. Taken together, these mechanisms make the gut microbiota a key neuromodulator of the gut-brain axis.

Thus, host genetic predisposition, along with environmental factors experienced throughout life, can modify the gut microbiome structure, affecting the activity of bacteria and contribute to differences in the developmental brain concerning cognitive and behavioral functions. Most observational studies in humans have focused on the impact of gut microbiota in neurodevelopmental disorders during the first years of the life and adulthood[280]. Research conducted during early life stages (pregnancy, infancy and toddlerhood) aim to determine whether early alterations in the gut microbiota can lead to long-term mental health disabilities[281] In this sense, several studies have shown that the gut microbiota may serve as a window of opportunity to prevent the development of neurodevelopmental disorders (NDDs) in childhood and adolescents as well as other more severe psychiatric consequences in adulthood[282–285].Regarding adulthood, several case-control studies have focused on mental disorders including Alzheimer's disease, Parkinson's disease, multiple sclerosis, and depression. In this regard, observational studies have revealed that the gut microbiota composition of adults with depression shows an increase in pro-inflammatory bacteria such as

species belonging to the genera *Eggerthella*, *Hungatella*, *Streptococcus*, *Alistipes*, *Flavonifractor*, and *Veillonella*, while showing a decrease in SCFA-producing bacteria compared to healthy controls[286–288]. However, findings observed at one age cannot be automatically generalized to other life stages and findings also differ across studies due to other interacting variables.

Childhood and adolescence are sensitive periods for brain development and for gut microbiota changes [236,254]. In fact, it is in childhood when learning disorders and difficulties in executive functions appear, evantually leading to onset of neurodevelopmental disorders as ASD or ADHD. Recent reviews have compiled and summarized current evidence on how the gut microbiota may influence the development and symptomatology of these neurodevelopmental disorders (NDDs) in children[289–291]. According to this, intestinal dysbiosis may provoke metabolic dysregulation involved changes in microbiota composition as well as SCFA and neurotransmitter production, contributing to aggravate symptomatology and behavioral problems[292,293]. Moreover, immune modulation, neuronal signaling across vagus nerve and alteration of the gut barrier integrity are additional mechanisms underlying neurodevelopmental disease[294–297]. Regarding microbiota composition, children with ADHD have been found to exhibit a lower abundance of *Faecalibacterium* and *Ruminococcus* genera, while genera such as *Odoribacter*, *Eggerthella*, and *Agathobacter* appear to be increased[295]. In children with ASD, a decrease in bacteria with potential anti-inflammatory properties—such as species of *Faecalibacterium, Collinsella*, *Bacteroidetes*, *Bifidobacterium*, and *Akkermansia muciniphila*—has been observed. Conversely, potential pro-inflammatory genera including *Corynebacterium*, *Clostridium*, *Desulfovibrio*, and *Sutterella* are often found in higher abundance, suggesting a microbial imbalance that may contribute to a neuroinflammatory processes [289].Concerning ASD children, disrupted synaptogenesis and atypical synaptic plasticity appear to be key neurobiological alterations, promoted by microglia [298]. In this sense, gut microbiota seems to play an important role in microglial maturation, identity, and function contributing to modulate microglial morphology and activation through metabolic pathways and inflammatory responses, thereby contributing to ASD progression and severity [30,299]. Furthermore, gastrointestinal symptoms have also been correlated with the severity of these disorders. This relationship has led to growing interest in therapies targeting the gut microbiota as a potential adjuvant or additional treatments[296,300] .

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Despite mood disorders and externalizing problems such as social relationships with peers are the most frequently examined psychological conditions in adolescence, studies investigating its association with the gut microbiota remain limited and their results inconsistent.[301].It is possible that other key factors during these sensitive developmental stages—such as brain maturation and hormonal changes—exert a stronger influence, potentially masking the effects of the gut microbiota. Notwithstanding, more recently studies have reported significant differences between depressive and non-depressive school-age children and adolescents[302–304]. In addition to variations in bacterial taxonomy, these studies have helped elucidate key outcomes resulting from microbiota–host interactions, including amino acids deficiency in the prefrontal cortex, altered immune activity with elevated levels of pro-inflammatory cytokines and chemokines and the activation of glycolysis/gluconeogenesis metabolic pathways in depressed children and adolescents. These findings highlight how alterations in the gut microbiota can induce metabolic and immune dysregulation within the gut–brain axis, thereby influencing mental health outcomes.

In summary, most observational studies that connected gut microbiota with mental features in children and adolescents are focused on specific neurodevelopmental disorders and only a few small-size studies are focused on subclinical cases and mental health evolution. This means that while there is relatively robust evidence linking gut microbiota alterations to the presence and severity of diagnosed disorders, there is limited information on how the gut microbiota relates to mental disease risk and mild or subclinical symptoms that can co-occur within different diagnosed psychological disorders [305,306].It is widely acknowledged the need to design effective strategies to promote psychological resilience through diet and gut microbiota modulation. However, cross-sectional studies that integrate diet, microbiome, and mental health outcomes are rather limited, especially regarding learning and academic achievements in middle-childhood and adolescence[229]. This thesis aims to address this knowledge gap, by providing information and knowledge in this field and during these sensitive age stages. The primary goal is to identify nutritional risk and protective factors and microbiota-based biomarkers, which may help design effective strategies for the prevention of mental illnesses throughout life.

NUTRITION AS A KEY MODULATOR OF THE GUT–BRAIN PATHWAY

It is well known that individual nutrients and dietary patterns affect the composition and diversity of our gut microbiota and the brain development and function. This relationship underscores the importance of diet as a modifiable factor in mental health and disease through direct and microbiota-mediated mechanisms[307].For instance, an immature microbiota caused by malnutrition during infancy and childhood can alter gut and brain metabolites as well as brain energy metabolism[308]. These changes may affect the reorganization of neural circuits, gene expression, behavior, synaptic pruning and myelination[309].This, in turn, can impact brain plasticity and contribute to cognitive abnormalities (Figure 3).

Emergent preclinical research have found bidirectional effects between dietary habits and psychological well-being, mediated by the gut-brain axis[310,311]. Among the various pathways through which diet modulates the gut microbiota, metabolites derived from microbial fermentation of nutrients is the most relevant, as they can impact the host's gut barrier, immune functions, the BBB and neurological functions[312].Carbohydrates, especially dietary fiber, promote a diverse gut microbiota, with abundance of bacteria producing SCFA, including butyrate, propionate and acetate[313].According to preclinical studies in juvenile mice and autism models, it seems that dietary fiber consumption provokes changes in the composition of the microbiota, inducing broad alterations in gene expression in the prefrontal cortex and amygdala related to synaptic activity and neuronal plasticity such as changes in neurotransmitters and BDNF levels. In addition, SCFA can modulate microglial activity, neuronal cell adhesion, inflammation and mitochondrial dysfunction [314–321].Additionally, an observational study in preadolescents suggests that reducing added sugar and increasing dietary fiber intake can improve creativity, likely through impact on hippocampal-dependent cognition probably mediated by intestinal microbes[322].

PUFAs, MUFAs, Vitamins and minerals are also important nutrients involved in metabolism of neurons and neurotransmitter and researches have also revealed that both omega-3 and cofactors have the ability to maintain at balanced microbial population[323]. For instance, in vitro studies have revealed that the deficiency in vitamin B9 affects negatively the gut microbiome impacting SCFAs metabolic paths[324]. Moreover, the antioxidant effect of olive and camellia oils, which are rich in MUFAs and promote increased levels of Ruminococcaceae_UCG014 in the gut microbiota, appears to prevent the mild cognitive impairment induced by aluminum chloride in rats[325].Meanwhile, a recent preclinical study shown

that a diet enriched with omega-3 fatty acids and vitamin A counteracts the negative effects of social stress during adolescence, restoring cognitive performance and intestinal microbiota composition in rats via increasing HPA axis activity and SCFAs and inducing changes in BDNF protein levels in the hippocampus[326]. Observational studies in children with autism found that lipid metabolism including steroid hormone levels or PUFAS, markers of mitochondrial dysfunction, and xenobiotic and phenolic metabolites are key pathways in the gut-brain axis[327]. On top of that, clinical human studies have found differences in behavioral changes among adolescent and adults with PUFAS n-3 deficiency.[328].For this reason, findings underscore the importance of targeting the promptly psychiatric symptom development, which can worsen if detected and treated later in life.

Additionally, recent studies have started to reveal the role of polyphenols in regulating the gut-brain axis, suggesting that these compounds may help restore gut microbiome dysbiosis by mitigating oxidative stress and inflammation[329].Thus, preclinical studies have demonstrated that polyphenols improve gut microbiota abundance and diversity, while also eliciting antidepressant and anti-autism-like effects in animal models through modulation of the HPA axis, enhancement of BDNF levels, increased SCFA production, and anti-inflammatory properties [330,331]. Similarly, clinical studies in adolescents disclosed that grape and blueberry may improve working memory, attention and anti-depressive behavior by means of changes in gut composition which may lead to regulate phenolic and neurotransmitters pathways such as GABA as well as increase SCFA levels and to reduce monoamine oxidase activity[332–335].

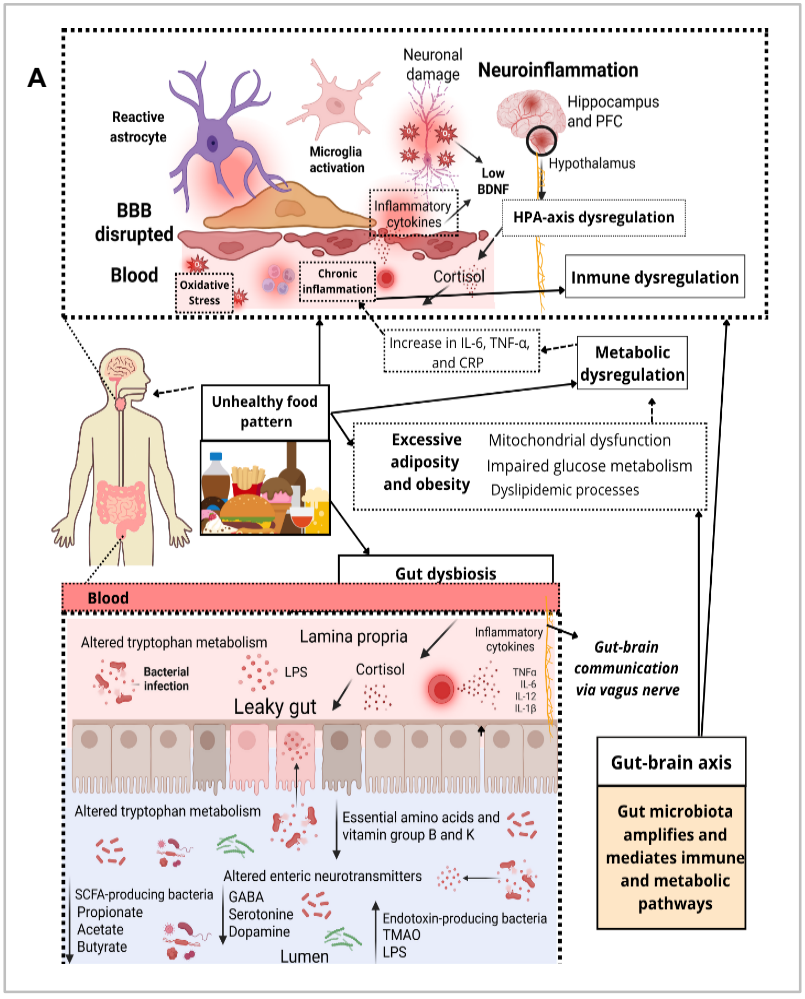
It is important to note that several studies have focused on examining dietary patterns instead of individual dietary compounds. These patterns help capture the combined effects of multiple foods and nutrients, which seem to more accurately reflect real-life eating behaviors[336]. In addition, dietary pattern and lifestyle factors appears to be more strongly associated with variations in the gut microbiota than individual features, with adolescence being a critical window of opportunity to establish healthy dietary habits that promote intestinal eubiosis[337]. Pertaining to this, a interventional study with fast food and Mediterranean dietary pattern found the maintenance of intestinal barrier integrity and the modulation of immune function through bacterial tryptophan metabolites are regulated and influenced by the gut microbiota composition, which is shaped by different dietary patterns[338]. Concerning this, a recent preclinical study in ADHD rats found that ketogenic diet, high in fat, low in carbohydrate, and moderate in protein enhance attention and hyperactivity at the same level as methylphenidate pharmacological treatment, involving mechanisms

that encompass gut microbiota and include amino acid and sugar metabolism [339]

Notwithstanding, the interplay diet, gut microbiota and brain during middle-childhood and adolescent is largely unstudied. As well, the largest number of interventions have been carried out in young patients with autism, ADHD or mood disorders[340–342]. Consequently, although various nutritional and lifestyle approaches including ketogenic diet, gluten- and casein-free diet, healthy (Mediterranean) or unhealthy (Western, ultra-processed, or additive-rich) dietary patterns, micronutrient supplements, and exercise have shown promising preliminary results, further research is still needed to confirm their effectiveness[116,343–346]

Additionally, human intervention with psychobiotics (probiotics), prebiotics, and symbiotics that support mental health have also emerged as potential adyuvant strategies to treatments for cognitive disorders in children and adolescents[347–352]. On the basis of these interventions, several hypotheses explain how probiotics could influence autism-related behaviors. The first is related to the theory that opioid peptides released from gluten and casein, could be digested by probiotics and also help strengthen the gut and BB barrier. Second, probiotics may restore intestinal dysbiosis, and third, may reduce inflammation elicited by dietary proteins[353]. However, it is important to note that current studies on this topic produce heterogeneous results due to significant methodological variations, including differences in study population, participant selection criteria, neuropsychological assessment of participants, identification of confounding factors, microbiome analysis techniques, probiotic dosage and combination, and duration of the intervention period[354–357]

Thus nowadays, dietary interventions are presented as a potential co-adjuvant therapy to improve mental health, partly through the gut microbiota. However, more cross-sectional, longitudinal and clinical trials as well as mechanistic studies are necessary to better understand the biological basis of the diet-gut microbiota-brain health connection and to prove causation in order to inform effectively dietary recommendations for protecting mental health specially in at risk populations and susceptible periods of the lifespan.



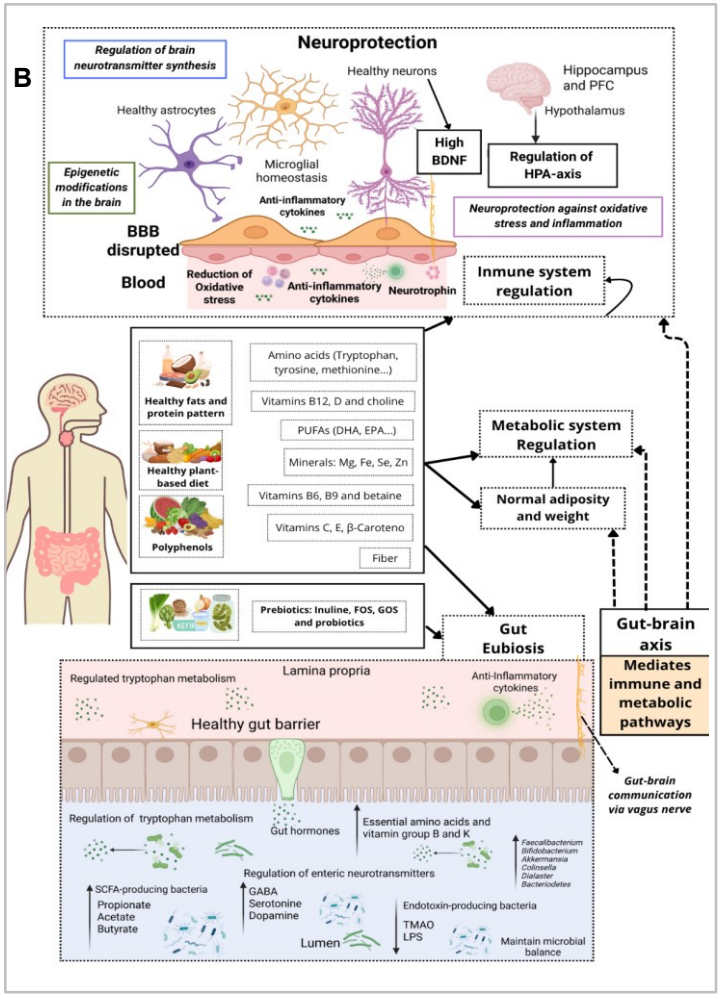


Figure 3. (A) Mechanistic pathways connecting unhealthy dietary patterns, gut microbiota alterations, and neurocognitive and behavioral outcomes in middle childhood and adolescence. (B) Mechanistic pathways through which healthy dietary patterns influence gut microbiota and promote neuroprotection during middle childhood and adolescence.

OBJECTIVES

Given the limited literature examining behavioural and cognitive outcomes in relation to both dietary factors and gut microbiota during this developmental stage in the Spanish population, this dissertation adopts an **exploratory approach** to investigate potential associations between dietary patterns and habits, gut microbiota composition, and behavioural or cognitive outcomes[323,358].

The **general aim** of this thesis is to establish robust associations between dietary intakes, the gut microbiota composition and function and mental health issues, with a focus on behaviour and cognitive functions, in children and adolescents of the Spanish population. The ultimate purpose is to contribute to the development of effective preventive and management nutritional strategies for mental disorders, based on a deeper understanding of diet-microbe associations and their impact on the gut-brain axis.

To this end, we divided the Thesis in three specific objectives:

SO1. To validate a food frequency questionnaire in order to ensure reliable, reproducible and useful data collection for nutritional research in Spanish children and adolescents

SO2. Identify dietary habits, and intestinal microbiota signatures, associated with behavioral problems in children and adolescents, considering other potential interacting variables (sociodemographic, anthropometric, perinatal, clinical, lifestyle factors, etc.).

SO3. Identify dietary habits, and intestinal microbiota signatures, which could be associated with learning difficulties and executive function impairments in children and adolescents, considering other potential interacting variables (sociodemographic, anthropometric, perinatal, clinical, lifestyle factors, etc.).

RESULTS

CHAPTER 1

VALIDITY AND REPRODUCIBILITY OF A SPANISH EPIC FOOD FREQUENCY QUESTIONNAIRE IN CHILDREN AND ADOLESCENTS

Ana Larroya, María Tamayo, María Carmen Cenit, Yolanda Sanz (2024). Validity and Reproducibility of a Spanish EPIC Food Frequency Questionnaire in Children and Adolescents. Nutrients 2024, 16, 3809. https://doi.org/ 10.3390/nu16223809

ABSTRACT

Background: Dietary habits are crucial for preventing many diseases, particularly in children and adolescents. Accurate assessment of dietary intake is essential for understanding the relationship between diet and health in these age groups. Objective: This study aimed to evaluate the reproducibility and validity of a Spanish version of the European Prospective Investigation into Cancer and Nutrition (EPIC) Food Frequency Questionnaire (FFQ) in 150 Spanish children and adolescents aged 10 to 17 using the average of 9 days of 24-h dietary recall (24H-DR) as a reference.Methods: Intraclass correlation coefficients (ICCs) were calculated to asses reproducibility and Spearman/Pearson correlation coefficients were calculated to assess validity and reproducibility. Results: The average ICCs were 0.41 for crude nutrients, 0.31 for food groups, 0.31 for energy-adjusted nutrients, and 0.4 for energy-adjusted food groups. Spearman/Pearson correlation coefficients averaged 0.39 and 0.41 for crude and energy-adjusted nutrients, respectively, and 0.51 and 0.47 for corresponding food groups. Regarding validity, the average correlation coefficient for crude, energy-adjusted, and de-attenuated nutrients was 0.32, 0.50, and 0.50, respectively. The highest crude coefficient was 0.50 for vitamin C and fiber, while the highest energy-adjusted coefficient was 0.76 for protein and carbohydrates. The highest de-attenuated coefficient was 0.72 for vitamin B6. Conclusions: Overall, these results suggest that the EPIC FFQ is a valid and reliable instrument for assessing dietary intake in Spanish children and adolescents.

1. INTRODUCTION

Childhood and adolescence are important developmental periods characterized by physical and mental changes. During this vulnerable phase, lifestyle factors, including dietary intake, physical activity, and nutritional status, play a pivotal role in preventing the onset of several diseases[1]. Poor dietary habits, in particular, can contribute to the onset of chronic conditions such as obesity, type 2 diabetes, cardiovascular disease, and nutritional deficiencies, which may increase the risk of neurodevelopmental disorders such as attention-deficit-hyperactivity disorder (ADHD)[2,3]. This highlights the urgent need for effective and reliable tools to measure and assess dietary characteristics in children and adolescents[2-4]. Common dietary assessment tools include the 24-h dietary recall (24H-DR), food records (FRs), weight food records (WFRs), food frequency questionnaires (FFQs), and, less frequently, blood or urine biomarkers of specific nutrients[5]. Each method has its own strengths and weaknesses, and the optimal choice depends on factors such as the size of the study population, economic constraints, research objectives, and the target population.

FFQs are dietary assessment tools that ask respondents to indicate how often they have consumed various foods and beverages over the last year. These questionnaires typically include between 5 and 227 food items with specified portion sizes and offer 9 frequency response options[5,6]. FFQs are practical, easy to administer, and inexpensive, making them ideal for assessing long-term dietary intake in large populations[7]. They are commonly used in nutrition research to estimate food groups and nutrient intakes and to classify individuals by dietary patterns. Due to their simplicity and cost-effectiveness, FFQs are the most widely used tools in epidemiological nutrition for studying relationships between dietary intake and health outcomes, including metabolic, mental, and immunological conditions[2,6-10]. In the Spanish population, validity and reproducibility studies for FFQs have focused primarily on adults[11-15], with relatively few studies on preschool[16,17] and school-aged18 children. Moreover, only a limited number of dietary assessment tools have been found to be both reproducible and valid in children and adolescents[4,5,18,19]. Validity studies in these populations have used small sample sizes and have not investigated the relationship between dietary risk factors and diseases.

The European Prospective Investigation into Cancer and Nutrition (EPIC) is a comprehensive study examining the links between diet, nutritional status, lifestyle, and environmental factors and the incidence of cancer and other chronic diseases across several European countries[15]. One valuable resource from this study is the EPIC Norfolk Food Consumption Questionnaire (FFQ), which covers a wide range of dietary items related to immunity, genetic predisposition, and environmental factors that contribute to inflammatory processes. This makes it an excellent tool for assessing dietary intake in younger populations. While the EPIC FFQ has been validated for use in Spanish adults[15,20,21], its reliability and validity for Spanish children and adolescents has not been established. To address this gap, we developed and adapted a Spanish version of the EPIC FFQ and evaluated its performance in this younger population. Once its reliability is confirmed, this adapted version can be used to investigate the relationship between nutrition and health.

2. MATERIALS AND METHODS

2.1. Study Population and Design

This was a longitudinal study designed to evaluate the validity and reproducibility of the EPIC FFQ adapted to Spanish language to assess dietary intakes of children and adolescents aged 10–17 years. Participants (n = 250) were recruited from the general population of two different schools located in Valencia (Spain) and excluded those with incomplete questionnaires, eating behavior problems or highly fluctuating dietary habits. To assess variability, we asked participants during each 24H-DR if their reported consumption differed from their typical eating patterns on other days. Data from the EPIC FFQ were collected twice: in December 2022 (FFQ1) and September 2023 (FFQ2). For the validity study, three 24H-DRs were collected on three non–consecutive days at three different points throughout the six-month study, and the average of these served as the reference[5,8]. A total of nine 24H-DRs were utilized as the reference method. The first 24H-DR (T1) was gathered in December together with the FFQ1, the second (T2) in March 2023, and the last (T3) in June 2023. Figure 1 shows a scheme of the study timeline. The study was approved by the Committee of the Spanish National Research Council (CSIC) on 3 of May 2022 (Reference 3 of 23 056/3 May 2022). Children, adolescents, and their parents gave written informed consent.

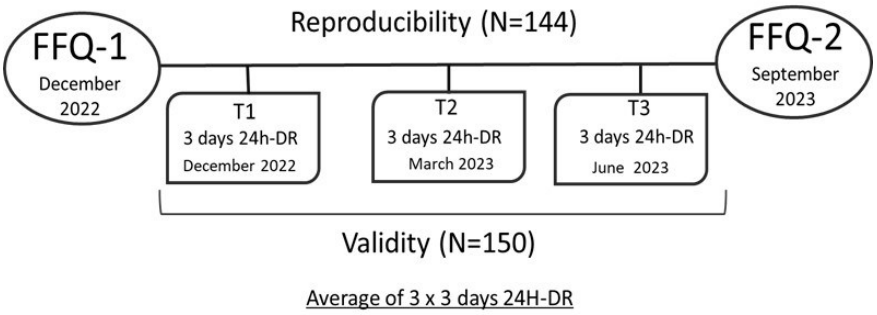


Figure 1. Timeline of questionnaire collection.

2.2. EPIC-Norfolk Food Consumption Frequency Questionnaire

The EPIC FFQ is a 137-item self-administered semi-quantitative food consumption frequency questionnaire[15]. It was translated into Spanish using a language understand able to children and adolescents and was adapted for online completion using LimeSurvey Community Edition Version 6.6.1+240806 (Hamburg, Germany: LimeSurvey GmbH n.d.). Participants accessed the questionnaire via a digital link.

The FFQ collects annual consumption data for each food through 9 consumption frequency responses: never or less than once, 1–3 times a month, 1 time per week, 2–4 times per week, 5–6 times per week, 1 time per day, 2–3 times per day, 4–5 times per day, and more than 6 times a day. To standardize and estimate portion sizes of the different food items referred to in the questionnaire, participants were shown the ENALIA photographic atlas (developed by the Spanish Agency for Food Safety and Nutrition (AESAN)[22]. If the portion size didn't match the amount shown in the atlas, they were asked to provide a photo, and the nutrition experts were the ones who calculated the amount. An additional questionnaire gathered sociodemographic (sex and age), anthropometric (weight and height), physical activity, and special diet use (e.g., gluten-free or vegetarian) information. Two trained nutritionists assisted with questionnaire completion (correct interpretation and training in completing the questionnaires) as well as in the use of the ENALIA photographic atlas. A total of 20 food group were evaluated, while the daily intakes of energy and 36 macro- and micronutrients were calculated using the National Higher Nutrition and Dietetics Education Center food tables (CESNID)[23],

Spanish food composition databases (RedBEDCA)[24], and the United States Department of Agriculture (USDA) nutrition and food composition tables[25].

2.3. Three-Day 24-h Dietary Recall (24H-DR)

Information from three non-consecutive days (including one day of the weekend and two working days) was gathered to use the 24H-DR as the reference method. The first three 24H-DRs were completed concurrently with the FFQ1 in December 2021; the second set of three 24H-DRs were completed three months later in March 2022; and the third set of three 24H-DRs were completed in June 2022. Two trained nutritionists explained to volunteers how to complete the 24H-DR and use the ENALIA photographic atlas[22].

Daily nutrient intake and energy data from 24H-DR were calculated using Dietopro dietary and nutritional management software (URL accessed on 17 July 2024)[26], and the same 36 macro- and micronutrients as in the EPIC FFQ were also assessed.

2.4. Statistical Analysis

IBM SPSS Statistics software (version 27.0) was used for statistical analysis. P values < 0.05 were considered statistically significant. The normality of the data distribution was evaluated using the Kolmogorov–Smirnov test. Quantitative variables were expressed as the mean and standard deviation (SD) or the median and interquartile range (IQR). Categorical variables were analyzed using the chi-square test and presented as percentages. To assess reproducibility, we calculated Spearman's rank correlation coefficient (r) for non–normally distributed data and Pearson's correlation coefficient for normally distributed data, after transformation of the values to their natural logarithm (ln), to compare energy, nutrients, and food groups between the FFQ1 and FFQ2. Values were interpreted as: $r > 0.50$ good, $0.2 < r < 0.49$ acceptable, and $r < 0.2$ poor[27]. The reliability of the EPIC FFQ was assessed using the test–retest method based on comparing the responses collected in FFQ1 and FFQ2 by calculating the interclass correlation coefficient (ICC) with a confidence interval of 95%. Values were interpreted as ICC > 0.75, good reliability; 0.75 < ICC < 0.4, moderate reliability; and ICC < 0.4, poor reliability[28]. All evaluations were conducted using crude and energy-adjusted variables

obtained through the residual method as advised by Willet[8]. This approach ensures that the values are not influenced by total energy intake, controlling the variability of dietary intake and reducing over- or underestimation bias. Finally, to evaluate the percentage of agreement between FFQ1 and FFQ2, we used misclassification and quartile agreement method.

To evaluate the validity of the EPIC FFQ, we compared the FFQ2 and the 24H-DR data. We calculated Spearman's or Pearson rank correlation coefficient (r) after transforming the values to their natural logarithm (ln). The criteria considered were the same as those in the reproducibility study. All analyses were carried out using crude, energy-adjusted, and de-attenuated coefficients. De-attenuated correlation coefficients were evaluated to eliminate random error caused by intra- and inter-person variability in the three 24H-DRs. Thus, we used the formula from Rosner and Willet[29] : $rt = r0\ \sqrt{1 + r\ (e\ \sigma w2/\sigma b2)/n}$; where r0 is the observed correlation between FFQs and 24H-DR; r is the rate of variation within- and between-person measured during three 24H-DRs; and n is the number of days of dietary recall (n = 3). We employed Bland–Altman plots to examine the agreement between the two types of dietary assessment across intake levels. A natural-log (ln) transformation was applied to refine the 95% limits of agreement (LOAs). Visualization of the limits of agreement (LOAs) (ln mean difference ± 1.96 SD) between the methods was performed by plotting the difference between the FFQ2 and the three 24H-DRs against the mean of the two methods[30].

3. RESULTS

A total of 250 children and adolescents from two different schools in Valencia (Spain) were recruited. Of these, 150 (60%) correctly completed all the questionnaires for the valid ity study, while 144 (57.6%) participated in the reproducibility study. The characteristics of the population included in the validity study are presented in Appendix A Table A1. The participants were almost evenly distributed between males and females (52.6% and 47.4%, respectively), with a mean age of 14.3 years (SD = 2.0). Most participants (75.3%) had an optimal weight, while 24.7% had overweight or obesity, with a mean weight of 58.0 kg (SD = 13.3), a mean height of 164.5 cm (SD = 12.6), and a mean body mass index (BMI) of 21.1 kg/m2

(SD = 3.2), all of which were calculated based on the z-score curves established by the World Health Organization (WHO) for school-aged children and adolescents[31]. In terms of education level, eight classrooms participated, including one primary (10–11 years old), five secondary (12–15 years old), and two high-school (16–17 years old) classes. Regarding levels of physical activity, 31.3% of participants reported low levels, 44.7% moderate levels, and 24% high levels of activity. Statistically significant sex related differences were observed only in weight levels ($p < 0.001$) and sports participation ($p < 0.001$).

3.1. Reproducibility

As noted previously, 144 individuals were included in the reproducibility study. When the EPIC FFQ was administered at two different time points, significant differences in daily nutrient intakes (g/day) and food group intakes (g/day) were observed (see Table 1 for nutrients and Appendix A, Table A2, for food groups).).

In general, FFQ1 reported higher intakes of several items, including energy, total protein, animal protein, vegetable protein, total fat, monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), saturated fatty acids (SFAs), cholesterol, carbohydrates, sodium, iron, calcium, selenium, zinc, magnesium, phosphorus, vitamin B1, vitamin B6, vitamin B3 (niacin), vitamin B9 (folate), vitamin A, and sugar confectionery. In contrast, FFQ2 reported higher consumption of fresh fruit, refined cereals, whole-grain cereals, and processed meat. These differences may be attributable to seasonal variation or may reflect both changes in participants' dietary habits and variability in data reporting.

To assess the reproducibility of the EPIC FFQ, we evaluated whether similar results were obtained when the measurements were repeated at two time points (FFQ1 and FFQ2) under similar conditions through different types of analysis. Table 2 shows the ICCs and Spearman and Pearson correlation coefficients between the two sets of data for crude and energy-adjusted nutrients, and Appendix A Table A3 shows the ICCs and correlation coefficients for the food groups. The ICCs of crude nutrient intake between the two FFQs ranged from 0.18 for vitamin D to 0.54 for energy; the ICCs of the crude food group intake ranged from 0.15 for pastries to 0.66 for processed meat. After adjusting for energy, the ICCs of

nutrient intake ranged from 0.08 for iron to 0.51 for vitamin C, and the ICCs of food group intake ranged from 0.11 for cheese to 0.60 for vegetables. The average ICCs of crude and energy-adjusted nutrient intake were 0.41 and 0.31, respectively. The average ICC was 0.40 for both the crude food groups and after adjustment for energy. Accordingly, we observed moderate reliability between the FFQ1 and FFQ2 for crude nutrients and food groups but poor reliability for nutrients when we adjusted for energy.

Table 1. Nutrient intake of the population in the reproducibility study (n = 144).

Nutrients	FFQ1						FFQ2						p*
	M *	Med *	SD *	25	50	75	M	Med	SD	25	50	75	
Energy (kcal)	2508.4	2328.7	1199.1	1720.4	2328.7	2878.6	2119.1	1974.1	1112.3	1399.8	1974.1	2529.3	<0.001
Total protein (g)	128.9	113.5	82.6	88.3	113.5	141.2	105.4	102.5	32	80.4	102.5	126.1	0.03
Animal protein (g)	87.3	78.7	59.9	58.9	78.7	98.7	74.1	69.3	30.7	50.9	69.3	93.4	0.014
Vegetable protein (g)	41.6	36.7	28	28.7	36.7	44.7	31.4	30	9.5	24.1	30	37.3	<0.001
Total fats (g)	110.5	96.6	78.2	74.7	96.6	121.4	86.7	85.5	21.7	70.9	85.5	103.3	0.04
MUFAs (g)	44.3	38.9	32.3	27.6	38.9	49.5	35.3	34.6	10.2	28	34.6	42.6	0.02
PUFAs (g)	18.8	16.3	13.5	12.3	16.3	20.6	14	13.7	4.2	10.8	13.7	16.5	<0.001
SFAs (g)	40.2	35.1	29.5	26.9	35.1	44.5	28.5	27.9	7.9	22.9	27.9	34.9	<0.001
Cholesterol (mg)	440.3	364.6	316	306.5	364.6	482.8	369.1	358.5	131.5	271.2	358.5	441.9	0.02
Carbohydrates (g)	316.2	277.4	203.3	236.2	277.4	324.9	236.3	229.4	57.7	187.4	229.4	284.8	<0.001
Total fiber (g)	28.6	25.2	19.7	18.4	25.2	32.9	22	20.2	9.1	15.3	20.2	25.7	0.03
Iodine (µg)	128.8	114.2	88.5	84.8	114.2	143.9	119.2	111.6	40.9	91.1	111.6	140.1	0.82
Sodium (mg)	3296.9	2948.9	2235.2	2220.2	2948.9	3592.9	2469.1	2286.4	750.5	1956.8	2286.4	3049.2	<0.001
Potassium (mg)	4350.9	3869.9	2851.1	2883.8	3869.9	4801.9	3551.4	3437.5	1001.8	2758.8	3437.5	4157.1	0.06
Calcium (mg)	1116.4	964	733.7	728.5	964	1283.9	831.2	800.7	284.8	643.6	800.7	977.4	**<0.001**
Magnesium (mg)	527.9	429.2	359.5	361.6	429.2	588.8	311.2	300.9	88.8	249.6	300.9	375.6	**<0.001**
Phosphorus (mg)	1759.1	1536.6	1142	1245.9	1536.6	1865.1	1409.1	1387.5	355.3	1127.5	1387.5	1661.3	**0.03**

Iron (mg)	30.4	24.4	21.2	19.1	24.4	32.9	16	15.9	4.6	12.4	15.9	18.9	**<0.001**
Zinc (mg)	15.3	13.4	10.2	11.1	13.4	16.6	12	11.4	3.4	9.2	11.4	14.1	**<0.001**
Selenium (µg)	127.5	113.8	80.3	90.8	113.8	140.1	97.5	94.8	31.9	72.9	94.8	114.1	**<0.001**
Vitamin B1 (mg)	2	1.7	1.2	1.4	1.7	2.2	1.7	1.6	0.5	1.2	1.6	2	**0.03**
Vitamin B2 (mg)	2.2	1.9	1.4	1.6	1.9	2.4	2	1.9	0.5	1.5	1.9	2.3	0.29
Vitamin B6 (mg)	3.1	2.7	2	2.2	2.7	3.5	2.6	2.5	0.8	2	2.5	3.1	**0.002**
Vitamin B12 (µg)	7.9	7.2	5.1	5.4	7.2	9	7.2	6.6	2.9	5.2	6.6	8.6	0.3
Vitamin B9 (µg)	370.1	318.5	251.4	235.6	318.5	416.6	281.2	262.9	113.8	198.1	262.9	350.8	**<0.001**
Vitamin B3 (mg)	42.5	38.1	27.4	30.5	38.1	44.7	31.6	31.5	9.9	24.3	31.5	37.9	**<0.001**
Vitamin C (mg)	222.7	171.3	193.2	98.8	171.3	274.2	148.8	124.3	95.2	74.9	124.3	207.9	**<0.001**
Vitamin A (µg)	1141.5	875.3	1067.5	592.9	875.3	1368.4	837.6	652.9	542.3	448.3	652.9	1088.2	**<0.001**
Vitamin D (µg)	3.3	2.6	2.8	1.5	2.6	4.1	3.4	2.8	2.1	1.9	2.8	4.5	0.51
Vitamin E (mg)	12	10.7	8.6	7.3	10.7	13.7	9.9	9.4	3.8	6.9	9.4	12.5	0.1

* Intakes are expressed as the mean (M), standard deviation (SD), median (Med), and interquartile range (P25:P75). *P-values of median differences were calculated using the Wilcoxon test. Differences are shown in bold ($p<0.05$).

With regards to the Spearman and Pearson coefficients, we observed that for crude nutrients, the correlation coefficients ranged from 0.19 for iron to 0.52 for vitamins B1 and B6. The correlation coefficients for crude food groups ranged from 0.30 for eggs and vegetable fats to 0.74 for processed meat. After adjusting for energy, the correlation coefficients for nutrient intake ranged from 0.14 for iron to 0.50 for vitamin C; and for food group intake, they ranged from 0.21 for eggs and sauces and processed food to 0.67 for vegetables and processed meat. The average correlation coefficients of the crude and energy-adjusted nutrients were 0.39 and 0.41, respectively. The average correlation coefficient of crude food group intake was 0.51, while the average correlation coefficient of energy-adjusted food group intake was 0.47. Consequently, between the FFQ1 and FFQ2, we observed acceptable consistency for crude nutrients and energy-adjusted nutrients and food groups, and good consistency for crude food groups.

Table 2. Reliability and consistency of nutrient intake between the FFQ1 and FFQ2.

Nutrients	ICCs		Pearson or Spearman Coefficient	
	r [a]	r-adjusted [b]	r [c]	r-adjusted [d]
Energy (kcal)	0.538 (0.44–0.465)	0.538 (0.44–0.465)	0.50	0.5
Total protein (g)	0.430 (0.290–0.560)	0.332 (0.180–0.470)	0.41	0.47
Animal protein (g)	0.511 (0.380–0.620)	0.447 (0.306–0.579)	0.44	0.43
Vegetable protein (g)	0.323 (0.169–0.482)	0.219 (0.058–0.369)	0.32 *	0.44
Total fats (g)	0.397 (0.250–0.526)	0.220 (0.060–0.371)	0.31	0.38
MUFAs (g)	0.412 (0.270–0.540)	0.244 (0.084–0.391)	0.32	0.32
PUFAs (g)	0.428 (0.285–0.550)	0.273 (0.115–0.420)	0.35	0.38
SFAs (g)	0.385 (0.240–0.515)	0.280 (0.122–0.424)	0.34	0.46
Cholesterol (mg)	0.398 (0.251–0.530)	0.243 (0.084–0.391)	0.35	0.38
Carbohydrates (g)	0.354 (0.203–0.490)	0.20 (0.028–0.342)	0.39	0.24 *
Total fiber (g)	0.414 (0.269–0.540)	0.342 (0.190–0.479)	0.41 *	0.48
Iodine (µg)	0.290 (0.137–0.436)	0.287 (0.130–0.430)	0.34	0.42
Sodium (mg)	0.359 (0.208–0.493)	0.239 (0.079–0.387)	0.37	0.38

Potassium (mg)	0.436 (0.290–0.560)	0.314 (0.159–0.454)	0.48	0.48
Calcium (mg)	0.394 (0.237–0.536)	0.280 (0.124–0.425)	0.32	0.40
Magnesium (mg)	0.310 (0.155–0.451)	0.234 (0.074–0.383)	0.32	0.32
Phosphorus (mg)	0.375 (0.225–0.507)	0.304 (0.149–0.445)	0.39	0.37 *
Iron (mg)	0.237 (0.070–0.385)	0.083 (−0.08–0.243)	0.19	0.14
Zinc (mg)	0.451 (0.311–0.572)	0.358 (0.207–0.492)	0.43	0.51
Selenium (µg)	0.384 (0.236–0.515)	0.302 (0.146–0.443)	0.38	0.36
Vitamin B1 (mg)	0.499 (0.366–0.612)	0.404 (0.258–0.532)	0.52	0.53
Vitamin B2 (mg)	0.399 (0.252–0.528)	0.345 (0.193–0.481)	0.33	0.51
Vitamin B6 (mg)	0.523 (0.394–0.632)	0.386 (0.238–0.517)	0.52	0.41 *
Vitamin B12 (µg)	0.433 (0.290–0.556)	0.387 (0.239–0.517)	0.45	0.46
Vitamin B9 or folate (µg)	0.460 (0.321–0.580)	0.380 (0.231–0.511)	0.48	0.47
Vitamin B3 or niacin (mg)	0.504 (0.374–0.616)	0.306 (0.150–0.447)	0.48	0.33
Vitamin C (mg)	0.514 (0.383–0.624)	0.428 (0.285–0.552)	0.5	0.46
Vitamin A (µg)	0.478 (0.341–0.595)	0.407 (0.267–0.534)	0.44	0.45
Vitamin D (µg)	0.178 (0.015–0.331)	0.178 (0.015–0.331)	0.23	0.20
Vitamin E (mg)	0.446 (0.305–0.568)	0.343 (0.190–0.479)	0.45 *	0.38
Average	0.41	0.31	0.39	0.41

FFQ1 vs. FFQ2 after 10 months. ICC: intra-class correlations coefficient. a r: ICC after nutrient or food group crude intake was transformed to the natural logarithm (ln). b r-adjusted: after nutrient intake or food group intake was adjusted for energy using the residual method. c r: correlation coefficient after nutrient or food group crude intake was transformed to the natural logarithm (ln). d r-adjusted: correlation coefficient after nutrient intake or food group intake was adjusted for energy using the residual method. * Pearson correlation.

To measure the percentage of agreement between the FFQ1 and FFQ2, we used misclassification rates and adjacent quartile agreement to measure the proportion of participants who were classified in different or in the same quartile in the two measurements by the same FFQ. The best agreement percentages between the FFQ1 and FFQ2 were 85.4% for

vitamin B1 and 89.4% for white meat. A misclassification into the opposite quartile >10% indicated low agreement between two FFQ assessments. The results showed that all foods and most nutrients had correct values (<10%) with the exception of iron (10.4%) (See Appendix A Table A4).

3.2. Validity

A total of 150 participants were included in the validity study (See Appendix A Table A1). We used the FFQ2, which captures dietary intake over the past 6 months, for comparison with the information collected during the same period using the 24H-DR.We found differences between the FFQ2 and 24H-DR questionnaire with regards to nutrient intake (g/day). The FFQ2 recorded higher intakes of energy, sodium, iodine, SFAs, cholesterol, potassium, magnesium, phosphorus, iron, selenium, zinc, vitamins B1, B2, B12, B9, C, and A, total fats, PUFAs, MUFAs, and total fiber than the 24H-DR (Table 3). This may suggest that the FFQ2 overestimates intake levels.

Table 3. Nutrient intake of the population in the validity study (*n* = 150).

Nutrients	3 × 3 Days 24H-DR					FFQ2					*P* ‡
	M *	SD *	Med *	P25 *	P75 *	M	SD	Med	P25	P75	
Energy (kcal)	1929.59	545.28	1892.47	1549.48	2200.15	2201.77	1288.6	1974.08	1412.54	2552.59	**0.01**
SFAs (g)	22.59	8.67	20.74	16.73	26.98	29.76	19.34	25.9	17.12	36.24	**<0.001**
Sodium (mg)	2192.87	754.41	2117.61	1658.8	2624.44	2532.67	1570.23	2161.81	1564.33	3108.67	**0.01**
Cholesterol (mg)	298.85	113.6	282.33	228.7	370.4	387.12	269.83	323.5	231.89	443.29	**<0.001**
Iodine (µg)	116.3	164.1	76.58	53.81	130.53	122.75	79.63	104.59	78.6	139.05	**0.04**
Potassium (mg)	2376.05	803.39	2278.82	1802.05	2855.08	3668.65	2424.79	3149.14	2283.77	4283.86	**<0.001**
Calcium (mg)	761.93	350.19	726.82	553.12	943.66	853.48	521.62	760.03	523.02	1015.57	0.18
Magnesium (mg)	268.79	165.12	249.86	202.5	303.94	326.19	208.21	269.11	204.39	382.06	**<0.001**
Phosphorus (mg)	1196.65	424.51	1154.05	926.83	1357.61	1443.95	826.76	1309.99	919.52	1664.39	**<0.001**
Iron (mg)	12.92	8.54	11.21	9.38	14.13	16.93	10.46	14.39	10.46	20.65	**<0.001**
Selenium (µg)	79.44	48.68	73.65	59.64	88.14	100.65	60.53	85.08	60.19	120.32	**<0.001**
Zinc (mg)	10.02	7.79	8.75	7.01	11.29	12.41	8.1	10.74	7.69	13.97	**<0.001**
Vitamin B1 (mg)	1.52	2.02	1.22	0.95	1.64	1.71	1.13	1.4	0.99	2.13	**<0.001**
Vitamin B2 (mg)	1.8	2.6	1.43	1.03	1.82	2	1.15	1.83	1.28	2.44	**<0.001**
Vitamin B6 (mg)	2.46	5.29	1.64	1.32	2.19	2.67	1.75	2.26	1.61	3.07	0.25

Vitamin B12 (µg)	5.96	4.49	5.19	3.9	7.18	7.52	5.48	6.34	4.25	8.7	**<0.001**
Vitamin B9 (µg)	242.27	299.99	193.82	144.08	258.71	296.94	230.89	242.98	174.08	354.24	**<0.001**
Vitamin B3 (mg)	31.68	31.66	24.93	20.06	33.15	32.53	19.99	28.62	19.5	38.13	0.08
Vitamin C (mg)	77.5	144.77	52.41	30.39	84.69	161.59	179.82	113.16	64.09	200.43	**<0.001**
Vitamin A (µg)	722.74	1194.4	495.93	304.3	831.4	926.93	890.56	616.4	393.15	1106.67	**<0.001**
Vitamin D (µg)	5	9.89	2.92	1.17	5.82	3.48	3.3	2.91	1.69	4.4	0.14
Vitamin E (mg)	10.91	24.71	7.17	5.57	9.09	10.34	7.55	8.44	5.87	12.71	0.42
Carbohydrates (g)	222.36	73.74	217.04	171.83	264.58	242.76	139.39	216.4	156.61	284.4	0.06
Total protein (g)	91.96	27.33	90.47	74.2	106.27	108.58	68.34	94.5	65.19	124.5	**0.001**
Total fats (g)	72.87	22.72	70.07	57.03	83.69	89.17	52.5	82.17	55.66	103.91	**<0.001**
PUFA (g)	11.32	4.27	10.82	8.66	13.36	14.21	8.03	12.66	9.04	17.19	**<0.001**
MUFA (g)	28.87	9.47	27.94	21.58	34.73	36.56	22.43	32.04	22.36	44.78	**<0.001**
Total fiber(g)	16.01	5.99	15.72	12.07	19.04	23.24	19.35	18.39	13.53	26.38	**<0.001**

Intakes are expressed as the mean (M), standard deviation (SD), median (Med), and interquartile range (P25:P75). ‡*p*-values of the median differences were calculated using the Wilcoxon test and *p* values of the mean were calculated using a paired *t*-test. Differences are shown in bold (p<0.05)

Table 4 summarizes the crude, energy-adjusted, and de-attenuated Pearson correlation coefficients of nutrient intake between the FFQ2 and 24H-DR. The attenuation correctionwas applied to eliminate intra- and interindividual variations. The Pearson correlation coefficient for crude nutrients ranged from 0.19 for SFAs and PUFAs to 0.50 for vitamin C and fiber. The coefficients of nutrients adjusted for energy ranged from 0.25 for SFAs and folate to 0.76 for total protein and carbohydrates. The de-attenuated Pearson correlation coefficient ranged from 0.30 for SFAs to 0.72 for vitamin B6. As expected, stronger correlations were observed for energy-adjusted nutrients and de-attenuated coefficients. The average correlation coefficients of the crude and adjusted-energy nutrients and the de-attenuated coefficient were 0.32, 0.50, and 0.50, respectively. Therefore, acceptable consistency was observed for crude nutrients between the FFQ and 24H-DR, while good consistency was observed when adjusting nutrients for energy and using de-attenuated coefficients.

Table 4. Validity of FFQ2 nutrient intake compared with 24H-DR and Bland–Altman agreement.

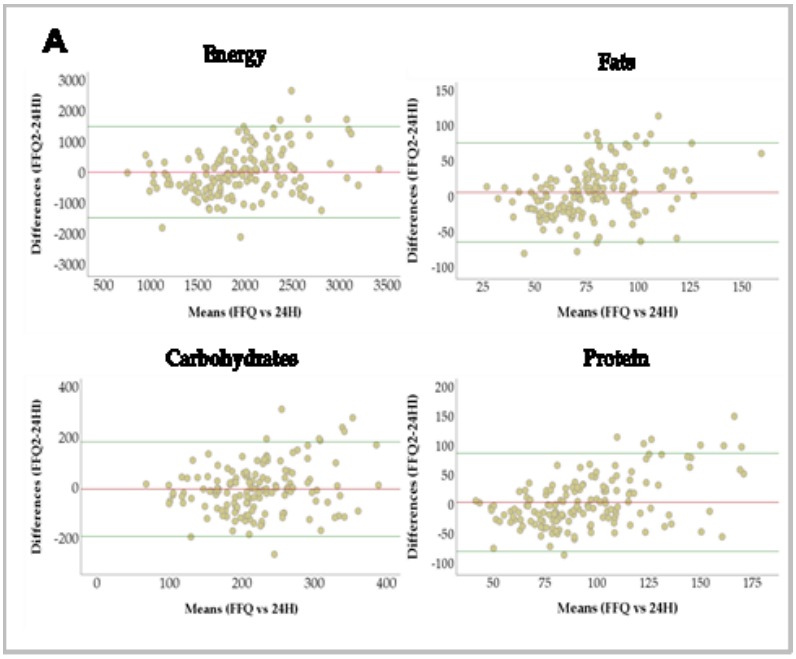
Nutrients	Pearson			Bland–Altman Index (%) ‡
	r [a]	r-adj [b]	r de-att [c]	
Energy (kcal)	0.374		0.44	4
SFAs (g)	0.19	0.25	0.3	8
Sodium (mg)	0.29	0.69	0.58	4.7
Cholesterol (mg)	0.21	0.46	0.36	4
Iodine (µg)	0.29	0.37	0.39	7.3
Potassium (mg)	0.32	0.65	0.38	3.3
Calcium (mg)	0.39	0.59	0.47	4.7
Magnesium (mg)	0.4	0.64	0.46	4
Phosphorus (mg)	0.33	0.7	0.39	6.7
Iron (mg)	0.4	0.58	0.49	8
Selenium (µg)	0.38	0.56	0.49	4
Zinc (mg)	0.2	0.59	0.31	6
Vitamin B1 (mg)	0.39	0.64	0.46	4.7
Vitamin B2 (mg)	0.36	0.51	0.53	4
Vitamin B6 (mg)	0.39	0.32	0.72	6
Vitamin B12 (µg)	0.28	0.32	0.5	5.3
Vitamin B9 or folate (µg)	0.37	0.25	0.46	4.7
Vitamin B3 or niacin (mg)	0.36	0.44	0.5	8
Vitamin C (mg)	0.5	0.27	0.58	6.7
Vitamin A (µg)	0.39	0.39	0.48	6
Vitamin D (µg)	0.235	0.4	0.55	6
Vitamin E (mg)	0.20	0.3	0.5	4
Carbohydrates (g)	0.31	0.76	0.38	6
Total protein (g)	0.33	0.76	0.39	5.3
Total fats (g)	0.2	0.67	0.31	6
PUFAs (g)	0.19	0.4	0.33	5.3
MUFAs (g)	0.24	0.5	0.36	6.7
Total fiber (g)	0.5	0.6	0.56	5.3
Average	0.32	0.51	0.45	5

[a] r: correlation coefficient after nutrient or food group crude intake was transformed to natural logarithm (ln). [b] r-adj: correlation coefficient after the nutrient intake or food group intake was adjusted for energy using the residual method. [c] r de-att: de-

attenuated correlation coefficients after log-transformation and energy adjustment.
‡ Percentage of subjects with values out of the limits of agreement.

Furthermore, we assessed the percentage of agreement between the FFQ2 and the 24H-DR questionnaire using misclassification rates and adjacent quartile agreement (See Appendix A Table A5). Once nutrients were divided into quartiles, the agreement percentage in the same or adjacent quartile between the FFQ2 and 24H-DR ranged from 65.56% for cholesterol and SFAs to 83.44% for vitamin C; the average percentage agreement for nutrient intake was 72.46%. Misclassification into the opposite quartile was <10% for all nutrients with the exception of total fats (10.60%).

Bland–Altman analysis was performed to quantify the agreement between the FFQ2 and 24H-DR. Table 4 shows that all nutrients remained within the LOA; less than 10% of subjects had values outside the LOA for all nutrients. Bland–Altman plots of energy and macronutrient intakes are shown in Figure 2A. These plots help to assess the agreement between the two methods of measuring dietary intake. Most of the data points closely aligned with the average line, with the exception of iodine, vitamin A, and vitamin D (Figure 2B), which reflects good agreement between the two questionnaires. The remaining nutrient data are shown in Appendix A (See Appendix A Figure A1).


A
Energy
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Means (FFQ vs 24H)
Fats
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Carbohydrates
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Protein
Differences (FFQ2-24HI)
Means (FFQ vs 24H)

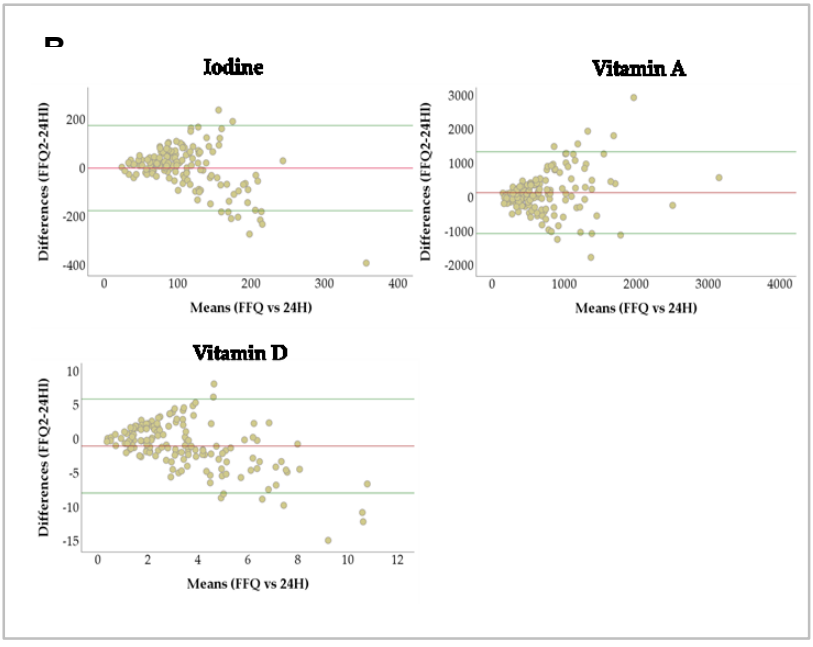
Iodine
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin A
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin D
Differences (FFQ2-24HI)
Means (FFQ vs 24H)

Figure 2. Bland–Altman plots showing the relationship between mean and differences in daily nutrient intake estimated with the average of FFQs and three 24H-DRs. (**a**) Energy, fats, protein and carbohydrates show good concordance. (**b**) Iodine, vitamin A and vitamin D did not show a good correlation. Red lines show the mean of the differences and green lines represent the Limits of Agreement (LOA)

3. DISCUSSION

The present study validates the Spanish-adapted EPIC FFQ in children and adolescents aged 10 to 17 years old, providing a reliable dietary assessment tool for this population. Compared with other methods such as FRs, WFRs, and biomarkers, 24H-DR is considered the most practical reference method for this age group due to its simplicity and ease of use[6,8]. To account for individual and stationary variations, we collected the three 24H-DRs three times over six months, spanning different seasons of the year. The sample sizes for the reproducibility and validity studies were 144 and 150, respectively, which are considered optimal[6]. The combination of extensive 24H-DR collection and large sample sizes has demonstrated strong validity and study quality[6].

In terms of reproducibility, we observed an overestimation of most nutrients (Refer to Table 1) and some food groups (Refer to Table A2) in the FFQ1, consistent with the findings of Rendo-Urteaga et al.[9]. The average ICC was 0.40 for nutrients and food groups. When we adjusted for energy, the ICC decreased to 0.31 for nutrients (Refer to Table 2), while it remained similar for food groups (Refer to Table A3). This decrease has been reported previously, which implies errors in the estimation of energy intake or variability in the subject's response[18,32,33]. Our findings are consistent with the results reported in previous studies involving children and adolescents where the correlation coefficient and ICC averages ranged from 0.3 to 0.5[18,34,35].However, other studies in this population observed higher correlation coefficients and ICCs, ranging from 0.5 to 0.9 [9,36,37]. These high correlations can likely be explained by the much shorter interval between the administrations of the two FFQs, ranging from two weeks to one month. By contrast, the nine-month gap in our study allows for seasonal dietary changes, potentially resulting in lower correlations[6,8]. Nevertheless, our results demonstrated acceptable reliability for nutrients and food groups, excluding vitamin D and iron.

We achieved a high level of agreement for nutrient (78.2%) and food group (81.9%) intake (Refer to Table A4), matching or surpassing previous studies[18,38]. This demonstrates that our EPIC FFQ is a reliable and valid tool for collecting and assessing dietary intake in Spanish adolescents and older children, aligning with established criteria [4–6, 8, 39].

Regarding validity, we found higher intake estimations for most nutrients from the FFQ2 compared with 24H-DR, with the exception of calcium, vitamin B6, vitamin B3, vitamin D, vitamin E, and carbohydrates (refer to Table 3). As reported in previous studies in children and adolescents, the FFQ tends to overestimate nutrient intake[18,35,38,40–45]. Inge Huybrechts et al.[21] suggest that this could be attributed to differences in data collection and questionnaire structure. It could also be due to the lack of collection of intakes from certain food groups by 24H-DR and difficulties in estimating portion sizes [21].

In the correlation analysis, we observed minor differences in Spearman and Pearson correlation coefficients, and, therefore, only Pearson's correlation was computed to adjust for total energy intake and intra- and inter-individual variation. The average crude intake was 0.32 (refer to Table 4), aligning with findings from similar studies [38,45] but exceeding those reported in others [18,35,46]. Upon adjusting for energy, the correlation increased to 0.5 (refer to Table 4). As expected, the stronger correlations when adjusting for energy were likely due to the close relationship between inter-subject intake variability and total energy intake[21]. The average de-attenuated correlation coefficient was 0.45 (refer to Table 4), reflecting an improvement when compared with crude intakes. This is likely attributed to intra- and inter-individual correction, as reported by Willet and Rosner [8,29]. The German EPIC study on FFQ reproducibility and validity also demonstrated that adjusting for attenuation due to within-person error in the reference method leads to more accurate and reliable correlation measurements[47].

The percentage of participants classified into the same or an adjacent quartile for nutrient intake was higher (72.46%) than that reported in a recent similar study[18] and equal to previous studies in the same population type[32,38,44,48]. Total fats were the only nutrient for which 10% or more of the children were classified into the opposite quartile (Refer to Table A5).

The Bland–Altman plots revealed no substantial bias in macronutrients and energy assessments between the two methods (refer to Figure 2a). For

micronutrients, the EPIC FFQ seems to overestimate vitamin A, vitamin D, and iodine intake compared with 24H-DR, showing proportional bias (refer to Figure 2b). The Bland–Altman index agreement showed a correct classification of all nutrients (<10%) (refer to Table 4). It is important to note that Bland–Altman plots provide only a visual interpretation[30], and their findings should be considered in conjunction with correlation coefficients and cross-classification into quartiles[18]. According to our results, both methods are in good agreement with each another.

The strengths of the present study include a suitable sample size (more than 100 participants), providing sufficient statistical power for dietary validation[3]; the use of 24H-DR as the reference method, which is the best available and most recommended method; the inclusion of data from three different time points of 24H-DR days, including a weekend day to have more representative data of the habitual diet, and the time interval between the different 24H-DR recording points captured seasonal variations; and the use of a photographic atlas to aid in food recognition and portion sizes. Limitations of the study include a lengthy questionnaire (137 food items) compared with other FFQs (fewer than 100), which may lead to participant fatigue and over-reporting of certain foods, potentially affecting accuracy[8]. While the long study duration (9- to-12-month data collection period) can be considered a strength regarding seasonality, it may also increase the likelihood of diet changes, potentially affecting reproducibility and validity. Although participants were trained and provided with a photographic food atlas, self-administration of the EPIC FFQ and 24H-DR may introduce some misreporting due to forgetfulness or difficulty recalling dietary intakes. Finally, the longer recall period of the FFQ over the 24H-DR may increase the risk of recall bias, leading to memory issues and potential inaccuracies[4].

4. CONCLUSIONS

The Spanish-adapted EPIC FFQ has been validated for all nutrients and demonstrated reproducibility for both food groups and nutrients in a large sample of Spanish children and adolescents. The online version of the questionnaire, which was developed and evaluated in this study, is more user-friendly and adaptable to the target population. The study design, which included questionnaires administered at different times of the year, confirms the reliability of the FFQ, including its ability to capture seasonal variations in dietary intake. However, further research is needed to develop new, shorter, and more accurate methods to assess dietary intake in this

population. Overall, our results suggest that the Spanish-adapted EPIC FFQ is a suitable method for assessing dietary intake in Spanish older primary school children and adolescents, which constitutes important progress in the availability of dietary assessment tools at crucial stages of development and in the understanding of the relationship between diet and health.

Author Contributions: Conceptualization, study design, and methodology, M.C.C. and A.L.; Research and resources, Y.S. and M.C.C.; Writing—original draft, A.L. and M.T.; Writing—revising and editing; A.L., M.T. M.C.C., and Y.S; administration of the FFQ in schools, A.L. and M.T.; supplementary activities in schools, A.L. and M.T.; FFQ EPIC analysis and statistical analysis, A.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Spanish National Research Council (CSIC) (Reference 056/3 may of 2022).

Informed Consent Statement: Written informed consent from parents and written informed consent from every subject was obtained for all students.

Data Availability Statement: Access to data can be requested from the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Suplemmentary tables

Appendix A

Table A1. Characteristics of the populations in the reproducibility (*n* = 144) study.

Charasteristics of participant	Overall (N=144)	Male (N=74, 51.4%)	Female (N=70, 46.8%)	P*
Age	14.42+1.69	14.55±1.7	14.29+1.67	0.343
Level of education				
third cycle of primary	17(11.8%)	9(12.2%)	8(11.4%)	
Secondary school	85(59%)	39(52.7%)	46(65.7%)	0.234
High school	42(29.2%)	26(35%)	16(22.9%)	
Weigh(kg)	58.2+13.2	62.6+13.8	53.7+10.8	**<0.001**
Height(cm)	164.7+12.6	169.8+12.5	159.2+10.1	**<0.001**
BMI (kg/m2)	21.2+3.1	21.5+3.3	21.0+2.9	0.312
Optimal	130(90.3%)	64(86.5%)	66(94.3%)	
Overweight	10.0(6.9%)	6(8.1%)	4.0(5.7%)	0.115
Obesity	4.0(2.8%)	4(5.4%)	0	
Activity level				
Low	46(31.9%)	7(9.5%)	39(55.7%)	
Moderate	65(45.1%)	39(52.7%)	26(37.1%)	**<0.001**
High	33(22.9%)	28(37.8%)	5.0(7.1%)	

P-values of the mean differences between males and females were calculated using the t-test or Mann-Whitney U test for continuous variables and the chi-square test for categorical variables. Differences are shown in bold ($p<0.05$)

Table A2. Food group intake of the population in the reproducibility study (*n* = 144).

Food Groups	FFQ1						FFQ2						*p* *
	M *	Med *	SD *	25	50	75	M	Med	SD	25	50	75	
Sugary beverages (g)	109.9	63.9	146.9	26.8	63.9	152.8	112.8	64.5	124.2	23.9	64.5	167.6	**0.05**
Fresh fruit (g)	458.1	358.3	451.6	197	358.3	568.1	461.2	407.8	342.4	196	407.8	612.5	**0.01**
Legumes (g)	25.2	21.5	25.1	11.4	21.5	32.9	26.2	22.7	23	12	22.7	33.1	0.23
Nuts and oil olive (g)	12.8	7.7	14.9	3.2	7.7	18.4	12.2	9.0	11.3	3.6	9.0	19.3	0.56
Vegetables (g)	329.2	241.4	318.2	117.9	241.4	449.2	339.3	278.1	293	144.5	278.1	443.1	0.49
Sauces and processed food (g)	143.5	133.4	89.1	89.1	133.4	174.9	146.8	132.9	70	98.4	132.9	185.8	0.08
Sugar confectionary (g)	83.9	54.9	104.8	25	54.9	92.5	76	56.4	63.5	29.3	56.4	104.3	**0.001**
Eggs (g)	16.9	7.9	16.2	7.9	7.9	23.7	17.2	13.1	14.4	7.3	13.1	25.8	0.21
Cheese (g)	30	17.2	35.3	5.7	17.2	40	29.2	22.1	28.9	6.5	22.1	41.4	0.1
Pastries (g)	55.7	34.8	74.7	18.5	34.8	57	51.6	39.4	46.8	21.7	39.4	66.4	0.42
Yogurt and milk (g)	233.4	204.2	199.1	86	204.2	308.3	245.4	196.2	219.6	91.1	196.2	339.1	0.10
Potatoes (g)	71.3	47.2	77.4	31.5	47.2	96	70.3	60.7	51.5	33.1	60.7	88.6	**0.05**
Whole grains (g)	41.7	34.1	40.3	22.6	34.1	52.4	44.2	35.2	37.1	22.8	35.2	57.6	**0.01**
Refined cereals (g)	112.7	92.4	81.9	53.1	92.4	169.1	114.5	106.6	71.1	62.2	106.6	56.4	**0.001**
Fish (g)	48.2	31.5	44.3	16.9	31.5	74.6	50.9	37.5	44	20.5	37.5	70.6	0.23
Red meat (g)	81.8	74.6	63.4	33.7	74.6	114.6	85.6	79.9	63.6	41.2	79.9	116	0.48
White meat (g)	53.3	64.5	45.5	21.5	64.5	64.5	57.5	48.7	46.2	26.4	48.7	74.8	0.87
Processed meat (g)	51.3	43.8	37.6	27.8	43.8	67.8	54.4	47.5	36.9	29.2	47.5	70.9	**0.03**
Animals fats (g)	1.7	0.9	2.7	0	0.9	2	1.9	1	3.2	0	1	2.3	0.50
Vegetable fats (g)	6.3	3.4	8.4	1.1	3.4	6.8	6.1	4.3	7.2	1.7	4.3	7.2	0.09

* Intakes are expressed as the mean (M), standard deviation (SD), median (Med), and interquartile range (P25:P75). * *p*-values of the median differences were calculated using the Wilcoxon test. Differences are shown in bold ($p<0.05$)

Table A3. Reliability and consistency of food group intake between FFQ1 and FFQ2.

Food Group	ICCs [1]		Pearson or Spearman Coefficient	
	r [a]	r-adjusted [b]	r [a]	r-adjusted [b]
Sugary Beverages (g)	0.540 (0.413–0.646)	0.459 (0.305–0.591)	0.58	0.59
Fresh fruit (g)	0.310 (0.154–0.450)	0.422 (0.276–0.549)	0.6	0.56
Legumes (g)	0.190 (0.028–0.342)	0.335 (0.168–0.483)	0.42	0.42 *
Nuts and oil olive (g)	0.226 (0.065–0.375)	0.407 (0.254–0.541)	0.48	0.46
Vegetables (g)	0.463 (0.324–0.582)	0.597 (0.477–0.695)	0.68	0.67
Sauces and processed food (g)	0.464 (0.325–0.583)	0.345 (0.191–0.482)	0.34	0.21 *
Sugar confectionary (g)	0.240 (0.08–0.388)	0.419 (0.273–0.545)	0.44	0.42 *
Eggs (g)	0.585 (0.467–0.683)	0.354 (0.189–0.499)	0.3	0.21
Cheese (g)	0.213 (0.052–0.363)	0.112 (−0.07–0.294)	0.6	0.61
Pastries (g)	0.147 (−0.016–0.303)	0.291 (0.133–0.435)	0.33	0.32
Yogurt and milk (g)	0.528 (0.399–0.636)	0.551 (0.422–0.658)	0.62	0.60
Potatoes (g)	0.489 (0.354–0.604)	0.341 (0.186–0.479)	0.5	0.35
Whole grain (g)	0.33 (0.180–0.470)	0.418 (0.266–0.55)	0.49	0.48
Refined cereals (g)	0.376 (0.227–0.508)	0.267 (0.106–0.414)	0.43	0.33
Fish (g)	0.482 (0.347–0.598)	0.449 (0.293–0.582)	0.69	0.69
Red meat (g)	0.511 (0.38–0.622)	0.418 (0.266–0.55)	0.67	0.64
White meat (g)	0.406 (0.261–0.534)	0.238 (0.075–0.389)	0.46	0.36
Processed meat (g)	0.664 (0.562–0.747)	0.508 (0.375–0.621)	0.74	0.67 *
Animals fats (g)	0.270 (0.112–0.415)	0.407 (0.181–0.592)	0.43	0.47
Vegetable fats (g)	0.201 (0.039–0.352)	0.308 (0.135–0.463)	0.3	0.28

FFQ1 vs. FFQ2 after 10 months. [1] ICC: intra-class correlation coefficient. [a] r: ICC after nutrient or food group crude intake was transformed to the natural logarithm (ln). [b] r-adjusted: after nutrient intake or food group intake was adjusted for energy using the residual method. * Pearson correlation.

Table A4. Agreement percentages for nutrients and food groups between FFQ1 and FFQ2 and misclassification (*n* = 144).

Nutrients	[1]Misclassification [1]		Agreement percentages [2]	
	N	%	N	%
Energy (kcal)	6	4.2	118	81.9
Total protein (g)	8	5.6	114	79.2
Animal protein (g)	6	4.2	114	79.2
Vegetable protein (g)	9	6.3	108	75
Total fats (g)	10	6.9	107	74.3
MUFAs (g)	9	6.3	105	72.9
PUFAs (g)	7	4.9	106	73.9
SFAs (g)	11	7.6	110	76.4
Cholesterol (mg)	8	5.6	110	76.4
Carbohydrates (g)	8	5.6	115	79.9
Total fiber (g)	7	4.9	114	79.2
Iodine (µg)	9	6.3	110	76.4
Sodium (mg)	10	6.9	111	77.1
Potassium (mg)	7	4.9	122	84.7
Calcium (mg)	13	9.0	111	77.1
Magnesium (mg)	11	7.6	111	77.1
Phosphorus (mg)	7	4.9	113	78.5
Iron (mg)	15	10.4	103	71.5
Zinc (mg)	7	4.9	110	76.4
Selenium (µg)	8	5.6	112	77.8
Vitamin B1 (mg)	7	4.9	123	85.4
Vitamin B2 (mg)	6	4.2	116	80.6
Vitamin B6 (mg)	8	5.6	121	81
Vitamin B12 (µg)	6	4.2	114	79.2

Vitamin B9 or Folate (µg)	5	3.5	117	81.3
Vitamin B3 or niacin (mg)	5	3.5	114	79.2
Vitamin C (mg)	4	2.8	117	81.3
Vitamin A (µg)	6	4.2	114	79.2
Vitamin D (µg)	12	8.3	103	71.5
Vitamin E (mg)	8	5.5	110	76.4
Nutrients Average	8.1	5.6	112.6	78.2
Sugary beverages	3	2.1	119	82.6
Fresh fruit	4	2.8	127	88.2
Legumes	6	4.2	115	79.9
Nuts and oil olive	4	2.8	119	82.64
Vegetables	1	0.7	127	88.2
Sauces and processed food	7	4.9	109	75.7
Sugar confectionary	6	4.2	106	73.6
Eggs	3	2.1	124	86.1
Cheese	3	2.1	119	82.6
Pastries	1	0.7	118	81.9
Yogurt and milk	1	0.7	121	84.03
Potatoes	4	2.8	114	79.2
Whole grain	1	0.7	108	75.0
Refined cereals	4	2.8	114	79.2
Fish	0	0.0	127	88.2
Red meat	1	0.7	114	79.2
White meat	0	0.0	129	89.6
Processed meat	4	2.8	126	87.5
Animals fats	4	2.8	110	76.4
Vegetables fats	6	4.2	114	79.2
Food group Average	3	2.2	118	81.9

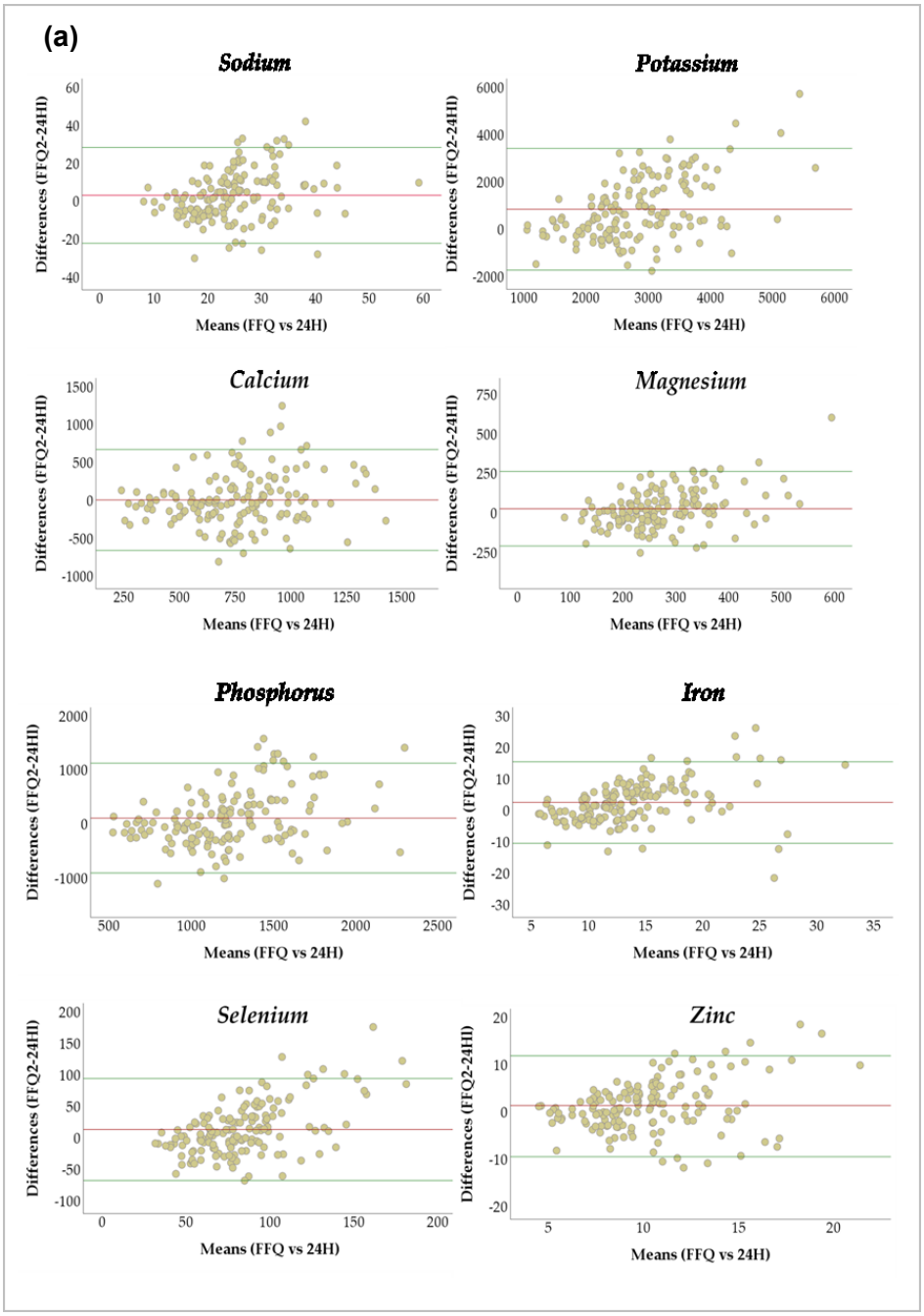
[1] Misclassification: percentage of participants belonging to the opposite quartile. [2] Percentage of participant belonging to the same or an adjacent quartile.

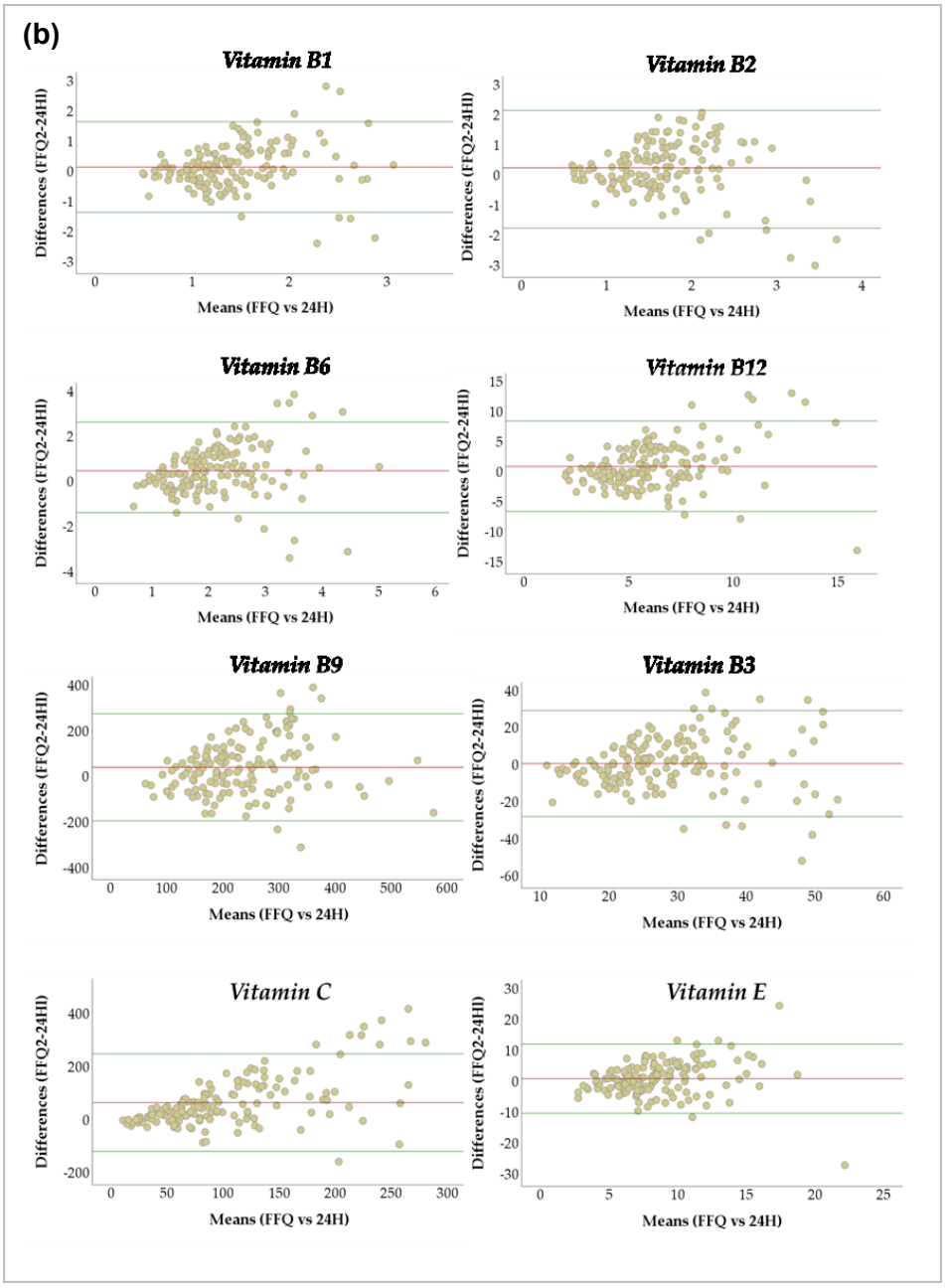
Table A5. Agreement percentages between FFQ2 and 24H-DR and misclassification (*n* = 150).

Nutrients	Misclassification [1]		Agreement percentages [2]	
	N	%	N	%
Energy (kcal)	14	9.3	110	72.9
SFAs (g)	14	9.3	99	65.6
Sodium (mg)	13	8.6	106	70.2
Cholesterol (mg)	8	5.3	99	65.6
Iodine (µg)	14	9.3	107	70.9
Potassium (mg)	11	7.3	106	70.2
Calcium (mg)	15	9.9	111	73.5
Magnesium (mg)	9	6.0	114	75.5
Phosphorus (mg)	15	9.9	113	74.8
Iron (mg)	8	5.3	113	74.8
Selenium (µg)	11	7.3	117	77.5
Zinc (mg)	14	9.3	103	68.2
Vitamin B1 (mg)	9	6.0	112	74.2
Vitamin B2 (mg)	10	6.6	116	76.8
Vitamin B6 (mg)	6	3.9	115	76.2
Vitamin B12 (µg)	9	6.0	105	69.5
Vitamin B9 or Folate (µg)	6	4.0	113	74.8
Vitamin B3 or Niacin (mg)	7	4.6	107	70.9
Vitamin C (mg)	6	4.0	126	83.4
Vitamin A (µg)	7	4.6	111	73.5
Vitamin D (µg)	13	8.6	106	70.2

Vitamin E (mg)	13	8.6	100	66.2
Carbohydrates (g)	11	7.3	108	71.5
Total protein (g)	11	7.3	109	72.2
Total fats (g)	16	10.6	108	71.5
PUFAs (g)	13	8.6	104	68.9
MUFAs (g)	14	9.3	112	74.2
Total fiber (g)	5	3.3	114	75.5
Average	10.8	7.1	109.4	72.5

[1] Misclassification: percentage of participants belonging to the opposite quartile. [2] Percentage of participants belonging to the same or an adjacent quartile.


(a)
Sodium
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Potassium
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Calcium
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Magnesium
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Phosphorus
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Iron
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Selenium
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Zinc
Differences (FFQ2-24HI)
Means (FFQ vs 24H)


(b)
Vitamin B1
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin B2
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin B6
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin B12
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin B9
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
Vitamin B3
Differences (FFQ2-24HI)
Means (FFQ vs 24H)
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Vitamin E
Differences (FFQ2-24HI)
Means (FFQ vs 24H)

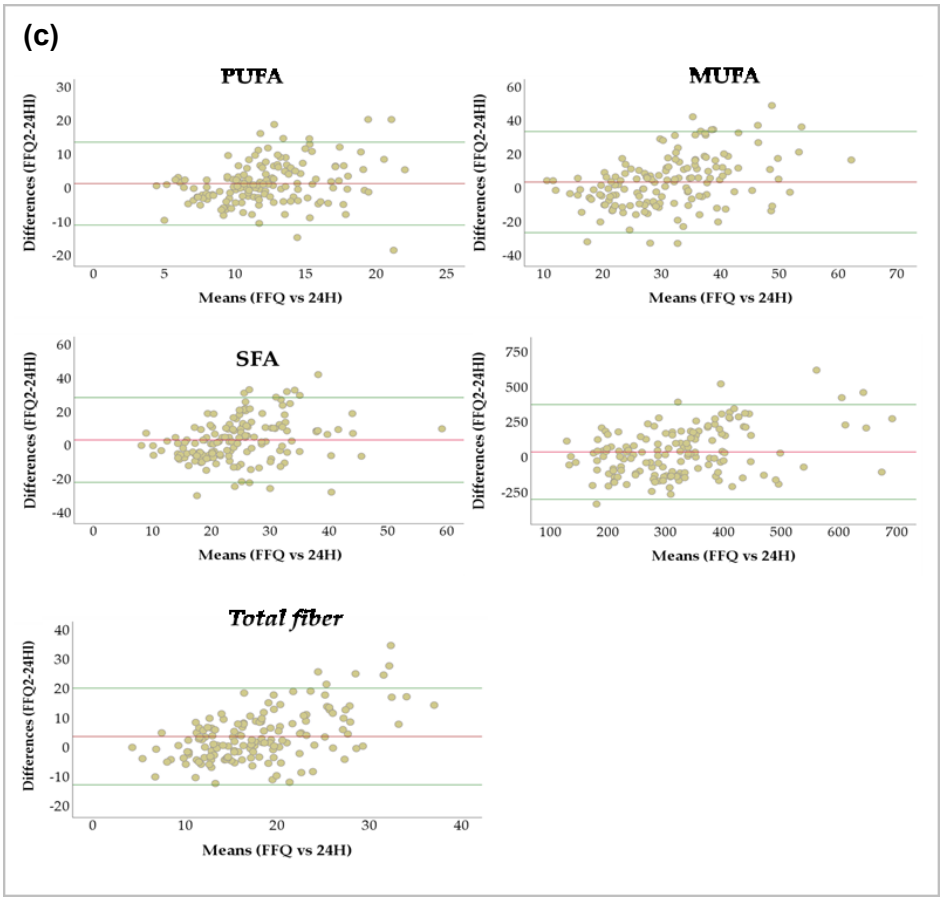

Figure A1. Bland–Altman plots showing the relationship between the means and differences in the daily nutrient intake estimated with the average of the FFQs and three 24H-DRs. (**a**) Plots showing sodium, potassium, calcium, magnesium, phosphorus, iron, selenium, and zinc. (**b**) Vitamin B1, B2, B6, B12, B9 or folate, B3 or niacin, C, and E. (**c**) Plots showing saturated fatty acids (SFAs), polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), cholesterol, and fiber. Red lines show the mean of the differences and green lines represent the Limits of Agreement (LOA).

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CHAPTER 2

GUT MICROBIOTA AND WESTERN DIETARY PATTERNS ASSOCIATED WITH BEHAVIORAL PROBLEMS IN CHILDREN AND ADOLESCENTS

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ABSTRACT

Background: Childhood and adolescence are crucial periods for brain development, influenced by gut microbiota and diet. However, the combined impact of those factors on neurodevelopment and mental disease risk remains largely unexplored. Here, we aimed to investigate the relationships between gut microbiota and the diet in predicting behavioral problems that may precede mental disorders in children and adolescents.

Methods: We performed a cross-sectional study, including data of 335 children and adolescents aged 5–17. Gut microbiota was analysed in stools by shotgun metagenomics. Dietary habits, lifestyle factors and emotional and behavioral difficulties were screened using validated questionnaires. Microbial diversity, differential abundance of bacterial species, dietary patterns, and food and nutrient intakes were compared among Healthy and Behavioral Problem participants. Moreover, we trained a series of Penalized Logistic Regression models to classify individuals into Healthy and Behavioral Problem groups.

Results: We found a consistent association between a Western diet and behavioral problems across all age groups, in line with a lack of compliance with dietary recommendations. Individuals with behavioral problems exhibited distinct gut microbiota profiles characterized by lower levels of short-chain fatty acid-producing bacteria (particularly butyrate-producing species) and higher levels of potential pathogens (e.g., Campylobacter coli and Lautropia mirabilis), linked to poor dietary choices. Furthemore, we revealed the mediation roles of the gut microbiota in the association between dietary patterns and food groups and behavioral problems. Thus, in adolescents, L. mirabilis was identified as a mediator between a Western diet and behavioral problems, while Anaerostipes rhamnosivorans mediated the relationship between fish consumption and behavioral problems. Gut microbiota data enhanced the predictive accuracy of logistic regression models for identifying individuals with behavioral problems over models based solely on dietary data.

Conclusion: Gut microbiota deviations, together with unhealthy dietary habits, are linked to behavioural problems and contribute to stratifying children and adolescents accordingly. Our findings may help to refine dietary interventions targeting the gut microbiota to improve mental health outcomes in these vulnerable populations.

Keywords: microbiota, gut-brain axis, behavior, Western diet, fibers, short-chain fatty acids.

1. INTRODUCTION

The 'gut-brain axis' describes a complex bidirectional communication network linking the gut microbiota and the brain, involving neural, neuroendocrine, metabolic, and neuroimmune pathways [1–3]. Studies in germ-free mice have provided compelling evidence for the profound impact of the microbiota on these pathways, influending various aspects of neurodevelopment such as behavior, social abilities, and emotions [4,5].

Gut bacteria also interacts with the diet playing a crucial role in producing neurotransmitters from amino acids (serotonin, ɣ-aminobutyric acid [GABA], etc.) and short-chain fatty acids (SCFAs) from fiber fermentation, which are involved in the modulation of neurophysiology, behavior, and immunity [6,7].These observations collectively suggest that the gut microbiota per se and through dietary interactions acts as a crucial biological mediator, significantly influencing individual variations in cognitive and behavioral functions [8].

While many clinical studies have explored the influence of the gut microbiota on neurodevelopmental and behavioral disorders in early childhood [9–13], research specifically examining its impact on these disorders during adolescence remains understudied. Furthermore, most observational studies linking the gut microbiome to mental health have primarily focused on specific disorders, such as autism spectrum disorder (ASD) and attention-deficit/hyperactivity disorder (ADHD) [14–20]. Fewer studies have investigated the association between the gut microbiome and broader emotional or behavioral difficulties, which may serve as precursors or co-occurring features of various neurodevelopmental and psychiatric disorders [21–23]. While one study found no significant associations between microbiota and behavioral issues in children, others observed links between social anxiety and decreased *Faecalibacterium* abundance in adolescents, and between externalizing behavior and increased *Prevotella* clusters, in both children and adolescents [22,23].

It is well-established that dietary factors influence both the gut microbiota composition and brain function [24,25]. For example, certain dietary fibers increase SCFA production, fostering a diverse gut microbiota with anti-inflammatory and immunomodulatory characteristics [26–28]. Furthermore,

dietary interventions have the potential to regulate levels of the inhibitory neurotransmitter GABA by modifying the gut microbiota, which may exert beneficial effects on mental well-being [29]. Cross-sectional studies have demonstrated links between unhealthy diets, such as the Western diet, and poorer mental health in children and adolescents [24]. However, only one study has simultaneously examined the interplay between dietary factors, gut microbiota, and mental health in this population, albeit specifically ASD symptoms [30]. Accordingly, a comprehensive understanding of how dietary components and patterns impact behavioral problems, particularly through gut-mediated mechanisms, is still lacking in childhood and adolescence.

Thus, the primary aim of this study is to enhance our understanding of the intricate interplay between diet, the gut microbiota, and behavioral health in children and adolescents. To this end, we investigated the cross-sectional associations between gut microbiota, lifestyle factors (particularly diet), and behavioral problems in those populations. We employed advanced shotgun metagenomic analysis to comprehensively characterize the gut microbiota. Additionally, we assessed the intake of individual nutrients, foods, diet quality indices, and dietary patterns and analyzed their relationships with both the gut microbiota and behavioral outcomes using sophisticated statistical methods. Our ultimate goal is to generate knowledge that could inform the development of more precise dietary recommendations for young people to improve mental well-being.

2. METHODS

Study design and data collection

This cross-sectional study included 335 Spanish participants, comprising 161 boys (48%) and 174 girls (52%) aged 5 to 17 years. This is part of a larger population study conducted in 15 schools that encompass ages 5 to 17 in Valencia, Spain. Recruitment occurred between November and December 2020 during the SARS-CoV2 pandemic, although none of the participants had been infected with the virus at that time according to specific antigen testing. Ethical approval was obtained from the Medical Ethical Committee of Hospital Dr Peset in Valencia (approval number 131/20). The study was conducted following the code of ethics of the World Medical Association (Declaration of Helsinki) and ensuring data protection. All parents of children and adolescents provided written informed consent. In addition, participants over 12 years of age also provided their consent.

This study aimed to investigate the association between environmental factors, the gut microbiota, and the physical and mental health of children and adolescents. We excluded participants with chronic inflammatory bowel disease, those who had received antibiotics within one month before stool sample collection and those who did not provide a stool sample or completed the dietary questionnaire incorrectly. Study design and assessments are depicted in Figure 1.

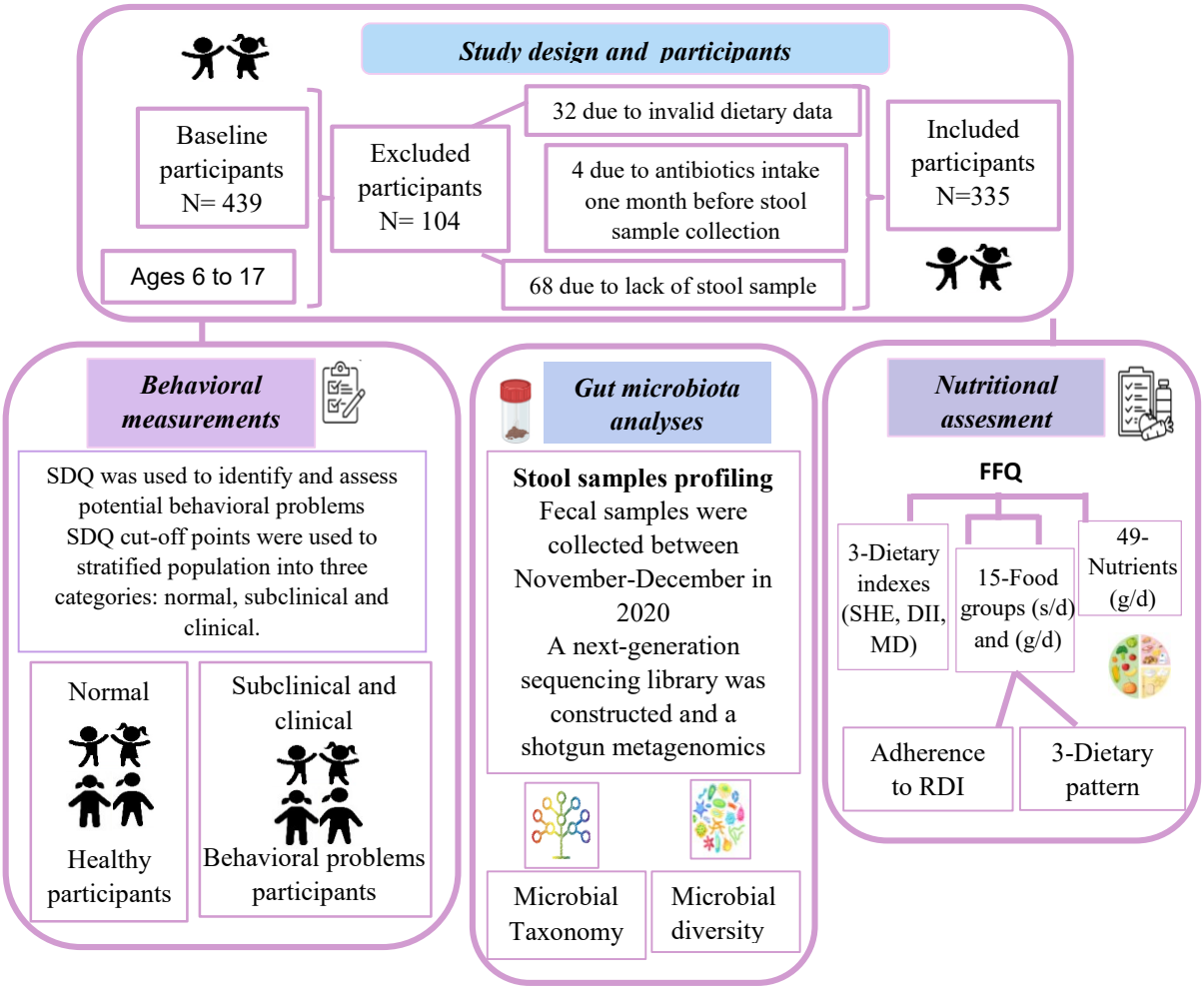


Figure 1. Flow chart of study design, number of participants and data collection. Abbreviations: SDQ: The Strengths and Difficulties Questionnaire; RDI: Recommended daily intake; SHEI: Spanish healthy eating index; DII: Dietary inflammatory index; MD: Mediterranean diet.

Parental reports provided data on sociodemographic factors (age, sex, parental education, marital status), pregnancy history (mode of delivery and breastfeeding, based on feeding practices during the first 6 months of life), bowel habits (assessed using the Bristol Stool Scale over the past month), sleep disorders, medical history, antibiotic or other medication use during the past six months (categorized by the Anatomical, Therapeutic, Chemical Classification System) and perceived stress levels [31]. (**Table 1**).

Anthropometric measures, including body mass index for-age z-score (z-BMI) and waist circumference (WC), were evaluated using international cut-offs based on z-score curves established by the World Health Organization (WHO). BMI z-scores for children and adolescents were calculated using AnthroPlus software, also developed by the WHO, and were categorized as follows: thinness ($-3 \leq$ z-score < -2), normal weight ($-2 \leq$ z-score $\leq +1$), overweight ($+1 <$ z-score $\leq +3$), and obesity (z-score $> +3$). This classification allowed for the evaluation and comparison of the nutritional status of the participants according to international criteria (NCD Risk Factor Collaboration (NCD-RisC, 2024). Height, weight, and WC were parent-reported based on measurements taken at home.

Physical activity was evaluated using multiple methods reported by parents: Assessment of Physical Activity Level Questionnaire (APALQ) [33] daily time spent on team sports, outdoor activities, screen-based activities, and a healthy lifestyle score[34].

Behavioral measurements

The Strengths and Difficulties Questionnaire (SDQ) was used as a screening tool to identify and assess potential behavioral or emotional problems. The SDQ evaluates 5 domains: emotions, behavior, hyperactivity, relationship problems with peers, and prosocial behavior. These domains are categorized into internalizing and externalizing subscales and a global scale. Each domain score ranges from 0 to 10. The externalizing score (sum of the behavior and hyperactivity) ranges from 0 to 20; the internalizing score (sum of the emotions and peer problem scales) ranges from 0 to 20. The total difficulties score (sum of the externalizing and internalizing subscales) ranges from 0 to 40. Higher scores indicate more behavioral problems. The population was classified

according to the SDQ cut-off points established for the Spanish child and adolescent population into three categories: normal (0-15 points), subclinical (16-18 points) and clinical (19-40 points) [35,36].

Nutritional assessments

To assess eating habits, parents of participants completed a 137-item semi-quantitative Food Frequency Questionnaire (FFQ). This FFQ was originally developed for the European Prospective Investigation into Cancer and Nutrition (EPIC) [37], and adapted and validated for use with children and adolescents [38].

Participants were categorized based on their adherence to the daily intake recommendations of the Spanish Society of Community Nutrition (in Spanish, Sociedad Española de Nutrición comunitaria (SENC)) [39]. This categorization compared the frequency of food consumption reported in the FFQ with the recommendations for children and adolescents outlined in the dietary guidelines of the Spanish Agency for Food Safety and Nutrition[40] Furthermore, the consumption of 15 specific food groups was evaluated quantitatively, assessing the amount consumed in grams per day (g/day). Food groups considered in the study were: fresh fruit and juices, sugar beverages, legumes, nuts and olive oil, vegetables, pastries and sugars, eggs and dairy products, potatoes, processed food, whole grains, refined cereals, fish, red meat, white meat, and processed meat. Food frequency consumption was converted into g/day using national (CESNID and RedBEDCA) [41 42] and international (USDA) food composition tables[43] and considering participant age and portion size, taking as reference the dietary guidelines of the SENC. Following these criteria, the intake of 49 micro- and macronutrients (g/day) was estimated. To account for variations in energy intake, we adjusted consumption (g/day) using Willet's residual method [44].

Dietary data were used to calculate the Dietary Inflammatory Index (DII), as described by Shivappa et al. (2014) and Hébert et al. (2019). Adherence to the Mediterranean Diet (MD) among children and adolescents was assessed using the validated Kidmed questionnaire [47]. Additionally, the quality of diets was evaluated using the Spanish Healthy Eating Index (SHEI)[48]. Higher scores on both the SHEI and MD indices indicate better diet quality and stronger adherence to the recommended dietary guidelines.

We conducted *a posteriori* dietary pattern analysis based on the intake of the 15 observed food groups. To characterize the dietary patterns of participants and stratify them into groups, we employed a statistical approach combining principal component analysis (PCA) with varimax rotation and k-means cluster analysis using IBM SPSS Statistics for Windows (v 27.0).

Gut microbiota analyses

Stool sampling, DNA extraction, sequencing, and data preprocessing

Stool samples were collected by parents and then stored at -80ºC after delivery at the clinic. Metagenomic DNA samples were isolated from approximately 300 mg of feces using the Magpure Stool DNA LQ Kit (Magen Biotech, Shenzhen, China). A next-generation sequencing library was constructed using the MGIEasy Universal DNA Library Prep Set (MGI Tech., Shenzhen, China) and shotgun metagenomics was performed using the MGI DNBSEQ-G50™ sequencing platform at the MGI Tech Co., Ltd manufacturing facility in Riga (Latvia). This generated paired-end short reads with a maximum of 150 base pairs and a sequencing depth of 20 million reads overage. The resulting reads underwent quality control analysis and subsequent trimming using FastQC (v0.12) [49], MultiQC (v1,13)[50] and Trimmomatic (v0.36)[51]. Reads shorter than 75 bases or with a base quality score below 20 were discarded, as well as the sequences of the corresponding adapters.

To remove human host genome sequences, reads were aligned against the human reference genome (GRCh38) using Bowtie2 (v2.4.5) [52]. The unmapped reads, presumed to be of non-human origin, were converted into FASTQ files and submitted to an additional quality control step, with the aforementioned software and read-discarding criteria, to identify and remove potential artifacts.

Taxonomic assignment was performed using Kraken2 (v2.1.2)[53] with a confidence score threshold of 0.1 and the default Standard Kraken2 database. Subsequently, Bracken (v2.6.2) [54] was used to refine the abundance estimates of bacterial taxa. To facilitate downstream analyses, KrakenTools scripts (v1.2) were used to convert the Bracken output into MetaPhlAn-styled files.

The taxonomical reads were normalized using rarefaction and further filtered from other non-human related taxa (for example, eukaryota and

viruses) using the phyloseq R package (v 1.62.2) [55]. Rarefaction depth corresponded to the minimum sample size of the metagenomic dataset.

Metataxonomic microbiota analysis

Microbial diversity was assessed using alpha diversity (within-sample diversity) metrics such as richness (Observed abundance, Chao1) and evenness (Shannon, Inverse Simpson), and beta diversity (between-sample dissimilarity) metrics such as Jaccard, Bray-Curtis, and Weighted Unifrac. A Shapiro-Wilk normality test was applied to the data before analyzing the statistical significance of the differences between the experimental groups in alpha diversity with either a Student T-test for normally distributed data or a Wilcoxon Rank Test for the rest." Beta diversity significant differences were determined using PERMANOVA (Anderson, 2001). The phyloseq package, along with fantaxtic (v2.0.1) and vegan (v2.6-4) [56], was used for data analysis and visualization. A p-value of <0.05 was considered statistically significant in all analyses.

Differentially-abundant species were identified using the DESeq2 (v1.39.8) R package [57]. Only species with a prevalence exceeding 5% in the dataset and a False Discovery Rate (FDR) <0.05, determined by Likelihood Ratio Test significance tests, were considered statistically significant. Log Fold-Change (LogFC) values represent the relative abundance of a taxon between "Healthy" and "Behavioral Problems" groups. Negative LogFC values suggest higher abundance in the "Healthy" group, while positive values suggest higher abundance in the "Behavioral Problems" group. The DESeq2 model was corrected for relevant confounding variables using the "reduce" parameter within the DESeq function. Differentially-abundant taxa values were normalized using the Variance-Stabilizing Transformation.

Data integration and statistical analyses

Exploratory analysis and variable preprocessing

Given the complex interplay of the study variables, first, we conducted exploratory analyses using SPSS (v28.0) and R (v4.3.2) to select the most relevant variables that potentially influence behavioral features and their relationship with gut microbiota and diet. The preliminary results highlighted the influence of age on both diet and microbiota composition

and, accordingly, the population was stratified into two groups: "Childhood" (ages 5-10) and "Adolescence" (ages 11 and older).

Behavioral difficulties were assessed using SDQ scales, resulting in three categories: clinical, subclinical, and normal. Exploratory analysis revealed no substantial differences between clinical and subclinical categories within each age group. Consequently, the population was dichotomized into two groups: "Healthy" individuals (categorized as "Normal" on all SDQ scales) and individuals with "Behavioral Problems" (combining subclinical and clinical categories).

To investigate the impact of microbial and dietary factors on behavior, we used the Global SDQ score as the primary outcome measure. This comprehensive score was selected after examining the three SDQ scales (emotional, conduct, and hyperactivity/inattention), and considering its overall balance and ability to capture a broad range of behavioral issues.

To account for potential confounding influences, we included the following variables: age, sex, z-BMI, stress, physical activity, parental education, parental marital status, bowel habits, and antibiotic intake. In the adolescent group, respiratory problems were added as a confounding variable based on its association with behavioral outcomes in exploratory analyses. To avoid spurious linear relationships in continuous variables such as age and z-BMI, they were stratified into two categorical groups (childhood and adolescents for age and normal/low weight or overweight/obesity for BMI z-scores).

Since most of the analyses performed are related to some degree to the linear or logistic relationship between Global SDQ and other variables, the adherence of the dataset to several statistical assumptions was assessed to ensure the validity of our analyses. First, we confirm the normality of the dataset, through Shapiro-Wilk formal testing. Second, we considered the independence of variable errors, examining autocorrelation through the Durbin-Watson test. Third, for continuous variables, we assumed linearity in their relationship with Global SDQ scores, evaluated through scatter plots. Fourth, we examined the absence of multicollinearity using correlation matrices, as high correlations between variables can lead to unreliable estimates. In cases where multicollinearity was identified, we performed a Variable Importance Analysis to select the most relevant variables for distinguishing between experimental groups. Finally, we identified significant outliers through leverage plots and Cook's distance,

carefully considering their removal to maintain data integrity while preventing undue influence on our results.

Regarding variable selection, comparisons and analysis of associations between the dependent variable and potentially independent variables were performed using parametric tests (Student's t-test) for continuous variables with normal distribution and non-parametric tests (Mann-Whitney U test and Wilcoxon signed-rank test) when distributions did not follow the normality. The Chi-squared test was used to compare and assess categorical variables. We considered statistical significance at a p-value <0.05.

Mediation analysis of diet and microbiota

Mediation analysis was conducted to test whether the relationship between diet and Global SDQ score was potentially mediated by specific gut microbiota species. We considered mediation when an independent variable (Exposure to a dietary component) influences a dependent variable (Outcome, the Global SDQ score) through the action of a third variable (Mediator, a specific bacterial species).

Mediation analysis was conducted following the Baron and Kenny framework [58]. This involved three steps: examining the direct effect of the dietary element on the SDQ score; examining the effect of the dietary element on the abundance of the microbial species; and examining the combined effect of the dietary element and the microbial species on the SDQ score. If all three regressions showed statistically significant relationships, the mediation effect was further examined for causality using the R mediation package (v4.5.0) (Tingley et al., 2014).

The direct effect (ADE) measures the influence of X on Y without going through M, while the Average Casual Mediation Effect (ACME) or indirect effect captures the influence of X on Y through M. Thus, the 'mediate' function estimates the indirect effect of Mediator and Exposure on Response and its p-value after specified bootstrapping repetitions of the model with different combinations of data (in this case, 5000 repetitions). The sum of the direct and indirect effects constitutes the total effect, providing an overall picture of how X affects Y both directly and through the mediator. Finally, a bootstrapping-based resampling method was employed to validate the identified relationships.

Predictive models

To classify individuals into Healthy and Behavioral Problem groups, we trained a series of Penalized Logistic Regression models. These models were built using a combined dataset of normalized, scaled, and trimmed microbial and dietary information (including nutrients, food groups, dietary patterns, and dietary indices). Penalization in these models reduces the influence of variables that are highly correlated with others or have little predictive power, based on predefined parameters.

We employed the Elastic Regression Technique [59] as our Penalization Model, implemented using the caret R package (v6.0-94) [60]. To obtain p-values and confidence intervals for predictor importance within this penalized model, we translated the models to the glmnet package (v4.1-8) [61] and utilized the hdi package (v0.1-9) [62] for analysis.

To enhance the performance of the Penalized Logistic Regression Models, we reduce the number of variables, first, by removing thoses exhibiting high levels of collinearity between microbial species and dietary elements and, second, by assessing variable importance for feature selections. For the last case, this involved incrementally adding features to a simplified logistic regression model and evaluating their contribution as classifiers using *a posteriori* Student's t-test. For each age group (children and adolescents), seven models were developed using the following variables: Differentially Abundant Species after feature selection, All Dietary Data Combined after adjusting for correlated variables, Differentially Abundant Species and All Dietary Data Combined, Differentially Abundant Species and Nutrients, Differentially Abundant Species and Food Groups, Differentially Abundant Species and Dietary Indices, and Differentially Abundant Species and Dietary Patterns.

To address the class imbalance between Healthy and Behavioral Problem groups of adolescents, we employed a down-sampling technique using the caret package in R. This involved reducing the number of samples from the overrepresented class. To prevent overfitting, we tested multiple down-sampled datasets with varying sample combinations. Furthermore, we implemented a 70/30 train-test split to partition the data for model training and evaluation. To further mitigate overfitting, three different cross-validation methods (5-fold cross-validation, 10-fold cross-validation, and "Leave One Out" cross-validation) were tested, enabling us to choose the most optimized model.

To evaluate the performance of our models, we utilized Receiving Operator Characteristic (ROC) curve analysis, which graphically illustrates the trade-

off between the model's sensitivity (correctly identifying true positives) and its specificity (correctly identifying true negatives). The Area Under the Curve (AUC) quantified the overall predictive power of the model. A higher AUC indicates superior model performance in distinguishing subjects with high Global SDQ scores from those with low scores.

3. RESULTS

Characteristics of children and adolescent groups

The study included 335 participants: 202 (61%) children from 5 to 10 years old and 133 (39%) adolescents from 11 to 17 years old. Participants were categorized as having "Behavioral Problems" or as "Healthy" based on cut-off values from the Global SDQ specific to each age group. This approach allowed for the identification of individuals with sub-clinical or clinical behavioral issues. Of the children, 88 (44%) exhibited behavioral problems, while 114 (56%) belonged to the healthy group. Among adolescents, 46 (36.4%) displayed behavioral problems and 87 (64.6%) were healthy participants. A small subset of participants (N=14) were diagnosed with neurodevelopmental disorders (e.g., autism, ADHD). Due to the limited sample size, no significant differences were found in analyses involving this group. Consequently, these individuals were included in the overall "behavioral problems" category for the main analyses.

When comparing the features of the categorized population (**Table 1**) we observed significant differences across several health indicators, including sleep disorders, gastrointestinal disorders and the DII, which all were higher in the behavioral problems group than in the healthy group. Further analysis by age group demonstrated that both children and adolescents with behavioral problems experienced elevated levels of sleep disorders and stress. However, increased respiratory disorders were only increased in adolescents with behavioral problems.

Behavioral problems are associated with perceived stress and sleep disorders

To investigate potential factors contributing to behavioral problems, we examined possible associations with sociodemographic, pregnancy-related, anthropometric, clinical, and lifestyle characteristics (**Table 2**). We found a strong association between daily stress and increased behavioral problems across both age groups, and adolescents. Sleep disorders were associated with behavioral problems in the overall population, while respiratory disease was associated with these problems specifically in the adolescent population. No other assessed confounders had a significant

effect. Since sleep disorders were not statistically associated with behavioral problems in the different age groups, it was not included as a confounder in subsequent analyses. By contrast, respiratory problems were deemed a potential confounder for adolescents, given their significant association with behavioral problems in this population

Alpha diversity, but not beta diversity, differs in subjects with behavioral problems compared to healthy individuals.

We investigated potential associations between gut microbiota differences and behavioral problems separately in the two age groups, given that dietary intakes, which significantly influence the gut microbiota, varied substantially between children and adolescents

Regarding alpha diversity, we observed increased alpha diversity, as measured by the inverse Simpson index, associated with behavioral problems in both age groups after pair comparisons adjusted by confounders (**Figures 2A and 2B)**. A similar significant trend was observed for the inverse of the Simpson index using regression analysis, after adjusting for confounders, in both children and adolescents (**Supplementary Table 1**). Conversely, we found no associations between beta diversity and global behavioral problems (**Figure 2A and 2B)**.

Table 1. Characteristics of the study population categorized by age and behavior

Age group	Overall (N=335)				p	Children (5–10 years) N= 202 (61%)				p	Adolescents (11–17 years) N=133 (39%)				p
Sociodemographic charasteristics*	*Healthy*		*Behavioral problems*			*Healthy*		*Behavioral problems*			*Healthy*		*Behavioral problems*		
	M/N	±SD/%	M/N	±SD/%		M/N	±SD/%	M/N	±SD/%		M/N	±SD/%	M/N	±SD/%	
Age mean ± SD	9.2	2.9	10.1	3.1	0.165	7.6	1.6	7.7	1.6	0.6	12.9	1.7	12.7	1.8	0.40
Sex															
Male N (%)	88	43.8	73	54.5	0.06	48	42.1	46	52.3	0.158	40	46.0	27	58.7	0.203
Female N (%)	113	56.2	61	44.6		66	57.9	42	47.7		47	54.0	19	41.3	
Parent education															
Below university N (%)	129	64.2	77	57.5	0.22	71	62.3	51	58.0	0.6	58	66.7	26	56.5	0.26
University degree N (%)	72	35.8	57	42.5		43	37.7	37	42.0		29	33.3	20	43.5	
Marital status															
Separated/single parent N (%)	69	34.3	36	26.9	0.19	39	34.2	23	26.1	0.22	30	34.5	13	28.3	0.56
Married or couple N (%)	132	65.7	98	73.1		75	65.8	65	73.9		57	65.5	33	71.7	
Delivery mode															
Vaginal N (%)	193	85.4	147	87.5	0.55	111	86	99	89.2	0.5	87	88.0	43	77.0	0.06
C-section N (%)	33	14.6	21	12.5		18	14	12	10.8		11	12.0	13	23.0	
Exclusive breastfeeding															
Formula or mixed N (%)	83	41.3	50	37.3	0.50	43	37.7	33	37.5	1.00	40	46.0	17	37.0	0.36
Maternal N (%)	118	58.7	84	62.7		71	62.3	55	62.5		47	54.0	29	63.0	
Bowel movements (per day)															
Not optimal < or > 3 N (%)	40	20.0	35	26.1	0.2	94	85.5	64	72.7	0.12	67	77.0	35	76.1	1.00
Optimal (1-3 bowels) N (%)	161	80.1	99	73.6		20	17.5	24	27.3		20	23.0	11	23.9	
Intestinal transit (Bristol scale)															
Diarrhea or constipation, N (%)	178	88.6	109	81.3	0.08	98	86	68	77.3	0.14	80	92.0	41.	89.1	0.75
Optimal transit, N (%)	23	11.4	25	18.7		16	14	20	22.7		7	8.0	5	10.9	

Self-reported sleep alteration															
Yes N (%)	13	7	28	21.0	**0.01**	8	7	18	20.5	**0.01**	5	5.7	10	21.7	**0.01**
No N (%)	188	94	106	79.1		106	93	70	79.5		82	94.3	36	78.3	
Respiratory problems															
Yes N (%)	80	45.6	105	57.7	0.19	62	70.5	87	76.3	0.35	18	20.7	18	39.1	**0.02**
No N (%)	96	54.4	54	42.3		27	23.7	26	29.5		69	79.3	28	61.0	
Gastrointestinal disorders															
Yes N (%)	19	10	25	18.7	**0.01**	13	11.4	18	20.5	0.11	6	6.9	7	15.2	0.14
No N (%)	182	91	109	81.3		101	88.6	70	79.5		81	93.1	3	84.8	
Antibiotics															
Yes N (%)	16	8	17	12.7	0.16	10	8.8	14	15.9	0.13	6	6.9	3	6.5	1.00
No N (%)	185	92.0	117	87		104	91.2	74	84.1		81	93.1	43	93.5	
Non-antibiotic drugs															
Yes N (%)	53	26	42	31.3	0.39	27	23.7	29	33.0	0.16	26	29.9	13	28.3	1.00
No N (%)	148	74	92	68.7		87	76.3	59	67.0		61	70.1	33	71.7	
ɣBody mass Index z-score (z-BMI) kg/m²															
z-BMI mean ± SD	17.8	0.3	18.0	0.3	0.99	16.9	3.9	16.9	3.0	0.9	19.1	3.6	20.1	3.9	0.9
Overweight/obesity N (%)	49	24.1	36	26.9	0.35	35	30.7	25	28.4	0.8	73	83.9	35	76.1	0.4
Normal or underweight N (%)	152	75.6	98	73.1		79	69.3	63	71.6		14	16.1	11	23.9	
Waist circumference (WC)(cm)															
WC mean ± SD	60.3	1.2	61.3	1.2	0.79	54.74	17.8	58.0	11.2	0.14	67.6	14.5	67.7	14.7	0.1
Obesity risk N (%)	44	22	34	25	0.51	29	25.4	23	26.1	1.0	15	17.2	11	23.9	0.4
No obesity risk N (%)	157	78	100	75		85	74.6	65	73.9		72	82.8	35	76.1	
Inventory of daily stressors perceived by parents (total stress)															
Stress N (%)	49	24	44	33.1	0.08	13	11.4	36	40.9	<0.0	17	19.5	27	28.7	**<0.001**
No stress N (%)	153	75.7	89	66.9		101	88.6	52	59.1	01	7	80.5	19	41.0	

Physical activity (APALQ)															
mean + SD	12.6	0.2	12.5	0.3	0.87	12.28	2.7	12.1	2.7	0.60	13	3.5	13.3	3.6	0.60
Sedentary N (%)	55.0	27.4	38.0	28.4	0.90	35	30.7	24	27.3	0.64	20	23	14	30.4	0.41
Moderately active or active	146.0	72.6	96.0	71.6		79	56.4	64	43.6		67	77	32	69.6	
Healthy lifestyle (HLS)															
mean + SD	5.9	0.1	5.7	0.2	0.2	5.9	1.7	5.7	1.7	0.42	5.9	1.6	5.6	1.8	0.42
Low or middle	123.0	61.2	91.0	67.2	0.09	70	61.4	63	71.6	0.2	53.0	60.9	27.0	58.7	0.12
High	78.0	38.8	44.0	32.8		44	38.6	25	28.4		34.0	39.1	19.0	41.3	
Daily use of screen-based activities (h/day)															
mean + SD	2.2	0.2	2.1	0.1	0.2	1.8	2.8	1.7	0.9	0.92	2.8	2.0	2.9	1.77	0.81
Daily time practice of team sports (h/day)															
mean + SD	4.53	0.3	4.13	0.3	0.4	4.5	4.1	3.7	3.1	0.2	4.6	2.9	5.0	2.99	0.2
Daily time practice of outdoor activities (h/day)															
mean + SD	2.4	0.2	2.1	0.1	0.70	2.40	2.0	2.3	1.7	0.9	2.4	3.2	1.7	1.18	0.9
Spanish healthy eating index (SHEI) mean + SD	76.8	9.1	75.9	8.6	0.7	78.1	8.4	76.0	7.1	0.1	75.1	9.7	75.9	10.6	0.1
Low or Need for improvement	119	59.2	87	64.9	0.3	64	56.1	59	67.0	0.1	55	63.2	28	61.0	0.9
Optimal N (%)	82	41.0	47	35.1		50	43.9	29	33.0		32	36.8	18	39.1	
Adherence Mediterranean diet (MD)															
mean + SD	7.0	2.4	6.8	2.3	0.5	7.3	2.2	6.7	2.2	0.10	6.7	2.6	6.5	2.5	0.2
Low or needs for improvement	117	58	81	60	0.68	66	57.9	51	58.0	1.00	51	58.6	30	65.2	0.58
Optimal,N (%)	84	42	53	40		48	42.1	37	42.0		36	41.4	16	34.8	
Diet Inflammatory Index score (DII)															
mean + SD	0.1	1.6	0.4	1.8	**0.04**	0.1	1.5	0.5	1.7	0.1	0.0	1.7	0.4	1.9	0.1
Pro-Inflammatory N (%)	38	18.9	37	27.6		21	18.4	26	29.5		17	19.5	11	23.9	
Neutral, N (%)	107	53.2	63	47.0	0.2	64	56.1	40	45.5	0.1	43	49.4	23	50.0	0.8
Anti-Inflammatory N (%)	56	27.9	34	25.4		29	25.4	22	25.0		27	31.0	12	26.1	

Considering as “Healthy” the subjects without behavioral problems measured by the SDQ.*Data are expressed as mean (SD) or percentage (N). Statistical tests: Mann-Whitney U, Kruskal-Wallis, and Student`s t-test were used for quantitative variables and Chi-squared for qualitative variables. Significant differences are in bold ($p<0.05$). ɣBMI z-scores were used to classify participants with obesity/overweight and normal weight/underweight following WHO international cut-offs.

Table 2. Associations between behavioral problems and other personal features and lifestyle factors

Overall population	Exp(β) (95% CI)	p-value	q-value
Daily perceived stressors	1.682 (0.96, 2.4)	<0.001	<0.001
Sleep disorders	3.39 (1.58, 7.30)	0.01	0.02
Childhood population	Exp(β) (95% CI)	p-value	q-value
Daily stressors perceived by parents	1.682 (0.96, 2.4)	<0.001	<0.001
Adolescent population	Exp(β) (95% CI)	p-value	q-value
Respiratory problems	2.7 1.14, 6.6)	0.03	0.04
Daily perceived stressors	1.76 (0.97, 2.56)	0.01	0.01

Exp (β) (95% CI) = Exponential of the coefficient β, representing an odds ratio. P = Not adjusted for covariables (p<0.05); Padj (q) = Model adjusted by age, sex, z-BMI, parent education and medication intake (P<0.05). Only variables associated with behavioral problems are shown.

Gut bacterial species differ in subjects with behavioral problems

Analysis of the gut microbiome revealed significant differences in the abundance of 19 bacterial species between children with behavioral problems and healthy children. Specifically, children with behavioral problems had a higher abundance of *Enterococcus durians, Campylobacter coli,* Lactobacillus *sakei*, and *Bifidobacterium pullorum*, while healthy children had higher levels of *Clostridium autoethanogenum*, *Acidaminococcus* spp., *Bacteroides* spp., *Coprococcus eutactus, Butyrivibrio fibrisolvens* and *Eubacterium* sp. MSJ 33, among other species (see Table 3 and Figure 2C).

Table 3. Differential abundance of bacterial species in children with behavioral problems

Bacterial species	**Log2FC***	**P**	**q***
Bacteroides nordii	-1.12	2.12E+02	1.38E+04
Bacteroides sp. M10	-1.33	5.82E-01	1.26E+02
Bacteroides thetaiotaomicron	-0.86	5.67E+02	2.80E+04
Campylobacter coli	0.61	1.26E+03	4.97E+04

Klebsiella pneumoniae	-2.39	2.49E+02	1.47E+04
Escherichia coli	-1.10	6.49E+02	2.81E+04
Berryella intestinalis	-1.17	3.73E+01	3.03E+03
Bifidobacterium pullorum	1.18	6.03E+02	2.80E+04
Megasphaera elsdenii	-1.83	1.88E+01	2.03E+03
Acidaminococcus fermentans	-1.95	9.47E-01	1.54E+02
Acidaminococcus intestini	-2.85	1.45E+03	4.97E+04
Enterococcus durans	3.35	3.24E+01	3.01E+03
Latilactobacillus sakei	2.71	4.35E-01	1.26E+02
Eubacterium sp. MSJ-33	-0.68	1.35E+03	4.97E+04
Intestinimonas butyriciproducens	-0.53	1.39E+03	4.97E+04
Clostridium autoethanogenum	-25.58	1.85E-12	1.20E-09
Oscillibacter sp. NSJ-62	-0.59	1.51E+02	1.09E+04
Butyrivibrio fibrisolvens	-0.76	9.68E+00	1.26E+03
Coprococcus eutactus	-0.91	4.88E+02	2.64E+04

Species associated with behavioral problems. *Note: Log2FC = Log2 fold-change representing the relative abundance difference in the behavioral problems group compared with the healthy group; positive values indicate greater abundance in the behavioral problems group and negative values indicate greater abundance in the healthy group. $p<0.05$. q is FDR-adjusted for age, sex, z-BMI, stress, antibiotics, and other medications.

Regarding adolescents, significant differences were found in the abundance of 22 bacterial species between adolescents with behavioral problems and healthy adolescents. Specifically, the gut microbiota of the behavioral problem group was enriched in taxa such as *Pyramidobacter psicolens*, *Parabacteroides johnsii*, *Lautropia mirabilis, Megasphaera elsdenii, Ruminococcus champanellensis*, *Parvimonas micra* and *Anaerostipes rhamnosivorans,* while *Muribaculum gordoncarteri*, *Butyricimonas* spp., *Phocaeicola coprophilus*, and *Bifidobacterium* spp., were more abundant in the healthy group (See **Table 4** and **Figure 2D**).

Table 4. Differential abundance of bacterial species in adolescents with behavioral problems

Bacterial species	Log2FC*	P	q*
Pyramidobacter piscolens	1.21	1.86E-03	4.26E-02
Treponema phagedenis	1.00	3.88E-04	2.04E-02
Muribaculum gordoncarteri	-1.80	2.62E-04	1.02E-02
Butyricimonas faecalis	-1.07	6.58E-04	1.95E-02
Butyricimonas virosa	-1.51	1.09E-03	4.46E-02
Parabacteroides johnsonii	1.54	7.12E-04	1.99E-02
Phocaeicola coprophilus	-2.26	1.53E-04	1.15E-02
Lautropia mirabilis	1.59	4.00E-04	2.04E-02
Parafannyhessea umbonata	-1.05	6.25E-04	1.95E-02
Thermophilibacter immobilis	-1.55	8.86E-04	4.02E-02
Parolsenella catena	-1.04	5.81E-05	5.94E-03
Bifidobacterium pseudolongum	-0.20	1.12E-03	2.92E-02
Bifidobacterium pullorum	-1.61	1.65E-03	4.95E-02
Bifidobacterium angulatum	-0.12	1.51E-03	3.62E-02
Megasphaera elsdenii	2.61	1.69E-04	1.15E-02
Parvimonas micra	1.57	3.08E+09	1.94E-03
Granulicatella elegans	1.40	1.26E-04	6.32E-03
Lactococcus garvieae	-0.23	2.31E+07	5.81E+09
Bulleidia sp, zg-1006	-1.50	3.27E-04	1.10E-02
Monoglobus pectinilyticus	1.53	1.59E-03	4.95E-02
Ruminococcus champanellensis	1.64	2.36E-06	4.82E-04
Anaerostipes rhamnosivorans	1.08	1.54E-05	2.10E-03

Species associated with behavioral problems. *Note: Log2FC = Log2 fold-change representing the relative abundance difference in the behavioral problems group compared with the healthy group; positive values indicate greater abundance in the behavioral problems group and negative values indicate greater abundance in the

healthy group. p<0.05. q is FDR-adjusted for age, sex, z-BMI, stress, respiratory disorders, antibiotics and other medications.

Behavioral problems are consistently associated with a Western dietary pattern and deficits in the intake of related nutrients and food groups

We first assessed adherence to SENC dietary guidelines and investigated its relationship with behavioral problems. The majority of children in our study adhered to the SENC recommendations regarding macronutrient and micronutrient intake. Notably, however, children with behavioral problems exhibited significantly higher energy and cholesterol intake and a lower intake of linoleic acid. By contrast, healthy children exhibited a lower intake of iodine. No differences related to nutrient intake were found in the adolescent population. Analysis of food group intakes revealed that children with behavioral problems did not adhere to dietary recommendations, as they consumed significantly higher amounts of processed food, milk and dairy products, and processed meat. Adolescents with behavioral problems also exhibited dietary non-compliance. They consumed significantly lower amounts of fruits and vegetables, and significantly higher amounts of fish than recommended (**Table 5**).

We also analyzed differences in nutrient and food group intakes between subjects with behavioral problems and healthy adjusted by energy consumption (**Supplementary Tables 2 and 3)**. We found that children with behavioral problems had a lower intake of total fiber, soluble fiber, insoluble fiber, and vitamin B9, and tended to have a high intake of fructose (**Supplementary Table 2**). Regarding food group intakes, we found differences in fresh fruit intake, which was lower in children with behavioral problems (**Supplementary Table 3**). In the adolescent group, we observed a higher intake of docosahexaenoic acid (DHA) in the behavioral problems group (**Supplementary Table 2**). In addition, adolescents with behavioral problems had a higher intake of fish, but a lower intake of legumes, nuts and oil olive, and vegetables (**Supplementary Table 3)**.

Figure 2. Metataxonomical analysis of the gut microbiome in children and adolescents. (A) Alpha diversity indices (Observed abundance, Chao1, Shannon and Inverse Simpson) in children and their statistical significance based on the Wilcoxon test ($p<0.05$) corrected for confounders. (B) Alpha diversity indices (Observed abundance, Chao1, Shannon and Inverse Simpson) in adolescents and their statistical significance based on the Wilcoxon test ($p<0.05$) corrected for confounders. (C) Differentially abundant bacteria between "Healthy" and "Behavioral Problems" groups in children. (D) Differentially abundant bacteria between "Healthy" and "Behavioral Problems" groups in adolescent

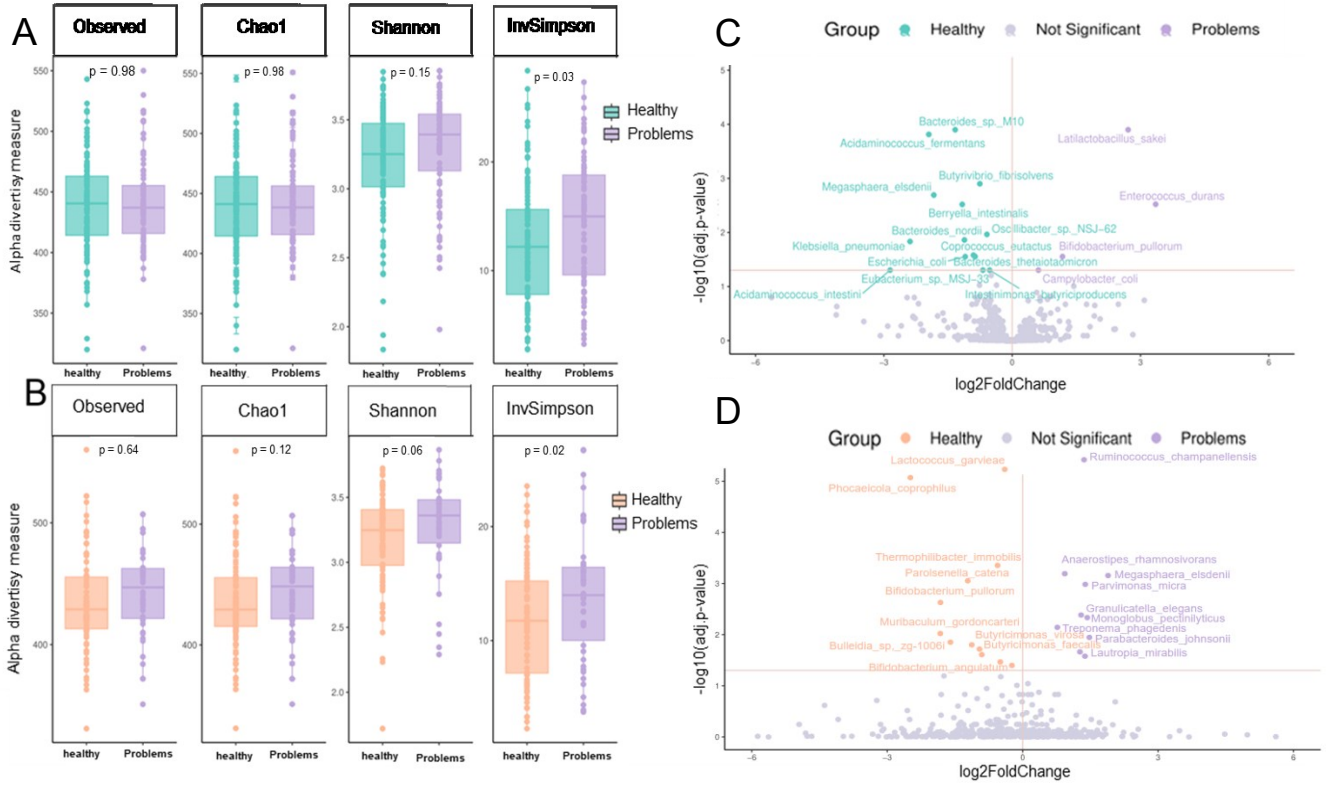


Table 5. Percentage of food group consumption (servings per day/week) and nutrients (g/day) according to participants' compliance with the recommended dietary intakes (RDI) established by dietary guidelines

Nutrients and food groups	Children (5–10 years) N=202 (61%)						p	Adolescents (11–17 years) N=133 (39%)						p
	Healthy			Behavioral problems				Healthy			Behavioral problems			
	Comply RDI *	Over RDI*	Below RDI*	Comply RDI	Over RDI	Below RDI		Comply RDI	Over RDI	Below RDI	Comply RDI	Over RDI	Below RDI	
Energy (kcal)	48.2	5.3	46.5	58	16	26.1	**0.00**	40.2	11.5	48.3	45.7	17.4	37	0.40
Protein (g)	63.2	36.8	0	64.8	35.2	0	0.96	55.2	44.8	0	67.4	32.6	0	0.39
Total fiber (g)	73.7	NA	49.1	71.6	NA	28.4	0.75	83.9	NA	16.1	80.4	NA	19.6	0.64
Fats (g)	50.9	49.1	0	61.4	38.6	0	0.16	57.5	42.5	0	67.4	32.6	0	0.35
Linolenic acid (g)	40	NA	60	55.3	NA	44.7	0.05	57.5	NA	42.5	58.7	NA	41.3	1.00
Linoleic acid (g)	58	NA	42	42	NA	58	**0.03**	73.6	NA	26.4	84.8	NA	15.2	0.14
DHA+EPA (mg)	74.6	NA	25.4	70.5	NA	29.5	0.53	85.1	NA	14.9	91.3	NA	8.7	0.42
SFA (g)	23.7	76.3	NA	23.9	76.1	NA	1.00	25.3	74.7	NA	28.3	71.7	NA	0.84
PUFAS (g)	14.9	NA	85.1	18.2	NA	81.8	0.57	78	NA	21.8	84.8	NA	15.2	0.36
MUFAS (g)	56.1	NA	44	51.1	NA	48.9	0.57	66.7	NA	33.3	56.5	NA	43.5	0.26
Cholesterol (mg)	63.2	36.8	NA	38.6	61.4	NA	**<0.00**	37.9	62.1	NA	28.3	71.7	NA	0.40
Iodine (µg)	34.2	NA	65.8	50	NA	50	**0.02**	58.6	NA	41	76.1	NA	24	0.06

Sodium (mg)	62.3	37.7	NA	48.9	51.1	NA	0.06	23	77	NA	11	89.1	NA	0.11
potassium (mg)	84.2	NA	15.8	92	NA	8	0.13	79.3	NA	20.7	84.8	NA	15.2	0.49
Calcium(mg)	36.8	NA	63.2	50	NA	50	0.06	35.6	NA	64.4	32.6	NA	67.4	0.85
Total folate (B9) (µg)	75.4	NA	24.6	78.4	NA	21.6	0.74	74.7	NA	25.3	69.6	NA	30.4	0.54
Vitamin A (µg)	81.6	NA	18.4	85.2	NA	14.8	0.57	85.1	NA	15	91.3	NA	8.7	0.42
Vitamin D (µg)	13.2	NA	86.8	19.3	NA	80.7	0.25	23	NA	77	30.4	NA	69.6	0.41
Vitamin E (mg)	71.1	NA	28.9	78.4	NA	21.6	0.26	65.5	NA	34.5	50	NA	50	0.10
Total polyphenols (mg)	60.5	NA	39.5	69.3	NA	30.7	0.24	73.6	NA	26.4	71.7	NA	28.3	0.84
Sugar Beverage (ser/day)	57	43	NA	48.9	51.1	NA	0.25	51.7	48.3	NA	56.5	43.5	NA	0.60
Fruit and vegetables (ser/day)	46.5	NA	53.5	44.3	NA	55.7	0.86	58.6	NA	41.4	37	NA	63	**0.02**
Legumes (ser/day)	58.8	15.8	25.4	51.1	20.5	28.4	0.52	47.1	31	21.8	43.5	43.5	13	0.27
Nuts and oil olive (ser/day)	65.8	NA	34.2	73.9	NA	26.1	0.22	62.1	NA	37.9	67.4	NA	32.6	0.53
Processed food (ser/day)	23.7	76.3	NA	11.4	88.6	NA	**0.03**	25.3	74.7	NA	19.6	80.6	NA	0.46
Pastries (ser/day)	10	90	NA	5.7	94.3	NA	0.30	13.8	86.2	NA	6.5	93.5	NA	0.21
Milk and diary products	94.7	5.3	0	85.2	14.8	0	**0.02**	90	10	0	89	11	0	0.76

(ser/day)													
Refined cereals (ser/day)	22	78	NA	19.3	80.7	NA	0.65	24.1	75.9	NA	26.1	73.9	NA	0.80
Whole grain cereals (ser/day)	2	NA	98	3.4	NA	96.4	0.45	4.6		95.4	6.5		93.5	0.64
Fish(ser/day)	39.5	41.2	19.3	26.1	52.3	21.6	0.13	24.1	59.8	16.1	32.6	34.8	32.6	**0.02**
Redmeat (ser/day)	26.3	73.7	NA	17	83	NA	0.12	17.2	82.8	NA	13	87	NA	0.53
White meat (ser/day)	93.9	6.1	NA	90.1	9.1	NA	0.43	94.3	5.7	NA	89.1	10.9	NA	0.29
Processed meat (ser/day)	15.8	84.2	NA	4.5	95.5	NA	**0.01**	3.4	96.6	NA	6.5	93.5	NA	0.42

Food group recommendations are expressed per serving and per day (ser/day) or per week (ser/wk). Nutrient recommendations are expressed per gram, milligram or microgram per day (g, mg or µg). * RDI: Recommendation dietary intakes. RDIs are expressed in percentage (%) of participants who comply or do not comply by over intake or below intake recommendation. Statistical differences were calculated using the Chi-squared test. Differences are shown in bold ($p<0.05$).

To identify data-driven dietary patterns, we conducted a PCA using 15 food groups as input variables, separately for children and adolescents. The factorial loading values for each generated dietary pattern are shown in **Supplementary Table 4**. Only components with eigenvalues greater than or equal to 0.4 were considered as those that substantially contribute to that specific dietary pattern.

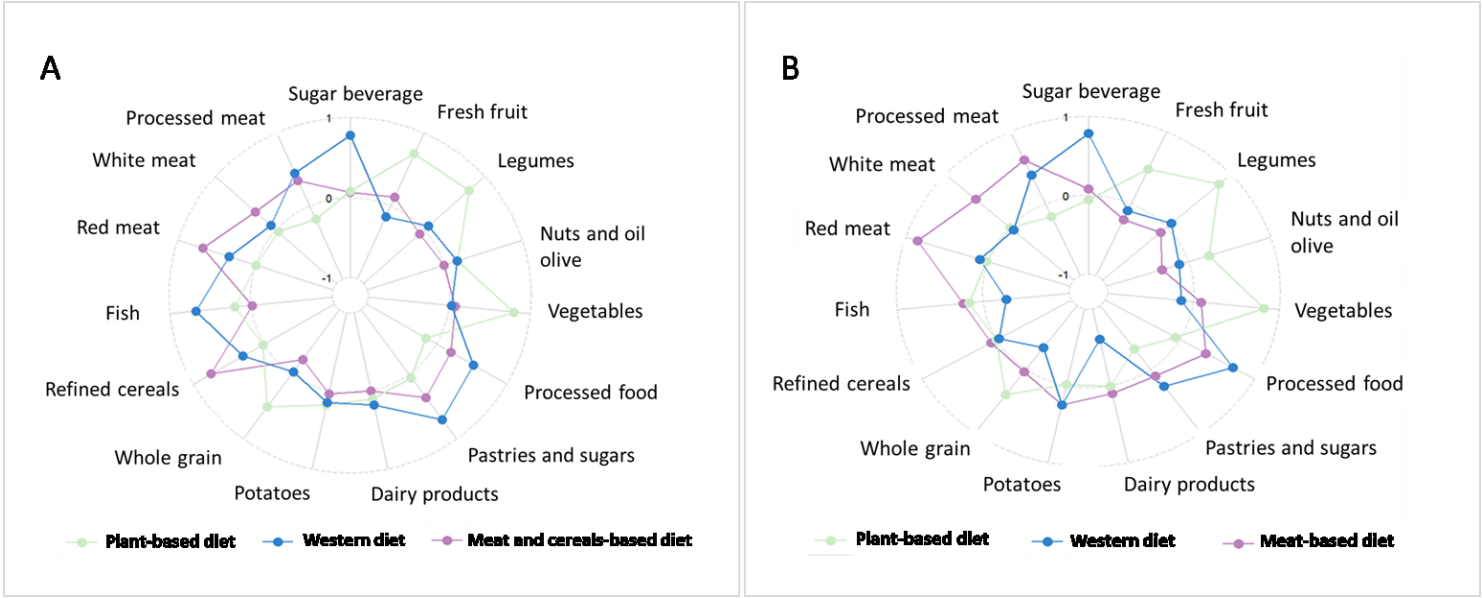
Three distinct dietary patterns emerged, exhibiting similar trends for both age groups: a “Plant-based diet”, rich in fruits, legumes, vegetables, and whole grains; a "Western diet”, rich in pastries, sugars, sugar beverages, and processed foods (and fish in adolescents); and a “Meat and Cereals based diet” for children or a “Meat-based diet” for adolescents (**Figure 3**). Adherence to the Western dietary pattern was significantly higher in children with behavioral problems than in healthy peers. Although not significant, a similar trend was observed in adolescents. No significant differences were found for the other two dietary patterns in either age group **(Table 6)**.

Table 6. Differences in dietary pattern adherence between children and adolescents with and without behavioral problems

Dietary patterns	Children (5–10 years) N= 202 (61%)					Adolescents (11–17 years) N=133 (39%)				
	Healthy		Behavioral problems		p	Healthy		Behavioral problems		p
	N	(%)	N	(%)		N	(%)	N	(%)	
Plant-based dietary pattern										
Low adherence	38.0	33.3	29.0	33.0		25.0	28.7	18.0	39.1	
Medium	38.0	33.3	30.0	34.1	1.0	33.0	37.9	13.0	28.3	0.40
High adherence	38.0	33.3	29.0	33.0		29.0	33.3	15.0	32.6	
Western dietary pattern										
Low adherence	47.0	41.0	20.0	22.5		35.0	40.2	10.0	21.7	
Medium	35.0	31.0	33.0	37.5	**0.02**	28.0	32.2	16.0	34.8	0.07
High adherence	32.0	28.0	35.0	40.0		24.0	27.6	20.0	43.5	
Meat dietary pattern										
Low adherence	44.0	38.6	23.0	26.1		30.0	34.5	14.0	30.4	
Medium	38.0	33.3	42.0	47.7	0.08	34.0	39.1	12.0	26.1	0.1
High adherence	32.0	28.1	23.0	26.1		23.0	26.4	20.0	43.5	

Values are presented as number (N) and percentage (%). “Healthy” refers to children/adolescents without behavioral problems, and “Behavioral problems” refers to children/adolescents with reported behavioral issues. p = p-value from statistical comparison across dietary patterns adherence levels. Differences are shown in bold ($p<0.05$)

Figure 3. **Dietary Patterns in children and adolescents.** Radar chart showing dietary patterns extracted from principal component analysis of 15 food groups in (**A**) children and (**B**) adolescents.



We used logistic regression models to investigate the association between behavioral problems and various dietary factors, including 15 food groups, 49 nutrients, several dietary indices (such as the SHEI and the DII), and adherence to the three distinct dietary patterns. In both populations, we found that behavioral problems were associated with adherence to the Western dietary pattern, even after adjusting for confounders. Also, a lower consumption of soluble fiber and vitamin B9 was associated with behavioral problems in children, and a lower consumption of legumes and nuts and olive oil was associated with behavioral problems in adolescents after adjusting for potential confounders. Finally, an increased intake of fish and DHA was associated with behavioral problems in adolescents (**Table 7**).

Table 7. Associations of nutrients, food groups and dietary patterns with behavioral problems

Childhood population	**Exp(β) (95% CI)**	**p**	**padj**
Soluble fiber(g)	0.33 (0.10, 0.61)	0.01	0.02
Vitamin B9 or folate(µg)	0.28 (0.09, 0.82)	0.02	0.07**
Western dietary pattern	1.57 (1.15, 2.10)	0.002	0.02
Adolescents population	**Exp(β) (95% CI)**	**p**	**padj**
Docohexaenoic acid (DHA) (mg)	2.34 (1.09, 4.74)	0.03	0.02
Legumes (g)	0.52 (0.30, 0.95)	0.04	0.04
Nuts and oil olive (g)	0.67 (0.47, 0.74)	0.03	0.01
Fish (g)	2.27 (1.16, 4.42)	0.02	0.01
Western dietary pattern	1.51 (1.15, 2.09)	0.04	0.04

Exp(β) (95% CI) Exponential of the coefficient B. It represents odds ratio. p: Model not adjusted by covariables. padj: Model adjusted by age, sex, z-BMI, parent education, bowels (Bristol score), APALQ, stress level, and medication and antibiotics intake. Only nutritional variables showing significant differences are included ($p<0.05$). Note ** ($p <0.1$)

Diet and behavioral associations are mediated by specific bacterial taxa

Analysis of the gut microbiotaas a mediator of the diet-behavior relationship revealed significant effects only in adolescents. Specifically,

after adjusting for confounders, the association between a Western diet and behavioral problems was significantly mediated by the abundance of *Lautropia mirabilis* (see indirect effect, Figure 4A), while the association between fish intake and behavioral problems was significantly mediated by the abundance of *Anaerostipes rhamnosivorans* (see indirect effect, Figure 4B). No mediating effects of the gut microbiome were found to explain the association between behavioral problems and the inverse Simpson diversity index in either children or adolescents. Furthermore, no significant mediating effects were observed for other tested gut microbiota or dietary variables.

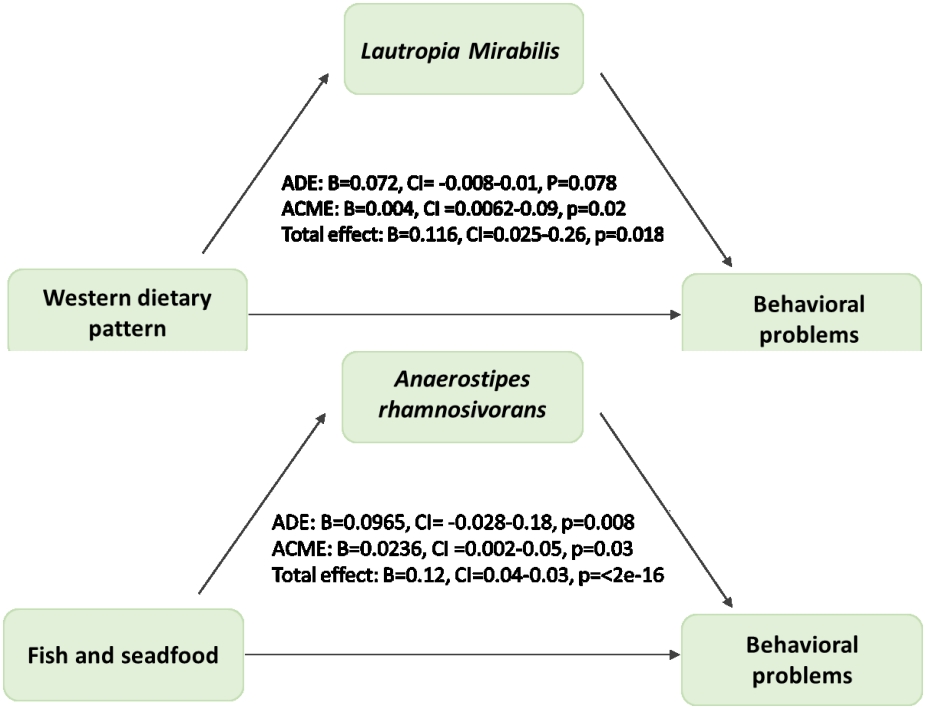


Figure 4. Mediation analysis in adolescents. (A) The mediating effect of Lautropia mirabilis on the relationship between adherence to a Western diet and behavioral problems, considering both the direct effect (ADE) of the Western diet and its interaction with the indirect effect (ACME) mediated through Lautropia mirabilis. (B) The mediating effect of Anaerostipes rhamnosivorans on the relationship between fish consumption and behavioral problems, considering both the direct effect (ADE) of fish consumption and its interaction with the indirect effect (ACME) mediated through Anaerostipes rhamnosivorans

Predictive models of behavior integrating dietary and microbiome data

To predict behavioral outcomes, we developed models integrating dietary and microbiota data. For each subject group (behaviorally healthy or with problems), we selected four bacterial species using logistic regression and variable importance based on Student's t-tests applied to the lists of the differentially abundant bacteria. These included the following: *Campylobacter coli, Bifidobacterium pullorum, Latilactobacillus sakei, Intestinimonas butyriciproducens,* and *Butyrivibrio fibrisolvens* for children; and *Butyricimonas virosa, Parolsenella catena, Ruminococcus champanellensis,* and *Anaerostipes rhamnosivorans* (also identified as relevant in the mediation analysis), for adolescents. Among these, *B. fibrisolvens* and *C. coli* were the most important predictors in the children group while *P.catena* and *R. champanellensi*s were the most important in the adolescents group. Relevant contributions of the remaining variables for each one of the models are shown in Supplementary Table 5 for children and 11 for adolescents.

Regarding the selection of dietary factors, in children, the most discriminant dietary factors were fructose and vitamin B9 at the nutrient level, and pastries, processed food, white meat, and processed meat at the food group level (Supplementary Tables 7 and 8). In adolescents, fiber (both total and insoluble), DHA, and eicosapentaenoic acid (EPA) were identified as highly discriminative. These findings partly support the results found for food groups, where legumes and fish (sources of fiber and DHA, respectively) were the more relevant items associated with behavioral problems (Supplementary Tables 13 and 14).

A total of fourteen models (seven for each age group) were trained using a different combination of bacterial and dietary data in each to assess their potential as classifiers (Figure 4). Models utilizing full dietary information alone demonstrated the lowest predictive performance (Figure 5A, B), with AUCs of 48% in both children and adolescents, aligning with the estimated p-values associated with the dietary components. By contrast, models using differentially abundant bacteria alone exhibited higher predictive performance, achieving AUCs of 58% in children and 65% in adolescents (Figure 5A, B). Notably, combining differentially abundant bacteria with all

dietary features did not improve predictive performance. However, models combining differentially abundant bacteria with individual dietary components (nutrients, food groups) achieved the highest AUC values, reaching 65% in children and 71% in adolescents (Figure 5A, B). This improvement in predictive performance aligns with the estimated p-values of these models, which demonstrate that while bacterial data generally contribute more to model predictive power, the inclusion of dietary components significantly improves model performance compared with models using only dietary data. Relevant contributions of the remaining variables for each one of the models are listed in Supplementary Tables 6–10 for children and 12–16 for adolescents.

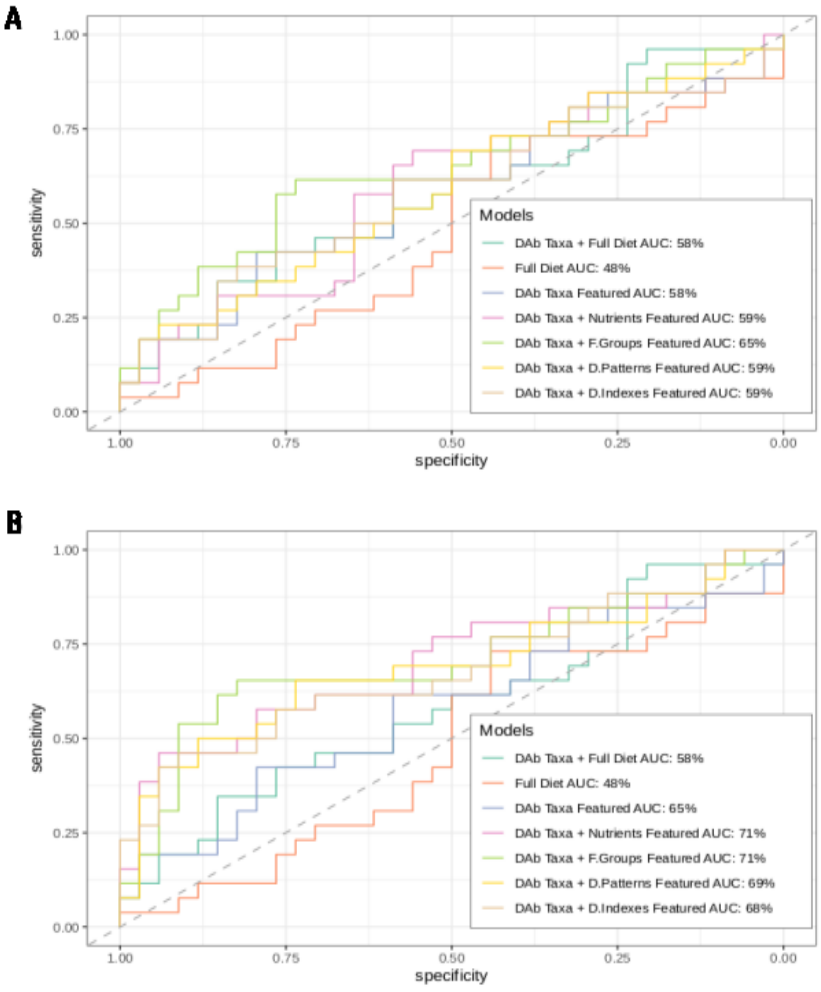


Figure 5 Receiving operating characteristic (ROC) curves of diet and microbiota penalized logistic regression models. Each curve corresponds to a different combination of dietary elements and meta-taxonomical items, considering the area under the curve (AUC) to address the discriminant power of the model between "Healthy" and "Behavioral Problems" groups. Models are based on the following data combinations: **DAb Taxa** (differentially abundant bacterial species); **Full Diet** (standalone combination of diet items, including nutrients, food groups, dietary patterns and dietary indexes); **DAb Taxa + Nutrients featured** (combination of feature-selected bacterial species and nutrients); **DAb Taxa + F.Groups featured** (combination of feature-selected bacterial species and food groups); **DAb Taxa + D.Patterns featured** (combination of feature-selected bacterial species and dietary patterns); **DAb Taxa + D.Indexes featured** (combination of feature-selected bacterial species and dietary indices). (**A**) ROC curves for children using previously-selected dietary and taxonomical elements through feature selection. (**B**) ROC curves for adolescents using previously-selected dietary and taxonomical elements through feature selection.

4. DISCUSSION

The present study contributes to our understanding of the complex interplay between long-term dietary habits, gut microbiota, and behavioral problems in children and adolescents, supporting a key role of the microbial communities in explaining behavioral problems.

Utilizing in-depth dietary analysis and shotgun metagenomics, our findings reveal that children and adolescents with behavioral problems exhibit increased alpha diversity within their gut microbiota. While higher alpha diversity is often associated with a healthy microbiota and improved mental health outcomes, such as temperament [12] and behavior [22], the quality and function of the microbial species within the gut ecosystem can be equally crucial for neurocognitive and neuropsychiatric outcomes. Consistent with our findings, studies have linked greater alpha diversity to major depression in adults [63] and autism in older children [64].

We observed increased levels of *C. coli* and *E. durans* in children with behavioral problems. *Campylobacter* spp. is one of the four major global causes of diarrheal disease [65], and its presence in the intestinal microbiota has been linked to child growth and neurodevelopment issues [66]. Similarly, preclinical studies show that oral ingestion of *Enterococcus* species can induce depression-like behavior and neuroinflammation [67]. While we also found increased levels of beneficial bacteria such as *Bifidobacterium* and

Latilactobacillus (*L. sakei* and *B. pollurum*) in this group, these are likely linked to dietary factors, particularly the consumption of fresh and processed meats [68], and are not necessarily linked to neurodevelopmental disorders in children [69,70].

Among the bacterial species found to be more abundant in healthy children than in peers with behavioral problems, notable examples include *B. nordii*, *B. thetaiotaomicron*, *C. eutactus* and *I. butyriciproducens*, which have been positively linked to normal cognitive functioning [17,71,72]. Notably, *C. euctatus* was also more abundant in healthy children than in peers with depressive and anxious behavior in a recent cross-sectional study (Xu et al., 2024). *Bacteroides* species and other commensals (e.g., *I. butyriciproducens*) are specialized in utilizing dietary fibers to produce SCFAs, which can strengthen the gut and brain barrier, reduce inflammation, and exert neuroprotective effects [73–76]. Furthermore, a preclinical study demonstrated that *C. eutactus* can produce serotonin from tryptophan, potentially influencing neuronal plasticity and glial activity in the prefrontal cortex [77], which could also be of help for children with mental health issues, where serotonin production is often impaired [78].

Notably, children with behavioral problems exhibited a strong association with a Western dietary pattern, characterized by a high intake of cholesterol- and energy-rich foods such as processed foods or meat, and a low intake of foods rich in soluble or insoluble fiber such as fruits. This was in line with a lack of full compliance with dietary recommendations. These children also showed lower intake of vitamin B9. A recent *in vitro* study [79] demonstrated that folic acid deficiency negatively impacts gut microbiota composition and SFCA production, suggesting a potential link between vitamin B9 deficiency and gut health in children with behavioral problems.

Consistent with our findings, recent cross-sectional studies in preschoolers and school-age children have described a positive association between Western dietary patterns and hyperactivity and inattention behavior [80–82]. Furthermore, deficiency in dietary fiber consumption coupled to a Western diet might underlie poor gut immune homeostasis and contribute to neurological disease development [83]. Conversely, a cross-sectional study in children and adolescents demonstrated that diets rich in fiber, vitamins and minerals, and low in saturated fats and cholesterol, are associated with improved cognitive function [84].

In adolescents, a notable finding of our study was the significant association between decreased levels of *Bifidobacterium* and *Butyricimonas* species and behavioral problems. This aligns with previous findings in adults, where a decreased abundance of the genus *Butyricimonas* was observed in patients with cognitive impairment [85]. Notably, several *Bifidobacterium* species identified in our study, including *B. angulatum* and *B. pseudolongum*, are known to produce GABA, an inhibitory neurotransmitter crucial for cognition, behavior, anxiety, and stress response [86–88]. Furthermore, *Butyricimonas faecalis* produces propionate and *Butyricimonas virosa* produces butyrate, which are SCFAs known to reduce BBB permeability and exert anti-inflammatory effects in the brain in mice.

Similar to children, we found associations between adherence to a Western diet and behavioral problems in adolescents. This is consistent with observations of reduced consumption of healthy foods, such as vegetables, legumes, nuts, and olive oil, among adolescents with behavioral issues [91]. These foods are rich in fiber and anti-inflammatory compounds, which are typically under-represented in Western diets. Recent research has demonstrated associations between Western dietary patterns and depressive behavior in adolescents [92].

Mediation analysis revealed that the association between behavioral problems and a Western diet in adolescents was partly mediated by *L. mirabilis*, a Gram-negative, facultative anaerobic bacterium commonly found in the human oral cavity and the upper respiratory tract [93]. *L. mirabilis* has been previously associated with infections in immunocompromised subjects and cystic fibrosis [94,95]. Unexpectedly, we also found an association between increased fish intake (and consequently higher DHA levels) and behavioral problems in adolescents. While DHA (an omega-3 fatty acid) is crucial for brain development and cognition [96], our analyses suggest that this association might be mediated (at least partly) by *A. rhamnosivorans,* a bacterium linked to respiratory conditions and inflammation [97]. A post-hoc analysis ruled out a significant influence of respiratory problems on this mediation, further supporting the role of *A. rhamnosivorans* as a diet-based mediator of the observed associations.

While fish is generally considered beneficial due to its omega-3 fatty acid content, it can also contain harmful substances such as heavy metals, trimethylamine-N-oxide (TMAO), dioxins, polychlorinated biphenyls, and microplastic particles. These contaminants can disrupt gut microbiota balance (dysbiosis) and permeability, potentially impacting behavior [98,99].

TMAO, produced by gut microbiota through dietary sources such as choline, L-carnitine, and phosphatidylcholine, has been linked to increased severity of ASD symptoms in children [100], and may contribute to neuroinflammation and cognitive dysfunction in rodents [101,102]. Although *A. rhamnosivorans* typically produces butyrate from fiber, in a low-fiber environment it might contribute to the production of other metabolites from fish that could negatively impact the gut-brain axis, immune function, and behavior [101]. Further mechanistic studies are crucial to fully understand the possible role of *A. rhamnosivorans* in mediating the relationship between fish intake and behavioral issues.

Our findings suggest that nut and olive oil consumption may have a protective effect against behavioral problems in adolescents. Walnuts are rich in alpha-linolenic acid, a short-chain omega-3 fatty acid involved in brain development [96], and may benefit cognitive development. Moreover, nut intake can modulate the gut microbiota, increasing the abundance of bacteria contributiong to butyrate production, such as *Bifidobacterium* species [25,103], associated with improved cognitive abilities in adolescents [84,104]. Preclinical and clinical trials in adults have reported that olive oil, rich in monounsaturated fatty acids and phenolic compounds, has positive effects on the gut microbiota by reducing pathogens and increasing SFCA- and GABA-producing bacteria, which may exert anti-inflammatory effects and potentially improve mental health

Regarding the Penalized Linear Model results, our findings demonstrate that combining stratified dietary data (nutrients, food groups, dietary indices, and dietary patterns) with microbial information better capture the behavioral differences between the two groups in both children and adolescents. This enhanced performance likely resulted from several factors: 1) reduced data complexity and redundancy through data stratification and feature selection; 2) mitigation of potential correlation among dietary variables; and 3) increased explanatory power by incorporating a broader range of relevant independent variables and potentially capturing important interaction effects between dietary and microbial factors.

Due to the nature of Elastic-Net Penalized models, pinpointing the exact interaction types between variables can be challenging. However, examining the estimated p-values for variables within each model provides valuable insights: a general improvement in p-values was observed across selected dietary elements and combined bacterial species when compared with their performance in standalone models, suggesting a complementary

effect between these two data types. This synergistic approach reveals complex relationships that might not be apparent when examining dietary components or microbial taxa in isolation. By integrating these data sources, we gain a deeper understanding of individual variability in response to diet, potentially explaining why individuals on similar diets experience different health or behavioral outcomes[105]. Therefore, the present results reveal the potential of gut microbiota data, when combined with dietary information, as a valuable tool for classification and diagnosis, an association previously recognized in literature but often overlooked in favor of single-centered dietary or taxonomical approaches [106].

This study has several limitations that must be acknowledged. First, as a cross-sectional study, it cannot establish causality between gut microbiota, diet, and behavioral problems. Second, parental reporting of behavioral problems may be subject to bias. Third, while dividing the general population into two age groups allowed us to study diet-gut-brain interactions effectively, this approach might have limited the statistical power due to the relatively reduced sample size within each group. The main strength of our study was the use of metagenomic data through shotgun sequencing, providing a more comprehensive understanding of microbial communities as compared with 16S rDNA sequencing used in most other studies. Furthermore, we controlled for key confounding variables influencing the gut microbiotaand employed advanced statistical and mathematical modeling to investigate the complex interplay between gut microbiota, diet and host behavior.

5. CONCLUSIONS

Our findings reveal consistent associations between dietary patterns, nutrients, and food group intakes and behavioral problems in children and adolescents. Adherence to a Western diet and a deficiency in fiber-rich foods emerged as key dietary determinants potentially impacting both the gut microbiota and behavior in both age groups. While specific food items and nutrients associated with behavioral problems differed between children and adolescents, these findings align with the notion that dietary patterns may better reflect diet-microbiota interactions than individual nutrients [107]. Observed dietary differences were mirrored by alterations in the gut microbiota, characterized by a decrease in SCFAs-producing bacterial species and an increase in potentially harmful bacteria in individuals with behavioral problems. These changes may contribute to a dysfunctional gut-brain axis. Although the role of gut microbiota in mediating the effects of diet on behavior was identified only in limited

cases, gut microbiota data demonstrated superior accuracy in categorizing individuals based on their behavioral phenotype compared with dietary data alone, highlighting the importance of the gut microbiota in understanding behavioral variations.

Competing interest

Authors do not have competing interest to declare

Authors contributions

Conceptualization: YS and PC; Experimental Investigation: AL, T-E V, R-R SM, S A- G, I S-C; Data curation and analysis: AL, R-G S, T-E V, R-R SM; Funding acquisition: YS; Original draft and edits: AL, R-G S, YS; Final editing and approval: all authors.

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Supplementary Tables

Supplementary Table 1. Associations between gut microbiota diversity and behavioral problems

Childhood	**β (95% CI)**	**P**	**q***
Chao1	0.002(-0.005, 0.008)	0.55	0.98
Shannon	0.68 (-0.07, 1.463)	0.08	0.15
Inv. Simpson	0.57 (0.008, 0.106)	**0.02**	**0.03**
Adolescence	**β (95% CI)**	**P**	**q***
Chao1	0.006 (0.004, 0.015)	0.23	0.12
Shannon	0.97 (-0.13, 2.07)	0.08	0.06
inv. Simpson	0.77 (0.008, 0.146)	**0.03**	**0.02**

β (95% CI) Coefficient of the independent variable, representing an odds ratio. p: Model not adjusted for covariables (P<0.05). q: FDR-adjusted by age, sex, z-BMI, parent education, bowel movements, APALQ, stress level and medication and antibiotic intake. Significant differences in bold (p<0.05).

Supplementay Table 2. Differences in nutrients intake between children and adolescents with and without behavioral problems

Nutrient intake (g/day)	Children (5-10 years) N=202 (61%)					Adolescents (11-17 years) N=133 (39%)				
	Healthy		Behavioral problems		p	Healthy		Behavioral problems		P
	M	SD	M	SD		M	SD	M	SD	
Total protein (g)	85.0	2.0	85.9	2.5	0.40	114.8	3.6	115.5	5.0	0.88
Vegetal protein (g)	33.0	1.6	30.5	1.7	0.07	37.7	1.9	35.9	2.3	0.72
Animal protein (g)	57.7	2.5	59.9	2.5	0.20	77.1	4.2	79.6	5.2	0.58
Carbohydrates(g)	220.0	5.6	210.3	4.8	0.17	245.5	7.6	246.4	9.5	0.84
Polysacchares (g)	130.4	5.3	126.2	5.6	0.31	147.3	7.2	158.4	10.7	0.54
Intrinsic sugars (g)	100.6	4.6	98.3	5.1	0.34	111.0	5.4	102.9	6.8	0.44
Fructose (g)	27.3	2.3	22.4	2.4	**0.06**	27.5	2.4	22.5	3.1	0.37
Glucose (g)	19.0	1.6	15.4	1.6	**0.09**	20.2	1.9	17.3	2.6	0.63
Sucrose (g)	3.3	0.2	3.0	0.3	0.13	4.3	0.4	4.6	0.6	0.87
Maltose (g)	26.8	1.6	25.3	1.6	0.34	28.7	2.2	24.9	2.5	0.59
Lactose (g)	32.1	2.8	37.2	3.4	0.38	38.0	3.7	37.6	5.0	0.29
Total fiber (g)	27.5	1.9	23.3	1.7	**0.04**	31.5	2.0	25.9	2.5	0.10
Soluble fiber(g)	7.0	0.4	5.9	0.5	**0.01**	8.1	0.5	6.9	0.6	0.13
Insoluble fiber (g)	19.9	1.5	16.6	1.4	**0.04**	22.1	1.5	18.1	1.9	0.13
Total fats (g)	68.8	1.7	71.8	1.7	0.16	89.5	2.7	84.3	3.7	0.26
Linolenic acid (ALA) (g)	1.4	0.2	1.2	0.1	0.93	2.2	0.2	2.0	0.3	0.75
linoleic acid (LA) (g)	4.8	0.3	4.5	0.2	0.79	5.8	0.4	5.3	0.4	0.46
Docohexaenoic acid (DHA) (mg)	391.8	35.3	327.4	37.4	0.21	460.7	55.9	688.0	91.4	**0.03**
Eicosapentaenoic Acid (EPA) (mg)	214.2	20.9	184.1	21.4	0.36	284.9	35.8	407.6	55.7	0.06
Saturated fats (SFA) (g)	22.5	0.7	24.3	0.9	0.22	29.5	1.2	27.6	1.6	0.40
Polyunsaturated fats (PUFA) (g)	10.1	0.3	10.8	0.5	0.28	13.3	0.6	12.9	0.9	0.37
Monounsaturated fats (MUFA) (g)	30.3	0.8	30.0	0.8	1.00	38.9	1.1	36.8	1.7	0.25
Cholesterol (mg)	310.2	14.1	340.0	16.2	0.09	352.7	15.9	352.6	20.7	0.92
Iodine (µg)	118.9	5.5	115.5	6.0	0.47	134.9	6.2	137.6	10.5	0.99
Sodium (mg)	1908.0	68.0	2003.5	78.0	0.32	2758.2	111.4	2847.6	170.6	0.84
Potassium (mg)	3332.9	118.7	3149.9	128.2	0.22	4091.9	163.5	3800.3	199.2	0.29
Calcium (mg)	914.1	42.4	932.6	55.0	0.93	1112.9	68.4	1043.2	88.0	0.63
Magnesium (mg)	341.9	10.3	327.3	11.4	0.32	403.4	15.2	383.4	24.1	0.25
Phosphorus (mg)	1235.6	28.4	1241.4	41.1	0.77	1481.4	43.1	1516.8	55.7	0.57
Iron (mg)	13.4	0.5	12.3	0.5	0.16	16.6	0.7	16.0	1.1	0.45
Zinc (mg)	9.7	0.2	9.8	0.3	0.74	12.5	0.4	12.1	0.5	0.57
Selenium (µg)	97.8	4.4	92.0	4.6	0.54	113.1	5.2	119.8	8.4	0.75
Vitamin B1 (mg)	1.6	0.1	1.5	0.1	0.15	2.0	0.1	2.0	0.1	0.86
Vitamin B2 (mg)	1.9	0.1	1.9	0.1	0.71	2.2	0.1	2.2	0.1	0.99
Vitamin B6 (mg)	2.4	0.1	2.2	0.1	0.14	3.0	0.1	3.0	0.2	0.71
Vitamin B12 (µg)	7.0	0.3	7.1	0.3	0.70	8.3	0.4	8.5	0.6	0.75
Vitamin B9 (µg)	351.8	23.5	282.8	17.6	**0.02**	377.1	23.4	337.9	33.4	0.33
Vitamin B3 (mg)	23.4	0.8	23.1	0.9	0.58	31.0	1.2	33.5	1.7	0.27
Vitamin C (mg)	191.0	16.8	160.2	15.5	0.25	255.8	23.0	199.0	31.3	0.15
Vitamin A (µg)	1335.2	123.3	1344.0	230.8	0.76	1551.2	136.9	1205.3	173.1	0.13
Vitamin D (µg)	4.2	0.4	4.8	0.6	0.79	6.0	0.6	6.5	0.8	0.60
Vitamin E (mg)	11.1	0.5	9.8	0.5	0.05	13.0	0.6	11.0	0.9	0.06

Flavonoids (mg)	839.6	96.1	985.2	106.2	0.36	1259.5	166.6	1309.6	256.7	0.96
Isoflavones (mg)	52.2	13.9	72.6	17.8	0.35	204.2	61.6	287.0	102.2	0.49
Antacionines (mg)	130.6	20.8	112.9	21.1	0.73	127.4	19.4	76.7	21.3	0.10
Resveratrol (mg)	0.3	0.0	0.3	0.1	0.41	0.4	0.1	0.2	0.1	0.15
Total polyphenols (mg)	2165.0	211.7	2088.8	214.2	0.86	2556.5	194.2	2466.5	279.3	0.79

* Mean (SD). Mann-Whitney U, Kruskal-Wallis and Student's t-tests were used for statistical analysis. Significant differences in bold (p<0.05).

Supplementary table 3. Differences in food group intake between children and adolescents with and without behavioral problems

	Children (5-10 years) N= 202 (61%)					**Adolescents (11-17 years) N=133 (39%)**				
	Healthy		**Behavioral problems**		**p**	**Healthy**		**Behavioral problems**		**p**
	M	**SD**	**M**	**SD**		**M**	**SD**	**M**	**SD**	
Sugar Beverages (g)	166.2	35.6	167.5	25.2	0.89	186.7	33.0	182.8	42.1	0.71
Fresh fruit and juices (g)	428.6	36.0	334.8	32.8	**0.05**	502.6	51.3	435.5	70.3	0.48
Legumes (g)	63.3	8.8	55.1	8.9	0.16	62.9	9.4	45.9	12.2	**0.04**
Nuts and oil olive (g)	11.8	2.1	10.0	2.2	0.20	11.2	2.3	9.3	3.2	**0.03**
Vegetables (g)	521.0	58.4	469.2	58.4	0.70	793.4	75.5	457.3	79.4	**0.04**
Processed food (g)	42.1	3.5	57.1	5.0	0.11	63.0	6.7	75.4	11.6	0.96
Eggs (g)	27.9	2.1	27.2	2.5	0.32	27.8	3.1	32.3	4.1	0.26
Cheese (g)	24.7	3.6	33.0	4.9	0.31	40.3	5.3	39.7	7.6	0.90
Pastries and sugars (g)	103.0	11.2	127.9	14.1	0.21	80.1	11.5	79.7	16.2	0.41
Yogurt and milk (g)	460.4	45.7	511.7	60.1	0.80	477.7	54.2	440.9	65.1	0.66
Potatoes (g)	71.5	7.4	87.1	9.1	0.20	97.8	12.6	116.8	19.1	0.16
Whole grain (g)	30.4	5.5	28.5	6.4	0.50	54.5	12.0	47.3	12.5	0.61
Refined cereals (g)	121.8	10.4	110.2	11.8	0.37	154.8	15.5	159.8	24.1	0.98
Fish (g)	72.1	5.7	64.2	6.7	0.20	97.3	12.3	135.6	17.9	**0.02**
Red meat (g)	58.7	6.8	64.2	7.8	0.50	103.7	14.1	106.9	17.3	0.57
White meat (g)	69.3	6.0	72.4	6.8	0.38	105.5	9.1	91.2	10.0	0.78
Processed meat (g)	19.5	1.8	24.6	2.3	0.23	49.9	9.0	48.9	13.5	0.75

* Data are expressed as means (SD); Mann-Whitney U, Kruskal-Wallis and Student's t-tests were used for statistical analysis. Significant differences are in bold (p<0.05).

Supplementary table 4. Factor loadings matrix for the three dietary patterns identified in the childhood and adolescent populations

	Childhood dietary patterns			Adolescence dietary patterns		
Food group	Plant-based diet	Western Diet	Meat and cereals-based diet	Plant-based diet	Western diet	Meat-based diet
Sugar Beverage (g)	0.1	**0.8**	0.1	-0.1	**0.8**	0.1
Fresh fruit(g)	**0.7**	-0.1	0.1	**0.5**	-0.1	-0.2
Legumes(g)	**0.7**	0.1	-0.1	**0.8**	0.1	-0.1
Nuts and oil olive(g)	0.2	0.2	0.0	0.2	-0.1	-0.3
Vegetables(g)	**0.8**	0.0	0.1	**0.8**	-0.1	0.1
Processed food(g)	-0.1	**0.5**	0.2	-0.1	**0.7**	0.3
Cheesee(g)	0.0	-0.1	0.1	0.0	0.3	-0.1
Pastries and sugars(g)	-0.2	**0.7**	0.2	0.2	-0.1	0.1
Yogurt and milk(g)	0.1	0.2	0.0	0.0	-0.1	0.0
Potatoes(g)	0.2	0.1	0.0	0.0	0.3	0.2
Whole grain(g)	**0.5**	0.0	-0.2	**0.4**	-0.3	0.0
Refined cereals(g)	0.0	0.3	**0.7**	0.0	0.0	0.1
Fish(g)	0.2	**0.7**	0.0	0.2	-0.3	0.2
Red meat(g)	0.0	0.3	**0.7**	0.0	0.1	**0.9**
Whitemeat(g)	0.0	0.1	0.3	0.0	-0.1	**0.5**
Processed meat(g)	-0.2	**0.4**	0.3	-0.2	**0.4**	**0.6**

The values presented are the factor loadings extracted by Principal Component Analysis (PCA) with varimax rotation. Loadings equal or greater than 0.4 in bold. Components are labeled based on the variables with the highest loadings on each factor.

Supplementary Table 5. Variable contribution summary in Elastic Net model for selected differentially abundant taxa in the children group

Variable	β-coefficients	Std. Error	Exp(β)	CI (95%)	*P value*
(Intercept)	-0.57	0.32	5.66E-01	[0.299, 1.05]	0.07
Campylobacter coli	0.49	0.17	1.64E+00	[1.2, 2.29]	**0.00**
Bifidobacterium pullorum	0.36	0.17	1.43E+00	[1.03, 2.02]	**0.04**
Latilactobacillus sakei	0.28	0.16	1.33E+00	[0.973, 1.82]	0.08
Intestinimonas butyriciproducens	-0.16	0.16	8.55E-01	[0.618, 1.18]	0.34
Butyrivibrio fibrisolvens	-0.48	0.17	6.16E-01	[0.435, 0.853]	**0.00**
Age	0.37	0.20	1.45E+00	[0.98, 2.16]	0.07
Sex	-0.48	0.32	6.18E-01	[0.329, 1.15]	0.13
Antibiotics	1.01	0.50	2.75E+00	[1.06, 7.53]	**0.04**

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 6. Variable summary in Elastic Net comparison for full diet in the children group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Total protein (g)	0.04	0.04	1.00	[0.97, 1.1]	0.26
Carbohydrates (g)	-0.01	0.01	0.99	[0.96, 1]	0.40
Polysacchares (g)	0.00	0.02	1.00	[0.97, 1]	0.88
Intrinsic sugars (g)	0.02	0.02	1.00	[0.99, 1.1]	0.24
Fructose (g)	-0.03	0.02	0.97	[0.94, 1]	0.09
Glucose (g)	0.01	0.04	1.00	[0.93, 1.1]	0.87
Sucrose (g)	-0.18	0.13	0.84	[0.65, 1.1]	0.16
Maltose (g)	-0.02	0.02	0.98	[0.95, 1]	0.39
Lactose (g)	0.11	0.12	1.10	[0.88, 1.4]	0.38

Total fiber (g)	0.03	0.27	1.00	[0.61, 1.8]	0.90
Soluble fiber(g)	-0.00	0.12	1.00	[0.79, 1.3]	0.98
Total fats (g)	-0.00	0.00	1.00	[0.99, 1]	0.22
Linolenic acid (ALA) (g)	0.01	0.01	1.00	[0.99, 1]	0.38
linoleic acid (LA) (g)	-0.04	0.07	0.96	[0.83, 1.1]	0.58
Docohexaenoic acid (DHA) (mg)	-0.01	0.07	0.98	[0.86, 1.1]	0.82
Eicosapentaenoic acid (EPA) (mg)	-0.01	0.01	0.99	[0.98, 1]	0.22
Saturated fats (SFA) (g)	0.00	0.00	1.00	[1, 1]	0.65
Polyunsaturated fats (PUFA) (g)	-0.00	0.00	1.00	[0.99, 1]	0.55
Monounsaturated fats (MUFA) (g)	0.00	0.00	1.00	[1, 1]	0.99
Cholesterol (mg)	-0.05	0.12	0.95	[0.76, 1.2]	0.65
Iodine (µg)	0.01	0.01	1.00	[0.98, 1]	0.61
Sodium (mg)	-0.31	0.57	0.73	[0.24, 2.2]	0.58
Calcium (mg)	0.20	0.89	1.20	[0.22, 7]	0.82
Magnesium (mg)	-0.29	0.75	0.75	[0.17, 3.3]	0.70
Phosphorus (mg)	-0.01	0.16	0.99	[0.72, 1.4]	0.95
Iron (mg)	0.06	0.07	1.10	[0.93, 1.2]	0.35
Selenium (µg)	-0.14	0.13	0.87	[0.68, 1.1]	0.29
Vitamin B2 (mg)	0.00	0.00	1.00	[1, 1]	0.44
Vitamin B12 (µg)	0.00	0.00	1.00	[1, 1]	0.49
Vitamin B9 (µg)	-0.01	0.00	0.99	[0.99, 1]	**0.01**
Vitamin B3 (mg)	0.11	0.06	1.10	[0.99, 1.3]	0.06
Vitamin C (mg)	0.00	0.00	1.00	[1, 1]	0.23
Vitamin A (µg)	0.00	0.00	1.00	[1, 1]	0.17
Vitamin D (µg)	0.31	0.65	1.40	[0.38, 4.9]	0.63
Vitamin E (mg)	-0.00	0.00	1.00	[1, 1]	0.42
Flavonoids (mg)	-0.05	0.11	0.95	[0.77, 1.2]	0.63

Isoflavones (mg)	-0.05	0.03	0.95	[0.9, 1]	0.12
Antacionines (mg)	-0.01	0.05	0.99	[0.89, 1.1]	0.88
Resveratrol (mg)	-0.00	0.08	1.00	[0.85, 1.2]	0.98
Total polyphenols (mg)	0.03	0.06	1.00	[0.92, 1.2]	0.59
Fresh fruit(g)	0.03	0.04	1.00	[0.94, 1.1]	0.56
Sugar Beverage (g)	-0.09	0.18	0.91	[0.65, 1.3]	0.61
Legumes(g)	0.04	0.06	1.00	[0.93, 1.2]	0.49
Nuts and oil olive(g)	-0.10	0.20	0.91	[0.61, 1.3]	0.64
Vegetables(g)	0.01	0.04	1.00	[0.94, 1.1]	0.76
Eggs (g)	0.02	0.02	1.00	[0.98, 1.1]	0.32
Cheese (g)	0.01	0.04	1.00	[0.94, 1.1]	0.83
Pastries and sugars(g)	0.02	0.03	1.00	[0.97, 1.1]	0.37
Yogurt and milk(g)	0.03	0.03	1.00	[0.98, 1.1]	0.24
Potatoes(g)	-0.01	0.04	0.99	[0.92, 1.1]	0.82
Processed food(g)	0.01	0.04	1.00	[0.93, 1.1]	0.87
Whole grain(g)	-0.03	0.07	0.97	[0.84, 1.1]	0.68
Refined cereals(g)	-0.02	0.03	0.98	[0.93, 1]	0.42
Fish(g)	0.06	0.05	1.10	[0.96, 1.2]	0.28
Red meat(g)	0.69	2.30	2.00	[0.023, 170]	0.76
Whitemeat(g)	0.21	0.48	1.20	[0.48, 3.1]	0.67
Processed meat(g)	-0.20	0.31	0.82	[0.44, 1.5]	0.53
Plant-based diet	-0.10	0.20	0.90	[0.61, 1.3]	0.61
Western diet	-0.00	0.04	1.00	[0.92, 1.1]	0.96
Meat and cereals diet	-0.12	0.57	0.88	[0.29, 2.7]	0.83
SHEI	0.83	0.55	2.30	[0.77, 6.7]	0.13
SHEIQ	0.00	0.25	1.00	[0.61, 1.6]	1.00
MDQ	1.90	0.64	6.90	[2, 24]	0.00
DII	0.16	0.23	1.20	[0.74, 1.8]	0.50

DIIQ	-0.13	0.27	0.88	[0.52, 1.5]	0.63

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 7. Variable contribution summary in Elastic Net model for selected differentially abundant taxa and nutrients in the children group

Variable	β-coefficients	Std. Error	Exp(β)	CI (95%)	z value	Pr(>|z|)
Campylobacter coli	0.48	0.17	1.61E+00	[1.17, 2.26]	2.85	**0.00**
Bifidobacterium pullorum	0.39	0.17	1.47E+00	[1.06, 2.09]	2.25	**0.02**
Butyrivibrio fibrisolvens	-0.46	0.17	6.30E-01	[0.443, 0.878]	-2.66	**0.01**
Antibiotics	1.25	0.53	3.50E+00	[1.28, 10.2]	2.38	**0.02**
Vitamin B9 (mg)	-0.38	0.18	6.88E-01	[0.468, 0.967]	-2.05	**0.04**

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 8. Variable contribution summary in Elastic Net model for selected differentially abundant taxa and food groups in the children group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Campylobacter coli	0.49	0.17	1.60	[1.2, 2.3]	**0.00**

Bifidobacterium pullorum	0.37	0.17	1.50	[1, 2]	**0.03**
Latilactobacillus sakei	0.26	0.16	1.30	[0.95, 1.8]	0.10
Intestinimonas butyriciproducens	-0.12	0.17	0.89	[0.64, 1.2]	0.47
Butyrivibrio fibrisolvens	-0.43	0.17	0.65	[0.47, 0.91]	**0.01**
Age	0.13	0.21	1.10	[0.75, 1.7]	0.53
Sex	-0.46	0.32	0.63	[0.34, 1.2]	0.15
Antibiotics	1.20	0.50	3.40	[1.3, 9]	**0.01**
z-BMI	-0.14	0.40	0.87	[0.39, 1.9]	0.73
Pastries (g)	0.26	0.17	1.30	[0.93, 1.8]	0.12
Potatoes (g)	0.27	0.17	1.30	[0.94, 1.8]	0.12
Processed food (g)	0.30	0.18	1.30	[0.95, 1.9]	0.09
Whitemeat (g)	0.23	0.17	1.30	[0.91, 1.7]	0.17

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 9. Variable Contribution Summary in Elastic Net model for selected differentially abundant taxa and dietary patterns in the children group

Variable	β-coefficients	Standard Error	Exp(β)	CI(95%)	*P value*
Campylobacter coli	0.48	0.16	1.60	[1.2, 2.2]	**0.00**
Bifidobacterium pullorum	0.34	0.16	1.40	[1, 1.9]	**0.04**
Latilactobacillus sakei	0.29	0.16	1.30	[0.98, 1.8]	0.06
Intestinimonas butyriciproducens	-0.15	0.16	0.86	[0.63, 1.2]	0.36
Butyrivibrio fibrisolvens	-0.44	0.17	0.64	[0.46, 0.89]	**0.01**
Age	0.30	0.20	1.40	[0.91, 2]	0.13
Sex	-0.42	0.31	0.66	[0.36, 1.2]	0.18
Antibiotics	1.10	0.49	3.20	[1.2, 8.2]	**0.02**
z-BMI	-0.06	0.38	0.94	[0.45, 2]	0.88
Western diet	0.36	0.16	1.40	[1, 2]	**0.03**

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 10. Variable contribution summary in Elastic Net model for selected differentially abundant taxa and dietary indexes in the children group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Campylobacter coli	0.51	0.16	1.70	[1.2, 2.3]	**0.00**
Bifidobacterium pullorum	0.35	0.17	1.40	[1, 2]	**0.04**
Latilactobacillus sakei	0.29	0.16	1.30	[0.98, 1.8]	0.06
Intestinimonas butyriciproducens	-0.11	0.16	0.89	[0.65, 1.2]	0.49
Butyrivibrio fibrisolvens	-0.44	0.17	0.65	[0.46, 0.9]	**0.01**
Age	0.33	0.20	1.40	[0.94, 2.1]	0.10
Sex	-0.36	0.32	0.70	[0.37, 1.3]	0.26
Antibiotics	1.10	0.49	2.90	[1.1, 7.5]	**0.03**
SHEI	-0.23	0.16	0.79	[0.58, 1.1]	0.16

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 11. Variable summary in Elastic Net comparison for selected differentially abundant taxa in the adolescent group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*

Butyricimonas virosa	-0.52	0.22	0.59	[0.39, 0.91]	**0.02**
Parolsenella catena	-0.67	0.26	0.51	[0.3, 0.86]	**0.01**
Ruminococcus champanellensis	0.59	0.22	1.80	[1.2, 2.8]	**0.01**
Anaerostipes rhamnosivorans	0.54	0.22	1.70	[1.1, 2.6]	**0.02**
Age	-0.52	0.36	0.59	[0.29, 1.2]	0.15
Sex	-0.37	0.42	0.69	[0.3, 1.6]	0.39
z-BMI	0.60	0.35	1.80	[0.93, 3.6]	0.08

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 12. Variable summary in ElasticNet comparison for full diet in the adolescents group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	P value
Total protein (g)	-0.03	0.03	0.97	[0.91, 1]	0.28
Carbohydrates(g)	-0.01	0.02	0.99	[0.95, 1]	0.71
Polysacchares (g)	0.01	0.02	1.00	[0.96, 1.1]	0.76
Intrinsic sugars (g)	0.01	0.03	1.00	[0.95, 1.1]	0.76
Fructose (g)	-0.02	0.03	0.98	[0.92, 1]	0.41
Glucose (g)	0.00	0.05	1.00	[0.9, 1.1]	0.96
Sucrose (g)	0.12	0.19	1.10	[0.78, 1.6]	0.53
Maltose (g)	-0.04	0.03	0.96	[0.91, 1]	0.20
Lactose (g)	-0.01	0.03	0.99	[0.94, 1]	0.73
Total fiber (g)	0.06	0.10	1.10	[0.88, 1.3]	0.53
Soluble fiber(g)	-0.36	0.28	0.70	[0.4, 1.2]	0.21
Total fats (g)	-0.07	0.06	0.93	[0.84, 1]	0.22
Linolenic acid (ALA) (g)	-0.10	0.31	0.91	[0.49, 1.7]	0.75
linoleic acid (LA) (g)	0.15	0.18	1.20	[0.82, 1.6]	0.39
Docosahexaenoic acid (DHA) (mg)	0.00	0.00	1.00	[1, 1]	0.44
Eicosapentaenoic acid (EPA) (mg)	-0.00	0.00	1.00	[0.99, 1]	0.33

Saturated fats (SFA) (g)	0.00	0.09	1.00	[0.84, 1.2]	0.98
Polyunsaturated fats (PUFA) (g)	0.01	0.12	1.00	[0.79, 1.3]	0.97
Monounsaturated fats (MUFA) (g)	0.07	0.12	1.10	[0.85, 1.3]	0.54
Cholesterol (mg)	-0.01	0.01	0.99	[0.99, 1]	0.31
Iodine (µg)	0.01	0.01	1.00	[0.99, 1]	0.30
Sodium (mg)	0.00	0.00	1.00	[1, 1]	0.62
Calcium (mg)	-0.00	0.00	1.00	[1, 1]	0.59
Magnesium (mg)	-0.00	0.00	1.00	[0.99, 1]	1.00
Phosphorus (mg)	0.00	0.00	1.00	[1, 1]	0.13
Iron (mg)	-0.04	0.13	0.96	[0.74, 1.2]	0.75
Selenium (µg)	0.00	0.02	1.00	[0.97, 1]	0.82
Vitamin B2 (mg)	-0.81	1.30	0.45	[0.03, 6.1]	0.54
Vitamin B12 (µg)	-0.12	0.26	0.88	[0.53, 1.5]	0.63
Vitamin B9 (µg)	-0.00	0.00	1.00	[0.99, 1]	0.65
Vitamin B3 (mg)	0.09	0.08	1.10	[0.92, 1.3]	0.31
Vitamin C (mg)	-0.00	0.00	1.00	[0.99, 1]	0.88
Vitamin A (µg)	0.00	0.00	1.00	[1, 1]	0.43
Vitamin D (µg)	0.07	0.12	1.10	[0.85, 1.4]	0.53
Vitamin E (mg)	0.09	0.15	1.10	[0.81, 1.5]	0.56
Flavonoids (mg)	-0.00	0.00	1.00	[1, 1]	0.24
Isoflavones (mg)	0.00	0.00	1.00	[1, 1]	0.09

Antacionines (mg)	-0.00	0.00	1.00	[0.99, 1]	0.59
Resveratrol (mg)	-0.20	0.89	0.82	[0.14, 4.7]	0.82
Total polyphenols (mg)	0.00	0.00	1.00	[1, 1]	0.35
Fresh fruit(g)	0.36	0.93	1.40	[0.23, 8.9]	0.70
Sugar Beverage (g)	0.01	0.04	1.00	[0.93, 1.1]	0.78
Legumes(g)	-0.03	0.05	0.97	[0.88, 1.1]	0.54
Nuts and oil olive(g)	-0.04	0.07	0.96	[0.84, 1.1]	0.51
Vegetables(g)	-0.00	0.03	1.00	[0.94, 1.1]	0.92
Eggs (g)	0.31	0.19	1.40	[0.94, 2]	0.10
Cheese (g)	0.01	0.05	1.00	[0.92, 1.1]	0.81
Pastries and sugars(g)	0.01	0.03	1.00	[0.96, 1.1]	0.79
Yogurt and milk(g)	-0.05	0.07	0.95	[0.83, 1.1]	0.48
Potatoes(g)	-0.01	0.03	0.99	[0.93, 1]	0.69
Processed food(g)	0.01	0.03	1.00	[0.95, 1.1]	0.66
Whole grain(g)	0.00	0.03	1.00	[0.95, 1.1]	0.96
Refined cereals(g)	-0.01	0.02	0.99	[0.95, 1]	0.68
Fish(g)	0.09	0.08	1.10	[0.94, 1.3]	0.27
Red meat(g)	-0.07	0.19	0.93	[0.64, 1.3]	0.71
Whitemeat(g)	0.00	0.03	1.00	[0.94, 1.1]	0.89
Processed meat(g)	-0.01	0.05	0.99	[0.9, 1.1]	0.88
Plant-based diet	-0.44	0.37	0.64	[0.31, 1.3]	0.24
Western diet	0.68	0.46	2.00	[0.8, 4.9]	0.14

Meat diet	-0.21	0.40	0.81	[0.37, 1.8]	0.61
SHEI	0.02	0.05	1.00	[0.92, 1.1]	0.71
SHEIQ	-0.19	0.84	0.83	[0.16, 4.3]	0.83
MDQ	-0.10	0.73	0.90	[0.22, 3.8]	0.89
DII	0.54	0.44	1.70	[0.73, 4.1]	0.22
DIIQ	0.10	0.42	1.10	[0.49, 2.5]	0.80
Age	-0.17	0.60	0.84	[0.26, 2.7]	0.78
Sex	-0.40	0.59	0.67	[0.21, 2.1]	0.50
Antibiotics	-1.30	1.30	0.27	[0.02, 3.5]	0.32
z-BMI	0.38	0.52	1.50	[0.53, 4]	0.47

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 13. Variable summary in ElasticNet comparison for selected taxa and nutrients in the adolescents group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Butyricimonas virosa	-0.45	0.21	0.64	[0.42, 0.97]	**0.04**
Parafannyhessea umbonata	-0.08	0.23	0.92	[0.58, 1.5]	0.72
Parolsenella catena	-0.74	0.26	0.48	[0.28, 0.8]	**0.00**
Ruminococcus champanellensis	0.59	0.22	1.80	[1.2, 2.8]	**0.01**
Anaerostipes rhamnosivorans	0.48	0.22	1.60	[1, 2.5]	**0.03**
Age	-0.37	0.36	0.69	[0.34, 1.4]	0.30
Sex	-0.29	0.42	0.75	[0.33, 1.7]	0.49
z-BMI	0.51	0.34	1.70	[0.86, 3.3]	0.13
Total fiber (g)	-0.54	1.00	0.58	[0.08, 4.2]	0.59
Insoluble fiber (g)	0.20	1.00	1.20	[0.17, 8.9]	0.84
DHA (mg)	0.33	0.21	1.40	[0.91, 2.1]	0.13

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary table 14. Variable Summary in Elastic Net comparison for selected taxa and food groups in the adolescents group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Butyricimonas virosa	-0.53	0.21	0.59	[0.39, 0.9]	**0.01**
Parafannyhessea umbonata	-0.04	0.24	0.96	[0.6, 1.5]	0.86
Parolsenella catena	-0.76	0.26	0.47	[0.28, 0.79]	**0.00**
Ruminococcus champanellensis	0.58	0.22	1.80	[1.2, 2.8]	**0.01**
Anaerostipes rhamnosivorans	0.46	0.22	1.60	[1, 2.4]	**0.04**
Age	-0.43	0.35	0.65	[0.33, 1.3]	0.22
Sex	-0.18	0.42	0.83	[0.37, 1.9]	0.67
Antibiotics	-0.43	0.81	0.65	[0.13, 3.2]	0.60
z-BMI	0.44	0.35	1.60	[0.78, 3.1]	0.21
Legum	-0.51	0.22	0.60	[0.39, 0.92]	**0.02**
Potatoes	0.22	0.20	1.20	[0.84, 1.8]	0.28
Fish	0.39	0.22	1.50	[0.96, 2.3]	0.07

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 15. Variable summary in Elastic Net comparison for selected taxa and dietary indexes in the adolescent group

Variable	β-coefficients	Standard Error	Exp(β)	CI (95%)	*P value*
Butyricimonas virosa	-0.52	0.22	0.60	[0.39, 0.92]	**0.02**
Parafannyhessea umbonata	-0.21	0.23	0.81	[0.52, 1.3]	0.36
Parolsenella catena	-0.59	0.25	0.55	[0.34, 0.91]	**0.02**
Ruminococcus champanellensis	0.65	0.22	1.90	[1.2, 3]	**0.00**
Anaerostipes rhamnosivorans	0.53	0.22	1.70	[1.1, 2.6]	**0.02**
Age	-0.39	0.38	0.68	[0.32, 1.4]	0.30
Sex	-0.32	0.42	0.72	[0.32, 1.7]	0.45
Antibiotics	-0.59	0.83	0.56	[0.11, 2.8]	0.48
z-BMI	0.57	0.35	1.80	[0.89, 3.5]	0.10
SHEI	0.01	0.04	1.00	[0.94, 1.1]	0.84
SHEIQ	0.05	0.60	1.00	[0.32, 3.4]	0.94
MD	0.08	0.15	1.10	[0.81, 1.5]	0.58
MDQ	-0.29	0.66	0.75	[0.2, 2.7]	0.66

DII	0.21	0.14	1.20	[0.94, 1.6]	0.13
DIIQ	0.04	0.29	1.00	[0.59, 1.8]	0.90

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

Supplementary Table 16. Variable summary in Elastic Net comparison for selected taxa and dietary patterns in the adolescent group

Variable	*β-coefficients*	*Standard Error*	*Exp(β)*	*CI (95%)*	*P value*
Butyricimonas virosa	-0.49	0.21	0.61	[0.41, 0.93]	**0.02**
Parafannyhessea umbonata	-0.15	0.23	0.86	[0.55, 1.3]	0.51
Parolsenella catena	-0.52	0.25	0.60	[0.37, 0.97]	**0.04**
Ruminococcus champanellensis	0.64	0.22	1.90	[1.2, 2.9]	**0.00**
Anaerostipes rhamnosivorans	0.48	0.22	1.60	[1.1, 2.5]	**0.03**
Age	-0.38	0.35	0.69	[0.34, 1.4]	0.28
Sex	-0.14	0.43	0.87	[0.38, 2]	0.75
Antibiotics	-0.64	0.82	0.52	[0.11, 2.6]	0.43
z-BMI	0.59	0.35	1.80	[0.9, 3.6]	0.09
Plant-based diet	-0.39	0.26	0.68	[0.41, 1.1]	0.14

Western diet	0.56	0.28	1.80	[1, 3]	**0.04**

β-coefficients, standard errors, exponentiated coefficients (Exp(β)), 95% confidence intervals (CI), and p-values are presented for each variable. Statistically significant results ($p < 0.05$) are shown in **bold**. Exp(β) represents the odds ratio associated with each predictor.

CHAPTER 3

DIET AND GUT MICROBIOTA ARE DIFFERENTLY ASSOCIATED WITH EXECUTIVE FUNCTION AND LEARNING DIFFICULTIES IN CHILDREN AND ADOLESCENTS

Ana-Larroya, Sergio Romera-Giner, Verónica Tolosa-Enguis, Sonia María Rodríguez-Ruano, Pilar Codoñer, Yolanda Sanz.Diet and Gut Microbiota are differently associated with Executive Function and Learning Difficulties in Children and Adolescents (2026).

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ABSTRACT

Evidence indicates that diet and gut microbiota influence cognitive processes during neurodevelopment, yet their interaction remains poorly understood. We conducted a cross-sectional study of 407 children and adolescents (6–17 years) to examine associations among dietary patterns, gut microbiota, and cognitive outcomes. Executive function problems (EFP) were assessed using the Behavior Rating Inventory of Executive Function, and learning difficulties (LD) were identified according to Diagnostic and Statistical Manual of Mental Disorders criteria. Diet was assessed using a validated Food Frequency Questionnaire, and the gut microbiota profiled using shotgun metagenomics of stool samples. Pairwise comparisons examined associations among diet, microbiota composition, and cognitive profiles. Key dietary contributors were identified using LASSO logistic regression, and multi-omics MintTea pipeline and mediation analyses were used to assess diet–microbiota–cognition interactions. Children with EFP consumed less B vitamins and iron and more sugar-containing foods, while those with LD adhered to proinflammatory diets rich in saturated fat and low in vegetables and antioxidants. Microbial taxa such as Bifidobacterium, Coprococcus, oral and lactic acid bacteria, differed across cognitive groups. Zinc, lauric acid, and dairy intake correlated with Bifidobacterium spp. in the LD group, whereas pastry intake was associated with oral bacterial species in EFP. Mediation analyses highlighted Segatella copri and Methanobrevibacter smithii as mediators of low oats and green vegetable intakes, and Lacticaseibacillus paracasei as a mediator of lauric acid associations with LD. Our findings indicate that specific diet-driven gut microbiota configurations may contribute to dietary effects on distinct cognitive deficits, warranting further mechanistic research.

keywords

Gut microbiota; Diet, Gut–brain axis; Executive function; Learning difficulties; Children and Adolescents

1. INTRODUCTION

The gut microbiota, together with diet, influences the gut-to-brain signaling (“gut-brain axis”), thus impacting brain development and function [1]. Studies in germ-free mice provided the strongest evidence for the role of the gut microbiota in cognitive functions and learning skills in early life[2–4]. In addition, preclinical interventions with antibiotics, fecal microbiota

transplantation, probiotics, and dietary components demonstrated that shifting the gut microbiota affects neuroplasticity, hippocampal structure, and function [5–9]. The underlying mechanisms involve immune, metabolic, hypothalamic-pituitary-adrenal (HPA), and vagal nerve pathways that mediate the gut-brain connection [10–12]. For example, gut microbiota has been shown to modulate cognitive processes, such as memory, by regulating the host gene expression of the brain-derived neurotrophic factor (BDNF) and generating neuroimmune metabolites from fiber fermentation (short-chain fatty acids), and neurotransmitters (serotonin, melatonin) and other immune modulators (e.g. indole lactic acid) from amino acids (tyrosine, tryptophan, etc.)[13,14]

In humans, links between changes in the gut microbiota and neurodevelopmental and behavioral disorders have also been established [15]. However, most observational research has concentrated on clinical conditions like autism spectrum disorders (ASD) or attention-deficit hyperactivity disorder (ADHD), while largely neglecting subclinical states and cognition-related issues that frequently occur as comorbidities in these neurodevelopmental conditions (16,17). Studies investigating factors influencing cognitive and EFP in healthy populations have primarily focused on the first years of life, as these are essential periods for neurocognitive development [18]. These studies have shown that prenatal and postnatal factors—such as gestational age, birth weight, mode of delivery or breastfeeding—as well as stressful events like parental separations, and lifestyle habits (including physical activity, sleep disorders, screen-based activities time, and dietary patterns) are significant contributors to alterations in both cognitive processes and gut microbiota[19–22]. However, studies in middle childhood and adolescence are much more limited, although these are also critical periods to cognitive development in which adaptability to the environment demands high neuroplasticity [23]. In fact, the neuroanatomical changes occurring during adolescence particularly affect the frontal cortex, a brain area implicated in cognition and EF [24]. In these developmental periods, the gut microbiota is also susceptible to changes as a consequence of increased exposure to stressors, social interactions, and independence, associated with dietary changes[23,24]

The aim of this study was to examine the role of the gut microbiota in the relationships between diet and EFP and LD among children and adolescents of the general population. Through a comprehensive analysis of the microbiota using shotgun metagenomics and by combining advanced statistics and machine learning methods, we provide new

insights into how the microbiota can act as a conduit of direct and indirect diet-mediated influences on the gut-brain axis and neurocognitive functions.

2. MATERIALS AND METHODS

2.1 Study design

This cross-sectional study comprises 407 children and adolescents, including 205 boys (50.4%) and 202 girls (49.6%), aged 6 to 17 years. They are part of a larger prospective study, which was conducted in 13 schools in Valencia (Spain). The participants were recruited between November and December 2020, during the SARS-CoV-2 pandemic, before they had been exposed to the virus. Other exclusion criteria included: having a chronic inflammatory bowel disease, having a diagnosis of a neurodevelopmental disorder, and having taken antibiotics one month prior to stool sample collection. Study design and assessments are depicted in Figure 1.

Parents of all participants provided information on sociodemographic variables (e.g., parental education and marital status), perinatal factors (e.g., mode of delivery), clinical and anthropometric parameters, and lifestyle factors related to physical activity and dietary quality. Detailed descriptions of each variable are provided in the **Additional Material (S1).**

The study was approved by the Ethics Committee of the Hospital Dr. Peset of Valencia, which was involved in the recruitment and clinical assessments. All parents of children and adolescents provided written informed consent. In addition, participants aged 12 years and older provided their own consent.

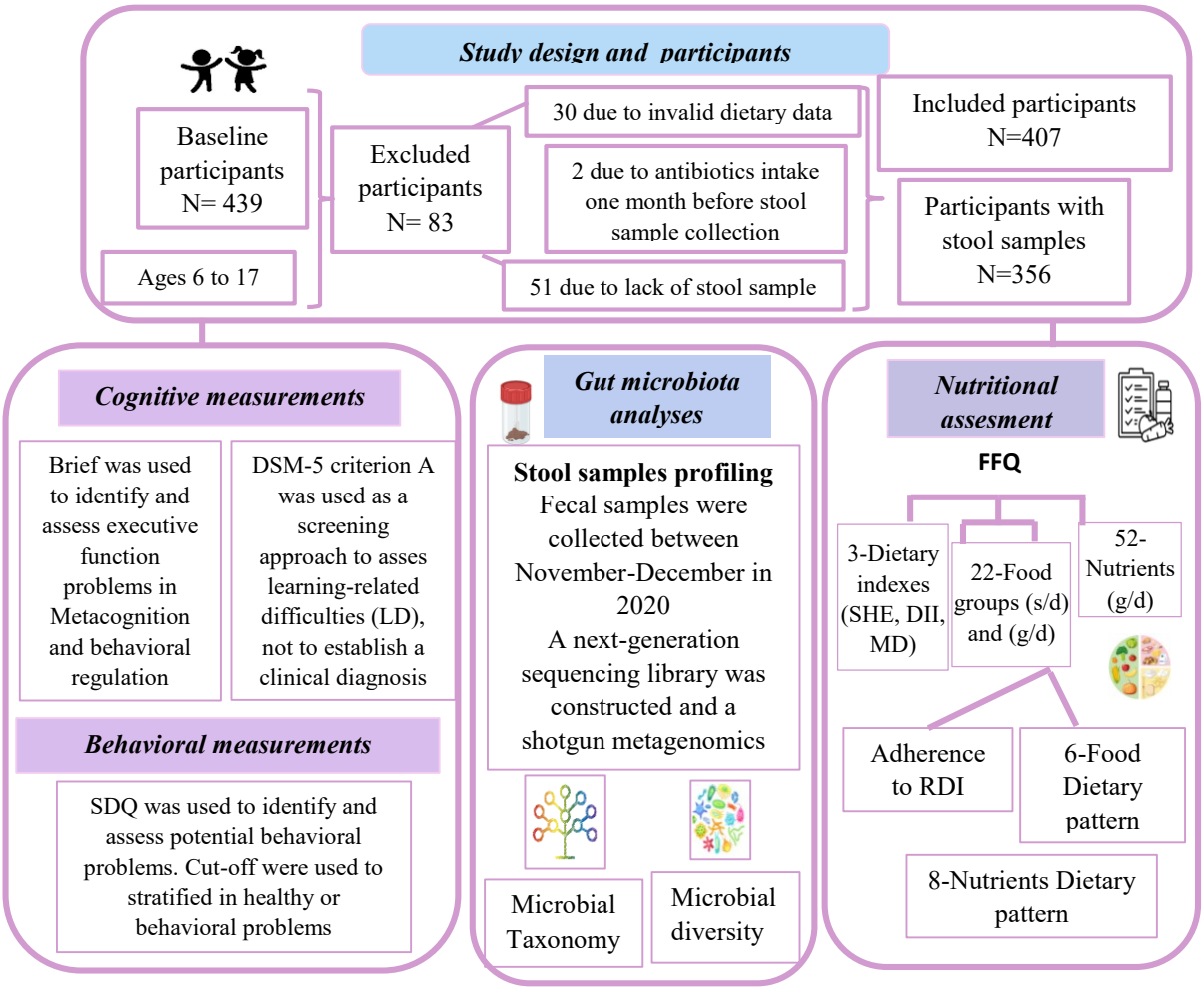


Figure 1. Flow chart of study design, number of participants and data collection. Abbreviations: SDQ: The Strengths and Difficulties Questionnaire; BRIEF: Behavior Rating Inventory of Executive Function DSM-V: Diagnostic and Statistical Manual of Mental Disorders. RDI: Recommended daily intake; SHEI: Spanish healthy eating index; DII: Dietary inflammatory index; MD: Mediterranean diet.

2.2 Cognitive impairment characterization

Assessment of Executive Functions and Learning Difficulties

Executive Functions (EF) skills were evaluated using the Behavior Rating Inventory of Executive Function (BRIEF) [25,26]. The test provides a profile of impairment across the facets of EF, including complex mental activities required to plan, organize, guide, review, regularize, and evaluate behaviors necessary for effective environmental adaptation and goal attainment. According to the BRIEF results, we stratified participants into two groups: those with EFP (combining clinical and subclinical impairments) and those without EFP.

Additionally, an assessment of learning difficulties (LD) according to the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (5th edition) (DSM-5) was included [27]. The categorization of LD enabled us to identify two groups: those with slight or significant difficulties (LD group) and those without difficulties. The control group consisted of subjects without any of the disorders listed above.

Other screening tools, such as the Strengths and Difficulties Questionnaire (SDQ) for the detection of externalizing and internalizing problems, were used for further stratification purposes [28]. Further information on the score-based stratification of subjects in those tests can be found in **Additional Material S1**.

2.3 Covariate characterization

A series of exploratory analyses was performed using R (v 4.3.2) to identify relevant risk factors and confounding variables for assessing associations with EFP or LD, including sociodemographic, clinical, perinatal, lifestyle, psychological, and anthropometric data. We also based our selection of covariates to be included in the adjusted models on previous literature, including sex, age, marital status, and parental education.

2.4 Nutritional assessments

Data on the participants' eating habits were collected by their parents using a 137-item semi-quantitative food frequency questionnaire (FFQ). Our

research group validated the Norfolk version of the EPIC-FFQ, which we adapted for use among Spanish children and adolescents [29]. Regarding analysis of food data, 52 nutrients, expressed in grams consumed per day (g/day) were evaluated using NutritionTools package [30] which included national [31] and international [32] food composition tables, considering the participant's age and portion size. In addition, we used the Phenol-Explorer database to evaluate polyphenol content and the CESNID food composition tables to complete information on animal and plant protein sources [33,34]. Complementary to nutrient analysis, a total of 22 food groups expressed in g/day and servings per day (s/day) were composed a priori, following the consumption and public health criteria recommended by the Spanish Agency for Food Safety and Nutrition (AESAN) [35]. Details on the nutrients and food groups evaluated can be found in the Methods section, **Additional Material S2**

Based on the dietary information of nutrients (g/day) and food groups (s/day), participants were further categorized based on compliance with the Recommended Dietary Allowance (RDA) established by the Spanish Society of Community Nutrition (SENC), and provided by AESAN, which are stratified according to sex and age [36]. Accordingly, participants were categorized as "Compliant" when their intake met the dietary guideline recommendations, and as "Below" or "Above" when they did not meet the daily intake recommendations

Unsupervised Dietary Patterns

To examine dietary patterns in our study population, we applied a statistical approach based on principal component analysis (PCA) with varimax (orthogonal) rotation. The analysis used intake of 20 food groups and 52 nutrients as input variables to identify data-driven, unsupervised (a posteriori) dietary patterns across study groups. Components with factor loadings greater than 0.40 were considered for interpretation. Higher loading values indicate a greater contribution of that food group or nutrient to the corresponding dietary pattern, reflecting higher intake. A detailed description of the identified patterns is provided in **Additional Material S2**.

Participants were subsequently characterized and stratified into tertiles of PCA-derived scores, representing levels of adherence to each pattern: T1 = low adherence, T2 = moderate adherence, and T3 = high adherence.

2.5 Gut microbiota analysis

Metagenomic sequencing and data processing

Stool samples were collected at home and stored at -80ºC after delivery until further processing. Metagenomic DNA was isolated from approximately 300 mg of feces using the Magpure Stool DNA LQ Kit. The NGS library was built using the MGIEasy Universal DNA Library Prep Set. Shotgun metagenomics was performed in the MGI's DNBSEQ-G50™ sequencing platform of the MGI Tech Co., Ltd manufacturing facility in Riga (Latvia), which generated paired-end short reads with a maximum of 150 base pairs (bp) and a sequencing depth of 20 million reads overage. Additional information can be found in the **Additional Material S3**. Samples of 356 participants were used, excluding 51 subjects from the original cohort due to technical and logistical reasons, ranging from non-delivery of the stool sample to failure to meet quality control criteria after sequencing.

Metataxonomic microbiome analysis

Microbial diversity of the samples was assessed using alpha- and beta-diversity indices. Alpha diversity metrics included Observed abundance, Chao1, Shannon and Inverse Simpson Indexes. Beta diversity was computed using Bray-Curtis and Jaccard distances. The study groups were compared to assess potentially significant intergroup differences. DESeq2 (v1.39.8) R package [37] was employed to identify differentially abundant species of each comparison. Further information about these indexes, their evaluation, and the differential abundance analysis can be found in the **Additional Material S3.**

2.6. Integrated dietary and microbiota data analyses

Module-based approach and interaction networks

Modules of interactions between dietary and taxonomic elements with strong associations with one another and with potential as classifiers for the selected cognitive status (either EFP or LD compared to controls) were identified using the MintTea pipeline [38]. Interactions were calculated by pairing taxonomic data with nutrients and food groups separately to avoid

the high correlation between the two dietary formats. Significant modules with an Area Under the Receiver Operating Characteristic (AUROC) > 0.6 and higher sparse canonical correlation values (sCC) were plotted into interaction networks, with relevant elements as nodes and interaction levels (ranging from 0.5 to 1) as edges. Additionally, a Spearman correlation analysis between the taxonomic and dietary elements of each module was performed to estimate the directionality (positive or negative) of their interactions, and the significance of these correlations was assessed using the cor.test function in the R stats base package. Further information about the pipeline can be found in the **Additional Material S4.**

Mediation analysis between dietary elements and microbiota.

A non-parametric, entropy-based mediation approach (NPEM) [39], was applied to check the possible interaction of relevant bacterial species over the effect of dietary components (nutrients, food groups and dietary patterns) on the binary cognitive outcomes (EFP versus Controls and LD versus Controls) More information regarding the NPEM and data preprocessing can be found in the **Additional Material S5**.

2.7 Statistical analysis

Relevant information of the statistical analysis, including relations with dependent variables, dietary data, covariates, and variable selection through Least Absolute Shrinkage and Selection Operator (LASSO) can be found in **Additional Material S6.**

3. RESULTS

3.1 Characteristics of the population and covariate selection

The population characteristics, group stratification by cognitive profiles, and pairwise comparisons of potentially relevant covariates are presented in Table 1.

According to the BRIEF, 45 participants (25 children [55.6%] and 20 adolescents [44.4%]) were categorized as having EFP. According to the DSM-5, 52 participants were identified as having LD difficulties (37 children [71.2%] and 15 adolescents [28.8%]). In addition, 27 participants, who had EFP problems (60% of the EFP group), also had LD. The remaining 310 participants (167 children [54%] and 143 adolescents [46%]) were considered as controls.

Covariates identified as relevant in Table 1 were subsequently included in the LASSO logistic regression model to select the most relevant ones, while accounting for multicollinearity. Based on the λ-1.SE penalization and λ.min criteria, the main contributors for the EFP group were Behavioral Regulation Index (β = 1.22), externalizing problems (β = 0.32), internalizing problems (β =0.45) and high stress (β = 0.1). Based on λ.min criterion, sleep disorders (β = 0.05), bowel movements (β = 0.07), marital status (β = 0.01), antibiotics (β =0.04) and non-antibiotic drugs (β =0.02) emerged as contributors to EFP (Table S2).

For the LD group, stress (β = 0.01; 0.005), internalizing problems (β = 0.61; 0.26), marital status (β = 0.60; 0.02) emerged as the strongest contributors under λ.min and λ-1.SE criterion, whereas parental education (β = 0.24), intestinal transit (β = 0.24) and DII (β = 0.24)) emerged as contributors according to λ.min. Among controls, sex and mode of delivery were identified as the main contributors compared to both, LD and EFP (Table S2).

Additionally, sex, under both penalization criteria (λ.min and λ.1se) as well as parental education, Behavioral Regulation Index, externalizing problems, DII and gastrointestinal disorders under the λ.min were the main variables differentiating the LD group from the EFP group (Table S2).

Table 1. Characteristics of the study population

Features	**Control (N=310)**	**EFP (n=45)**	**LD (n=52)**	**P-value[a]**	**P-value[b]**	**P-value[c]**
Age stage						
Children (6-10 years) N (%)	167 (53.9%)	25 (55.6%)	37 (71.2%)	0.9	**0.03**	0.2
Adolescents (11-17 years) N (%)	143 (46.1%)	20 (44.4%)	15 (28.8%)			

Sex						
Male N (%)	149 (48.1%)	32 (71.1%)	24 (46.2%)	**0.005**	0.9	**0.02**
Female N (%)	161 (51.9%)	13 (28.9%)	28 (53.8%)			
Parent education						
University degree N (%)	201 (64.8%)	26 (57.8%)	19 (36.5%)	0.6	**0.002**	0.05
Below university N (%)	109 (35.2%)	19 (42.2%)	33 (63.5%)			
Marital status						
Married or couple N (%)	276 (89%)	34 (75.6%)	39 (75%)	**0.04**	**0.01**	1.0
Separated/single parent N (%)	34 (11%)	11 (24.4%)	13 (25%)			
Gestational age						
Optimal (37-42 weeks) N (%)	260 (83.9%)	38 (84.4%)	41 (78.8%)	0.8	0.5	0.6
No optimal N (%)	50 (16.1%)	7 (15.6%)	11 (21.2%)			
Mode of delivery						
C-section N (%)	195 (62.9%)	36 (80%)	37 (71.2%)	**0.04**	0.3	0.4
Vaginal N (%)	115 (37.1%)	9 (20%)	15 (28.8%)			
Exclusive breastfeeding,						
Maternal, N (%)	187 (60.3%)	23 (51.1%)	27 (51.9%)	0.3	0.3	1
Formula or mixed N (%)	123 (39.7%)	22 (48.9%)	25 (48.1%)			
Childbirth complications						

No N(%)	236 (76.1%)	29 (64.4%)	39 (75%)	0.2	0.9	0.4
Yes N (%)	74 (23.9%)	16 (35.6%)	13 (25%)			
Maternal age at birth *Mean ± SD*	33.7±3.9	33.1±4.6	32.2±6.4	1.0	1.0	1.0
Birth weight						
Normal or high N (%)	299 (96.5%)	45 (100%)	49 (94.2%)	0.4	0.7	0.3
Low weight N (%)	11 (3.5%)		3 (5.8%)			
Self-reported sleep alteration						
No N (%)	273 (88.1%)	33 (73.3%)	43 (82.7%)	**0.02**	0.4	0.4
Yes N (%)	37 (11.9%)	12 (26.7%)	9 (17.3%)			
Intestinal transit (Bristol scale)						
Optimal, N (%)	256 (82.6%)	32 (71.1%)	37 (71.2%)	0.2	0.08	0.7
No optimal, N (%)	54 (17.4%)	13 (28.9%)	15 (28.8%)			
Bowel movements per day						
Optimal N (%)	288 (92.9%)	37 (82.2%)	45 (86.5%)	0.06	0.3	0.8
No optimal N (%)	22 (7.1%)	8 (17.8%)	7 (13.5%)			
Chronic gastrointestinal disorders						
No N (%)	273 (88.1%)	34 (75.6%)	46 (88.5%)	**0.03**	1.0	0.2

Yes N (%)	37 (11.9%)	11 (24.4%)	6 (11.5%)			
Allergy						
No N (%)	289 (93.2%)	40 (88.9%)	46 (88.5%)	0.6	0.4	1.0
Yes N (%)	21 (6.8%)	5 (11.1%)	6 (11.5%)			
Respiratory diseases						
No N (%)	227 (73.2%)	31 (62.2%)	43 (82.7%)	0.4	0.2	0.2
Yes N (%)	83 (26.8%)	14 (31.1%)	9 (17.3%)			
Antibiotics						
No N (%)	283 (91.3%)	39 (86.7%)	47 (90.4%)	0.4	1.0	0.8
Yes N (%)	27 (8.7%)	6 (13.3%)	5 (9.6%)			
Non antibiotic drugs						
No N (%)	220 (71%)	24 (53.3%)	30 (57.7%)	0.2	0.07	0.8
Yes N (%)	90 (29%)	21 (46.7%)	22 (42.3%)			
Body mass index (z-BMI)$^{\chi}$ *Mean ± SD*	18.1+3.7	17.4+3.7	17.8+3.8	0.4	0.9	0.8
Waist Circumference (WC) *Mean ± SD*	63.1±13.6	60.3±15.3	62.44±13.6	0.9	0.5	1.0
Ponderal status						
Normal/underweight, N (%)	233 (75.2%)	37 (82.2%)	36 (69.2%)	0.2	0.5	0.2
Overweight/obesity N (%)	77 (24.8%)	8 (17.8%)	16 (30.8%)			

Physical activity (APALQ)						
Mean + SD	12.6+2.9	11.9+3.1	12.03+2.99	0.2	1.0	0.1
Moderately active or active N (%)	231 (74.5%)	29 (64.4%)	34 (65.4%)	0.2	0.2	1.0
Sedentary N (%)	79 (25.5%)	16 (35.6%)	18 (34.6%)			
Screen-based activities (hours according to WHO)						
Optimal N (%)	221 (71.3%)	27 (60%)	38 (73.1%)	0.1	1.0	0.2
Non optimal N (%)	89 (28.7%)	18 (40%)	14 (26.9%)			
Outdoor activities (Hours according to WHO)						
Optimal N (%)	71 (22.9%)	11 (24.4%)	12 (23.1%)	0.8	1.0	1.0
No optimal N (%)	239 (77.1%)	34 (75.6%)	40 (76.9%)			
Healthy lifestyle score (HLS)						
Optimal lifestyle N (%)	181 (58.4%)	25 (55.6%)	25 (48.1%)	0.9	0.2	0.6
Low or medium N (%)	129 (41.6%)	20 (44.4%)	27 (51.9%)			
Healthy Eating Index (HEI) *Mean ± SD*	76.15±8.7	74.2±9.7	73.8±9.5	0.4	0.2	1.0
Optimal, N (%)	110 (35.5%)	15 (33.3%)	12 (23.1%)	1.0	0.1	0.4
Low or Needs improvement N	200	30	40 (76.9%)			

(%)	(64.5%)	(66.7%)				
Adherence Mediterranean Diet Index, *Mean ± SD*	6.9±2.6	6.7±2.5	6.48±2.25	1.0	0.4	1.0
Optimal, N (%)	131 (42.3%)	18 (40%)	16 (30.8%)	1.0	0.2	0.5
Suboptimal, N (%)	179 (57.7%)	27 (60%)	36 (69.2%)			
Diet Inflammatory Index (DII)						
Anti-inflammatory, N (%)	250 (80.6%)	35 (77.8%)	30 (57.7%)	0.9	<0.001	0.06
Neutral or Pro-inflammatory, N (%)	60 (19.4%)	10 (22.2%)	22 (42.3%)			
Inventory of daily stressors						
No stress N (%)	241 (77.7%)	18 (40%)	31 (59.6%)	**0.005**	0.1	**0.008**
Stress N (%)	69 (22.3%)	27 (60%)	21 (40.4%)			
Initiative						
No problems N (%)	288(94%)	21(46.7%)	46 (88.5%)	**<0.001**	**0.04**	**<0.001**
Problems in EF N (%)	12(6%)	24(53.3%)	6 (11.5%)			
Working memory						
No problems N (%)	283(98%)	7(15.6%)	41 (78.8%)	**<0.001**	**0.001**	**<0.001**
Problems EF N (%)	17(2%)	38(84.4%)	11 (21.2%)			

Plan and organize						
No problems N (%)	280(95.8%)	17(33.4%)	48 (92.3%)	**<0.001**	0.05	**<0.001**
Problems in EF N (%)	20(4.2%)	28(37.8%)	4 (7.7%)			
Monitor						
No problems N (%)	280(95.8%)	15(33.4%)	47 (90.4%)	**<0.001**	0.3	**<0.001**
Problems EF N (%)	20(4.2%)	30(66.6%)	5 (9.6%)			
Material organized						
No problems N (%)	286(92.8%)	27(60%)	49 (94.2%)	**<0.001**	0.7	**0.002**
Problems EF N (%)	14(7.2%)	18(40%)	3 (5.8%)			
Behavioral Regulation Index (BRI)						
No problems N (%)	276(92%)	22(48.8%)	47 (90.4%)	**<0.001**	0.5	**<0.001**
Problems in EF N (%)	24(8%)	23(51.2%)	5 (9.6%)			
Emotional regulation index (ERI)						
No problems N (%)	266(92.1%)	28(62.2)	44 (84.6%)	**0.006**	0.2	**0.02**
Problems in EF N (%)	34(7.9%)	17(37.8%)	8 (15.4%)			
Externalizing problems (SDQ)						
No problems N (%)	280(94.5%)	14(31.2%)	30 (57.7%)	**<0.001**	0.2	**0.01**
Problems N (%)	20(5.5%)	31(68.8%)	22			

			(42.3%)			
Internalizing problems (SDQ)						
No problems N (%)	193(64.3%)	18(40%)	25 (48.1%)	**<0.001**	**0.001**	0.5
Problems N (%)	107(35.7%)	27(60%)	27 (51.9%)			
Learning difficulties (LD)						
No N (%)	310 (100%)	18 (40%)	0			
Yes N (%)	0	27 (60%)	53(100%)			
Metacognition index (MI)						
No problems N (%)	310 (100%)	27 (60%)	53(100%)			
Total problems in EF N (%)	0	18 (40%)	0			

Controls are those subjects without any cognitive process problems measured by the BRIEF (EFP) or DSM-V (LD). Data are expressed as mean and standard deviation (SD) for continuous variables, and as frequencies and percentages (%) of the total sample for categorical variables. Statistical tests: Mann-Whitney U and Student's t-test were used to compare quantitative variables, and the chi-square test was used to compare qualitative variables. Pairwise comparisons were conducted to examine between-group differences. Statistical significance was established at $p < 0.05$ for the following pair-comparisons: [a] for Control vs. EFP, [b] for Control vs. LD, and [c] for EFP vs. LD. [ɣ]BMI z-scores were used to classify participants with obesity/overweight and normal weight/underweight following WHO international cut-offs. Abbreviations: EEP: Executive function problems; LD: Learning difficulties; WHO: World Health Organization

3.2 Differences in dietary indexes and RDA compliance across cognitive groups

Fewer than half (40%) of children and adolescents demonstrated good adherence to the Mediterranean diet, with no significant differences across the study groups. However, exploratory analysis of dietary index scores showed the LD group exhibited significantly higher adherence to a pro-

inflammatory dietary pattern ($p < 0.001$) (Table 1). Consistently, in the LASSO logistic regression model, the DII emerged as the main contributor distinguishing the LD group from both controls and the EFP (Table S2).

In the analysis of RDA compliance, the majority of the population showed an increase in the consumption of processed foods (refined cereals, processed meat, miscellaneous, pastries and dairy desserts) and a decrease in vegetables, fresh fruit, legumes, nuts and whole grains intakes (Table S3). However, several dietary components were identified as the strongest contributors to differences across groups, especially for the LD group. While intakes above RDA of pastries and optimal of potassium were the only dietary factors associated with the EFP group, intakes below the RDA for oily fish ($\beta = 0.15$), nuts ($\beta = 0.36$), vegetables ($\beta = 0.16$) and vitamin C ($\beta = 0.12$), as well as intakes exceeding the RDA for dairy products ($\beta = 0.43$), miscellaneous foods ($\beta = 0.11$), total fats ($\beta = 0.0$), and the LA/ALA ratio ($\beta = 0.38$) were among the dietary factors contributing most strongly to distinguishing the LD group from controls. Conversely, high consumption of refined cereals ($\beta = -0.22$) and an intake below the RDA for LA ($\beta = -0.10$) characterized controls compared to the LD group (Table 2).

Regarding the comparison between LD and EFP groups, consumption of refined cereals ($\beta = -0.38$), pastries ($\beta = -0.10$) was higher in the EFP group, while consumption of oily fish ($\beta = 0.10$) below the RDA and of miscellaneous foods ($\beta = 0.50$) and white fish and seafood ($\beta = 0.19$) above the RDA contributed to the LD group. In addition, higher total protein ($\beta = -0.50$) intakes were observed in EFP group, whereas lower intakes of vitamin B6 ($\beta = 0.008$) and ALA ($\beta = 0.68$) were found in the LD group (Table 2).

Table 2. Pairwise comparisons of groups for dietary patterns and RDA compliance using Lasso logistic regression

Dietary patterns & RDA variables	*Differences across cognitive groups		
	EFP vs control[a]	LD vs control[b]	EFP vs LD[c]

	λ.min	λ.1se	λ.min	λ.1se	λ.min	λ.1se
Food dietary pattern (Ref =moderate)						
Whole-Grain Cereals (Low)	0.00	0.00	0.00	0.00	0.00	0.00
Whole-Grain Cereals (High)	0.00	0.00	0.00	0.00	-0.50	0.00
Fish and Dairy (Low)	0.00	0.00	-0.20	0.00	0.00	0.00
Fish and Dairy (High)	0.00	0.00	0.00	0.00	-0.79	0.00
Nutrients dietary pattern (Ref =moderate)						
Plant-Based Nutrient (Low)	0.00	0.00	0.60	0.00	0.00	0.00
Plant-Based Nutrient (High)	0.00	0.00	0.00	0.00	0.51	0.00
Group-B vitamins and Iron (High)	-0.32	0.00	0.00	0.00	0.00	0.00
Group-B vitamins and Iron (Low)	0.00	0.15	-0.15	0.00	-1.0	0.00
Trans fatty acids and starches (Low)	0.00	0.00	-0.26	0.00	0.00	0.00
Trans fatty acids and starches (High)	0.00	0.00	0.40	0.00	0.1	0.00
Food (RDA) (Ref = Complies)						
Nuts (Below)	0.00	0.00	0.36	0.00	0.00	0.00
Vegetables (Below)	0.00	0.00	0.16	0.00	0.00	0.00
Dairy products (Above)	0.00	0.00	0.43	0.00	0.00	0.00
Refined cereals (Above)	0.00	0.00	-0.22	0.00	-0.38	0.00
White Fish and seafood (Above)	0.00	0.00	0.00	0.00	0.19	0.00
Oily Fish (Below)	0.00	0.00	0.15	0.00	0.10	0.00
Miscellaneous (Above)	0.00	0.00	0.11	0.00	0.50	0.00
Pastries (Above)	0.25	0.00	0.00	0.00	-0.10	0.00
Nutrients (RDA) (Ref = optimal)						
Protein (Below)	0.00	0.00	0.00	0.00	0.00	0.00
Protein (Above)	0.00	0.00	0.00	0.00	-0.50	0.00
Total Fats (Above)	0.00	0.00	0.10	0.00	0.00	0.00
ALA (Below)	0.00	0.00	0.00	0.00	0.68	0.00
LA (Below)	0.00	0.00	-0.10	0.00	0.00	0.00
Ratio LA/ALA (Below)	0.00	0.00	0.00	0.00	0.00	0.00
Ratio LA/ALA (Above)	0.00	0.00	0.38	0.00	0.00	0.00
Potassium (Below)	-0.12	0.00	0.00	0.00	0.20	0.00
Vitamin B6 (Below)	0.00	0.00	0.00	0.00	0.01	0.00

Vitamin C (Below)	0.00	0.00	0.12	0.00	0.00	0.00

For each pairwise comparison (EFP vs controls; LD vs control and EFP vs LD), results are shown for the optimal penalization parameter (λ.min) and the parsimonious penalization parameter (λ.1se). For food-based dietary patterns "moderate" indicates the reference intake. For food groups, "complies" corresponds to the reference intake, whereas for nutrients, "optimal" corresponds to the reference intake. Coefficients correspond to the adherence to dietary patterns and RDA compliance selected by LASSO under each λ. Low and high refer to the adherence level; Below and above refer to RDA levels. [a]Positive coefficients indicate greater contribution to the EFP group; negative coefficients indicate greater contribution to the control group.[b]Positive coefficients indicate greater contribution to LD group; negative coefficients indicate greater contribution to the control group. [c]Positive coefficients indicate greater contribution to the LD group and negative coefficients indicate greater contribution to the EFP group. Abbreviations: EFP = executive function problems; LD = learning disorders; RDA = recommended daily intake.

3.3 Differences in dietary patterns across cognitive groups.

A total of six food-based dietary patterns and eight nutrient-based dietary patterns were identified (Additional material S3), with similar trends across both age groups (children and adolescents) (Figure S1).

Comparison of adherence to dietary patterns identified by the descriptive analysis revealed lower adherence to the vitamin-B group and iron pattern in the EFP compared to controls (p=0.006). Moreover, higher adherence to the trans fatty acids and starches pattern (p = 0.004), along with lower adherence to the plant-nutrients pattern (p = 0.01), was most strongly linked to LD (Table S4).

The LASSO model confirmed that lower adherence to vitamin Group-B and iron pattern was the main contributor to EFP, whereas lower plant-based nutrient adherence (β = 0.60) and higher adherence to trans fatty acids and starches pattern (β = 0.40) were the main contributors to LD. Conversely, low adherence to high-fat and cholesterol, fish and dairy

products intake pattern, and whole grains and oats pattern were linked to the control group (Table 2).

LASSO-logistic regression comparing EFP and LD showed similar trends in dietary pattern adherence to those observed when comparing each cognitive group with controls. However, when comparing EFP with LD, we also found lower adherence to the animal and refined cereals pattern associated with LD (Table 2).

3.4 Differences in individual nutrients and food intakes across cognitive groups

Comparison of individual nutrient and food intakes, adjusted for energy intake, identified in the descriptive analysis revealed lower consumption of vitamin B1 and pantothenic acid and higher consumption of eggs in the EFP group than in controls (Table S5). Meanwhile, we observed higher intakes of fatty acids, including arachidonic acid and other SFAs, sugars and starch, which were associated with lower consumption of fermentable carbohydrate-rich vegetables (e.g., broccoli, spinach, beetroot), soluble and insoluble fiber, and essential micronutrients such as potassium, magnesium, vitamin B6, folate, vitamin C, anthocyanins and polyphenols in the LD group compared with controls (Table S5)

The analysis of energy-adjusted foods and nutrient intakes by the LASSO-logistic regression also revealed that potassium ($\beta = 0.10$) and eggs ($\beta = 0.05$) were the main contributors to EFP. Meanwhile, the most influential individual dietary contributors to the LD group were higher intakes of saturated fatty acids (particularly palmitic acid; $\beta = 0.23$), as well as trans fatty acids ($\beta = 0.11$) and arachidonic acid ($\beta = 0.14$). A higher intake of vitamin C was more associated with control (Table 3).

Additionally, LASSO logistic regression models identified distinct individual nutrients and foods as contributors to EFP and LD: vitamin E, Vitamin C, biotin, fish and seafood, eggs, refined cereals and processed meet intake were more strongly associated with EFP. However, greater intake of oleic, arachidonic and stearic acid, sodium, pantothenic acid, dairy desserts, oats, pastries and white meat were among the leading dietary items associated with LD (Table 3).

Table 3. Pairwise comparisons of groups for energy-adjusted nutrient and food intakes using Lasso logistic regression

Food and nutrients (g/day)	*Differences across cognitive groups					
	EFP vs control[a]		LD vs control[b]		EFP vs LD[c]	
	λ.min	λ.1se	λ.min	λ.1se	λ.min	λ.1se
Eggs	0.05	0.00	0.00	0.00	0.00	-0.03
Pastries	0.00	0.00	0.00	0.00	0.00	0.21
Dairy desserts	0.00	0.00	0.00	0.00	0.00	0.03
Refined cereals	0.00	0.00	0.00	0.00	-0.14	-0.24
Seed bread and oat	0.00	0.00	0.00	0.00	0.10	0.07
Fish (lean and oily) and seafood	0.00	0.00	0.00	0.00	0.00	-0.19
Processed Meat	0.00	0.00	0.00	0.00	0.00	-0.34
Fatty acids, total trans (g)	0.00	0.00	0.40	0.00	0.33	0.19
Fatty acid 16:0 (palmitic acid) (g)	0.00	0.00	0.50	0.00	0.00	0.00
Fatty acid 20:4 n-6 (arachidonic acid) (g)	0.00	0.00	0.05	0.00	0.00	0.53
Fatty acid, 18:0 (stearic acid) (g)	0.00	0.00	0.00	0.00	0.00	0.25
Fatty acid 18:1 n-9 cis (oleic acid) (g)	0.00	0.00	0.00	0.00	0.00	0.20
Vitamin E (tocopherol) (mg)	0.00	0.00	0.00	0.00	-0.16	-0.15
Biotin (ug)	0.00	0.00	0.00	0.00	0.00	-0.39
Pantothenic acid (mg)	0.00	0.00	0.00	0.00	0.00	0.32
Vitamin C, total ascorbic acid (mg)	0.00	0.00	-0.08	0.00	-0.24	-0.05
Pantothenic acid (mg)	0.00	0.00	0.00	0.00	0.00	0.32
Vitamin A, RAE (ug)	0.00	0.00	0.00	0.00	0.00	0.008
Potassium, K (mg)	0.10	0.00	0.00	0.00	0.00	0.00
Sodium, Na (mg)	0.00	0.00	0.00	0.00	0.00	0.53

* For each pairwise comparison (EFP vs controls; LD vs control and LD vs EFP), results are shown for the optimal penalization parameter (λ.min) and the parsimonious penalization parameter (λ.1se). Coefficients correspond to the nutrients and food selected by LASSO under each λ. [a]Positive coefficients indicate greater contribution to the EFP group; negative coefficients indicate greater contribution to the control group.[b]Positive coefficients indicate greater contribution to LD group; negative coefficients indicate greater contribution to the control group. [c]Positive coefficients indicate greater contribution to the LD group and negative coefficients indicate greater contribution to the EFP group. Abbreviations: EFP = executive function problems; LD = learning disorders.

3.5 Alpha and beta diversity differences across cognitive groups

We found no differences in alpha diversity between groups with EFP (Figure S2A) or LD (Figure S2B) and control individuals. However, we observed higher alpha diversity, as indicated by the Shannon ($p = 0.02$) and Inverse Simpson ($p = 0.02$) indices (Figure 2A), in the group with EFP than in the group with LD.

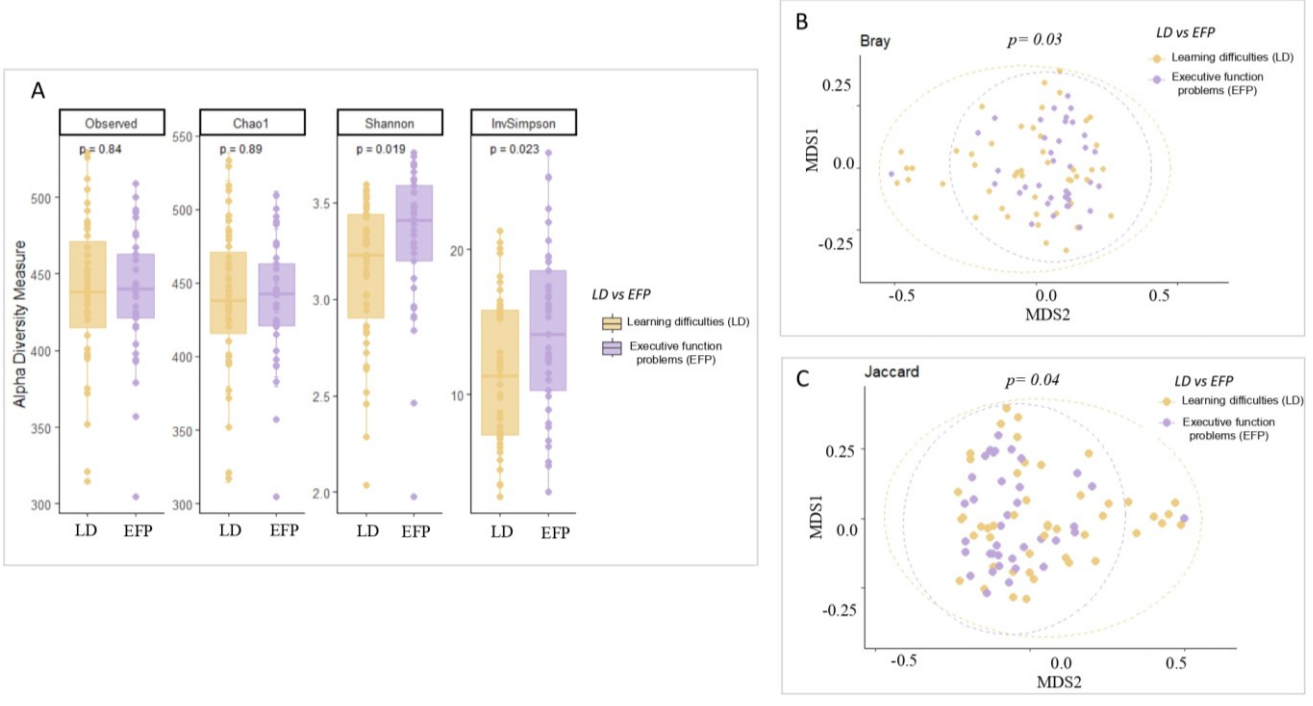
Regarding beta diversity, differences in beta diversity were not found between the EFP group and control group (Figure S2C-D), whereas we found weaker differences ($p < 0.1$) between the LD group and controls using Bray-Curtis and Jaccard distance (Figures S2E-F). However, we found significant differences between the EFP and LD groups in beta diversity assessed through both Bray-Curtis ($p=0.03$) and Jaccard distances ($p=0.04$) (Figure 2B-C).

3.6 Differences in the abundance of gut bacterial species across cognitive groups

Differentially abundant species were found in the three study groups: 35 species related to EFP compared to control (17 enriched, 18 depleted), 43 species related to LD compared to control (19 enriched, 24 depleted) and 48 related to LD compared to EFP group (23 enriched and 25 depleted). The complete comparisons list can be found in Tables S6-S8 and Figure S3.

Individuals with EFP show an enrichment in *Alistipes onderdonkii*, Clostridium baratii, Staphylococcus aureus, and species of the genera Bacteroides, Suterella, and Dialister. There is also a notable depletion in lactic acid bacteria (LAB) such as *Streptococcus thermophilus*, *Leuconostoc mesenteroides* and *Lactobacillus delbrueckii* compared to control individuals (Table S6).

Figure 2. Alpha and beta diversity analysis of the gut microbiota from subjects of different cognitive impairment groups (executive function problems and learning difficulties). (A) Alpha diversity indexes and statistical relevance through Wilcoxon test. **(B and C)** Beta diversity indexes (Bray-Curtis and Jaccard, respectively) and statistical relevance based on PERMANOVA permutations.



Regarding LD, we found an enrichment in Bifidobacterium species, mainly B. dentium, B. breve and B. longum along with *Blautia pseudococcoides* and oral bacteria (*Haemophilus pittmaniae, Rothia aeria* and *Parvimonas micra*), and a depletion of *Paraprevotella xylaniphila* and Coprococcus spp (Table S7).

When comparing the two groups with cognitive impairments, lactic acid bacteria (LAB) such as *Lactobacillus delbrueckii* and *Streptococcus termophilus* remained depleted in individuals with EFP relative to those with LD. Additionally, *Akkermansia muciniphila*, *Enterobacter ludwigii*, and *Sellimonas intestinalis* was increased in the LD group, whereas *Faecalibacterium duncaniae* and *Segatella copri* were enriched in the EFP group (Table S8).

3.7. Diet -microbiota interactions accounting for different cognitive groups

Modules based on food groups' interactions with microbial taxa, which were relevant for comparisons of EFP and LD with controls, exhibited a highly connected interaction network with more dietary items than bacterial species (Figure 3). In the EFP comparison with controls, a relevant module was identified for bacterial taxa and food groups, comprising 22 nodes (14 corresponding to food groups and 8 to bacterial species), with an AUROC = 0.63 and a median sCC = 0.05 (Figure 3A).

Within the EFP group, a main cluster is centered on *Streptococcus sanguinis*, *Actinomyces naeslundii*, and *Mogibacterium diversus*, which were positively correlated with sugar-based elements, such as sugar confectionery and sugar beverages, based on Spearman correlation indices, with *A. naeslundii* showing a significant positive correlation with pastries ($p < 0.05$). All cluster elements also showed significant negative correlation with fresh fruits and juices natural ($p < 0.01$) (Figure 4A).

LD-based interactions include a module with 22 nodes (15 food groups and 7 bacteria), with AUROC = 0.61 and median sCC = 0.05. The main clusters are centered on *Coprococcus catus*, with a high interaction with fruits, seed bread and oat, dairy desserts, potatoes, and refined bread, pasta, and rice, and on Bifidobacterium spp. (*B. breve, B longum*) and *Blautia pseudococcoides*, which interact with yogurt, milk and cheese, dairy desserts, legumes, fish and seafood, refined pasta and rice and seed bread and oats (Figure 3B). In the case of Bifidobacterium species, there are significant positive correlations with dairy desserts ($p < 0.05$) and significant negative correlations with refined pasta and rice ($p < 0.001$).

Despite not having significant correlations values, *C. catus* shows a tendency to correlated positively with the fresh fruit and juices and seed bread and oat groups, but negatively with food groups like yogurt, milk and cheese, fish and seafood and vegetables (Figure 4B).

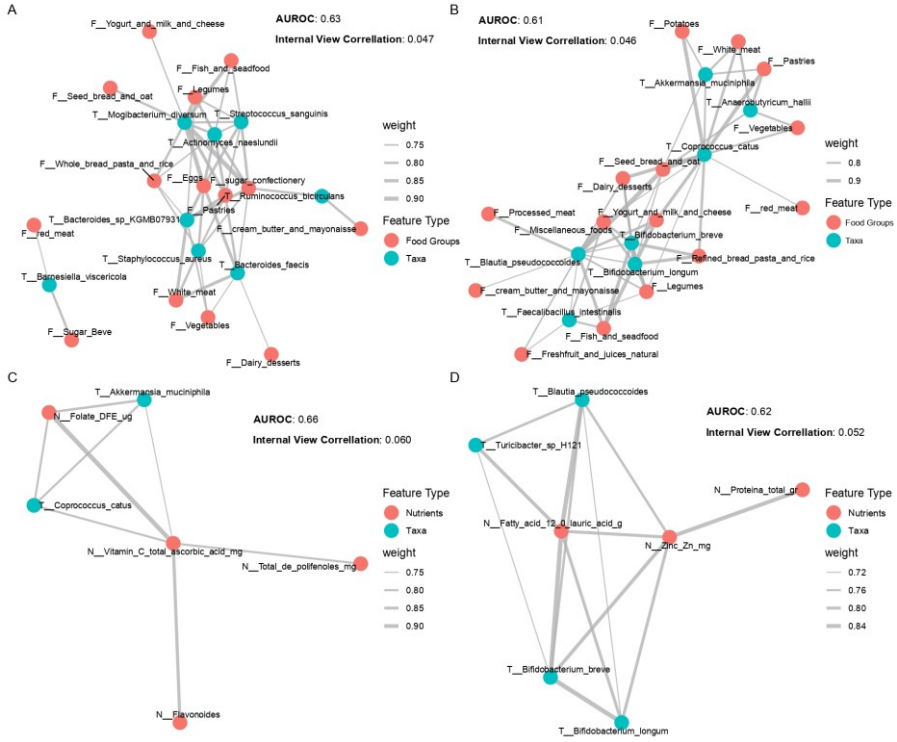


Figure 3. Interaction network modules based on correlations between bacterial taxa and dietary elements. Each one of the modules is composed of nodes representing bacterial data (T, blue nodes) and dietary data, either food groups (F, red nodes) or Nutrients (N, red nodes). The thickness of the edges connecting the network nodes is related to the importance (weight) of the interaction. Each module is accompanied by its Area Under the Receiver Operating Characteristic Curve (AUROC), which reflects the network's ability to classify between controls and cognitively impaired individuals. The Internal View Correlation corresponds to the median sparse Canonical Correlation (sCC) across all interactions in the network. (**A)** Interaction network module of bacterial species and food groups related to the executive function problems (EFP) group versus

controls comparison. **(B)** Interaction network of bacterial species and food groups related to the learning difficulties (LD) versus controls comparison. **(C)** and **(D)** Interaction network modules of bacterial species and nutrients related to the LD versus Controls comparison.

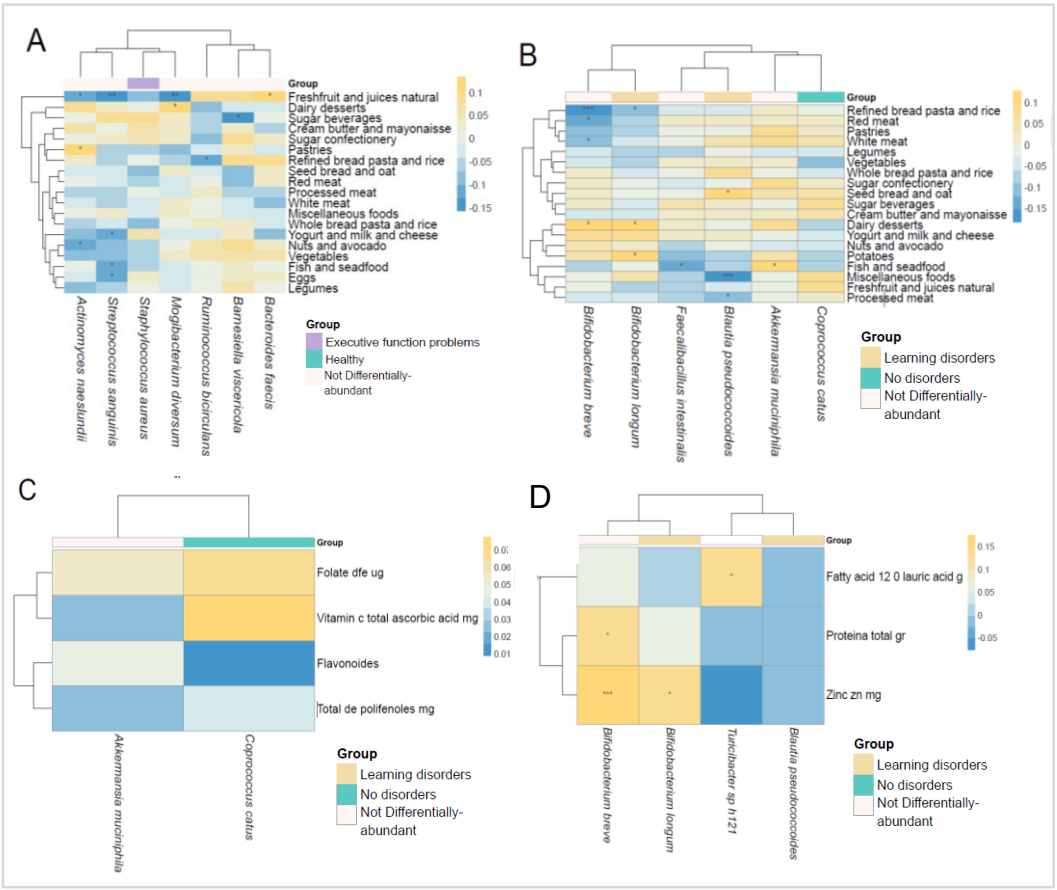


Figure 4. Spearman correlation coefficient heatmaps of the taxonomic and dietary features for the significant interaction modules. Selected bacterial taxa that appeared as differentially abundant are highlighted based on the group comparison. (A) Correlation heatmap corresponding to the interaction module of bacterial species and good groups related to the executive function problems (EFP) versus controls comparison. (B) Correlation heatmap corresponding to the interaction module of bacterial species and food groups related to learning

difficulties (LD) versus controls comparisons. (C) and (D) Correlation heatmaps corresponding with the interaction network modules of bacterial species and nutrients related to the LD versus controls comparison. * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Regarding nutrient-based interactions, two modules were identified exclusively in the LD group compared to the control group. The first module includes 4 nutrients and 2 bacterial species (AUROC = 0.66, median sCC of 0.06) (Figure 3C), with vitamin C linked to flavonoids (weight = 0.85), polyphenols (weight = 0.80) and folate (weight = 0.90), A. muciniphila (weight = 0.75) and C. catus (weight = 0.80); while the second includes 7 nodes (3 nutrients and 4 bacteria, AUCROC = 0.62, median correlation = 0.05) (Figure 3D) connected through Bifidobacterium spp. (B. breve and B. longum) (weight = 0.840), zinc (weight = 0.80), lauric acid (weight = 0.84), and *B. pseudococcoides* (weight = 0.76), but only *B. breve* is correlated with *Turicibacter sp. H121*, while total protein node is only connected to zinc (weight = 0.80)

Although not significant, all relations with folate and vitamin C show a positive Spearman correlation coefficient with *A. municiphila* and *C. catus* in the first module (Figure 4C). In the second module, the Bifidobacterium species show a significant positive correlation with zinc ($p < 0.001$), while *Turicibacter sp H121* shows a significant positive correlation with lauric acid ($p < 0.05$) (Figure 4D).

In the mediation analysis, four relevant mediation effects appeared within the LD group at the nutrients, food groups, and food patterns levels (Figure 5). At the nutrient level, the lauric acid association (gamma = 0.042, p = 0.039) appears significantly mediated by *Lacticaseibacillus paracasei* (beta=0.086, p = 0.007) (Figure 5A). Regarding food groups, seed bread and oats groups (gamma = 0.042, $p < 0.001$) appears mediated by *Methanobrevibacter smithii* (beta = 0.131, p = 0.049) (Figure 5B) and Segatella copri (beta = 0.13, p = 0.03) (Figure 5C), which also mediated the association of fermented vegetables (gamma = 0.02, p = 0.02) (Figure 5D).

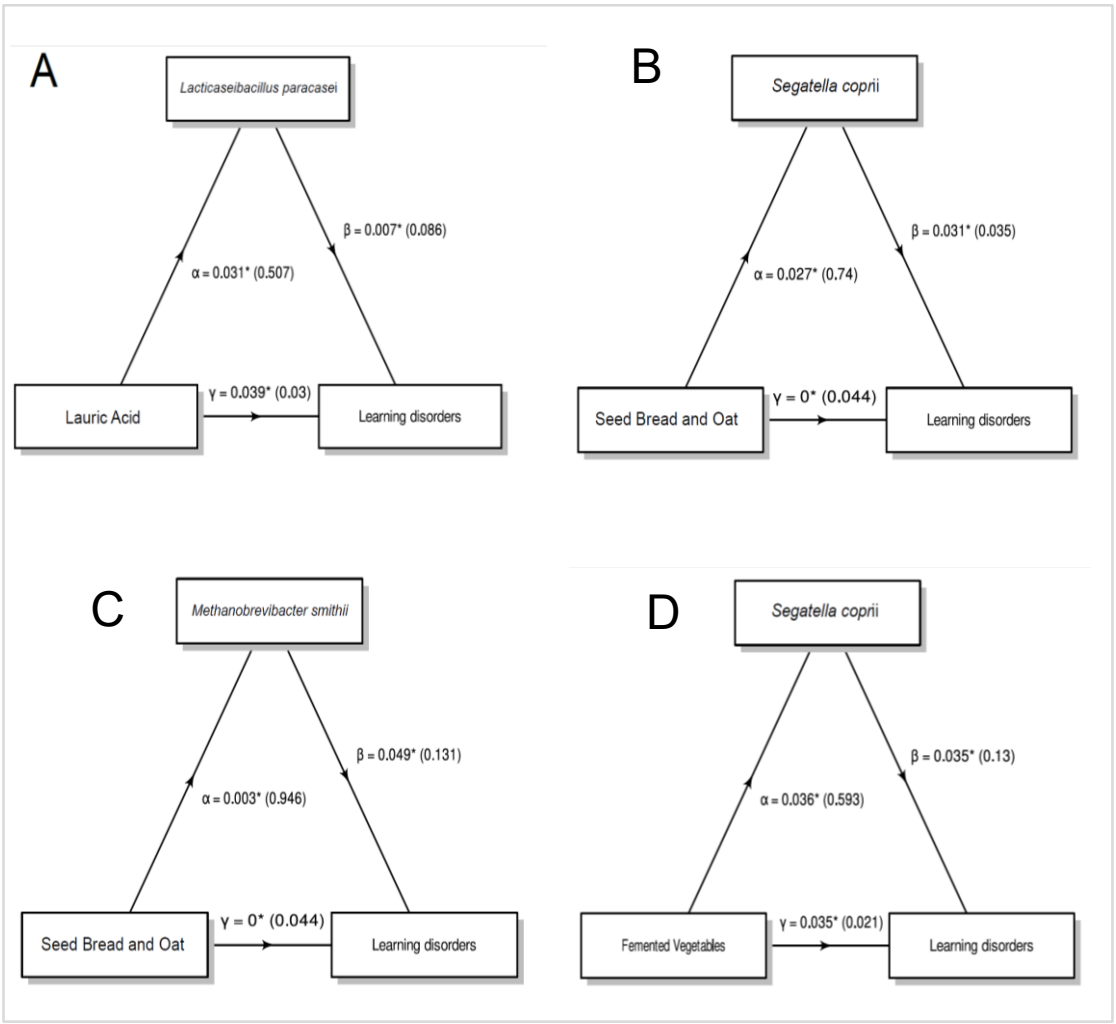


Figure 5. Non-parametric mediation analysis of dietary elements through bacterial taxa within cognitive-impairment groups. Alpha (α) reefers of the effect of the dietary element (effector) over the bacterial species (mediator); Beta (β) points to the effect of the mediator over the cognitive impairment classification of cases and controls (outcome) and Gamma (γ) shows the direct effect of the effector over the outcome. (**A)** mediation of *Lacticaseibacillus paracasei* over the

lauric acid effect on the Learning difficulties (LD) outcome. (**B)** mediation of *Segatella copri* over the seed bread and oat effect on the LD outcome. (**C)** mediation of *Methanobrevibacter smithii* over the seed bread and oat effect on the LD outcome. (**D)** mediation of *Segatella copri* over the effect of fermented vegetables over the LD outcome.

4. DISCUSSION

Our study provided new insights into the relationship among diet, gut microbiota, and the diet-gut microbe intersection with early signs of cognitive impairments in children and adolescents. Our findings especially highlight the role of the diet in subjects with LD, who exhibited the lowest healthy dietary intake and the most distinct dietary habits, characterized by a high pro-inflammatory eating profile. Differential abundance of gut bacterial species effectively discriminates among the three study groups (controls, EFP, and LD), although the main differences in whole microbiota structure (alpha- and beta-diversity) were observed between EFP and LD. Consistent with this, diet–microbiota interactions were predominantly observed in the LD group, suggesting that specific bacteria mediate the effects of certain dietary components, particularly on learning impairments. Collectively, these results provide new evidence on how diet–microbiota interactions influence different neurocognitive profiles in young populations.

Despite extensive overlap among prevalent taxa in healthy gut microbiomes [40] and the fact that our study investigated subclinical cognitive impairments in an otherwise healthy population, we identified specific species that discriminate among different cognition profiles. Notably, the EFP group showed enrichment for opportunistic pathogens, including *Staphylococcus aureus* and *Sutterella* and *Dialister* species[41,42], which were also previously associated with poorer cognitive performance [43]. Moreover, the EFP group showed a depletion of LAB species (e.g. *Streptococcus thermophilus, Leuconostoc mesenteroides*, and *Lactobacillus delbrueckii*), which have potential roles in reducing inflammation and synthesizing neurotransmitters (GABA and acetylcholine) linked to cognitive abilities [44,45]. In particular, supplementation with *L. delbrueckii* has been associated with cognitive improvements in older adults with mild cognitive impairment by modulating

neuroinflammatory pathways via the production of indole-derived metabolites [46]. The microbial metabolite indole-3-propionic acid has also been suggested to ameliorate social and cognitive memory deficits characteristic of neurodevelopmental disorders, such as ASD, in rodent studies [47].

Regarding the group with LD compared to controls, there was a notably enrichment of *Bifidobacterium* species (*B. breve*, *B. animalis* and *B.longum*) and *Blautia pseudococcoides* . However, *Bifidobacterium* species have been acknowledged for their potential neuroprotective capabilities playing roles in neuroplasticity and hippocampal protection in mouse models [45,48–50]. In spite of that, the effects of *B. longum* could be context-dependent, exacerbating ADHD-like behaviors in young adults [51], but improving social and cognitive function in older adults [52]. In this context, the possibility that the observed disparity in effects is due not only to the experimental models used but also to strain differences has not been investigated. Moreover, the associations found in our study may be attributable to the LD group's higher consumption of dairy products, often containing bifidobacteria, and they do not necessarily imply causation for cognitive impairment.

Conversely, depletion of *Paraprevotella xylaniphila* and *Coprococcus species* (*C. euctatus* and *C. catus*), which are known to thrive in plant-based diets [53] was found in LD group which is line with low consumption of related foods and nutrients. *Coprococcus* spp. may mediate neuroprotective effects via SCFA production (specifically butyrate) as well as via dopamine and tryptophan synthesis pathways [54,55], and the depletion of this was linked to depression in adolescents and LD in children [56,57]. Notably, *Alistipes ondedonkii,* previously proposed as a biomarker for neurological disorders [58,59], appeared enriched in both EFP and LD, hinting a common ground for both cognitive-impairment profiles.

Concerning the group with LD compared to EFP, some *Bacteroides* species (*B. faecis* and *B. stercoris*) were overrepresented in the EFP group and, among others, *Akkermansia municiphila* and *Enterobacter ludwigii* in the LD group. In line with this, *A. municiphila* and *B. stercoris* were previously associated with impaired language, memory and social isolation, and *B. faecis* was linked to ADHD [60–63]. Whereas animal models suggest that *Enterobacter ludwigii* may influence fundamental processes of neuronal development [64]

The particularities of the dietary intakes could also explain differences in the composition of the gut microbiota [65,66], which in turn could mediate part of the dietary effects on the EFP [67,68], as well as in LD and broader behavioral, and emotional processes [14,69] In fact, in the EFP group, the diet shows interesting interactions with microbial taxa. Thus, a pattern of high sugar confectionery and pastries consumption, both refined sugar-related food groups, correlates with the abundance of opportunistic pathogenic bacteria of the oral cavity and upper intestinal tract, including *Streptococcus sanguinis*, *Actinomyces naeslundii*, and *Mogibacterium diversus*. The prevalence of these species is correlated with periodontal diseases and poor oral health status [70], with the latter species identified as a potential contributor to impairments in speech, language, learning, and school performance in younger populations [71–73]. Similarly, *Dialister* species enriched in the EFP group were correlated with processed meat intake, which is highly consumed by this group and may promote a pro-inflammatory milieu that favors the overgrowth of opportunistic pathogenic species in the gut [74].

Regarding the LD comparison, the first interaction module shows a positive interaction between Vitamin C (ascorbic acid), polyphenols (highlighting flavonoids), and folate, with *A. municiphila* and *C. catu*s. Antioxidant compounds such as polyphenols and vitamin C, as well as methyl donors as folate, are critical nutrients for neurodevelopment [75] and can influence gut microbiota composition through intestinal mucosal integrity and the production of microbiota-derived metabolites, particularly SCFAs [76,77].

Following with the LD-comparison, the food group-bacteria module was formed by *C. catus, A. muciniphila* and *Anarobutyricum hallii*, which positively correlated with fruits, seed bread and oat and also with higher intake of fish and seafood.

As previously mentioned, depletion of *C. catus* has been related to a reduced intake of a plant-based, fruit-rich dietary pattern [78], which could explain our positive correlation, but non-significant, of this bacterial species with fruits and vitamin C. By contrast, *Akkermansia* is enriched, probably due to a slight positive correlation with fish and seafood, dairy desserts, and pastries, which were consumed in greater quantities in the LD group. In this regard, it appears that *Akkermansia* has greater adaptability to pro-inflammatory dietary environments[79,80].

Another co-occurring food group-bacteria cluster formed by *B. longum, B. breve* and *B. pseudococoides* was identified, positively correlated with dairy products and negatively with refined grains and other animal foods in LD group. The increased consumption of dairy products (mostly dairy-based desserts, as seen through the significant Spearman coefficient) probably explain the increased abundance of bifidobacteria in the LD group, as speculated before.

In line with these findings, the nutrient-microbiota module showed that lauric acid and zinc coexist with *Bifidobacterium* spp., *B. pseudococoides*, and *Turicibacter*, reinforcing previous observations that the intake of dairy products, which are known sources of these nutrients [81], could help explain the composition of the gut microbiota in the LD group. Also, the anti-microbial properties of lauric acid could explain an increase of some commensals such as *Turicibacter* species [82].

Although these network associations should be interpreted as co-occurrence of dietary-microbial patterns rather than causal biological effects, these findings allow the identification of interactions underlying potential mechanistic hypotheses.

Mediation analyses confirmed lauric acid and oats as relevant dietary factors in the LD group. Lauric acid's associations appeared mediated by *Lacticaseibacillus paracasei*, which could be related to the higher dairy foods intake. Moreover, consistent evidence links dairy intake to reduced *Coprococcus* abundance, as well as higher *Lactobacillus* and *Bifidobacterium* levels (80), as well as improved cortical morphology and enhanced executive functioning [83–85]. Furthermore, LAB-derived L-lactate may support synaptic plasticity, while excess D-lactate may impair cognitive function [44]. This paradox indicates that the presumed beneficial roles of bifidobacteria, LAB, and dairy metabolites may be outweighed by other nutrient–microbe interactions in the LD group.

Furthermore, in the group with LD, the intake of high-fiber foods such as fermented vegetables and oats, mediated effects through *Methanobrevibacter smithii* and *Segatella copri,* both taxa responsive to fiber intake [86,87], which theoretically could generate neuroprotective metabolites (e.g SCFAs) [88].

Although these relationships may be bidirectional, our mediation models, partly supported by prior preclinical and epidemiological evidence [89–91], favor a pathway in which diet shapes the microbiota, which then influences cognitive outcomes. Anyway, these findings remain

exploratory, underscoring the need for longitudinal and intervention studies to confirm causality and disentangle diet–microbiota–cognition mechanisms.

Our study has several limitations that should be considered when interpreting the findings. First, its cross-sectional design precludes causal inference. Our results suggest links between dietary patterns, gut microbiota profiles, and cognitive difficulties, but the directionality of these associations cannot be determined, and reverse causality remains possible. Second, characterizing EFP and LD in children and adolescents using dietary and meta-taxonomic profiles is inherently challenging, given the heterogeneity of cognitive phenotypes and the complexity of the interacting variables used for classification. Third, the identified associations between diet, microbial taxa, and cognitive outcomes were modest, and longitudinal, hypothesis-driven studies are needed to validate these results.

5. CONCLUSIONS

Stratification between EFP, LD, and controls revealed group-specific differences in the gut microbiota composition, particularly in oral and LAB species and SCFA-producing bacterial taxa such as *Bifidobacterium and Coprococcus,* and *Faecalibacterium*, suggesting potential targets for monitoring and intervention. Meanwhile, dietary pattern differences revealed that the LD group adhered more to a pro-inflammatory diet, rich in processed foods, fats, and sugars, but low in plant-based foods, whereas the EFP group showed lower adherence to vitamin B and iron-rich foods, whole grains, and oats.

The integration of dietary components and microbial data highlighted nuanced, group-specific patterns, revealing interactions between opportunistic oral pathogens and pro-inflammatory dietary components, including sugars and meats, while antioxidant nutrients were connected to butyrate producers and bacterial species involved in the regulation of intestinal mucosal integrity. These findings suggest that diet-induced shifts in gut microbial communities may contribute to inflammation-mediated cognitive outcomes, while also supporting a role for bacterial taxa responsive to plant-based foods (e.g. oats and green vegetables) and their metabolites in gut–brain-axis communication. Together, these results highlight diet as a possible strategy to modulate both microbial and

neurochemical pathways underlying cognitive function[14]. Nevertheless, further longitudinal and mechanistic studies are needed to progress toward causality and the underlying mechanisms of those microbiota–cognition interactions.

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AUTHORS' CONTRIBUTION

Conceptualization: YS and PC; Experimental Investigation: AL, T-E V, R-R SM, S A- G, I S-C; Data curation and analysis: AL, R-G S, T-E V, R-R SM; Funding acquisition: YS; Original draft and edits: AL, R-G S, YS; Final editing and approval: all authors.

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Supplementary material and methods

S1. Data Collection

Parents of all participants provided information on sociodemographic variables, including age, sex, marital status, and parents' education. Perinatal and postnatal variables (gestational age, exclusive breastfeeding, maternal age at birth, childbirth complications, weight at birth, and delivery mode), clinical variables (intestinal transit evaluated by Bristol scale, sleep, allergy, respiratory disorders, chronic inflammatory bowel disease (IBD), antibiotics and non-antibiotic drugs according to Anatomical, Therapeutic, Chemical (ATC) Classifications System).

Anthropometric measures recorded included waist circumference (WC) (cm) and body mass index (BMI) (Kg/m2). The BMI z-score was calculated using international cutoff values according to World Health Organization (WHO) criteria. These z-scores were then classified into the following categories: thinness (-3 ≤ z-score < -2), normal weight (-2 ≤ z-score ≤ +1), overweight (+1 < z-score ≤ +3), and obesity (z-score > +3). This categorization provided a standardized framework to evaluate and compare the ponderal status of the participants[1)]

Concerning lifestyle factors, daily time spent on team sports, outdoor activities, and screen-based activities was assessed in accordance with World Health Organization (WHO) recommendations. The Childhood Daily Stress Inventory (IECI) was used for assessing the stress level of the population[2].

Physical activity was assessed using the Physical Activity Level Questionnaire (APALQ), a self-reported instrument that assesses the frequency, duration, and intensity of physical activity across contexts such as school, leisure time, and sports participation. The APALQ provides an overall physical activity index that classifies participants into low, moderate, or high activity levels based on adherence to international physical activity recommendations for children and adolescents[3].

Dietary quality was assessed using three complementary indices. Adherence to the Mediterranean diet was measured using the KIDMED index, a 16-item questionnaire assigning +1 for Mediterranean-favorable behaviors and –1 for unfavorable ones, with higher scores indicating better adherence.[4].Overall diet quality was evaluated through the Spanish

Healthy Eating Index (SHEI), which includes 10 food-group components scored from 0 to 10, summing to a total of 0–100 points, where higher scores reflect healthier dietary patterns[5]. Finally, the Dietary Inflammatory Index (DII), developed by Shivappa and Hébert, was used to estimate the inflammatory potential of the diet based on up to 45 dietary parameters; higher DII values denote a more pro-inflammatory diet, while lower or negative scores indicate a more anti-inflammatory profile[6,7].

Regarding psychological variables, the Strengths and Difficulties Questionnaire (SDQ) was used as a screening tool to facilitate early detection and evaluation of potential behavioral or emotional problems in children and adolescents aged 3 to 17. The SDQ assesses five domains: emotional and behavioral problems, hyperactivity, relationship problems with peers, and prosocial behavior, categorized into internalizing and externalizing subscales and a global scale. For each of the five domains, the score ranges from 0 to 10. The externalizing score ranges from 0 to 20 and is the sum of the behavior problems and hyperactivity scales. The internalizing score ranges from 0 to 20 and is the sum of the emotional and peer problems scales. Higher scores represent more behavioral problems. The population was classified according to the SDQ cut-off points established for the Spanish child and adolescent population into three categories: normal (0-15 points), subclinical (16-18 points) and clinical (19-40 points) [8,9].

The Behavior Rating Inventory of Executive Function (BRIEF) test provides a profile of impairment across the facets of Executive Functions (EF), including complex mental activities necessary for planning, organizing, guiding, reviewing, regulating, and evaluating behaviors required to adapt effectively to achieve goals. BRIEF consists of 86 items with three possible answers (1 = Never; 2 = Sometimes; 3 = Frequently) which evaluate three main indices composite by eight domains: Behavioral regulation Index (shift, emotional control and inhibit), Metacognition Index (initiation, working memory, plan/organize, task-monitor and organization of materials) and Global Index of Executive Function (Behavioral Index and Metacognition Index). The raw score was transformed into a T-score (mean = 50, SD = 10) by age and sex. A higher T-score indicates greater EF impairment: T-score values less than 60 indicate "no problem in the EF"; between 60 and 65 indicate "subclinical problems in the EF"; and 65 and higher indicate "clinical problems in the EF".

We used the first Diagnostic and Statistical Manual of Mental Disorders (DSM-5) diagnostic criterion for Specific Learning Disorders, which focuses on persistent academic difficulties in reading, writing, or mathematics. This criterion was operationalized through six items rated on a three-point scale (0 = no difficulties, 1 = slight difficulties, 2 = major difficulties). We used these indicators solely to identify potential subclinical learning difficulties, not for clinical diagnosis, as the remaining DSM-5 criteria (duration, response to intervention, and impact on daily functioning) were not assessed.

S2. Nutritional assessments: food, nutrients and dietary patterns

Food groups. The 22 food groups evaluated were: sugars beverages, fresh fruit, legumes, nuts, oils and margarine, vegetables, vegetables high in fermentable CHO, miscellaneous, sugar confectionery, eggs, pastries, dairy desserts, dairy products, potatoes, whole-grain cereals, refined cereals, seeded bread and oats, fish and seafood, red meat, white meat, processed meat, animal fats (butter, cream).

Nutrients. The 52 nutrients evaluated were: Water (g), total lipid (fat) (g), sugars (g), sucrose (g), starch, total (g), carbohydrate, by difference (g), fatty acids, total polyunsaturated (g), fatty acid 12:0 (lauric acid) (g), cholesterol (mg), fatty acids, total trans (g), fatty acid 16:0 (palmitic acid) (g), fatty acid 18:1 n-9 cis (oleic acid) (g), fatty acid 18:3 (g), fatty acid 20:4 n-6 (arachidonic acid) (g), fatty acids, total monounsaturated (g), fatty acids, total saturated (g), fatty acid 14:0 (myristic acid) (g), fatty acid 18:0 (stearic acid) (g), fatty acid 18:2 (g), fatty acid 20:5 (eicosapentaenoic acid) (g), fatty acid 22:6 n-3 (docosahexaenoic acid) (g), thiamin (mg), vitamin E (alpha-tocopherol) (mg), vitamin B-6 (mg), biotin (µg), niacin (mg), riboflavin (mg), vitamin D (D2 + D3) (µg), vitamin B-12 (µg), vitamin C, total ascorbic acid (mg), folate, DFE (µg), pantothenic acid (mg), vitamin A, RAE (µg), calcium, Ca (mg), potassium, K (mg), selenium, Se (µg), sodium, Na (mg), iron, Fe (mg), iodine (µg), magnesium, Mg (mg), zinc, Zn (mg), phosphorus, P (mg), total protein (g), animal protein (g), vegetal protein (g), total fiber (g), total soluble fiber (g), total insoluble fiber (g), flavonoids, isoflavones, anthocyanins, total polyphenols (mg)

Food-based dietary patterns identified. The first pattern (P1), termed the Plant-Based pattern, was characterized by high intakes of vegetables,

legumes, fruits, vegetable fats, and whole grains. The second pattern (P2), termed Animal and Refined Cereals, was characterized by refined cereals and red, white, and processed meats. The third pattern (P3) named Sugars and Processed Food included processed foods, sugar-sweetened beverages, pastries, and confectionery products. The fourth pattern (P4), named Whole-Grain Cereals, was mainly composed of whole grains, seed breads, and oats. The fifth pattern (P5) Fish and Dairy comprised fish and seafood, dairy products and dairy desserts. The sixth pattern (P6), Eggs and Animal Fats, was characterized high intake of eggs, cream, and butter (Figure S1A).

Nutrient-based dietary patterns identified. The first pattern (P1), termed the Plant-Based Nutrient pattern, included vegetal protein, total, soluble, and insoluble fiber, polysaccharides, digestible carbohydrates, total polyphenols, natural sugars, potassium, magnesium, biotin, and vitamins B6, B9, C, A, and E. The second pattern (P2), High-Fat and Cholesterol pattern, comprised total fats, cholesterol, monounsaturated fatty acids (MUFAs), saturated fatty acids (SFA), and zinc. The third pattern (P3), Unsaturated Fatty Acids (FAs), was characterized by polyunsaturated fatty acids (PUFAs), including ALA, LA, and oleic acid. The fourth pattern (P4) included Omega-3 PUFAs (EPA, DHA), Selenium and Zinc. The fifth pattern (P5), Lauric and Myristic Saturated FAs (C12-14 SFAs) and Other Minerals (iodine, calcium, phosphorus). The sixth pattern (P6) was defined by Vitamins B2, B12, and D. The seventh pattern (P7), Group-B vitamins and Iron, included group B vitamins (B1, B3, B5, and B6) and iron, while the eighth pattern (P8) was characterized by Trans Fatty Acids and Starches (Figure S1B).

S3. Metagenomic data processing

De novo assembly

Raw sequencing reads were first trimmed and quality-assessed using Trimmomatic v0.36 (1). During this step, adapter sequences were removed, and only reads longer than 75 bp and with a minimum base quality score of 20 were retained. This filtering ensured that the dataset consisted exclusively of high-quality reads suitable for subsequent analyses. To exclude host-derived sequences, the cleaned reads were aligned against the human reference genome (GRCh38) using Bowtie2 v2.4.5(2). Alignment results were converted from SAM to BAM format using SAMTools v1.14(3). Reads that did not map to the human genome—

representing potential microbial or other non-host sequences—were isolated and reconverted into separate FASTQ files. These files then underwent a secondary quality control check to confirm data integrity prior to assembly.

Metataxonomic analysis

Taxonomic profiling of the metagenomic reads was performed using Kraken2 v2.1.2(4) coupled with Bracken v2.6.2(5). Kraken2 classified sequences according to the Standard Kraken2 database with a confidence threshold of 0.1. The abundance of taxa at various hierarchical levels (species, genus, and phylum) was subsequently refined by Bracken, which normalized read length to 75 bp. Bracken output tables were reformatted into MetaPhlAn-compatible (MPA) style using scripts from KrakenTools v1.2, and all samples were merged into a consolidated abundance matrix. Before proceeding with downstream analyses, a rarefaction step was applied to retain bacterial taxa with prevalence greater than 1%. The rarefaction depth corresponded to the dataset's smallest sample size (5,240,270 reads), ensuring consistent sampling effort across all samples.

Diversity analysis

Alpha and Beta diversity were assessed using the phyloseq package as well as supporting packages such as fantaxtic (v0.20) and vegan (v2.6-4), for figure plotting and community-related analysis. Alpha-diversity indices included Observed abundance, Chao1, Shannon, and Inverse Simpson. To evaluate the statistical significance between the experimental groups using these four indexes, a Shapiro-Wilk normality test was performed to address the normality, applying a Student T test in normally distributed indexes and a U de Mann-Whitney Test for the rest, deeming significance at a p-value < 0.05

Beta-diversity was analyzed based on Jaccard's Similarity Index and Bray-Curtis Dissimilarity to establish possible statistical relevance with the permutational multivariate analysis of variance (PERMANOVA) test of the vegan package (v2.7.1)[10].

Differential abundance analysis

Differential abundance analysis of bacterial taxa was performed through the DESeq2 package, using the Likelihood Ratio Test approach (LRT) to identify differences between specific groups (e.g. EFP versus Control groups) and also adjusting for each one of the relevant confounding variables found in the exploratory analysis. This allows to identify differentially-abundant bacteria that are truly affected by the main variable comparedand not by the confounding factors. Only species with a prevalence exceeding 3% in the whole dataset and a False Discovery Rate (FDR) < 0.05 adjusted by the Benjamini-Hochberg method were considered as statistically significantly different.

S4. Multi-omics based MintTea pipeline

Each possible module is associated with an average sparse canonical correlation (sCC) value between its components to address the compositional nature and interrelations among taxonomic and dietary features. Additionally, a Random Forest classification model is trained on the aforementioned features, using the resulting Area Under the Receiver Operating Characteristic (AUROC) value to assess whether the elements in the interaction network are relevant for classifying between control and problem groups. Relevant modules are then compared with 99 randomly selected combinations of dietary and taxonomic elements, which undergo the same sCC value and AUROC calculations for validation: modules that show an AUROC > 0.60 and a better sCC and AUROC performance than the 95% of the random combinations were deemed significant.

Due to the lack of directionality values resulting from the sCC analysis, a non-compositional Spearman correlation analysis is proposed between the dietary and taxonomic elements of each relevant module to estimate the nature of their interactions (positive or negative) and their statistical significance.

S5. Mediation analysis

Prior to the NPEM analysis, the meta-taxonomic data are transformed into relative abundances and then via the Central Log Ratio (CLR). Species with a prevalence >1.5% in the samples and a detection threshold >0.0015 relative-abundance values were filtered to improve consistency. Dietary data were integrated in the analysis, considering nutrients, food groups, and dietary patterns separately to avoid high levels of correlation.

S6. Statistical analysis

Bivariate analyses were performed to examine associations between the dependent variables (EFP and LD) and dietary and microbial variables, and other covariates evaluated. Parametric tests (Student's t-test) were used for continuous variables following a normal distribution, while non-parametric tests (Mann–Whitney U test and Wilcoxon signed-rank test) were applied when normality was not met. The Chi-squared test was used to compare categorical variables. A p-value < 0.05 was considered statistically significant.

Regarding dietary characteristics, the Willett residual method was used to adjust nutrient or food intakes, controlling for total energy intake[11]. Additionally, extreme values were handled using winsorization[12], with variables log-transformed prior to further analyses. Dietary patterns were generated using PCA with varimax rotation in R version 4.3.3, using the psych package[12].

Model discrimination and variable selection were performed using the Least Absolute Shrinkage and Selection Operator (LASSO) method using the glmnet packages[13] instead of traditional stepwise selection, following recommendations from previous literature[14,15]. Concerning LASSO models, both the regularization parameter that minimizes the cross-validated mean squared error (λ.min) and the one–standard-error rule (λ-1.SE) were examined to obtain a more parsimonious model. Three models were generated as follows: a first model including the covariates evaluated in the study (Table 1), a second including categorical variables related to dietary adherence (PCA-derived dietary patterns and compliance with SENC and AESAN RDI), and a third including continuous variables

corresponding to daily intake of nutrients and food groups (g/day). Importantly, within each of the three main models, three submodels were fitted, each comparing a different pair of cognitive groups: (1) EFP vs. Control, (2) LD vs. Control, and (3) EFP vs. LD. Selected coefficients were visualized in a bar plot to illustrate the magnitude and direction of associations. Positive coefficients indicate a higher probability of belonging to EFD or LD group compared to control, whereas negative coefficients reflect an inverse association. All tests were performed at the 0.05 significance level.

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Supplementary Tables

Table S1. Factor loadings from principal component analysis (PCA) used to derive dietary patterns based on foods or nutrients.

	Factor Loadings*					
Foods	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Sugars Beverages	0.02	0.08	**0.62**	-0.01	-0.16	-0.2
Fresh fruit	**0.67**	-0.05	-0.12	0.16	-0.02	-0.01
Legumes	**0.72**	-0.01	0.03	0.02	0.13	-0.19
Nuts	**0.6**	-0.09	0.13	0.13	-0.07	0.26
Oils and margarine	0.16	0.09	0.06	-0.09	0.03	**-0.56**
Vegetables	**0.85**	0.04	-0.03	0.1	-0.01	0.06
Miscellaneous	-0.02	0.44	**0.6**	-0.06	-0.13	-0.03
Sugar Confectionary	-0.04	0.02	**0.69**	-0.06	0.2	0.08
Eggs	0.19	0.15	-0.12	0.07	0.21	**0.63**
Pastries	0	0.04	**0.59**	0	0.16	0.1
Dairy desserts	-0.19	0.13	0.34	0.18	**0.42**	-0.12
Dairy products	0	0.01	0.16	-0.14	**0.75**	0.11
Potatoes	0.23	0.39	0.28	0.19	0.1	0.15
Whole grains cereals	0.21	-0.08	-0.07	**0.83**	-0.01	0.02
Refined cereals	0.21	**0.51**	0.13	-0.35	-0.17	0.09
Seed bread and oat	0.19	0.04	0.04	**0.79**	-0.11	0.14
Fish and sea food	0.32	0.23	-0.08	-0.01	**0.55**	0.03
Red meat	-0.1	**0.69**	0.07	0.09	0.07	-0.05
White Meat	-0.05	**0.63**	-0.09	0.02	0.36	0.03
Processed Meat	-0.05	**0.7**	0.18	-0.12	0.01	0.03
Animals' fats (butter, cream)	0.1	0.12	0.41	-0.06	-0.04	**0.58**

Nutrients	P1	P2	P3	P4	P5	P6	P7	P8
Water (g)	**0.83**	-0.02	-0.18	0.07	0.35	-0.09	0.11	-0.1
Total lipid (fat) (g)	-0.26	**0.8**	0.4	-0.04	0.17	0,00	-0.12	0.18
Sugars (g)	0.41	-0.15	0.31	-0.12	0.11	0.01	-0.04	0.16
Sucrose (g)	**0.54**	-0.29	0.18	-0.17	-0.02	-0.06	0.32	-0.15
Starch, total (g)	0.08	-0.09	-0.04	-0.01	-0.1	-0.1	0.07	**0.81**
Carbohydrate, by difference (g)	0.19	**-0.88**	-0.19	-0.26	-0.14	0.06	-0.01	-0.08
Fatty acids, total polyunsaturated (g)	-0.09	0.4	0.29	0.05	-0.32	-0.01	-0.15	**0.66**
Fatty acid 12:0 (lauric acid) (g)	-0.17	-0.11	0.21	-0.13	**0.59**	-0.11	-0.12	0.09
Cholesterol (mg)	-0.38	0.39	0.23	0.42	0.16	0.08	-0.14	-0.12
Fatty acids, total trans (g)	-0.3	0.1	0.24	-0.03	0.27	0.07	-0.11	**0.71**
Fatty acid 16:0 (palmitic acid) (g)	-0.44	0.26	0.46	0.12	**0.54**	0.08	-0.03	0.27
Fatty acid 18:1 n-9 cis (oleic acid) (g)	-0.38	0.31	**0.61**	0.16	0.22	0.03	-0.06	0.3
Fatty acid 18:3 (g)	0.04	0.21	**0.68**	-0.03	0.04	-0.03	-0.05	-0.07
Fatty acid 20:4 n-6 (arachidonic acid) (g)	-0.13	0.03	**0.65**	0.02	-0.03	0.01	0.08	0.05
Fatty acid, total monounsaturated (g)	-0.11	**0.83**	0.34	-0.07	-0.09	-0.03	-0.09	0.12
Fatty acid, total saturated (g)	-0.33	**0.62**	0.31	-0.09	**0.52**	0.07	-0.08	-0.12
Fatty acid, 14:0 (myristic acid) (g)	-0.22	0.06	0.16	-0.04	**0.88**	0.07	-0.06	0,00
Fatty acid, 18:0 (stearic acid) (g)	-0.46	0.27	**0.5**	0.11	0.4	0.09	-0.02	0.34
Fatty acid, 18:2 (g)	-0.13	0.24	**0.79**	-0.06	-0.03	0.06	-0.08	0.12
Fatty acid, 20:5 (eicosapentaenoic acid) (g)	0.08	0.02	0.12	**0.86**	-0.03	0.03	0.07	0.12
Fatty acid, 22:6 n-3 (docosahexaenoic acid) (g)	0.12	-0.04	0.05	**0.85**	-0.06	0.05	0.04	0.13
Thiamin (mg)	0.36	0.07	0.07	0.19	-0.07	-0.18	**0.77**	-0.04
Vitamin E (alpha-tocopherol) (mg)	**0.83**	0.1	-0.05	-0.14	-0.14	-0.07	0.16	0.11
Vitamin B-6 (mg)	**0.60**	0.09	-0.26	0.22	-0.07	-0.12	**0.62**	0.01
Biotin (ug)	**0.64**	-0.04	0.21	0.08	-0.09	-0.11	-0.08	-0.02
Niacin (mg)	0.07	0.26	-0.07	**0.58**	-0.04	-0.05	**0.65**	-0.03
Riboflavin (mg)	-0.11	-0.01	0.02	-0.04	0.04	**0.97**	-0.01	-0.01
Vitamin D (D2 + D3) (ug)	-0.11	-0.02	0.03	0,00	0.02	**0.98**	-0.01	-0.01
Vitamin B-12 (ug)	-0.19	-0.01	0.03	0.05	0.04	**0.96**	-0.03	-0.04
Vitamin C, total ascorbic acid (mg)	**0.85**	-0.09	-0.02	-0.02	0.03	-0.02	0.2	-0.09
Folate, DFE (ug)	**0.77**	-0.09	-0.18	-0.03	0.03	0.22	0.39	-0.08
Pantothenic acid (mg)	0.07	-0.23	0.01	-0.16	0.04	0.19	**0.81**	0.02
Vitamin A, RAE (ug)	**0.66**	0.14	-0.21	-0.03	0.15	0.05	0.2	-0.12
Calcium, Ca (mg)	0.25	0.23	-0.26	-0.13	**0.73**	0.07	0.07	-0.29
Potassium, K (mg)	**0.87**	0.06	-0.21	0.13	0.15	-0.14	0.18	0.07
Selenium, Se (ug)	-0.29	-0.23	-0.13	**0.65**	-0.23	-0.07	-0.22	-0.23
Sodium, Na (mg)	-0.47	0.23	0.05	0.18	-0.32	-0.18	0.09	-0.23
Iron, Fe (mg)	**0.54**	-0.06	-0.35	0.14	-0.17	-0.15	**0.58**	-0.02
Iodine (ug)	0.17	0.14	-0.21	0.39	**0.62**	0.11	0.02	-0.04
Magnesium, Mg (mg)	**0.82**	-0.03	-0.34	0.04	-0.01	-0.2	0.14	-0.01
Zinc, Zn (mg)	0.24	**0.56**	-0.3	0.33	0.08	-0.03	0.26	-0.18
Phosphorus, P (mg)	0.36	0.34	-0.36	0.41	**0.53**	-0.12	0.18	-0.14
Protein total (gr)	-0.09	0.4	-0.22	**0.71**	0.12	-0.12	0.19	-0.15
Protein animal(g)	-0.44	0.46	0.04	**0.6**	0.21	0.02	0.03	-0.16
Protein vegetal (g)	**0.59**	-0.32	-0.35	-0.07	-0.21	-0.2	0.2	0.03
Total fiber (g)	**0.86**	-0.15	-0.23	-0.01	-0.22	-0.18	0.13	-0.01
Fiber soluble total(g)	**0.74**	-0.22	-0.27	-0.05	-0.34	-0.19	0.06	-0.06
Fiber insoluble total (g)	**0.88**	-0.12	-0.21	0,00	-0.17	-0.17	0.16	0.01
Flavonoids	**0.73**	-0.18	0.12	-0.01	-0.07	0.26	-0.08	-0.19
Isoflavones	0.38	0.09	-0.18	-0.09	-0.03	-0.06	0.06	0.15
Anthocyanins	**0.69**	-0.16	0.14	0.08	-0.03	-0.1	-0.13	-0.1
Total de polyphenols (mg)	**0.85**	-0.19	-0.01	0,00	-0.07	0.04	0.12	-0.15

*Factor loadings derived from PCAs with varimax rotation. Positive loadings indicate variables that contribute positively to the component, whereas negative loadings indicate variables that vary in the opposite direction to the component. All

food groups were energy-adjusted and standardized prior to analysis. Loadings greater than 0.50 are highlighted in bold.

Table S2. Covariates selected by LASSO-logistic regression for cognitive groups*.

Pairwise comparison	EFP vs control[a]		LD vs control[b]		EFP vs LD[c]	
Penalization criteria	λ.min	λ.1se	λ.min	λ.1se	λ.min	λ.1se
Behavioral Regulation Index	1.57	1.22	0.00	0.00	-1.5	-1.18
Externalizing problems	0.51	0.32	0.00	0.00	-0.27	0.00
Internalizing problems	0.78	0.45	0.61	0.26	0.00	0.00
Sex (Female)	-0.53	-0.13	-0.35	0.00	0.49	0.05
Healthy eating index (SHEI)	0.00	0.00	0.04	0.00	0.00	0.00
Dietary inflammatory index (DII) (Proinflammatory)	0.00	0.00	0.24	0.00	0.43	0.00
Marital status (Separated)	0.01	0.00	0.60	0.02	0.00	0.00
Parents education	0.00	0.00	0.24	0.00	0.35	0.00
Delivery mode	-0.76	-0.28	-0.04	0.00	0.00	0.00
Stress	0.01	0.01	0.01	0.005	0.00	0.00
Bowels movements	0.07	0.00	0.00	0.00	0.00	0.00
Intestinal transit (Bristol scale)	0.00	0.00	0.24	0.00	0.00	0.00
Sleep disorders	0.05	0.00	0.00	0.00	0.00	0.00
Gastrointestinal disorders	0.00	0.00	0.00	0.00	-0.1	0.00
Respiratory disorders	0.00	0.00	-0.28	0.00	0.00	0.00
Antibiotics	0.04	0.00	0.00	0.00	0.00	0.00
Non-antibiotic drugs	0.02	0.00	0.00	0.00	0.00	0.00

*For each pairwise comparison (EFP vs controls; LD vs control and LD vs EFP), results are shown for the optimal penalization parameter (λ.min) and the parsimonious penalization parameter (λ.1se). Coefficients correspond to the covariates selected by LASSO under each λ [a]Positive coefficients indicate greater contribution to the EFP group; negative coefficients indicate greater contribution to the control group. [b]Positive coefficients indicate greater contribution to LD group; negative coefficients indicate greater contribution to the control group. [c]Positive coefficients indicate greater contribution to the LD group and negative coefficients indicate greater contribution to the EFP group. EFP = executive function problems; LD = learning disorders.

Table S3. Pairwise comparison of RDA compliance, considering nutrients and food group intakes*, among control, EFP, and LD groups

Food and nutrients category	Compliance	Total population	Controls (N=308)	EFP (N=45)	LD (N=53)	p-value[a]	p-value[b]	p-value[c]
Fresh fruit	Complies	169 (41.6%)	130 (42.2%)	23 (51.1%)	16 (30.2%)	0.23	0.22	0.06
Fresh fruit	Below	237(58.4%)	178 (57.8%)	22 (48.9%)	37 (69.8%)			
Legumes	Complies	108(26.6%)	86 (27.9%)	10 (22.2%)	12 (22.6%)	0.6	0.62	1
Legumes	Below	298(73.4%)	222 (72.1%)	35 (77.8%)	41 (77.4%)			
Nuts	Complies	133(32.8%)	110 (35.7%)	12 (26.7%)	11 (20.8%)	0.45	0.02	0.74
Nuts	Below	273(67.2%)	198 (64.3%)	33 (73.3%)	42 (79.2%)			
Oil olive	Complies	307(75.6%)	231 (75%)	37 (82.2%)	39 (73.6%)	0.36	0.56	0.27
Oil olive	Below	99(24.4%)	77 (25%)	8 (17.8%)	14 (26.4%)			
Vegetables	Complies	175(43.1%)	143 (46.4%)	15 (33.3%)	17 (32.1%)	0.1	0.07	1
Vegetables	Below	231(56.9%)	165 (53.6%)	30 (66.7%)	36 (67.9%)			
Eggs	Complies	375(92.4%)	285 (92.5%)	41 (91.1%)	49 (92.5%)	0.97	1	1
Eggs	Above	31(7.6%)	23 (7.5%)	4 (8.9%)	4 (7.5%)			
Dairy products	Complies	309(76.1%)	243 (78.9%)	32 (71.1%)	34 (64.2%)	0.52	0.03	0.56
Dairy products	Above	97(23.9%)	65 (21.1%)	13 (28.9%)	19 (35.8%)			
Whole grains	Below	451(111.1%)	326 (80.3%)	80 (19.7%)	45 (11.1%)			
Refined cereals	Complies	71(17.5%)	53 (17.2%)	5 (11.1%)	13 (24.5%)	0.32	0.87	0.11
Refined cereals	Above	335(82.5%)	255 (82.8%)	40 (88.9%)	40 (75.5%)			
Fish seafood	Complies	223(54.9%)	161 (52.3%)	27 (60%)	35 (66%)	0.57	0.25	0.72
Fish seafood	Below	183(45.1%)	147 (47.7%)	18 (40%)	18 (34%)			
BlueFish	Complies	257(63.3%)	200 (64.9%)	30 (66.7%)	27 (50.9%)	0.74	0.04	0.16
BlueFish	Below	149(36.7%)	108 (35.1%)	15 (33.3%)	26 (49.1%)			
Red meat	Complies	79(19.5%)	61 (19.8%)	10 (22.2%)	8 (15.1%)	0.77	0.98	0.46
Red meat	Above	327(80.5%)	247 (80.2%)	35 (77.8%)	45 (84.9%)			
White meat	Complies	93(22.9%)	71 (23.1%)	8 (17.8%)	14 (26.4%)	0.5	0.96	0.37
White meat	Above	313(77.1%)	237 (76.9%)	37 (82.2%)	39 (73.6%)			
Potatoes	Complies	392(96.5%)	299 (97.1%)	42 (93.3%)	51 (96.2%)	0.41	0.61	0.81
Potatoes	Above	14(3.4%)	9 (2.9%)	3 (6.7%)	2 (3.8%)			
Miscellaneous	Complies	40(9.9%)	32 (10.4%)	6 (13.3%)	2 (3.8%)	0.57	0.56	0.15

Miscellaneous	Above	366(90.1%)	276 (89.6%)	39 (86.7%)	51 (96.2%)			
Pastries	Complies	22(5.4%)	19 (6.2%)	0	3 (5.7%)	0.18	0.65	0.31
Pastries	Above	384(94.6%)	289 (93.8%)	45 (100%)	50 (94.3%)			
Dairy Sugars	Complies	187(46.1%)	146 (47.4%)	19 (42.2%)	22 (41.5%)	0.7	0.56	0.98
Dairy Sugars	Above	219(53.9%)	162 (52.6%)	26 (57.8%)	31 (58.5%)			
Processed meat	Complies	278(68.4%)	273(88.6%)	3(6.7%)	2(3.8%)	0.48	0.13	0.81
Processed meat	Above	128(31.5%)	35(11.3%)	42(93.3%)	51(96.2%)			
Total Carbohydrates	Below	242(59.6%)	180 (58.4%)	30 (66.7%)	32 (60.4%)	0.53	0.91	0.33
Total Carbohydrates	Above	46(11.3%)	38 (12.3%)	5 (11.1%)	3 (5.7%)			
Total Carbohydrates	Complies	118(29.1%)	90 (29.2%)	10 (22.2%)	18 (34%)			
Total protein	Below	9(2.2%)	8 (2.6%)	1 (2.2%)	0	0.61	0.45	0.12
Total protein	Above	127(31.3%)	98 (31.8%)	17 (37.8%)	12 (22.6%)			
Total protein	Complies	270(66.5%)	202 (65.6%)	27 (60%)	41 (77.4%)			
Vegetal:animal protein (%)	Animal	381(93.8%)	286(93%)	44(98%)	51(96%)	0.23	0.65	0.81
Vegetal:animal protein (%)	Vegetal	25(6.1%)	22(7%)	1(2%)	2(4%)			
Total Fats	Above	220(54.2%)	160 (51.9%)	25 (55.6%)	35 (66%)	0.96	0.07	0.39
Total Fats	Complies	186(45.8%)	148 (48.1%)	20 (44.4%)	18 (34%)			
Sugar (%)	Above	360(66.7%)	98 (32%)	14 (31.2%)	17 (32.1%)	1	0.94	1
Sugar (%)	Complies	46(11.3%)	210 (68%)	31(68.8%)	36(67.9%)			
PUFAs(%)	Below	98(24.1%)	75(24.35%)	12(26.6%)	11(20.7%)	0.87	0.69	0.65
PUFAs(%)	Complies	308(75.9%)	233(75.5%)	33(73.4%)	42(79.3%)			
MUFAs(%)	Above	230(56.6%)	197(63%)	11(24%)	22(41.5%)	0.17	0.54	0.09
MUFAs(%)	Complies	176(43.3%)	111(36%)	34(75%)	31(58.5%)			
SFA(%)	Above	349(85.9%)	261(84.7%)	49(92.4%)	39(86%)	0.9	0.2	0.54
SFA(%)	Complies	57(14%)	47(15.2%)	4(7.6%)	6(13%)			
ALA (%)	Below	337(83%)	240 (84.4%)	40 (11%)	53 (15.6%)	0.9	0.08	0.08
ALA (%)	Complies	72(17%)	68(100)	4 (25%)	0			
LA (%)	Below	350(86.2%)	256 (82.3%)	48 (17.7%)	39 (10.9%)	0.78	0.96	0.77
LA (%)	Complies	100(24.6%)	70 (74.5%)	5(25.5%)	6 (11.7%)			
Ratio LA/ALA	Below	40(9.8%)	31 (10.1%)	5 (11.1%)	4 (7.5%)	0.96	0.15	0.75
Ratio LA/ALA	Above	56(13.8%)	38 (12.3%)	6 (13.3%)	12 (22.6%)			
Ratio LA/ALA	Complies	310(76.3%)	239 (77.6%)	34 (75.6%)	37 (69.8%)			

DHA	Below	70(17.2%)	52(16.8%)	8(17.7%)	10(18.8%)	1	0.87	1
DHA	Complies	336(82.7%)	256(83.1%)	37(82.3%)	43(81.2%)			
EPA+DHA	Below	180(44.3%)	142 (46.1%)	16 (35.6%)	22 (41.5%)	0.24	0.56	0.68
EPA+DHA	Complies	226(55.6%)	166 (53.9%)	29 (64.4%)	31 (58.5%)			
Trans fatty acids	Above	252(62.1%)	181 (58.8%)	16 (35.6%)	23 (43.3%)	0.4	0.02	0.55
Trans fatty acids	Complies	154(37.9%)	127 (41.2%)	29 (64.4%)	30(56.6%)			
Total fiber	Below	112(27.6%)	83 (26.9%)	12 (26.7%)	17 (32.1%)	1	0.64	0.71
Total fiber	Complies	294(72.4%)	225 (73.1%)	33 (73.3%)	36 (67.9%)			
Cholesterol	Above	241(59.4%)	181 (58.8%)	28 (62.2%)	32 (60.4%)	0.79	1	1
Cholesterol	Complies	165(40.6%)	127 (41.2%)	17 (37.8%)	21 (39.6%)			
Iodine	Below	247(60,8%)	194 (63%)	23 (51.1%)	30 (56.6%)	0.21	0.16	0.73
Iodine	Complies	159(39.1%)	114 (37%)	22 (48.9%)	23 (43.4%)			
Sodium	Above	360(88.7%)	272 (88.3%)	42 (93.3%)	46 (86.8%)	0.42	1	0.46
Sodium	Complies	46(11.3%)	36 (11.7%)	3 (6.7%)	7 (13.2%)			
Potassium	Below	132(32.5%)	105 (34.1%)	8 (17.8%)	19 (35.8%)	0.04	0.58	0.07
Potassium	Complies	274(67.5%)	203 (65.9%)	37 (82.2%)	34 (64.2%)			
Calcium	Below	267(67.8%)	208 (67.5%)	24 (53.3%)	35 (66%)	0.08	0.25	0.28
Calcium	Complies	139(34.2%)	100 (32.5%)	21 (46.7%)	18 (34%)			
Magnesium	Below	114(28.1%)	89 (28.9%)	9 (20%)	16 (30.2%)	0.28	0.67	0.35
Magnesium	Complies	292(71.9%)	219 (71.1%)	36 (80%)	37 (69.8%)			
Phosphorus	Below	11(2.7%)	8 (2.6%)	1 (2.2%)	2 (3.8%)	1	0.78	1
Phosphorus	Complies	395(97.3%)	300 (97.4%)	44 (97.8%)	51 (96.2%)			
Iron	Below	93(22.9%)	73 (23.7%)	9 (20%)	11 (20.8%)	0.75	0.85	1
Iron	Complies	313(77.1%)	235 (76.3%)	36 (80%)	42 (79.2%)			
Zinc	Below	75(18.5%)	60 (19.5%)	7 (15.6%)	8 (15.1%)	0.73	0.49	1
Zinc	Complies	331(81.5%)	248 (80.5%)	38 (84.4%)	45 (84.9%)			
Selenium	Below	10(2.5%)	9 (2.9%)	0	1 (1.9%)	0.53	0.72	1
Selenium	Complies	396(97.5%)	299 (97.1%)	45 (100%)	52 (98.1%)			
Vitamin B1	Below	48(11.8%)	35 (11.4%)	8 (17.8%)	5 (9.4%)	0.29	1	0.36
Vitamin B1	Complies	358(88.2%)	273 (88.6%)	37 (82.2%)	48 (90.6%)			
Vitamin B2	Below	33(8.1%)	25 (8.1%)	5 (11.1%)	3 (5.7%)	0.63	1	0.54
Vitamin B2	Complies	373(91.8%)	283 (91.9%)	40 (88.9%)	50 (94.3%)			
Vitamin B6	Below	7(1.7%)	5 (1.6%)	0	2 (3.8%)	0.74	0.9	0.54

Vitamin B6	Complies	399(98.3%)	303 (98.4%)	45 (100%)	51 (96.2%)			
Pantothenic acid	Below	27(6.6%)	21 (6.8%)	3 (6.7%)	3 (5.7%)	1	1	1
Pantothenic acid	Complies	379(93.3%)	287 (93.2%)	42 (93.3%)	50 (94.3%)			
Vitamin B12	Below	7(1.7%)	7 (2.3%)	0	0	0.74	0.4	0.41
Vitamin B12	Complies	399(98.3%)	301 (97.7%)	45 (100%)	53 (100%)			
Folate	Below	94(23.1%)	74 (24%)	8 (17.8%)	12 (22.6%)	0.47	0.8	0.73
Folate	Complies	312(76.9%)	234 (76%)	37 (82.2%)	41 (77.4%)			
Niacin	Below	6(1.5%)	5 (1.6%)		1 (1.9%)	0.83	1	1
Niacin	Complies	400(98.5%)	303 (98.4%)	45 (100%)	52 (98.1%)			
Vitamin C	Below	32(7.8%)	21 (6.8%)	4 (8.9%)	7 (13.2%)	1	0.29	0.72
Vitamin C	Complies	374(92.1%)	287 (93.2%)	41 (91.1%)	46 (86.8%)			
Vitamin A	Below	65(16%)	50 (16.2%)	5 (11.1%)	10 (18.9%)	0.48	0.74	0.43
Vitamin A	Complies	341(84%)	258 (83.8%)	40 (88.9%)	43 (81.1%)			
Vitamin D	Below	134(33%)	105 (34.1%)	16 (35.6%)	13 (24.5%)	0.84	0.33	0.33
Vitamin D	Complies	272(67%)	203 (65.9%)	29 (64.4%)	40 (75.5%)			
Vitamin E	Below	196(48.3%)	150 (48.7%)	19 (42.2%)	27 (50.9%)	0.49	0.7	0.5
Vitamin E	Complies	210(51.7%)	158 (51.3%)	26 (57.8%)	26 (49.1%)			
Total polyphenols	Below	187(46%)	140 (45.5%)	19 (42.2%)	28 (52.8%)	0.71	0.58	0.39
Total polyphenols	Complies	219(54%)	168 (54.5%)	26 (57.8%)	25 (47.2%)			

Data of nutrients and food are presented as frequencies and percentages. Group differences were assessed using the chi-square test. Pairwise comparisons were conducted to examine between-group differences. Statistical significance was established at $p < 0.05$ for the following pair-comparisons: [a] for Control vs. EFP, [b] for Control vs. LD, and [c] for EFP vs. LD. Statistically significant p-values are shown in bold. Abbreviations: RDA Recommended Dietary Allowances; SFA, saturated fatty acids; ALA, α-linolenic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Table S4. Pairwise comparison of dietary pattern* adherence among control, EFP, and LD groups

Food dietary pattern	Adherence	Controls (N=308)	EFP (N=45)	LD (N=52)	p-value[a]	p-value[b]	p-value[c]
Plant-based pattern	High	109 (35.4%)	16 (35.6%)	14 (26.4%)			
Plant-based pattern	Low	100 (32.5%)	13 (28.9%)	20 (37.7%)	0.86	0.44	0.54
Plant-based pattern	Moderate	99 (32.1%)	16 (35.6%)	19 (35.8%)			
Animal and refined cereals	High	107 (34.7%)	17 (37.8%)	14 (26.4%)			
Animal and refined cereals	Low	103 (33.4%)	11 (24.4%)	19 (35.8%)	0.46	0.47	0.36
Animal and refined cereals	Moderate	98 (31.8%)	17 (37.8%)	20 (37.7%)			
Sugars and processed food	High	109 (35.4%)	11 (24.4%)	19 (35.8%)			
Sugars and processed food	Low	98 (31.8%)	19 (42.2%)	15 (28.3%)	0.24	0.85	0.29
Sugars and processed food	Moderate	101 (32.8%)	15 (33.3%)	19 (35.8%)			
Whole-Grain Cereals	High	105 (34.1%)	13 (28.9%)	21 (39.6%)			
Whole-Grain Cereals	Low	107 (34.7%)	16 (35.6%)	10 (18.9%)	0.74	0.08	0.16
Whole-Grain Cereals	Moderate	96 (31.2%)	16 (35.6%)	22 (41.5%)			
Fish and Dairy	High	101 (32.8%)	14 (31.1%)	23 (43.4%)			
Fish and Dairy	Low	109 (35.4%)	16 (35.6%)	9 (17%)	0.88	0.03	0.10
Fish and Dairy	Moderate	98 (31.8%)	15 (33.3%)	21 (39.6%)			
Eggs and Animal Fats	High	105 (34.1%)	16 (35.6%)	17 (32.1%)			
Eggs and Animal Fats	Low	100 (32.5%)	12 (26.7%)	21 (39.6%)	0.62	0.57	0.37
Eggs and Animal Fats	Moderate	103 (33.4%)	17 (37.8%)	15 (28.3%)			
Plant-Based Nutrient	High	110 (35.7%)	15 (33.3%)	13 (24.5%)			
Plant-Based Nutrient	Low	94 (30.5%)	13 (28.9%)	27 (50.9%)	0.75	**0.01**	0.08
Plant-Based Nutrient	Moderate	104 (33.8%)	17 (37.8%)	13 (24.5%)			
High-fat and cholesterol	High	112 (36.4%)	10 (22.2%)	18 (34%)			
High-fat and cholesterol	Low	101 (32.8%)	14 (31.1%)	18 (34%)	0.07	0.94	0.27
High-fat and cholesterol	Moderate	95 (30.8%)	21 (46.7%)	17 (32.1%)			
Unsaturated fatty acids	High	105 (34.1%)	15 (33.3%)	20 (37.7%)	0.73	0.33	0.85

Unsaturated fatty acids	Low	107 (34.7%)	13 (28.9%)	13 (24.5%)			
Unsaturated fatty acids	Moderate	96 (31.2%)	17 (37.8%)	20 (37.7%)			
Omega-3 PUFAs (DHA, EPA), selenium and zinc	High	103 (33.4%)	18 (40%)	18 (34%)			
Omega-3 PUFAs (DHA, EPA), selenium and zinc	Low	100 (32.5%)	14 (31.1%)	20 (37.7%)	0.68	0.65	0.75
Omega-3 PUFAs (DHA, EPA), selenium and zinc	Moderate	105 (34.1%)	13 (28.9%)	15 (28.3%)			
Lauric and myristic Saturated FAsand other minerals	High	100 (32.5%)	16 (35.6%)	22 (41.5%)			
Lauric and myristic Saturated FAs and other minerals	Low	103 (33.4%)	14 (31.1%)	17 (32.1%)	0.96	0.38	0.73
Lauric and myristic Saturated FAs and other minerals	Moderate	105 (34.1%)	15 (33.3%)	14 (26.4%)			
Vitamins B12, D and B2	High	103 (33.4%)	15 (33.3%)	22 (41.5%)			
Vitamins B12, D and B2	Low	107 (34.7%)	14 (31.1%)	11 (20.8%)	0.93	0.13	0.47
Vitamins B12, D and B2	Moderate	98 (31.8%)	16 (35.6%)	20 (37.7%)			
Group-B vitamins and Iron	High	113 (36.7%)	7 (15.6%)	19 (35.8%)			
Group-B vitamins and Iron	Low	99 (32.1%)	24 (53.3%)	11 (20.8%)	**0.006**	0.37	**0.002**
Group-B vitamins and Iron	Moderate	96 (31.2%)	14 (31.1%)	23 (43.4%)			
Trans fatty acids and starches	High	94 (30.5%)	17 (37.8%)	28 (52.8%)			
Trans fatty acids and starches	Low	110 (35.7%)	14 (31.1%)	10 (18.9%)	0.86	**0.004**	0.25
Trans fatty acids and starches	Moderate	104 (33.8%)	14 (31.1%)	15 (28.3%)			

*Data of dietary patterns are presented as frequencies and percentages. Group differences were assessed using the chi-square test. Pairwise comparisons were conducted to examine between-group differences. Statistical significance was established at $p < 0.05$ for the following pair-comparisons: [a] for Control vs. EFP, [b] for Control vs. LD, and [c] for EFP vs. LD. Statistically significant p-values are shown in bold. LD, learning difficulties; EFP, executive function problems; SFA, saturated fatty acids; ALA, α-linolenic acid; LA, linoleic acid; PUFAs, polyunsaturated fatty acids; MUFAs, monounsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Table S5. Energy-adjusted intake* of individual nutrients and foods among control, EFP, and LD groups

Food and nutrients category	Controls (N=308)					EFP (N=45)					LD (N=53)					p[a]	p[b]	p[c]
	Mean	SD	Q1	median	Q3	mean	SD	Q1	median	Q3	mean	SD	Q1	median	Q3			
Sugars Beverages	58.5	70.1	10.3	33.3	90.6	63.2	73.5	14.64	33.6	93.65	65.95	73.46	0.27	33.99	105.24	0.43	0.77	0.7
Fresh fruit	329.2	179.6	192.3	305.4	433.6	339.4	185.1	219.7	308.6	497.71	303.6	180.09	166.38	274.29	424.07	0.55	0.92	0.22
Legumes	38.4	32.7	19.7	30.2	45.6	47.75	82.9	18.39	30.2	44.63	32.18	20.52	19.91	30.1	42.99	0.88	0.8	0.89
Nuts	5.4	84	0.03	2.6	7.7	4.5	6.5	0.03	2.0	4.32	4.85	8.45	0.02	1.99	3.6	0.6	0.19	0.75
Oils and margarine	10.5	3.9	9.8	10.1	10.3	10.08	1.2	9.8	10.1	10.28	10.6	2.78	9.89	10.14	10.74	0.45	0.15	0.27
Vegetables	344.7	203.7	179.8	313.6	515.6	315.5	194.1	190.9	249.3	424.9	354.4	183.8	230.8	367.9	469.5	0.24	0.85	0.71
Vegetables high in fermentable CHO	32.8	51.2	0	18.2	41.6	36.1	67.2	0	16.5	36.85	20.63	25.87	0	12.53	32.03	0.8	**0.001**	0.12
Miscellaneous	48.2	46.9	23.24	37.1	56.9	46.1	36.2	25.1	34.9	50.53	44.44	33.55	24.51	34.75	56.43	0.84	0.54	0.59
Sugar Confectionary	300.3	190.1	168.2	269.8	420.8	301.6	189.8	152.9	288.5	497.87	335.68	192.28	186.86	308.21	534.5	0.9	0.47	0.47
Eggs	64.8	50.4	26.8	54.3	84.4	61.6	32.5	36.3	56.1	83.66	63.36	39.74	30.4	53.33	83.3	**0.04**	0.42	0.32
Pastries	12.5	19	0	2.5	19.9	16.2	10.6	0.08	0.3	10.09	10.45	16.43	0.05	2.88	14.55	0.17	0.98	0.10
Dairy desserts	92.1	56.6	58.2	79.4	112.4	88.8	51.6	53.5	82.1	110.58	94.07	43.87	70.87	91.25	104.33	0.25	0.99	**0.05**

Dairy products	33.31	24.36	15.79	29.35	43.58	35.2	26.74	14.69	34.44	46.26	31.46	18.62	21.02	30.27	46.62	0.68	0.75	**0.09**
Potatoes	18.01	11.37	7.63	20.8	24.55	22.05	13.19	8.26	22.86	26.8	17.93	10.47	8.19	21.45	24.34	0.24	0.22	0.66
Whole-grain cereals	31.01	42.45	0.6	15.86	39.64	31.61	33.72	0.55	22.17	44.39	32.06	36.97	5.24	21.05	37.51	0.56	0.63	0.44
Refined cereals	7.22	16.4	0	0.22	5.64	4.68	11.12	0.03	0.33	3.54	8.44	14.53	0.1	2.1	7.15	0.90	0.27	**0.05**
Seed bread and oat	59.63	38.27	30.39	52.27	78.96	50.39	27.38	30.02	43.9	73.42	70.7	40.87	38.81	73.43	92.23	0.52	0.41	0.14
Fish and sea food	17.75	27.75	0.01	4.65	23.41	12.58	18.6	0.01	3.72	22.74	18.89	25.06	0.03	6.94	30.6	0.09	0.24	**0.01**
Red meat	53	44.41	21.45	42.99	76.87	55.88	37.69	23.59	47.11	74.07	62.1	61.81	35.62	47.22	70.07	0.94	0.55	0.93
White Meat	51.25	24.2	44.22	54.5	59.95	50.13	23.75	21.22	53.23	59.92	49.97	19.49	44.01	52.73	58.58	0.13	0.56	0.71
Processed Meat	35.57	26.77	19.25	30.57	44.46	39.82	24.07	23.16	39.73	50.71	35.07	17.75	21.53	34.66	45.67	0.81	0.52	**0.06**
Animals' fats (butter, cream)	3.35	5.47	0.19	1.56	3.88	3.79	6.73	0.25	1.67	5.55	2.91	4.33	0.1	1.32	3.31	0.81	0.52	0.82
Water (g)	1096.91	297.66	926.17	1065.42	1222.89	1080.62	257.82	893.81	1115.36	1228.12	988.6	272.06	782.77	972.85	1186.66	0.56	0.08	0.09
Total lipid (fat) (g)	75.81	9.85	69.39	75.72	82.49	76.29	10.63	71.4	75.9	80.77	79.03	10.43	72.42	78.25	85.49	0.58	0.42	0.19
Sugars (g)	14.25	9.86	7.69	12.06	18.41	14.93	8.13	9.75	14.22	20.79	15.09	8.29	8.7	13.79	20.63	0.91	**0.03**	0.87
Sucrose (g)	14.79	7.38	9.99	13.88	18.44	13.95	7	10.29	12.7	18.2	13.45	6.32	8.42	13.03	16.29	0.83	0.35	0.92
Starch, total (g)	16.31	7.36	11.44	15.07	20.12	17.83	9.8	12.21	13.71	22.64	18.86	7.28	13.76	18.92	24.4	0.98	**0.04**	0.27

Carbohydrate, by difference (g)	212.2	25.75	197.22	212.68	228.82	210.53	26.53	203.3	210.76	220.38	207.33	25.55	192.5	209.36	225.31	0.73	1	0.59
Fatty acids, total polyunsaturated (g)	10.04	2.1	8.63	9.94	11.18	10.45	3.27	8.84	9.87	10.93	10.7	2.46	8.79	10.82	12.66	0.95	0.19	0.51
Fatty acid 12:0 (lauric acid) (g)	0.79	0.48	0.51	0.7	0.93	0.76	0.57	0.5	0.65	0.91	0.82	0.57	0.55	0.72	0.95	0.4	0.76	0.55
Cholesterol (mg)	322.4	69.38	278.8	324.5	363.0	333.6	71.72	279.0	324.2	382.3	329.5	62.73	279.3	334.2	367.1	0.77	0.74	0.82
Fatty acids, total trans (g)	1.33	0.66	0.91	1.19	1.56	1.6	1.03	1.02	1.34	1.67	1.79	0.93	1.13	1.56	2.4	0.43	0	0.26
Fatty acid 16:0 (palmitic acid) (g)	8.57	2.3	7.1	8.56	10.06	8.77	2.04	6.71	8.57	10.37	9.93	2.17	8.18	10.18	11.31	0.73	0	**0.001**
Fatty acid 18:1 n-9 cis (oleic acid) (g)	16.71	3	14.83	16.52	18.43	16.61	2.71	14.69	17.14	18.46	18.2	2.73	16.51	18.1	19.58	0.51	**0.02**	**0.004**
Fatty acid 18:3 (g)	0.37	0.21	0.24	0.31	0.4	0.36	0.25	0.23	0.27	0.37	0.35	0.16	0.24	0.32	0.41	0.31	0.95	0.37
Fatty acid 20:4 n-6 (arachidonic acid) (g)	0.4	0.32	0.2	0.33	0.5	0.4	0.36	0.12	0.31	0.44	0.53	0.37	0.28	0.39	0.77	0.6	**0.05**	**0.05**
Fatty acid, total monounsaturated (g)	24.26	4.42	21.44	24.13	26.76	24.19	4.96	21.77	23.75	25.48	24.85	4.77	21.36	24.03	28.22	0.42	0.82	0.47
Fatty acid, total saturated (g)	26.52	4.99	23.06	26.99	30.03	26.94	4.94	23.8	27.23	29.69	28.01	4.99	24.61	27.89	31.43	0.66	0.58	0.28
Fatty acid, 14:0 (myristic acid) (g)	1.42	0.58	1.02	1.32	1.83	1.49	0.6	1.05	1.41	1.91	1.61	0.61	1.13	1.57	2.07	0.85	**0.04**	0.31
Fatty acid, 18:0 (stearic acid) (g)	3.84	1.1	3.21	3.78	4.47	3.88	1.03	2.99	3.9	4.38	4.47	1.07	3.74	4.29	5.04	0.58	0	**0.005**

Fatty acid, 18:2 (g)	4.41	1.95	3.06	4.07	5.21	4.57	2.27	3.08	4.02	5.5	4.64	1.68	3.42	4.31	5.96	0.93	0.24	0.56
Fatty acid, 20:5 (eicosapentaenoic acid) (g)	0.1	0.05	0.06	0.09	0.13	0.11	0.07	0.06	0.1	0.15	0.1	0.05	0.06	0.09	0.12	0.47	0.63	0.22
Fatty acid, 22:6 n-3 (docosahexaenoic acid) (g)	0.22	0.12	0.13	0.19	0.29	0.25	0.17	0.12	0.19	0.39	0.2	0.11	0.12	0.17	0.28	0.33	0.58	0.12
Thiamin (mg)	1.46	0.34	1.25	1.45	1.61	1.35	0.3	1.14	1.35	1.49	1.4	0.29	1.15	1.37	1.62	**0.03**	0.18	0.35
Vitamin E (alpha-tocopherol) (mg)	9.75	2.62	8.14	9.36	11.18	9.36	2.07	8.02	9.06	10.3	9.01	1.78	7.8	9.02	9.82	0.93	0.1	0.40
Vitamin B-6 (mg)	2.6	0.58	2.26	2.59	2.88	2.51	0.62	2.11	2.45	2.92	2.38	0.39	2.1	2.32	2.69	0.49	**0.01**	0.29
Biotin (ug)	0.8	0.8	0.3	0.58	1.12	0.82	0.67	0.2	0.74	1.24	0.57	0.47	0.26	0.41	0.80	0.39	0.09	**0.06**
Niacin (mg)	26.84	4.51	23.49	26.51	29.79	26.91	4.56	23.87	26.75	29.51	26.27	3.29	23.62	26.42	29.03	0.61	0.58	0.56
Riboflavin (mg)	52.14	61.62	2.46	33.33	83.06	55.67	72.49	1.91	39.31	94.55	64.99	70.94	5.15	71.53	90.80	0.75	0.13	0.15
Vitamin D (D2 + D3) (ug)	73.95	87.47	3.8	47.71	117.11	79.12	102.43	2.68	55.31	132.94	91.51	99.73	8.61	100.29	128.74	0.78	0.17	0.17
Vitamin B-12 (ug)	19.93	16.52	6.56	15.66	28.84	21.21	20.47	6.33	15.66	31.45	24.52	21.02	8.53	25.64	30.75	0.87	0.08	0.16
Vitamin C, total ascorbic acid (mg)	169.3	95.3	114.84	153.72	198.35	160.91	75.51	123.77	156.22	180.22	129.51	64.41	81.06	111.49	166.36	0.67	**0.01**	**0.02**
Folate, DFE (ug)	357.31	127.64	282.42	335.87	402.3	337.08	91.07	283.3	325.13	395.13	320.82	84.81	268.72	293.86	360.60	0.92	**0.03**	0.44
Pantothenic acid (mg)	1.43	1.5	0.54	0.88	2.06	1.13	1.42	0.41	0.63	1.13	1.4	1.11	0.51	1.03	1.88	**0.05**	0.95	**0.07**

Vitamin A, RAE (ug)	1107.51	540.18	694.04	1012.71	1352.56	1026.21	480.63	698.28	855.68	1304.56	952.81	421.23	647.89	860.54	1228.73	0.64	0.1	0.47
Calcium, Ca (mg)	831.04	208.59	687.58	814.62	953.33	849.31	225.98	705.72	862.77	988.33	824.43	228.03	654.69	835.3	1000.55	0.58	0.69	0.60
Potassium, K (mg)	2900.08	340.28	2735.38	3082.53	3138.19	2910.97	310.54	2726.55	3084.99	3138.19	2748.87	389.19	2461.95	2858.73	3138.19	0.61	**0.02**	**0.04**
Selenium, Se (ug)	87.85	20.98	74.25	86.26	100.15	90.9	19.34	79.62	92.33	104.97	86.85	20.69	73.93	82.24	92.08	0.12	0.57	0.35
Sodium, Na (mg)	2065.17	413.33	1776.39	2026.18	2338.89	1987.14	416.66	1719.61	1920.97	2165.51	2120.95	451.2	1790.9	2031.27	2289.87	0.14	0.38	0.11
Iron, Fe (mg)	14.86	2.93	12.69	14.44	16.6	14.54	3.52	12.36	14.05	16.24	13.93	2.27	12	14.04	15.39	0.48	0.06	0.42
Iodine (ug)	112.39	27.95	94.78	108.92	128.19	113.32	27.43	90.05	117.27	133.02	112.11	27.18	93.42	113.85	129.86	0.94	0.94	0.80
Magnesium, Mg (mg)	302.02	61.26	263.31	296.33	334.22	294.27	55.08	257.55	302.32	322.87	282.78	51.97	240.88	282.77	311.97	0.95	**0.03**	0.30
Zinc, Zn (mg)	10.12	1.17	9.29	10	10.76	10.07	1.16	9.3	10.08	10.98	10.01	1.3	9.12	9.84	10.6	0.99	0.24	0.77
Phosphorus, P (mg)	1289.75	152.53	1191.75	1283.16	1382.88	1305.01	176.15	1185.97	1326.82	1433.26	1274.49	156.99	1153.12	1275.55	1383.09	0.33	0.68	0.42
Protein total (gr)	91.77	11.95	83.83	90.83	98.84	92.97	11.63	85.14	94.64	99.03	90.65	10.43	82.58	89.6	95.63	0.35	0.41	0.33
Protein animal(g)	61.86	15.09	52.98	62.53	69.94	64.14	13.48	54.66	65.59	72.13	62.88	11.7	55.16	61.79	68.94	0.36	0.83	0.72
Protein vegetal (g)	29.92	8.14	24.05	28.93	34.29	28.87	7.92	24.25	27.36	33.01	27.81	6.67	23.16	26.57	30.98	0.78	0.12	0.55
Total fiber (g)	25	9.01	19.26	24.27	28.8	24.51	8.61	19.58	23.1	28.2	21.22	6.17	16.57	19.53	26.53	0.7	**0.02**	**0.05**
Fiber soluble total(g)	7.18	2.15	5.61	6.99	8.33	7.03	2.42	5.57	6.76	8.52	6.12	1.62	5.01	5.94	7.09	0.73	**0.01**	**0.08**

Fiber insoluble total (g)	17.78	7.1	13.48	17.07	20.53	17.44	6.45	13.81	16.28	20.25	15.07	4.7	11.38	13.91	18.86	0.7	**0.01**	**0.05**
Flavonoids	478.7	253.38	313.73	433.87	581.57	474.14	264.67	280.47	407.82	616.21	400.17	214.12	227.75	379.26	521.87	0.75	0.07	0.18
Isoflavones	2.8	9.83	0.01	0.05	0.22	2.9	11.35	0.01	0.04	0.11	1.03	3.47	0.01	0.02	0.19	0.38	0.22	0.66
Anthocyanins	44.19	48.28	11.88	27.81	66.05	46.9	44.89	7.63	36.52	75.29	27.78	30.62	4.93	15.28	52.77	0.17	**0.04**	**0.07**
Polyphenols Total (mg)	1639.51	650.47	1198.8	1573.82	1935.78	1625.53	653.88	1290.59	1588.91	1808.12	1353.92	606.5	909.01	1260.28	1725.56	0.48	**0.01**	**0.03**

*Data of food and nutrients are expressed as mean and standard deviation (SD) and median (Q1–Q3, interquartile range). Pairwise comparisons were conducted to examine between-group differences. Statistical tests: Mann-Whitney U and Student's t-test were used to compare the quantitative variables. Statistical significance was established at $p < 0.05$ for the following pair-comparisons: [a] for Control vs. EF problems, [b] for Control vs. LD, and [c] for EF vs. LD. Abbreviations: EFP, executive function problems; LD, learning difficulties

Species	Mean	*Log2-fold Change	Standard Error	Likelihood-Ratio Statistic	*P*	*P(Adjusted)*
Raoultella_planticola	195.58	-0.02	0.18	11.81	5.90E-04	1.13E-02
*Bacteroides*_sp._PHL_2737	1,590.96	-0.03	0.18	13.38	2.55E-04	5.04E-03
Enterococcus_durans	534.67	-0.04	0.18	18.07	2.13E-05	5.49E-04
Clostridium_sporogenes	257.71	-0.04	0.18	16.52	4.81E-05	1.14E-03
Klebsiella_grimontii	196.31	-0.06	0.18	32.08	1.48E-08	8.80E-07
Klebsiella_aerogenes	365.09	-0.07	0.18	27.70	1.41E-07	5.25E-06
Enterococcus_casseliflavus	734.76	-0.07	0.18	22.91	1.70E-06	5.62E-05
Leuconostoc_mesenteroides	389.13	-0.08	0.21	30.58	3.21E-08	1.59E-06
Anaerococcus_mediterraneensis	29.39	-0.19	0.43	1,856.98	0.00E+00	0.00E+00
Lactobacillus_delbrueckii	145.18	-0.25	0.36	28.15	1.12E-07	4.44E-06
Peptoniphilus_harei	145.60	-0.26	0.37	3,528.95	0.00E+00	0.00E+00
Prevotella_buccalis	945.31	-0.68	0.36	6,672.64	0.00E+00	0.00E+00
Bifidobacterium_breve	5,550.50	-0.68	0.25	28.30	1.04E-07	4.40E-06
Lacrimispora_sphenoides	19.44	-0.87	0.26	10.33	1.31E-03	2.29E-02
Anaerostipes_caccae	201.84	-1.01	0.31	17.05	3.64E-05	9.00E-04
Streptococcus_thermophilus	14,795.11	-1.33	0.35	16,157.15	0.00E+00	0.00E+00
Monoglobus_pectinilyticus	6,414.87	-1.44	0.36	14.78	1.21E-04	2.66E-03
Sellimonas_intestinalis	2,502.82	-1.46	0.30	18.10	2.10E-05	5.49E-04
Schaalia_turicensis	298.10	0.02	0.36	14.85	1.17E-04	2.66E-03
Enterococcus_lactis	590.36	0.02	0.18	59.22	1.41E-14	1.05E-12
Methanosphaera_stadtmanae	228.47	0.03	0.18	10.77	1.03E-03	1.86E-02
Megamonas_funiformis	1,971.97	0.05	0.18	10.89	9.67E-04	1.79E-02
Phascolarctobacterium_faecium	11,863.17	0.05	0.23	18.99	1.31E-05	3.90E-04
Oxalobacter_formigenes	193.69	0.08	0.18	31.89	1.63E-08	8.80E-07
Dialister_massiliensis	1,060.11	0.08	0.18	30.41	3.49E-08	1.60E-06

Bacteroides_stercoris	22,405.53	0.30	0.26	18.84	1.42E-05	4.02E-04
Dialister_hominis	921.75	0.11	0.18	33.24	8.14E-09	5.37E-07
Prevotella_nigrescens	68.74	0.12	0.38	13.38	2.55E-04	5.04E-03
Sutterella_megalosphaeroides	44.40	0.18	0.20	26.75	2.32E-07	8.11E-06
Acidaminococcus_fermentans	209.66	0.26	0.31	185.41	3.19E-42	3.79E-40
Alistipes_onderdonkii	69,205.21	0.34	0.34	123.93	8.74E-29	7.42E-27
Clostridium_baratii	82.46	0.61	0.46	13.72	2.13E-04	4.51E-03
Staphylococcus_aureus	269.36	0.63	0.41	21.54	3.47E-06	1.09E-04
Streptococcus_suis	1,561.84	0.80	0.26	10.26	1.36E-03	2.31E-02
Sutterella_faecalis	45.34	0.94	0.46	137.87	7.77E-32	7.69E-30
*Bacteroides*_sp._CACC_737	3,154.77	1.20	0.34	9.22	2.40E-03	4.95E-02

*Log2FC = Log2 fold change indicates change in the EFP group abundance related to the control group; positive values indicate greater abundance in the EFP group and negative values indicate greater abundance in the control. p <0.05. q is FDR-adjusted by age, sex, stress, internalizing problems, Behavioral Regulation Index problems, externalizing problems, delivery mode, and antibiotic use. Abbreviations: EFP, executive function problems; LD, learning difficulties.

Table S7. Species differentially abundant in subjects with learning difficulties (LD) compared to controls

Species	Mean	*Log2-fold Change	Standard Error	Likelihood-Ratio Statistic	*P*	*P(Adjusted)*
Enterobacter_hormaechei	1,116.93	-0.01	0.14	23.15	1.50E-06	5.31E-05
Shigella_flexneri	233.68	-0.04	0.14	10.44	1.23E-03	2.12E-02
Acidaminococcus_fermentans	209.66	-0.04	0.25	118.63	1.26E-27	4.46E-25
Phascolarctobacterium_faecium	11,863.17	-0.04	0.18	33.01	9.17E-09	5.88E-07
Methanosphaera_stadtmanae	228.47	-0.04	0.14	13.18	2.84E-04	5.41E-03
Klebsiella_pneumoniae	18,411.70	-0.07	0.14	158.76	2.11E-36	1.49E-33
Acidaminococcus_intestini	202.97	-0.07	0.14	18.61	1.60E-05	4.18E-04
Sarcina_sp._JB2	298.40	-0.14	0.30	8.60	3.37E-03	4.95E-02
Ruminococcus_bovis	7,942.51	-0.15	0.28	27.31	1.74E-07	8.74E-06
Muribaculum_gordoncarteri	313.37	-0.16	0.22	17.18	3.40E-05	8.26E-04
Parafannyhessea_umbonata	1,579.90	-0.20	0.23	48.67	3.03E-12	3.56E-10
Turicimonas_muris	72.92	-0.28	0.30	16.16	5.81E-05	1.32E-03
Turicibacter_sanguinis	1,140.47	-0.28	0.32	19.55	9.82E-06	2.89E-04
Ligilactobacillus_ruminis	1,591.55	-0.40	0.34	15.97	6.44E-05	1.42E-03

Streptococcus_sp._oral_taxon_064	5.89	-0.40	0.36	20.64	5.55E-06	1.70E-04
Streptococcus_vestibularis	79.84	-0.42	0.29	23.58	1.20E-06	4.98E-05
Coprococcus_catus	71,534.47	-0.48	0.13	13.18	2.83E-04	5.41E-03
Herbinix_luporum	8.78	-0.69	0.21	10.19	1.41E-03	2.33E-02
Streptococcus_equi	18.40	-0.75	0.24	8.64	3.28E-03	4.93E-02
Olsenella_uli	117.60	-0.79	0.36	38.37	5.86E-10	5.90E-08
Coprococcus_eutactus	15,949.88	-0.85	0.23	10.83	9.96E-04	1.76E-02
Thermophilibacter_immobilis	47.68	-1.26	0.25	21.07	4.42E-06	1.42E-04
Prevotella_xylaniphila	3,362.23	-1.30	0.29	16.31	5.37E-05	1.26E-03
Streptococcus_equinus	32.63	-1.48	0.32	14.58	1.34E-04	2.79E-03
Corynebacterium_argentoratense	429.45	0.00	0.14	12.98	3.15E-04	5.84E-03
Gemella_sp._oral_taxon_928	6.37	0.01	0.24	26.68	2.40E-07	1.13E-05
Enterococcus_durans	534.67	0.01	0.14	82.69	9.58E-20	1.69E-17
Alistipes_onderdonkii	69,205.21	0.02	0.26	37.39	9.68E-10	7.58E-08
Lacticaseibacillus_rhamnosus	394.99	0.03	0.14	26.49	2.64E-07	1.17E-05
Klebsiella_grimontii	196.31	0.04	0.14	30.59	3.19E-08	1.87E-06
Latilactobacillus_curvatus	209.35	0.04	0.17	8.80	3.01E-03	4.62E-02
Megamonas_funiformis	1,971.97	0.05	0.14	36.07	1.90E-09	1.34E-07
Salmonella_enterica	361.08	0.07	0.14	14.74	1.23E-04	2.64E-03
Bifidobacterium_animalis	3,414.48	0.07	0.15	37.77	7.94E-10	7.00E-08
Leuconostoc_mesenteroides	389.13	0.08	0.16	19.38	1.07E-05	3.03E-04
Enterococcus_sp._DA9	139.87	0.17	0.14	27.36	1.69E-07	8.74E-06
Klebsiella_oxytoca	120.75	0.27	0.14	84.95	3.05E-20	7.18E-18
Bifidobacterium_dentium	2,874.49	0.37	0.25	61.40	4.65E-15	6.55E-13
Blautia_pseudococcoides	793.85	0.40	0.13	10.18	1.42E-03	2.33E-02
Haemophilus_pittmaniae	3.11	0.48	0.35	18.44	1.75E-05	4.41E-04
Rothia_aeria	31.21	0.57	0.18	10.09	1.49E-03	2.39E-02
Parvimonas_micra	127.95	0.73	0.25	9.79	1.76E-03	2.75E-02
Bifidobacterium_longum	236,126.02	0.81	0.22	13.26	2.71E-04	5.41E-03
Lacrimispora_sphenoides	19.44	0.86	0.20	21.47	3.60E-06	1.21E-04
Bifidobacterium_breve	5,550.50	1.03	0.19	11.20	8.20E-04	1.48E-02
Sellimonas_intestinalis	2,502.82	1.05	0.23	18.99	1.31E-05	3.57E-04
Vaginimicrobium_propionicum	32.33	1.18	0.24	23.42	1.30E-06	5.09E-05
Aminipila_luticellarii	161.39	1.23	0.25	23.14	1.51E-06	5.31E-05

* Log2FC = Log2 fold change indicates change in the LD group abundance related to the control group; positive values indicate greater abundance in the LD group and negative values indicate greater abundance in the control. p <0.05. q is FDR-adjusted by age, sex, stress, internalizing problems, parental education, marital status, proinflammatory dietary index, and intestinal transit. Abbreviations: EFP, executive function problems; LD, learning disorders

Table S8. Species differentially abundant in subjects with learning difficulties (LD) compared to those with executive function problems (EFP).

Species	Mean	*Log2-fold Change	Standard Error	Likelihood-Ratio Statistic	P	P(Adjusted)
Bifidobacterium_pseudolongum	121.13	-0.19	1.21	78.36	8.60E-19	4.13E-17
Clostridium_baratii	155.04	-0.23	0.58	26.14	3.17E-07	8.06E-06
Bacteroides_stercoris	29,506.31	-0.30	3.37	10.61	1.12E-03	1.99E-02
Sutterella_wadsworthensis	2,184.78	-0.50	3.37	11.91	5.59E-04	1.01E-02
Coprobacter_secundus	455.67	-0.56	0.53	987.52	9.27E-217	1.89E-214
Segatella_copri	31,784.15	-0.56	3.37	45.39	1.62E-11	6.28E-10
Oxalobacter_formigenes	205.60	-0.57	3.39	29.41	5.85E-08	1.70E-06
Dialister_massiliensis	1,294.60	-0.61	3.39	173.80	1.09E-39	1.11E-37
Novosphingobium_sp._EMRT-2	54.28	-0.70	0.62	20.94	4.75E-06	1.05E-04
Megasphaera_massiliensis	127.32	-0.81	3.40	89.32	3.37E-21	1.96E-19
Bacteroides_faecis	17,968.28	-0.85	3.37	30.95	2.65E-08	8.31E-07
Corynebacterium_argentoratense	434.71	-0.94	3.39	142.35	8.15E-33	7.39E-31
Paraprevotella_xylaniphila	2,445.71	-0.95	2.45	29.34	6.07E-08	1.71E-06
Dialister_hominis	1,263.35	-0.95	3.39	241.07	2.30E-54	2.68E-52
Acidaminococcus_fermentans	702.44	-1.11	0.74	1,645.35	0.00E+00	0.00E+00
Eubacterium_sp._MSJ-33	1,834.63	-1.12	0.35	9.96	1.60E-03	2.72E-02
Clostridium_autoethanogenum	75.67	-1.18	3.40	61.53	4.37E-15	1.87E-13
Phascolarctobacterium_faecium	12,222.54	-1.25	3.37	58.37	2.17E-14	8.86E-13
Turicimonas_muris	129.69	-1.44	3.34	87.55	8.22E-21	4.47E-19
Streptococcus_ruminantium	43.08	-1.48	1.38	87.25	9.58E-21	4.89E-19
Faecalibacterium_duncaniae	11,310.18	-1.51	3.37	21.68	3.23E-06	7.52E-05
Ruminococcus_bovis	3,559.43	-1.66	0.46	2,303.30	0.00E+00	0.00E+00
Sutterella_megalosphaeroides	74.36	-1.84	3.39	104.98	1.23E-24	8.39E-23
Bacteroides_sp._PHL_2737	1,736.08	0.13	3.38	20.99	4.61E-06	1.05E-04
Desulfovibrio_fairfieldensis	832.15	0.13	3.38	41.03	1.50E-10	5.31E-09
Limosilactobacillus_mucosae	3,284.29	0.15	3.40	33.35	7.69E-09	2.51E-07
Lactobacillus_helveticus	297.30	0.15	1.62	27.52	1.56E-07	4.23E-06
Latilactobacillus_sakei	2,001.42	0.29	3.37	20.70	5.38E-06	1.16E-04
Enterococcus_sp._DA9	422.79	0.43	3.40	10.43	1.24E-03	2.15E-02
Raoultella_ornithinolytica	151.21	0.45	3.40	22.58	2.02E-06	4.85E-05
Clostridium_novyi	110.05	0.51	3.40	26.46	2.69E-07	7.08E-06
Bifidobacterium_animalis	6,230.45	0.56	3.37	30.32	3.66E-08	1.11E-06
Enterococcus_lactis	574.27	0.59	3.39	19.52	9.96E-06	2.08E-04
Lawsonella_clevelandensis	157.27	0.62	2.40	90.48	1.87E-21	1.17E-19
Klebsiella_oxytoca	477.20	0.65	3.39	19.24	1.15E-05	2.35E-04
Citrobacter_portucalensis	40.10	0.73	3.40	12.30	4.53E-04	8.39E-03
Enterococcus_durans	371.91	0.77	3.39	113.32	1.84E-26	1.36E-24
Akkermansia_muciniphila	505,402.08	0.78	3.37	41.76	1.03E-10	3.82E-09

Klebsiella_grimontii	234.15	0.78	3.40	37.38	9.73E-10	3.31E-08
Anaerococcus_vaginalis	28.70	0.91	0.76	310.29	1.89E-69	2.57E-67
Clostridium_beijerinckii	1,211.06	0.91	3.37	13.46	2.43E-04	4.84E-03
Leuconostoc_mesenteroides	571.12	1.37	3.38	123.19	1.26E-28	1.03E-26
Enterococcus_casseliflavus	81.69	1.42	1.76	26.09	3.26E-07	8.06E-06
Lacrimispora_sphenoides	26.27	1.59	0.42	12.52	4.02E-04	7.64E-03
Sellimonas_intestinalis	3,795.35	1.74	0.44	13.25	2.72E-04	5.29E-03
Enterobacter_ludwigii	61.95	12.53	3.40	76.18	2.60E-18	1.18E-16
Streptococcus_thermophilus	11,891.06	2.15	0.54	3,701.34	0.00E+00	0.00E+00
Lactobacillus_delbrueckii	128.95	2.45	0.87	865.31	3.41E-190	5.56E-188

*Log2FC = Log2 fold change indicates change in the LD group abundance related to the EFP group; positive values indicate greater abundance in the LD group and negative values indicate greater abundances in the EFP. $p < 0.05$. q is FDR-adjusted by age, sex, parental education, Behavioral Regulation Index, externalizing problems, proinflammatory dietary index and gastrointestinal disorders. Abbreviations: EFP, executive function problems; LD, learning disorders.

Supplementary Figures

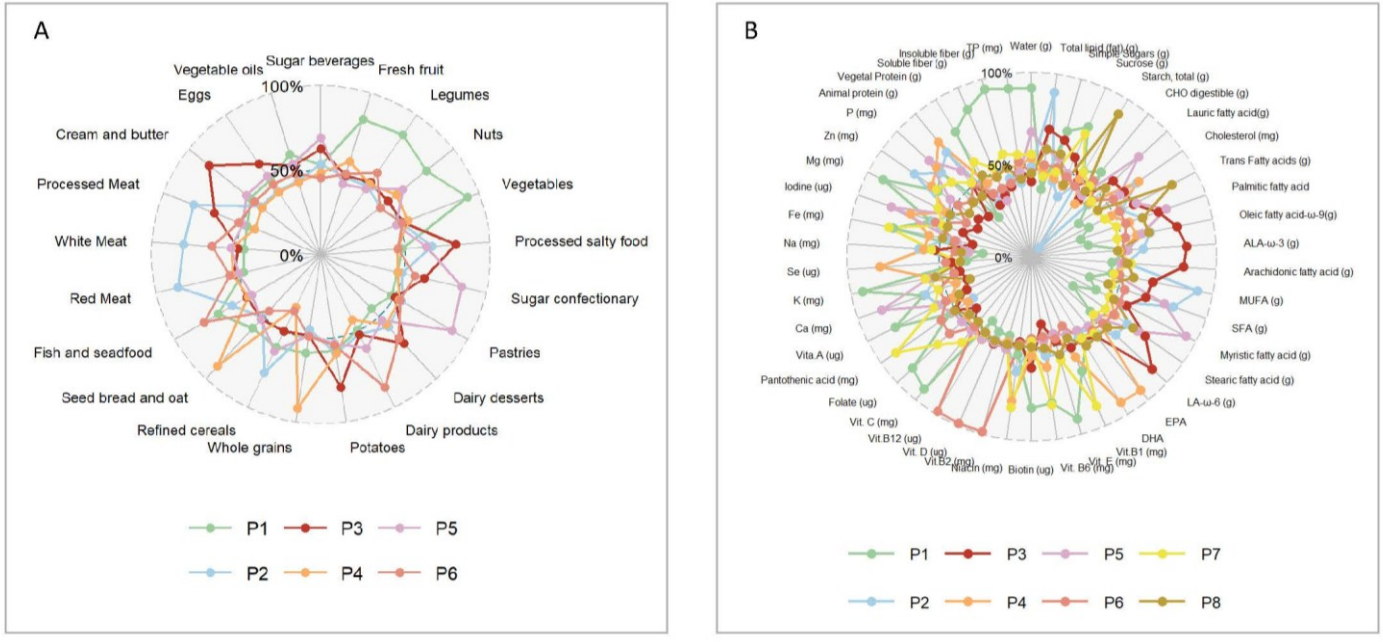


Figure S1. **Dietary Patterns in children and adolescents.** Radar chart showing dietary patterns extracted from principal component analysis of 20 food groups (**A**) and 52 nutrients (**B**).

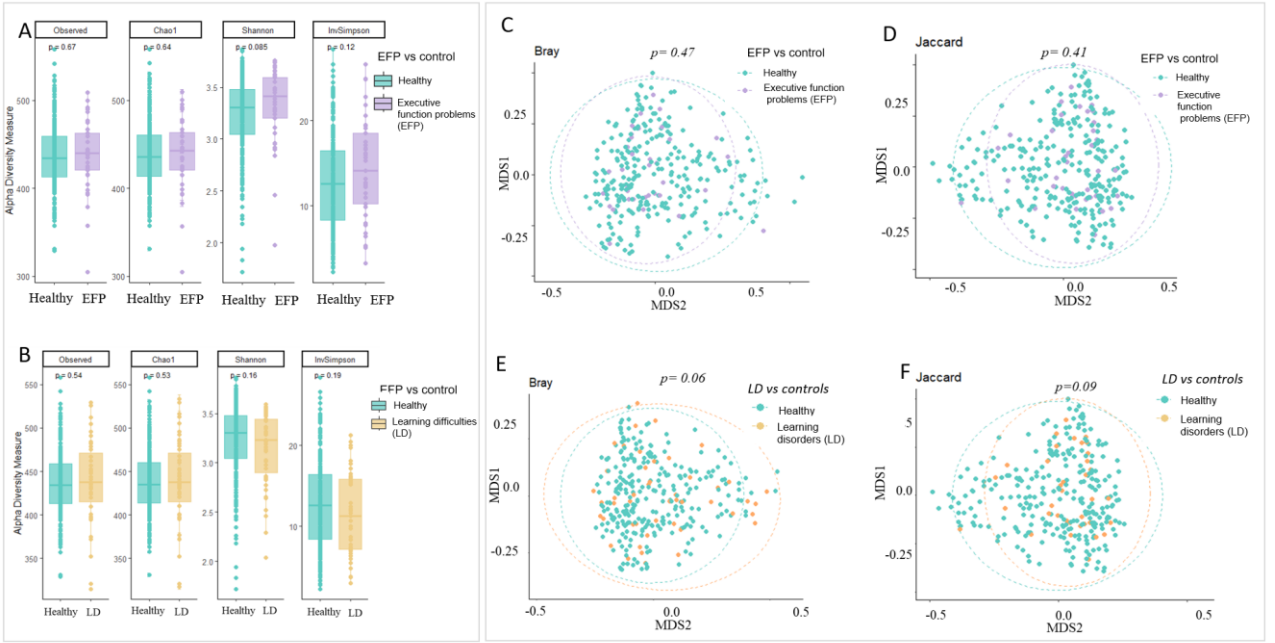


Figure S2. Alpha and beta diversity analysis of the gut microbiome from subjects of the executive function problems (EFP) group. (A) Alpha diversity indexes and statistical relevance through Wilcoxon test in executive function problems (EFP) vs controls. (B) Alpha diversity indexes and statistical relevance through the Wilcoxon test in LD vs control. (B and C) Beta diversity indexes (Bray-Curtis and Jaccard, respectively) and statistical relevance based on PERMANOVA permutations in learning difficulties (LD) vs control.

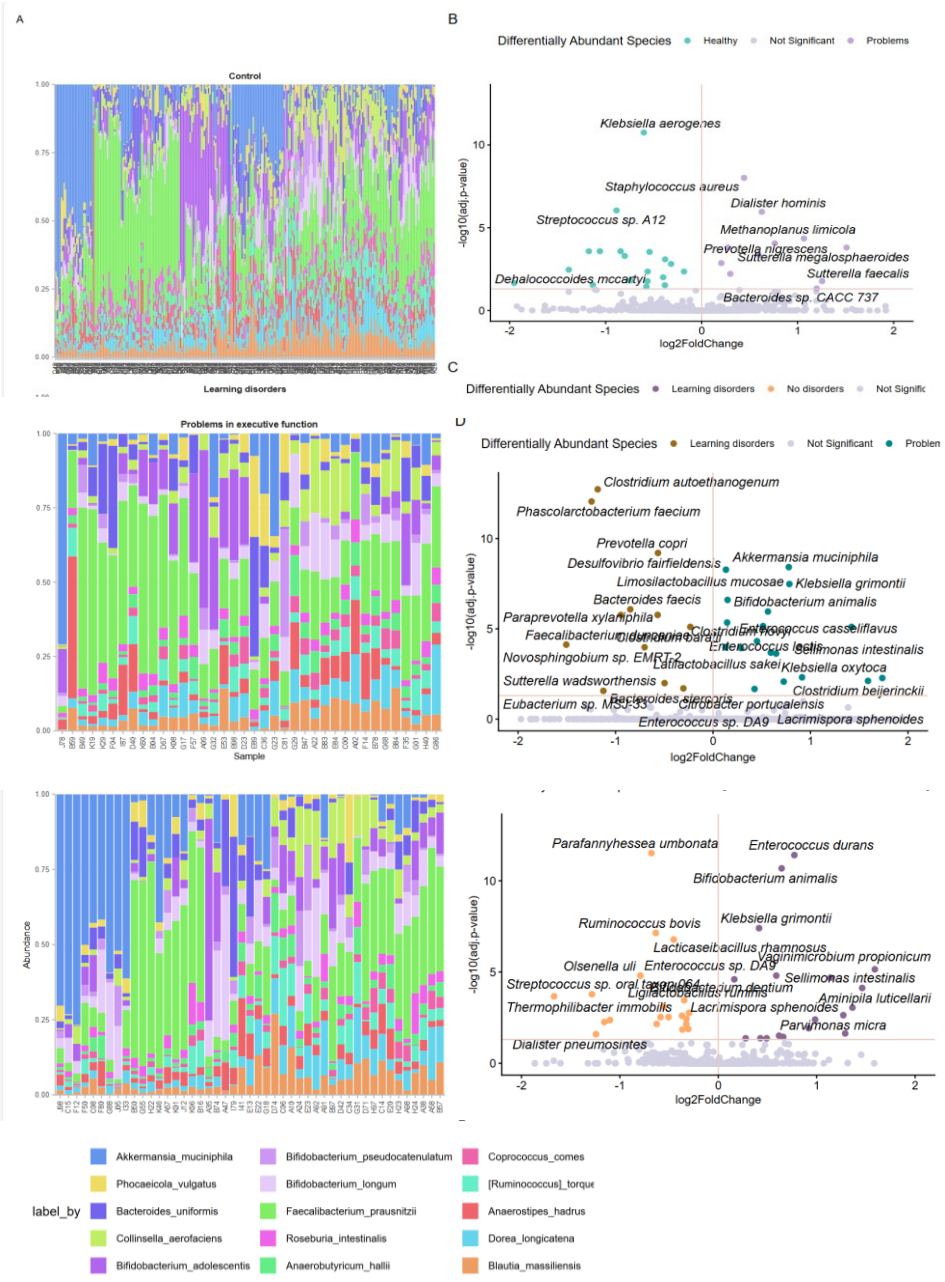
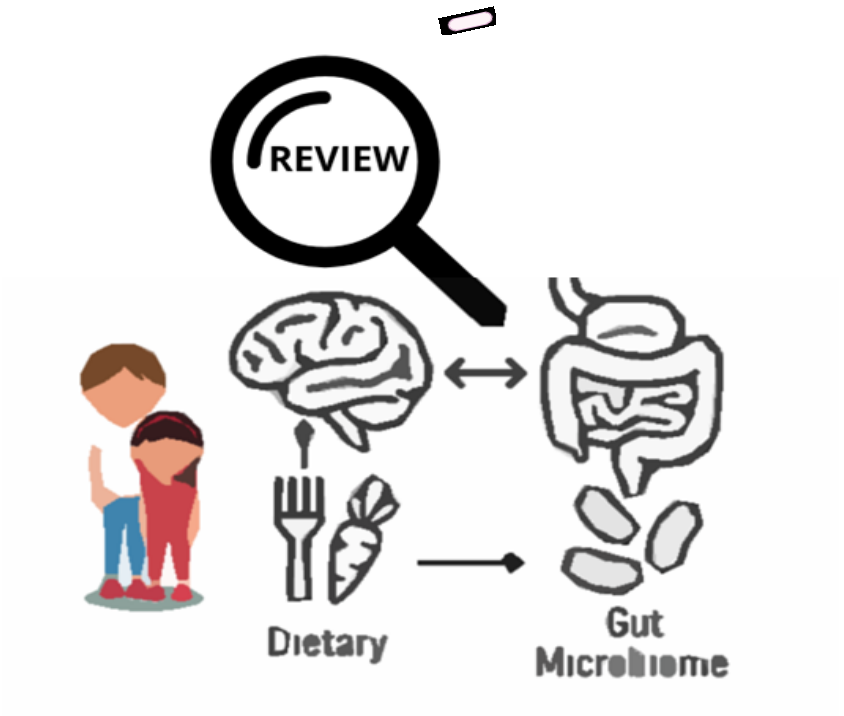


Figure S3.Metataxonomical analysis across the cognitive-impairment groups and their comparison. (A) More prevalent taxa across the control, executive function problems (EFP) and learning difficulties (LD) groups. **(B)** Differentially-abundant species from the comparison of the EFP and control groups. **(C)** Differentially-abundant species from the comparison of the LD and control groups. **(D)** Differentially-abundant species from the comparison of the EFP and LD groups

CHAPTER 4

TOWARDS PERSONALIZED MICROBIOME AND DIETARY APPROACHES FOR NEURODEVELOPMENTAL DISORDERS: SETTING THE THEORETICAL BASIS



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ABSTRACT

Mental health is determined by a complex interplay between the Neurological Exposome and the Human Genome. Multiple genetic and non-genetic (exposome) factors interact early in life, modulating the risk of developing the most common complex neurodevelopmental disorders (NDDs), with potential long-term consequences on health. To date, the understating of the precise etiology underpinning these neurological alterations, and their clinical management pose a challenge. The crucial role played by diet and gut microbiota in brain development and functioning would indicate that modulating the gut-brain axis may help protect against the onset and progression of mental-health disorders. Some nutritional deficiencies and gut microbiota alterations have been linked to NDDs, suggesting their potential pathogenic implications. In addition, certain dietary interventions have emerged as promising alternatives or adjuvant strategies for improving the management of particular NDDs, at least in particular subsets of subjects. The gut microbiota can be a key to mediating the effects of other exposome factors such as diet on mental health, and ongoing research in Psychiatry and Neuropediatrics is developing Precision Nutrition Models to classify subjects according to a diet response prediction based on specific individual features, including microbiome signatures. Here, we review current scientific evidence for the impact of early life environmental factors, including diet, on gut microbiota and neuro-development, emphasizing the potential long-term consequences on health; and also summarize the state of the art regarding the mechanisms underlying diet and gut microbiota influence on the brain–gut axis. Furthermore, we describe the evidence supporting the key role played by gut microbiota, diet and nutrition in neurodevelopment, as well as the effectiveness of certain dietary and microbiome-based interventions aimed at preventing or treating NDDs. Finally, we emphasize the need for further research to gain greater insight into the complex interplay between diet, gut microbiome and brain development. Such knowledge would help towards achieving tailored integrative treatments, including personalized nutrition.

Keywords: gut microbiota, gut bacterial microbiome, virome, diet, precision nutrition, neurodevelopment, psychiatry and mental health

INTRODUCTION

Neurodevelopmental disorders (NDDs) are linked to the disruption of coordinated events leading to neurodevelopmental processes, and constitute a cluster of disorders characterized by the inability to reach cognitive, emotional, and motor developmental milestones1. Although NDDs have an early onset, they frequently persist into adulthood, potentially leading to significant long-term health consequences. Characteristically, NDDs constitute a serious health problem in our society, affecting roughly 3% of children worldwide. In addition, the prevalence of these conditions is rising, which poses a serious challenge.

Human genetics is a well-known key factor involved in the etiology of the most common NDDs; however, both environmental and gene-environment interactions appear to strongly influence their clinical manifestation, which would explain the lack of heritability of these disorders1, 2. Despite many years of research, studies have failed to fully identify all determinants and mediators leading to the onset and progression of these multifactorial conditions and, thus, their precise etiologies remain uncertain. High comorbidity among NDDs is frequently observed, which in turn worsens their prognosis3. In this regard, the identification of the shared pathogenic mechanisms among the different NDDs will ultimately help to lead to effective treatment to combat this high comorbidity as well as to monitor their progress and to anticipate future complications.

The diagnosis of these conditions, based mainly on clinical symptoms, and their management is highly challenging. Pharmaceutical treatment, the first-line therapy of choice for clinical management, is often associated with several side effects, and other concerns about long-term effects and efficacy exist 4. Therefore, new alternatives are required for the diagnosis, prevention and treatment of these disorders. In this respect, dietary intervention ranks very highly among such strategies and has the added benefit of being acceptable by most people as more “benign” and potentially free of side effects.

The bidirectional nature of the gut–brain axis is well- recognized, with gut microbiota emerging as a key player controlling this two-way communication system5. Importantly, the gut microbiota establishes its symbiotic relationship with the host early in life and the initial perturbations

of this connection can impact neurodevelopment, potentially leading to adverse mental health consequences later in life6. In-depth knowledge of this critical early microbial– neural window of opportunity will open new insights for novel microbiota modulation-based therapeutic interventions in early life, targeting neurodevelopmental impairments and brain disorders 7. In fact, recent evidence suggests that dysregulation of the gut-brain axis may be a crucial factor contributing to many mental health related disorders. In this regard, animal models such as germ-free (GF) mice, entirely devoid of microbiota, as well as animals treated with antibiotics or with specific bacterial species have shed light on how the gut microbiota and its genome (gut microbiome) are involved in regulating brain development and function and, consequently, cognition and behavior 8, 9.

Specifically, studies using GF mice have established that the total absence of microbiota impairs social behavior 10, as well as other types of behavior such as anxiety and stress response11–13. Furthermore, certain behaviors linked to the lack of gut microbiota correlate with neurochemical changes in different brain regions, with specific alterations in neurotransmitter pathways14. Additionally, fecal microbiota transplantation has demonstrated its ability to transfer certain behavioral traits, suggesting a causal link and thus pointing to alterations in the microbiota as a trigger of particular phenotypes15. Moreover, a growing number of human cross- sectional studies have investigated the microbiota composition in individuals with certain neurological disorders or impairment vs. healthy age-matched individuals, and identified gut microbiota alterations associated with neurological alterations. However, these studies only provide a snapshot in time, which is a strong limitation to establishing causality9 and, thus, there is a clear need for well-designed human prospective studies to identify the specific human gut microbial members or gut microbiota profiles able to trigger NDDs or to modify their prognosis.

Diet has been recognized as one of the main factors shaping the gut microbiota and consequently its modulation is considered one of the easiest ways to develop and maintain a healthy gut microbiome. In fact, gut microbiome modulation through dietary interventions has been widely suggested as a robust and attainable strategy to prevent disorders, including mental conditions 16–18, and to improve human health19, 20. In this regard, modulation of gut microbiota functionalities has been proposed as a key mediator of the dietary effects on mental health 21. However, diet

is also known to be involved in brain development and functioning through both microbiota-dependent and independent mechanisms22. Thus, scientific evidence indicates that diet and gut microbiota can interact or act synergistically to provide resilience against disorders, or conversely work together in the progression from health to disease.

In the context of NDDs, some dietary interventions, including dietary advice and specific diets or certain dietary components and supplements, may influence the onset and manifestation of neurodevelopmental impairments in childhood neurological disorders, at least in specific cohorts 23–27. However, a high inter-individual variation in the clinical response to different dietary interventions has been well-documented, and patient subgroups with the same diagnosed condition respond differently to certain dietary interventions26. Accordingly, within the framework of Nutritional Psychiatry, research has focused on “precision nutrition,” which is currently working on the design of tailored microbiome-based and dietary intervention strategies to promote the development and maintenance of a healthy brain26–29. It is important to note that potential new tailored dietary-based intervention strategies could pave the way to preventing the development of NDDs in subjects with risk indicators and constitute also new alternatives for improving the clinical management of subjects diagnosed with NDD.

In this context, we should highlight that the relationship between diet and mental health is complex and bidirectional, and studies report less healthy dietary choices among patients with NDDs 30, 31. In this regard, subjects suffering NDDs might also find it more difficult to adhere to potentially beneficial dietary interventions. Therefore, there is an urgent need to decipher the mechanisms by which dietary intervention exerts beneficial effects, with a view to designing safer and easy-to- implement targeted intervention strategies for patients with mental disorders.

Here, we review the current scientific evidence for the impact of early life environmental factors on gut microbiota and neurodevelopment, and highlight the long-term consequences of early nutrition on physical and mental health. We also summarize the state of the art regarding the mechanisms by which gut microbiota influences the brain–gut axis. In addition, we describe the evidence supporting the key role played by gut microbiota, diet and nutrition in neurodevelopment, as well as the effectiveness of certain dietary and microbiome-based interventions aimed at preventing and treating NDDs. Finally, we highlight the need for further

research to advance towards achieving essential tailored integrative treatments, including personalized nutrition.

IMPACT OF EARLY LIFE ENVIROMENTAL FACTORS ON GUT MICRIOBIOTA AND NEURODEVELOPMENT: KEY ROLE OF DIET

One of the main promises of the Human Genome Project (HGP) was to increase our understanding of the mechanisms underlying the etiology of complex diseases, thereby furthering their prevention and treatment. However, after HGP completion, human genetics was found to explain merely 20–30% of the etiology of these traits, with the rest owing to environmental factors and their interactions with the human genome[32].

The exposome can be defined as the measure of all types of exposure an individual is subjected to in a lifetime and include insults from the external environment, which interact with the internal environment (biological factors such as metabolism, gut microbiota, inflammation, and oxidative stress) impacting on health[33, 34]. The host is especially vulnerable to the effects of environmental risk factors during organ development in the prenatal period, infancy, childhood and adolescence. Consequently, the early life Exposome-Genome interplay leads to crucial long-term consequences on mental and overall health[35]. Additionally, it is well-established there are periods where gut microbiota is more unstable and, therefore, at greater risk of suffering alterations due to exposure to environmental stressors. These periods can also be considered as windows of opportunity to positively influence mental health through gut microbiota modulation[7].

Remarkably, the critical gut microbiota developmental period occurs in parallel with brain development[2] and, although it may experience changes later in life despite greater resilience, gut microbiota colonization of the infant is more dynamic. This process begins prenatally, and the symbiotic link between the host and the microbiota is established early in life during the perinatal period and continues through infancy, childhood and early adolescence [7].

Well-documented factors associated with the risk of NDD onset and contributors to the individual's microbiota are those related to maternal

health (stress, infections, gestational age, obesity and exposure to medication, including antibiotics and other drugs) [36] and many others related to infant and childhood health[37–41] such as mode of delivery, early type of feeding, medication, stress and infections (Figure 1). In fact, maternal health, diet and nutrition during pregnancy can modulate the offspring microbiome and neurodevelopment as well as brain characteristics of the offspring, both positively and negatively, by regulating neurotransmitter pathways, synaptic transmission and signal-transduction pathways[42]. Evidence from animal models has demonstrated that maternal high-fat- diet (MHFD)-induced obesity alters gut microbiota composition in the offspring and impacts behavioral programming, thereby triggering impairments in social/emotional behavior and cognitive development (43). Recent studies in mice have shown that some probiotics reverse the effects of stress caused by maternal separation[42] while timely dietary interventions can rescue maternal obesity-induced behavioral deficits and neuroinflammation in offspring[44, 45].

By contrast, several studies have associated maternal adherence to a Mediterranean diet with favorable offspring behaviors. Important to note it is that many of the Exposome factors playing a role in the etiology of NDDs, including diet, are known to be closely related with the risk of developing disorders or conditions through epigenetic mechanisms[46, 47]. Mode of delivery has also been reported as one of the main regulators of the early-life gut microbiota composition, and a recent study in mice demonstrated that alterations in gut microbial community induced by Cesarean-section (C-section) are associated with anxiety and social and cognitive impairment in the offspring [48]. In this regard, the authors also reported that supplementation from birth with a *Bifidobacterium* breve strain, or with a dietary prebiotic mixture that promotes bifidobacterial growth, reverses selective behavioral alterations in C-section mice. These results strongly suggest that adjunctive microbiota-targeted therapies may be developed to help avert the long-term negative behavioral consequences associated with C-section. In consistency with this, very recently in a human prospective study it has been reported the impact of breastfeeding on the modulation of the longitudinal impact of delivery mode on the gut microbiota composition, with an observed increase in the relative abundance of *Bacteroides fragilis* and *Lactobacillus* in Cesarean-delivered infants with longer duration of breastfeeding [49]. This finding strongly supports the fact

that duration of breastfeeding might plays a critical role in restoring a health-promoting microbiome.

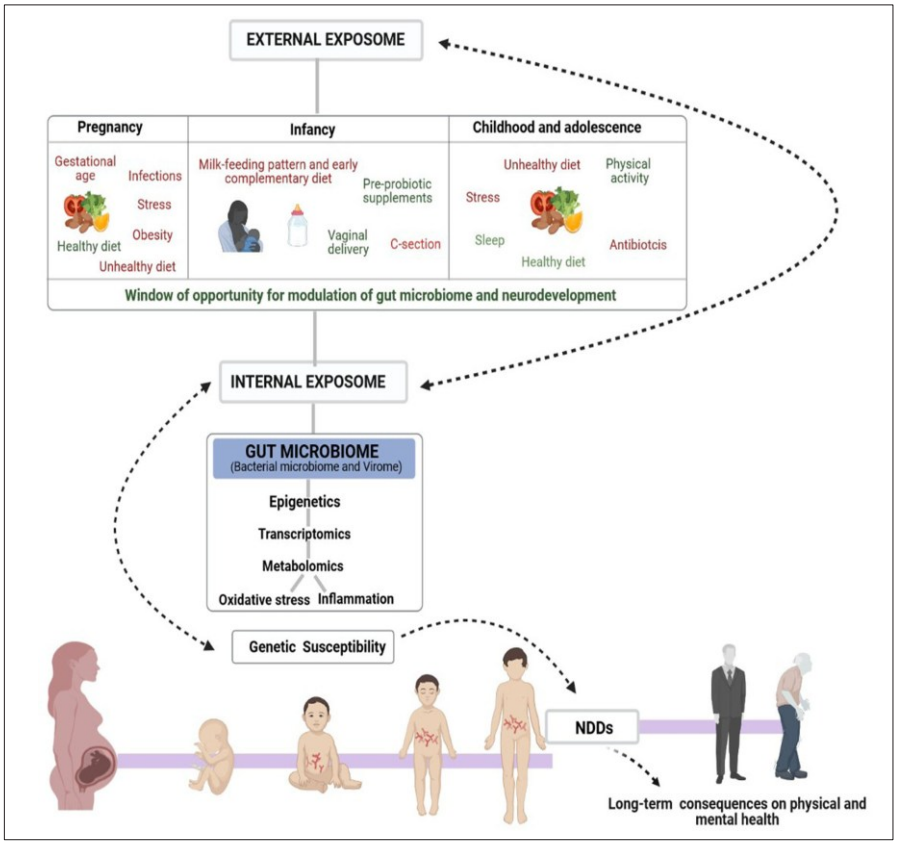

FIGURE 1 | Schematic representation of the complex Exposome-Genome interplay in human neurological development. The figure also shows the early window of opportunity for gut-microbiome and neurodevelopmental modulation to influence mental health, mainly through dietary interventions. Early non-genetic risk and protective factors of the external exposome (prenatal, infancy, childhood and adolescence lifestyle factors) interact with the internal exposome (biological factors such as gut microbiota, metabolism, inflammation, and oxidative stress) controlling the onset of the most common NDDs in genetically susceptible individuals and consequent physical and mental health in adult life.

Other environmental exposures such as antibiotics administration during pregnancy, in the first months of life, childhood or adolescence may

also strongly impact gut microbiota colonization and determine neurocognitive impairments later in life[39–41, 50]. A recent study on the effects of 3-weeks microbiota depletion in antibiotic-treated mice reported that transient microbiota depletion during adolescence, a particularly vulnerable time for gut microbiota composition and when the brain is highly responsive to certain environmental factors, has long-lasting effects on microbiota composition and increases anxiety-like behavior; however, this was not the case in adulthood [51].

Nonetheless, the precise positive and negative consequences of gut microbiota manipulation, mainly through dietary interventions in adolescent mice, have yet to be fully understood, and require more in-depth study. In this respect, human dietary interventions have already emerged as promising alternatives or adjuvant therapies for the prevention and clinical management of NDDs, at least for specific subsets of patients [23–27]. However, further human trials should be conducted to investigate the precise role of dietary modification in rescuing behavioral and cognition alterations induced in early life, and to understand whether gut microbiota modulation mediates these effects. Currently, the potential beneficial effects of early-life dietary supplementation with omega-3 polyunsaturated fatty acids (PUFAs) on neurocognitive development are being extensively explored. To date, research has shown the importance of omega-3 PUFA supplementation during the prenatal and early-life period as a potential protective factor for the onset of neurological impairments[52–54].

It is important to highlight that the full understanding of the early interaction between gut microbiota, environment and host through well-designed prospective human studies at early stages in life would open new insights for microbiome-based therapeutic interventions. Such strategies could prevent the development of NDDs in individuals at risk, or improve the treatment of NDD diagnosed subjects.

GUT MICROBIOTA MECHANISMS MODULATING GUT-BRAIN AXIS

Although further understanding of how the microbiota regulates gut-brain communication is required in order to establish the rationale behind microbiota-based interventions, numerous mechanisms by which gut microbiota regulates the gut–brain axis and subsequently modulates brain development and function have been described, including immune, metabolic, endocrine and neural pathways[9] (Figure 2). Remarkably, gut

microbiota can modulate eating behavior by, for example, influencing reward and satiety pathways [55], which may, in turn, potentially change microbiota composition and function[56]).

The gut microbiota can substantially impact on host metabolomics profile. In this context, certain gut microbiota members produce or modulate the levels of different neuroactive molecules, such as essential neurotransmitters and other specific neuromodulators[5]. Neurotransmitters in the intestinal lumen can induce epithelial cells to release molecules, including hormones and cytokines, potentially modulating neural signaling within the enteric nervous system and, consequently, controlling brain function, cognition and behavior. Additionally, the brain can influence gut microbiota composition and function through the release of hormones or neurotransmitters, which act on gut physiology and microbiota environment, resulting in preferential growth of certain microbial communities[5]. Some animal studies have also shown that different bacterial strains mediate their effects on brain function and behavior via the vagus nerve. Regarding neurotransmitters, tryptophan is an essential amino acid precursor of the neurotransmitter serotonin and also the precursor of metabolites related to the kynurenine pathway, whose metabolism is controlled by gut microbiota[57]. Furthermore, the downstream metabolites of the kynurenine pathway have neuroactive properties and can also modulate neurotransmission[5].

Furthermore, some studies in germ-free (GF) mice have revealed that gut microbiota modulate the development of the HPA axis, impacting stress response. In this respect, some studies have proven the effectiveness of microbiota-based interventions in increasing stress resilience in healthy subjects[58]. Other signals to the brain that originate from the gut microbiome are likely mediated through a variety of bacterial neuroactive metabolites, such as short-chain fatty acids (SCFAs) butyrate, acetate, and propionate, which bind free fatty acid receptors in the brain. Specifically, in rats, the administration of high doses of propionate, a common preservative found in foods, induces neuroinflammation and behavioral alterations associated with NDDs[59].

Research has also revealed that the gut microbiota largely impacts host gene expression by modulating epigenetic modifications, such as methylation, histone acetylation or non- coding RNAs (ncRNAs), which are

involved in neurogenesis, neuronal plasticity, learning and memory[60]. Thus, modulation of the microbiota might enable us to shape our epigenome. Indeed, methylation levels of genes involved in inflammatory responses have been linked to gut microbiota profiles. Several studies have defined a link between the levels of specific microRNAs and microbiota[61], while others have also reported that SCFAs can inhibit histone deacetylases (HDACs), activating the gene expression of previously deacetylated genes that impact on crucial physiological processes[60]. In this regard, butyrate has been demonstrated to exert beneficial behavioral effects, through an epigenetic mechanism related to histone deacetylase inhibition[62].

The gut microbiota is well-known to play a central role in modulating oxidative stress as well as immune system development and function[63, 64]. The involvement of the relationship between dietary constituents, gut microbiota and gut immunity has been emphasized in the overall homeostasis of inflammation and oxidative stress, pathways with important implications on the central nervous system and involved in the mechanisms of action and therapeutic effects of omega-3 PUFAs and other dietary supplements on mental health[65]. In addition, dysregulation of microglial functions, such as synaptic pruning, are involved in disorders associated with cognitive impairment and gut microbiota plays a crucial role in the development and function of this specialized macrophage cell population in the central nervous system[66]. In fact, the administration of SCFAs has been reported to rescue the microglial function previously compromised in GF animals[67].Through the effect of SCFAs, the microbiota has also been shown to modulate the levels of brain-derived neurotrophic factor (BDNF), a neurotrophin with important effects on brain development and plasticity, which is dysregulated in patients with NDDs [68]. Therefore, further studies are warranted to explore whether gut microbiota could be modulated to positively impact neurotrophin levels in the context of neurodevelopment[69]. Finally, it is also important to note the role of gut microbiota in modulating gut permeability, as well as the permeability of the blood-brain barrier by regulating the expression of tight-junction proteins[70].

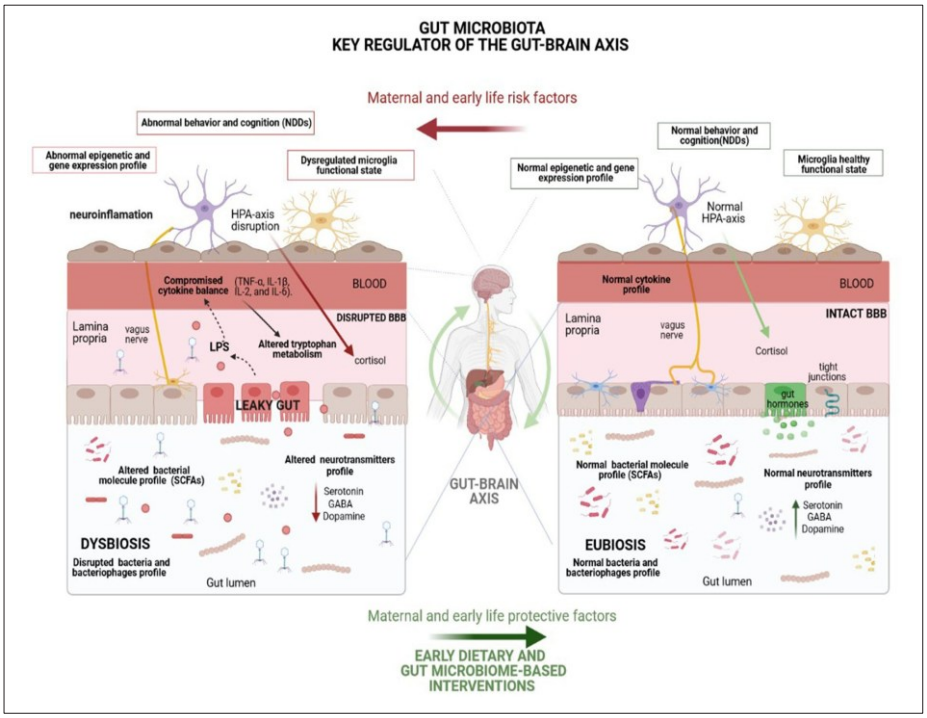

FIGURE 2|Representation of the underlying mechanism, including immune (cytokines balance and microglia function), metabolic (short-chain fatty acids), endocrine (cortisol) ans neural (vagus nerve and enteric nervous system-ENS) pathways, by which the gut microbiota modulates the gut-brain axis and, consequently, brain function and behavior. Many environmental factors may disturb gut microbiota homeostasis, leading to a microbial imbalance (dysbiosis) which enhance the risk of NDDs mainly through gut permeability disruption, as well as by the disturbance of the host stress hormones and cytokine profile and the alteration of neurotransmitter metabolism and levels of other crucial neuroactive molecules. Highlighted potential of healthy dietary interventions to curb the hamrful effects of the life stressors on gut microbiota composition and mental health. HPA, Hypothalamic-pituitary-adrenal; BBB, Blood brain barrier.

THE POTENTIAL ROLE OF THE UNDEREXPLORED VIROME IN NEURODEVELOPMENT

Nowadays, it is clear that the human gut is inhabited not only by bacteria but also by less explored gut prokaryotic and eukaryotic viruses. With the growth of next-generation sequencing technologies, our knowledge about the role of gut bacterial microbiome in health and disease has rapidly expanded[71]. By contrast, the role of the genome of gut viruses (gut viral microbiome or virome) on health still remains underexplored due to the important challenges facing gut virome research. Notwithstanding, in recent years these microorganisms have gained their own "omics," becoming known as (meta) viromics[72].

In studying the role of viruses, researchers have traditionally focused on eukaryotic viruses due to their well-known impact on human health. A few years ago they began to realize the relevance of gut bacteriophages for gut microbiome dynamics, and to pay attention to the previously ignored relationship between bacteriophages and human diseases. In fact, cumulative evidence has started to suggest that gut bacteriophages known as phages, which are prokaryotic viruses that dominate over other viruses in the gut ecosystem and infect gut bacteria, can also strongly impact human health, either beneficially or detrimentally[72]. In fact, despite the challenges facing research into the role of the virome in health, compelling evidence is emerging for bacteriophage involvement in several diseases, such as Inflammatory bowel disease (IBD)[73], Type 1 diabetes (T1D) [74], Type 2 diabetes (T2D) [75] and Clostridium difficile infection (CDI) [76], among others. Through predation, bacteriophages can influence the abundance of specific bacterial taxa, with indirect effects on the rest of the microbial community. Moreover, in certain cases, bacteriophages can be responsible for driving bacterial evolution to adopt more or less virulent phenotypes, by inserting their genomes into the bacterial chromosome[77]. The human gut virome develops rapidly after birth and during early development and, like the bacteriome, it is tremendously dynamic[78] with gut virome inter-individual variability largely due to environmental rather than genetic factors[79]. Specifically, recent research reports an expansion of the eukaryotic virome and the bacterial microbiome from birth to 2 years of age, during healthy development, closely accompanied by a decrease and shift in bacteriophage composition[78]. Additionally, in recent years it is becoming evident that alterations in the virome early in life, similarly to early alterations in the bacterial microbiome, could set the stage for the infant's future health and disease risk. In this regard, although there is still a knowledge gap in mother–infant virome transmission, various studies have indicated that mode of delivery, breastfeeding and early

nutrition are among the main environmental factors involved in shaping the infant virome [78].

Furthermore, ongoing studies are indicating the high potential of bacteriophages to modulate bacteria linked to the intestinal mucosa and related to diseases[80, 81]. For instance, IBD patients seem to display a significant expansion of bacteriophages from the order Caudovirales[75], while Cornault et al. found that prophages of Faecalibacterium prausnitzii, a bacterium repeatedly found depleted in IBD patients, are more abundant in the fecal samples of IBD patients compared to healthy controls[82]. Since IBD patients generally have less *F. prausnitzii* in their gut microbiota than healthy controls, the higher abundance of some of phages infecting major human gut commensal bacteria, such as *F. prausnitzii*, may indicate the potential role of these phages in IBD pathophysiology[75]. Additionally, recent scientific evidence indicates that the virome profile at the time of transplant can influence treatment success of FMT. In this regard, bacteriophage transfer during FMT in Clostridium difficile infection has been associated with treatment outcome[83]. These findings pave the way for further studies investigating whether bacterial microbiome modulation through bacteriophages may improve the effectiveness of therapeutic strategies, such as dietary interventions. In relation to mental health, extensive evidence has supported the fact that exposure to viruses can alter cognition and behavior[84, 85]. Furthermore, several common human herpesvirus and some bacteriophages, including the lactobacillus phage phi-adh, are more predominant in individuals with schizophrenia compared to controls, and their abundance has also been associated with different clinical patterns and medications[84]. In particular, the high abundance of a chlorovirus, specifically Acanthocystis turfaea chlorella virus-1 (ATCV-1), has been associated with decreased cognitive functioning in both mice and healthy subjects without an underlying psychiatric disorder[85]. ATCV-1 exposure in mice was also found to result in an altered expression of genes involved in pathways related to synaptic plasticity, learning and memory, in the hippocampus[85].Thus, it is important to highlight the need for further investigation into the role of the virome in shaping the gut ecosystem during early life, as well as the influence of external factors on the long-term evolution of bacteriome– virome interactions, and the involvement of these interactions in neurodevelopment and mental health. In addition, we have yet to elucidate the precise mechanisms through which viruses could influence human health, including brain functioning. Greater knowledge about the role of the virome in brain development and function may promote future approaches toward more accurate manipulation of the

human gut microbiota through the modulation of viruses to improve mental health.

GUT MICROBIOTA, BEHAVIOR AND COGNITION

The gut microbiota is known to play a critical role in essential brain processes such as myelination, neurogenesis and microglial activation involved in behavior, mood and cognition[14]. Several studies using GF mice have demonstrated that gut microbiota is key to social behavior[10] and other types of behavior such as anxiety[86] and stress response[87]. Moreover, the ability to transfer behavioral traits by FMT supports the fact that some microbiota changes could rather be a driver rather than a consequence of the behavioral alteration[15]. Additionally, recent studies using GF animals have also shown that the microbiome can control neurogenesis [88], a key process in learning and memory, as well as function of the amygdala[89], a brain area for social and fear-related behaviors, and prefrontal cortical myelination[90]. On the other hand, administration of certain probiotics to healthy rats and mice can modulate behavior, accomplishing beneficial effects on stress- related behaviors by reducing anxiety-like and depressive-type behaviors[91]. The gut microbiota assembly and dynamic progression in early life is shown to be accompanied by dramatic changes in the brain and the emergence of a number of cognitive functions[7]. However, the effect of diet and gut microbiota on the development and progression of cognitive functions is still largely unknown[92]. To date many studies in animal models have indicated the short-term and long-term effects of diet and eating habits on cognitive functions. For instance, a high-fat diet, which changes gut microbiota composition leading to gut dysbiosis, has been widely associated with cognitive decline, while experimental manipulation of gut microbiota in rodents has been shown to impact on cognition[93]. Proper HPA- axis functioning has been demonstrated essential for cognitive processes, such as memory and learning, and specific microbiota components beneficially modulate stress-induced alteration of the HPA axis[94].

However, similar evidence in humans is scarce. An observational study investigating 77 children at 18–27 months of age showed some associations between gut microbiome composition and child temperament characteristics, specifically reporting a positive correlation of extraversion and gut microbiota diversity in both boys and girls, as well as an association between fear and a high abundance of *Rikenellaceae* in girls[95].

Regarding gut microbiome and cognition, a cross-sectional study reported a positive correlation between gut microbial composition and adult cognition[96]. A specific association has been described between the 1-year-old gut microbiome and cognitive outcomes at one and 2 years of age[97]. Particularly, a prospective study predicted better cognitive development in infants with higher alpha gut microbiota diversity, as well as with higher abundance of *Bacteroides* putative beneficial microorganisms, which have been linked to vaginal delivery[97]. However, studies have not yet addressed when exactly this relationship between gut microbiota and cognition emerged.

GUT MICROBIOTA, DIET AND ADHD

Attention deficit hyperactivity disorder (ADHD) is currently the most prevalent neurodevelopmental complaint. It is characterized by inappropriate levels of hyperactivity, impulsivity and/or attention difficulties[98]. Current ADHD standard treatment is based on a multimodal approach combining psychotherapy and pharmacotherapy. However, in clinical trials 20–35% of ADHD subjects showed an inadequate response to the treatment[99]. Moreover, pharmacotherapy is frequently associated with adverse side effects and its long-term efficacy is still questionable[100]. Therefore, safer and alternative treatments for ADHD are clearly needed. In this regard, several environmental factors, including diet, associated with the risk of developing ADHD, are linked to shifts in gut microbiota composition, which suggests a link between gut microbiota and ADHD etiology[101]. In fact, some studies have found gut microbiota alterations linked to ADHD. However, findings provided by these studies have been largely inconsistent and the specific gut microbiota members or profiles involved in ADHD onset and prognosis have yet to be elucidated[102]. To date, there is no evidence on whether gut microbiota-directed interventions can help in ADHD management, although preclinical evidence on gut microbiota-ADHD causality has recently been reported for fecal microbiota transplant in mice[103]. Regarding the potential effects of probiotics on neurobehavioral outcomes in children and adolescents, a recent systematic review reported that prenatal and early infant probiotic supplementation with *Lactobacillus rhamnosus GG (LGG)* is associated with a lowered abundance of gut *Bifidobacteria* in infancy, and a decreased risk of developing ADHD[104].

Research into the effect of food on ADHD started[105] 40 years ago when pediatric allergist Benjamin Feingold hypothesized that both artificial food additives and foods rich in salicylates would contribute to ADHD development and manifestation. Later, the Feingold studies were followed by other elimination diet studies, investigating the effects of either artificial food color (AFC) elimination or of a diet eliminating numerous foods and additives, called the few-foods diet (FFD), and by studies investigating the effects of supplements, vitamins, minerals, and PUFAs on ADHD[106]. Some of these studies showed the protective effect of increased intake of specific nutrients such as iron, zinc, iodine and PUFAs, while others confirmed the adverse effect of excessive ingestion of food coloring agents, preservatives and sugar. In addition, different consistent evidence for the fact that ADHD might be triggered by particular foods, at least in a subgroup of subjects[26]. As a result of these studies, the potential benefit of certain dietary interventions was proposed for the prevention and treatment of ADHD.

More recently, meta-analyses have evaluated the efficacy of dietary interventions on children with ADHD. They showed that the clinical effects of PUFA supplementation and food additive elimination were small to modest, while the effects of the FFD on ADHD were substantial, pointing to the existence of a food- induced subtype of ADHD[105]. Indeed, the FFD approach is often applied in clinical practice, and a recent study showed its effectiveness, leading to a clinically relevant reduction of ADHD and Oppositional Defiant Disorder (ODD) symptoms[26]. Additionally, recent meta-analyses reported that omega- 3 PUFA supplementation seems to provide small benefits for ADHD symptoms[106], effects that seem to be largely modulated by different genetic and non-genetic factors. On the whole, these alternatives offer new opportunities for non-treated ADHD or for children with ADHD who do not respond to medication.

Unhealthy dietary patterns and ADHD show a positive correlation[31], suggesting that healthy dietary patterns might protect against or successfully treat ADHD, while an unhealthy diet might potentially increase ADHD risk. Consistent with this, two studies recently reported an association between ADHD and low adherence to the Mediterranean diet, suggesting that the Mediterranean diet might help prevent ADHD development[107,108]. The relationship between diet and mental health is bidirectional, which often implies unhealthy dietary choices among patients with mental disorders. Thus, the consumption of unhealthy foods might be a

consequence of ADHD rather than a determinant of the disorder, since certain foods, especially those with high sugar content, are activators of the reward system and consequently lead to high intake[109]. In fact, several studies have shown a higher prevalence of eating disorders[110, 111] and obesity[112] in individuals with ADHD, which often implies the greater difficulty of these subjects to adhere to potentially beneficial dietary interventions. So far, there is not irrefutable evidence about diet causality on ADHD and, in this regard, future studies are needed. Longitudinal and interventional randomized controlled trials, instead of cross-sectional designs, should be implemented to investigate the potential beneficial effect of a healthy diet on ADHD symptomatology, and to generally investigate the causal role of diet in ADHD onset and manifestation.

Besides the reported ADHD-diet association, there is also scientific evidence for an association between other lifestyle factors and ADHD. According to research, children with ADHD are almost twice as likely to demonstrate less healthy behavior, which leads to the previously described association between ADHD and obesity, even after adjustment for other multiple parameters such as age, sex, medication and comorbidities [113]. Given this close association between ADHD and obesity, clinicians should initiate nutrition counseling with families of children with ADHD shortly after diagnosis to prevent obesity and its comorbidities. In this regard, generally improved lifestyle choices may provide more substantial benefits to children with ADHD than dietary modifications alone and, thus, future research is required to assess the effects of a combined healthy lifestyle-diet intervention on ADHD[113].

Observational studies have also demonstrated some associations between specific micronutrient levels in subjects with ADHD and the presence or severity of symptoms; however, these studies do not allow conclusions to be drawn on causal relationships. In addition, as demonstrated in omega-3 PUFAs supplementation, the therapeutic benefits of supplementation may depend on baseline nutrient status and thus may likely be narrowed to individuals with specific micronutrient deficiencies[114]. Furthermore, the Ketogenic diet has recently been suggested as a dietary intervention with potentially therapeutic effects for ADHD, warranting greater investigation into its role in prevention and treatment of the disorder.

GUT MICROBIOTA, DIET AND SOCIAL BEHAVIOR: FOCUS ON AUTISM SPECTRUM DISORDERS

Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by the presence of stereotypical behavior, exhibiting communication and social interaction deficits. Although the precise ASD etiology remains unknown, both genetic and environmental factors are recognized to play a key role in its pathogenesis. Many environmental factors linked to the risk of developing ASDs have been associated with gut microbiota shifts (dysbiosis) as dysbiosis-related gastrointestinal alterations are often associated with ASDs. Furthermore, a strong positive correlation has been demonstrated between ASD severity and the severity of the comorbid gastrointestinal dysfunction linked to gut dysbiosis[115]. Regarding investigation into the role of gut microbiota in social interaction and communication, it is important to highlight that studies have demonstrated that GF-mice display impairment in social behavior and increased repetitive traits. These alterations are normalized following bacterial colonization. This fact strongly suggests that a healthy composition of gut microbiota is essential for normal social behavior and that autism-like symptoms would be favored when normal gut bacterial species are absent[10]. In addition, fecal microbiota transplantation from human donors with ASD or from typically developing (TD) control subjects into GF mice also revealed that the colonization with ASD gut microbiota is sufficient to induce autistic behavior in mice[116]. In particular, the mice with gut microbes from ASD donors showed lower levels of specific compounds produced by gut bacteria, such as the amino acids 5-aminovaleric acid (5AV) and taurine, which affect brain function by increasing the activity of the brain's γ-aminobutyric acid (GABA) receptors. Interestingly, the transfer of these two missing metabolites (5AV and taurine) to mice with autism-like symptoms was also reported to improve core deficits in social interaction and repetitive behavior. Cryan *et al.* also reported that mice with an autism-like condition showed lower levels of *Bifidobacteria* and *Blautia* gut bacteria, and their guts produced fewer compounds, such as bile acid and tryptophan,needed to produce serotonin[117]. It is important to note that we require a greater understanding of the mechanisms underlying the social deficits, which may include modulation of immune cell cytokine release, changes in vagal nerve activity and neuroendocrine function. Such knowledge could potentially lead to the emergence of novel and more effective therapies to counteract symptoms in the social domain.

The administration of a single bacterial strain can modulate the social behavior of animals beneficially. Either *Bacteroides fragilis*[70] or *Lactobacillus reuteri*[118] could reverse many of the behavioral and

gastrointestinal alterations reported in ASD. Interestingly, a study using the maternal immune activation (MIA) mouse model (animal model known to display features of ASD as well as gastrointestinal barrier alterations and microbiota changes) demonstrated that the oral treatment of offspring with the human commensal *Bacteroides fragilis* was able to correct gut permeability, restore microbial composition, and ameliorate alterations in communicative, stereotypic, anxiety-like and sensorimotor behaviors[70]. The same study also revealed that MIA offspring displayed an altered serum metabolomic profile, and that *B. fragilis* supplementation was able to positively modulate levels of several metabolites[70]. These findings strongly indicate the strong potential of probiotic therapy for GI and specific behavioral symptoms linked to neurodevelopmental disorders, such as ASD.

Many studies in humans have shown gut microbiota alterations in ASD subjects. Despite the lack of consistency in some of the gut microbiome changes reported across studies, various cross-sectional studies comparing the gut microbiome of neurotypical children vs. children with ASDs have reported increased levels of Clostridiales in the latter. In addition, Luna *et al.* showed increased levels of *Clostridiales* (*Lachnospiraceae* and *Ruminococcaceae*) in ASD children with gastrointestinal pain [119]. Furthermore, children with autism have also consistently shown lower levels of *Veillonellaceae, Coprococcus,* and *Prevotella* gut bacteria than those without the condition. Additionally, cumulative causal evidence from preclinical studies has revealed that gut microbiota and SCFAs seem to play an important role in gastrointestinal disorders and ASD. In this regard, Liu *et al.* recently observed a decreased abundance of key butyrate-producing taxa (*Ruminococcaceae, Eubacterium, Lachnospiraceae, and Erysipelotrichaceae*) in autistic children, together with low levels of SCFAs detected in patients' fecal samples[120], suggesting that butyrate-producing bacteria could be a promising strategy for ASD treatment. On the other hand, *Clostridium perfringens* strains isolated from the microbiota of children with ASDs were found to express the gene encoding b2 toxin, cpb2, to a greater extent than the same strain isolated from the microbiota of neurotypical children[121]. This toxin is associated with various gastrointestinal diseases, which may help to explain the comorbid gastrointestinal symptoms frequently observed in ASD individuals. Furthermore, Clostridia bacterial pathogens have been reported to generate propionic acid in the gut, a compound known to disrupt the production of neurotransmitters and to causes autism-like symptoms in rats [122]. Of note, whereas animal studies have

reported promising results for the direct connection between early life gut microbiota and neurobehavioral development related to ASD, data in human populations reporting causality are still scarce, with only two recent prospective studies supporting a potential causal association between infant gut microbiome and early childhood neurodevelopment [123, 124].

In this regard, a very recent study has explored this causal association by analyzing gut microbiota and measuring early childhood neurodevelopment with the Ages and Stages Questionnaire, third edition (ASQ-3), and suggests that the infant gut microbiome may be associated with subsequent development of communication, personal and social, and fine motor skills in 3-year-old children and with delays. In fact, the main finding was the potential association between *Clostridiales* and poorer communication, personal and social scores, and with increased likelihoods of delay in the development of these skills[124]. Consistent with these results, another recent study aimed to explore the direct link between gut bacteria and the onset and manifestation of ASD. The authors also found potential associations between the early-childhood gut microbiome and social behaviors. By investigating the associations between the infant/toddler gut microbiome at 1, 2, and 3 years and ASD- related social behaviors at the age of 3 years, several taxa were found to be associated with the Social Responsiveness Scale 2 (SRS-2) performance, including many in the *Lachnospiraceae* family. Particularly, higher relative abundance of *Adlercreutzia equolifaciens* and *Ruminococcus torques* at 1 year were associated to poorer SRS-2 performance[123]. One small-scale pilot study of microbiota-transfer therapy in patients with ASD also showed promising results [125]

Children with ASD receiving microbiota transfer therapy were reported to have significantly reduced gastrointestinal disruptions, and significant improvements in ASD-related behavior, which persisted for at least 2 years after the end of treatment. The authors also noted a significantly reduced bacterial diversity and significantly increased abundance of *Bifidobacterium, prevotella,* and *desulfovibrio* after treatment.

Some studies investigating the effects of potential probiotics in subjects with ASD have reported promising results. Indeed, several studies suggest that probiotic supplementation in children with ASD might reduce intestinal inflammation and permeability, and restore the abnormal gut microbiota linked to ASD, leading to an improvement in GI and ASD symptoms. Particularly, a recent 4-weeks, randomized, double-blind, placebo-controlled study investigated the effects of *Lactobacillus Plantarum PS128*

on ASD symptoms, a psychobiotic strain with beneficial effects on anxiety, and demonstrated that a 28-days intervention with the probiotic significantly ameliorated certain behavior, specifically opposition/defiance, in boys with ASD[126].

Regarding the potential use of probiotic supplementation for ASD prevention, in a recently published review[104] researchers reported only one study demonstrating that specific probiotic supplementation with *Lactobacillus rhamnosus GG* during pregnancy and during the first year of life was associated with a reduction in the risk of developing Asperger's síndrome [127]. It is worth noting that the reduction in disease onset risk was coupled with a lowered abundance of gut *Bifidobacterium* in infancy and, although the mechanisms underlying the benefits of this probiotic supplementation remain unclear, a potential explanation lies in the modulation of gut microbiota composition through probiotic supplementation. Furthermore, the administration of the prebiotics galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) increases social interaction in chronically stressed mice, positively influencing the relative abundance of the key bacterial genera *Akkermansia, Bacteroides, and Desulfovibrio*[128]. However, further human intervention studies are warranted to determine whether probiotic/prebiotic supplementation can be useful for pregnant mothers as preventive tool for ASD.

Regarding nutritional deficiencies linked to the onset of ASDs, several preclinical studies have reported that early life dietary deficiency of omega-3 fatty acids leads to deficits in social recognition[129]. Conversely, omega-3 supplementation during childhood and adolescence has been demonstrated to improve social behavior in both animal and human studies[130]. Particularly, a recent pilot trial found a significant improvement in ASDs symptoms after omega-3 supplementation in preterm children showing early signs of ASD[131]. Additionally, a variety of other dietary deficiencies, including deficiencies of vitamins A, C, B6, B12, D, and folate[132], have also been suggested as potential triggers of ASDs onset. As a result, observations that these deficiencies may be linked to ASDs have led to several trials to study the effects of supplementation on treating ASD symptoms. Remarkably, multiple studies have also documented both reduced methylation and antioxidant biomarkers, such as S-adenosylmethionine and glutathione (GSH), in individuals with ASD. Specifically, Methyl-B12, vitamin B6, and 5-Metiltetrahidrofolato are all essential cofactors involved in pathways related to methylation and oxidative stress, and the supplementation with these cofactors showed a significant improvement in ASD symptomatology, especially in a subgroup of ASD children with baseline methylation and antioxidant deficits[132].

Gluten-free diet is often combined with casein-free diet into a single gluten-free and casein-free (GFCF) diet, which has gained popularity in recent years for ASD treatment[133]. The rationale for the use of GFCF diet in ASD largely stems from the need to eradicate the effects of some bioactive food- derived opioid peptides, released by the partial digestion of both gluten and casein peptides by intestinal enzymes, such as β-casomorphin-7 (BCM7) among others, with negative consequences on mental health[134]. These food-derived opioid peptides can pass through the permeable intestinal membrane, eventually crossing the blood-brain barrier and binding to opioid receptors in the brain, acting as neuromodulators and affecting neurotransmission. Emerging evidence indicates that opioid receptors may indeed be involved in regulating aspects of social behavior, and contribute to the pathogenesis of ASD[134].

In recent years, the literature has reported that besides the human genome, the gut microbiome can also largely influence dietary protein digestion by the expression of peptidases and, thereby, contribute to the change in the host response to the peptides affecting host physiology[135]. Indeed, different bacterial strains can degrade food-derived peptides and could consequently be useful to prevent diseases triggered by these peptides. In this regard, bacterial strains commonly found in the infant intestine (e.g., strains of *Bifidobacterium*) have shown particularly high dipeptidyl peptidase IV activity, the primary degrading enzyme of BCM7, suggesting they may limit BCM7 activity during early development. Taking into account the reported evidence and despite the need for further studies, gut microbiota might be considered to positively impact peptidases levels in the context of neurodevelopment and, thus, on ASD. Selected strains of *B. longum subsp. Infantis,* and *B. bifidum,* among others with high degradative capabilities, might be potential probiotic microorganisms able to abolish food-derived opioid peptides and, thereby, contribute to host health[135]. Due to the fact that gut microbiota can also impact gluten and casein digestion, it would be interesting to investigate the gut microbiota profile as a potential biomarker for the efficacy of GFCF dietary intervention.

There is still a lack of conclusive evidence for the efficacy of the GFCF diet for ASD treatment, although some positive results have been reported in systematic reviews evaluating the effect of GFCF diet by interventional studies[132, 136,137]. Importantly, the results have described an important time dependency for the detection of beneficial GFCF dietary effects or noticeable improvements. This is probably because gut dysbiosis,

inflammation, and perturbations in intestinal motility and permeability encountered in many patients with ASD need a substantial time to normalize after prolonged dysfunction[138]. In addition, a high inter-individual variation in response to GFCF diet has been observed[136], and thus studies are warranted to accurately identify biomarkers for patient populations that can benefit from a GFCF dietary intervention.

The Ketogenic diet (KD) is the dietary intervention with the most proven therapeutic effect in Nutritional Psychiatry, especially for the treatment of refractory pediatric epilepsy and with a high therapeutic potential for many mental health and metabolic disorders. The KD has recently been proposed as a promising metabolism-based dietary intervention for the prevention and treatment of ASD. In this respect, a recent systematic review of the literature examining the interplay between KD and ASDs revealed encouraging findings, particularly from animal studies, which support the potential to improve core behavioral symptoms of ASD by using KD or analogous metabolic strategies[139]. In fact, various preclinical studies using different animal models have strongly supported the beneficial effect of KD to ameliorate behavioral symptoms associated with ASD[140, 141]. In addition, it is important to note that although further studies are warranted to investigate the role of gut microbiota as a potential underlying modulator of the effects of KD on ASD, there are reports of the impact of KD in remodeling the gut microbiome of the BTBR mouse model of ASD[142]. Human studies into the potential therapeutic effects of KD in ASDs have also provided promising results[143] although additional studies are warranted to truly understand the short and long-term effects of KD in individuals with ASD, and to investigate the underlying mechanisms.

GUT MICROBIOTA, DIET, ANXIETY, SOCIAL PHOBIA, AND DEPRESSION

Extensive scientific evidence advocates that unhealthy dietary patterns may increase the risk of developing depression or anxiety, while a healthy dietary pattern and some specific dietary components may do the opposite. This would suggest that dietary interventions could help to prevent, or be an alternative or adjunct therapy for depression and anxiety. However, the relationship between diet and depression and anxiety is complex and further studies are essential to understand the nature of this relationship, especially in individuals vulnerable to these conditions[21]. Regarding the effects of specific gut microbiota-related dietary components in anxiety and depression, experimental studies and clinical trials in humans suggest that probiotics and prebiotics might provide anxiolytic effects.

Preclinical studies have revealed potential gut-brain pathways by which gut microbiota exerts beneficial effects on these conditions. For instance, Bravo *et al.* demonstrated that the administration of the lactic acid bacteria *Lactobacillus rhamnosus* in mouse models modulated GABA receptor expression and induced anxiolytic behavioral effects via a vagus nerve-dependent pathway[144]. Similarly, Bercik et al. also showed that probiotic intake reduced gut inflammation-induced anxiety and the association of these anxiolytic effects with changes in BDNF, and dependent on the vagus nerve[145]. Additionally, in a clinical placebo-controlled trial Tillisch et al. demonstrated that the consumption of a fermented milk product containing a combination of probiotics during a 4-weeks period can modulate brain activity, acting on brain processes of negative social stimuli[146]. Additional clinical trials have also demonstrated anxiolytic effects of specific probiotics and prebiotics in patients with chronic fatigue syndrome[147], irritable bowel syndrome[148] and in healthy volunteers from the general population[149].

Regarding the role of diet in preventing the onset of social anxiety disorder or social phobia, a study in 710 young adult students[150] suggested that fermented foods, which typically contain probiotics, may offer some protection against the development of social anxiety disorder in subjects with greater vulnerability to the condition. Although additional research would be necessary to determine the direction of causality, the results advocate that consumption of fermented foods may serve as a low-risk intervention for reducing social anxiety.

GUT MICROBIOTA, DIET AND SCHIZOPHRENIA

As proposed by the neurodevelopmental hypothesis of schizophrenia (SZ), the abnormal trajectory of brain development linked to this disorder is established during gestation and early life, long before the onset of the clinical symptoms of disease, which often occur in early adult life. SZ is a chronic mental illness characterized by positive symptoms (delusions, hallucinations and disorganized thoughts and speech), negative symptoms (apathy, anhedonia, loss of motivation and lack of social interest) and cognitive alterations (impairments in attention, working memory, or executive function). The disease typically progresses gradually, with early warning signs (prodromal stage) developing before the first severe episode (psychosis) when positive symptoms started to be experienced. Interestingly, the shortened life expectancy observed in patients with SZ has been attributed to many other medical disorders often comorbid to the disease, such as cardiovascular diseases and diabetes.

It is well-established that different early life environmental factors, many of which are linked to gut microbiota shifts, might trigger the onset of this psychotic disorder in genetically susceptible individuals. Indeed, preclinical studies have shown that maternal stress and maternal immune activation (MIA) during pregnancy result in SZ-like behavior in offspring[151].In addition, immune dysregulation and infections have been suggested as crucial risk factors for SZ development[152]. Dopamine dysregulation has been traditionally considered as a main etiological factor of SZ and it has been used as a target to develop drugs for managing the condition. However, the use of antipsychotic drugs is often associated with important side effects and, therefore, alternative treatments for SZ are clearly needed. Increasing evidence suggests that gut microbiota may be involved in the development of SZ through immune and inflammatory mechanisms[153]. In addition, dopamine can be produced by microbes, and this fact together with the increased gastrointestinal inflammation associated to the disease has strongly suggested the potential role played by gut microbiota in modulating the risk of developing SZ and its prognosis[153].

Recently, a fecal microbiota transplant study showed that the SZ gut microbiome itself can alter the neurochemistry and neurologic function of GF mice in ways that may be relevant to SZ pathology[154]. Furthermore,

several studies have investigated the microbiome composition in SZ patients compared to healthy control and they identified some significant potential microbiota biomarkers of the disease [155]. In addition, it has been suggested that the gut dysbiosis described in SZ patients may affect gut integrity, with increased susceptibility to infection and inflammation and thus contribute to SZ development and its severity. Consistent with this, the abundance of specific bacterial genera, such as *Succinivibrio* and *Corynebacterium,* has been associated with severe symptoms of SZ[156]. However, the studies conducted in humans have all been cross-sectional and a causal relationship could not be inferred. Notwithstanding, the potential role played by the gut microbiome in the etiology of SZ strongly motivates the development of microbiome-targeted interventions for SZ prevention and/or treatment. In this respect, there is still inconclusive evidence for the efficacy of pro/prebiotic supplementation in the prevention or treatment of SZ and further research and more clinical trials are needed to test the validity of modulating the gut microbiome to improve the clinical management of SZ.

It is well-recognized that diet plays a key role in controlling inflammation, and research has described the anti- or pro- inflammatory potential of specific foods, dietary components and dietary patterns to be of particular importance[157]. In this regard, studies strongly suggest that chronic inflammation, driven partially by dietary factors, might impact on SZ development and aggravate the course of the disease[30]. In fact, the dietary inflammatory index (DII), a standardized score related to the inflammatory potential of diet, was reported to be significantly higher in SZ patients in a recent population-scale study[30]. Particularly, the intake of total energy, carbohydrates, total fat, saturated fat, and sugar, known as pro-inflammatory components, were found significantly higher in SZ subjects compared to controls[158].

In general, unhealthy dietary intake patterns and increased prevalence of immune and metabolic dysfunction have been observed in subjects with SZ [159]. Regarding lifestyle and in the setting of SZ, research has described the relationship between diet quality and other lifestyle factors (physical activity, smoking, and sleep quality). For instance, a correlation between poorer diet quality and smoking in SZ patients has been reported [28]. In addition, a pro-inflammatory diet and a poor quality of life could aggravate neuroinflammation and the disease, thereby encouraging the detrimental role of unhealthy foods and poor life-style. By contrast, although

clinical trials have not yet evaluated the beneficial impact of the Mediterranean diet or other healthy life-style factors for SZ, some researchers hypothesize that they could improve metabolic and other disease outcomes related to premature mortality in SZ [159].

Some nutritional deficiencies have been proposed to play a role in the etiopathogenic mechanisms of SZ, and particular dietary interventions have been proposed to reverse the SZ- associated neurobiological changes established in the early phases, at least for certain cohorts of SZ patients [160]. Regarding nutritional deficiencies, evidence has shown that PUFAs deficits are associated with both negative and positive symptoms of SZ. Additionally, several studies also showed blood levels of PUFAs, particularly DHA, negatively correlated with the severity of SZ symptoms. Nervonic acid (NA) is a monounsaturated omega-9 fatty acid with an important role in myelin biosynthesis, and low NA levels in the red blood cell membrane have emerged as an early risk factor for the development of psychosis in adolescents at high clinical risk[161]. Furthermore, omega-3 fatty acid supplementation has been shown to reduce psychotic conversion rates and to improve both positive and negative symptoms as well as global functions in high-risk adolescents[162, 163]. Indeed, several randomized double-blind placebo-controlled trials have reported that during the prenatal period, childhood and adolescence, the administration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce the levels of pro-inflammatory cytokines and ameliorate positive and negative SZ symptoms. However, in patients with chronic psychotic disease, no differences between treatment and placebo groups were observed for psychotic illness improvement, suggesting that omega-3 supplementation would be more effective when prescribed in early life[163].

Regarding micronutrients, several studies have also pointed out particular vitamin deficiencies as risk factors for SZ development. Specifically, folic acid (vitamin B9), piroxidine (vitamin B6), and cobalamine (vitamin B12) deficiencies have been reported common in SZ patients.Total plasma homocysteine (Hcy) level is considered as a sensitive measure of these deficiencies and elevated Hcy levels have been widely associated with the etiology of numerous health impairments, especially cardiovascular diseases and several mental health disorders such as Alzheimer's disease and SZ [164] and some studies have reported a positive correlation between Hcy levels and SZ illness severity[165].In this regard, several randomized double-blind placebo-controlled trials have shown the effectiveness of

homocysteine-reducing strategies, with folic acid and vitamin B supplementation improving both positive and negative symptoms, mostly related to attention/vigilance, and especially in chronic SZ patients with hyperhomocysteinemia[166].

Additionally, low serum levels of vitamin D have also been observed in SZ patients having a negative correlation with psychosis severity[167]. Likewise, a randomized, placebo-controlled trial reported a significant improvement in SZ cognitive impairments after 8 weeks of vitamin D supplementation[168]. Furthermore, a low dietary intake of vitamin C, which has important anti-oxidative properties, has also been associated with an increased risk of SZ, while vitamin C has been linked to the significant improvement of several SZ-related psychiatric scores[169, 170]. Taking into account all the aforementioned evidence, diet should be considered as one of the most crucial factors for the clinical management of SZ patients [160].

Other studies highlight the association between SZ and celiac disease[171] and there have been several small controlled studies in which a GFD showed promising results because approximately 10% of patients ameliorated SZ symptoms. However, large-scale epidemiological studies and clinical trials are needed to investigate the extent and the underlying mechanisms of this gluten-SZ association, as well as to identify biomarkers to predict the clinical response of SZ patients to a GFD[172]. Interestingly, it has been reported that patients with SZ tend to eat more carbohydrates immediately before a psychotic episode. This fact points to carbohydrate intake as a potential risk factor for clinical disease progression, and a recent study reported a low-carbohydrate KD as a therapeutic approach to managing longstanding SZ symptoms[173]. Subsequently, several case studies have demonstrated that the KD induced significant improvement in psychiatric symptoms, as well as in metabolic dysfunctions observed among patients with SZ [173]. In particular, KD has been described to modulate the ratio of key neurotransmitters involved in brain functioning, especially γ-aminobutyric acid (GABA) and glutamate [174]. Consistent with this, the modulation of the GABA/glutamate ratio could be a powerful therapeutic mechanism by which KD might act in SZ, since these neurotransmitters may be involved in regulating the altered dopamine levels observed among SZ patients.

GUT MICROBIOTA, DIET AND EPILEPSY

There is strong scientific evidence for the role of gut microbiota in seizure susceptibility [175]. Firstly, several GF mice studies as well as studies investigating antibiotic-treated mice and humans indicated the specific role of gut microbiota in synaptic changes of key brain areas involved in epileptogenesis. Indeed, Braakman et al. described how five out of six cases of drug-resistant epilepsy became seizure-free during the course of antibiotic treatment[176]. Additionally, a prospective study in the pediatric population reported a relationship between rotavirus infections and neonatal seizures. This study also described a significantly close association between probiotic administration after birth and reduction of rotavirus-associated neonatal seizures [177]. Furthermore, a recent study also described how gut microbiota transfer by fecal microbiota transplantation (FMT) in rats was able to modulate the risk of seizure, supporting gut microbiota causality [178]. This experimental finding was clinically supported by a case study where a patient with Crohn's disease, with a 17-year-long history of seizures, became seizure- free for at least 20 months, despite discontinuing antiepileptic drug treatment with sodium valproate, after a FMT[179]. Additionally, several studies have shown patients suffering epilepsy have an imbalance in gut microbiota (dysbiosis), with a greater abundance of potentially pathogenic bacteria such as those belonging to *proteobacteria* [180].

Regarding dietary interventions with a proven efficacy for epilepsy, KD can be considered the most effective diet-based alternative therapy for children and adults with refractory epilepsy. This condition affects more than one-third of epileptic individuals and is defined by a failure to reach seizure control with the currently available anti-epileptic drugs. Additionally, the positive impact of KD has been demonstrated in children and adolescents with refractory epilepsy in terms of mood, as well as social behavior and cognitive functioning, with a beneficial effect on sustained attention and alertness. In a clinical setting, more than half of the patients following a KD respond favorably to this diet, with at least 50% seizure reduction, and different studies have already pointed to some potential biomarkers to predict clinical response to KD in epilepsy[181, 182].

Additionally, several studies in infants and animals with epilepsy have reported the impact of KD on gut microbiota composition[182–184]. In this regard, most of the studies concluded that KD triggers important changes in gut microbiota, with a loss of microbial diversity but a positive balance

between potentially beneficial genera (such as *Bacteroides* and *Bifidobacterium*) and potentially harmful bacteria (*proteobacteria*). By contrast, in a recent study published by Lindefeld et al., the authors draw attention to the potential negative effects of KD on gut microbiota, describing a decrease in *Bifidobacteria* and an increase in *E. coli* [184].

Some studies have evaluated the beneficial effects that KD-induced modulation of gut microbiota might play in epilepsy. Concerning this issue, a recent study in two mouse models for refractory epilepsy revealed the novel and crucial role for gut microbiota in mediating the anti-seizure effects of the KD[174]. This study reported that both GF mice and mice treated with antibiotics were resistant to KD- mediated seizure protection compared with the anti-seizure effects of KD observed in conventionally colonized mice. Furthermore, the authors identified specific members of the intestinal microbiota with essential roles in mediating the KD-anticonvulsant effects. Specifically, authors demonstrated that KD intervention modified gut microbiota composition by significantly increasing the bacterial species *Akkermansia muciniphila* and *Parabacteroides merdae* and, most remarkably, they demonstrated that the gnotobiotic colonization of GF mice with these two bacteria conferred similar protection against epileptic seizures as KD[174]. The authors also investigated the underlying mechanisms of the effects of combined administration of *Akkermansia muciniphila* and *Parabacteroides merdae* and found an increase in the GABA/glutamate ratio in the hippocampus, which could explain the changes observed in behavior. These findings have not been confirmed in humans yet, but if established, this microbiota-based intervention strategy (*Akkermansia muciniphila* and *Parabacteroides merdae* co-supplementation) could be recommended to replace KD for refractory epilepsy treatment. Although KD is highly valued to treat epilepsy and has therapeutic potential for the prevention and treatment of other mental health and metabolic disorders[185;] its widespread implementation remains limited due to the potential long-term side effects[143]. We have yet to clarify the precise mechanisms by which KD exerts its beneficial neuroprotective and metabolic effects in certain disorders, and studies are needed to identify biomarkers for clinical responses to KD, as well as those related to KD side-effects. The mechanisms by which KD appears to exert its anti-seizure effects seem to differ from those by which antiepileptic pharmacological treatments act, suggesting that KD might also be considered as an adjuvant treatment to improve disease management[175].

Regarding the use of probiotics, a study in an animal model of epilepsy known as the Pentylenetetrazol-kindling model found that the administration of other combinations of different probiotic strains (*Lactobacillus rhamnosus, Lactobacillus reuteri,* and *Bifidobacterium infantis*) in PTZ-kindled rats, reduced seizure severity and epileptic activity when compared to seizures in controls[186]. Interestingly, in this study the anti-epileptic effects of probiotic administration were accompanied by an increase in GABA levels in the brain, as well as a decrease in brain oxidative stress. This was the first preclinical report showing a positive effect of probiotic bacteria on seizure- induced neurological alterations such as learning difficulties and memory deficits. In another recent study in humans, a probiotic treatment was shown to reduce seizure frequency by 50% in 29% of patients with drug-resistant epilepsy [187]. Furthermore, different studies have reported that the gut microbiome of patients with drug- resistant epilepsy differ from that of patients with drug-sensitive epilepsy. Specifically, an increase in alpha diversity has been described, with a greater relative abundance of rare bacteria, mainly belonging to the phylum Firmicutes, in drug-resistant patients compared to drug-sensitive patients and healthy controls [180]. Furthermore, an inverse correlation was reported between the relative abundance of *Bifidobacteria a*nd *Lactobacilli* and the number of seizures per year in both patient groups.

It is noteworthy that the intestinal microbiota is increasingly recognized as an important factor in biotransformation of xenobiotics, including medications[188, 189]. Regarding the role of the gut microbiome in the response to antiepileptic drug therapy, several studies have reported that Zonisamide, a common antiepileptic drug, is metabolized to 2-sulfamoylacetylphenol by the intestinal microbiota[190], supporting the role of gut microbiota in modulating anti- epileptic drug metabolism and, thus, in the clinical response to anti-epileptic drugs. In turn, treatment with anti-epileptic drugs may also impact on gut microbiota composition. More specific studies are needed to investigate the true significance of the interaction between anti-epileptic drugs and the intestinal microbiome in epilepsy treatment.

TOWARDS "PRECISION NUTRITION" IN PSYCHIATRY AND NEUROPEDIATRICS: WHERE ARE WE NOW AND WHERE SHOULD WE GO?

It is important to highlight that even though there are food intake guidelines and general dietary recommendations for the population as a whole, a high inter-individual variation has been documented in clinical response to diet[191]. The concept of "Precision Nutrition" arose when the scientific community agreed that the observed inter-individual variability in response to diet was not exclusively based on genetics (nutrigenetics), but rather on the interaction between the host's genetic make-up and the environment[192, 193]. Regarding Nutritional Psychiatry, scientific evidence strongly supports that subject stratification based on multiple individuals' specific variables, including gut microbiota profile modulated by multiple factors such as diet and other lifestyle factors (physical activity, smoking, medication and other features), would increase the effectiveness of particular dietary interventions for improving mental health (Figure 3).

To highlight the relevance of the gut microbiota profile for precision nutrition, in the context of cardiovascular diseases (CVDs) often associated to mental health disorders, researchers few years ago reported the role of the gut microbiota in modulating the relationship between red meat consumption and atherosclerosis and other cardiovascular disorders. In particular, several studies in mice and humans have reported increased fasting plasma levels of trimethylamine (TMA), a compound produced by the gut microbiota metabolism, and its proatherogenic metabolite trimethylamine-N-oxide (TMAO), concomitant with an increased risk of atherosclerosis, after oral intake of L-carnitine and phosphatidylcholine depending on the gut microbiota profile[194]. Additionally, an algorithm that integrates gut microbiota profile and other parameters, such as blood, dietary habits, anthropometrics and physical activity, was devised to accurately predict personalized postprandial glycemic response to real-life meals[195]. Thus, tailored dietary strategies may successfully modify elevated postprandial blood glucose and its metabolic and mental health consequences.

Of note, relevant also for the precision nutrition field, is the fact that the interaction between diet and host genetic background likewise modulate gut microbiota composition. In this regard, a recent study including three

independent Dutch population cohorts found a gene-diet interaction between a functional variant at the lactase locus and the intake of dairy products modulating *Bifidobacterium* abundance [196] an important aspect to be considered in Precision Nutrition.

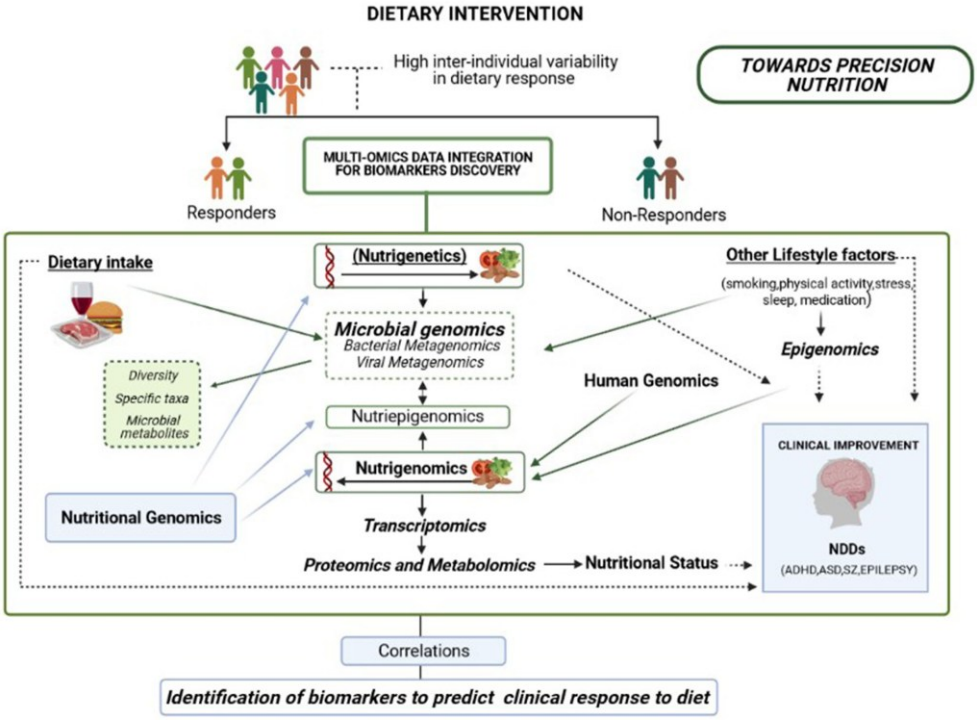

FIGURE 3. Overview of a multi-omics integration analysis to reveal biomarkers able to predict clinical reponse to diet, including genomics, metagenomics, epigenomics, transcriptomics, proteomics and metabolomiscs. An integrative approach is necessary to improve the effectiveness of dietary interventions aiming to promote healthy brain development according to multiple individual features, including baseline microbiome signatures.

Accordingly, in the context of Nutritional Psychiatry, several studies have been conducted aiming to design tailored dietary and gut microbiome-based interventions to promote the development and maintenance of a healthy brain. Remarkably, regarding different responses to the effect of the omega-3 PUFA supplementation on mental health, a direct link was reported between lower endogenous PUFA levels associated with the inflammatory status and an increased risk of developing interferon-alpha-induced depression[197]. In addition, pre- treatment with EPA was demonstrated to reduce the onset of depression induced by this

inflammatory cytokine[198]. In this regard, Rapaport et al. found antidepressant effects of EPA administration only in patients with high baseline inflammation, indicating that the effective supplementation of EPA might depend largely on the pathogenesis and underlying mechanisms of depression [199].

Consistent with this framework, another recent study found that youths with the lowest levels of baseline endogenous EPA show the largest improvement in cognitive function following EPA administration[29]. Also, two clinically distinct endophenotypes in SZ patients determined by PUFA levels have been described and SZ patients with low PUFA levels have more negative symptoms than those with high levels, and they show a better clinical respond to omega-3 intervention[200]. In addition, a study reported that the attention improvements in ADHD children with EPA supplementation appear to occur more frequently in children with comorbid ODD[201], children who respond poorly to medication, and children with comorbid learning difficulties[202]. This would suggest that ADHD-specific symptoms may respond differently to a dietary intervention based on the presence of comorbid disorders. Moreover, an ADHD subgroup, particularly with high inflammation or low baseline levels of LC omega-3 PUFAs, may profit more from omega-3 PUFA supplementation[29]. Some studies have already indicated that the inter-individual variability in omega-3 PUFA clinical response seems to be largely explained by genetic variants and epigenetic modifications located within human genes involved in PUFA metabolism and associated with the risk of ADHD and ASD such as FADS1, FADS2, ELOVL5 and ELOVL2 [203, 204]. Genetic and epigenetic variations in these genes related to the long chain -omega-3 PUFA endogenous synthetic pathway has been directly associated to serum and tissue levels of these fatty acids[205, 206]. The epigenetic modulation of gene expression is related to many environmental factors, therefore the environment may shape the individual's ability to metabolize and accumulate omega-3 PUFAs in tissues. Interestingly, the ability of gut microbiota to produce PUFA-derived bacterial metabolites was also reported some years ago, while recent studies have highlighted the important role of gut microbiota in the metabolism of PUFAs[207]. Particularly, Djuric *et al.* investigated whether the individual microbiota profile may influence the efficacy of omega-3 PUFA supplementation in reducing colonic prostaglandin E2, and they found a subgroup of individuals showing high abundance of *Prevotella* who were resistant to omega-3 PUFA anti-inflammatory effects[208]. This observation strongly suggests that the individual gut microbiota profile may be an additional

factor able to influence the efficacy of omega-3 PUFA supplementation. Moreover, variable intake of non-lipidic dietary factors has also demonstrated to affect PUFAs metabolism. The metabolic antagonism of omega-3 PUFAs by high levels of linoleic acid (LA) is well-known, as is the correlation between low levels of linolenic acid (LNA) and the scarce production of EPA and DHA omega-3 PUFAs. On the other hand, high levels of EPA and DHA lead to feedback repression of omega-3 PUFA synthesis[209]. Thus, based on the current evidence, it is fundamental to highlight the need for future studies to consider baseline PUFA status together with other features, such as age, sex, and genotype among others, which may modulate the effects of omega-3 PUFAs on behavior, mood and cognition. Taking into account all the above, the final goal should be to identify specific factors/biomarkers or rather the network of factors that could accurately predict those subjects that might truly benefit from sustained long-chain omega-3 supplementation in clinical practice.

Regarding the role of gut microbiota on modulating the effectiveness of KD, Zhang *et al.* described how gut microbiota could be considered as a biomarker for KD efficacy in epilepsy. Specifically, authors reported some microbial taxa such as *Clostridiales, Ruminococcaceae, Rikenellaceae, Lachnospiraceae,* and *Alistipes* related to a poor KD clinical efficacy. Additionally, some genetic variants have also been associated with the neurological outcomes and the degree of weight loss in KD intervention studies[210] and studies have cited baseline ketones levels in blood as potential biomarkers for the KD response [181]. It is thus very clear that both genetic and dynamic markers of KD response may help to identify individuals most likely to benefit from KD and to pinpoint individuals at higher risk of adverse KD-related health outcomes (biomarkers of contraindication for KD intervention). The underlying mechanisms of gene– microbiota-diet interactions involved in KD response are still unknown but they might involve differences in KD-mediated gene expression changes in the brain. In this regard, KD-induced improvements in SZ symptoms are reported to be accompaniedby reduced levels of TNF-α in plasma and IL-1β in the brain[211], which would suggest the more favorable effects of KD in SZ patients with comorbid inflammation. In conclusion, future multi-center and long-term studies are warranted to accurately identify all KD response biomarkers, including those related to adverse effects, for all the mental disorders that may benefit therapeutically from KD.

Regarding the effectiveness of homocysteine-reducing strategies for the treatment of SZ, different factors has been largely linked to the efficiency of Hcy catabolism, such as the availability of folic acid, vitamin B12 and vitamin B6, and to the modulation of the response to the dietary intervention. In addition, the presence of different common polymorphisms (C677T and A1298C) located in the methylenetetrahydrofolate reductase (*MTHFR*) gene, which modulates MTHFR enzyme activity, and other factors such as age, gender, smoking and certain medications have been associated with Hcy levels[212]. In consistency with this, a recent study reported that female SZ patients with hyperhomocysteinemia and affective psychosis were associated with greater improvements in attention/vigilance following B-vitamin supplementation[213]. Thus, further studies are needed to identify all the individual features that could, when combined, interact with folic acid and B-vitamin treatment, determining specifically intervention outcomes.

Regarding gluten-free and casein-free (GFCF) regime as therapeutic approach for ASD, a recent systematic review of four randomized controlled trials on GFCF diets found a 50% efficacy rate of treatment, and reported that the GFCF diet may provide more substantial clinical benefits in individuals with low GSH levels and in those with preexisting GI symptoms[136]. Research into gut microbiota shows its strong involvement in the digestion of both gluten and casein[135] and this indicates the importance of analyzing the gut microbiota profile as a potential biomarker for the efficacy of GFCF dietary interventions. In this respect, further studies are warranted to identify all biomarkers, including gut microbiota, which may help to accurately predict patients able to benefit from a GFCF dietary intervention. Regarding the therapeutic effects of diet in ADHD, recent double-blind research investigating the effects of a few-foods diet (FFD) demonstrated a significant decrease in ADHD symptoms in many patients adhering to this particular dietary approach. Indeed, in the most recent randomized controlled trial, 64% of children with ADHD responded favorably to the FFD, with its use being strongly recommended in cases of ADHD comorbid food allergy symptoms (27). Currently, ongoing research is focusing on the identification of baseline molecular signatures or biomarkers that could help to accurately predict whether FFD participants will belong to the FFD-responder or non-responder group. Likewise, ongoing research also aims to provide a better understanding of the mechanism(s) underlying the FFD response and to study the role of gut microbiota changes in modulating the effects of this dietary approach. The research underway is based on an unbiased approach, integrating

multiomics data of microbiota-gut-brain axis parameters, and including profiling of the microbiome, transcriptome, metabolome, methylome and proteome. Results could enable researchers to accurately identify molecules that might (co)determine FFD effects based on the individual's microbiota-gut-brain (MGB) axis configuration.

CONCLUSIONS, KNOWLEDGE GAPS, AND FUTURE PERSPECTIVES

Currently, preclinical and cross-sectional studies have strongly established that the gut microbiota influences cognition, mood, and behavior. In this regard, research highlights that early life perturbations of gut microbiota function can profoundly impact neurodevelopment, with potentially crucial long-term consequences on health. However, there is still a critical need for population-based prospective cohort studies to provide a comprehensive understanding of the early temporal variability in the host-microbe dynamic interactions, which determine the shift from mental health to mental disorder, and vice versa. In this regard, gut microbiota profiles and determinants of those profiles linked to the onset of NDDs should be extensively investigated, and further studies are essential to design gut microbiome- targeted intervention strategies to promote the development and maintenance of a healthy brain. Thus, to develop more effective microbiome-based interventions and innovative dietary supplements targeting NDDs, we require a greater understanding of the beneficial gut microbiota members for mental health. These would include bacteria as well as bacteriophages, which are active against particular bacteria, and the underlying mechanisms by which they act, including bioactive molecules (postbiotics). In the near future, such potential novel intervention strategies could pave the way toward preventing the development of NDDs in subjects with risk indicators, and provide new alternatives to improve the clinical management of subjects diagnosed with NDD.

Diet is also considered as an amendable factor that can help to protect against the onset and manifestation of mental health disorders by controlling the gut-brain axis. To date, studies have reported a prominent inter-individual variation in the clinical response of NDDs to beneficial dietary interventions, and consequently a major challenge currently faced

by nutritional interventions is to identify biomarkers that can accurately predict the efficacy of the response.

The gut microbiota may be a key mediator of dietary effects on mental health, although diet is also known to be involved in brain functioning through other microbiota- independent mechanisms. In this respect, exhaustive studies are needed to decipher the precise mechanisms by which certain dietary interventions exert their effects, with the main goal mechanisms. Finally, it is also important to note that although diet is a major component of an individual's lifestyle, data on other lifestyle factors and the Human Genome provide fundamental information that should also be taken into account when studying the determinants of mental health, besides the bases of dietary effects on mental health. In fact, scientific evidence has indicated that the effects of diet on the host may be modulated by variations in the human genome as well as by other lifestyle factors that may, likewise, influence gut microbiota and human genome expression. Therefore, in the current -omics era, further studies based on multi-omics data integration are greatly needed in the field of Nutritional Psychiatry in order to identify baseline molecular signatures and/ lifestyle determinants (diet and other lifestyle factors) that may (co)determine or predict the specific effects of dietary interventions. The results provided by these studies will likely lead to refined future dietary strategies to prevent or improve the treatment of NDDs, and, in this way, help Psychiatry progress towards tailored nutrition and personalized medicine.

AUTHOR CONTRIBUTIONS

MC and AL wrote the first draft of the manuscript. JP, PC-F, and MC have contributed substantially to the writing and revising of the manuscript. MC designed the aim of the editorial. All authors have made substantial intellectual contributions, took responsibility to the manuscript, and give final approval of the version to be submitted.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERAL DISCUSSION

Globally, according to the World Health Organization (WHO)[3], the European Mental Health Platform[359], and recent data from the Spanish Ministry of Health[4], there has been a substantial rise in mental health problems among children and adolescents, particularly in the aftermath of the COVID-19 pandemic[360,361]. In light of this increase, the Sustainable Development Goals (SDGs) recognise the promotion of mental health and well-being as an important health priority, which can be achieved through the development of early disease risk detection and preventive strategies that integrate multiple health determinants, such as nutrition, social environment, and emotional well-being[5].

A healthy lifestyle, including dietary, physical activity, and screen time management, may represent a preventive approach to reducing the prevalence of mental conditions in early stages of life. This Doctoral thesis has contributed to the identification of lifestyle factors, with particular emphasis on the diet, which may help ameliorate behavioral and learning disorders in children and adolescents, and develop easier educational and community preventive nutritional strategies and policies to reduce mental problems.

Furthermore, this thesis also supports the idea that these relationships are biologically grounded, showing that lifestyle factors—especially diet—can influence body systems that are directly involved in brain development and mental health. In this context, we have explored associations among dietary patterns, the gut microbiota, and mental health outcomes, thereby disentangling the role of the gut-brain axis in behavior, executive functions and learning difficulties.

In the first chapter of this doctoral thesis, we emphasize the need to develop and implement optimal tools for accurately assessing dietary intake in epidemiological studies, particularly in children and adolescents. In the second and third chapters, we establish robust associations between the diet, the gut microbiota and behavioural and executive function problems. These findings set the basis for future prospective and mechanistic studies to advance the understanding of how dietary factors and gut microbiota interact and may impact and help prevent neurodevelopment disorders. Finally, we include a review of previous studies focused in clinically diagnosed neurodevelopmental disorders.

1. DIETARY ASSESSMENT TOOLS FOR NUTRITIONAL RESEARCH

The results of the first chapter contribute to nutritional research by assessing, for the first time, the validity and reproducibility of the EPIC-FFQ in a Mediterranean Spanish child population. This study helps address an important methodological gap by adapting and validating a dietary questionnaire for children and adolescents, thereby providing a critical tool for future research on diet and health in early life in our country.

Although assessing dietary intake remains challenging due to the limitations of commonly used self-report tools—such as food frequency questionnaires (FFQs), food diaries, and 24-hour dietary recalls—we selected the FFQ because it allows us to obtain information on the long-term habitual consumption pattern in large populations. This approach is also less burdensome to complete and may reduce reporting errors among children and adolescents, although the potential for recall bias remains[75]. Moreover, the FFQ provides a comprehensive overview of dietary intake, which, although it sacrifices absolute precision, captures inter-weekly and inter-seasonal variability, thereby offering valuable insights into food consumption from a broader perspective. This aspect is of particular importance in the study of the diet–microbiota relationship, since although it has been suggested that the latter can respond to short-term dietary changes, such effects appear to be temporary. However, while it remains unclear whether prolonged dietary changes can induce permanent alterations in the gut microbiota, it is considered that long-term or habitual dietary habits exert a great influence on its composition and metabolic activity[362]

Additionally, we assessed the validity and reproducibility of the EPIC-FFQ, given several advantages of its design. The EPIC-FFQ provides accurate estimations of food portion sizes and includes nine frequency categories for food consumption. This structure enables precise assessment of daily nutrient and food intake, capturing key dietary components (e.g., types of fiber, vitamins, amino acids, and PUFAs) that may modulate immunometabolic pathways and microbial composition, with potential effects on brain function. In addition to measuring individuals' usual food and nutrient intake, FFQs can be used to construct dietary indices and patterns, thereby allowing quantification of dietary variability and identification of dietary patterns adhered to by individuals or populations [336,363]. Importantly, the EPIC-FFQ comprises 125 food items, a large number that, according to Cui et al. (2021), is crucial for ensuring high-quality dietary data records[364].

From a methodological and logistical perspective, we automated the EPIC-FFQ to reduce costs, improve efficiency, facilitate self-administration, and increase engagement and acceptability among older children and adolescents. Evidence from studies comparing adolescents' and children's experiences with web-based versus paper-based dietary tools indicates that online formats are perceived as more independent and user-friendly. In addition, web-based questionnaires are associated with shorter completion times and lower rates of misreporting, ultimately enhancing the overall quality of dietary data collection[365].

Concerning data analysis, it should be noted that although our average ICC values are below the threshold of 0.5 (an ICC > 0.5 is recommended to distinguish real changes from measurement errors), several studies conducted in complex populations, such as children and adolescents, consider an ICC > 0.4 to be "moderately acceptable"[366]. Furthermore, for validation, we collected multiple 24-hour dietary recalls over 9 months, allowing us to account for seasonal variation in food intake. Reproducibility was assessed one year later. According to Cui et al., a recall period of 12 months or more increases confidence in the results and improves the ability to distinguish true dietary changes from measurement errors, thereby strengthening the detection of associations between diet and disease[364] In line with our findings, other EPIC-FFQs have also demonstrated moderate to good long-term reproducibility, as observed in the EPIC-Heidelberg cohort[367]. Finally, reproducibility is essential when FFQs are used in longitudinal studies and is particularly crucial for international comparability, as it facilitates cross-cohort comparisons and data harmonization in multicenter nutritional research[77.]

In epidemiological nutritional research on mental health, recent studies highlight the importance of validating dietary questionnaires in specific study populations, as eating patterns, relationships with food, and factors influencing diet can differ significantly in individuals with mental disorders or neurodevelopmental conditions, such as autism, compared with the general population[115].Although our purpose was to use this questionnaire in a population of healthy children and adolescents, we acknowledge this as a possible limitation of our study. Assessing the validity and reproducibility of questionnaires in clinical or subclinical settings can also be important for obtaining more reliable nutritional data to evaluate comprehensive dietary habits and identify key nutritional risks. In turn, this is key for designing targeted interventions and developing specific nutritional guidelines for children and adolescents with or at risk of mental health conditions[368,369].

In line with our approach, nutritional psychiatry research should move beyond the mere validation of dietary questionnaires and focus on adapting these tools to specific populations. This would allow the identification of population-specific dietary patterns and provide a more accurate understanding of how real-world eating behaviors—rather than isolated nutrients, influence the mental health of children and adolescents [204].Such an approach is particularly relevant given the persistent prevalence of unhealthy dietary patterns in this age group

In line with our own study and the complex dietary data analysis performed in this Thesis, nutritional psychiatry should investigate the role of dietary patterns to enable the understanding how the combination and frequency of food intake in the diet, and not just the sum of isolated nutrients, influences mental health in children and adolescents, where there is a clear persistence of unhealthy dietary patterns.[204]

2. IMPACT OF COVID-19 ON PSYCHOLOGICAL WELL-BEING, MICROBIOME COMPOSITION AND LIFESTYLE FACTORS

In relation to chapters 2 and 3, exploring the relationship between the diet, the gut microbiota, and mental health outcomes in children and adolescents, it is worth noting that the data were collected between October and November 2020, approximately seven months after the first wave of the COVID-19 pandemic. This period has been well documented to have caused significant lifestyle disruptions and potential subclinical and clinical effects on children's and adolescents' mental health, providing a particularly relevant context for the interpretation of our results[10,360,361].On this matter, a cross-sectional study conducted in Spain concluded that lifestyle factors such as screen-based activities, sleep, diet, or physical activity, as well as behavioral and emotional problems, worsened seven months after the first confinement in Spain, coinciding with the timeframe of our data collection[370]

In this context, data collected during this time window has offered a unique opportunity to examine the pandemic's potential as a risk factor for mental health, gut health, and lifestyle habits in the general population of children and adolescents. This timing allowed us to capture early, yet potentially lasting, changes in subclinical markers of psychological well-being. In other words, within our initially general population of healthy children and adolescents, and considering the potential impact of the pandemic as a risk factor for mental health, gut health, and lifestyle habits, we were able to stratify participants based on the presence of subclinical

symptomatology versus healthy profiles. This allowed us to examine diet- and microbiota-related associations across these subgroups.

To assess mental health risks, we used three screening tools: the SDQ to assess difficulties with externalising and internalising behaviours; the BRIEF to evaluate executive function problems; and the DSM-5 criteria to screen for learing/academic difficulties. A recent review showed that these methodologies are commonly used to understand the interplay among the diet, gut and mental health[342].

Considering evidence that supports the effect of COVID-19 on lifestyle factors and the role these factors play in shaping mental health outcomes[66,360] we systematically evaluated lifestyle factors susceptible to change during the COVID-19 pandemic, such as physical activity, level of stress, sleep disorders, daily use of screen-based activities, daily time practice of team sports, and outdoor activities. Our results consistently suggest a potential association between impaired cognitive and behavioral functioning and changes in dietary pattern, sleep difficulties, and stress, as we showed in the results of chapters 2 and 3.

Additionally, when we evaluated the lifestyle index score, measured as a composite of physical activity, BMI, HEI, adherence to MD, screen use, and time spent in outdoor activities, we observed that almost half of the population (45%) presented a medium or low lifestyle score. Specifically, as expected, 80% of the population reported low daily engagement in outdoor activities, whereas 48% of adolescents, but not children (15%), showed excessive screen use. In addition, fewer than half (40%) of our child and adolescent population maintain good adherence to the Mediterranean diet and an overall healthy eating quality. For this reason, and as expected, no differences were observed between participants with executive function or behavioral difficulties and healthy participants when considering other lifestyle factors such as physical activity, adherence to the Mediterranean diet, and screen time [65,371,372]

Concerning adherence to dietary pattern, several cross-sectional studies have evaluated adherence to the Mediterranean diet in populations from Spain, Italy, Greece, and Portugal. Although in general, Spanish children and adolescents showed better adherence, possibly due to greater intake of home-cooked meals rather than fast food and increased parental control over eating habits eatings[373,374], some studies align with our results by reporting a decline in adherence among certain groups of children[373] . This decline was associated with increased consumption of processed foods (miscellaneous and pastries) and reduced intake of fresh fruit, legumes, nuts, and whole grains, as we also observed in our sample.

All studies attribute these unhealthy eating patterns to the emotional impact of the lockdown[375]. In this regard, our results in Chapter 2 show that participants with behavioral problems are more associated with adherence to Western and pro-inflammatory eating patterns. Meanwhile, in Chapter 3, we were able to specify that this association with unhealthy eating patterns was associated with learning disorders and internalizing problems.

In addition, we observed a notable increase in adolescents presenting behavioral problems, stress, and respiratory problems. Previous research has shown that respiratory conditions such as asthma or pneumonia can activate the HPA axis and immune system, increasing the risk of mental health impairments, for example, in executive functioning. This supports the idea of a lung–brain axis, where respiratory health may directly influence neurodevelopment and behavior, particularly under stressful conditions[376]

Taken together, although we lack pre-pandemic biological samples and lifestyle data, we hypothesize, based on existing literature, that the gut microbiota profile, as well as stress levels, unhealthy dietary patterns, and sleep disturbances in participants with subclinical symptoms, may have been partially influenced or aggravated by the COVID-19 pandemic as an adverse environmental event

While the negative consequences of the pandemic are evident, several recent cross-sectional and longitudinal studies have explored how healthy lifestyle factors- including optimal physical activity, non smoking, non alcohol consumption, good sleep status and adherence to healthy dietary pattern rich in fruit, vegetables and anti-inflammatory nutrients prior, during and after stressful events, may play a protective role against the psychiatric comorbidities and neurocognitive consequences associated with COVID-19 in adults [377–379]. These findings support the growing hypothesis that engaging in positive health behaviors may act as resilience mechanisms during periods of heightened psychosocial stress, promoting better mood and well-being in healthy adults. However, there remains a significant research gap regarding children and adolescents. To date, only one longitudinal study in children and adolescents has explored the effects of excessive screen time combined with low physical activity during the pandemic on the emotional health of this population [63].

It is worth noting that, in addition to the cross-sectional data collected during the first wave of the COVID-19 pandemic, a second round of data collection was carried out 12 months later, coinciding with the gradual return to normality. In this context and based on the preliminary findings of

this thesis, the next step will be to develop a predictive model aimed at better understanding how lifestyle behaviors during the pandemic, particularly dietary habits and gut microbiota status, alongside low physical activity and excessive screen exposure, may influence long-term mental health outcomes in children and adolescents.

Importantly, although the COVID-19 pandemic served as the stress-inducing event in this study, the insights gained are broadly applicable to other adverse contexts that compromise children's and adolescents' mental health. The future findings may support the development of targeted dietary strategies to mitigate the long-term consequences of stress on youth development.

3. ASSOCIATIONS OF DIET AND GUT MICROBIOTA WITH BEHAVIORAL AND COGNITIVE PROCESSES IN CHILDHOOD AND ADOLESCENCE

The second and third chapters of the thesis examine the relationship between the diet-gut-brain axis in children and adolescents. Most of the observational studies published so far describe associations between dietary patterns or nutrients with diagnosed psychiatric disorders in children and adolescents, but a direct connection between dietary patterns or nutrients and subclinical symptoms in these populations has not yet been established [228,380]. A substantial body of observational, preclinical and clinical studies conducted in subjects with diagnosed mental disorders has consistently demonstrated that the gut microbiota and diet play a significant role in cognition, mood, and behavior. Notwithstanding, while these findings have been fundamental to advancing our understanding, there is a lack of studies exploring whether such associations are also present in subclinical cases[381]. For this reason, in the second chapter, we examined the relationship between diet and the gut microbiota in children and adolescents presenting with behavioural or cognitive alterations, which may indicate heightened vulnerability. This approach can help identify early risk or protective factors, thereby informing the development of preventive strategies and early interventions to support mental health.

3.1 How the granularity of the dietary assessment contributes to explaining behavioral, executive functional and learning difficulties alterations

It is worth noting that our study incorporated detailed dietary influences, including dietary patterns, specific nutrients, food groups, and adherence to the recommendations of the Spanish dietary guidelines, not limiting the analysis to general indicators or summary scores[382]. In this way, we were able to provide a more comprehensive perspective on modifiable environmental factors, such as diet, that are involved in mild behavioral and cognitive disorders and increase vulnerability to mental diseases in the long term.

The dietary analyses conducted in this thesis combined a priori and a posteriori approaches to capture different dimensions of diet with the aim of generating new hypotheses through the exploration and identification of specific dietary patterns in our child and adolescent population with mild cognitive and behavioral impairments, which are not reflected in traditional dietary guidelines[383].

Following current methodological recommendations[383–385], an a priori approach was first applied to evaluate overall dietary quality using three indices: the Spanish Healthy Eating Index (SHEI), Mediterranean Diet adherence score, and the Dietary Inflammatory Index (DII). These indices allowed standardized comparisons of global dietary quality across study groups[382,386].

Complementarily, a data-driven (a posteriori) approach based on Principal Component Analysis (PCA) with orthogonal rotation was used to identify population-specific dietary patterns. A factor loading threshold > 0.4 was applied to define relevant contributors to each pattern[385]. These approaches are widely used in the literature and enhance comparability with other studies[383,385]. Importantly, a posteriori pattern analysis has recently shown greater ability to explain diet–gut microbiota relationships than single-nutrient approaches[336].

At the same time, emerging evidence emphasizes that integrating dietary patterns with analyses of individual foods and nutrients provides deeper insight into biological mechanisms[387,388].Consistent with this, the interaction and mediation models presented in Chapters 2 and 3 revealed strong associations and correlations when nutrients and food groups were examined individually, while also identifying links between Western dietary patterns and specific microbiota species, and behavioral outcomes. Nutrient-level findings are particularly useful for generating hypotheses that can be tested in preclinical models and translated into feasible dietary interventions, as small food changes are often more realistic than modifying entire dietary patterns[389].

This thesis illustrates how each approach can provide helpful information. On the one hand, the dietary-pattern analyses, as discussed in chapter 2, allowed us to identify clinically relevant associations between Western dietary patterns and behavioral outcomes in children and adolescents. In addition, Chapter 3 showed that the learning-difficulties group, which was characterized by higher levels of internalizing problems, was associated with a more pro-inflammatory dietary pattern. These findings are partly consistent with previous observational studies showing that pro-inflammatory diets are associated with an increased risk of anxiety, depression, stroke, sleep disorders, dementia, and schizophrenia in adult populations[122].

Nevertheless, the association between dietary patterns and executive functions or learningis more inconsistent across current literature. Most of the evidence available in Spanish children and adolescents focuses almost exclusively on the effects of adherence to the Mediterranean diet on academic performance [390]. Earlier studies also found a relationship between the consumption of ultra-processed foods and the deterioration of executive functions and learning[391,392]. However, more recent research shows weaker or non-significant associations, and to our knowledge only one study conducted in a Colombian pediatric cohort—has examined dietary patterns and quality in relation to executive functioning [393].It is possible that the lack of studies in this field has so far prevented the identification of strong links between dietary intake and cognitive or executive functions. In this context, our study provides new insights by evaluating a posteriori dietary patterns and their association with learning and executive-function profiles. Specifically, we identified four dietary patterns that most clearly differentiated cognitive groups: (1) a “Fish and Dairy Products” pattern, (2) a Fiber and vegetal-protein and micronutrients pattern, (3) a "trans fatty acids and starches” and (4) “Group B-vitamins and Iron” pattern.

On the other hand, when we focused on specific nutrients and foods, we can detect reductions in the intake of specific fibre-rich foods such as vegetables, legumes and nuts. In adolescents with behavioral problems and learning difficulties, whereas children with behavioral problems and learning difficulties showed higher consumption of processed foods and pastries. No differences were observed in the group with executive function impairments.

Addionally, we were able to detect specific differences related to omega-3 that were not detectable when assessing dietary patterns .In relation to the findings presented in Chapter 2, we observed that intake of EPA, DHA,

and fish, which were highly correlated, was higher among adolescents with greater behavioral difficulties.At first glance, this seems contradictory to previous literature, which describes the essential role of omega-3 fatty acids in synaptic plasticity, dopaminergic and serotonergic neurotransmission, hypothalamic–pituitary–adrenal axis regulation, and hippocampal[328]. This apparent discrepancy can be explained by considering the gut microbiota as a mediator or variable that interacts with this nutrient in machine learning models (which we will discuss in the next section).

Regarding cognitive outcomes, children with greater learning difficulties showed lower ALA consumption, possibly due to lower intake of oily fish and ALA-rich nuts, despite compensatory intake of olive oil. This was accompanied by an increase in the proinflammatory LA/ALA ratio.In this context, previous studies have linked lower ALA intake and higher LA/ALA ratios to poorer academic and neurocognitive performance in healthy Spanish children and adolescents [206,394] underscoring the importance of ALA-rich dietary patterns for optimal cognitive development[395].

B-group vitamins, such as folate, vitamin B6 or B1, polyphenols, and vitamin C also emerged as relevant nutrients in the learning difficulties group.Importantly, these nutrients and foods are widely recognized for their roles in neurodevelopment and cognitive function. B-vitamins are essential for neurotransmitter synthesis and antioxidant compounds, including vitamin C and omega-3 fatty acids, are involved in brain function and the regulation of neuroinflammatory processes, although some inconsistencies remain in the evidence[396–398]

Insights from nutrient-level analyses and microbiota interactions

When we focused on specific nutrients and foods, we could hypothesize on diet-microbe interactions and the generation of metabolites with potential protective effects on mental health. For example, reduced intake of fiber-rich foods may decrease production and intake of anti-inflammatory and neuroprotective phytochemicals, which may explain behavioural profiles in young people. In line with this, existing literature shows that certain dietary patterns ,particularly Western diets, and plant-based patterns rich in fiber, vegetal protein, and micronutrients are strongly associated with emotional and behavioral outcomes in children and adolescents[389,399–401].

Extending this mechanistic perspective, it is possible that adherence to a Western dietary pattern, observed among participants with more pronounced behavioral difficulties and characterized by higher

consumption of animal-derived foods and ultra-processed products, may have contributed to higher blood and urinary TMA or TMAO concentrations. This metabolites, generated from dietary precursors such as choline, betaine, and L-carnitine by the gut microbiota, has been associated with potentially adverse effects on brain health, as it can cross the blood–brain barrier and modulate processes related to neuronal function[402]. However, recently studies have found that TMA, the microbial precursor of TMAO, compromises the integrity of BBB, whereas physiological and prolonged levels of TMAO appear to protect it against inflammatory triggers[403].This dietary context mediated by the gut microbiota may help explain the absence of the expected direct neuroprotective effects of omega-3 fatty acids, since their action may depend on the metabolic and microbial context. However, this hypothesis must be confirmed by intervention studies that simultaneously evaluate omega-3 consumption and the functionality of the gut microbiota and metabolic pathways in different dietary contexts, together with analyses focused on TMA/TMAO synthesis in different cognitive and behavioral phenotypes.

Therefore, approaches that cover pattern-, food-, and nutrient-specific analyses provide complementary information and should be combined to generate meaningful insights into the overall role of the diet in mental health, as well as to guide further translational mechanistic and interventional trials that help elucidate specific diet–microbiome–host mechanisms and solutions[404].

3.2 Gut Microbiota Signatures of Behavior, Executive Functional and Learning Alterations.

The majority of observational studies on the relationship between subclinical mental health problems and the gut microbiota have been conducted in adults presenting mild depressive and anxiety symptons[405,406]. Even though, a recent observational study in adolescents has identified a potential link between subclinical neuropsychiatric symptoms, particularly those related to behavior and emotional regulation, and changes in gut microbiota composition, consistent with the findings presented in Chapter 2 of this thesis. Thus, Brown et al. (2025) reveal stronger and clearer associations with microbial functional pathways than with microbial diversity[407]. In contrast, as found in chapters 2 and 3, children and adolescents with subclinical behavioral problems and cognitive impairments exhibited increased alpha diversity, but no

significant alterations in the functional profile of the gut microbiota (results not shown in Chapters 2 and 3 due to lack of statistical significance).

Regarding the absence of results in the functional profile in our study, we conducted a broad and exploratory functional analysis using PICRUSt2 combined with MetaCyc pathways. However, recent studies suggest that predicting functional profiles using PICRUSt2 for broad phenotypes, such as global behavioral problems, may require larger sample sizes, external validation, and possibly complementary targeted approaches such as those employed by Brown et al. and Hu et al[408]. For instance, the use of PICRUSt2 combined with HUMAnN2, along with selected MetaCyc metabolic pathways based on existing literature, may be sufficient to confirm and refine functional associations[407,409]. For this reason, narrowing the analysis to specific functional pathways could yield more robust and interpretable results and should be considered in future research. Furthermore, separating the evaluation of behavioral and emotional problems as distinct clinical dimensions (internalizing or externalizing problems) may be a useful strategy for detecting functional differences in the microbiome that could be overlooked when analyzing a broad and global phenotype. These findings highlight the importance of recognizing and addressing subclinical symptoms as potential intervention points to detect, prevent, or mitigate major mental disorders[407].

However, as detailed in Chapter 3, even after stratifying the cohort by neurodevelopmental domain, no significant differences were observed in the predicted microbial functional profiles. Subclinical symptoms are typically shaped by a combination of environmental and biological factors with small effect sizes and substantial inter-individual heterogeneity[410].Under these conditions and according to recent multi-omic work, disease-relevant microbial functions often emerge only when integrating microbial composition with dietary inputs and metabolite availability, rather than when analysing global functional pathways alone[410] In line with this reasoning, a more sensitive approach would have been to anchor the functional inference to the specific dietary determinants identified in the cohort (e.g., higher adherence to a Western dietary pattern; lower intake of whole grains, green leafy vegetables, oats, or soluble-fiber sources) and examine whether these dietary gradients aligned with microbial metabolic pathways.

Concerning microbial diversity, while high microbial diversity has been associated with general health and healthy dietary patterns, such as Mediterranean and plant-based diets [411,] other research in children and adolescents has found increased alpha diversity associated with

neurological disease and poorer dietary patterns[282,342,412]. Similar findings were found and discussed in Chapter 2.

In this context, when analysing microbial diversity in relation to executive function, learning difficulties and healthy participants ((Chapter 3), subtle yet meaningful differences emerged particularly among the groups with cognitive difficulties[413]. This suggests that the presence and dominance of certain bacteria may be more important than overall diversity for optimal neurodevelopment[414].

Therefore, to disentangle which genera were driving these diversity shifts across groups, differential abundance analyses were performed. As we found in chapter 2 and 3, children (6-10 years old) and adolescents (11-17 years old) with behavioral problems as well as participants with executive function problems had increased levels of well-characterized pathogenic bacteria and bacteria from the oral cavity and upper intestinal tract. These include *Shigella* spp*., Escherichia* spp*., Campylobacter* spp*., Salmonella enterica., Staphylococcus aureus Enterobacter* spp., and *Enterococcus* spp*., Sutterella faecalis*, *Dialister* spp and *Parvimonas micra*, compared to control groups[415,416] These microbial signatures have previously been associated with low-grade inflammation and impaired intestinal barrier integrity, a phenomenon described in neurodevelopmental disorders as well as in metabolic conditions and chronic stress[417–421]. Among the environmental factors capable of disrupting intestinal microbial homeostasis, exposure to antibiotics, as discussed in Chapter 4, is particularly significant in light of recent preclinical studies indicating that antibiotic administration can have long-lasting effects on the composition of the gut microbiota and can alter behavioral phenotypes during childhood and adolescence [269,271]. In this context, and consistent with this evidence, our findings from Chapters 2 and 3 further support this hypothesis, showing that antibiotic use emerges as a significant factor associated with both behavioral difficulties and EFP in children and adolescents.

In contrast, the group with learning difficulties exhibited a distinct microbial pattern, characterized by enrichment of *Bifidobacterium* spp., but accompanied by a reduction in carbohydrate- and fiber-utilizing bacteria, such as *Coprococcus* and *Prevotella*, including *S.Copri* and *P.xylaniphila* taxa typically associated with diets rich in complex polysaccharides and overall healthy dietary patterns[422–424]. The reduction of these genera has been widely reported in populations adhering to a more pro-inflammatory or Western-type dietary pattern, consistent with the lower intake of fiber, and plant-based foods observed in this cohort. In this context, the loss of these key fiber consumers may indicate an altered SCFA metabolism,

potentially affecting metabolic and immunological pathways relevant to neurodevelopment.

It is important to highlight that, across the analyses presented in Chapters 2 and 3, *Coprococcus eutactus* consistently appeared depleted in children and adolescents with both cognitive difficulties and behavioral problems compared with healthy participants. Recent mechanistic studies in animal models have begun to unravel the pathways through which *C. eutactus* may exert its effects on neurotransmission[425]. The studies demonstrating the capacity of this bacterium species to inhibit key enzymes in the kynurenine pathway, restore intestinal integrity, modulate neurotransmitter signaling in the brain, influencing GABAergic, serotonergic, and dopaminergic pathways and influence SCFA-mediated signaling indirectly via cross-feeding with other beneficial genera[425,426]. These findings provide additional evidence supporting the potential role of *C. eutactus* in internalizing problems and learning difficulties in children and adolescents. However, intervention studies are needed to directly test whether modulation of this taxon can causally influence behavior or learning outcomes.

While *Prevotella* spp. showed higher levels in healthy children than in those at risk of learning difficulties in our cohort, the published evidence to date remains inconclusive regarding the role of this genus in neurocognitive development[427,428]. In contrast to taxa such as *Coprococcus*, for which more consistent associations have been reported, findings related to *Prevotella* appear to be highly context-dependent. In this regard, recent studies suggest that the relationship between *Prevotella* and cognitive function may be modulated by dietary patterns, developmental stage, and differences at the species or strain level, which should not be disregarded [429,430] .

Additionally, the depletion of LAB observed in the executive-function group—compared with both the control and learning-difficulties groups—may be a potential contributor to executive-function impairments in this population. The results suggest that reductions in LAB may be more specifically linked to executive dysfunction and externalizing problems rather than to learning difficulties alone. According to previous findings, LAB have been proposed to contribute to gut homeostasis and to influence neurotransmission (GABA, acetylcholine) and neuronal function through metabolites (e.g., indol-3-propionic acid)[431–434], which can restore synaptic transmission in children and adolescents by modulating neuroinflammatory and epigenetic pathways[435.]

Furthermore, our learning-difficulties group showed enrichment for acetate-producing bacteria, including *Bifidobacterium spp*. and *A. muciniphila*, accompanied by a depletion of butyrate producers, such as C. catus and C. eutactus. This imbalance—higher acetate availability but reduced butyrate-generating capacity—may reflect a suboptimal functional microbiome for supporting neurocognitive processes that rely on anti-inflammatory, barrier-protective, and neuroactive functions typically associated with butyrate production. For instance, environments characterized by high acetate levels have been associated with improvements in inattention—but not in hyperactivity—in ADHD populations[436], demonstrating that both acetate and butyrate influence cognitive-related networks through multiple mechanisms. These include inflammatory signaling pathways and neurotransmission systems, including dopaminergic, serotonergic, and glutamatergic[437,438].

Finally, *Alistipes onderdonkii* was enriched across cognitive difficulties groups compared to healthy controls. This species has been associated with increased IL-6 and high-sensitivity C-reactive protein. Although its specific function in the gut–brain axis remains unknown, numerous preclinical and clinical studies suggest a potential role for the genus Alistipes in gut–brain communication. This appears to be mediated by changes in metabolites, such as amino acids, vitamin B, and bile acids, as well as by functional routes involving alterations in gray matter morphology, spontaneous brain activity, and white matter integrity[439,440]. Therefore, the role of *Alistipes* species from our cohort, in the gut–brain axis, warrants further investigation.

At first glance, these microbial profiles appear to vary according to developmental stage, suggesting that age-related differences may play a key role in shaping the microbiota–brain relationship during neurodevelopment. Furthermore, the subclinical mental phenotypes also exhibited differences in microbial composition, showing a more dysbiotic profile or higher abundance of opportunistic and pathogenic bacteria in the case of global behavioral problems and executive functions groups. Additionally, differences in the abundance of key SCFA-producing taxa were also observed betweenthe learning difficulties and the healthy group.

3.3 Modeling Relationships Between Diet, Microbiota, and Brain Outcomes

We have explored the interactions between diet and microbiota that could contribute to subclinical behavioural and cognitive outcomes in children and adolescents through different approaches: (i) Machine Learning (ML), (ii) mediation analysis.

Machine Learning

It is important to note that, our ML approach was conceived with an exploratory rather than strictly predictive purpose, aligning with the methodological constraints and opportunities of cross-sectional designs and aimed at identifying candidate biomarkers and interaction pathways rather than producing clinically deployable predictive models[441].

Additionally, and in line with recent literature recommending the combination of linear models for their transparency and variable-selection capabilities with non-linear models for their ability to capture complex interactions characteristic of gut–brain axis data, we applied both types of algorithms[442,443], the results of which are presented in Chapters 2 and 3.Thus, in Chapter 2, we applied linear models such as sPLS and Elastic Net to ensure interpretability and robust feature selection across gut microbiota, dietary variables, and covariates for the classification of behavioral problems.

Specifically, these results showed that both behavioral problems group, and *Campylobacter spp* co-occurred with a Western dietary profile characterized by higher intake of processed meats, pastries and ultra-processed foods. This pattern is consistent with prior evidence linking high animal-fat/processed food exposure to increased intestinal colonization by pro-inflammatory and barrier-disruptive taxa[385] .

Conversely, Butyrivibrio fibrisolvens and folate (vitamin B9) showed inverse associations with behavioral problems, aligning with the broader role of fiber/legume-rich diets and B-vitamin adequacy in supporting butyrate-linked fermentation and neurodevelopmental processes[277]. As introduced in this thesis, folate is also closely linked to proper neurodevelopment, particularly through its involvement in epigenetic regulation[444].Whereas, at a global level, we found tha that integrating dietary and microbial features improves discriminative power compared to analyzing each domain separately, highlighting joint diet–microbiota patterns linked to behavioral difficulties, executive functioning and learning performance.

In parallel, non-linear algorithms (Random Forest and Gradient Boosting) were implemented to capture complex and potentially non-linear diet–microbiota interactions predicting differences in executive functions and learning, as we demonstrate in Chapter 3. This findings found as dietary components typical of proinflammatory diet—especially added sugars and processed meats—were strongly linked to oral and opportunistic taxa (e.g., *Streptococcus* and *Staphylococcus aureus*), reinforcing the idea that diet

may shape not only metabolic capacity but also the likelihood of oral–intestinal translocation and barrier stress in externalizing problems and executive-function difficulties[445].

It is important to note that these results should be interpreted as concurrent changes in microbial composition associated with low-quality diets which may coincide with other ecological and host physiology changes in immune–metabolic and neural pathways (cytokines, neuroactive metabolite production such as butyrate, propionate, indoles, GABA) that, together, can affect behavior and executive performance[446] and would required further investigations.

Across cognitive phenotypes, the learning difficulties group displayed a distinct diet–microbiota configuration, consistent with reduced intake of antioxidant, polyphenol and fiber-rich foods (e.g., leafy vegetables and fruit). In this profile, machine-learning interaction mapping highlighted positive links between polyphenols, vitamin C and folate and taxa such as *Coprococcus spp*.

Likewise, supplementation with vitamin C exerts regulatory effects on the gut microbiota by reducing oxidative stress and promoting the enrichment of barrier-supporting commensals[447,448].Within the context of our findings, these associations may indicate that dietary patterns rich in micronutrients and antioxidants, such as leafy green vegetables, could promote microbial configurations that support barrier function and overall gut health. We hypothesized that the depletion of *C.eutactus* and *C.catus* was linked to a pro-inflammatory dietary pattern, whereas the enrichment of *Akkermansia* could be explained as an adaptation to unhealthy dietary conditions, as this bacterial taxon is know to survive using host glycans under low-fibre diets [449].

Additionally, as discussed in Chapter 3, the increased abundance of *Bifidobacterium* observed in the learning-difficulties group was more strongly and positively correlated with the consumption of dairy-based desserts than with dairy products such (yoghurt, milk and cheese). In light of these findings and considering evidence that dairy products can modulate cortical structure,[398] future studies should investigate how different dairy products, varying in fat composition and fermentation characteristics, differentially shape gut microbiota composition and metabolic output, thereby exerting distinct effects on learning through the gut–brain.

Moreover, zinc intake was positively correlated with *Bifidobacterium breve*, in the learning-difficulties group. While zinc has been widely

associated with beneficial effects on cognition and neuronal plasticity during neurodevelopment, growing evidence indicates that its biological effects are strongly modulated by the intestinal ecosystem, influencing microbial composition, metabolite production, inflammation, and gut permeability[450]. In line with this, preclinical studies in ASD and ADHD models further indicate that zinc supplementation, particularly under zinc-deficient conditions, can improve gut microbial diversity and partially normalize disorder-associated dysbiosis[451]. Together, these observations generate a testable hypothesis in which the cognitive effects of zinc may depend on microbiota-mediated mechanisms, warranting further investigation into zinc–microbiota interactions within the gut–brain axis.

Our exploratory analyses generate human-data-driven hypotheses about how interactions between diet and the microbiota may contribute to behavioural and learning difficulties. However, these mechanistic hypotheses remain to be confirmed and validated through longitudinal and intervention studies that are essential for elucidating the factors and biological pathways involved.

Mediation analysis

In mediation analyses, we found that the association between fish consumption, but not EPA and DHA, and behavioral problems was captured within a bacterial network that included *Anaerostipes rhamnosivorans* as a mediator. Although *A. rhamnosivorans* is a butyrate producer, evidence suggests that in more dysbiotic and proinflammatory ecological states, it may contribute to a proinflammatory profile, especially when other SCFA-producing bacteria are reduced[452–454]This underscores the importance of community context and substrate availability when interpreting which could be “beneficial” taxa.

Furthermore, emerging evidence indicates that the genus *A. rhamnosivorans* is enriched in behavioral phenotypes associated with ADHD[455]. Therefore, we hypothesized that, within the proinflammatory dietary environment of the affected population, *A. rhamnosivorans* could adapt its metabolism to the available nutrients, without necessarily leading to SCFA production.

A similar logic applies to *Segatella Copri*: although it is a butyrate-producing bacterium capable of thriving in pro-inflammatory dietary environments and it have been associated with poorer cognitive performance in healthy school children[428]; it is metabolically highly versatile[456]. In our population, *S. copri* appeared to mediate the association between intake of fiber-rich foods, including oats and fermented

vegetables, and learning outcomes. This suggests that, under conditions of a fiber-rich diet, *S. copri* may contribute to a favorable gut environment that supports the production of metabolites or signals beneficial for the central nervous system and the gut–brain axis.

Methanobrevibacter smithii was identified as a putative mediator linking oats to improved learning outcomes. *M. smithii* has been associated with improved cognitive responses through metabolic pathways regulation inclusing bile acid metabolism, ALA/LA metabolism, histidine, phenylalanine and butyrate [457].

We also identified *Lacticaseibacillus paracasei* as a mediator of the association between lauric acid and learning difficulties. *L. paracasei* is a bacterial species commonly present in fermented dairy products such as yogurt and cheese, which has been associated with downstream SCFA production and reduced HPA axis activity, contributing to lower stress levels and potentially supporting cognitive performance[458]. Building on this finding, and considering the positive correlation between Bifidobacterium prevalence in the learning-difficulties group and dairy-derived sugars, we further emphasize again that the specific type of dairy consumed may be critical in shaping gut microbiota composition and metabolite production in the subclinical population with executive function problems or learning difficulties

In summary, following pro-inflammatory Western-style dietary patterns may reduce the abundance of key SCFA-producing taxa (*Faecalibacterium, Bifidobacterium, Coprococcus*), increase the abundance of oral-derived pathogens (*Streptococcus, Parvimonas, L. mirabilis*), and promote low-grade inflammation, which could affect executive function and behavior. Conversely, fiber- and plant-rich eating habits may increase the abundance of other taxa such as *M. smithii*, *S. copri*, as well as indole- and SCFA-producing bacteria that reinforce intestinal barrier integrity and neurochemical signaling (e.g., GABA, serotonin), offering potential protection against difficulties related to learning and executive functions. Nevertheless, under specific dietary contexts, some bacterial species, such as *S. copri* and *A. rhamnosivorans*, may persist and activate metabolic pathways that could negatively influence mental health. Finally, the consumption of dairy products may contribute to improved learning and executive function performance, potentially through the enrichment of the microbiota with *L. paracasei* and potentially other *Lactobacillus* species.

4.FROM ONE-SIZE FITS ALL TO PERSONALIZED NUTRITION

Short- and long-term changes in gut microbiota in response to diet have been observed, suggesting that diet-based strategies could help restore optimal nutrient intake and mitigate deviations in the gut microbiota associated with mental health risks, thereby preventing brain health issues [459]. As also discussed in Chapter 4 dietary patterns explored to influence the gut–brain axis include ketogenic diets, which aim to modulate metabolic and microbial pathways through high-fat and low-carbohydrate intake, as well as balanced, nutrient-rich diets emphasizing diversity and adequate intake of vitamins, fiber, and omega-3 fatty acids. Although these interventions show potential to influence microbiota composition, inflammation, and neurocognitive outcomes, evidence of the possible microbiome-mediated effects remains limited and inconsistent, particularly in pediatric and adolescent populations. Alongside this, as discussed in Chapter 4, a significant challenge in translating psychobiotic and diet-based interventions into clinical practice is the considerable inter-individual variability in treatment response. Many individuals may benefit from dietary or psychobiotic strategies (responders), while others show minimal or no improvement (non-responders). Baseline microbiota, endogenous nutrient status, genetics, inflammatory state, and comorbidities appear to modulate responsiveness. For instance, omega-3 PUFA supplementation appears most effective in individuals with low baseline EPA levels or high inflammation, and ADHD children with comorbid Oppositional Defiant Disorder (ODD) or learning difficulties may respond better to specific diets such as EPA supplementation or a few-foods diet (FFD)[204,460]. Similarly, ketogenic diets, B-vitamin interventions, and gluten/casein-free diets show variable effectiveness, which, among other factors, may depend on the microbiota, genetic background, and metabolic status. Therefore, stratification strategies could be essential for identifying at-risk subjects and patients who are more likely to respond to specific dietary interventions, thereby guiding precision or personalized nutrition.

Although existing studies demonstrate that diet and the gut microbiota can modulate the progression of mental and neurocognitive conditions, there is a notable lack of studies on subclinical populations, despite the fact that they play a critical role in prevention[461]. Most available research has concentrated on individuals with a clinical diagnosis, limiting our understanding of the early processes that precede the mental disorders

and the possible efficacy of dietary interventions and microbiome modulation strategies to reverse the trajectory from health to disease[461]

In fact, several reviews indicate that initiating interventions during childhood and adolescence, periods characterized by high plasticity, is more effective than intervening later, when individuals have already consolidated patterns of behavior and thinking that are more resistant to change[462].In this sense, international public health policies have increasingly recognized the value of lifestyle-based interventions—such as diet and physical activity—both for preventing and managing mental and physical health problems in young populations[463].

Although there is growing evidence that various nutrients and foods (e.g. B vitamins and polyphenols) can indirectly influence neurocognitive development through microbiota-mediated pathways—including microbially derived metabolites such as SCFAs and indoles—these mechanistic hypotheses have not yet been sufficiently tested in specific subclinical phenotypes[461]. Our exploratory analyses help bridge this gap by generating hypotheses on how interactions between diet and the microbiota may contribute to learning difficulties and executive and behavioral problems in a subclinical population of children and adolescents.

In particular, our data suggest microbial signatures specific to each phenotype studied, such as a higher Westernized dietary load, deficits in plant-derived nutrients implicated in cognition, such as fiber and ALA; or an increased intake of dairy products in children with learning difficulties; and specific nutritional deficiencies (B vitamins, iron, whole grains) in those with executive problems and externalizing behavior. These differences were linked to observed alterations in the prevalence of SCFA- or lactic acid–producing bacteria and pathogens. Yet, there is a need for longitudinal, multi-omic studies to establish causal direction and elucidate the metabolic mechanisms linking dietary patterns, microbiota changes, and neurocognitive function in subclinical youth populations.

Given the importance of nutrition, the dietitian-nutritionist, as part of multidisciplinary teams, may play a key role in the detection, prevention, and management of cognitive aspects in children and adolescents. Nutritional interventions can contribute to emotional well-being and overall quality of life by establishing dietary strategies that promote mental health and strengthen psychological resilience during critical developmental stages[464] . At the same time, addressing the complexity of the diet–microbiota–brain axis requires the combined expertise of dietitians, psychologists, neuroscientists, clinicians, and bioinformaticians. Such

multidisciplinary collaboration is crucial for developing personalized, preventive, and therapeutic strategies that integrate nutrition, microbial ecology, and behavioral science, ultimately enabling more precise interventions tailored to individual developmental and biological profiles[465]

Future directions

Our study provides new insights into how specific dietary patterns, foods, nutrients, and gut microbiota signatures are associated with behavioural and executive dysfunction in children and adolescents, supporting the role of nutrition in mental health and generating new hypotheses. However, the results should be interpreted with caution, considering the inherent limitations of cross-sectional studies and relatively modest sample sizes of the subgroups under study.

Future advances in the field will require longitudinal studies that integrate not only metagenomic analysis but also other omics (e.g. metabolomics) to clarify the temporal direction of the effects and provide a solid basis for understanding how the diet, partly through microbiota-mediated mechanisms, shapes the neurobiological processes involved in cognitive and behavioral phenotypes in children and adolescents

Additional translational studies will be essential to establish causality and elucidate the mechanistic pathways within the gut-brain axis that may worsen or protect mental health.

Ultimately, this approach could support the development of refined dietary recommendations and personalized interventions aimed at improving the prevention and treatment of mental disorders in childhood, where few efforts have been made so far.

CONCLUSIONS

1. A dietary assessment tool has been validated for the first time in Spanish children and adolescents, contributing to generating a culturally adapted, sensitive instrument capable of capturing the unique eating patterns characteristic of our population at these life stages. This represents a significant methodological advance for nutritional and epidemiological research in paediatric populations.

2. Alterations in cognitive domains (e.g., working memory, planning/organization, and monitoring) are more strongly associated with externalizing behavioral problems and deficits in behavioral control (e.g., hyperactivity and impulsivity), whereas learning difficulties are primarily linked to working memory as an executive function domain, as well as to internalizing behavioral problems.

3. Dietary and microbiota differences related to behavioral problems were age-dependent. The low intake of folate and soluble fiber combined with higher adherence to an unhealthy dietary pattern explained behavioral problems in children, while low intake of legumes and nuts but higher fish explains behavioural problems in adolescents.

4. The impact of fish consumption (highly correlated with EPA and DHA intake) on global behavioral problems was mediated by the microbiota species *A. anaerostipes*, whereas learning difficulties and cognition may be more directly influenced by ALA availability and the LA/ALA ratio, independently of the gut microbiota

5. Our findings reveal distinct diet–microbiota signatures associated with different neurocognitive phenotypes. Specifically, adherence to an unhealthy dietary pattern was linked to the presence of pathogenic bacteria in children and adolescents with executive function problems whereas lower adherence to a healthy pattern—rich in vegetables, walnuts, and fruits—was associated with the depletion of butyrate-producing bacteria in participants with learning difficulties.

6. The associations of dietary features with behavioral problems and learning difficulties appear to be mediated—at least in part—by the gut microbiota, whereas this mediation was not observed for executive function.

7. Species such as *C. eutactus*, *C. catus*, and *Alistipes onderdonkii* have been postulated as bacterial candidates with potential effects on the gut–brain axis, representing promising targets for future studies aimed at elucidating their role in cognitive and behavioral outcomes.

8. Differences in the abundance of lactic acid bacteria (LAB) and *Bifidobacterium* were observed between the EFP and LD groups. Specifically, a negative association was found between dairy product consumption and executive function, whereas *Bifidobacterium* was positively correlated with the intake of

dairy-based desserts. These observations generate a testable hypothesis in which the type of dairy consumed may influence gut microbiota composition and, in turn, cognitive outcomes during childhood and adolescence.

9. The classification models based on machine learning developed in this work highlight the need to study diet and gut microbiota as an integrated system to better understand their combined role in cognitive and behavioral outcomes.

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CONTRIBUTIONS

ABBREVIATIONS

ADHD	Attention-Deficit/Hyperactivity Disorder
ALA	Alpha-Linolenic Acid / Ácido α-linolénico
ASD	Autism Spectrum Disorder
BBB	Blood–Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
BRIEF	Behavior Rating Inventory of Executive Function
CLR / CRL	Centered Log Ratio
CNS	Central Nervous System
COVID-19	Coronavirus Disease 2019
DHA	Docosahexaenoic Acid
DII	Dietary Inflammatory Index
DMM	Dirichlet Multinomial Mixture
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
Elastic Net	Elastic Net Regularization
ENS	Enteric Nervous System
EPA	Eicosapentaenoic Acid
EPIC	European Prospective Investigation into Cancer and Nutrition
EPIC-FFQ	EPIC Food-Frequency Questionnaire
FFQ	Food-Frequency Questionnaire
FMT	Fecal Microbiota Transplantation
GABA	Gamma-Aminobutyric Acid
GF	Germ-Free
GWAS	Genome-Wide Association Study
HEI	Healthy Eating Index
HFD	High-Fat Diet
HMs	High Methanobrevibacter smithii Group
HPA axis	Hypothalamic–Pituitary–Adrenal Axis
HPLC-ESI-MS/MS / LC-MS	High-Performance / Liquid Chromatography–Mass Spectrometry
ICC	Intraclass Correlation Coefficient
IL-6	Interleukin-6
IPA	Indole-3-Propionic Acid
LA	Linoleic Acid
LAB	Lactic Acid Bacteria
LNAA	Large Neutral Amino Acids
LPS	Lipopolysaccharides

LASSO	Least Absolute Shrinkage and Selection Operator
MD	Mediterranean Diet
ML	Machine Learning
MRI	Magnetic Resonance Imaging
MUFA	Monounsaturated Fatty Acids
NDDs	Neurodevelopmental Disorders
NHS	National Health System
PCA	Principal Component Analysis
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
SCFA	Short-Chain Fatty Acids
SDGs	Sustainable Development Goals
SDQ	Strengths and Difficulties Questionnaire
SNP	Single Nucleotide Polymorphism
SPF	Specific-Pathogen-Free
sPLS	Sparse Partial Least Squares
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
UNICEF	United Nations International Children's Emergency Fund
WHO	World Health Organization

LIST OF PUBLICATIONS

The present Doctoral Thesis is composed of three original articles and one review. The third and fourth manuscripts were under revision at the moment of the Thesis

1. Larroya A, Pantoja J, Codoñer-Franch P, Cenit MC. Towards Tailored Gut Microbiome-Based and Dietary Interventions for Promoting the Development and Maintenance of a Healthy Brain. Front Pediatr. 2021 Jul 1;9:705859. 10.3389/fped.2021.705859

2. Larroya A, Tamayo M, Cenit MC, Sanz Y. Validity and Reproducibility of a Spanish EPIC Food Frequency Questionnaire in Children and Adolescents. Nutrients. 2024 Nov 7;16(22):3809. 10.3390/nu16223809.

3. Larroya A, Romera-Giner S, Tolosa-Engís V, Rodríguez-Ruano SM, Andrés-García S, Soro-Conde I, Codoñer P, Sanz Y. Gut microbiota and Western dietary patterns associated with behavioral problems in children and adolescents: A cross-sectional study. (Journal Nutrition). **Manuscript accepted, under review**; **2025**.

4. Larroya A, Romera-Giner S, Tolosa-Engís V, Cenit-Maria Carmen, Rodríguez-Ruano SM, Andrés-García S, Soro-Conde I, Codoñer P, Sanz Y. Cross-Sectional Associations of Diet and Gut Microbiota with Executive Functions and Learning Outcomes in Children and Adolescents. **Manuscript in Submission 2026**

Congres participation

I have disseminated some of my research results in different congresses, symposiums workshops and seminars:

1. Larroya A, Romera-Giner S, Tolosa-Engís, Sanz Y “Intestinal microbiota and dietary patterns associated with cognitive and behavioral alterations in children and adolescents.” VII Congress on Food, Nutrition and Dietetics & I International Congress. Madrid, Spain. October 2025. (Oral presentation)

2. Larroya A, Romera-Giner S, Tolosa-Engís, Sanz Y “Association of gut microbiota and dietary patterns with behavioral alterations in the adolescent population”.VI Congress on Food, Nutrition and Dietetics. November 2023. (Poster presentation)

3. Larroya A, Tolosa-Enguis, Veronica, Cenit, María Carmen, Sanz Y “Descriptive analysis of dietary patterns related to behavioral difficulties in children population” PhD student symposium CSIC October 2022. IATA-CSIC, October 2022. (oral and poster presentation)

4. Larroya A, Tolosa-Enguis, Veronica, Cenit, María Carmen, Sanz Y “Translational research for the development of tailored gut microbiome-based strategies to target neurodevelopmental disorders”en las I Research Conference of the Doctoral Program in Food Science, Technology, and Management, January 2022 (oral presentation).

ACHIVEMENTS, COURSES AND DIVULGATION ACTIVITIES

Throughout the PhD period, I actively participated in a wide range of teaching, training, and scientific outreach activities that complemented and enriched my research trajectory. I contributed to academic training through the design and development of the online course on the gut–brain axis offered by IATA-CSIC for clinical professionals, and by coordinating and delivering talks and workshops on eating behavior disorders in secondary schools across the Valencian Community.

I took part in multiple initiatives aimed at promoting scientific literacy among students and the general public, including guided visits within the “Fórmate con Ciencia” program, participation in the “Women in Science” (WINS) initiative, and the organization of the activity “I Gut the Power” during the annual “ExpoCiencia” open day from 2022 to 2024.

My training also included several specialized courses and workshops, such as advanced applied statistics, multivariate analysis, R programming, survey design with LimeSurvey, microbiome research techniques, microbial cultivation using minibioreactors, and accredited animal experimentation training. Altogether, these activities demonstrate a strong commitment to scientific communication, academic development, and continuous methodological training.