Universitat de València

Programa de doctorado: 3139 Medicina

Evaluación de la respuesta *in vitro* de la microbiota intestinal a diversos alimentos y del impacto de una intervención nutricional personalizada

Tesis Doctoral presentada por:

Alberto Manuel Lerma Aguilera

Dirigida por:

Dra. M. Pilar Francino Puget

Dra. María José Gosalbes Soler

Tutora:

Dra. Dolores Corella Piquer

Abril 2024

VNIVERSITAT DE VALÈNCIA Facultat de Medicina i Odontologia

INFORME DIRECTORAS Y TUTORA PARA DEPÓSITO DE TESIS

Directoras:

1.- Apellidos y nombre: Francino Puget, María Pilar. N.I.F. 46346544A, Departamento/Instituto: Área de Genómica y salud. Centro: FISABIO – Salud Pública

2.- Apellidos y nombre: Gosalbes Soler, María José. N.I.F. 19844439Q, Departamento/Instituto: CIBER Centro: FISABIO-Salud Pública

Tutora

Apellidos y nombre. Corella Piquer, Dolores. N.I.F. 52790127K, Departamento/Instituto: Medicina Preventiva y Salud Pública. Centro: Facultad de Medicina, Universitat de València

Directoras y tutora, respectivamente, de la tesis doctoral: "Evaluación de la respuesta *in vitro* de la microbiota intestinal a diversos alimentos y del impacto de una intervención nutricional personalizada"

de D. Alberto Manuel Lerma Aguilera,

estudiante del Programa de Doctorado **3139 Medicina** (RD99/2011) en Medicina de la Universitat de València, emiten informe favorable para la realización del depósito y la defensa de la tesis doctoral.

Fecha: 22 abril 2024

Fdo.:	Fdo.:	Fdo.:
Directora María Pilar Francino Puget	Directora María José Gosalbes Soler	Tutora Dolores Corella Piquer

ESCUELA DOCTORAL
UNIVERSITAT DE VALÈNCIA

“Tengo respuestas aproximadas, posibles opiniones
y diferentes grados de certeza sobre distintas cosas,
pero no estoy absolutamente seguro de nada”

Richard Feynman

Agradecimientos

En primer lugar, quiero agradecer a mis directoras, Pilar y Pepa. Gracias por haber confiado en mí y haberme brindado la oportunidad de formarme y desarrollarme como investigador. Gracias por haberme acogido como uno más desde el principio y por vuestra motivación cuando las cosas no salían como debían. Ha sido un honor teneros como mentoras y siempre os estaré agradecido por vuestro apoyo y por vuestra guía todos estos años.

Pepa, gracias por preocuparte y defender siempre lo mejor para mí y para esta tesis. Gracias por tu exigencia y por incentivarme a hacer las cosas siempre de la mejor manera posible.

Pilar, gracias por tu apoyo incondicional y tener siempre un "momentito" disponible para mí cuando lo necesitaba. Tu optimismo siempre ha ayudado a llevar mejor el trabajo y tu manera de darle la vuelta a los problemas para aprovecharlos son dos actitudes que espero llevarme conmigo.

Gracias a Sandra, Daniel y Sonia, mis increíbles compañeros de grupo, por estar siempre disponibles cuando les necesitaba. Habéis hecho mucho más amena esta tesis. A Nicole, por ser prácticamente otra compañera de grupo solo a cambio de arreglar los bugs en R, me llevo una amiga para siempre. A Carlos, Neris, Irving, Bea, Martha, Xue y todos los que pasasteis por la sala por todos los ratos memorables, las risas e historias que llevaré siempre en el recuerdo. Y gracias siempre por vuestro apoyo y ayuda durante todos estos años, llegamos a formar una gran familia para superar el doctorado juntos.

Gracias a Nuria y Loles por toda vuestra ayuda en el procesamiento y secuenciación de las muestras, ha sido una gran cantidad de trabajo que

no podría haber llevado adelante sin vosotras. Gracias a Artacho por tus consejos estadísticos. Y gracias a toda la gente que está y ha pasado por FISABIO por haber hecho mis paso tan agradable.

Gracias a toda la gente que ha participado en el proyecto Stance4Health, por su trabajo duro, a pesar de las dificultades de la pandemia.

Gracias a la Prof. Adamantini Kyriacou y Georgia por acogerme con los brazos abiertos en su laboratorio, ahora una parte de mí estará siempre en Grecia.

A Silvia, por descubrirme el inmenso poder que tienen los taninos. Gracias por haber visto siempre potencial en mí y haber confiado en mí más que yo mismo.

Gracias a Aleh, Emilio, Fer y Jorge, mi familia Valenciana no Valenciana, que he tenido el honor y placer de haber podido elegir. Hemos vivido de todo y generado innumerables recuerdos, que llevaré siempre conmigo. Compartir nuestros doctorados nos ha ayudado a todos a seguir adelante. Gracias por todas las tardes de jueguitos y las tonterías que hemos creado que son solo nuestras. Sé que nunca encontraré amigos como vosotros, pero también sé que seguiréis siendo la familia que elegí. Gracias por todo vuestro apoyo, un cachito de esta tesis es vuestra.

Gracias a Laura, esta tesis es tan mía como tuya, ya tenemos dos. Eres la mejor compañera que jamás pude soñar para hacer este viaje, por hacer que cada paso fuera una aventura con la que crecer, aprender y pasárselo bien. Por compartir todos los buenos momentos y por tu apoyo en los más difíciles, por tu confianza y por creer en mí. Por compartir ilusiones, risas y aficiones. Por tu felicidad e ilusión por las cosas que te apasionan. Y, en definitiva, gracias por ser mi gran apoyo y el amor de mi vida, sin duda esto no hubiera sido posible sin ti.

Gracias a mis padres, Emi y Manolo, por simplemente ser los mejores. Por vuestro apoyo incondicional, por creer siempre en mí y darme todo vuestro amor. Por ser mis mayores mentores, siempre aprendo cosas nuevas con vosotros. Gracias por todo vuestro esfuerzo para que haya podido llegar hasta aquí. No podría estar más orgulloso de vosotros

Gracias Ernesto, por estar siempre ahí animando, por tu alegría y por tu apoyo. Gracias por todas las horas felices, por haber compartido tantos buenos momentos.

Gracias a María José e Irene, por haberme acogido siempre con los brazos abiertos como uno más de la familia, por contar conmigo para todo y por hacer siempre una ración extra de croquetas.

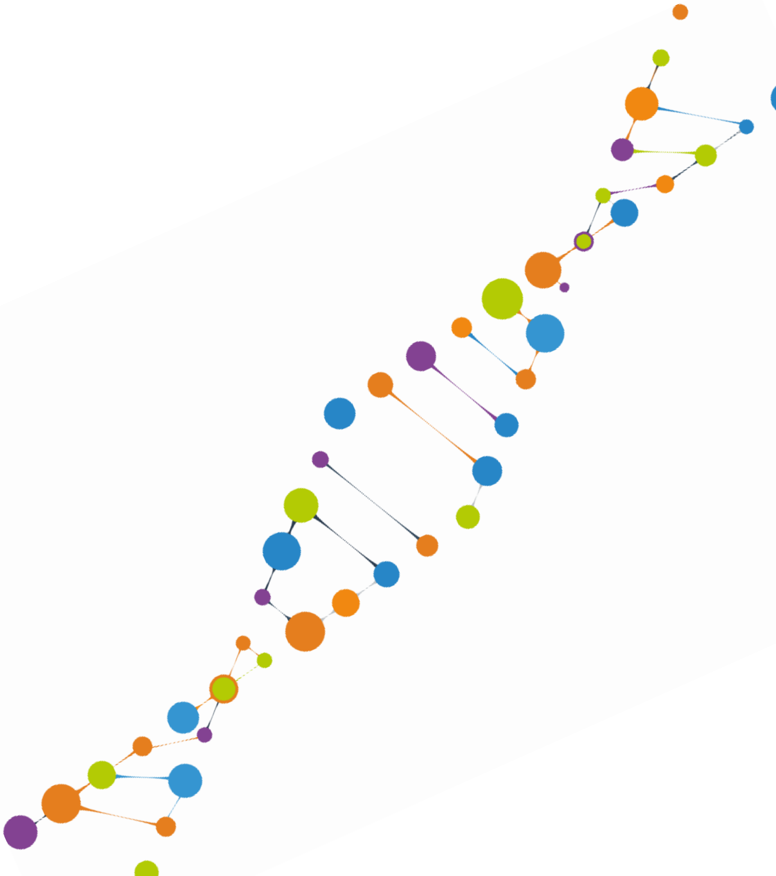
Gracias a mi tita Carmen, la mejor que se puede pedir, la más fuerte y siempre dispuesta a tirar del carro. Gracias por ser como una segunda madre para mí.

Gracias a mi abuelo, el Sr. Ing. Francisco Aguilera, por haberme inculcado desde pequeño el amor por la ciencia, por hacer que tenga curiosidad sobre el porqué de todo que me rodea. Sin ti seguramente no habría seguido este camino.

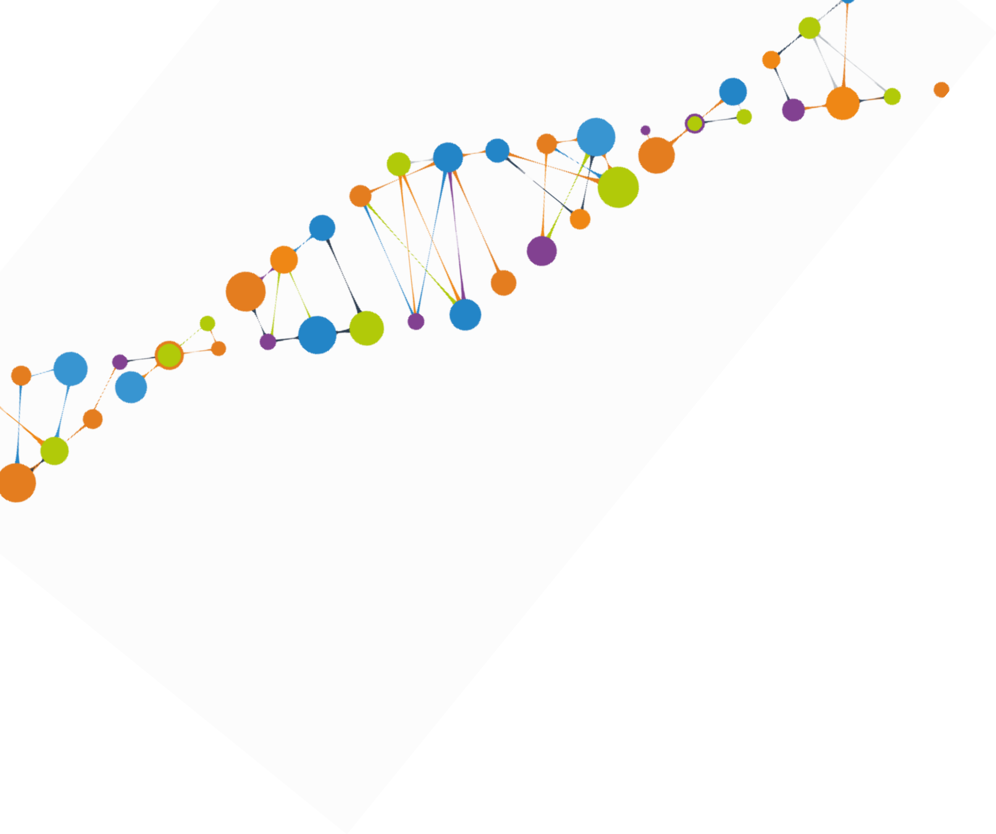
Gracias a toda mi familia por vuestro apoyo a pesar de lo complicado del tema, siempre habéis creído y confiado en mí.

Y gracias a Valencia por haberme acogido todos estos años, tus almuerzos y tu sol son especiales.

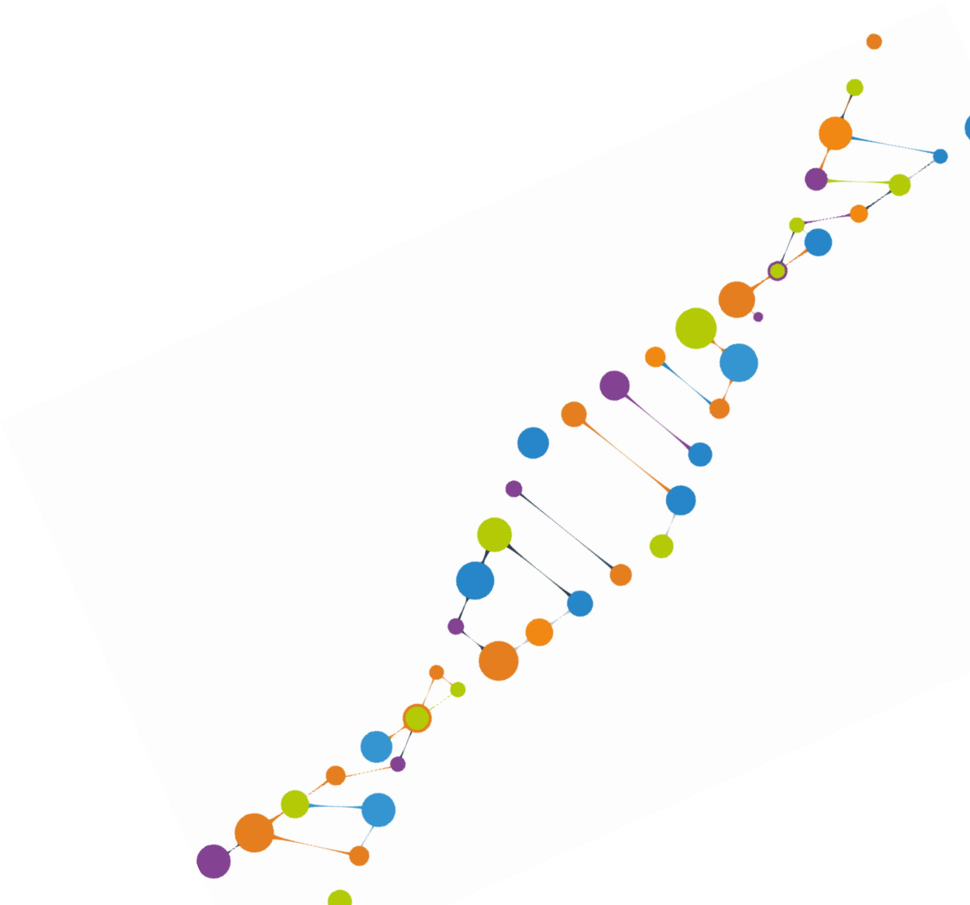
Index



Index



Resumen



Resumen

Introducción

La microbiota intestinal consiste en la comunidad microbiana que habita en el intestino formando un consorcio simbiótico que coopera con el hospedador. Esta es una comunidad dinámica que realiza funciones protectoras, metabólicas y estructurales, jugando un papel clave en el correcto funcionamiento del organismo.

La composición de la microbiota intestinal depende de una combinación de factores tanto intrínsecos (p. ej. genética, sistema inmune y metabolismo) como extrínsecos (p. ej. estilo de vida, medicación y dieta). Debido a la gran cantidad de funciones esenciales que realiza en el cuerpo humano y su papel en numerosas patologías, resulta de gran interés estudiar cómo puede ser modulada la microbiota por factores extrínsecos. La dieta es el factor ambiental que mayor impacto tiene sobre la composición de la microbiota intestinal, por lo que resulta prometedor el uso de una alimentación personalizada para modular con precisión la composición de la microbiota hacia una con mayor presencia de bacterias beneficiosas y menor de aquellas perjudiciales.

También, se han desarrollado probióticos (microorganismos que estimulan efectos beneficiosos para la salud), prebióticos (sustancias no digeribles que promueven la proliferación de bacterias y mejora la salud del hospedador) o simbióticos (una combinación de ambos anteriores) que son capaces de alterar la composición del microbioma. Otras muchas sustancias como antioxidantes, polifenoles o medicación también es capaz de alterar su composición.

Sin embargo, es necesario profundizar en el estudio de los efectos de los alimentos en la microbiota intestinal, especialmente teniendo en cuenta que la composición de la misma puede ser muy diferente en función estado de salud del individuo y la gran variabilidad que existe en la microbiota de los individuos.

Objetivos

La presente tesis tiene como objetivos evaluar el efecto modulador de diferentes alimentos en la composición de la microbiota intestinal de adultos y niños usando digestiones gastrointestinales y fermentaciones colónicas *in vitro* acompañadas con secuenciación de alto rendimiento. Además, se pretende evaluar el impacto de una aplicación móvil de nutrición personalizada en la composición de la microbiota intestinal a través de una intervención dietética. Asimismo, se han planteado como objetivos específicos determinar el efecto de diferentes alimentos y métodos de cocinado en la microbiota intestinal de adultos sanos, investigar el efecto diferencial de varios alimentos y métodos de cocinado en la microbiota de niños sanos, obesos, celíacos y alérgicos, estudiar la respuesta diferencial de la microbiota intestinal entre adultos y niños saludables, y por último evaluar el impacto de una suplementación con taninos en la composición de la microbiota intestinal de individuos sanos a través de una intervención dietética usando una aplicación de nutrición personalizada

Metodología

La presente tesis se ha dividido en 4 capítulos en los que los tres primeros se analizan datos procedentes de fermentaciones colónicas *in vitro* y en el cuarto se analizan datos de microbiota obtenidos tras una intervención nutricional.

En los capítulos del uno al tercero, se emplearon 55 alimentos diferentes, representativos de la dieta occidental y mediterránea que fueron cocinados de hasta 5 maneras diferentes y sometidos a un proceso de digestión y fermentación *in vitro*. Para ello se emplearon como inóculo heces procedentes de tres individuos diferentes por cada grupo. Estas fermentaciones se llevaron a cabo por duplicado y usando agua como control de fermentación. Tras finalizar las fermentaciones, las bacterias presentes fueron aisladas y conservadas en frío hasta el proceso de extracción de ADN.

Para poder simplificar el análisis de la gran cantidad de datos obtenidos se clasifican los alimentos en tres niveles 1) alimentos basados en plantas o en animales, 2) 15 diferentes categorías de alimentos (carne, productos lácteos, huevos, pescado, grasas animales y vegetales, fruta, productos basados en granos, legumbres, frutos secos, tubérculos, azúcar, vegetales, bebidas alcohólicas y bebidas basadas en agua) y 3) los alimentos individuales.

Se reclutaron 107 participantes fueron reclutados para una intervención nutricional en la que debían seguir las recomendaciones dietéticas proporcionadas a través de una aplicación móvil que tenía en consideración para generarlas sus gustos, hábitos y necesidades energéticas. Esta intervención se llevó a cabo durante un periodo de 12 semanas en las que se tomó una muestra de heces basal y otra cada 6 semanas para evaluar el impacto de esta en la microbiota intestinal. Estas muestras fecales fueron procesadas lo más rápidamente posible y conservadas en frío hasta el proceso de extracción de ADN.

Para todas las muestras bacterianas tanto de las fermentaciones como de la intervención se siguió el mismo protocolo de extracción de ADN empleando la plataforma MagNaPure LC JE379 y se comprobó la integridad a través de una electroforesis en gel de agarosa y cuantificó empleando un Qubit 3.0. El ADN genómico fue conservado a -20ºC hasta el proceso de secuenciación.

Para la identificación de las bacterias presentes en las muestras se realizó una secuenciación del amplicón del gen del RNA ribosomal 16S (16S rRNA). Para ello se amplifica la región hipervariable V3-V4 y siguiendo las recomendaciones del fabricante se incluyeron los adaptadores de Illumina Nextera XT. Las muestras fueron secuenciadas empleando la plataforma Illumina Miseq en carreras de final pareado de 2x300 ciclos.

Los datos procedentes de la secuenciación fueron analizados empleando el paquete de R DADA2, generando las *Amplicon Sequence Variants* (ASV). Se llevó a cabo un filtrado por la longitud de las lecturas y recorte de las mismas. Y se fijó un solapamiento mínimo de 15 nucleótidos y un error máximo de 1 para la unión de las lecturas.

La identificación taxonómica de los ASVs se realizó empleando el paquete informático DADA2 de R y la base de datos de referencia SILVA v.138. El programa MegaBLAST fue utilizado para la identificación más allá de la ofrecida por DADA2, requiriendo al menos un 97% de identidad para la asignación a nivel de especie y una diferencia mínima del 2% entre la primera y segunda coincidencia. Los ASVs con un total de conteos menor de 10 fueron eliminados.

Los análisis estadísticos empleados variaron en función de los objetivos de cada capítulo. Para analizar la alfa diversidad se emplearon el

índice de diversidad de Shannon y el estimador Chao1. Se utilizó el método Bray-Curtis para calcular la disimilitud entre las muestras y analizar la beta diversidad mediante métodos como PCoA o CCA, analizados con el método PERMANOVA que se encuentra en el paquete de R *Vegan.* Para los análisis de abundancia diferencial se han empleado 2 versiones diferentes, el método ANCOM y ANCOM-BC2.

Resultados

Capítulo I

La alimentación es capaz de influenciar la estructura de la microbiota intestinal tanto en periodos de tiempo cortos como largos. Los patrones dietéticos son capaces de dar forma a la microbiota intestinal e inducir cambios que pueden contribuir en el desarrollo de diferentes enfermedades. Además, se ha observado que no solo los alimentos, sino también los métodos de cocinado que se aplican en los alimentos tienen un impacto en la microbiota intestinal.

El objetivo de este capítulo es estudiar cómo la composición de las comunidades microbianas que viven en nuestro intestino son modificadas por acción de los alimentos y los métodos de cocinado empleando un sistema de digestión y fermentación *in vitro* de 55 alimentos variados que se encuentran en la dieta occidental y mediterránea y que además han sido cocinados de hasta 5 manera diferentes. Para ello se emplearon como inóculo heces de 3 adultos saludables y se analizó la composición de la microbiota resultante mediante la secuenciación del gen 16S rRNA.

Tras analizar los datos resultantes se encontró que los principales cambios observados eran debidos a la gran variabilidad en la composición de la microbiota de los individuos participantes, mientras que los cambios que podían producir los alimentos en este corto periodo de fermentación estaban enmascarados. Sin embargo, fuimos capaces de observar cambios que eran comunes entre los tres individuos, sugiriendo un efecto robusto de estos alimentos.

Las grasas animales y vegetales fueron la categoría de alimentos que produjeron una mayor cantidad de cambios en la microbiota intestinal, produciendo un aumento de diversas bacterias del filo Firmicutes y un disminución de *Bacteroides* y *Bifidobacterium.* También se encontró un aumento de abundancia de taxones potencialmente beneficiosos como *Faecalibacterium*, *Roseburia*, *Blautia* y *Lachnospira.* Cabe destacar que algunos de los análisis presentados en esta tesis fueron realizados previamente a la publicación de la actualización en la organización de filos por parte de la *International Committee on Systematics of Prokaryotes* (ICSP), por lo que con la finalidad de mantener una coherencia en la nomenclatura a lo largo del texto se ha decidido continuar con la organización previa.

Los productos lácteos y pescado también produjeron numerosas diferencias como el aumento de *Lachnoclostridium*, asociado con la producción de compuestos beneficiosos como los ácidos grasos de cadena corta y encontrado en otros estudios como aumentado en dietas ricas en grasas.

El efecto de los métodos de cocinado en la microbiota se observó que dependía en gran medida del alimento en sí, puesto que, al tener diferente perfil nutricional, era alterado de manera diferente por los diferentes cocinados. La fritura destacó como el método de cocinado que produjo una mayor cantidad de diferencias, debido en parte a que el alimento retiene una mayor cantidad de grasa que en los otros y a que la mayor temperatura que se alcanza produce una mayor cantidad de cambios en su composición, por ejemplo, a través de la reacción de Maillard. Por otro lado, métodos como el cocido condujo a menores abundancias de varios géneros del filo Firmicutes, posiblemente debido a la dilución de nutrientes en el agua que se pierden y a la ausencia de la reacción de Maillard al alcanzarse menores temperaturas, que disminuye la disponibilidad de nutrientes para estas bacterias.

Capítulo II

La microbiota intestinal es conocida por jugar un papel esencial en funciones como la protección del intestino, inmunológicas y metabólicas, participando en el metabolismo de lípidos, vitaminas y otros nutrientes como carbohidratos. Por este motivo, aquellos trastornos que se caracterizan por la presencia de una microbiota alterada, inclusive de una disbiosis, puede provocar un desajuste en estas funciones, pudiendo producir efectos perjudiciales en la salud. Algunos ejemplos de trastornos que están asociados a alteraciones de la microbiota intestinal son la obesidad, la celiaquía y la alergia.

En este capítulo se plantea el estudio de los efectos de alimentos y métodos de cocinado en la microbiota de niños en edad escolar con diferentes patologías como son la obesidad, celiaquía y alergia alimenticia, junto con un grupo control de individuos sanos delgados para poder avanzar en el desarrollo de terapias de nutrición personalizada dirigidas a dichos grupos. Para ello se empleó la misma metodología del capítulo anterior de digestión y fermentación *in vitro* empleando como inóculo heces de niños de estos grupos.

A pesar de no mostrar diferencias significativas entre la diversidad y composición de la microbiota inicial, tras la fermentación se observó una mayor diversidad de las muestras del grupo sano delgado frente al resto, pudiendo indicar esto que la microbiota presente en este grupo es más diversa y capaz de adaptarse a las condiciones presentes en la fermentación

En un primer lugar se compararon los alimentos basados en animales y los basados en plantas. Los géneros *Barnesiella*, *Odoribacter* y *Alistipes*, con efectos beneficiosos para la salud, vieron reducida su abundancia por acción de los alimentos basados en animales en los grupos celiaco y alérgico, incluso en el grupo obeso para el último taxón mencionado. Algunos de los cambios significativos detectados fueron la disminución de la abundancia de *Lachnoclostridium* y *Solobacterium* a causa de alimentos basados en animales en celíacos, la reducción de *Faecalibacterium prausnitzii*, *Roseburia* y *Monoglobus* en alérgicos también a causa de alimentos basados en animales o el aumento de *Bifidobacterium longum* en este grupo por alimentos basados en plantas. También se encontró que el grupo de individuos obesos reaccionaba a alimentos basados en plantas mediante una disminución en la abundancia de *Dorea* y *Dialister*.

Al estudiar el efecto de los alimentos organizados por categorías, encontramos que la respuesta de los diferentes microorganismos a estos alimentos es muy dependiente del grupo de estudio. Por ejemplo, encontramos que algunos de los alimentos basados en animales (como los productos lácteos, huevos, pescado y carne) producen una reducción en la abundancia de géneros descritos como beneficiosos como son *Coprobacter*, *Haemophilus* y *Alistipes* en el grupo de alérgicos. Sin embargo, también se ha encontrado que la microbiota del grupo celiaco se ve afectada por la carne y productos lácteos aumentando la abundancia de bacterias beneficiosas como *Butyricicoccus* y *Ruminococcus* o potencialmente perjudiciales como *Lachnoclostridium.* Además, hemos encontrado resultados llamativos como es la disminución de *Agathobacter* y *Dorea* en el grupo obeso por acción de la fruta y las legumbres, a pesar de ser estos géneros bacterianos conocidos por su capacidad de crecer en presencia de carbohidratos fermentables; sin embargo, efectos como este pudiera ser beneficioso debido a que taxones como *Dorea* han sido asociados en la bibliografía con IBS en personas con obesidad.

Dado que la composición química de los alimentos es diferente, las alteraciones por los métodos de cocinado también lo son, por lo que es necesario estudiar su efecto para cada tipo de alimento. Como es el ejemplo de *Alistipes*, cuya abundancia se vio disminuida por efecto de la fritura en los vegetales en los grupos obeso, celiaco y alérgico; o el caso de *Butyricicoccus*, *Coprococcus*, *Dorea* y *Lachnoclostridium* que aumentan su abundancia por efecto de la fritura de carne en el grupo celiaco. Asimismo, muchas de estas diferencias dependen del grupo, debido a las diferencias iniciales de la composición de su microbiota.

A pesar de las limitaciones que presenta este estudio, como puede ser las evidentes diferencias con un modelo *in vivo*, su simplicidad nos da la oportunidad de desentrañar el comportamiento de la microbiota con los alimentos sin la presencia de factores externos que puede introducir una gran cantidad de variables de las que carecemos de control y puede dificultar en gran medida la interpretación de los resultados. Sin embargo, a pesar de la necesidad de otras investigaciones que validen nuestros hallazgos, esta investigación supone un avance en el conocimiento de cómo

la microbiota es modificada por los alimentos y abre la puerta a investigar sobre su modulación intencionada como posible terapia.

Capítulo III

La microbiota intestinal humana juega un papel clave en el correcto funcionamiento del organismo. Una etapa clave en su desarrollo es la colonización del intestino que tiene lugar durante el periodo prenatal pero que se ve influenciado por factores como la genética, la salud, la dieta de la madre, el modo del parto y si la lactancia es materna o de fórmula.

Los estudios indican que la microbiota intestinal de los niños hasta los 2-3 años presenta una baja diversidad que junto a la composición evolucionan gradualmente hacia la de un adulto, siendo crucial un establecimiento adecuado, ya que una parte significativa se mantiene estable a lo largo de la vida. Numerosas investigaciones han explorado la asociación entre el cambio en la composición y diversidad del microbioma en niños y diversas enfermedades gastrointestinales y no gastrointestinales. Algunos de ellos han encontrado que casos de disbiosis o tratamientos con antibióticos en niños producen un mayor riesgo de desarrollar enfermedades como asma, enfermedad de Crohn o celiaquía en etapas posteriores.

Estudios con cohortes amplias han profundizado en las diferencias en el microbioma entre niños y adultos, así como el impacto de las dietas a corto y largo plazo en el microbioma infantil, determinando que se pueden prevenir efectos negativos en niños inducidos por los antibióticos o que alimentos específicos o cambios de dieta pueden tener efectos más profundos en niños que en adultos, sugiriendo una mayor maleabilidad del microbioma infantil frente a cambios ambientales o dietéticos.

El presente capítulo se compara la influencia de los alimentos y los métodos de cocinado en la microbiota intestinal de niños y adultos sanos. Se busca proporcionar una referencia básica para el desarrollo de estrategias de modulación dietética dirigidas al microbioma en poblaciones adultas e infantiles.

Se observó que la alfa diversidad en el grupo de niños era significativamente inferior que la del grupo de adultos, existiendo una diferencia significativa en la composicionalidad de ambos grupos. Esto demuestra que la microbiota intestinal de los niños se encuentra aún en fase de desarrollo.

La comparación entre los efectos producidos por los alimentos basados en plantas y animales resultó en una gran cantidad de bacterias que aumentaron su abundancia de manera significativa a causa de los alimentos basados en animales. Estas diferencias se encontraron en el grupo de adultos o el de niños, pero ninguna fue encontrada en ambos grupos. Dentro de estos, en el grupo de adultos aumentaron *Colidextribacter*, *Anaerostipes*, *Lachnoclostridium* y *Romboutsia*, mientras que en el grupo de niños aumentaron *Blautia, Collinsella, Dorea, Erysipelotrichaceae UCG 003, Solobacterium* y *Subdoligranulum.*

Comparando el efecto de los grupos de alimentos en la microbiota fermentativa de los grupos adulto y niño, se observó que pocos microrganismos se vieron afectados en ambos grupos al mismo tiempo, entre ellos se observó un aumento de *Lachnospira* (*L. pectinoschiza*) y *Anaerostipes* (*A. hadrus*) por acción de la grasa animal y vegetal, así como *Adlecreutzia* (*A. equolifaciens*), aumentado por la anterior y por el pescado. Sin embargo, en el grupo de adultos aumentó por acción de los alimentos ricos en grasas y proteínas la abundancia de *Anaerostipes* y *Lachnoclostridium*; mientras que estos alimentos en el grupo de niños produjeron un aumento de *Roseburia*, Erysipelotrichaceae UCG 003, *Blautia*, Oscillospiraceae UCG002 y *Subdoligranulum* entre otros.

Al examinar los alimentos en particular, se encontraron resultados similares a los encontrados anteriormente, siendo la mantequilla y el queso los que más efectos producen en adultos y mantequilla, queso, aceite de oliva, aceite de girasol y salmón en niños. Alimentos como la mantequilla produjeron un aumento de *Lachnospira*, *Blautia*, *Anaerostipes*, y *Ruminococcus gauvreauii* group en ambos grupos, mientras que el aceite de oliva aumentó la abundancia de *Adlecreutzia*, *Ruminococcus gauvreauii* group, *Ruminococcus torques* group, *Blautia*, *Roseburia* y *Subdoligranulum* en el grupo de niños. Otros alimentos como la carne de cordero tuvieron efecto en ambos grupos sobre una bacteria no muy frecuente en el resto de los alimentos como son *Latilactobacillus* y *Hafnia-Obesobacterium*, o el caso de *Carnobacterium* en el salmón.

Como ya se ha descrito anteriormente, los métodos de cocinado tienen la capacidad de modificar los componentes nutricionales de los alimentos y por tanto de modificar cómo estos alteran a la microbiota intestinal. En este estudio se ha comparado el efecto de los métodos de cocinado en la composición de la microbiota de los niños y adultos. Bacterias como *Roseburia* destacaron por estar aumentadas en varios alimentos por acción de la fritura con aceite de oliva, lo que concuerda con lo visto anteriormente sobre el aumento de su abundancia por acción de alimentos ricos en grasas y en concreto las grasas animales y vegetales en el grupo de niños. Otro ejemplo es el de *Carnobacterium*, que aumenta en el pescado al cocinarse a la plancha en ambos grupos.

Los resultados hallados muestran que los niños en edad escolar poseen una microbiota intestinal aún en desarrollo sensible a numerosos factores, siendo los alimentos uno de los principales. Mantener un correcto desarrollo de la microbiota en esta edad es clave para reducir el riesgo de padecer ciertas enfermedades futuras y la nutrición personalizada se presenta como una herramienta muy prometedora para utilizar en esta ventana de tiempo en que la microbiota intestinal es más maleable.

Capítulo IV

Las dietas poco saludables son uno de los principales factores que afectan a la prevalencia de enfermedades no transmisibles. Por ello, tradicionalmente se han emitido recomendaciones dietéticas a la población de manera general, sin tener en cuenta la gran variabilidad que existe entre individuos a nivel de genoma, proteoma, metaboloma y microbioma. Por este motivo aparece la nutrición personalizada con el objetivo de realizar a cada individuo las recomendaciones dietéticas que más beneficio le puede proporcionar a su salud de manera precisa y adaptándose a una gran cantidad de factores como sus preferencias, objetivos, hábitos y necesidades energéticas. Además, dado el carácter dinámico de la microbiota intestinal y la gran cantidad de procesos en los que participa, así como las patologías en las que se ha demostrado su participación, intervenciones dietéticas dirigidas al microbioma tienen un potencial considerable para que la nutrición personalizada pueda aprovecharla. Para ello es esencial una exhaustiva caracterización de cómo la microbiota intestinal responde a la dieta, sugiriendo muchos autores que tecnologías como el *machine-learning* o la inteligencia artificial pueden jugar un papel clave para identificar dichas interacciones. Asimismo, la aparición de modelos metabólicos a

escala genómica surge como una aproximación muy prometedora para poder simular el comportamiento de la microbiota frente a los alimentos.

Los taninos son polifenoles descritos por sus propiedades antioxidantes y antinflamatorias que han sido relacionadas con la estimulación de la producción de ácidos grasos de cadena corta por parte de la microbiota intestinal. En nuestro grupo hemos publicado diversos estudios sobre su efecto en la microbiota intestinal mediante fermentaciones *in vitro* y una intervención dietética.

En este capítulo se analiza la microbiota intestinal de un de los participantes en un ensayo clínico piloto aleatorizado y ciego con 107 participantes a los que se les ha proporcionado a través de una aplicación móvil recomendaciones dietéticas personalizadas. Esta intervención se llevó a cabo durante un periodo de 12 semanas en las que se tomó una muestra de heces basal y otra cada 6 semanas para evaluar el impacto de esta en la microbiota intestinal. Estas muestras fecales fueron analizadas mediante la secuenciación del gen 16S rRNA para determinar la composición de la microbiota intestinal de los participantes.

Gracias al uso de la aplicación móvil se pudo monitorizar el uso de la misma, permitiendo clasificar a los participantes en un grupo de bajo uso de la app y otro de alto uso. Para ello se emplearon dos métodos de clasificación, siendo el primero en función de su actividad en la aplicación como la consulta de dietas, actualización de peso, etc. El segundo método consiste en la medición del compromiso en el uso de la app, medido mediante las interacciones con la app para modificar las dietas sugeridas, registro de ingestas no programadas, etc. Ambos métodos de clasificación se emplearon debido a sus diferencias en parámetros medidos y de individuos registrados en cada uno.

En los grupos de alto uso de la app, en los que se espera un mayor seguimiento de las recomendaciones dietéticas, no se observaron apenas diferencias en la diversidad bacteriana, pero se observaron numerosas diferencias de abundancia a lo largo del periodo del estudio. Entre ellas destacan el aumento de *Ruminococcus* y *Coprococcus comes*, bacterias asociadas con efectos positivos en la salud mediante su capacidad de producción de ácidos grasos de cadena corta. Entre las bacterias diferencialmente abundantes solo según uno de los métodos de clasificación de uso de la app podemos encontrar el aumento de Lachnospiraceae ND3007 o *Agathobacter* entre las bacterias descritas como beneficiosas para la salud, y el aumento de *Lachnoclostridium* o *Actinomyces* y la disminución de *Gemella* y *Desulfovibrio* entre las bacterias descritas en la literatura como potencialmente perjudiciales.

Además del uso de la app, en esta intervención se quiso evaluar el efecto que los taninos tienen en la microbiota intestinal de estos participantes, por lo que a un grupo de ellos se le suministró unas pastillas con taninos para suplementar su dieta. A pesar de ver un ligero aumento de la diversidad ya descrito en la bibliografía, este no fue suficiente para ser significativo. La suplementación con taninos mostró un aumento de la abundancia de *Succinatimonas* y *Butyricimonas* frente al resto de grupos, al mismo tiempo que se encontró que el grupo que tomó taninos mostró un aumento de *Collinsella* y disminución de *Bilophila wadsworthia.* Entre las bacterias diferencialmente abundantes solo según uno de los métodos de clasificación de uso de la app podemos encontrar el aumento de *Roseburia hominis, Flavonifactor, Ruminococcus torques* group,

Bifidobacterium, Ruminococcus, Eubacterium hallii, Solobacterium mooeri y *Dorea.*

En vista de los resultados queda patente la necesidad de profundizar en la creación de nuevos métodos para evaluar los cambios de la microbiota por su interacción con los diversos componentes de la dieta y con el hospedador. Resulta esencial la aparición de nuevos proyectos observacionales que utilicen métodos estandarizados para evaluar de manera exhaustiva a la población y obtener una gran cantidad de información sobre todos los factores que intervienen en la microbiota intestinal y poder integrarlos para utilizarlos en una nutrición personalizada que considere la microbiota intestinal de cada individuo.

Conclusiones

- La microbiota intestinal fermentativa de adultos y niños, a pesar de la variabilidad interindividual, muestra cambios comunes debidos al efecto de diversos alimentos.
- Los alimentos de origen animal y las grasas animales y vegetales influyen significativamente en la microbiota fermentativa de adultos y niños.
- Muchos cambios de la microbiota inducidos por los alimentos son específicos de los individuos o limitados a adultos o niños, especialmente aquellos relacionados con los métodos de cocinado.
- Los métodos de cocción son capaces de influir en el efecto que los alimentos tienen sobre la microbiota fermentativa, y varían según el alimento, siendo la fritura el que tiene un mayor impacto.

- La microbiota fermentativa de los niños muestra mayores diferencias mediadas por los alimentos que la de los adultos, lo que indica una mayor maleabilidad a través de la dieta.
- Los niños con patologías asociadas, o producidas, por alteraciones dietéticas muestran diferencias en la composición de su microbiota intestinal, la cual puede directamente influir su respuesta a los alimentos.
- Los efectos específicos de los alimentos y los métodos de cocción en la microbiota intestinal deberían tenerse en cuenta para elaborar dietas personalizadas que modulen la microbiota intestinal de forma dirigida.
- En una intervención de alimentación personalizada en la que se emplee una aplicación, el compromiso de los participantes a un alto uso de la app es un factor crucial para el éxito de la intervención y debería ser considerado para identificar los efectos reales de la intervención
- Las métricas empleadas para estudiar el uso de la app pueden alterar sustancialmente la evaluación de los resultados de la intervención, pareciendo las métricas basadas en la implicación, centradas en el número de modificaciones del menú realizadas por los usuarios, como la más adecuada para establecer el uso de la app.
- El alto uso de la aplicación móvil i-Diet probablemente refleja una mayor adherencia a las recomendaciones dietéticas personalizadas, resultando en la modulación de la microbiota intestinal con un aumento de bacterias beneficiosas como *Ruminococcus* y *Coprococcus comes*
- La suplementación con taninos modifica la microbiota intestinal, aumentando la abundancia de bacterias beneficiosas productoras de ácidos grasos de cadena corta y disminuyendo las patobiontes.

Abstract

In recent decades, the intestinal microbiota has gained considerable attention for its important contribution in numerous physiological processes and its involvement in pathologies including celiac disease, food allergies, and obesity. It has been described that both the diversity and composition of the intestinal microbiota play significant role in these conditions, and their modulation may attenuate some of their adverse effects. Diet stands out as a major environmental factor capable of modifying the composition of the microbiota, suggesting personalized dietary interventions as a strategy for managing certain pathologies.

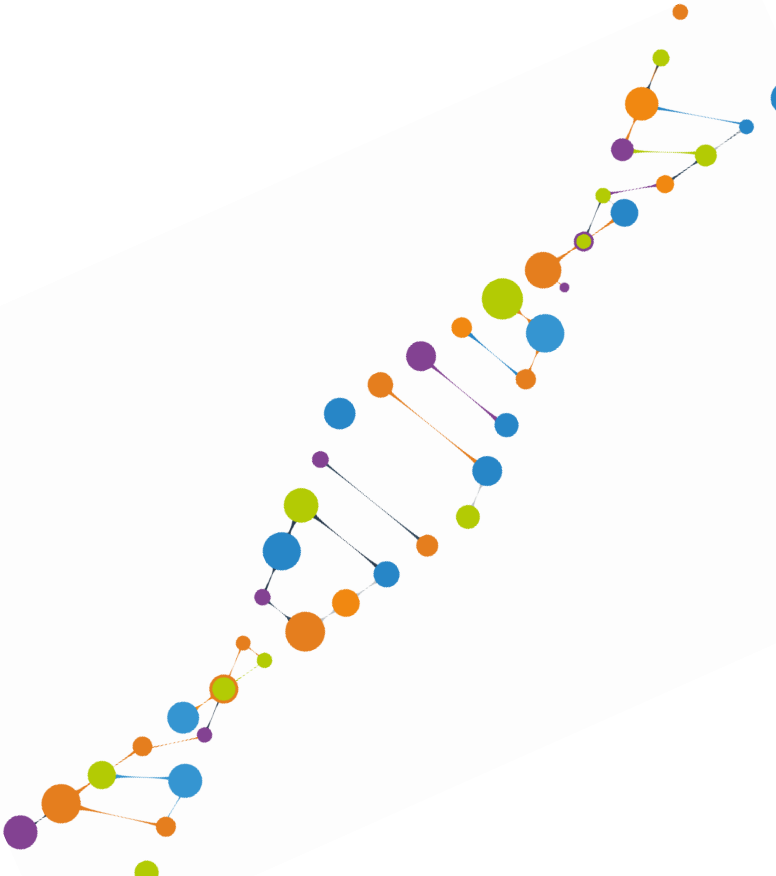
To study the impact of food on the gut microbiota, this thesis aimed to investigate the modulatory effects of different foods prepared using different cooking methods on the gut microbiota composition of adults and children. In order to achieve this aim, we used *in vitro* gastrointestinal digestion and colonic fermentation techniques, coupled with high throughput sequencing.

Our study involved the *in vitro* fermentation of 55 foods prepared using up to 5 cooking methods and revealed distinct effects on the gut microbiota of children and adults. Many of these effects proved to be individual-specific, with animal and vegetable fats having a significant influence. Cooking methods showed different effects on the gut microbiota depending on the food, with frying having the most pronounced effect. Noteworthy, the gut microbiota of children showed greater malleability in response to dietary changes compared to adults. In addition, individuals with obesity, celiac disease, food allergies and those considered healthy

showed different responses to the foods tested due to their different baseline gut microbiota composition.

In addition, we aimed to elucidate the influence of a dietary intervention on the gut microbiota, facilitated by a personalized nutrition application, over a 12-week period. The metrics of app usage facilitated the categorization of individuals based on their level of engagement, which emerged as a key factor in the interpretation of the results. Importantly, the level of app engagement significantly influenced the observed outcomes, highlighting the importance of participant adherence monitorization in dietary interventions.

Introduction



Introduction

1 Gut microbiota and health

When the Human Genome Project was completed in 2001, it was argued that this milestone would remain incomplete until there was a better understanding of the relationship between humans and the trillions of microorganisms that inhabit them. These microorganisms form a symbiotic consortium that cooperates with its human host and are collectively known as the microbiota. Extensive efforts have been applied to understand the complex relationship between humans and their associated microorganisms by numerous initiatives such as the Human Microbiome Project, Metagenomics of the Human Intestinal Tract (MetaHIT) or My New Gut[1–4].

The microbiota inhabiting the gastrointestinal tract, commonly known as the gut microbiota, represents a dynamic microbial community specifically adapted to colonize the intestinal environment. This community is composed by archaea, eukarya, viruses, and predominantly bacteria, represented mostly by the phyla Bacillota, Bacteroidota, Actinomycetota, Fusobacteriota, Pseudomonadota, Verrucomicrobiota, and Cyanobacteriota [5]. It is important to note that some of the analyses presented in this thesis were performed prior to the publication of the updated phyla rankings by the International Committee on Systematics of Prokaryotes (ICSP), thus, in order to maintain consistency of nomenclature throughout the text, the previous rankings have been maintained (Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteriota, Proteobacteria, Verrucomicrobia, and Cyanobacteria)[6].

These microorganisms benefit from the nutrient rich gut environment and perform protective, metabolic, and structural functions. Remarkably, the intestinal microbiota has the enzymatic capacity to degrade carbohydrates resistant to host enzyme digestion, as well as proteins, lipids, and other molecules such as bile salts and polyphenols, scavenging between 10 and 30% of the ingested energy [7]. The intestinal microbiota plays an important role as a protective agent through its interaction with the mucus layer, which is used as a source of carbohydrates and peptides for commensal bacteria and acts as a barrier between them and the intestinal cells. In addition, the close relationship of the microbiota with the immune system is crucial, influencing the immune maturation process. The microbiota is subject to the innate and adaptive immune responses, which can be exacerbated in the event of a microbiota imbalance, commonly referred to as dysbiosis. The microorganisms within the gut microbiota also have the ability to produce metabolites that modulate immune response and inflammation, such as short-chain fatty acids (SCFAs)[7,8]. Butyric acid is the primary energy source for colonic epithelial cells and plays important roles in the regulation of various physiological and immune processes. In addition, gut bacteria are known for their ability to produce several neurotransmitter-like metabolites, including γ-aminobutyric acid, acetylcholine, and serotonin. These substances can directly affect brain function, demonstrating their critical role in the gut-brain axis [9].

1.1 Gut microbiota from birth to adulthood

The microbial colonization of the infant gut involves the *de novo* formation of a complex microbial community that is influenced by a variety of environmental and host-related factors. In recent years, there has been increasing evidence that this colonization process begins before birth through the presence of bacteria in the placenta, amniotic fluid, and other *in utero* sites. Several factors, including delivery method, feeding practices, use of antibiotics, diet, and environment, exert a significant influence on the colonization process[10].

Infants delivered vaginally are exposed to maternal vaginal and fecal microbiota, which significantly influence the colonization process. In contrast, infants born by C-section lack exposure to this maternal microbiota and are more likely to be colonized by environmental microorganisms from maternal skin and the hospital environment. Studies indicate that C-section delivered infants have a less complex microbiota, with lower levels of *Bifidobacterium* and *Bacteroides.* While these differences gradually diminish over the first 12 months, some persist into childhood, as observed in children up to 7 years of age[10–14] Feeding is another key factor in the colonization process, as differences in microbial composition have been identified between formula-fed and breast-fed infants [15]. Formula feeding has been associated with lower levels of beneficial bacteria and higher levels of pathogens, as well as with greater microbiota diversity, as documented by Milani C. *et al.*[10]. Additionally, environmental factors such as maternal diet, the presence of siblings, geographic location, and host genetics play a critical role in influencing the gut microbiota of infants during colonization[10].

After delivery, the intestinal tract is rapidly colonized by facultative and aerotolerant oxygen-consuming bacteria, allowing the subsequent establishment of a complex microbial community composed predominantly of anaerobic bacteria [12]. Nevertheless, the neonatal microbiota shows substantial inter-individual variation greater than that observed in adults, with these differences gradually decreasing over time[14]. The age at which the microbiota reaches an adult-like structure remains unclear, with divergent findings suggesting around 3 years of age; however, some research suggests that although both are similar in terms of their global composition, certain microbial groups require longer periods of time to fully develop, such as *Bifidobacterium* which has been reported to gradually decrease until adulthood [16–19].

1.2 Role of the gut microbiota in pathology

As previously described, the gut microbiota plays a crucial role in several physiological functions and exerts a key influence that could affect the proper functioning of the organism if not in balance. There exist numerous factors that could alter the balance in the gut microbiota and cause dysbiosis, such as dietary habits, use of medications (such as non-steroidal anti-inflammatory drugs and antibiotics), the state of the immune system, oxidative stress resulting from inflammatory processes, or the presence of bacteriophages and bacteriocins [20]

Dysbiosis in the intestinal microbiota has been associated with a growing list of diseases. Notably, inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease have been widely associated with dysbiosis. Additionally, dysbiosis has been reported to be implicated in neurological disorders and autoimmune diseases[20–24]. In animal models the transplantation of intestinal microbiota from an affected animal to a healthy one has been shown to replicate several of these diseases, inducing the disease or increasing the risk of developing it, thus confirming the contribution of dysbiosis. This phenomenon is exemplified in well-documented cases such as obesity and atherosclerosis in mice [25,26].

Besides genetics and environmental factors, the gut microbiome has risen as a fundamental contributor to metabolic diseases, including obesity, diabetes, and non-alcoholic fatty liver disease. Obesity is a condition that is strongly associated with metabolic dysregulation and chronic inflammation. Many research studies have revealed differences in the composition and diversity of the gut microbiota of obese people in comparison with healthy individuals [27,28]. In addition, it has been suggested that dysbiosis may contribute to obesity through distinct potential pathways, such as diminishing the ability of the microbiota to produce short-chain fatty acids, resulting in negative effects on the organism.[26,29].

Further diseases closely associated with a dysbiosis of the gut microbiota include IBD, with substantial evidence suggesting that the onset of intestinal inflammation is contributed by the gut microbiota [30,31]. Furthermore, some studies indicate that the production of toxic metabolites by the gut microbiota could be directly involved in cancer development, or indirectly through inflammatory or immunosuppressive processes [29].

The intestinal microbiota plays an essential role in the development and maturation of the immune system. This has led to extensive research into its influence on immune system diseases. For instance, in celiac disease, an autoimmune disorder triggered by the ingestion of gluten, studies have identified intestinal dysbiosis and elevated levels of pro-inflammatory bacteria. Although additional research is required to clarify the role of microbiota in the loss of gluten tolerance, some studies have identified factors, such as breastfeeding, with a protective action against celiac disease [32,33]. Similarly, food allergies, a pathological immune response triggered by ingesting a food protein antigen, have been widely associated with alterations in the intestinal microbiota, particularly during early childhood, which is a critical period for the development of both the immune system and the intestinal microbiota [34,35]. For instance, various studies have revealed that children diagnosed with milk allergy exhibit an altered gut microbiome, such as lower concentration of *Bifidobacterium* [34,36].

Of particular note is the increasing evidence regarding the importance of the microbiota in children, emphasizing that an imbalance or inadequate stimulation during these critical years may have long-lasting effects, including inflammatory responses associated with IBD or an increased risk of allergies, celiac disease, or metabolic disorders [10,37–39].

2 Modulation of the microbiota

2.1 Factors modifying the gut microbiota

The gut microbiota is shaped by a combination of intrinsic (e.g., host genetics, immunity, and metabolic regulation) and extrinsic (e.g., lifestyle, medication and diet) factors. However, it is known that environmental factors have a predominant effect over host genetics. In particular, it has become apparent that diet plays a predominant role in shaping the gut microbiota. Another important extrinsic factor that alters the gut microbiota is the use of different medicines. The most representative example of this is antibiotic use, which is known to cause short-term disturbances in the human gut microbiome, but it has also been described to have a long-term impact that remains for up to 2 years [40]. In addition, the circadian rhythm, which is related to fluctuations in nutrient availability, among other changes, causes changes in the gut microbiota that occur very rapidly along the day [41].

With the increasing evidence of its potential impact on human health, there has been increased interest in understanding and modulating the composition and functionality of the gut microbiota.

Probiotics are non-pathogenic microorganisms that do not cause diseases and stimulate beneficial effects in the host. These effects are very diverse and dependent on the microorganisms. During the probiotic treatment the gut microbiota is altered, reflecting survival during the transit through the digestive tract. Traditionally, lactic-acid-producing bacteria such as lactobacilli and bifidobacteria isolated from dairy products have been used as probiotics. With the development of affordable complete genome sequencing, next-generation probiotics have been developed such as *Roseburia intestinalis, Faecalibacterium prausnitzii* and *Akkermansia muciniphila,* which offer physiological functions such as the production of butyrate, propionate and other bioactive substances. For instance, *A. muciniphila* is one of the more promising candidates and initial studies have shown to improve several metabolic parameters[42]. In addition, some fermented foods are the most common natural source of potentially probiotic strains of lactic acid bacteria, associated with important health benefits including reduced risk of type 2 diabetes and cardiovascular diseases[43]. Also fermented milk products can contain many probiotic bacteria, and they have been associated with higher microbiota evenness and increased levels of butyrate-producer bacteria[44]. Effects associated with probiotic consumption include the improvement of digestion, the stimulation of the immune system, the alleviation of psychopathologies, a decrease in antibiotic resistance, and anti-obesity effects among others[44,45].

Prebiotics are indigestible substances that modulate the gut microbiota by promoting the proliferation of beneficial bacteria or promoting the generation of beneficial metabolites in the colon for improving host health. Two of the best known and studied prebiotics are oligofructose and inulin. They have been shown to reduce food consumption and facilitate weight loss[46]. Prebiotics are broadly found across plants and are generally not hydrolyzed in the intestine, inducing metabolic activities in the colon by stimulating bacterial growth. However, there exist other prebiotic substances that are produced by animals (such as oligosaccharides present in milk) and yeasts. Although many prebiotics are complex saccharides, others are non-carbohydrate substances including polyphenols, fatty acids, and other micronutrients. Polyphenols as bioactive antioxidants can help in the treatment of dysbiosis, modulating oxidative stress to improve the intestinal permeability, thereby ameliorating the chronic activation of the inflammatory response [47]. Symbiotics consist of a mixture of prebiotics and probiotics with the aim of improving health[43,48].

In addition, the composition of the gut microbiota is influenced by habits and lifestyle, including physical activity. Exercise has been reported to be beneficial for the gut microbiota by supporting butyrate-producing bacteria. Nevertheless, excessive and intense exercise can also lead to gastrointestinal disorders[47,49,50].

There are more invasive and drastic methods for altering the gut microbiota composition, such as fecal microbiota transplantation. This method consists of transferring feces from a healthy donor into the gastrointestinal tract of the receiver with the aim of restoring the healthy balance of the gut microbiota. It is meant to be used to treat dysbiosis and has been described as effective in the treatment of recurrent *Clostridium difficile* infections[51]. However, additional research is necessary to evaluate its efficacy and safety in various patient populations[45].

2.2 Influence of diet on the gut microbiota

Diet is the environmental factor that produces the greatest effect on gut microbiota composition and is also the most studied one. Gut microbiota composition is highly variable between individuals; however, diet can explain over 20% of the microbiota's structural variation, highlighting the potential for gut microbiota modulation through diet for disease management [52].

Foods consumed in the diet are essential to provide the necessary nutrients to the organism, but they also serve as a substrate for the microbial community of the gut microbiota. The main macronutrients used as energy sources in the human diet are carbohydrates, proteins, and fats. However, their digestibility and accessibility to the microbiota can vary considerably. The non-digestible carbohydrates that constitute dietary fiber reach the colon partially degraded or undigested where they will be metabolized by the microbiota, yielding SCFAs. Individuals with high-fiber diets have higher levels of fermentable substrates available, while a low-fiber diet deprives the gut microbiota of external energy sources and forces it to use endogenous fermentable substrates such as mucin. Dietary fiber has been widely described as an essential nutrient for maintaining the diversity of the microbiota [53]. Reduced gut microbiota diversity has been correlated with several of the chronic inflammatory conditions mentioned above, including obesity, IBD, and ulcerative colitis [54–56].

Besides macronutrients, micronutrients also play a vital role in the proper functioning of the host organism. This category includes substances such as vitamins and minerals that are commonly found in the diet, and whose deficiencies can have severe consequences. Some of the micronutrients that reach the microbiota can be used by these microorganisms to grow and therefore have some effect on the composition and functionality of the intestinal microbiota. For instance, supplementation with vitamins B, C, D and E changes the composition of the microbiota by promoting the growth of *Bifidobacterium*, *Lactobacillus* and *Roseburia*. Similarly, minerals such as calcium, iron, zinc, magnesium and phosphorus may have the potential to influence the microbiota [45,57,58]. Likewise, the gut microbiota is involved in the synthesis of several vitamins, such as biotin, folate, vitamin K, and some B vitamins, which are necessary for many physiological processes [59]. The gut microbiota also influences the absorption of essential minerals such as calcium, iron, and magnesium by increasing their bioavailability [60].

Also, there are thousands of polyphenols from vegetables and fruits that reach the colon intact, and each type may interact differently with the gut microbiota. Polyphenols may act as potential prebiotics, but some of them have been described to have antioxidant capacity and antimicrobial activity. Therefore, polyphenols are also thought to play an important role in modulating the gut microbiota, as they can inhibit the growth of specific taxa and enhance the growth of others when they are metabolized. For instance, cranberry-rich extracts have been shown to stimulate *A. municipphila* and to provide antimicrobial action against pathogens[43,61]. The evidence about the associations of polyphenols with reduced risk of chronic diseases has substantially increased[62]. This is the case of tannins, which are one type of polyphenols synthesized by plants, associated with beneficial effects in health through their antioxidant activity and the stimulation of SCFA production [61,63,64].

Cross-sectional studies of populations have shown that long-term dietary patterns are strongly associated with gut microbiota composition [65]. This suggests that these patterns contribute to shaping each individual's stable microbiota profile. It is necessary to investigate how microbial composition changes in response to long-term diets in order to improve dietary strategies for sustained microbiota shifts. The long-term diet-microbiome relationships have been widely studied through the use of food frequency questionnaires in epidemiological studies. This method allows evaluating the effect of diet on the gut microbiota through different approaches such as the use of dietary indices, or the analysis of gut microbiota associations with nutrients, foods and food groups. A high dietary diversity has been associated with microbial stability, particularly the number of different types of plant-based foods consumed has been associated with higher microbial alpha-diversity and higher presence of the SCFA-producing bacteria *Faecalibacterium prausnitzii* and *Oscillospira.* The use of dietary diversity indices may help the study of diet diversity-microbial relationships[41,66,67].

The nutrient profiles derived from the questionnaires are also used to study the effects of diet on the gut microbiota. With this approach, fiber is the most commonly accepted nutrient that produces a beneficial effect on microbiota composition. The long-term elimination or drastic reduction of fiber from the diet results in microbial losses that are difficult to reverse

[68–70]. Claesson M. *et al.* studied individuals following a low fat/high fiber diet and described that it was associated with improved health parameters and with the most diverse microbial profiles in comparison with individuals following moderate fat/low fiber or high fat/low fiber diets [71]. In other studies, fiber was found to increase the fecal abundance of *Bifidobacterium* and *Lactobacillus* species but not the overall alpha diversity of the microbiota[72]. In 4-8 year-old children, a dietary pattern enriched in fiber-rich foods including grains, dairy, legumes, nuts and seeds was associated with higher abundances of *Cyanobacteria* and *Phascolarctobacterium* and lower abundances of *Dorea* and *Eubacterium* [41,73]. Fiber source also has an influence, as fruit and grain fibers have been shown to have significantly different impacts on the gut microbiota [66,72,74].

In terms of overall dietary patterns, the Western diet, characterized by a reduced dietary diversity and a low dietary fiber intake, has been strongly associated with alterations in gut microbiota composition and with related pathologies such as obesity and metabolic diseases, although the biological mechanisms are still unclear [75]. In children between 2 and 9 years of age, Western dietary patterns defined by high consumption of refined carbohydrates, ultra-processed foods, and high-fat animal products, were associated with alterations in microbial composition. These included a negative correlation of *Bacteroides* with the amount of animal protein consumed, while the genus displayed a positive correlation with the amount of whole grains [76]. In contrast, the Mediterranean diet pattern has been shown to have beneficial effects on the gut microbiota and overall health. For instance, consumption of a Mediterranean diet partially restored gut microbiota dysbiosis to a healthier state after 2 years in patients with obesity at different levels of metabolic dysfunction [77]. On the other hand, diets that exclude essential nutrients, as occurs in diets low in Fermentable Oligo-Di-Monosaccharides and Polyols (FODMAP) or gluten-free diets, may decrease the diversity of the gut microbiota. For example, it has been reported that gluten-free diets decrease the abundance of healthy bacteria, whereas unhealthy bacteria increase due to the reduction in polysaccharide intake [68–70].

Short-term dietary interventions involving severe dietary restriction have been shown to be able to rapidly (24h period) alter the gut microbiota in humans. This was reported by David et al. comparing the effect of two dietary regimens in a cross-over design, one almost exclusively plant-based and the other almost exclusively animal-based for 5 days. However, these shifts were temporal and did not persist beyond a few days[54]. The gut microbiota can also rapidly respond to altered macronutrient levels and novel food components, as Wu *et al.* showed by investigating the effect of high fat/low fiber and low fat/high fiber interventions, where the participants displayed substantial but temporal shifts[65]. However, the impact of short-term dietary interventions on the gut microbiota is likely to be relatively modest compared to the large inter-individual variability existing across microbial profiles.

The microbiota's resilience to perturbations depends on the responsiveness of the core taxa. This may protect against the loss of critical taxa but may also inhibit the improvement of an established dysbiotic microbiota. Long-term changes in the core gut microbiota are required to affect health outcomes, likely needing sustained dietary changes. It is unclear how long dietary interventions would need to last to permanently shift the ecological homeostasis of the microbiota community, creating a new state that would then be maintained [41]. However, it has been observed that the function and composition of the gut microbiota may be affected by variation at the scale of yearly seasons, as occurs in hunter-gatherers [78].

Moreover, there is considerable evidence that individuals are highly heterogeneous with respect to their ability to respond to dietary interventions. Thus, one of the greatest challenges to understanding the relationship between diet, the microbiota and health is deciphering the high variability in individual responses to dietary intake, leading to conflicting results in studies aimed at finding diet-specific effects[41]. This may be due to the high interindividual variability in the basal composition of the gut microbiota and in dietary habits, along with the aforementioned high resilience to change [79]. For instance, Healey G. et al. showed that low basal concentrations of bifidobacteria result in a greater increase after inulin intervention [80]. Therefore, the responsiveness of the gut microbiota to an increase in fermentable substrate may be influenced by an individual's previous dietary intake and resulting basal microbiota composition [65].

2.3 Methodological approaches to study the gut microbiota

The techniques available to study the gut microbiota have evolved along history, particularly in recent years with the arrival of next-generation sequencing techniques (NGS). The NGS revolution resulted in the identification of an extraordinary number of new microbial genes and taxa, which were enriched by extensive metagenomic sequencing projects such as the MetaHIT and the Human Microbiome Project [7,81]. Among the methods used to study the microbiota, the first approaches were based on cultivation-dependent techniques that allowed the identification and characterization of microorganisms, although these methods only capture a low proportion of bacteria present in the gut due to the suboptimal growth environment[7]. As cultured-based methods are insufficient, many other molecular-based techniques were developed such as Denaturing Gradient Gel Electrophoresis, DNA microarrays or Fluorescence *In Situ* Hybridization. Currently, the main methods to analyze the human microbiome include the sequencing and analyzing of the hypervariable regions of the 16S rRNA gene, which is the gold standard phylogenetic marker; shotgun metagenomics, which allows the sequencing of a sample of the genes present in the microbiome; and metatranscriptomics, which studies gene expression in the microbiome by sequencing the mRNA. These methods not only allow to identify the composition of the gut microbiota, but to study the function and expression of its genes providing information on how the functionality of the microbiome is altered by different conditions. In addition, in the last decade, advanced sequencing technologies such as PacBio and Nanopore have been introduced, demonstrating considerable improvements over previous generations of sequencing platforms. These technologies provide longer reads that can cover whole genomes, addressing computational challenges such as genome assembly and transcript reconstruction, while introducing the capability of real-time analysis. For instance, they increase the taxonomic resolution obtained by 16S rRNA amplicon analysis by enabling sequencing of the entire gene. Although these innovations require further improvements in error correction, sequencing depth and cost, when combined with previous technologies, they allow deep studies of bacterial communities proving to be a promising research tool [7,81,82].

To study the relationships between gut microbiota and food components or other substances, different *in vitro* fermentation models have been developed. These models involve the use of pure cultures, mixed cultures, or fecal inocula, and can range from simple batch fermentations to sophisticated multistage continuous culture systems. Great advances have been made in these methods over the years by optimizing the environmental conditions to better mimic the physiological conditions of the host. This optimization has allowed for higher rates of bacterial growth, providing a more accurate representation of the gut microbial communities and allowing the study of how different factors such as foodstuffs affect them. In addition, there is a wide variety of models that have been developed. The batch fermentation models are closed systems in which microorganisms grow in a fermenter after cell inoculation. These methods are inexpensive and easy to set up, being especially useful for fast testing of different substrates and to study rapid and short-term changes in the fermentative microbiota. In addition, multiple and simultaneous samples can be studied at once. These models have proven to be useful for screening of different foods or substances, despite their lower physiological similarity and the accumulation of bacterial waste [83–85]. Another important factor to consider when performing the fermentation is whether to perform a prior in vitro digestion. This may affect considerably the outcome as introduce the presence of enzymes and bile salts, that would break down the food structure, making nutrients more accessible for microbes during the fermentation, as well as can be used and transformed by the microbes. Additionally, several different *in vitro* batch fermentation approaches have been used, with variation in conditions such as the composition of the medium used (minimum or nutrient-rich medium), among others. Although the specific conditions need to be tailored to the experiment at hand, it is also important to highlight the need for a standardized methodology for *in vitro* digestion and fermentation. A proposed solution to this challenge has been published by Perez-Burillo S. et al 2022, which we have implemented in our research [83].

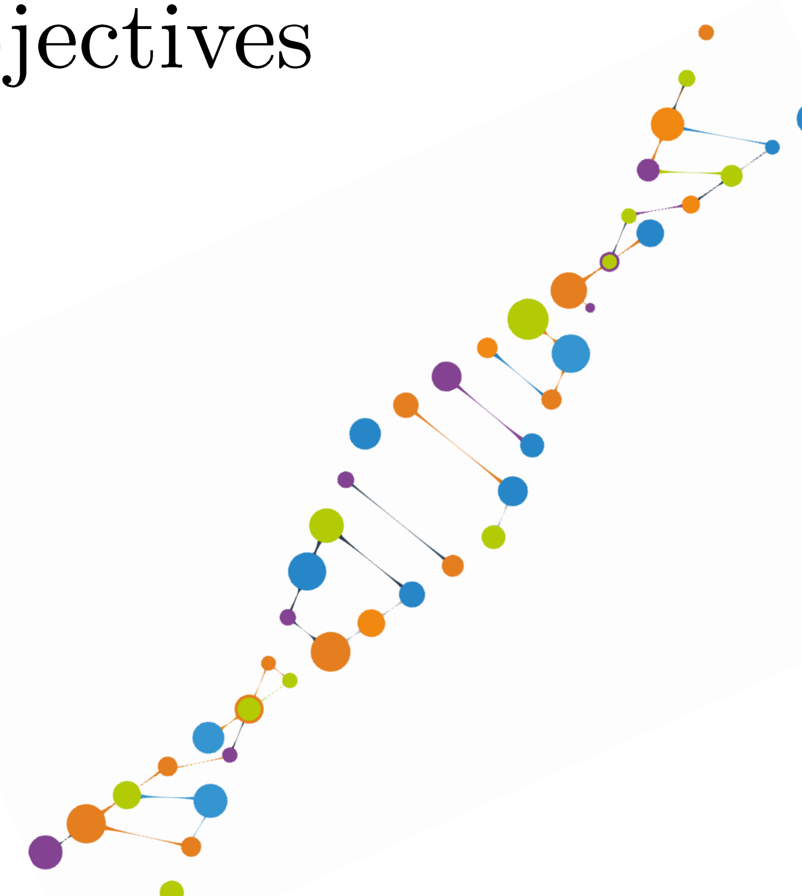
In addition, there are more complex systems with uninterrupted medium flow through continuous fermentation models, which are considered to better mimic physiological conditions. In these continuous fermentation chemostat models, the fresh medium is added, and the spent medium is removed, allowing long-term testing. These systems can have different complexity levels from single-stage models to multi-stage models that simulate multiple parts of the gastrointestinal tract[86]. For instance, the continuous system Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is one of the most complex systems that allows to obtain physiological conditions more similar to the real ones during long fermentation processes. It consists of five stages, two of which mimic gastrointestinal digestion and three that mimic colonic fermentation (ascending, transverse and descending). Due to the first SHIME designs being limited to modeling the luminal microbial community, some improvements have been developed to also mimic mucosal microbial colonization, allowing to culture both the luminal and mucus-associated microbial communities over periods of several weeks[87]. It is noteworthy that the main disadvantage of these models is the inability to study numerous samples at the same time[86,88–91]. Furthermore, the various types of systems can complement each other, with batch fermentations being used for initial screening and continuous systems for a subset of experiments.

Along with *in vitro* experiments, it is necessary to perform *in vivo* experiments in animal models or interventions in humans to validate their findings. Among the advantages of the use of *in vivo* experiments over *in vitro* systems is the higher physiological relevance to humans due to the presence of host immunological interactions, epithelial mucosa and neuroendocrine system. Animal models need to be used to study the effects of extreme diets in the gut microbiota, due to the technical and ethical complexities of performing such trials in humans. Among the most used models we can find worms, flies, zebrafish, mice, and pigs. The mouse is the most common animal model used for research on interactions among food, host, and microbes, including effects of dietary interventions and the role of the gut microbiota in a pathological context. Mice have many similarities to humans in physiology, genetics and gut microbiota composition, and germ-free mice provide a simplified way to investigate the role of a controlled gut microbiota in many processes. However, it is important to note that there exist some physiological and gut microbiota composition differences between humans and all animal models that must be considered during result interpretation[92].

Despite the aforementioned difficulties that may emerge when conducting gut microbiota studies with humans, the growing evidence about the participation of the gut microbiota in numerous physiological processes and associated pathologies points to the need to perform such studies. Human studies are required to address the real complexity of the human gut environment and how the microbiota interacts with epithelial and immune cells, as well as the intricate biochemical conditions. Then, in the last years, there has been a substantial increase in both epidemiological and experimental studies investigating the impact of the gut microbiota in health, as well as the impact of habits, diets or supplements on the gut microbiota composition and function. These studies have significantly contributed to the understanding of the function of the gut microbiota and its relationship with the host. Most of the performed interventions are non-targeted trials with the aim to assess the impact of specific nutrients, personalized diets, or habit changes such as increased physical activity on the intestinal microbiota of the participants [93,94]. During these interventions, participants are divided into different groups, which, depending on the objective of the study, are assigned to a specific dietary pattern, set of habits, or level physical activity to follow for a certain period of time. The participants are examined in detail through questionnaires to assess their progress. In some cases, biological samples may be collected through the study, such as stool samples, to gain insight into how the different parameters vary between the groups. With the rise of technology and personalized nutrition, some studies are appearing to evaluate the effect of dietary recommendations provided automatically through mobile devices using these nutritional interventions [95,96]. Furthermore, some trials are designed to identify a set of bacteria or metabolites as biomarkers for a specific pathology or to clarify their role in pathogenesis by studying a group of participants with this pathology [9,97]. There are also interventions that aim to evaluate the potential of food ingredients or bacteria that could be used as pre- or probiotics to promote a healthier composition of the gut microbiota or an improved metabolite production. This includes examples such as the use of inulin as a prebiotic or *Faecalibacterium prausnitzii* as a probiotic, both of which have widely evidenced beneficial effects on health [98,99]. Also, trials have been performed to explore the effectiveness of fecal microbiota transplants as a treatment for chronic infections, such as those caused by *Clostridium* *difficile* [100]. In addition, targeted interventions can be performed with bacteria genetically modified to express certain proteins or to produce specific metabolites, or with bacteriophages modified to selectively target a set of microbes that may be causing dysbiosis. However, the use of genetically modified organisms in humans presents an ethical dilemma that regulatory authorities have to resolve [101].

The present thesis examines the effects of various foods on the human intestinal microbiota. To this end, *in vitro* digestion and fermentation techniques are employed on a wide variety of foods representative of the Mediterranean and Western diets, which have been subjected to different cooking methods. The resulting fermentative microbiota are analyzed to identify the differential effects of different foods and cooking methods. Inocula from healthy adults and children, as well as obese, celiac, and food-allergic children, have been utilized for this purpose. Additionally, the impact of a nutritional intervention based on personalized dietary recommendations provided via a mobile application on the intestinal microbiota has been evaluated.

Hypotheses & Objectives



Hypotheses

Our hypotheses are:

1. Different foods and cooking methods employed in the Mediterranean and Western diets affect the human gut microbiota in specific ways, which differ between adults and children with different pathologies, and their effects can be evidenced in an *in vitro* fermentation system.
2. An intervention employing an application providing nutritional recommendations based on the Mediterranean diet, accompanied or not with a supplementation with tannins, can modify the gut microbiota of participants in a beneficial manner.

Objectives

This thesis seeks to achieve the following general objectives:

- To evaluate the modulatory effect of different foods on the gut microbiota composition of adults and children using *in vitro* gastrointestinal digestion and colonic fermentation coupled with high throughput sequencing

- To assess the impact of a personalized nutrition app on gut microbiota composition via a dietary intervention

The proposed specific objectives are:

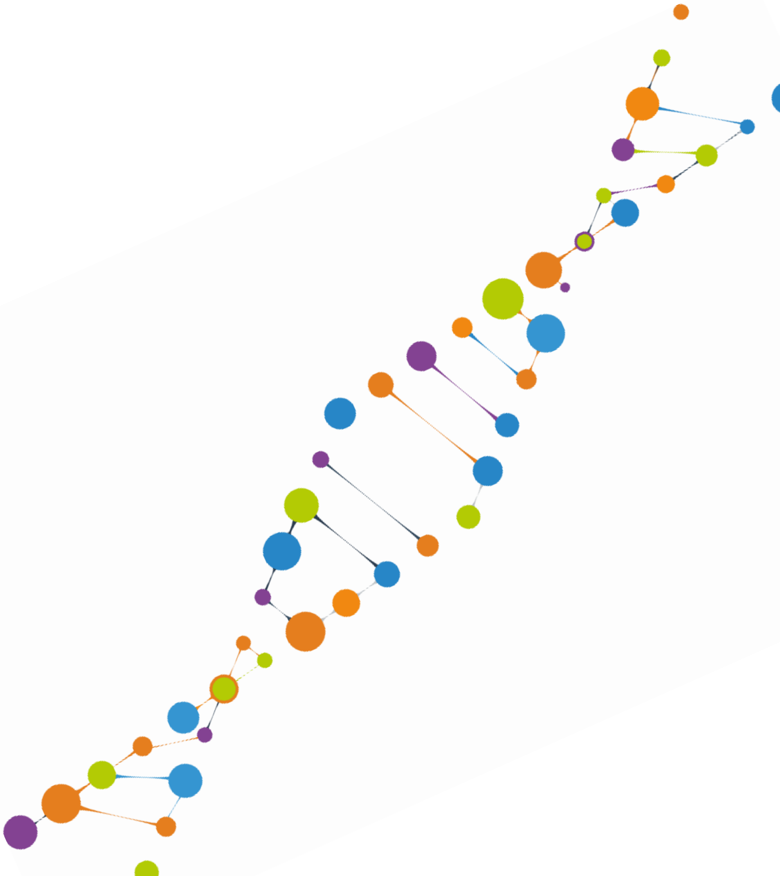
- To determine the effects of different foods and cooking methods on the gut microbiota of healthy adults

- To investigate the differential effects of various foods and cooking methods on the gut microbiota of obese, celiac, allergic, and healthy lean children

- To study the differential responses of the gut microbiota between healthy adults and children

- To evaluate the impact of using a personalized nutrition app and a tannin supplementation on the composition of the gut microbiota of healthy adults

Chapter I



Chapter I. Evaluation of the *in vitro* effects of foods and cooking methods on the gut microbiota.

1 Introduction

Long and short-term dietary intake influence the structure and activity of the gut microbiota, as confirmed by the shifts in microbial community structure caused by short-term animal or plant-based diets [54], and by the strong association between enterotypes and long-term diets [65]. Dietary patterns such as the Western diet alter the composition and metabolic activity of the microbiota, inducing changes suspected of contributing to growing epidemics of chronic illnesses such as obesity and type 2 diabetes [102]. Although there are only a few studies to date, growing evidence suggests that the chemical alterations undergone by foods during cooking may also play an important role in modulating the gut microbiota, for example by modifying phenolic compounds [103–108]. These modulatory effects will depend on both the cooking method and the food, so that they should be studied separately for different foods.

Dietary interventions are likely tools for modulation of the gut microbiota. However, the large inter-individual variability in gut microbiota composition, due to differences in diet and other factors, leads to different host responsiveness to interventions. Furthermore, the responsive bacterial taxa may differ among individuals. Therefore, host and microbiota response to an intervention are difficult to predict; and this variability may influence the results of intervention studies and interfere with reproducibility [79]. Ongoing research focuses on finding dietary manipulations aimed at promoting beneficial microorganisms that take into account host-microbiota relationships, with the aim of using personalized nutrition in therapeutic interventions and as a solution to tackle the variability challenge.

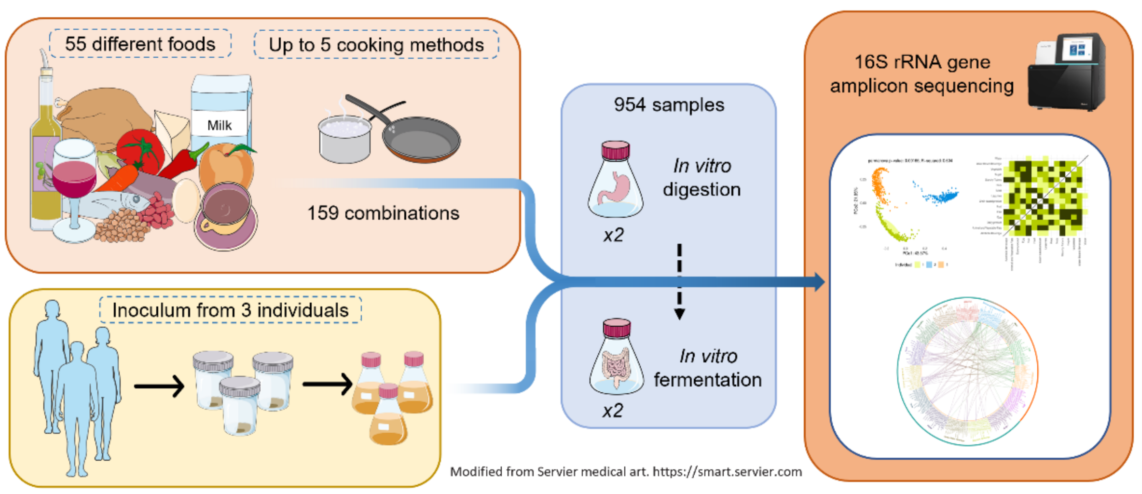
Nevertheless, intervention studies focused on gut microbiota modifications are difficult to perform, and the impact of a particular food cannot be assessed. For this reason, a variety of *in vitro* digestion and fermentation models that mimic human processes have been developed, allowing the assessment of the direct effects of foods on the microbiota [109]. Each approach has advantages and limitations, as, although *in vivo* studies provide more relevant physiological information, *in vitro* models are key for testing specific foods and for initial screenings. Furthermore, batch fermentation models enable short-time parallel cultures to carry out many fermentations simultaneously. Therefore, this is the best approach to characterize the behavior of the gut microbiota when fermenting many different foods, which can be tested one by one without the confounding factors of other foods.

The research of the present chapter has been already peer-reviewed and published [110]. The aim of this work is to study how the composition of gut microbiota communities responds to a wide variety of foods representative of Western and Mediterranean diets, as well as the influence of cooking methods. We have performed over 900 *in vitro* digestion and fermentation assays to test the effect of 55 different foods, raw or cooked with up to five different methods, on the microbiota of three healthy adult individuals. The characterization of the resulting fermentative microbiota has revealed which bacteria tended to increase or decrease when exposed to specific foods. This knowledge will be useful to refine dietary interventions aimed at modulating the gut microbiota, moving towards the goal of personalized nutrition.

2 Material and methods

2.1 Study design

Fifty-five different foods, raw or cooked with up to five cooking methods, resulting in 159 total combinations, were *in vitro* digested, and fermented to study their effect on the gut microbiota. Stools from three healthy adults were used as separate inocula and *in vitro* fermentations were performed in duplicate. A graphical summary of the study design is shown in **Figure I-1**.



Modified from Servier medical art. https://smart.servier.com

Figure I-1. Graphic summary of study design.

Due to the large number of foods used, they have been grouped at three different levels for the analysis of results: i) Animal-based and plant-based foods, ii) Food categories and iii) Individual foods. The foods included at each level are described in **Table I-1**.

2.2 Food and cooking methods

The foods and cooking methods employed are representative of Western and Mediterranean diets [111]. We investigated 42 plant foods belonging to 10 different categories (alcoholic drinks, fruit, grain-based products, legumes, nuts, starchy tubers, sugar, vegetables, vegetable fats and water-based beverages), and 11 animal foods belonging to 4 categories (dairy products, egg, fish, and meat) (**Table I-1**). In addition, the beverages cola and light cola were also investigated. Foods were bought in Spanish supermarkets and stored at room temperature or under refrigeration for a maximum of 2 days before cooking.

The food samples were submitted to different culinary treatments: boiling, grilling, roasting, frying, and toasting. In addition, some foods were also investigated in raw form. Milk was commercially processed by ultra-high temperature.

Boiling was performed at 100ºC for 20 min at a water/food ratio of 5:1. Roasting was performed at 180ºC for 10 min. Extra virgin olive oil was used as a cooking medium for grilling and frying. Grilling was performed at 220–250ºC for 3 min on each side at an oil/food ratio of 0.5:1 and frying at 180ºC for 8 min at an oil/food ratio of 5:1. Toasting was performed in a Grunkel TS140H toaster at the fourth level at 200ºC for 3 min at 900 W, following the manufacturer's instructions.

Cooking times and food/medium ratios were based on Ramírez-Anaya *et al.*, 2015 [112] and adapted to our equipment and laboratory conditions. Once cooked, all samples were homogenized and stored under nitrogen atmosphere at -80ºC to avoid oxidation.

Plant based/ animal-based	Food category	Food	Raw	Fried	Boiled	Roasted	Grilled	Toasted
Animal-based	Meat	Chicken		●	●	●	●	
		Beef		●	●	●	●	
		Lamb		●	●	●	●	
		Pork		●	●	●	●	
	Dairy Product	Milk	●					
		Yogurt	●					
		Gouda	●	●		●	●	
	Egg	Egg		●	●	●	●	
	Fish	Salmon	●	●	●	●	●	
		Cod fish		●	●	●	●	
	Animal and Vegetable Fats	Butter	●	●				
Plant-based	Animal and Vegetable Fats	Olive oil	●	●				
		Sunflower oil	●	●				
	Fruit	Apple	●	●		●	●	
		Banana	●	●		●	●	
		Orange	●	●		●	●	
		Grapes	●	●		●	●	
		Plum	●	●		●	●	
		Peach	●	●		●	●	
		Olives	●					
	Grain based product	Bread	●	●				●
		Bread whole grain	●	●				●
		Penne			●			
		Penne whole grain			●			
		Rice longo			●			
		Rice longo whole grain			●			
		Biscuits	●					
		Biscuits whole grain	●					
		Breakfast Cereal whole grain	●					
		Breakfast Cereal	●					
	Legumes	Beans Kidney			●	●	●	
		Lentils			●	●	●	
	Nuts	Nut mix	●	●		●		
		Peanuts			●	●	●	
	Starchy Tubers	Potato		●	●	●	●	
		Sweet potato		●	●	●	●	
	Sugar	Dark Chocolate	●					
		Nutella	●					
	Vegetable	Zucchini	●	●	●	●	●	
		Capsicum	●	●	●	●	●	
		Carrot	●	●	●	●	●	
		Eggplant	●	●	●	●	●	
		Onion	●	●	●	●	●	
		Cauliflower	●	●	●	●	●	
		Spinach	●	●	●	●	●	
		Garlic	●	●	●	●	●	
		Tomato	●	●	●	●	●	
		Cabbage	●	●	●	●	●	
		Lettuce	●					
	Alcoholic Beverage	Red wine	●					
		Beer	●					
	Water Based Beverage	Coffee	●					
		Coffee instant	●					
	Water Based Beverage	Cola	●					
		Cola light	●					

Table I-1. Foods employed in this study during *in vitro* digestions and fermentations. Different cooking methods performed with each food are indicated with a dot. Information is provided on which foods belong to which categories of the two food classifications employed. These two classifications as well as individual foods will be used in subsequent analyses.

2.3 Fecal material collection

Fecal samples were obtained from three healthy donors, who had not taken antibiotics, prebiotics, or probiotics for three months prior to the assay, with a mean body mass index of 21.3. At least three fecal samples of each donor were obtained and pooled together to reduce intra-individual daily variability. Fecal donors followed a regular Mediterranean diet before sample collection.

Stools were deposited in a sterile recipient, stored in a home refrigerator, and transported to the laboratory in a cooler bag within 4 h. Upon arriving at the laboratory, the feces were mixed with a water:glycerol solution (20% vol/vol) and stored at -80°C.

2.4 *In vitro* gastrointestinal digestion and fermentation

All foods were submitted to *in vitro* batch digestion-fermentation mimicking physiological processes in the human gut, according to previously described protocols [83,113]. The *in vitro* fermentations were performed at 37°C for 20 h using the digested foods, the fecal microbiota and fermentation medium as described [83]. The fermentation medium is composed by peptone solution (14 $g\ L^{-1}$) at pH 7, 0.312 $g\ L^{-1}$ of cysteine, 0.312 $g\ L^{-1}$ sodium sulfide and 1.25 $mL\ L^{-1}$ of resazurin solution at 0.1% (wt/vol). This oligotrophic medium was suitable for testing the different foods as the main source of energy and nutrients for the microbiota, highlighting the bacterial community involved in their metabolism. For each food (in duplicate), 5g were added to Falcon tubes and the three digestion phases were performed sequentially under agitation at 37ºC: oral (adding α-amylase for 2 min), gastric (adding pepsin for 2 h at pH 2–3) and intestinal (adding bile salts and pancreatin for 2 h at pH 7). The *in vitro* fermentations were performed at 37ºC for 20 h using the digested foods, the fecal microbiota and fermentation medium (peptone, cystine and resazurin) [83]. A fermentation control was performed using water in place of the digested substrate. Fermentations were centrifuged, and bacterial cells were recovered for taxonomical analysis by high throughput sequencing.

2.5 DNA extraction

Bacterial pellets derived from *in vitro* fermentations were lysed with 0.1mg/ml lysozyme during 30 min at 37ºC. DNA extraction was performed with the MagNaPure LC JE379 platform and DNA Isolation Kit III (Roche). DNA was quantified with a Qubit 3.0 Fluorometer (Invitrogen), while agarose gel electrophoresis (0.8 % w/v agarose in Tris-borate-EDTA buffer) was used to determine DNA integrity. DNA was stored at -20ºC until further processing.

2.6 16S rRNA gene amplicon sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using 12ng of DNA, following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. PCR was performed with forward primer (5'-TCGT CGGC AGCG TCAG ATGT GTAT AAGA GACA GCCT ACGG GNGG CWGCA-G3') and reverse primer (5'-GTCT CGTG GGCT CGGA GATG TGTA TAAG AGAC AGGA CTAC HVGG GTAT CTAA TCC3'), fitted with adapter sequences for compatibility with the Illumina Nextera XT Index kit. Amplicon libraries were pooled and sequenced in an Illumina Miseq sequencer in 2 x 300 cycles paired-end runs (MiSeq Reagent kit v3).

2.7 Bioinformatic analysis

The DADA2 (v1.8.0) package as implemented in R (v3.6.0) was employed for sequence read processing and forward and reverse merging as well as clustering into Amplicon Sequence Variants (ASVs) [114]. Filtering and trimming parameters were as follows: maxN=0, maxEE=c(2,5), truncQ=0, trimLeft=c(17,21), truncLen=c(270,220), and rm.phix=TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for read merging.

Taxonomic identification was assigned to ASVs using DADA2 and the SILVA v.138 reference database. The MegaBLAST tool from BLAST v(2.10.0) was further used for those ASVs identified only at genus level with DADA2, requiring at least 97% identity for species-level assignation and a minimum difference of 2% between the first- and second-best matches. ASVs with a total number of counts lower than 10 were removed.

2.8 Statistical analysis

Alpha and beta diversity indices (Shannon, Chao1 and Bray-Curtis) were computed using the vegan package (v2.5-2) in the R platform. Differences in alpha-diversity were tested using the Wilcoxon rank-sum test. The Bray-Curtis dissimilarity index was employed to quantify the overall dissimilarity between two microbial communities and was used in permutational multivariate analysis of variance (PERMANOVA) and Principal Coordinates Analysis (PCoA). PERMANOVA was performed using the adonis function from Vegan with 600 permutations. The

Benjamini-Hochberg (BH) procedure was applied for false discovery rate control.

Analysis of the composition of microbiomes (ANCOM) was used to identify differentially abundant taxa among samples and test significance was determined using the BH procedure for false discovery rate control, as described in Kaul et al., 2017. ANCOM was performed at the level of individual foods and at the two different levels of food classification for each individual, and significance was assigned at $q < 0.05$. Differential abundance tests were performed at the genus and species level, with species differences between parentheses indicating when that genus was also different for the same comparison, and without when that difference was only found at the species level.

3 Results

16S rRNA gene amplicon sequencing of the microbiota in the 159 food combinations, after fermentation in duplicate with 3 different inocula, generated a total of 89,826,329 reads, averaging 93,277 reads per sample, 76,205,997 of which were assigned at genus-level. Taxonomic assignation identified reads belonging to 10 phyla, 65 families, 175 genera and 147 species.

3.1 Microbiota composition

Significant differences in bacterial composition (**Figure I-2A**) and diversity (Shannon index and Chao1 estimator, $q < 0.001$) were detected among the microbiota of the three analyzed individuals. Moreover, most of the variance in microbiota composition when all samples were considered was caused by differences among individuals. Therefore, the effect of foods and cooking methods was studied separately for each individual in all following analyses.

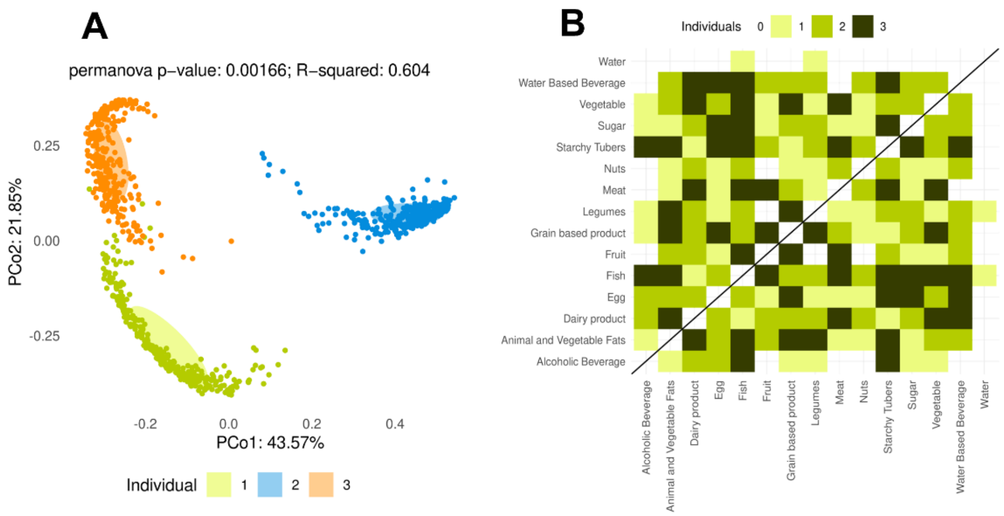


Figure I-2. Analysis of overall microbiota composition after *in vitro* fermentations. A) Principal Coordinates Analysis (PCoA) based on the Bray Curtis dissimilarity index for all fermented food samples. Colors represent individuals 1, 2 and 3. PERMANOVA reveals significant differences in overall microbiota composition among individuals (p = 0.00166). B) Number of individuals with significant (q < 0.05) PERMANOVA between food categories based on the Bray Curtis dissimilarity index.

PERMANOVA revealed that, in each individual, the overall composition of the microbiota was significantly different (q < 0.01) between fermentations of plant and animal-based foods. At the level of lower food categories, few significant differences in overall microbiota composition were detected between individual categories and the water control (for water-based beverages, legumes, eggs and dairy products, in one individual only), but numerous differences existed between different food categories (**Figure I-2B**). This may be due to different foods modifying the microbiota composition in different directions, resulting in larger differences between two foods than between each and the water control. In all three individuals, starchy tubers and fish resulted in significantly different microbiota compositions when compared to the largest number of other foods (**Figure I-2B**).

3.2 Differential abundance of taxa

Of 175 taxa identified at genus level, 78 were found in all individuals. Of these, 6 taxa were significantly more abundant in the fermentations of animal-based foods than in those of plant-based foods in each individual (**Figure I-3A**): *Faecalibacterium*, *Lachnoclostridium*, Lachnospiraceae UCG 004, *Fusicatenibacter*, *Romboutsia* and *Actinomyces.* In addition, 21 other taxa were significantly different between these fermentations for two individuals, and many more differences were specific to a single individual (**Figure I-3A**). Taxa present across individuals could have differential abundances between animal- and plant-based foods in one individual but not in others, as most of the taxa that were differential in only one individual were also present in the other two. At food category level, 49, 59 and 25 genera were differentially abundant for a given comparison in one, two or three individuals, respectively (**Figure I-3B**). Again, taxa present across all three individuals could behave differently in each. Of the 25 genera in which differences were detected for a given comparison in all individuals, the following stand out for their higher abundance: *Bacteroides*, *Faecalibacterium*, *Lachnoclostridium*, *Ruminococcus*, *Bifidobacterium* and *Agathobacter* (**Figure I-3C**).

A genus-level summary of the food category comparisons significant in all the individuals is shown in **Figure I-4**; also, all significant comparisons for food categories and individual foods can be found in further detail in **Supplementary Material I-1** to **I-4**, at both genus and

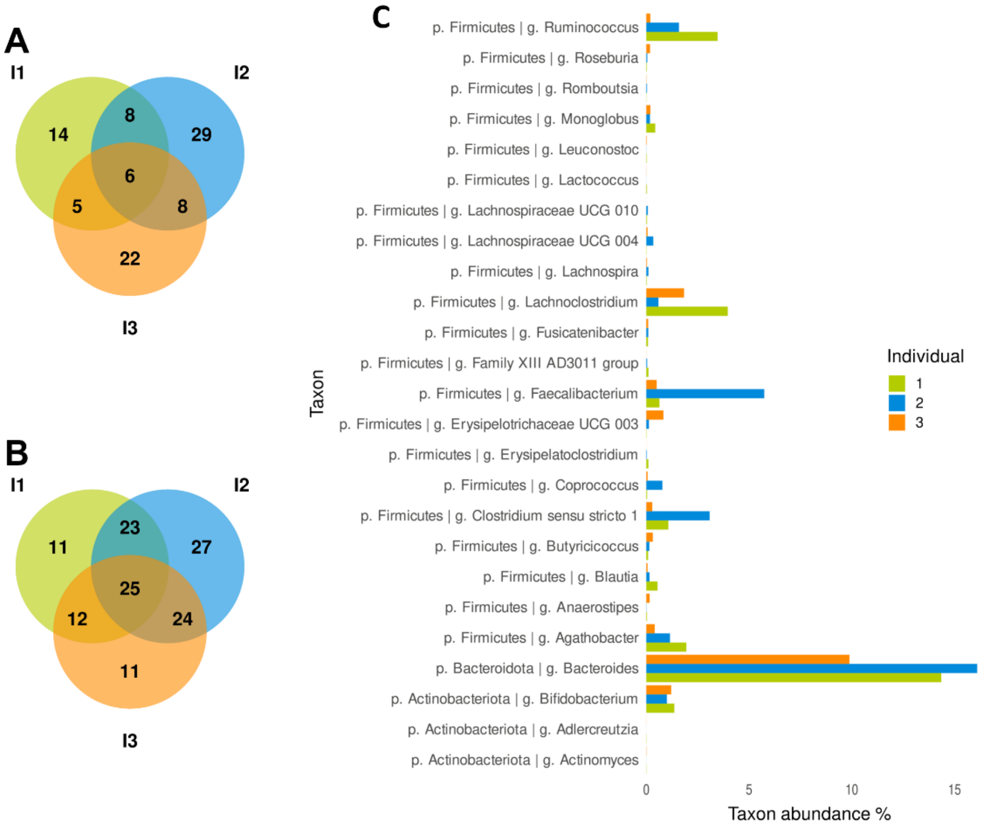


Figure I-3. Differentially abundant genera shared among individuals. A Venn diagrams between plant-based vs animal-based foods and **B** different food categories. Differential abundances were assessed with the ANCOM method and the BH procedure for false discovery rate control (q <0.05). Differences are considered common among individuals when changes take place in the same direction. **C** Relative abundance of differentially abundant genera in food category comparisons. The Y axis presents the 25 genera detected as differentially abundant for a given food comparison in all individuals. Abundances were normalized by Total Sum Scaling. The mean relative abundance for all samples from an individual is presented.

species level. In the following sections we review the food categories having the strongest impacts on microbiota composition, indicating those taxa whose abundance is differentially affected across all three individuals, and highlighting relevant differences among specific foods within each category.

3.2.1 Effect of animal and vegetable fats

Animal and vegetable fats was the category that resulted in the most significant effects on taxon abundance. The abundance of the Lachnospiraceae genera *Blautia*, *Lachnoclostridium*, *Lachnospira* and *Roseburia*, and of the Oscillospiraceae genus *Faecalibacterium* (*F. prausnitzii*), increased with animal and vegetable fats compared with fruits, legumes, grain-based products (excluding *Roseburia*) and water-

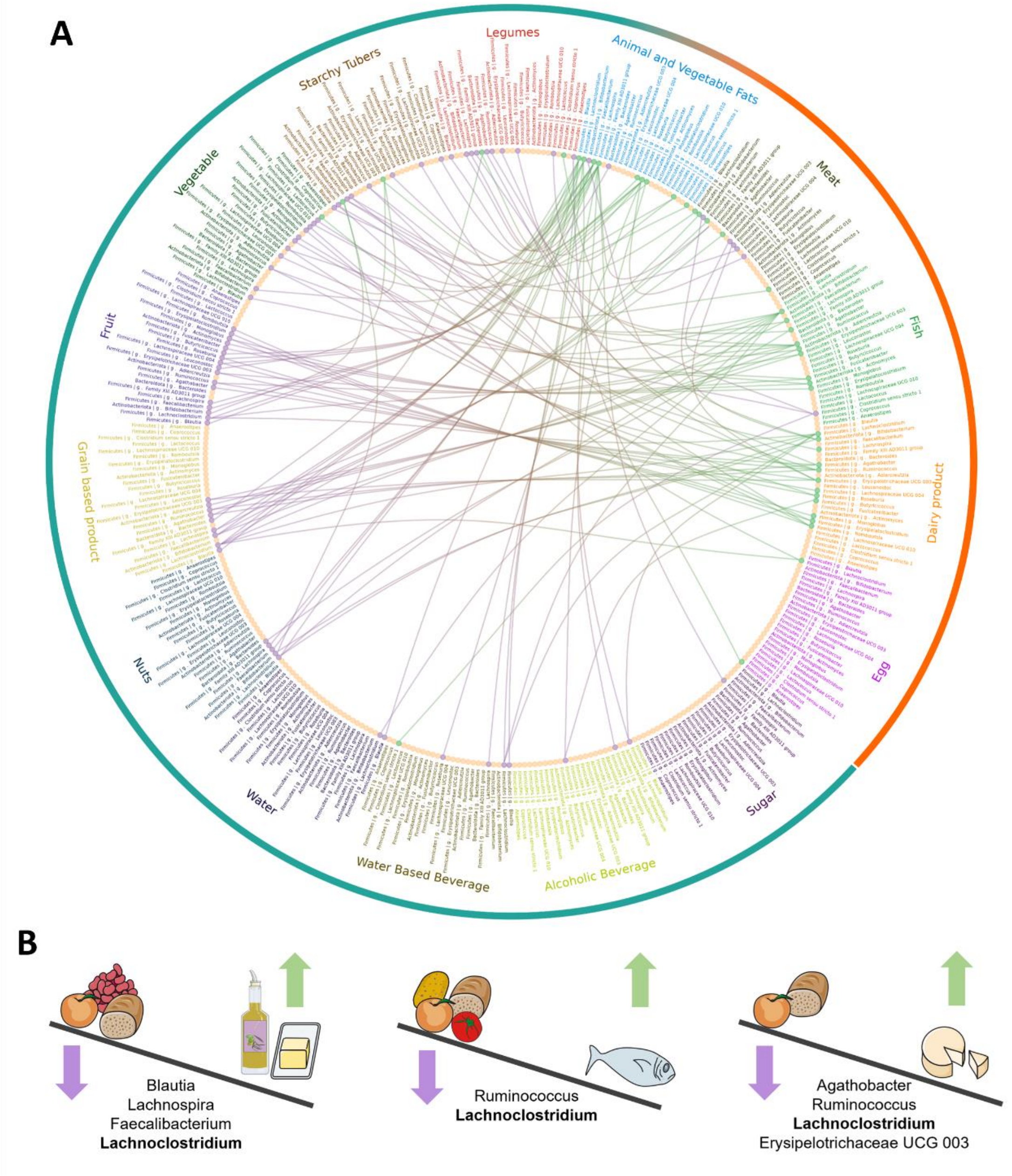


Figure I-4. Genus-level abundance differences between food categories. **A)** circos plot showing differentially abundant genera between food categories. The same 25 genera, with the same order, in which common differences have been detected across all individuals, are represented in each food category. The fifteen food categories are shown with different colors and the wider level of classification, animal and plant-based foods, is shown in orange and blue in the outermost circle. The lines represent comparisons significant across all individuals between two food categories for a given taxon. Green and purple indicate higher and lower abundance in the food category. The dots for each taxon are colored to indicate whether most of their differences were increased abundances (green), decreased abundances (purple) or whether there was no difference (yellow). **B**) Graphical summary of main differential outcomes in relative taxon abundance between food categories. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

based beverages (excluding *Faecalibacterium*). In particular, butter showed the highest number of significant comparisons of all foods tested in any food category, resulting in higher abundances of *Faecalibacterium* and the aforementioned Lachnospiraceae genera, as well as *Agathobacter* and *Fusicatenibacter*, in comparison with many other foods. In general, olive oil also induced significantly higher abundances of most of these genera, but only in comparison to a smaller array of foods. However, *Blautia* was not found at higher abundances in olive oil in comparison to any other food. Regarding sunflower oil, the number of significant comparisons was lower than for the other fats, with only *Faecalibacterium* and *Agathobacter* showing higher abundances in comparison to various other foods. *Faecalibacterium* was increased in all three types of fats for a similar number of comparisons to foods belonging to a variety of plant-based categories.

3.2.2 Effect of fish

Fish fermentations led to higher abundances of numerous genera, most notably *Lachnoclostridium* and *Ruminococcus*, which were elevated with respect to 6 and 5 plant-based categories, respectively. Several genera showed higher abundances in fish fermentations when compared to those of fruits, including the two aforementioned plus Erysipelotrichaceae UCG 003, *Fusicatenibacter*, *Actinomyces* and *Monoglobus.* In contrast, *Clostridium sensu stricto* 1 had lower abundances in fish fermentations in comparison to vegetables, legumes, and water-based beverages.

At food level, both salmon and cod induced higher abundances of *Lachnoclostridium* and *Ruminococcus* in comparison to a variety of plant-based foods. In addition, cod was involved in a very high number of significant comparisons, nearing those identified with butter, often resulting in higher abundances of *Faecalibacterium*, *Monoglobus* and *Bifidobacterium.*

3.2.3 Effect of dairy products

Dairy products resulted in a higher abundance of *Bifidobacterium* compared with animal and vegetable fats, meat, and nuts. Firmicutes genera, such as *Agathobacter*, *Lachnoclostridium*, *Ruminococcus* (*R. bromii*), *Romboutsia* and Erysipelotrichaceae UCG 003, were also often increased in dairy products in comparison to plant-based food categories, such as fruit and grain-based products, and, to a lesser extent, vegetables, and legumes. In particular, all of these genera were more abundant in dairy product fermentations than in those involving fruits. At food level, gouda was involved in a high number of significant comparisons, showing higher abundances of *Lachnoclostridium*, *R. bromii*, *Romboutsia* and Erysipelotrichaceae UCG 003 when compared to a variety of plant-based foods. In contrast, yogurt resulted in a single significant comparison, with *Roseburia* in lower abundance in comparison to butter, while no significant comparisons involving milk fermentations were common among the three individuals.

3.2.4 Effect of fruit

Fruit reduced the abundance of a large number of genera (17) in comparison to at least one other food. However, most of these genera were reduced only with respect to one or two food categories. *Lachnoclostridium* stands out as the only taxon reduced in comparison to three categories, namely animal and vegetable fats, fish and dairy.

Apple was the fruit resulting in the most differences, all representing reduced abundances. *Agathobacter* was reduced in the largest number of comparisons, with animal-based foods such as butter, salmon, beef and chicken, but also with plant-based foods such as biscuits, nuts, vegetable oils, cabbage, carrot, and plum. *Lachnoclostridium*, *Faecalibacterium*, *Fusicatenibacter*, *Dorea* and *Butyricicoccus* were also reduced in apple fermentations compared to various other foods. The comparisons of apple against butter and gouda showed the highest number of reduced taxa (> 12 in each). Similar results were obtained for bananas, which showed reduced abundances of 7 taxa in relation to butter and gouda, with lower levels of *Lachnoclostridium* in comparison to several additional foods. Also, oranges, grapes, plums and peaches showed reduced abundances in various taxa in comparison to butter. However, plums were unique among fruits in increasing the abundances of *Bacteroides* and *Ruminococcus* in relation to several other foods. On the other hand, peaches induced a significantly higher abundance of *Lachnoclostridium* than most other fruits, tomato, and whole-grain bread.

3.2.5 Effect of vegetables

Similarly, to fruits, vegetables affected the abundance of a relatively large number of genera (11) but only in respect to one or two food categories in each case. In most instances, vegetables showed lower abundances of taxa in comparison with animal and vegetable fats, dairy products or fish.

At food level, results were also similar to those obtained with fruits; most of the significant comparisons were against butter, gouda, codfish and salmon. Onion resulted in lower abundances of *Lachnoclostridium*, *Ruminococcus* and *Faecalibacterium* compared with these four foods, while tomato significantly reduced *Lachnoclostridium* and *Dorea* in comparison to these foods and several others. Garlic is a singular case as it showed differences against a large number of foods, including lower abundances of *Ruminococcus* and *Fusicatenibacter*, and higher abundances of *Bacteroides.* In particular, *Bacteroides* increased in garlic in relation to other plant foods such as capsicum, apple, nut mix and sweet potato, as well as animal foods such as gouda and salmon.

3.2.6 Effect of grain-based products

As in the cases of fruits and vegetables, grain-based products reduced the abundances of several genera (9) only in respect to one or two food categories, mainly animal and vegetable fats, dairy products, or fish. *Lachnoclostridium* stands out as being reduced not only in relation to these three food categories, but also in comparison to egg and meat.

Most of the individual foods within this category, similarly to fruits and vegetables, differed in many taxa in relation to butter and gouda, and, to a lesser degree, in relation to salmon, cod and olive and sunflower oils.

Interestingly, whole-grain bread, but not regular bread or whole-grain biscuits, reduced *Lachnoclostridium* in relation to a variety of foods, including butter, gouda, cod, salmon, peach and plum.

3.3 Effects of cooking methods on the fermentative microbiota

When taking together all food categories, only the comparison between fried and roasted foods yielded a significant difference in taxon abundance, specifically for the low-abundant Ruminococcaceae UBA1819 group, which was significantly reduced in fried foods in all individuals. Since the effects of cooking methods could be masked by the effects of the different food categories, we analyzed them separately within each.

No significant differences in bacterial abundance were found in all individuals when comparing cooking effects within each food category, but a few common differences were found in two individuals and many in single individuals. All genus-level significant differences are shown in **Figure I-5** and **Supplementary Material I-5**, and **Supplementary Material IV-6** presents the significant differences at species level. In the following sections, we review the food categories for which cooking methods had a stronger impact, indicating those taxa that were the most affected. A summary of the effects of the most differential cooking methods within each food category is presented in **Figure I-6**.

3.3.1 Cooking methods in fish

Salmon and cod were boiled, fried, roasted, and grilled. Differences in taxon abundance depending on the cooking method were observed in individuals 1 and 3, but not in individual 2. Boiling resulted in the most marked differences, mostly in comparison to roasting in individual 1 and to grilling in individual 3. Overall, differences were observed mainly in the

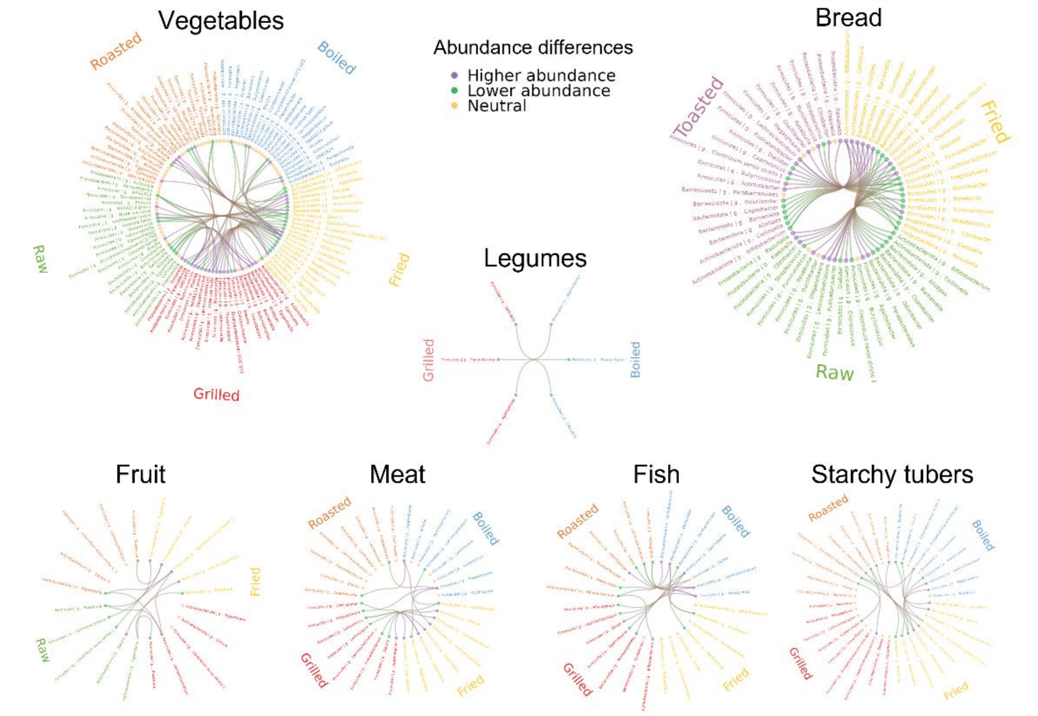


Figure I-5. Circos plots of genus-level abundance differences between cooking methods by food category. Cooking methods and genera involved in significant comparisons for at least one individual are shown for each food category. Cooking methods are represented with different colors and differentially abundant genera are shown in the same order for all cooking methods. The lines represent significant comparisons between two cooking methods. Green and purple indicate higher and lower abundance of the corresponding genus. The dots for each genus are colored to indicate whether most of the differences were increased abundances (green), decreased abundances (purple) or whether there was no difference (yellow).

abundances of members of the Firmicutes, with boiling producing lower abundances of numerous genera, particularly in the Lachnospiraceae, Ruminococcaceae and Erysipelotrichaceae families. In particular, *Monoglobus* and *Lachnoclostridium* were reduced in both individuals 1 and 3 in comparison to roasting. The abundances of some Proteobacteria, as well as those of *Bacteroides* and *Bifidobacterium,* were also impacted by boiling, mostly in comparison to roasting in individual 1. Only 3 genera increased in boiled fish compared with other cooking methods: *Agathobacter* in individual 1 in relation to roasting, and *Dorea* and *Parabacteroides* in individual 3 in relation to grilling.

3.3.2 Cooking methods in meat

Like fish, meats were boiled, fried, roasted and grilled. Again, boiling resulted in the most marked differences, mainly consisting of

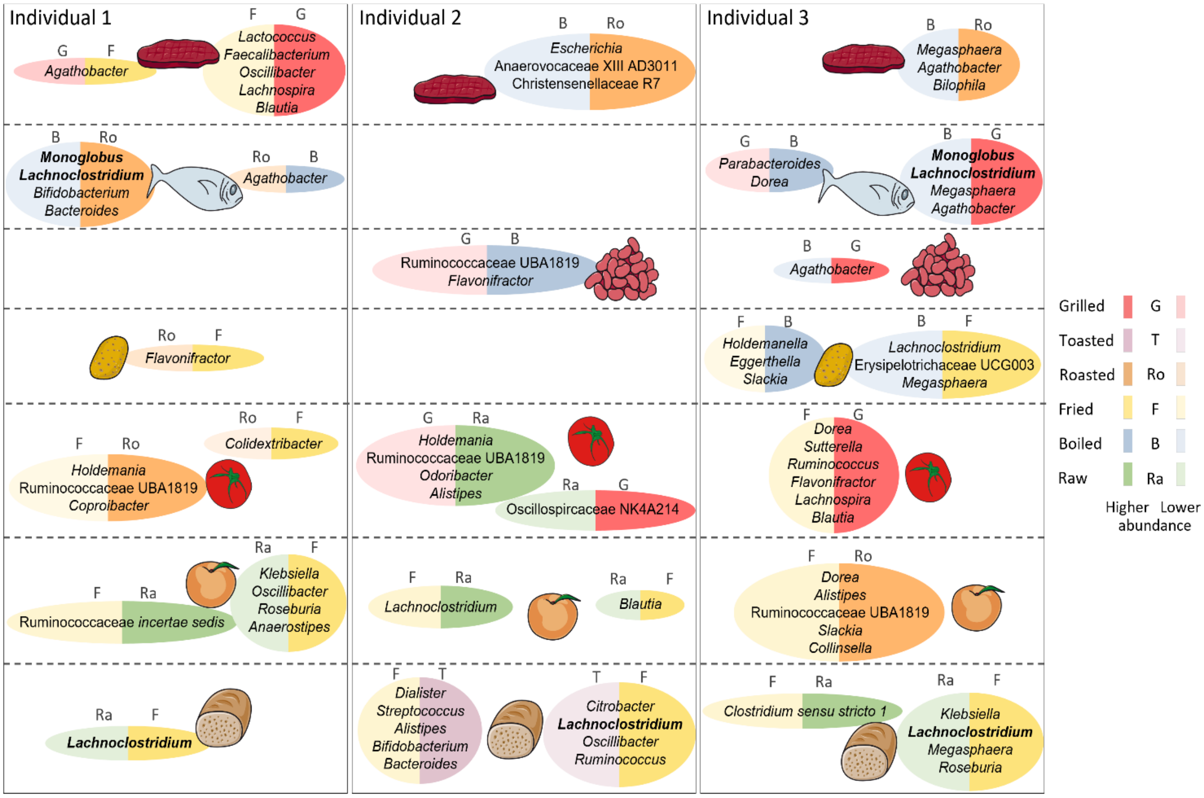


Figure I-6. Graphical summary of main differential outcomes in relative taxon abundance between cooking methods. Differences are shown per individual and food category. For each individual, the two most differential cooking methods for each food category are shown, along with representative taxa whose abundance is reduced with the corresponding cooking method. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. This figure is available in higher quality through the link in **Supplementary Material.**

reduced abundances of Firmicutes taxa in comparison to roasting in individuals 2 or 3. Of notice, in individual 3 boiling had a strong impact on the abundance of *Megasphaera*, which was reduced in relation to all other cooking methods. Only *Lachnoclostridium* increased in boiled meat compared to other methods, augmenting in individual 1 in relation to frying. Notable differences were also obtained when comparing frying and grilling, with frying reducing the abundances of the Firmicutes genera *Faecalibacterium, Oscillibacter, Lachnospira* and *Blautia* in individual 1 and of *Lactococcus* in both individuals 1 and 2. In contrast, *Agathobacter* was increased in fried versus grilled meat in individual 1, as well as in fried versus boiled meat in individual 3.

3.3.3 Cooking methods in fruit

The effects of fruits (excluding olives, which were only analyzed raw) were compared when raw, fried, roasted or grilled. Frying and grilling resulted in a variety of differences in comparison to raw fruit, while there were no differences in roasted fruit versus raw. Fried fruits resulted in the most marked differences. In particular, frying decreased the abundances of many taxa in individual 3 in relation to roasting, including those of various Ruminococcaceae, *Dorea*, *Clostridium sensu stricto 1*, *Alistipes*, *Slackia* and *Collinsella*. Differences between fried and raw fruits were observed in all individuals, although affecting different taxa in each: in individual 1, frying resulted in increases of *Klebsiella, Oscillibacter, Roseburia* and *Anaerostipes* and a reduction of Ruminococcaceae *incertae sedis*, in individual 2 it resulted in the increase of *Blautia* and the reduction of *Lachnoclostridium,* whereas in individual 3 it resulted in the reduction of Ruminococcaceae UBA1819, *Clostridium sensu stricto* 1 and three Eggerthellaceae genera.

In turn, grilled fruit also resulted in a variety of differences when compared to raw, some of which were also detected in comparison to roasted. Shared differences included reduced abundances of Ruminococcaceae UBA1819 and *Clostridium sensu stricto* 1 in individual 3. In addition, *Caproiciproducens*, *Peptoniphilus* and Ruminococcaceae *incertae sedis* were reduced in grilled fruits in comparison only to roasted, in individuals 1, 2 and 3, respectively. Individual 1 also had increases of many taxa in grilled fruits in comparison to raw, including *Parasutterella*, *Lachnoclostridium*, *Oscillibacter* and Oscillospiraceae UCG005. Finally, grilled fruits differed from fried only in having a lower abundance of *Clostridium sensu stricto* 1 in individual 1.

3.3.4 Cooking methods in Vegetables

The effects of vegetables (excluding lettuce, which was only analyzed raw) were compared when raw, boiled, fried, roasted or grilled. As for fruits, raw vegetables presented a variety of differences in comparison to those fried and grilled, while no differences were detected against those boiled or roasted. Again, frying resulted in the most marked differences, resulting mainly in taxon increases, including those of numerous genera of the Firmicutes, mostly in the Lachnospiraceae. The microbiota of individual 3 was particularly impacted by frying, showing a large number of differences in comparison to grilling, mostly not detected in the other individuals. These included mainly increases of genera in the families Ruminococcaceae, Oscillospiraceae and Lachnospiraceae. Among the latter, many were increased in individual 3 also when fried vegetables were compared to other cooking methods, with *Lachnoclostridium* and *Blautia* augmented in relation to all. Frying also resulted in decreases of some genera, including the Bacteroidetes *Alistipes, Coprobacter* and

Barnesiella, the Actinobacteriota *Eggerthella*, and various Firmicutes. Among these, Ruminococcaceae *incertae sedis* is of note, as it was decreased in fried vegetables in relation to raw, boiled and roasted in individuals 1 and 3.

In contrast to frying, grilling resulted mainly in lower abundances of taxa in relation to raw vegetables and other methods of cooking. Of note, Ruminococcaceae UBA1819 was reduced in grilled vegetables in relation to raw, boiled and roasted in one or two individuals. In individual 3, numerous other taxa were also reduced with grilling in relation to one or two cooking methods, whereas in individual 2 other taxa were reduced with grilling but only in relation to raw. On the other hand, some taxa increased in grilled vegetables in relation to other methods, notably various members of the Oscillospiraceae, such as *Oscillibacter* in relation to roasted (individual 1), *Oscillospira* in relation to raw (individual 2) and Oscillospiraceae NK4A214 in relation to both raw and fried (individual 2). In addition, Lachnospiraceae NK4A136 and Ruminococcaceae *incertae sedis* increased in relation to boiled in individual 3 and to fried in individual 1, respectively, although they were decreased in relation to other cooking methods.

3.3.5 Cooking methods in legumes

Kidney beans and lentils were assayed boiled, roasted, and grilled. Differences were only observed between boiling and grilling, with the former causing a reduction of *Agathobacter* in individual 3 and an increase of Ruminococcaceae UBA1819 and *Flavonifractor* in individual 2.

3.3.6 Cooking methods in starchy tubers

Potatoes and sweet potatoes were assayed fried, boiled, roasted, and grilled. Roasting displayed the most differences with other methods, although nearly all were detected only in individual 3. In particular, roasting resulted in reductions of *Flavonifractor*, *Roseburia* and *Lachnoclostridium* and in increases of Erysipelotrichaceae UCG003 and *Holdemanella* in relation to frying, as well as in increases of Erysipelotrichaceae UCG003 in relation to boiling, and of *Holdemanella* and Clostridium sensu stricto 1 in relation to grilling. Individual 3 also presented several differences between frying and boiling, with reductions of *Holdemanella*, *Slackia* and *Eggerthella* and increases of *Megasphaera*, *Lachnoclostridium* and Erysipelotrichaceae UCG003 with the former method. Therefore, for individual 3 the Erysipelotrichaceae (Erysipelotrichaceae UCG003 and *Holdemanella*) and Eggerthellaceae (*Eggerthella* and *Slackia*) appear to be particularly sensitive to the cooking methods employed for starchy tubers.

3.3.7 Cooking methods in bread

Among grain-based products, comparisons of cooking methods could only be performed for breads (white and whole grain) since other products were not exposed to different cooking procedures. Frying caused several differences in relation to both raw and toasted breads, mostly in individual 2, while the latter methods did not differ. Such differences included reductions in *Dialister*, *Streptococcus*, *Clostridium sensu stricto* 1, *Alistipes, Odoribacter, Coprobacter, Barnesiella, Collinsella* and *Bifidobacterium,* as well as increases of *Raoultella, Citrobacter, Lachnoclostridium, Fusicatenibacter, Coprococcus* and *Agathobacter.* In addition, several taxa were increased in fried bread in individual 2 only in relation to toasted (*Ruminococcus, Oscillibacter* and *Butyricicoccus*). In individual 3, only *Lachnoclostridium* was increased in relation to both raw and toasted, while *Fusicatenibacter, Klebsiella, Megasphaera, Roseburia* and *Parabacteroides* were increased only in relation to raw, and *Bifidobacterium* was decreased only in relation to toasted.

4 Discussion

Here, we have examined in which specific ways representative foods of Mediterranean and Western diets can alter gut microbial communities. The experimental design employed has certain limitations, such as the fact that we can only observe changes that occur rapidly; however, the power of diet to alter the composition of the gut microbiota in the short term has been demonstrated [54]. Also, our set up reflects mainly the punctual effects of drastically limiting the substrates available to the microbiota to those present in a single food. Although effects on overall parameters such as alpha diversity may indeed mostly reflect this immediate change, nevertheless, the fermentation experiments reveal those bacterial taxa that are most affected by the availability of a given food.

The initial composition of the microbiota of the three individuals defined most of the compositional variability in our data after the food fermentations. Moreover, many taxon abundance changes were detected in a single individual, suggesting that they may be composition-dependent. Dietary intervention studies have also suggested that the response of the gut microbiota presents high inter-individual variability, and that certain microbial taxa can be responsive or resistant to dietary changes [79,115]. Further studies with expanded numbers of individuals will be required to understand composition-dependent changes and the taxa that drive them. Nevertheless, we have detected several shared trends across individuals, which likely represent the more robust effects. These include compositional differences between all plant-based and animal-based food fermentations, as well as differences in comparisons at food category and individual food level. In the following paragraphs, we discuss the significant composition differences that occurred in all three individuals.

Animal and vegetable fats was the food category that produced the most changes in the gut microbiota, generally resulting in higher abundances of bacteria of the phylum Firmicutes and lower abundances of the genera *Bacteroides* and *Bifidobacterium*, in accordance with the results of [116] for mice fed a high-fat diet. Although there are not many studies on the effects of dietary fatty acids on the microbiota *in vitro*, the ability of the microbiota to grow on fatty acids has been reported, as well as a large effect of these substrates on microbiota composition [88]. Our results showed that the use of fats (especially butter) as substrates induced an increase in the abundance of potentially beneficial taxa such as *Faecalibacterium* (mainly *F. prausnitzii*), *Roseburia* (*R. inulinivorans*) and *Blautia*, as previously described in *in vivo* studies with a medium to low percentage of fatty acids in the diet [77]. Although *F. prausnitzii* is considered one of the main butyrate producers in the human gut and has been attributed significant anti-inflammatory properties, evidence on the effect of diet on the modulation of this species is still conflicting. Nevertheless, monounsaturated fatty acids have been associated with its increase [77,117]. *Blautia* and *Roseburia* are members of the Lachnospiraceae, a phylogenetically and morphologically heterogeneous family that contains many members described as having the ability of hydrolyzing carbohydrates to produce butyrate and other SCFAs [118]. *Blautia* has been negatively associated with visceral fat accumulation [118] and decreases in diseases such as diabetes, Crohn's disease or colorectal cancer [119,120]. It has also been reported to increase in high-fat diets [117,121], in accordance with our results; however, there is no consensus, as other research suggests the opposite [122]. In addition, animal and vegetable fats also increased the abundance of *Lachnospira*, a butyrate producer mainly reported to increase with high-fiber foods, although it has also been found to increase with unsaturated fats and to correlate with olive oil consumption in vegans and vegetarians [121].

Many studies have assessed the effects of high fat diets and even of saturated and unsaturated fats on the gut microbiota (reviewed in Yang *et al.*, 2020). Broadly, saturated, and unsaturated fats have been reported to have opposite effects on gut microbiota modulation, with the latter producing an increase in the abundance of beneficial taxa such as *Akkermansia* and *Bifidobacterium*, and a decrease in potentially detrimental taxa such as *Streptococcus* and *Escherichia*. Our analyses also indicate some differences between saturated and unsaturated fats. Butter, which contains mainly saturated fatty acids, produced some of the greatest effects of all foods, consistently resulting in higher abundances of the Lachnospiraceae *Blautia*, *Roseburia*, *Lachnospira*, *Agathobacter*, *Fusicatenibacter* and *Lachnoclostridium* in comparisons to a large variety of foods. In contrast, Lachnospiraceae genera were detected to increase in vegetable oils, which contain mainly unsaturated fatty acids, only in a much smaller range of comparisons. Moreover, *Blautia* did not increase in fermentations with either olive or sunflower oil, while *Roseburia*, *Lachnospira* and *Fusicatenibacter* increased in some comparisons involving olive oil, but not with sunflower oil.

Numerous differences in the fermentative microbiota were also detected in comparisons involving dairy products or fish. Some previous studies have assayed the effects of fish oil on the gut microbiota, although without considering the contribution of the whole fish, detecting increases in Bacteroidetes, Lachnospiraceae and *Bifidobacterium*. In our fish fermentations, we mainly detected high abundances of *Lachnoclostridium* and *Ruminococcus*. These genera are known SCFAs producers, but they have also been correlated with trimethylamine N-oxide (TMAO), which is generated in the liver from trimethylamine (TMA) produced by the gut microbiota from choline and carnitine (present in sources such as eggs, beef, pork and fish). In agreement, *Lachnoclostridium* and *Ruminococcus* have been associated with risk of atherosclerosis and cardiovascular disorders [123,124]. In addition, *Lachnoclostridium* has been reported to increase in high-fat diets, and to be positively correlated with visceral fat through decreasing circulating acetate levels [121]. On the other hand, *Ruminococcus* has been associated with various contrasting dietary patterns, including long-term fruit and vegetable consumption, low dietary fiber intake and omnivore diets [54,123].

Our results also revealed increases in the abundances of the genera *Lachnoclostridium* and *Ruminococcus* in fermentations of dairy products, along with increases of *Bifidobacterium*, *Agathobacter*, Erysipelotrichaceae UCG 003 and *Romboutsia*. In a systematic review, Aslam *et al.*, 2020 [125] reported a generalized increase with dairy consumption of *Bifidobacterium* and *Lactobacillus*, which are widely considered to be beneficial to the host, although they highlighted a lack of consensus due to the wide variety of dairy products with very different nutritional composition. On the other hand, members of the Erysipelotrichaceae have also been positively associated with dairy intake [126]. However, elevated abundances of these organisms are likely detrimental since they are enriched in the intestines of obese humans and mice, have been associated with symptoms of the metabolic syndrome and promote obesity in gnotobiotic mice [127]. The dairy food that produced the greatest variation in the microbiota in our experiments was gouda cheese, which has a high fatty acid content. This further supports a fundamental role for fatty acids in modifying the composition of the gut microbiota. Furthermore, choline and choline phospholipids, like phosphatidylcholine, are particularly enriched in high-fat dairy products such as gouda and butter, which likely contributed to the significant effect of these two foods on *Lachnoclostridium* abundances.

It is remarkable that most elevated taxon abundances in the microbiota were detected in fermentations with animal and vegetable fats, dairy products, and fish. Overall, fermentations of these foods often produced higher abundances of taxa of the Lachnospiraceae family, with *Lachnoclostridium* standing out in the three food categories. In contrast, significant differences involving fruits, vegetables and grain-based products mostly represented reduced abundances of genera in comparison to these animal-based food categories. This may suggest that the short fermentation processes used in our experiments were not sufficient to favor increases of specific genera when carbohydrate-rich foods were employed. As most intestinal bacteria are mainly adapted to growth in the presence of the complex polysaccharides and fibers that reach the colon, many taxa present in the inoculum must have been able to take advantage of these substrates. Therefore, a short fermentation may not have provided enough time to select for taxa having relatively small growth advantages with respect to other carbohydrate fermenters. Longer fermentation times may thus be needed to amplify growth rate differences among taxa with specific carbohydrate-rich foods. In contrast, the ability to grow on proteins and, especially, fats are likely more unevenly distributed across gut bacteria, so that short fermentations with foods rich in these substrates may more readily select for subsets of specialized bacteria, resulting in significant increases in their abundances. In this respect, it is noteworthy that *Lachnoclostridium* is a known protein degrader and is the most abundant gut bacterium capable of metabolizing the choline obtained from high-fat foods [124]. David *et al.* 2014 also reported that animal-based diets had a greater impact on gut microbiota composition, particularly an increase in the abundance of bile-tolerant microorganisms, presumably due to increased bile acid secretion as a result of the high fat intake linked to such diets. However, in our *in vitro* experiments there is no regulation of bile acid level depending on the food, suggesting that the effects of animal-based products on gut microbiota composition are also influenced by other factors. In accordance, the taxa favored by animal-based diets *in vivo* in the work by David *et al.* are the bile-resistant *Bilophila*, *Bacteroides* and *Alistipes*, which were not increased by fatty foods in our experiments.

Regarding cooking methods, it is well established that they affect food digestibility and nutrient bioaccesibility [103,128], and they may impact extensively on gut microbiota structure and functionality [104]. On one hand, cooking methods that apply high temperatures, such as frying or grilling, favor cellular break-down and release of chemicals into the environment [106,107,129]. This may favor bacterial growth by facilitating the uptake of bioactive compounds. However, released phytochemicals may be harmful for some bacteria [130], while others may have the enzymatic equipment to metabolize such phytochemicals and grow on them [59]. On the other hand, cooking favors specific chemical reactions, such as chemical browning in protein and sugar-rich foods. Chemical browning involves a plethora of chain reactions that yield numerous compounds, such as furanic compounds, which have inhibitory effects on some bacteria [131]. Chemical browning can also yield melanoidins, known to have a fiber-like behavior and therefore promote the growth of some beneficial species [132]. Accordingly, cooking may have a complex effect on gut microbial communities.

However, to date, few studies have been conducted on the effect of cooking methods on the composition of the gut microbiota [103,104,108,133]. Although these studies have collectively evaluated the effect of different cooking processes on meats, legumes, vegetables, cereals, and fruits, none has addressed this issue at a large scale by analyzing a wide range of foods within the same study. Here, we have examined separately the effects of cooking methods on a variety of different food types.

In our experiments, frying resulted in a lower abundance of Ruminococcaceae UBA1819 in comparison with roasted foods for all food categories considered together and in all individuals. This taxon is a butyrate producer known to increase with consumption of soluble fibers and to correlate negatively with subcutaneous and epidermal fat, cholesterol and weight gain [134], suggesting that its decrease with fried foods is undesirable. This result is particularly remarkable in that one could expect the effects of cooking methods to be masked by those of food composition, suggesting that frying and roasting have strong effects across food categories. Previous work in our group identified frying as the cooking method that produced the highest amounts of furosine and furfural (used as indicators of the Maillard reaction) and found it to be correlated with the abundance of different taxa depending on the food [104].

Our results also highlighted several trends when comparing cooking methods within the different food categories. However, these results were not detected across individuals, indicating that they are likely dependent on the composition of the microbiota and suggesting that cooking method effects may be highly personalized. Frying resulted in significant taxon abundance differences with other methods within various food categories, mainly fruit, vegetables and bread, resulting mostly in higher and lower abundances of genera within the Lachnospiraceae and Ruminococcaceae families, respectively, including lower abundances of the aforementioned Ruminococcaceae UBA1819. Similar results were also obtained for grilling, a method that resembles frying in reaching elevated temperatures and employing oil. These high temperature treatments have strong effects on the fibers and other complex carbohydrates present in plant-based foods, on which members of both the Lachnospiraceae and Ruminococcaeae are specialized to grow [135], but such effects might facilitate the utilization of these substrates for some bacteria while hindering it for others. Olive oil retained in grilled and fried foods may also have contributed to Lachnospiraceae increases, as genera of this family were detected at high abundances in olive oil fermentations. However, grilling and frying did not result in Lachnospiraceae increases in meat. Another relevant observation regarding the effect of frying on meat is that *Lactococcus*, particularly *L. piscium*, known to be one of the predominant taxa within spoilage microbial communities in cold-stored meat products, was detected at lower abundance in fried meat, suggesting that frying may contribute to reduce the presence of these bacteria.

Boiling was another cooking method that yielded significant differences against other methods in various food categories, mainly fish and meat. Generally, we detected a lower abundance of many Firmicutes genera when comparing boiled foods with other methods. This result may be due to several complementary causes. On one hand, the loss of hydrosoluble nutrients such as vitamins and minerals may limit the growth of some bacteria. On the other, the lower temperature reached during boiling will result both in lower levels of cellular breakdown and in a paucity of Maillard reaction products, which would otherwise be abundant when protein-rich foods such as fish and meat are prepared at higher temperatures. The lack of Maillard reaction products such as melanoidins, which are fiber-like structures that can be metabolized by some bacteria, along with the lower levels of cellular breakdown, may have reduced the substrates available for the growth of Firmicutes genera in boiled fish and meat.

The *in vitro* approach followed in this work has revealed the short-term effects of specific foods on the gut microbiota of different individuals. Many of the detected effects have been particular to single individuals, highlighting likely dependencies on initial gut microbiota composition and the highly personalized nature of responses to food. However, some shared trends across all individuals have also been manifested, such as the significantly different impacts of foods derived from plants and animals. Importantly, the fact that this is detected *in vitro* implies that bile-acid levels can not be the only factor affecting the composition of the gut microbiota when following an animal-based diet. Our findings also highlight the substantial effects of vegetable and animal fats, especially butter, which have a positive impact on the abundance of potentially advantageous taxa including *Faecalibacterium*, *Roseburia*, and *Blautia*. However, butter and other high-fat animal foods, such as dairy products and fish, also resulted in higher abundances of *Lachnoclostridium*, which has been associated to several diseases.

We also identified that frying and boiling produced the most distinct effects on the microbiota when contrasted to other methods of cooking a particular food, maybe due to their extreme positions in the spectrum of generation of Maillard reaction products. However, the impact of cooking methods was highly varied across individuals, highlighting the need for further research that takes into account individual variation to unravel the complex effects of cooking on the gut microbiota. This knowledge will pave the way to a personalized modulation of the microbiota for precision nutrition.

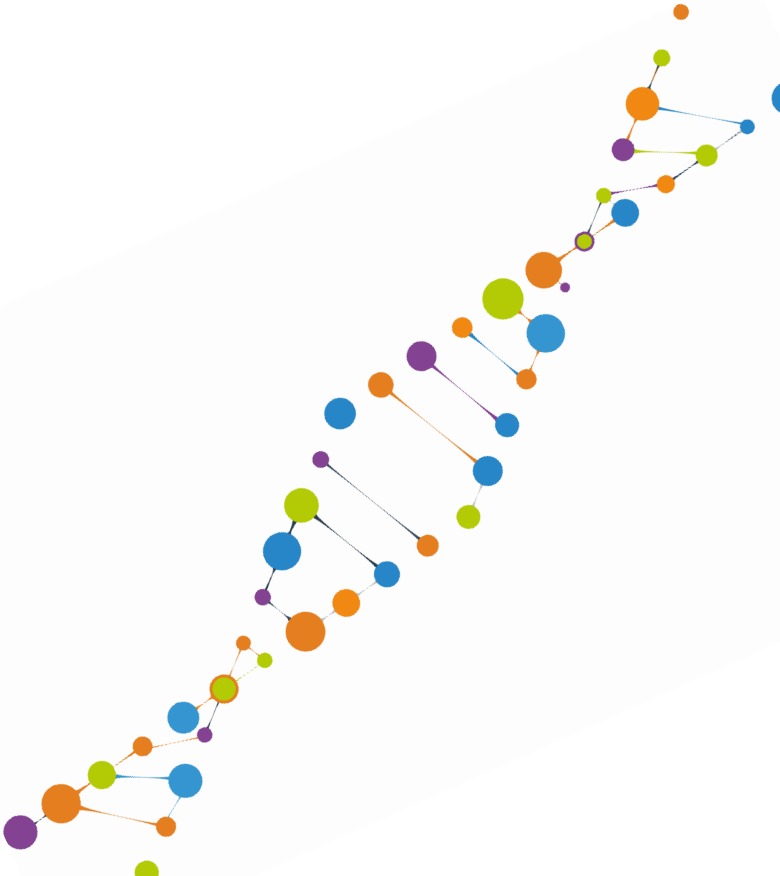
5 Conclusions

1. The fermentative gut microbiota, despite inter-individual variability, shows several common changes due to the effect of various foods.

2. Animal-based foods and animal and vegetable fats significantly impact the fermentative microbiota.

3. Cooking methods are capable of influencing the effect that foods have on the fermentative microbiota Their influences vary according to the food, with frying having the greatest impact.

Chapter II



Chapter II. Evaluation of the *in vitro* effects of foods on the gut microbiota of lean, obese, celiac, and allergic children.

1 Introduction

The gut microbiota and gut mucosa maintain a symbiotic relationship that contributes to substantial metabolic, immunological, and gut protective functions. The gut microbiota plays an essential role in nutrient metabolism, participating in lipid metabolism, vitamin synthesis, etc. and ferments the dietary polysaccharides and polyphenols that arrive to the colon poorly digested producing SCFAs, which are used as a source of energy by the intestinal cells[136,137]. The gut microbiota also has the ability to metabolize xenobiotics and drugs, and plays an important role in antimicrobial protection, immunomodulation and maintaining the integrity of the gut barrier[136].

In recent years, there has been a growing body of evidence linking several diseases, directly or indirectly related to dietary patterns, to the gut microbiota [138]. This is notably exemplified by obesity, defined by the World Health Organization as the condition of having a body mass index exceeding 30, which represents one of the most significant global health challenges. Obesity is strongly associated with metabolic dysregulation, chronic inflammation, oxidative stress and an increased risk of cardiovascular disease and diabetes among others [136]. Following the finding that the microbiota of obese mice could increase the fat content of germ-free mice, research efforts have progressively revealed differences in the composition of the gut microbiota between obese individuals and their healthy counterparts, as well as variations in microbial diversity

[27,28,139,140]. It has been shown that the obesity-associated microbial imbalance can be reverted through, for example, prebiotics/probiotics and balanced dietary therapy, improving some of the related symptoms such as insulin resistance[141,142].

Celiac disease is an autoimmune condition characterized by a specific serological and histological profile triggered by the ingestion of gluten in genetically predisposed individuals (with high familial recurrence of 10-15%). In recent years, the number of diagnoses has increased, mainly due to the greater availability of sensitive and specific screening tests, reaching a reported prevalence of 0.5-1%. Innate immunity plays a critical role in the initiation of celiac disease, releasing proinflammatory cytokines, followed by the adaptive immune response [143]. Numerous studies suggest that extensive exposure to non-pathogenic commensal microorganisms during early life is associated with protection against celiac disease. Specific differences in the presence of microorganisms, including *Lactobacillus*, have been recorded. However, it is necessary to emphasize the need for further research to elucidate how the composition of the gut microbiota may influence the gluten tolerance loss [143–145]. In such cases, the primary treatment approach involves following a gluten-free diet, which, combined with the multiple alterations that occur in the gut, can profoundly disrupt the composition of the microbiota. This can frequently result in intestinal dysbiosis in these patients, which is identified by a decrease in probiotic species and an elevation in pro-inflammatory bacteria, thereby contributing to disease progression [32,33,146].

Food allergies are also a pathological immune response triggered by ingesting a food protein antigen. Their prevalence rates range from 0.7 to 19.9% as verified and self-reported food allergy in European children

[147,148]. Four categories of immune-mediated food allergies have been identified, including IgE-mediated, non-IgE-mediated, mixed or cell-mediated reactions. Cow's milk allergy is the most prevalent food allergy in infancy and early childhood. It has been found to be mediated by both IgE-dependent and independent pathways [35]. The prevalence of allergic disease has risen significantly in recent decades due to environmental and lifestyle changes, such as high-fat and high-protein diets, as well as a limited exposure to microbes and/or antibiotic administration in early life. It is widely acknowledged that the early microbial colonization of the gut has a significant impact on overall health throughout one's life. For example, reduced diversity of gut microbes during early infancy has been linked to increased risk of allergic diseases in childhood, as well as subsequent food sensitization [35,149]. Numerous studies have revealed that children diagnosed with milk allergy exhibit an altered gut microbiome, such as lower concentration of *Bifidobacterium,* but it remains uncertain whether dysbiosis is the cause or consequence of disease [34,36]. In recent years, an increasing number of research studies have been published that advocate treating this pathology by modulating the intestinal microbiota through probiotics, prebiotics, synbiotics or fecal microbiota transplantation [35].

Many pathologies have been observed to be significantly associated with changes in gut microbiota composition or diversity, often leading to dysbiosis. Since dietary intake is a major factor affecting the composition of the gut microbiota, and the diets of many individuals suffering from these pathologies are disturbed, it is believed that personalized nutrition could be a valuable method for deliberately shaping the composition of the gut microbiota. To this aim, obtaining a thorough comprehension of the response of microorganisms to specific dietary components is essential. Therefore, taking into account the diversity and the composition of the intestinal microbiota across various pathological states, we carried out *in vitro* food fermentations to determine the changes of the gut microbiota according to the characteristics of each food item. In this instance, the inocula were obtained from healthy children and those with obesity, celiac disease, and allergy. The primary aim was to identify dietary effects on the gut microbiota in function of the microbiota composition of each group of children.

2 Material and methods

Some of the methods used in this chapter, such as food procurement, handling, and cooking methods, fecal material collection, *in vitro* gastrointestinal digestion and fermentation, DNA extraction and 16S rRNA gene amplicon sequencing, have been previously described in **Chapter I –Material and methods** (page 30).

2.1 Study design

Fifty-five different foods, raw or cooked with up to five cooking methods, were *in vitro* digested and fermented to study their effects on the gut microbiota. Children were divided into 4 groups: obese, celiac and allergic and healthy (lean). Stool samples from 12 different children (3 children from each group) were used as separate inocula for the *in vitro* fermentations, which were performed in duplicate. Altogether, 159 combinations of different foods and cooking methods were tested per duplicate, this resulted in 318 samples per individual (321 considering controls and inocula) and 3852 fermentations in total.

Children had not taken antibiotics, prebiotics or probiotics for the three months prior to the assay and their age ranges between 8 and 10 years. At least three fecal samples of each donor were obtained and pooled together to reduce intra-individual daily variability. Fecal donors followed a regular Mediterranean diet before sample collection. Inclusion criteria for lean children were based on having a Body Mass Index (BMI) between the 5th and 85th percentile for their gender and age; and for obese children a BMI higher than the 95th percentile. Inclusion criteria for celiac children were diagnosis of celiac disease and elimination of gluten from the diet; while inclusion criteria for allergic children were diagnosis of food allergy.

Due to the large number of foods used, they were grouped at three different levels for the analysis of results: i) Animal-based and plant-based foods and ii) Food categories. The foods included at each level are described in **Table I-1**(**Chapter I**).

To validate that samples used as inoculum are representative of each group, 25 samples of individuals with the same inclusion criteria of each group were also sequenced and analyzed together with the inocula.

2.2 Bioinformatic analysis

The DADA2 (v1.8.0) package as implemented in R (v3.6.0) was employed for sequence read processing and forward and reverse read merging as well as clustering into ASVs [114]. Filtering and trimming parameters were as follows: maxN=0, maxEE=c(5,5), truncQ=0, trimLeft=c(17,21), truncLen=c(265,225), and rm.phix=TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for read merging. Reads were aligned against the human genome (GRCh38.p13) using Bowtie2 (v2.3.5.1) [150] and matches were discarded.

Samples sequenced in different sequencing runs were processed separately and joined using the “mergeSequenceTables” function from DADA2.

Taxonomic identification was assigned to ASVs using DADA2 and the SILVA v.138 reference database [151]. The MegaBLAST tool from BLAST v(2.10.0) was further used for those ASVs identified only at genus level with DADA2, requiring at least 97% identity for species-level assignment and a minimum difference of 2% between the first- and second-best matches. ASVs with a total number of counts lower than 10 were removed.

Fermentation duplicates were aggregated in the ASV count tables. In this process, for each taxon we multiplied the relative abundance in each of the duplicates by the mean of the total reads of both, then we took the mean of the values obtained for each duplicate. In addition, count tables at different taxonomic levels were created by adding those counts within the same taxonomic group.

2.3 Statistical analysis

Alpha and beta diversity indices (Shannon, Chao1 and Bray-Curtis) were computed using the vegan package (v2.5-2) [152] in the R platform. Differences in alpha-diversity were tested using the Wilcoxon rank-sum test. The Bray-Curtis dissimilarity index was employed to quantify the overall dissimilarity between two microbial communities, and it was used in PERMANOVA. PERMANOVA was performed using the adonis function from Vegan with 600 permutations. The BH procedure was applied for false discovery rate control. Canonical Correspondence Analysis (CCA) as well as ANOVA like permutation test to assess the significance was also conducted using the cca and anova.cca function from Vegan package.

Analysis of Composition of Microbiome with Bias Correction (ANCOM-BC2) was used to identify differentially abundant taxa among samples. This new ANCOM version provides an approach to correct the bias induced by unobservable differential sampling fractions across samples and takes into account the compositionality of the microbiome data. As it has implemented a regression framework, it can perform multigroup comparisons and consider fixed and random effects of the covariates. For the food comparisons, we tested the three food levels we have defined, including the food or food category to which a sample belongs as covariate in the fixed effects formula, and information on the individual as covariate in the random effects formula. To test the effect of the cooking methods, the covariate describing the cooking method employed was added to the fixed effects. The default parameters were used with prv_cut=0. In order to correct for multiple testing and multiple pairwise comparisons, ANCOM-BC2 has implemented a mixed directional false discovery rate method, and we used BH as control method. In addition, a normalized abundance table was generated using ANCOM-BC2 and was used for the beta diversity analysis based on the Bray-Curtis dissimilarity index.

3 Results

The 16S rRNA gene amplicon sequencing of the fermentative microbiota yielded a total of 348.150.860 reads, averaging 90.381,84 (±39.336,96) reads per sample (3852 samples in total). Taxonomic assignment identified reads belonging to 17 phyla, 88 families, 230 genera and 245 species. 264.578.262 reads were assigned at genus-level, averaging 68.685,95 (±29.020,18) reads per sample.

3.1 Microbiota composition

For *in vitro* fermentations, fecal samples from three individuals were employed for each of the analyzed groups: obese, celiac, allergic, and healthy lean children as reference group. Consequently, we first verified the representativeness of the samples utilized as inocula by comparing them with 25 additional samples in each group. As illustrated in **Figure II-1**, the samples corresponding to the inocula exhibit similar alpha and beta diversity values to those of the other individuals within the group. This observation signifies that the samples we employed as inocula are indeed representative of their respective group.

In order to have a better understanding of the differences in the effects of the foods among the different groups we are studying, we first analyzed the diversity and composition of the baseline microbiota (inocula) of the three children of each group who participated in the study. As shown in **Figure II-2**, the diversity and richness in the obese group, as well as the diversity in the allergic group, are lower than those in the lean reference group, although these differences do not reach statistical significance, likely due to the reduced number of samples per group. When comparing the microbiota composition with CCA and PERMANOVA (**Figure II-2C**), similar results were found. There are no compositional differences among the groups according to PERMANOVA, although the obese samples appear slightly segregated and CCA revealed significant differences (p-value 0.011) among the four children groups in terms of microbiota composition (**Figure II-2C**). However, the samples among the groups are moderately scattered, suggesting a great inter-individual heterogeneity.

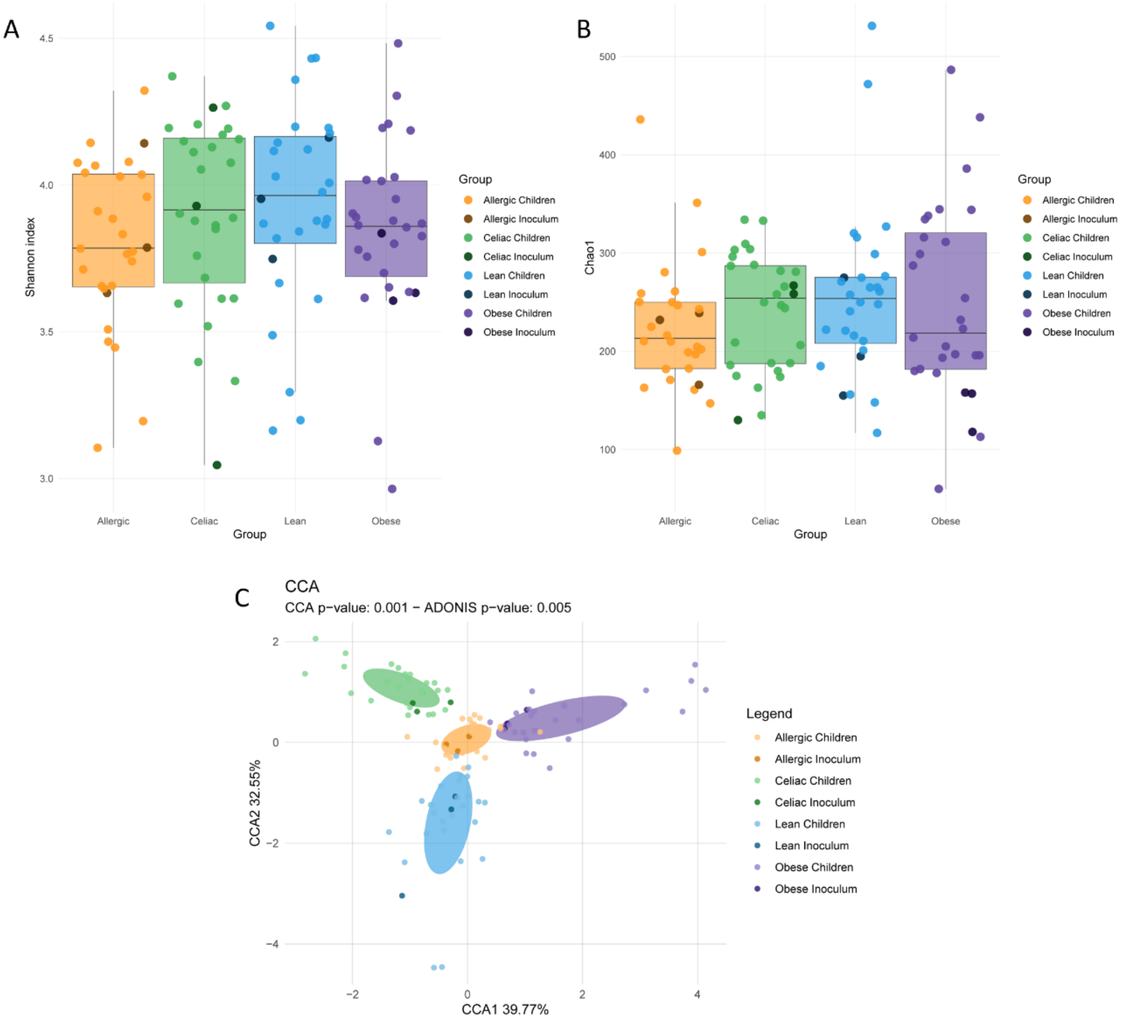


Figure II-1. Microbiota diversity of children by group. Individuals whose fecal samples were used as inocula are marked with darker colors. A) Shannon diversity index and B) Chao1 richness estimator of the gut microbiota grouped by allergic, celiac, lean, and obese. C) CCA of samples by group with PERMANOVA test for compositional comparisons. Colors indicate the group of the samples according to the legends. All these analyses were performed at the ASV level.

Afterwards, we proceeded to analyze the diversity of the samples fermented with the different foods and cooking methods (**Figure II-3**). Particularly, we compared the diversity and richness we observed in each group with that of the lean reference group and found significantly higher diversity in the lean group in both Shannon index and Chao1 compared to the rest.

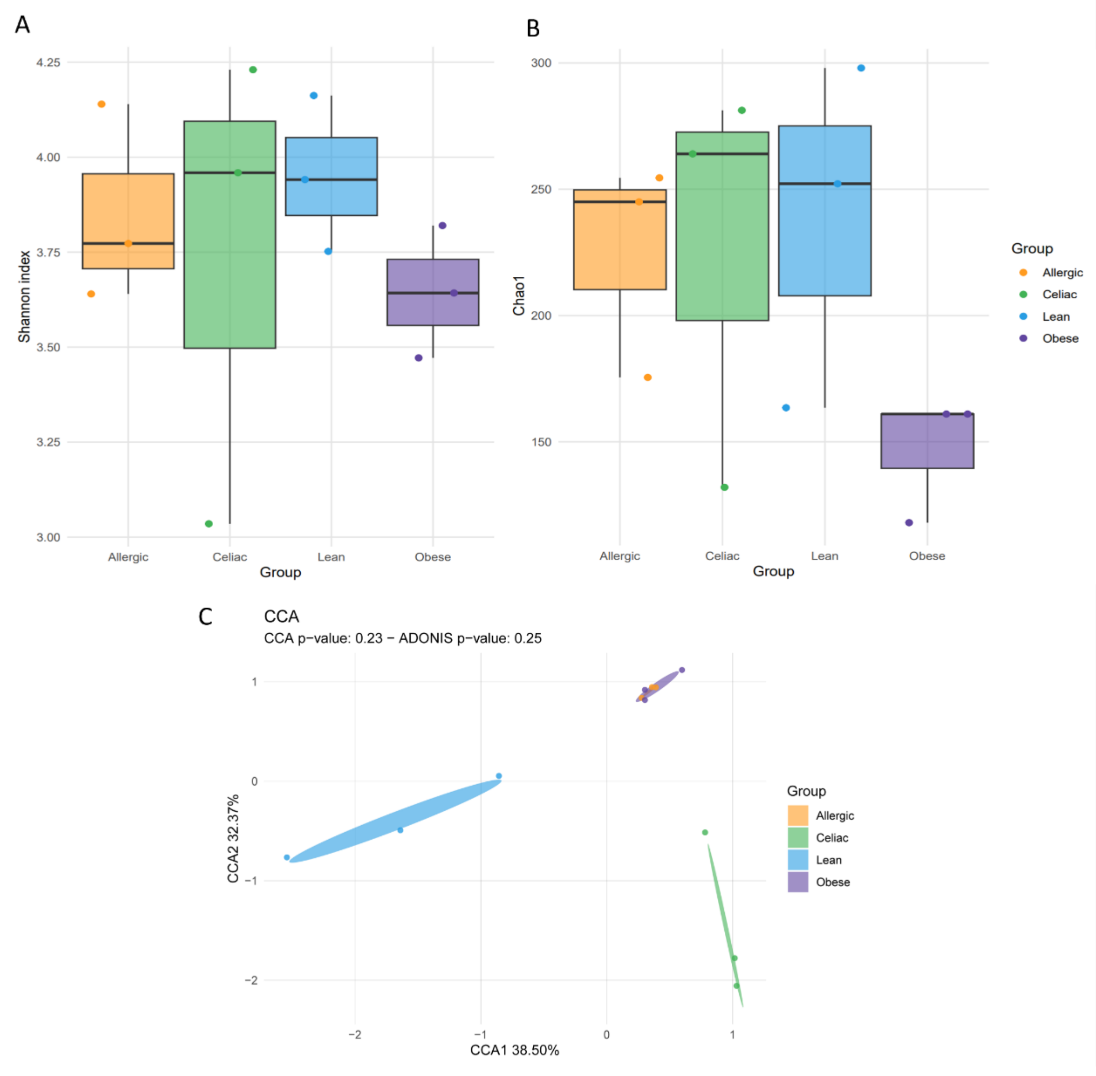


Figure II-2. Baseline microbiota diversity of children by group. A) Shannon diversity index and B) Chao1 richness estimator of baseline samples of each individual grouped by allergic, celiac, lean and obese. C) CCA of baseline samples of each individual by group with PERMANOVA test for compositional comparisons. Colors indicate the group of the samples according to the legends. All these analyses were performed at the ASV level.

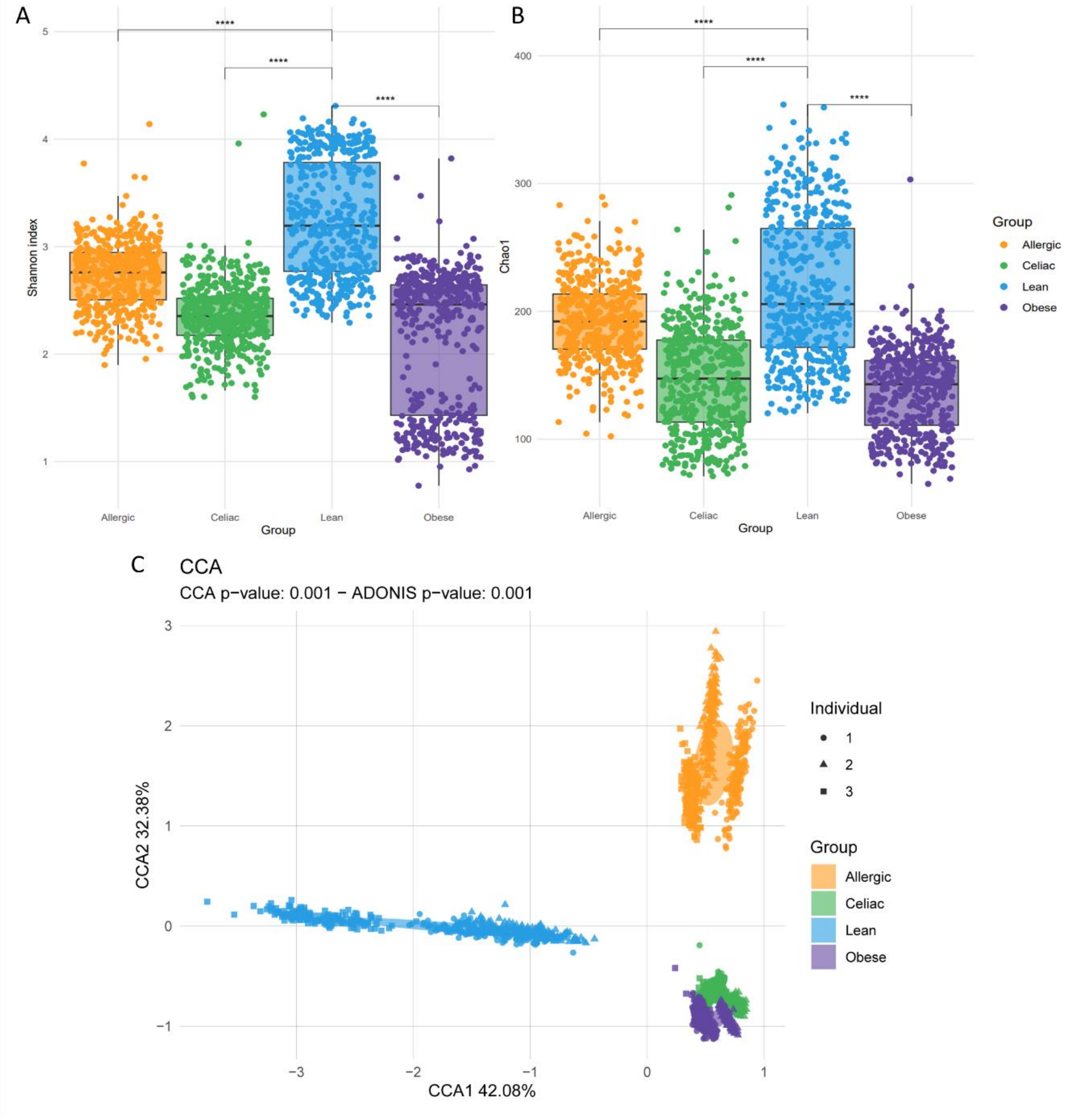


Figure II-3. Diversity of fermented microbiota by group. A) Shannon diversity index and B) Chao1 richness estimator of fermented samples grouped by allergic, celiac, lean, and obese. Wilcoxon test between groups and lean condition (as reference group) was performed to test for differences. BH procedure was used to correct for multiple testing. **** indicates q value < 0.0001. C) CCA of samples of each individual with PERMANOVA test for compositional comparisons. Colors indicate the group of the samples according to the legends. All these analyses were performed at the ASV level.

Moreover, most of the variance in microbiota composition when all samples were considered was caused by differences among individuals (**Figure II-3C**). Although the individual lean – 3 is substantially separate from the rest, the other groups remain also mildly stratified. Therefore, to study the effect of foods and cooking methods, we have considered these inter-individual variations.

In order to determine the effects of the different foods on the overall composition of the gut microbiota after fermentation, we first compared the growing bacteria in the food fermentations to those growing in a control fermentation containing water instead of food. We compared animal- and plant-based foods as well as each different food category with water (**Figure II-4A**). Although some variation in the level of dissimilarity with the control could be observed, none of the differences in overall microbiota composition reached statistical significance.

3.2 Differential abundance of taxa

To investigate in more detail the potential shifts of the fermentative microbiota we studied the differential abundance of taxa among different food fermentations using the ANCOM-BC2 methodology. As our main aim is to detect the differential microbiota responses of the groups to foods, we tested the bacterial abundance differences between foods and water in each group and compared them to the response in the reference healthy lean group. In addition, we established the baseline differences in microbiota composition among groups, by identifying differentially abundant taxa in the fermentation inoculum between the lean group and the rest (**Figure II-5**). We identified 5 differentially abundant genera, including *Anaerostipes* with higher abundance in all groups against lean, or *Butyricimonas* with lower abundance in obese and celiac against lean.

Comparing the growing microbiota in fermentations of animal and plant-based foods with those of water, we detected 48 differentially abundant genera in at least one food type and group (**Figure II-6**), with most of these differences being present in only one group. If we focus on differences that are observed exclusively with one type of food, the cases of *Odoribacter*, *Barnesiella*, and *Alistipes* stand out. These genera showed a decrease in abundance in fermentations of animal-based foods in the

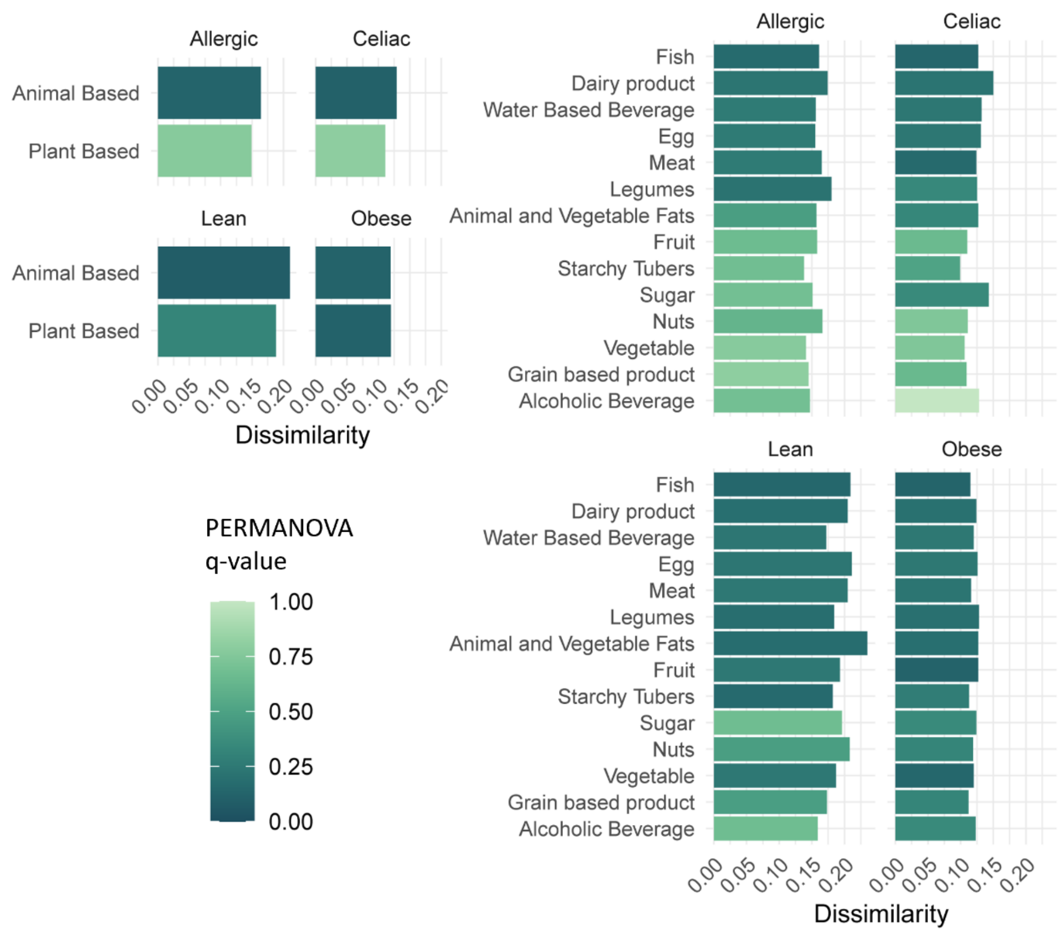


Figure II-4. Barplot representing the mean Bray Curtis dissimilarity of A) Animal- and Plant-based foods and B) Food categories against water. Colors represent q-value from PERMANOVA after multiple testing correction. No significant differences were found. Bray Curtis dissimilarity index was calculated from the ASV-level ANCOM-BC2 normalized counts table.

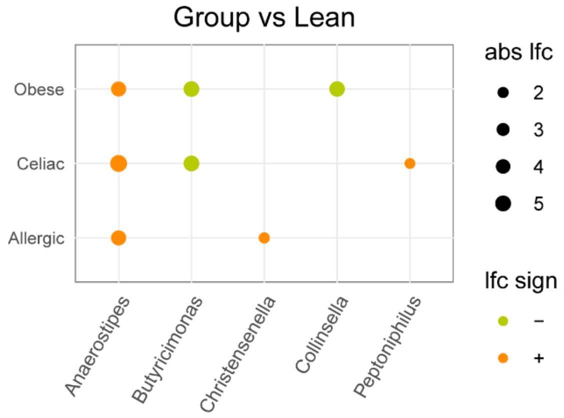


Figure II-5. Differentially abundant genera when comparing inoculum microbiota of obese, celiac, and allergic vs. lean. Dots reflect significant differences, representing the absolute value of log fold change (lfc) by size. Green and orange color indicate negative/positive lfc when comparing groups vs. lean.

celiac and allergic groups, and also in obese individuals in the case of *Alistipes*. The microbiota of the celiac group is further impacted by animal-based foods, resulting in an increase of *Ruminococcus*, *Butyricicoccus*, the Family XIII AD3011 group, Erysipelotrichaceae UCG 003, *Lachnoclostridium (L. edouardi)*, *Solobacterium (S. moorei)* and *Gemella*, as well as by plant-based foods, with a decrease of *Romboutsia* and an increase of *Bifidobacterium longum*. Likewise, the bacterial community of the allergic group is influenced by animal-based foods, leading to a decrease of *Faecalibacterium (F. prausnitzii)*, *Holdemania*, *Monoglobus*, *Roseburia*, Oscillospiraceae UCG 003, *Lachnospira* and *Negativibacillus (N. massiliensis)*, as well as an increase of *Romboutsia,* while plant-based foods resulted only in a decrease of *Bifidobacterium*. The microbiota of the obese group was modulated by plant-based foods lowering the *Dorea (D. formicigenerans)* and *Dialister (D. invisus)* abundances and by animal-based foods by decreasing the *Enterococcus* abundance. Detailed differential abundances at species level for the animal and plant-based food comparisons against the water control can be found at **Supplementary Material II-1**.

3.2.1 Different responses in the microbiota of obese and lean children

In order to identify those different responses when fermenting the diverse tested food categories between groups, we compared the abundance

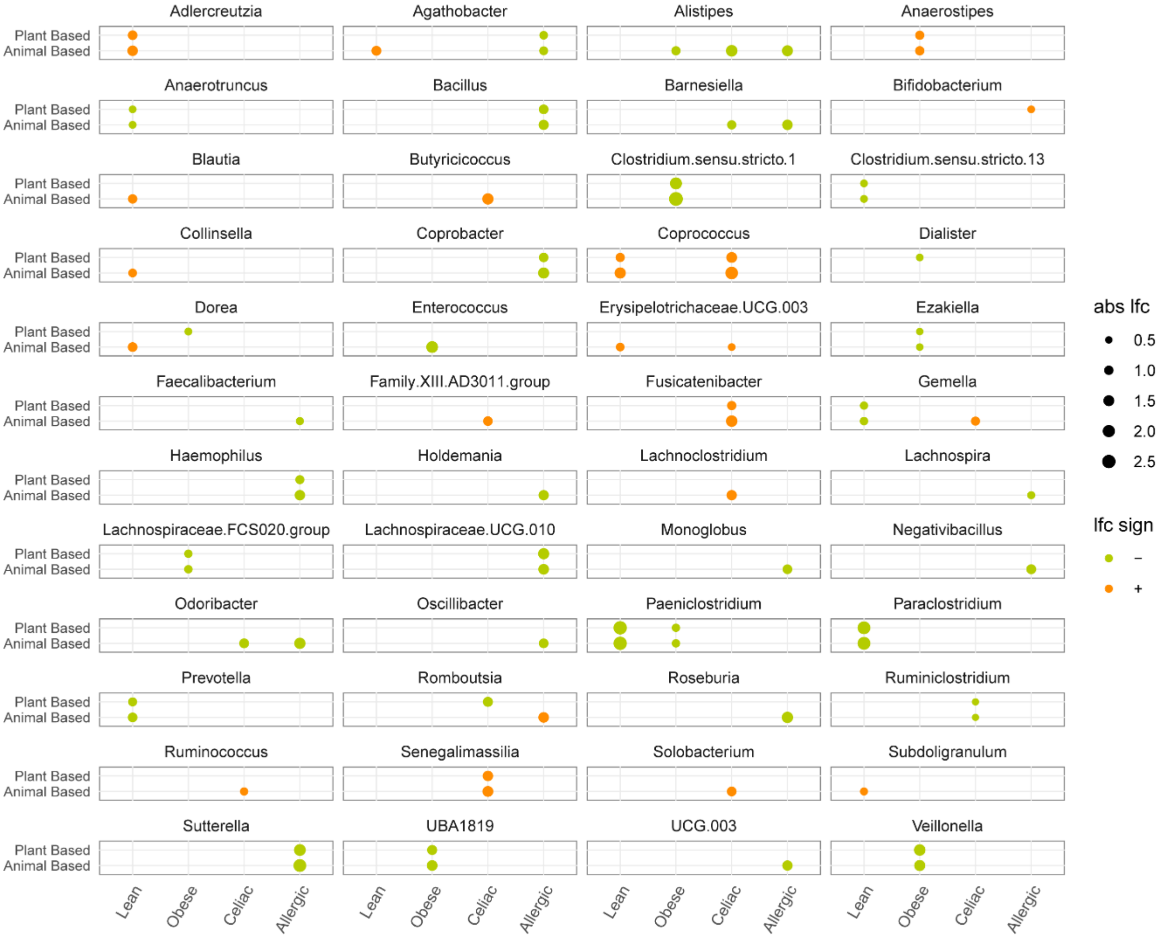


Figure II-6. Differentially abundant genera when comparing animal- and plant-based foods with water. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing animal-/plant-based foods vs. water.

differences of each food category against water of obese and lean group. As we can observe at **Figure II-7**, certain taxon differences were identified across the majority of the food categories, suggesting that these taxa are either favored or disfavored by the incorporation of any food during the fermentation as compared with the fermentation control. However, only *Paeniclostridium* decreased with all foods in both the obese and lean groups. In contrast, *Paraclostridium* and *Anaerotruncus* decreased only in the lean group, while *Coprococcus* and *Adlercreutzia* increased. On the other hand, the Lachnospiraceae FCS020 group, *Veillonella* and *Clostridium sensu stricto 1* decreased only in the obese group.

In the lean group, animal and vegetable fats and animal-based foods such as dairy products and fish resulted in increases of Oscillospiraceae UCG 002, Erysipelotrichaceae UCG 003, *Coprococcus*, *Adlercreutzia*, *Blautia*, *Agathobacter*, *Roseburia*, *Dorea* and *Collinsella*. In the obese group, these increases were not detected, but numerous other taxa were differentially abundant with respect to the water control. Most notably, *Anaerostipes*, especially *A. hadrus*, and *Roseburia intestinalis* were found with higher abundance exclusively in the obese group with animal and vegetable fats, dairy products, egg, nuts, and sugar, while *Weissella* and *Lactobacillus* increased with grain-based products. In contrast, *Alistipes* and Ruminococcaceae UBA1819 abundances decreased by the action of animal and vegetable fats, fish, grain-based products, and nuts, while *Agathobacter* and *Dorea* decreased with fruit and legumes. Detailed differential abundances at species level between water and food categories can be found at **Supplementary Material II-2**.

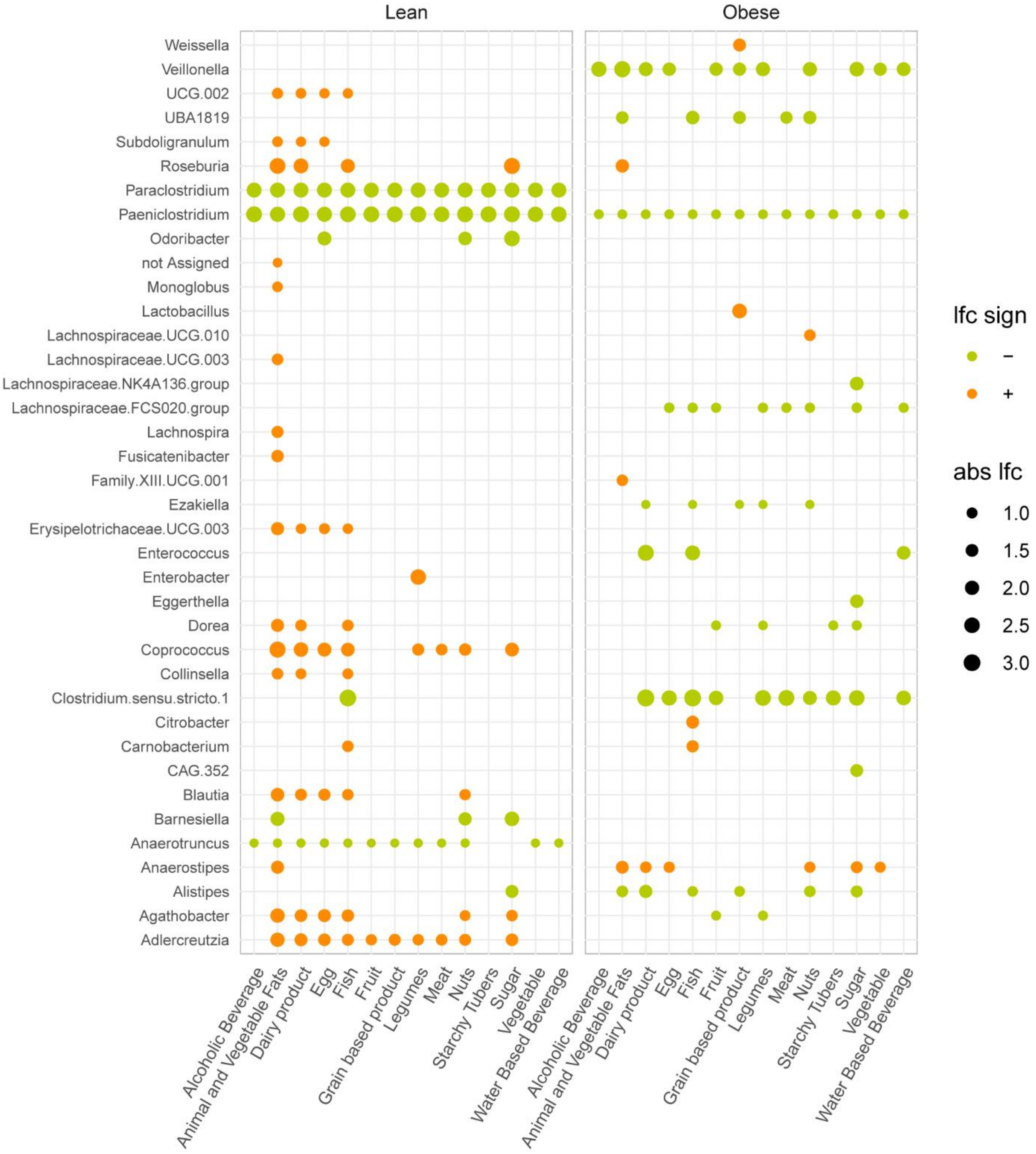


Figure II-7. Differentially abundant genera when comparing food categories in the x axis with water in lean and obese groups. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and Orange color indicate negative/positive lfc when comparing food categories vs. water.

3.2.2 Different responses in the microbiota of celiac and lean children

When comparing the responses between lean and celiac groups, we observed (**Figure II-8**) that only *Coprococcus* responded in both groups in most of the tested food categories (with an abundance increase). In addition, *Senegalimassilia* and *Fusicatenibacter* showed higher abundances in most food categories in the celiac group only. Moreover, in the celiac group, several taxa behaved similarly to what was observed in the obese group: *Anaerostipes* increased its abundance with animal and vegetable fats, dairy products, egg, and sugar, while Ruminococcaceae UBA1819 and *Alistipes* decreased their abundances with animal and vegetable fats, fish, grain-based products, and nuts (among others). Other responses that were unique to the celiac group include decreases of *Romboutsia* (with fruit, grain based products, nuts, starchy tubers, sugars, and vegetables) along with *Flavonifractor* and *Faecalibacterium* (with grain based products and starchy tubers), as well as increases of the Family XIII AD3011 group, *Ruminococcus*, *Lachnoclostridium* and *Butyricicoccus* (all increased with dairy products and meat, while fish also increased *Lachnoclostridium* and *Butyricicoccus* and animal and vegetable fats also increased *Ruminococcus* and *Butyricicoccus*).

3.2.3 Different responses in the microbiota of allergic and lean children

The microbiota response of the allergic group showed almost no common features with the lean group (**Figure II-9**). *Bacillus* and Lachnospiraceae UCG 010 were decreased by almost all food categories only in the allergic group.

Figure II-8. Differentially abundant genera when comparing food categories in the x axis with water in lean and celiac groups. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and Orange color indicate negative/positive lfc when comparing food categories vs. water.

Figure II-9. Differentially abundant genera when comparing food categories in the x axis with water in lean and allergic groups. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and Orange color indicate negative/positive lfc when comparing food categories vs. water.

In addition, abundance decreases were identified in the allergic group in the animal-based food categories for *Coprobacter*, *Haemophilus*, *Odoribacter* and *Alistipes*, the latest sharing decreases in several food categories with the obese and celiac groups. Furthermore, egg resulted in decreases of numerous genera in the allergic group, including *Negativibacillus*, *Lachnospira*, the Lachnospiraceae NK4A136 group, *Holdemania*, *Roseburia*, *Barnesiella*, *Butyricimonas* and *Faecalibacterium.* Fish also resulted in decreases of most of these taxa, while animal and vegetable fats decreased only *Barnesiella*, *Butyricimonas* and *Faecalibacterium* and legumes decreased only *Holdemania* and *Roseburia.*

3.3 Differential effects of cooking methods on taxon abundance

In order to be able of detect the differential effects of cooking methods on each tested group, we compared the effects of those foods in each food category that were cooked with the same methods against the control condition. A summary of the most representative changes induced by the cooking methods by food category and groups is shown in **Figure II-10**, while detailed results can be found in **Supplementary Material II-3**.

Most of the detected effects on taxon abundance took places for all the cooking methods within a specific food category and group, indicating that these changes were mainly attributable to the foods and not so much to the cooking methods. Here we will highlight only those changes that were influenced by the cooking methods.

3.3.1 Bread

In the obese group *Roseburia*, Family XIII UCG 001 and *Citrobacter* were increased by fried bread, while the frying process decreased the abundance of *Veillonella* and *Faecalibacterium*. On the other hand, toasting increased *Citrobacter* and decreased *Agathobacter*, *Colidextribacter* and *Veillonella*.

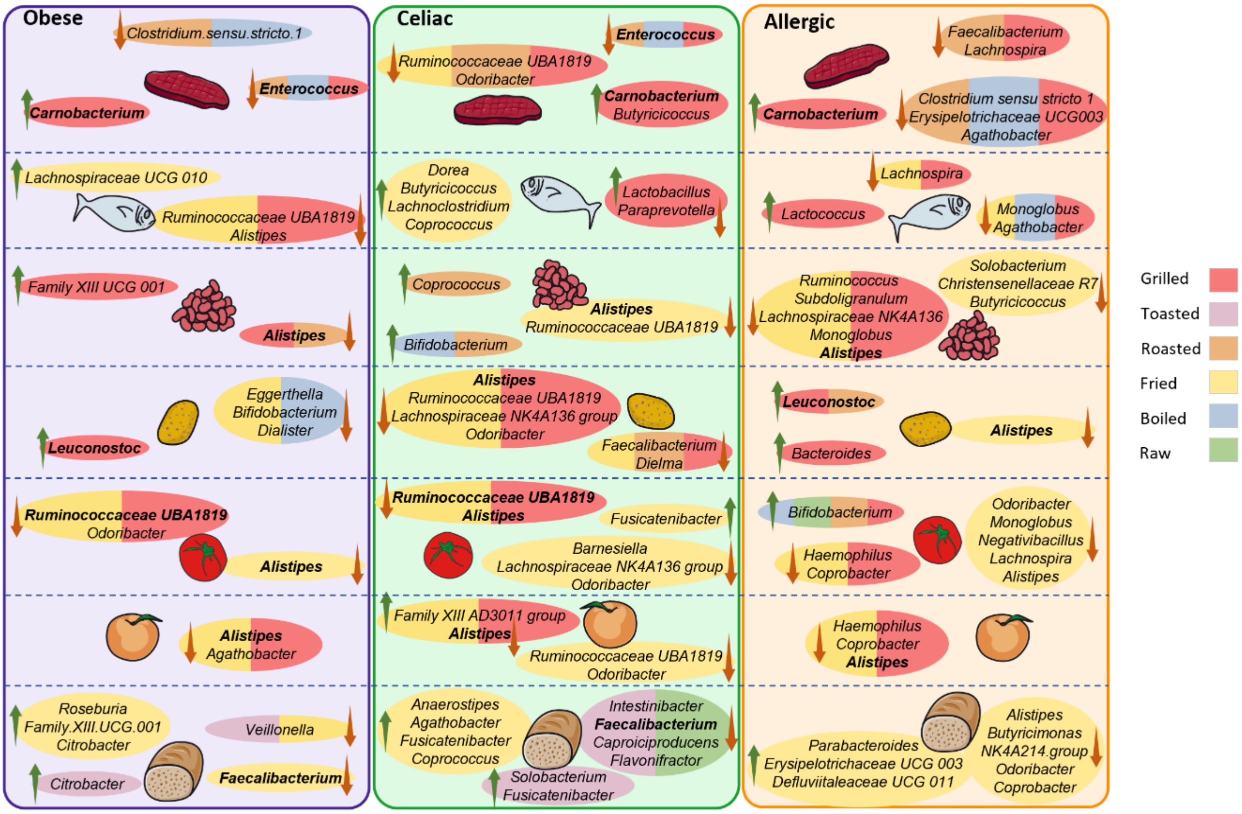


Figure II-10. Graphical summary of main differential outcomes in relative taxon abundance between cooking methods and water by food. Differences are shown per group and food category. For each group, the most representative taxa whose abundance is reduced or increased with the corresponding cooking method are shown. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. This figure is available in higher quality through the link in **Supplementary Material.**

In the celiac group we detected the largest number of differences associated with bread cooking methods. Frying increased the abundance of *Fusicatenibacter*, *Coprococcus*, *Agathobacter* and *Anaerostipes.* Raw and toasted bread produced a decrease in the abundance of *Anaerostipes*, *Caproiciproducens*, *Faecalibacterium*, *Flavonifractor* and *Intestinibacter*, while only raw bread resulted in a decrease of *Agathobacter,* and toasted bread resulted in increases of *Solobacterium* and *Fusicatenibacter.*

The allergic group were mainly influenced by bread when fried, with decreases in the abundance of *Alistipes*, *Butyricimonas*, *Coprobacter*, the NK4A214 group and *Odoribacter,* and increases in the abundance of *Parabacteroides*, Erysipelotrichaceae UCG 003 and Defluviitaleaceae UCG 011 (also increased by toasting).

3.3.2 Fruit

This food category stands out by the effect of frying and grilling as reducers of *Alistipes* abundance in obese, celiac, and allergic groups, as well as *Agathobacter* in the obese, and *Coprobacter* and *Haemophilus* in the allergic.

3.3.3 Vegetables

The abundance of *Alistipes* was reduced by fried vegetables in obese, celiac and allergic groups, while UBA1819 was reduced by both frying and grilling only in the obese and celiac groups.

The obese group showed reduced abundances of *Megasphaera* with boiled vegetables and of *Clostridium sensu stricto 1* with roasting and grilling. In the celiac group, the frying process seems to have a strong influence by diminishing the abundance of *Odoribacter*, the Lachnospiraceae NK4A136 group and *Barnesiella* as well as increasing the abundance of *Fusicatenibacter*. In addition, raw and boiled vegetables reduced the abundance of *Roseburia* in the celiac group. The allergic group response to vegetables was also very affected by the frying process, which lowered the abundance of *Odoribacter, Negativibacillus, Monoglobus, Lachnospira, Haemophilus* and *Coprobacter* (the latter two also lowered by grilling). Furthermore, *Bifidobacterium* abundance in the allergic group was increased by all cooking methods except frying.

3.3.4 Starchy Tubers

Leuconostoc increased with grilling and roasting of starchy tubers in the allergic group, and only with grilling in the obese. In the obese group, frying and boiling of starchy tubers decreased the abundances of *Eggerthella*, *Dialister* and *Bifidobacterium.* In contrast, *Bifidobacterium* increased with frying and boiling in the lean group. In the celiac group frying and grilling decreased *UBA1819*, *Odoribacter*, the Lachnospiraceae NK4A136 group, Ruminococcaceae *Incertae Sedis*, *Alistipes*, *Dielma* and *Faecalibacterium*, while roasting decreased only the latter two genera. All cooking methods except roasting reduced *Coprobacter* abundance in the allergic group, while frying also reduced that of *Alistipes.*

3.3.5 Legumes

Most of the abundance differences detected with legumes were produced independently of the applied cooking method. Some exceptions were seen in the obese group, where *Alistipes* was reduced only by grilling and roasting and Family XIII UCG 001 was increased only by grilling. Furthermore, in the celiac group, *Alistipes* and UBA1819 were found with lower abundance only in grilled legumes, while *Bifidobacterium* was increased in roasted and boiled legumes and *Coprococcus* only in roasted. The allergic group presented most of the differences, the majority of which were independent of the cooking method. The Lachnospiraceae NK4A136 group, *Monoglobus*, *Ruminococcus*, *Subdoligranulum, Butyricicoccus*, the Christensenellaceae R7 group and *Solobacterium* were reduced by roasted and grilled legumes, while the latter three genera were reduced by roasting only.

3.3.6 Animal and Vegetable fats

This is one of the food categories with the largest number of significant abundance differences. Some of them like *Anaerostipes* and *Alistipes* were increased and decreased respectively by fried and raw fats in obese, celiac, and allergic groups.

3.3.7 Meat

In the obese group, frying and grilling meat decreased the abundance of *Alistipes* and UBA1819, while frying only increased the abundance of Lachnospiraceae UCG 010. *Butyricicoccus*, *Coprococcus*, *Dorea* and *Lachnoclostridium* increased their abundances with fried meat in the celiac group, which also showed an increase of *Lactobacillus* and a decrease of *Paraprevotella with* grilled meat. In the allergic group frying, grilling, and boiling meat resulted in reductions in *Agathobacter*, *Monoglobus, Lachnospira* (except boiling), and the Lachnospiraceae NK4A136 group (except grilling), while grilling resulted in a *Lactococcus* increase.

3.3.8 Fish

We found many common responses among groups to the different cooked fish. *Carnobacterium* increased in all groups with grilled fish, while *Enterococcus* decreased with grilled, roasted, and boiled fish in the obese and celiac groups and *Clostridium sensu stricto 1* decreased with roasted and boiled fish in the obese and allergic groups. *Bifidobacterium* was also reduced by fried fish in the obese group. In the celiac group, frying, grilling, and toasting reduced the abundance of *Odoribacter*. On the other hand, increases of various genera were detected with different cooking methods, including *Butyricicoccus* with grilling, Erysipelotrichaceae UCG 003 with grilling and roasting, *Gemella* with frying and boiling and *Klebsiella* with roasting and boiling. Furthermore, in the allergic group grilling and roasting resulted in decreases of *Lachnospira*, *Faecalibacterium*, *Agathobacter*, *Clostridium sensu stricto 1*, and Oscillospiraceae UCG 003, while boiling also resulted in the decrease of the latter three.

4 Discussion

We have examined in which specific ways some representative foods of Mediterranean and Western diets affect gut bacterial communities from lean, obese, celiac, and allergic children and we have evaluated how differently the microbiota of obese, celiac, and allergic children responded to foods compared with the reference group, that of healthy lean children.

Several studies have described the presence of alterations in the composition of the gut microbiota in obese, celiac, and allergic children [28,56,153–155]. However, when analyzing the inocula used for the fermentations, we only observed in the obese group a trend towards a lower diversity so that its overall microbiota composition was distinct from those of the other groups, while the allergic and celiac groups were more similar to each other and to the lean individuals. This could be due to the low number of children analyzed per group and to the great interindividual heterogeneity among the children in each group, as previously reported in the literature [79,115].

The differences between groups increased when the *in vitro* fermentations were carried out, showing that the microbiota of the lean individuals was able to adapt more effectively to the fermentation process and the provided foods, resulting in a greater diversity than that of the other groups and suggesting that the initial microbiota is a key factor to bear in mind in personalized nutrition.

Numerous differences among the groups emerged during taxon differential abundance analysis, primarily driven by the intricate interplay between food and the fermentative microbiota. Consequently, a more insightful approach entails focusing on the differences arising with a single food or a specific subset of foods.

An illustrative example is the case of *Alistipes*, a genus with evidence of protective effects against some diseases such as colitis but also with a pathogenic potential described in other studies [156]. In our research, a reduction of this genus abundance occurred in response to animal-based foods in the obese, celiac, and allergic groups. This effect gathers attention, given that *Alistipes* has been described to increase due to diets based in animal products [54]. One potential explanation for this difference with *in vivo* studies is that our *in vitro* model does not involve a food-dependent bile regulation system, and therefore does not provide a positive selective pressure for this bile-tolerant bacterium. However, experiments in mice have shown that *Alistipes* increases with high fat diets even in animals without bile glands and that have been administered bile acid sequestering agents[157]. On the other hand, *Barnesiella* has been associated to anti-inflammatory actions[158], and a reduction of *Odoribacter* abundance has been linked to non-alcoholic fatty liver disease and IBD [159]. Thus, the lower abundance of these two genera within celiac and allergic groups when applying animal-based foods suggests that this food category could adversely affect allergic and celiac children through the decrease of these highly beneficial anti-inflammatory bacteria.

The celiac group suffered the growth of detrimental bacteria by the action of animal-based foods such as *Lachnoclostridium* and *Solobacterium* [160–162]. Similarly, animal-based foods resulted in the allergic group in the reduction of beneficial bacteria such as *Faecalibacterium prausnitzii, Roseburia* and *Monoglobus* among others [163–170]. In addition, plant-based foods raised the healthful *Bifidobacterium longum*, used frequently as probiotic [171,172]. Regarding the obese group, *Dorea* and *Dialister,* which have been described as increased in obese people, were lowered by plant-based foods.

As in the animal and plant-based food comparisons, *Alistipes* is the only taxon that showed an abundance change by a variety of food categories, mainly animal and vegetable fats, dairy products, fish, nuts and sugar, in all the studied groups except the lean reference group.

The microbiota responses of the obese group to the tested food categories were varied. Increases of the potentially beneficial bacterium *Anaerostipes* with the capacity to produce SCFAs from dietary fibers, resulted from a variety of foods [173–175]. It is remarkable to find that bacteria such as *Agathobacter* and *Dorea*, described in the literature for their ability to grow in the presence of fermentable carbohydrates, were reduced by the action of fruit and legumes; however, the fact that *Dorea* has been associated with IBS or found to be increased in obese people leads us to believe that this effect may be beneficial to this group. Grain-based products showed an increase of positive bacteria frequently used as probiotics, such as *Weissella* and *Lactobacillus*. Furthermore, the latter has shown the potential to help in weight loss in overweight and obese people [176–179]. In addition, although *Roseburia* is a genus known for its capacity for butyrate production through the fermentation of complex polysaccharides, the microbiota from obese individuals showed an abundance increase of this taxon by animal and vegetable fats. However, such an increase has been previously reported and could be helpful for the obese individuals due to the association of *Roseburia* with various health benefits [166,180,181].

It has been shown that a low-gluten diet, by reducing the amount of polysaccharides, leads to a decrease in gut-healthy bacteria and a parallel increase in unhealthy bacteria [68]. For example, *Faecalibacterium* is usually reduced in people with celiac disease, and it is interesting to find that it is reduced by the effect of grain-based products in the celiac group [32,182]. We have also found that foods such as meat and dairy products have caused an increase in the proportion of beneficial bacteria such as *Butyricicoccus* [183] or, along with animal and vegetable fats, of potentially harmful bacteria such as *Lachnoclostridium* and *Ruminococcus,* which have been associated with unhealthy states [162,184]. *Ruminococcus* has been described to increase by animal protein intake, however it has been found to be decreased in celiac patients, suggesting that further research is needed to shed light about the role of this taxon in celiac individuals [185,186].

The response of the microbiota from allergic individuals to nuts as well as to several animal-based foods, such as animal and vegetable fats, dairy products, eggs, fish, and meat, has been characterized by a reduction in the abundance of potentially beneficial genera such as *Coprobacter*, *Haemophilus*, *Odoribacter* and *Alistipes* [187–189]. These genera have been reported to decrease in response to a high-fat diet. In addition, *Odoribacter*, has been associated with the amelioration of food allergies following treatment with cyanidin-3-O-glucoside [190]. Furthermore, *Roseburia* and *Lachnospira*, which have been described in the literature to be increased in children with more food-allergy tolerance and in non-allergic people [153,191,192], were decreased by fish and egg, along with others beneficial bacteria. Overall, our findings suggest that the intake of animal-based foods by allergic individuals may decrease the presence of beneficial bacteria.

Different cooking methods have varied effects depending on the food, as the chemical alterations that occur are determined by the composition of the food itself. Moreover, these effects differ significantly between the studied groups given their different baseline microbiota composition, resulting in variable responses to foods and modifications induced by cooking processes. The disparities resulting from the different cooking methods employed for different foods will serve as a basis for future considerations in the development of personalized dietary regimens. This strategic approach aims to intentionally shape the composition of the gut microbiota, potentially offering significant assistance to individuals with gut microbiota dysbiosis, facilitating the transition to a healthier microbial composition. In general, it has been observed that frying induces the most significant changes in the microbiota, exhibiting the greatest variability among cooking methods for different foods. This phenomenon can be attributed to the high temperatures reached during frying, coupled with the immersion effect, which leads to a substantial increase in the temperature of the food, resulting in more pronounced chemical changes.

In addition, the residual oil adhering to the food can also influence the microbiota. Similarly, the next cooking method to produce notable discrepancies in the composition of the microbiota is grilling, which is characterized by elevated temperatures, although with a smaller surface area exposed to these conditions.

An illustrative example of the relevance of cooking methods is the frying of vegetables, which leads to a significant reduction in the abundance of various bacteria, an effect that is not observed with other cooking methods and is particularly noticeable in the allergic group. Remarkably, in this group, frying is the only cooking technique that does not increase the presence of *Bifidobacterium* in vegetables. The frying of vegetables also causes a decrease in several beneficial bacterial taxa, including *Odoribacter*, *Monoglobus* and *Lachnospira*.

The case of starchy tubers in individuals with celiac disease is also intriguing, as the processes of frying and grilling have been observed to lead to a reduction in numerous bacteria, the majority of which are considered beneficial[193–195], such as *Odoribacter*, Lachnospiraceae NK4A136 group, *Faecalibacterium*, or *Alistipes*. However, the effect of fried bread on the microbiota of individuals with celiac disease yielded noteworthy results, with frying increasing the abundance of two butyrate-producing genera such as *Anaerostipes* and *Agathobacter*[173,196].

Thus, the variations in the composition of the gut microbiota among the groups play a critical role in determining their responses to different foods and even different cooking methods. Despite the inherent limitations of the *in vitro* assays, this approach provides us the opportunity to unravel the intricacies of these interactions without the presence of external factors that may introduce confounding variables into the systems. Although further evidence and validation through other types of research, such as a nutritional intervention, are needed, gaining insight into these differential responses at this level will facilitate our progress toward the desired targeted modulation of gut microbiota composition and its substantial and beneficial therapeutic potential.

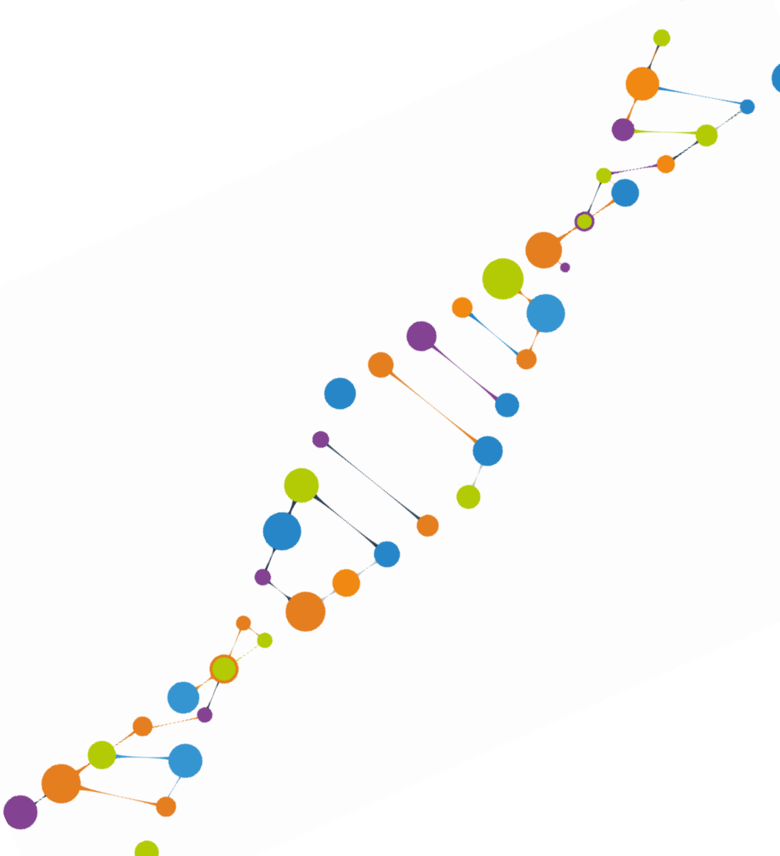
5 Conclusions

1. Individuals afflicted with pathologies associated with, or resulting from, dietary disturbances exhibit discernible differences in the composition of their gut microbiota, which directly influence their response to dietary intakes.

2. The cooking method can have a significant influence on the response of different microbial taxa to dietary components, and this response is closely linked to the individual's unique microbiota composition.

3. The use of dietary choices and cooking techniques emerges as a promising approach to achieve targeted and fully personalized modulation of microbiota composition.

Chapter III



Chapter III. Comparison of the *in vitro* effects of foods on the gut microbiota of children and adults

1 Introduction

The human gut microbiota has been widely studied, exhibiting a key role in the normal functioning of the host organism through many diverse mechanisms of interactions. These interactions take place since the establishment of the gut microbiota that may be influenced by several factors including genetics, maternal health and diet, delivery mode and breast or formula feeding. Gut microbiota colonization begins during the antenatal period due to the presence of bacteria in the amniotic fluid, placenta or meconium [197–199]. The mother's diet before and during pregnancy may impact the child's gut microbiota, while the neonatal and childhood periods are critical for gut microbiota development. The gut microbiota of children remains less diverse than that of the adult until 2-3 years of age, when diversity and composition become similar to the adult ones[200,201]. After this period, the diversity and composition of the microbiota continue to evolve, but much fewer studies have focused on later childhood and adolescence compared to infancy and the toddler years. Proper microbiota establishment is crucial, because about 60-70% of bacterial members of the microbiota remains unchanged throughout life [202,203].

In recent years, studies with large cohorts have been published that examine differences in the microbiota between children and adults, as well as the impact of short- and long-term diets on children microbiota [17,204,205]. Several studies have indicated that diet can prevent certain changes induced by antibiotics can be prevented in children. In addition, it has been observed that the intake of probiotics, specific foods or changes in dietary patterns can induce more profound changes in the gut microbiota of children than in that of adults[206–210]. These observations suggest that the microbiota of children exhibits greater malleability in response to environmental or dietary changes than that of adults. Such investigations provide valuable insights into understanding the variations in the microbiota during development and in assessing their impact on various diseases and on microbiota modulation through diet.

As elucidated in previous chapters, dietary choices and cooking methods exert a significant influence on the gut microbiota. Consequently, the current chapter seeks to explore the differential response of the gut microbiota in adults and children to these foods and cooking methods, using the same *in vitro* fermentation approach employed previously. This study aims to provide a basic reference for the development of different dietary modulation strategies targeting the microbiota in adult and child populations.

2 Material and methods

Some of the used methods such as food procurement and handling and cooking methods, fecal material collection, *in vitro* gastrointestinal digestion and fermentation, DNA extraction and 16S rRNA gene amplicon sequencing have been previously described in **Chapter I –Material and methods** (page 30).

The data from the three adult individuals featured in chapter 1 and the three lean children in chapter 2 were employed in this study. Furthermore, 87 samples of individuals of each group were also sequenced and employed to validate the representativeness of the samples used in the fermentation process for their respective groups. All of them complied with the same inclusion criteria required for the studies in Chapter I and II. i.e., they had not taken antibiotics, prebiotics or probiotics for the three months prior to the assay. At least three fecal samples of each donor were obtained and pooled together to reduce intra-individual daily variability.

Due to the large number of foods used, they have been grouped at three different levels for the analysis of results: i) Animal-based and plant-based foods, ii) Food categories and iii) Individual foods. The foods included at each level are described in **Table I-1**(**Chapter I**).

3 Results

After 16S rRNA gene amplicon sequencing of the fermentative microbiota within each of the 159 distinct food combinations, which were fermented in duplicate using six distinct inocula (three per group), resulting in 1926 samples considering the fermentation control, a grand total of 181.339.097 reads were acquired. This yielded an average of 94.153 reads per sample with a standard variation of 52.428. Upon application of length filtering, 143.116.141 reads underwent taxonomic assignment, resulting in an average of 74.307 reads per sample, assigned at the genus level, with a standard deviation of 42.136. The taxonomic assignment of the reads resulted in the identification of 12 phyla, 78 families, 237 genera and 213 species.

3.1 Microbiota composition

In the context of the *in vitro* fermentation, due to the great number of fermentations performed, only fecal samples from three individuals were used for each group. Consequently, we conducted an initial validation to determine the representativeness of the samples used as inoculants within each group. To achieve this, we compared a set of 90 samples, which included the three samples used as inoculants for each group.

As illustrated in **Figure III-1**, some of the samples corresponding to the inocula exhibit alpha diversity values below the average although similar to their corresponding groups. In addition, the CCA in **Figure III-1C** showed beta diversity values similar to those of the other individuals within the group. No significant differences were found between the inocula and the other group samples based on the PERMANOVA and CCA analyses. These observations suggest that the samples we employed as inocula are representative of their respective group, although the adult's samples are on the low end of the adult diversity range. Additionally, it was noted that the Shannon diversity index and Chao1 richness were significantly greater in adults than children, while the composition of the gut microbiota was significantly different according to the PERMANOVA. The alpha and beta diversity analyses of the baseline microbiota (inocula) of the adults and children who participated in the fermentation study showed no significant differences between the groups, likely due to the small number of samples in each group.

Afterwards, we analyzed the beta diversity of the samples that had undergone fermentation under different foods and cooking methods. Most of the variance in microbiota composition was caused by differences among individuals (**Figure III-2**). Therefore, in order to study the impact of foods and cooking methods, we have considered these inter-individual variations in those analyses that supports this type of corrections such as ANCOM-BC2.

As one of the purposes of this study is to determine the effects of the different foods on the growth of the gut microbiota, we compared the microbial composition in water fermentations, using it as a control, with the fermented food. **Figure III-3A** shows the dissimilarity levels of

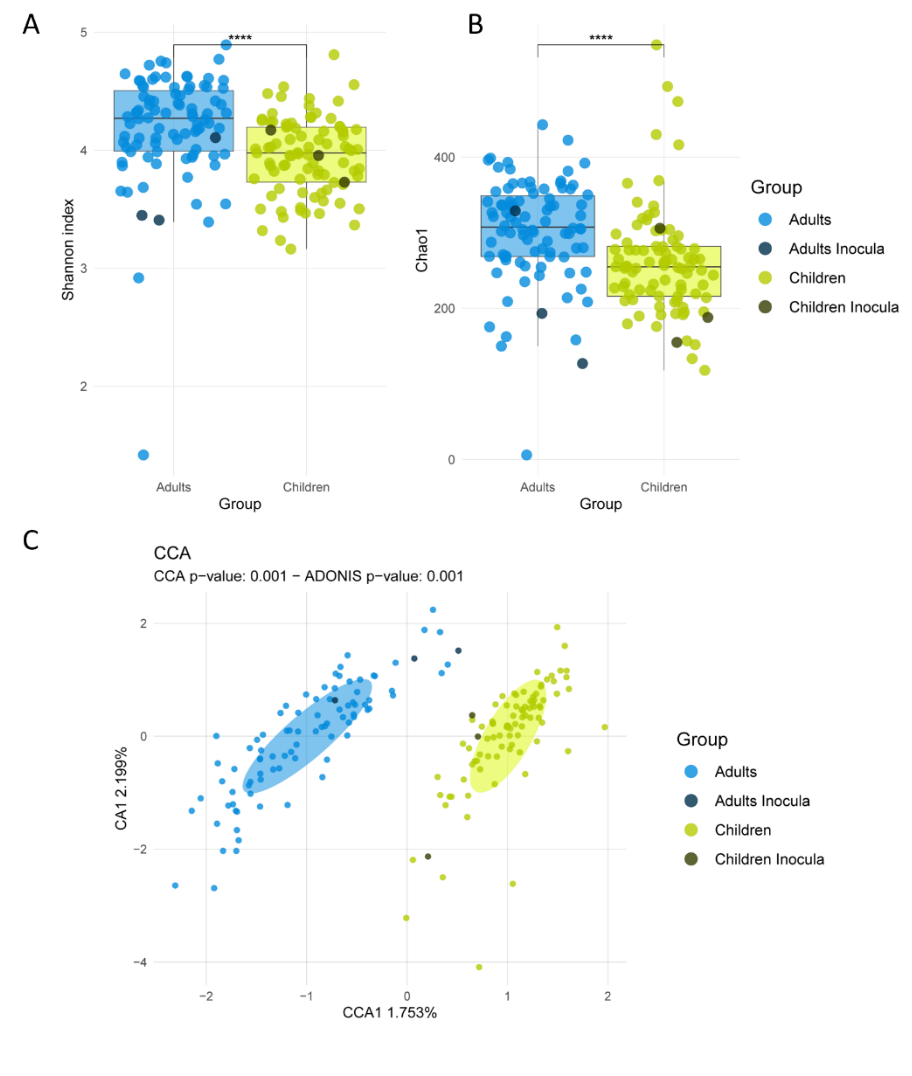


Figure III-1. Microbiota diversity of adults and children. Individuals whose fecal samples were used as inoculums are marked with darker colors. A) Shannon diversity index and B) Chao1 richness estimator of gut microbiota grouped by adults and children. The mark **** indicate p-value < 0.0001. C) CCA of samples by group with PERMANOVA test for compositional comparisons. Colors indicate the group of the samples according to the legends. All these analyses were performed at the ASV level.

animal-based and plant-based foods with water. Although differences with the water fermentation do not reach significance, lower dissimilarity values for plant-based foods were obtained in both groups and higher dissimilarity levels were found in the children's group. The comparison of the different categories of food versus water (**Figure III-3B**) yielded results equivalent to the above, without significant differences and with higher dissimilarity levels in children. In addition, the food categories' dissimilarity against water within each group remains at similar levels, although it is mostly higher for those that are animal-based.

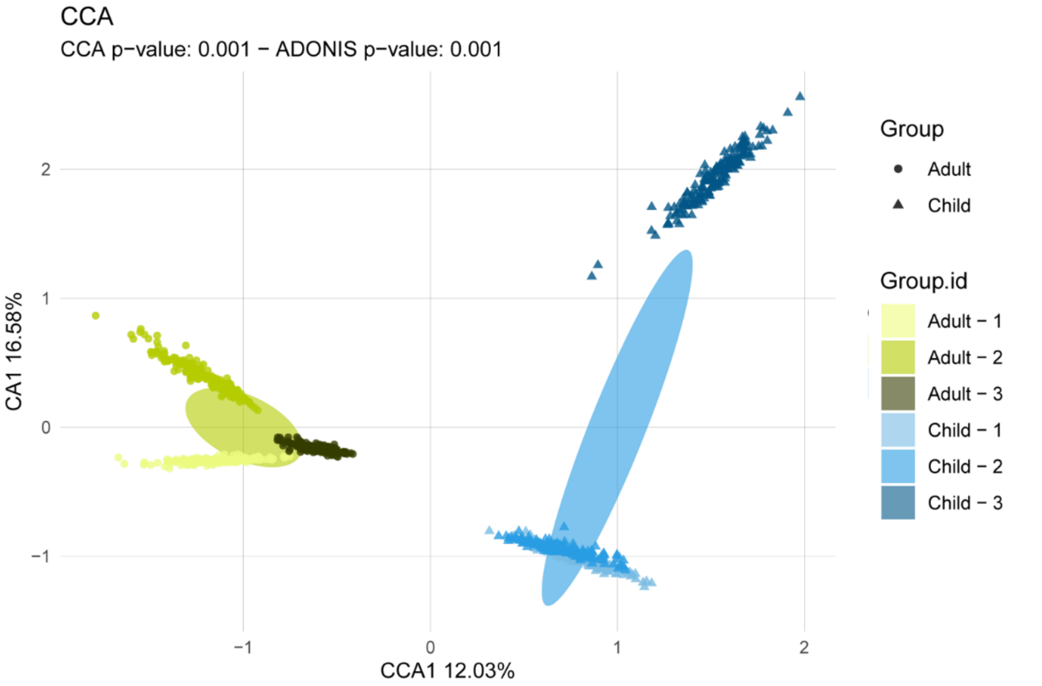


Figure III-2. Beta diversity analysis of fermented microbiota of adults and children individuals. CCA of samples of each individual with PERMANOVA test for compositional comparisons. Colors indicate the group and individuals of the samples according to the legends. All these analyses were performed at the ASV level.

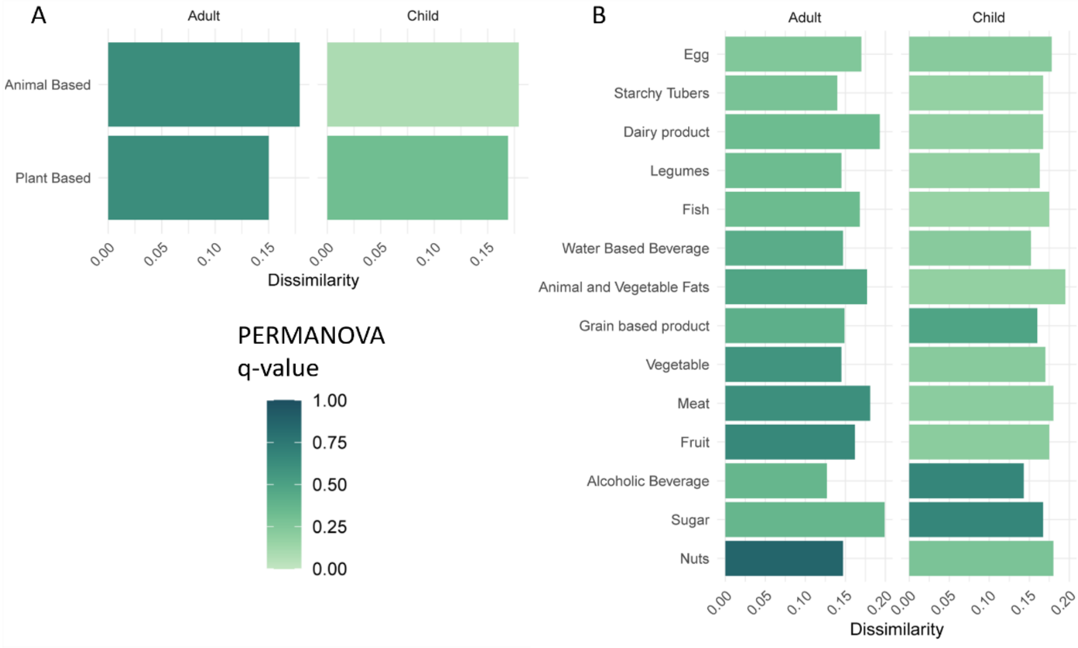


Figure III-3. Barplot representing the mean Bray Curtis dissimilarity of A) animal- and plant-based foods and B) food categories against water. Colors represents q-value from PERMANOVA after multiple testing correction, no significant difference were found. Bray Curtis dissimilarity index was obtained from ASV-level ANCOM-BC2 normalized counts table.

3.2 Differential abundance of taxa in the microbiota of adults and children

In order to improve our understanding of the dynamics associated with the fermentative microbiota, our investigation focused on the evaluation of taxonomic abundance differences using the ANCOM-BC2 methodology. Our main goal is to identify any distinct responses of these bacteria to dietary components in adults and children. To achieve this goal, we systematically examined the differences in taxonomic abundance between the product of the fermentation of these dietary components and water, contrasting both groups for comparative analysis.

3.2.1 Differential response to animal- and plant-based foods between adults and children

In the comparative analysis of animal and plant-based foods with water, our research revealed the presence of 25 different genera that exhibited differential abundance in at least one specific condition or group (see **Figure III-4**). Notably, a large portion of these observed differences were limited to a single group, for both the animal and plant-based foods. In addition, our analysis identified 23 specific species as being differentially abundant, which are detailed in **Supplementary Material III-1**.

Further examination of these differences, particularly those associated exclusively with either animal or plant-based food, revealed that animal-based foods increased the abundance of *Anaerostipes (A. hadrus)*, *Colidextribacter*, *Lachnoclostridium (L. edouardi), Romboutsia, Megasphaera massiliensis* and *Blautia faecis* in adults, and increased the abundance of *Blautia*, *Collinsella (C. aerofaciens)*, *Dorea*, Erysipelotrichaceae UCG 003, *Roseburia*, *Solobacterium, Subdoligranulum (S. variabile), Coprococcus comes* and *Coprococcus eutactus* in children. It is noteworthy that animal-based foods increased the abundance of the genus *Blautia* in children as well as that of *Blautia faecis* in adults.

In contrast, plant-based foods exerted a more limited effect, manifested as a reduction in the abundance of *Erysipelatoclostridium and Ruminococcus callidus* in the adult group. However, *Erysipelatoclostridium ramosum* showed a reduced abundance in response to the animal-based foods.

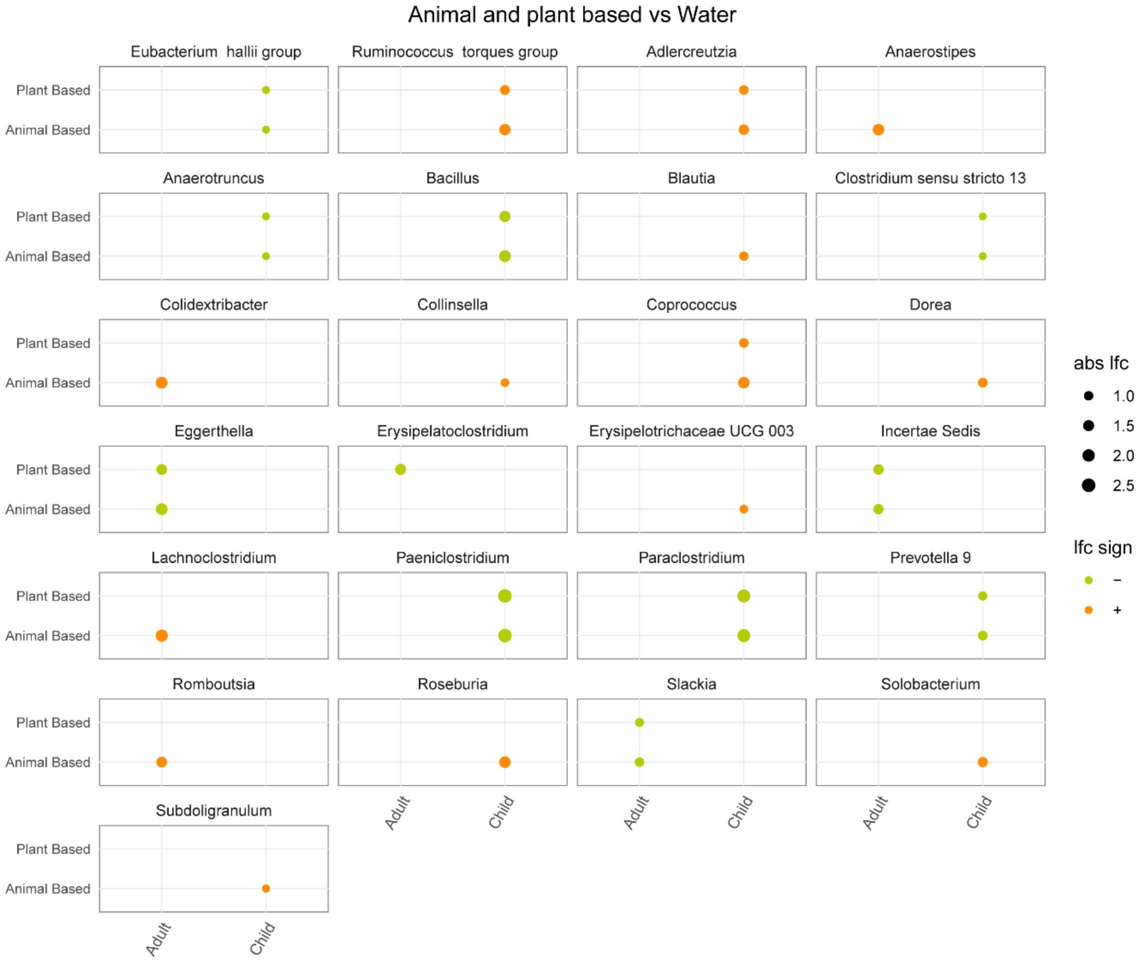


Figure III-4. Differentially abundant genera when comparing Animal- and Plant-based foods with water. Results are arranged by group in x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing animal-/plant-based foods vs. water.

3.2.2 Differential response to food categories between adults and children

To identify how different food categories affected the abundance of microorganisms within our experimental groups, we conducted a comparative analysis of the abundance differences between each food type and a water control for both adults and children. Abundance differences at genus and species level are detailed at **Figure III-5** and **Figure III-6**.

There were few common differences observed between adults and children across the different food categories. Particularly, the fermentation of animal and vegetable fats resulted in both in an increased presence of *Lachnospira* (*L. pectinoschiza*) and *Anaerostipes* (*A. hadrus*), as well as *Adlecreutzia* (*A. equolifaciens*), which was also increased by the effect of fish fermentation. These differences in microbial abundance were found to be statistically significant at both the genus and species level, highlighting their marked influence on both the adults' and children's groups. In addition, it is also worth noticing in both groups the almost complete lack of significant differences for beverage categories, both alcoholic and water-based. This indicates that the lack of nutrients in these beverages results in their having an effect almost indistinguishable from that of the water control.

The adult group stands out by the effect of animal and vegetable fats, dairy products, egg, and fish increasing the abundance of *Anaerostipes* and *Lachnoclostridium* (the latter also increased by meat). At species level, differences related to these foods, with the exception of egg, were found for *Anaerostipes hadrus*, *Lachnoclostridium edouardi* and *Blautia faecis*. The latter two species were also increased by meat. Moreover, animal and vegetable fats and dairy products also increased the abundances of *Eubacterium hallii* and Lachnospiraceae UCG 004. Fish also increased the abundances of *Romboutsia*, *Citrobacter* and *Carnobacterium*. There were only two cases of decreased abundances found in adults, i) Ruminococcaceae *Incertae Sedis* in response to egg, grain-based products, meat, nuts, starchy tubers and sugar, and ii) *Eggerthella* (*E. lenta*) in response to alcoholic beverages, animal and vegetable fats, egg, meat and sugar.

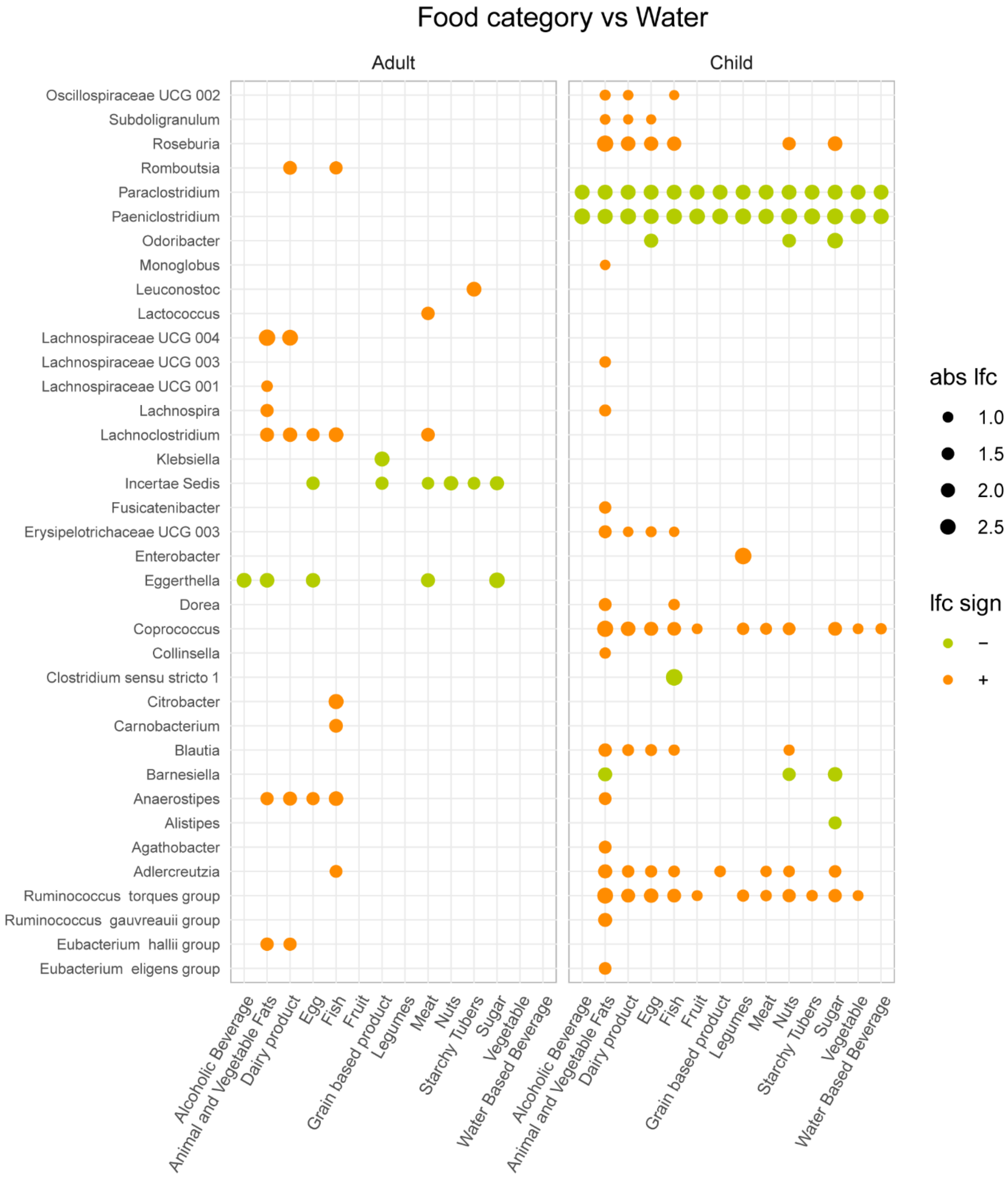


Figure III-5. Differentially abundant genera when comparing food categories at x axis with water in adult and child groups. Results are arranged by group in x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing food categories vs. water.

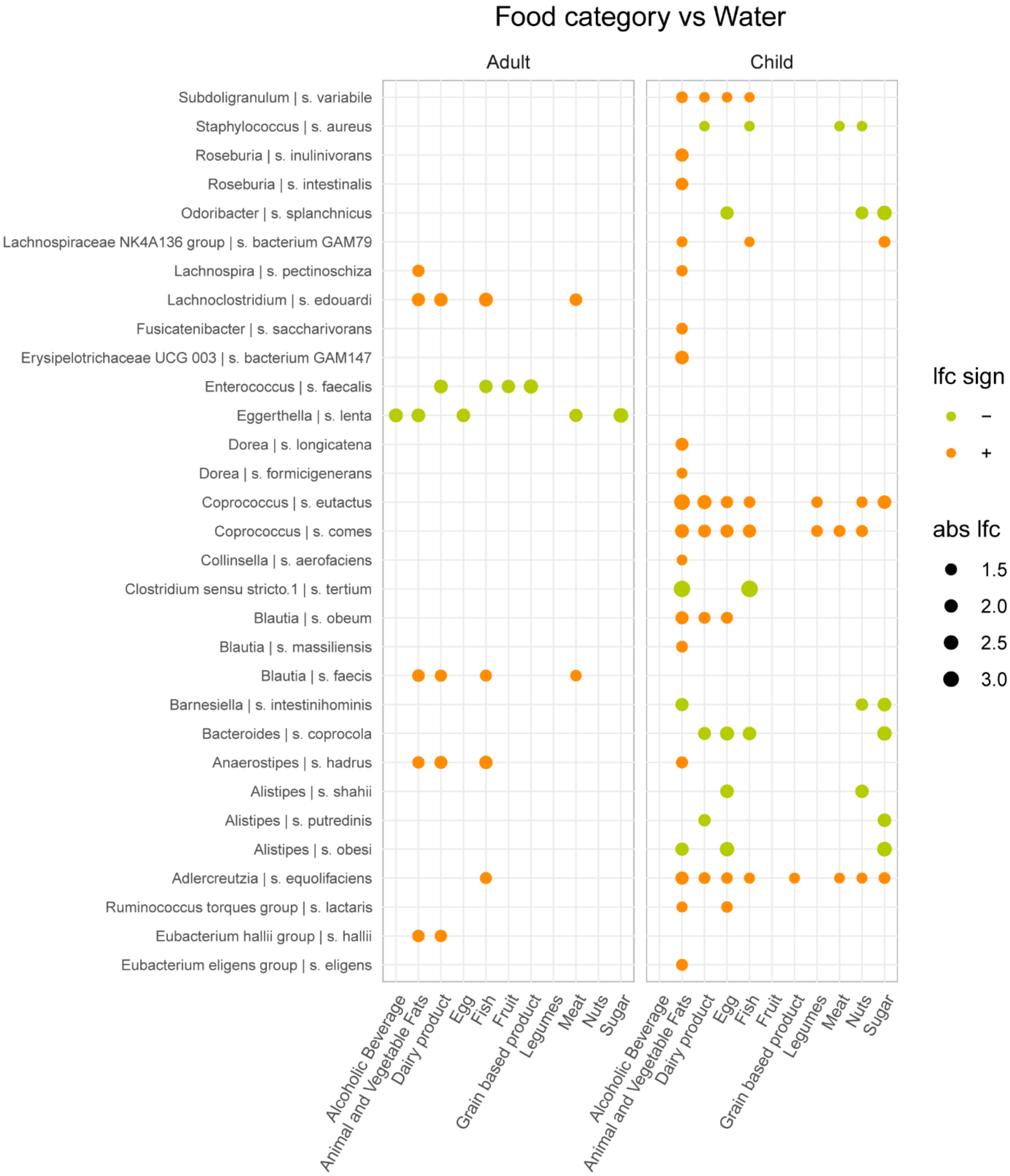


Figure III-6. Differentially abundant species when comparing food categories at x axis with water in adult and child groups. Results are arranged by group in x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing food categories vs water.

The children group presented a larger number of differences in microbial abundance across food categories. The abundances of some taxa were increased in a considerable number of the categories. This suggests that these differences are not very specific to particular foods, but rather might be linked to the fermentation of many of the added food substrates, as opposed to the limited fermentation of those substrates present in the inoculum that might be going on in the water control. This was the case of *Adlecreutzia* (*A. equolifaciens*), *Ruminococcus torques* group (*R. lactaris*) and *Coprococcus* (*C. eutactus* and *C. comes*). In contrast, *Paraclostridium* and *Paeniclostridium* displayed decreased abundances in all food categories. This suggests that these taxa may be particularly apt at growing on the limited substrates derived from the inoculum that would be available in the water control.

As observed in the adults' group, animal and vegetables fats was the food category associated with the largest number of significant abundance differences. It increased the abundance of *Monoglobus*, Lachnospiraceae UCG 003, *Lachnospira* (*L. pectinoschiza*), *Fusicatenibacter* (*F. saccharivorans*), *Collinsella* (*C. aerofaciens*), *Anaerostipes* (*A. hadrus*), *Ruminococcus gauvreauii* group, *Eubacterium eligens* group (*E. eligens*), *Dorea*, Oscillospiraceae UCG 002, *Subdoligranulum* (*S. variabile*), *Roseburia*, Erysipelotrichaceae UCG 003, and *Blautia*. In contrast, animal and vegetable fats decreased the abundance of *Barnesiella* (*B. intestinihominis*). Other categories that affected various taxa were the animal-based dairy products, fish and egg, which, like animal and vegetable fats, increased the abundances of *Subdoligranulum* (*S. variabile*), *Roseburia*, Erysipelotrichaceae UCG 003, and *Blautia*. Oscillospiraceae UCG 002 also increased with two of these food categories (dairy and fish), while *Dorea* did so only with fish. On the other hand, fish reduced the abundance of *Clostridium sensu stricto 1* (*C. tertium*), while egg reduced that of *Odoribacter splanchnicus.* Regarding plant-based food categories, both nuts and sugar decreased the abundance of *Odoribacter splanchnicus* and *Barnesiella* (*B. intestinihominis*), while sugar only decreased that of *Alistipes* (*A. putredinis* and *A. obesi*). Finally, legumes increased the abundance of Erysipelotrichaceae UCG 003

Examining the impacts on the fermentative microbiota of individual foods, as illustrated in **Figure III-7**, various results consistent with those obtained at the food category level are observed. First, in the children's group *Coprococcus*, *Adlercreutzia* and *Ruminococcus torques* group exhibited substantial shifts across foods characterized by very different nutrient composition. Second, the children's group presented a larger number of differences in microbial abundance. Third, foods belonging to fats and animal-based categories resulted in the largest number of differences, with butter and gouda having the most effects in adults and butter, gouda, olive oil, salmon and sunflower oil in children.

In the category of animal and vegetable fats, butter arose as the food with the largest number of abundance differences and the only one with specific differences occurring in both the adult's and children's groups, including the increases of taxa such as *Lachnospira*, *Blautia*, *Anaerostipes*, and *Ruminococcus gauvreauii* group. Otherwise, most of the observed differences occurred in the children's group, especially in response to vegetable oils. In the children's group, certain bacteria such as *Adlecreutzia*, *Ruminococcus gauvreauii* group, *Ruminococcus torques* group, *Blautia*, *Roseburia* and *Subdoligranulum* experienced a rise in abundance in response to all fats i.e., olive oil, sunflower oil and butter. Others like

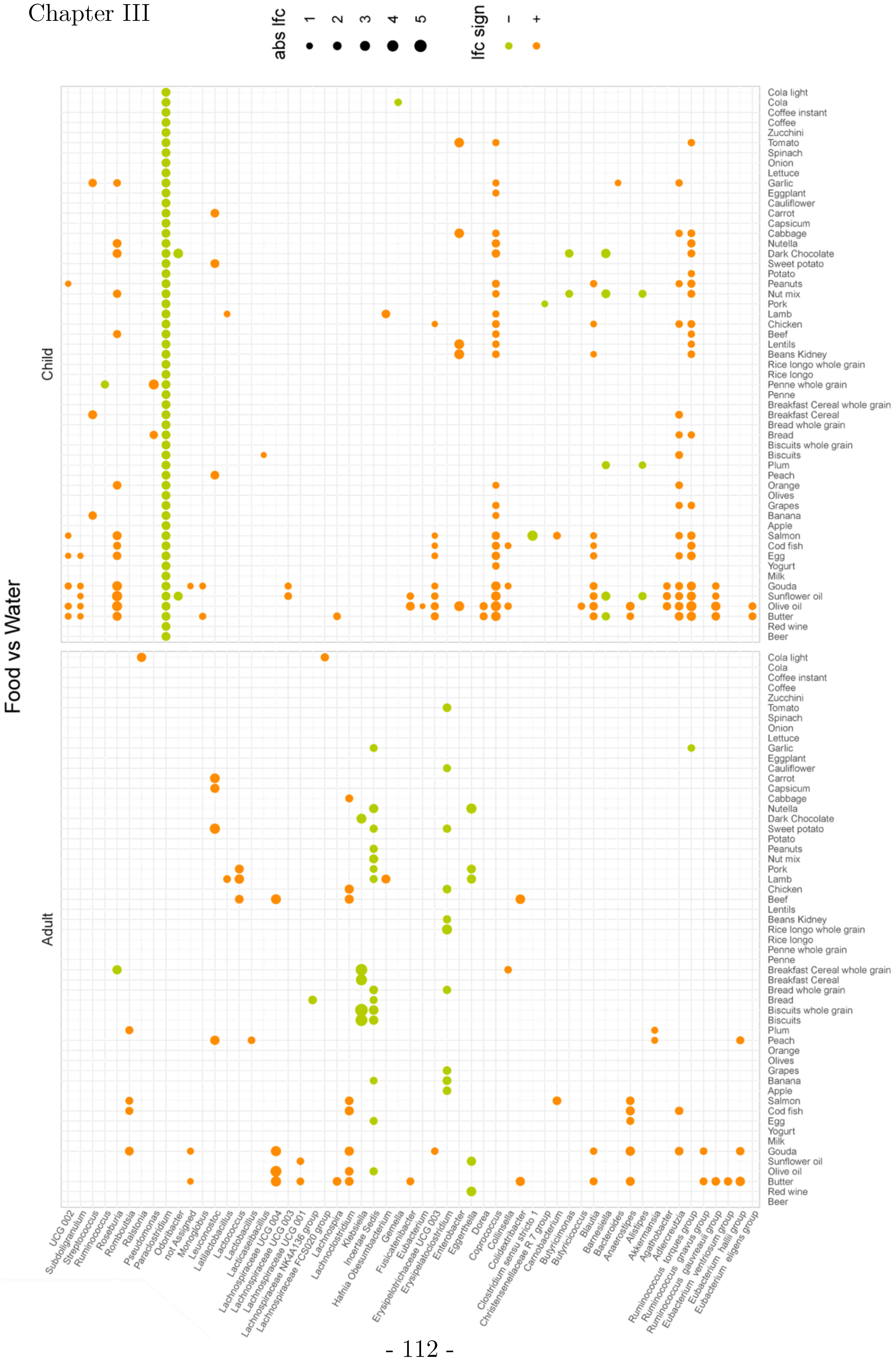

abs lfc
1
2
3
4
5
lfc sign
-
+
Food vs Water
Child
Adult
Cola light
Cola
Coffee instant
Coffee
Zucchini
Tomato
Spinach
Onion
Lettuce
Garlic
Eggplant
Cauliflower
Carrot
Capsicum
Cabbage
Nutella
Dark Chocolate
Sweet potato
Potato
Peanuts
Nut mix
Pork
Lamb
Chicken
Beef
Lentils
Beans Kidney
Rice longo whole grain
Rice longo
Penne whole grain
Penne
Breakfast Cereal whole grain
Breakfast Cereal
Bread whole grain
Bread
Biscuits whole grain
Biscuits
Plum
Peach
Orange
Olives
Grapes
Banana
Apple
Salmon
Cod fish
Egg
Yogurt
Milk
Gouda
Sunflower oil
Olive oil
Butter
Red wine
Beer
UCG 002
Subdoligranulum
Streptococcus
Ruminococcus
Roseburia
Romboutsia
Ralstonia
Pseudomonas
Paraclostridium
Odoribacter
not Assigned
Monoglobus
Leuconostoc
Latilactobacillus
Lactococcus
Lactobacillus
Lacticaseibacillus
Lachnospiraceae UCG 004
Lachnospiraceae UCG 003
Lachnospiraceae UCG 001
Lachnospiraceae NK4A136 group
Lachnospiraceae FCS020 group
Lachnospira
Lachnoclostridium
Klebsiella
Incertae Sedis
Hafnia Obesumbacterium
Gemella
Fusicatenibacter
Eubacterium
Erysipelotrichaceae UCG 003
Erysipelatoclostridium
Enterobacter
Eggerthella
Dorea
Coprococcus
Collinsella
Colidextribacter
Clostridium sensu stricto 1
Christensenellaceae R 7 group
Carnobacterium
Butyricimonas
Butyricicoccus
Blautia
Barnesiella
Bacteroides
Anaerostipes
Alistipes
Akkermansia
Agathobacter
Adlercreutzia
Ruminococcus torques group
Ruminococcus gnavus group
Ruminococcus gauvreauii group
Eubacterium ventriosum group
Eubacterium hallii group
Eubacterium eligens group

Figure III-7. Differentially abundant genera when comparing individual foods at x axis with water in adult and child groups. Results are arranged by group in x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing foods vs. water. This figure is available in higher quality through the link in **Supplementary Material.**

Dorea and *Anaerostipes* were augmented only by butter and olive oil, while *Barnesiella* was reduced only by butter and sunflower oil, and *Agathobacter* was increased only with vegetable oils.

Gouda, a dairy product characterized by high fat content induced increases in the abundance of *Blautia*, Erysipelotrichaceae UCG 003 and *Adlercreutzia* in both the adult's and children's groups. In addition, *Anaerostipes*, *Rombutsia*, *Ruminococcus gnavus group, Eubacterium hallii group*, *Lachnoclostridium*, and Lachnospiraceae UCG 004 presented higher abundances exclusively in the adults' group, while *Subdoligranulum*, Oscillospiraceae UCG 002, *Monoglobus*, *Roseburia*, Lachnospiraceae UCG 003, *Agathobacter*, *Ruminococcus torques* group, *Ruminococcus gauvreauii* group, and *Collinsella* exhibited increased abundances only in the children's group.

Salmon generated substantial differences in taxonomic abundance, particularly in the case of *Carnobacterium,* which increased, especially in the children's group. Additionally, salmon increased the abundance of Oscillospiraceae UCG 002, *Roseburia*, *Coprococcus*, *Blautia*, *Adlecreutzia*, *Ruminococcus torques* group and Erysipelotrichaceae UCG 003, in the children's group, while decreased the abundance of *Clostridium sensu stricto.*

Lamb stands out among meat products because of its effect of increasing *Latilactobacillus* and *Hafnia Obesumbacterium*, which was observed exclusively in response to lamb in both the adult's and children's groups. Adults also experienced a decrease in the abundance of *Eggerthella* by the action of pork and lamb, as well as an increase of *Lactococcus* by the action of pork, lamb, and beef (also increased by meat at food category level analysis).

Moreover, in the adults' group, the fermentation of plum and peach increased the abundance of *Akkermansia*, and garlic similarly elevated the abundance of *Bacteroides*. Additionally, in the children's group, the action of sunflower oil, nut mix and dark chocolate reduced the abundances of *Barnesiella*, *Butyricimonas* (only in the case of the latter two) and *Alistipes* (only in the case of the first two).

3.2.3 Differential effects of cooking methods on taxon abundance

To discern the differential impacts of cooking methods, we separately analyzed foods cooked in different manners, individually and grouped by category, comparing them with a water control condition. **Figure III-8** depicts a summary of some changes induced by the cooking methods across food categories and groups. Further comprehensive results concerning food categories can be found in **Supplementary Material III-2**, while detailed results referring to specific foods can be found in **Supplementary Material III-3**.

Notably, most of the observed differences consistently occur across all cooking methods within a given food, suggesting that these changes are predominantly due to the inherent properties of the foods themselves rather than the cooking methods employed. Therefore, our focus is to clarify the changes associated with cooking methods.

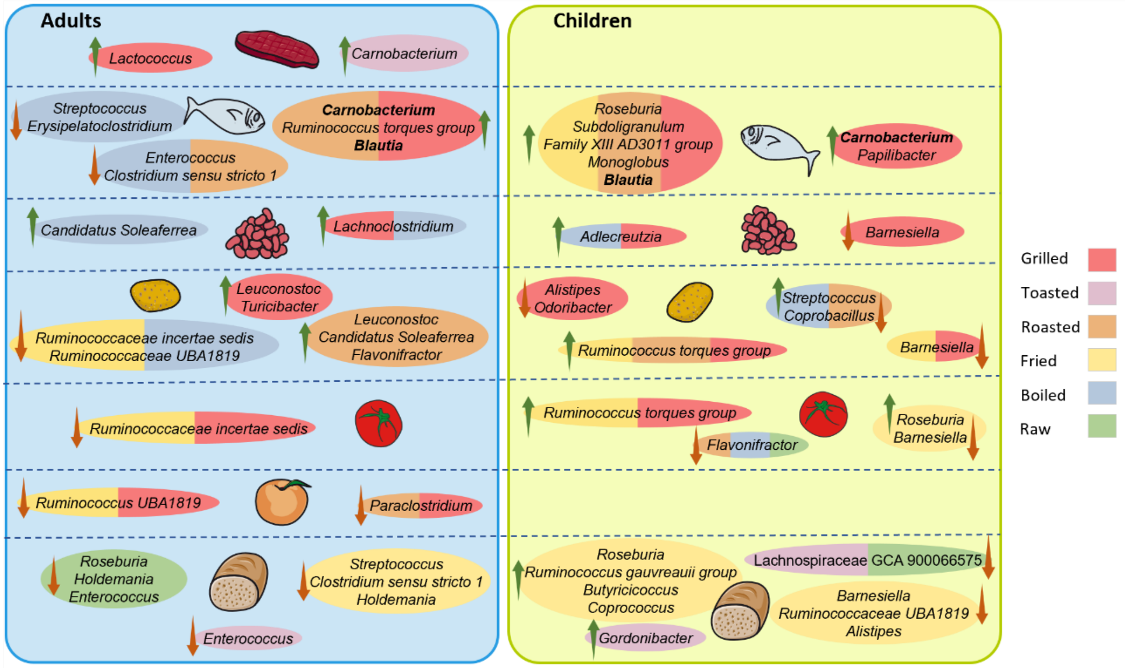


Figure III-8. Graphical summary of main differential outcomes in relative taxon abundance between cooking methods and water by food category. Differences are shown per group and food category. For each group, the most representative taxa whose abundance is reduced or increased with the corresponding cooking method are showed. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. This figure is available in higher quality through the link in **Supplementary Material.**

Meat

Cooking methods have resulted in relatively few changes in the effects of meat as a food category on the fermentative microbiota, which have been detected only in the group of adults. The grilling method increased *Lactococcus* levels, while the roasting method led to an increase in *Carnobacterium*. Examining specific types of meat, *Lactococcus* exhibited higher levels after grilling and roasting in pork, lamb and beef but not in chicken in the adults' group, as well as after grilling in pork and beef in the children's group. Additionally, *Carnobacterium* increased in response to roasting in pork, chicken, and lamb within the adults' group. Furthermore, in both groups *Latilactobacillus* increased in chicken with grilling, while it increased in lamb with both grilling and roasting. It is also noteworthy that *Hafnia-Obesumbacterium* increased exclusively in chicken and lamb due to grilling in the adult's group.

Fish

As previously noted, fish is distinguished as one of the food categories that produce the highest number of abundance differences in the fermentative microbiota, especially in children. Notably, fish appears to be particularly influenced by the cooking methods used because major differences were only observed with methods other than boiling. For instance, the children's group displayed increased levels of *Roseburia*, *Monoglobus*, *Family XIII AD3011 group* and *Blautia* in fried, roasted and grilled fish, as well as an increase in *Carnobacterium* with grilling. *Blautia* was found to be elevated in the adult group after the grilling and roasting of fish. Regarding specific fish, both salmon and cod fish displayed an increase in *Carnobacterium* due to grilling and roasting in both groups, except for roasted cod fish in children. Furthermore, it is noteworthy that roasting led to an increase in *Blautia* in the children's group for salmon, while this effect was observed in the adult's group for cod fish.

Animal and vegetable fats

Within this food category, only raw and fried foods were examined, and all observed differences manifested in both cooking methods, indicating their independence from food treatment effects. Specifically, *Roseburia* and *Blautia* exhibited an augmented presence in both groups. Notably, raw sunflower oil was found to induce heightened levels of *Solobacterium*, *Parasutterella*, *Parabacteroides*, *Monoglobus* and *Eubacterium nodatum* group in the children's group. Furthermore, fried olive oil showed an increase in *Butyricicoccus* among the children's group and an increase in *Acetanaerobacterium* in that of adults. Regarding butter, in the adults' group raw butter resulted in decreases of *Streptococcus* and *Enterococcus*, accompanied by an increase in *Candidatus Soleaferrea*; whereas fried butter resulted in an increase of *Colidextribacter*.

Legumes

In adults, grilling had a decreasing effect on Ruminococcaceae UBA1819 and an increasing effect on *Lachnoclostridium*, whereas boiling increased *Lachnoclostridium* and *Candidatus Soleaferrea* (exclusively in lentils). In children, the results were different, where grilling had a decreasing effect in *Barnesiella* while boiling and grilling had an increasing effect in *Adlercreuztia*. Boiled kidney beans were found to increase the abundance levels of *Parabacteroides*, *Enterococcus*, *Coprococcus*, *Collinsella*, and *Blautia* in the children's group.

Starchy tubers

The influence of starchy tubers on the microbiota was found to vary widely depending on the cooking method. At food category, roasting increased *Leuconostoc, Flavonifactor* and *Candidatus Soleaferrea* in the adult's group, but individual food analyses showed that the latter two genera increased only in potatoes but not in sweet potatoes. In the children's group *Streptococcus* levels increased for botch potatoes and sweet potatoes with boiling, but only for potatoes with roasting; in contrast, only roasted or grilled sweet potatoes resulted in an increase of *Leuconostoc*.

Vegetables

Cooking methods had a small effect on how vegetables affected the fermentative microbiota in the adults' group, where the only difference was that frying and grilling led to a decrease in Ruminococcaceae *incertae sedis*. For children, frying resulted in fewer *Barnesiella* and more *Roseburia*, and both frying and grilling caused an increase in *Ruminococcus torques* group. Additionally, the children's group presented a reduced abundance of *Flavonifractor* associated with raw, roasted, and boiled vegetables.

Examining the effects of cooking method individual vegetables, the most recurrent observation was the increase in *Leuconostoc* abundance, observed in both adults and children, with various cooking methods. Particularly noteworthy is the case of garlic, which caused a reduction in the abundance of many taxa in the adult's group with different methods of cooking. In contrast, in the children's group garlic induced an increase in *Bacteroides* abundance, regardless of whether it was raw, boiled or fried.

Fruit

This category of foods showed limited differences among the effects of cooking methods. Differences occurred mainly in the adult's group, including the reduction of Ruminococcaeae UBA1819 by frying and grilling and of *Paraclostridium* by grilling and roasting. Nonetheless, each specific fruit showed different effects. For example, frying significantly affected plums in the adult's group, leading to an increase in the presence of *Akkermansia*, *Intestinimonas*, *Fusicatenibacter*, *Blautia*, *Romboutsia*, *Lachnoclostridium*, *Anaerostipes*, and *Eubacterium halii* group, with grilling also increasing the abundance of the latter four genera. However, apples had opposite effects in the adult's group, where taxa such as *Ruminococcus*, *Parabacteroides*, *Enterococcus*, *Eggerthela*, and *Agathobacter* were decreased by frying.

Bread

Cooking methods affected the effects of bread in the group of adults, with frying reducing the amount of *Streptococcus*, *Clostridium sensu stricto 1*, and *Holdemania*. Additionally, raw bread showed lower levels of *Roseburia*, *Holdemania*, and *Enterococcus*, with the latter also decreasing in toasted bread. Frying had the strongest impact in the child group, raising the levels of *Roseburia*, *Coprococcus*, *Butyricicoccus*, and *Ruminococcus gauvreauii*, while decreasing Ruminococcaceae UBA1819, *Barnesiella*, and *Alistipes*. Raw bread resulted in higher *Haemophilus* and lower Lachnospiraceae GCA 900066575 in the children's group, a reduction also observed in toasted bread, while toasted bread increased the prevalence of *Gordonibacter*.

4 Discussion

In continuity with the previous chapter, we have examined the distinctive ways in which certain representative foods of the Mediterranean and Western diets, coupled with their characteristic cooking methods, may affect the microbial communities within the gut microbiota. Particularly, our investigation here focuses on the characterization of the microbiota profiles in healthy adults and children, clarifying the diverse responses exhibited by their respective microbial ecosystems.

The samples of adult and pediatric individuals utilized in the present study suggest that the microbiota of school-age children is still in the process of development, as evidenced by the lower diversity observed in children compared to adults, along with a clear difference in compositional profiles between the two groups. Some studies have described that from the age of 3 years onward, the intestinal microbiota of children acquires a structure similar to that of adults [19,211]. However, other studies report a lower diversity of intestinal microbiota in children compared to adults, supporting our findings, and indicating that such development may take more time [17,18]. This developmental timeline is crucial, as there is substantial evidence suggesting that alterations in the microbiota during this developmental phase may have consequences at a later stage [10,212].

This developmental time frame gains significance when considering that the Bray-Curtis dissimilarities between food-fermenting microbiota and that of a water control tend to be greater for the microbiota of children than for that of adults, suggesting that the children's microbiota is more influenced by foods. Therefore, given that diet is a key factor in development and has a significant influence on the microbiota, the study of microbiota modulation through diet in children becomes a promising tool to ensure a healthy development, avoiding extrapolations from adult data, as occurred, for example, in the case of fiber recommendations [54,213].

In addition, we have found that gut microbiota from children and adults are impacted differently in the food fermentations. This could be caused by the differences in the composition of the initial microbiota as well as the initial diversity. The gut microbiota diversity is an important factor that may contribute to the resilience of the gut microbiota to shifts caused by external factors. In general, diverse communities are less susceptible to invasion by new species because they use limiting resources more efficiently, with different species specialized to each potentially limiting resource. Therefore, the lower diversity of the children's microbiota could partially explain the higher impact of foods[5].

Animal-based foods as a whole have demonstrated their ability to alter the abundance of numerous bacteria in the microbiota of both adults and children. While in all cases these foods led to increases in taxon abundances, no bacterial taxon increased in both groups. It is noteworthy, however, that both the taxa increased in the adults' group, such as *Anaerostipes, Colidextribacter, Lachnoclostridium*, and *Romboutsia*, and those increased in the children's group, such as *Blautia, Dorea, Collinsella, Roseburia, Solobacterium*, and *Subdoligranulum*, contain bacteria that are potentially beneficial or detrimental in both groups. Hence, it is essential to investigate in detail the effects of similar foods to discern their specific impacts.

Similarly, to animal-origin foods taken as whole food categories such as animal and vegetable fats, dairy products, eggs, and fish resulted in the most significant changes in abundance, mostly increases, and these changes affected children more prominently than adults. The increased taxon abundances resulting from high-fat or high-protein foods may be attributed to the fact that the intestinal microbiota is adapted to obtain its primary energy from the fermentation of complex carbohydrates and dietary fiber. The presence of fats and proteins, which are typically absorbed earlier in their passage through the intestine, may favor the growth of bacteria with the capacity to metabolize such nutrients, leading to an increase in their abundance.

Among the bacteria whose abundance has been increased in both age groups due to the use of high-fat foods (such as animal and vegetable fats or fish) are *Lachnospira*, *Anaerostipes*, and *Adlercreutzia*, all described as potentially beneficial to health. Particularly noteworthy are the first two due to their ability to produce SCFAs and their reduced levels in allergic children, those at a higher risk of asthma or with other diseases [153,174,175,214–216]. Their increase is interesting since these bacteria are known to use dietary fiber and complex polysaccharides as a source of energy, yet no previous reports have indicated an increase in their abundance due to fat consumption or high-fat diets [76,123,173]

Furthermore, most of the changes occurring exclusively in the adults' group took place due to foods with high-fat and/or high-protein content (animal and vegetable fats, dairy products, eggs, and fish), affecting *Lachnoclostridium*, being described as increased in high-fat diets with potentially negative health effects on individuals [121,162]. Fish also led to an increase in *Carnobacterium* and *Citrobacter* in adults. *Carnobacterium* has been identified as a potential probiotic, suggested for application in the dairy, meat, and fish industries due to its ability to produce bacteriocin. This characteristic helps in combating the growth of pathogenic bacteria through competition and exclusion [217–219].

On the other hand, the same high-fat and protein-rich foods have produced even more unique effects in children, such as increases in *Roseburia,* *Subdoligranulum,* *Erysipelotrichaceae* UCG-003, *Oscilospiraceae* UCG-002, and *Blautia*; among these, the first two genera have been described as beneficial to health whereas the latter three could be considered potentially harmful. Members of the *Erysipelotrichaceae* have been found to be associated with obesity and with symptoms of the metabolic syndrome[127]. While *Blautia* has been documented as increased in high-fat diets, *Roseburia*, conversely, has been described as increased in vegetarian diets and reduced in diets based on animal-based foods [54,121,220].

It is noteworthy that while butter induces similar changes in both age groups, sunflower oil and, to a greater extent, olive oil have a more pronounced impact on the microbiota of children, increasing the abundance of mostly beneficial genera such as *Ruminococcus torques* group*, Coprococcus, Adlercreutzia, Roseburia*, and *Subdoligranulum*, which are also increased by butter. Additionally, *Fusicatenibacter* and *Agathobacter,* also reported as beneficial, are increased solely by the action of olive oil[196].

Although most of the changes detected in our experiments could be attributed to the effect of the food itself, numerous differences were also found to depend on the effects of the cooking method used. Cooking methods can be expected to produce different effects on the microbiota depending on the nutritional composition of the food to which they are applied. In accordance, many cooking method effects were only detected in our experiments for specific foods. As an example, *Carnobacterium* increased in both age groups only with grilled and roasted salmon. Interestingly, raw salmon did not exhibit any significant impact on *Carnobacterium* levels, indicating that the cooking methods were required to favor its proliferation. It is notable that both grilling and roasting are non-immersive and result in relatively low internal food temperatures.

However, in some cases, specific cooking methods were associated with similar effects across various food types. For instance, in the children's group the butyrate producer *Roseburia* showed an increase in different types of food as a result of frying. In this group *Roseburia* was also increased with high-fat foods, including the animal and vegetable fats category and olive oil specifically. Thus, its increased abundance as a consequence of being cooked by immersion in olive oil is in agreement with these results.

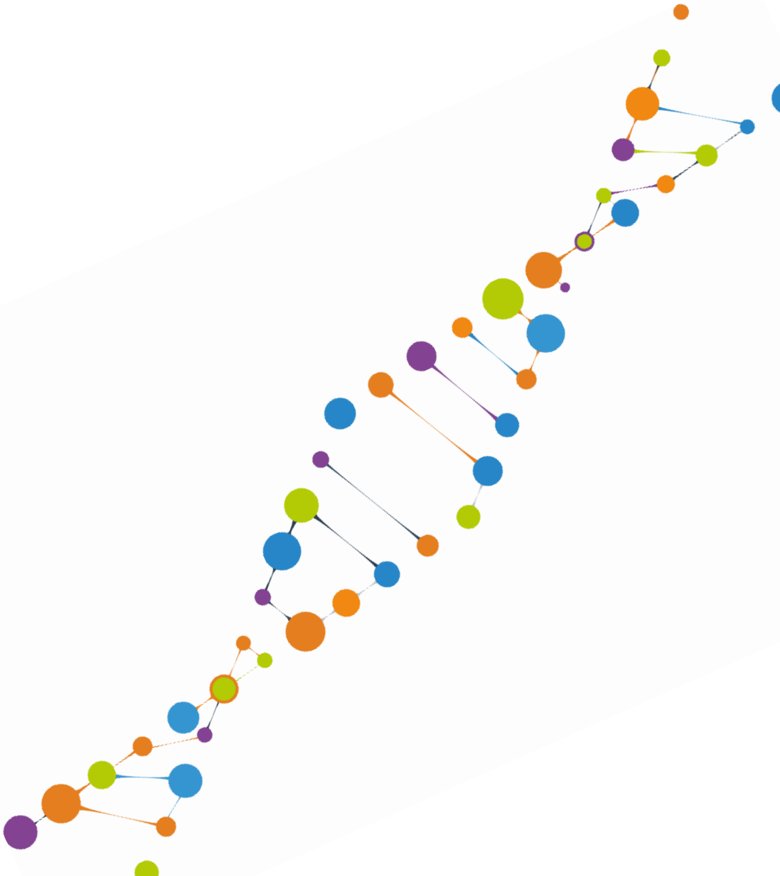
The findings presented in the current study have demonstrated that the microorganisms within the gut microbiota of school-aged children, which is still developing but is becoming more adult-like in structure, respond differently to different foods and cooking methods. Furthermore, this influence on microbiota composition differs significantly from that observed in adults, highlighting the need to further explore these differences in order to develop personalized strategies for each group. Such strategies aim to enhance the potential of foods as modulators of the microbiota. In this context, while the primary effect on the microbiota can often be attributed to the food itself, the cooking method may indeed have significant and valuable effects that should be considered and further studied, taking into account that they may vary across individual foods. Reaching optimized dietary strategies is particularly important in children given the developmental stage of this population, which opens a window of opportunity with significant potential to mitigate the risk of future diseases.

5 Conclusions

1. The foods and the cooking methods have a differential effect on the response of the gut microbiota of adults and children.

2. The fermentative microbiota of children shows greater food-mediated differences than that of adults, indicating greater malleability through diet.

Chapter IV



Chapter IV. Modulation of the gut microbiota through a nutritional intervention involving a personalized dietary app and tannin supplementation

1 Introduction

Unhealthy diets have been widely identified as a major factor implicated in the prevalence of non-communicable diseases such as obesity, diabetes, cardiovascular diseases, and cancer [221]. In response to these concerns, scientific and health organizations have developed nutritional programs during the last years with the aim of promoting healthy diets through generalized dietary recommendations and guidelines. However, this approach has typically ignored the significant inter-individual variability that exists within the population at the genome, proteome, metabolome, and microbiome level, thus resulting in a wide range of responses to these recommendations [222,223]. This has led to a growing interest in precision or personalized medicine, a very important branch of which is personalized nutrition for individuals, as is illustrated by the European Food4me project, one of the largest studies carried out in this field [224–226]. Due to the differential response of individuals to foods and nutrients [227], personalized diets can be more beneficial and efficient for each person. Additionally, these diets can be tailored to an individual's preferences, goals, and behavior, which can increase motivation and adherence to make changes in their dietary habits [224].

The human gut microbial community is also able to modulate the absorption and energy harvest from the diet, modifying the host exposure to dietary components. Dietary interventions often result in participants being separated into responder and non-responder groups, likely in part due to high inter-individual variability in the gut microbiota composition and highlighting the importance of addressing this through personalized nutrition[228]. An illustrative example is the intervention conducted by Zeevi D. *et al.* (2015), where the individualized glycemic response to specific foods was successfully predicted by integrating data such as gut microbiota composition, clinical information from participants, and nutritional data. This approach effectively addressed the high inter-individual glycemic response variability when consuming the same food that [229,230]. In addition, due to the dynamic nature of the gut microbiota, tailored interventions to modulate the microbiome emerge with a huge potential to help personalized nutrition to accurately provide the individual's nutritional needs. Hence, it is necessary to accurately and comprehensively characterize the diet-responsive microbiota in order to optimize the effectiveness of dietary interventions[45].

Dietary supplementation with substances such as prebiotics and other functional compounds can have beneficial effects on health through interactions with the gut microbiota. In particular, plant polyphenols present beneficial effects on human health through their antioxidant activity and their prebiotic effect by stimulating the production of SCFAs [61,64]. In recent years, our group has conducted research on the effect of tannins on the gut microbiota through *in vitro* fermentations, by combining them with different foods or evaluating different methods of tannin release, as well as through *in vivo* studies involving short-term supplementation in healthy patients [64,231,232].

In addition to the nutrients obtained through diet, supplementation with other substances such as prebiotics or probiotics are usually taken due to its beneficial effect on health by interacting with the gut microbiota. Thus, the polyphenols synthesized by plants, present beneficial effects on human health through their antioxidant activity and their prebiotic effect by stimulating the production of SCFAs [61,64]. In recent years, our group has conducted research on the effect of tannins on gut microbiota through *in vitro* fermentations, by combining them with different foods or evaluating different methods of tannin release; or *in vivo* studies by short-term supplementation in healthy patients [64,231,232]. Understanding how these compounds can work as prebiotic and how they interact with the gut microbiota is important because they can be used in personalized diets to achieve targeted effects for each individual. Understanding how these compounds can work as prebiotics and how they interact with the gut microbiota is important because they can be used in personalized diets to achieve targeted effects for each individual.

Many researchers highlight that the future of personalized nutrition depends on the development of tools that create diets rationally or by incorporating machine-learning or other artificial intelligence methods. Moreover, these tools could incorporate functions to identify the beneficial foods according to the metabolites that the microbiota of the individual produces. In addition, these methods can also take into account factors such as clinical parameters, genetic predisposition, personal objectives, and lifestyle [96,233]. In this context, the technological expansion of the last decade has increased the accessibility to smart devices such as smartphones that prove to be very helpful for registering various clinic and physical parameters as well as enabling dietary self-monitoring of the individuals' food intake. In addition, these devices allow the monitoring in real time of those parameters and the adjusting of the dietary recommendations to be aligned to the specific needs [95,96,234].

This chapter assesses the impact on the gut microbiota of a 12-week human intervention involving the use of the dietary app i-Diet and a tannin supplementation. Through the i-Diet mobile app, participants received personalized diet recommendations according to their preferences, personal objectives, habits, and energy needs. The aim is to shed light on the impact of this personalized nutrition strategy on the gut microbiota of the participants. This will support the development of improved models that also take into account the complex interactions between diet, host, and gut microbiota.

2 Material and methods

For the present study, a randomized clinical trial was conducted during 12 weeks in a healthy adult population to evaluate the impact of a mobile application designed to formulate personalized diets. In addition, a subset of the participants received a simultaneous tannin supplementation to study its effects on the gut microbiota.

Some of the methods employed here such as fecal material collection, DNA extraction and 16S rRNA gene amplicon sequencing have been previously described in **Chapter I –Material and methods** (page 30).

2.1 Study design and nutritional mobile app

The single-blind, randomized trial was conducted in Oviedo by the University of Granada and is registered with the ISRCTN clinical trial registry https://www.isrctn.com/ISRCTN63745549. All data have been obtained and utilized with the permission of the researchers responsible for the study. Data processing adhered to the provisions outlined in Regulation (EU) 2016/679, the “General Data Protection Regulation (GDPR)”, concerning the protection of individuals regarding the processing of personal data and the free movement of such data. Confidentiality was strictly maintained, ensuring anonymity for the subjects. Informed consent was obtained from all subjects, who voluntarily decided to participate in the study, in accordance with the Declaration of Helsinki on research ethics. The study protocol was approved by the Research Ethics Committee of the University of Granada and was registered under the number 1080/CEIH/2020.

The study included healthy adult subjects of both sexes, between the ages of 20 and 65 years, with a BMI in the range of 20 to 28 kg/m2, maintaining a stable weight, being able to use a smartphone, and having access to an internet connection. Exclusion criteria included the presence of chronic gastrointestinal disorders, celiac disease, or chronic conditions such as diabetes or other metabolic disorders. Individuals were also excluded if they were currently pregnant or lactating within six weeks prior to study entry, or if they indicated an intention to become pregnant within the next 12 weeks. Other exclusion factors included recent inflammation and/or prolonged use of anti-inflammatory medications, adherence to a medically prescribed diet or special dietary regimen for any reason (e.g., high-protein diet, vegetarianism, veganism, etc.), antibiotic treatment within three months prior to study entry, use of antioxidant, prebiotic, or probiotic supplements within one month prior to study entry, intense physical activity exceeding 10 hours per week, and alcohol consumption exceeding 21 drinks per week for men and 14 drinks per week for women.

The nutritional intervention is based on the use of the i-Diet app that estimates menus based on the nutritional values of foods and recipes obtained from a food composition database specifically developed in the framework of the Stance4Health project [235], taking into account some personal factors, including sex, age, height, weight, objectives, health status, and incorporating factors such as physical activity, dietary reference values, total energy expenditure, and basal metabolism[236]. Thus, the app generates personalized dietary plans encompassing breakfast, lunch, dinner, and snacks based on Mediterranean dietary patterns encouraging fruits, vegetables, and whole grain foods and giving participants specific advice about nutrition and lifestyle. The users have the possibility of interacting with the app, i.e. of making variations in the proposed menu, recalculating then the next meals accordingly. Additionally, in a blindly selected group of participants, the diet was supplemented with tannin pills, which were chosen for their proven efficacy in modulating gut microbiota composition and functionality. Two different natural tannin extracts were chosen to formulate the dietary supplement. One of the extracts is obtained from quebracho colorado (*Schinopsis lorentzii spp.*) and is characterized by the presence of condensed tannins. The other is composed of hydrolyzable gall oak tannins. Tannin capsules (Welltan active) were provided by Silvateam Spa (San Michele di Mondoví, Italia), using as formulation two thirds quebracho extract and one third gall oak extract. [64,231,232,237].

As the app enabled monitoring of participants' engagement, the participants were classified in low and high app use groups following two different approaches. The two approaches were suggested by Stance4Health partners who specialize in analyzing consumer behavior. The first evaluated the "Activity" in the use of the app by quantifying all the interactions of the users with the app (such as modifying their weight, recipes and any other personal or dietetic data); the second evaluated specifically the number of changes in the suggested weekly menus and thus the "Engagement" of the user with the app (assuming that engaged users are more likely to modify the proposed diet according to their preferences and tastes). After the intervention, the participants were classified according to the app use according to these two approaches, assuming a correlation between high app use and high adherence to the recommendations. The number of participants included in each app-user group and the number of shared participants between the groups are illustrated in **Figure IV-1**. As a very low number of participants with low app use received tannin pills, and since their low use of the app suggested overall low compliance with the intervention, a group of "low app use plus tannin" was not considered for analysis.

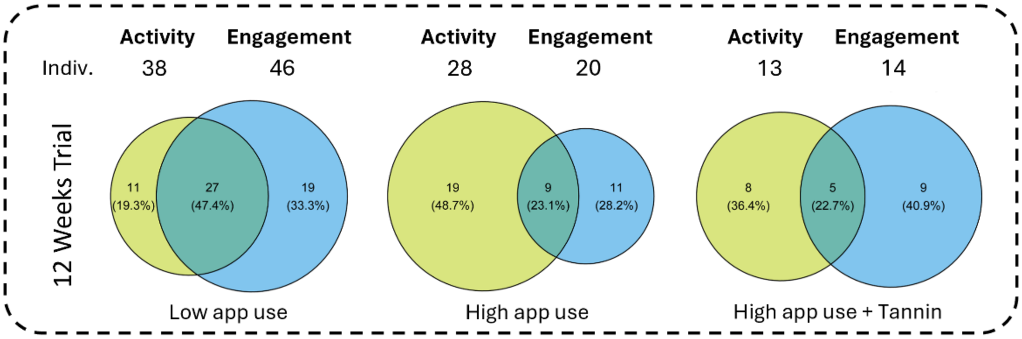


Figure IV-1 Number of participants of each group according to the app usage classification. Venn diagrams represent the number of shared participants between the two app usage classification methods employed.

At the start of the intervention, fecal samples were collected from all participants (T0) for 16S rRNA gene sequencing, allowing the analysis of the baseline composition of their gut microbiota. The intervention consisted of a 12-week period during which fecal samples were collected at six-week intervals (T1 and T2). The structure of the groups and the timeline used in this study are illustrated in **Figure IV-2**.

2.2 Bioinformatic analysis

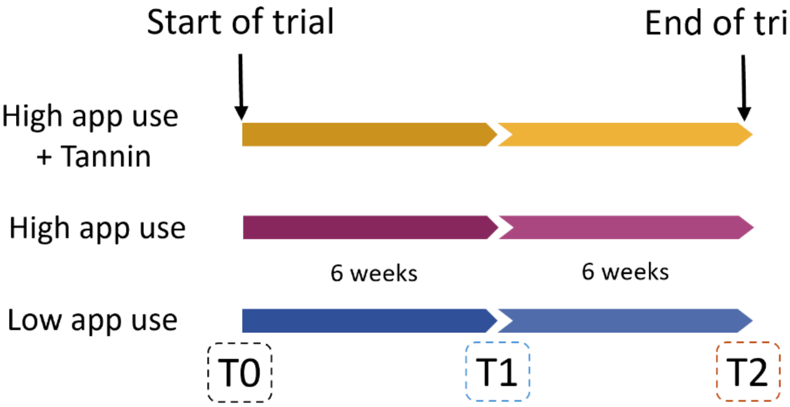


Figure IV-2. Design of the trial groups: The low app use group consists of participants who rarely interacted with the mobile app. The high app use group consists of participants who used the app very frequently. The high app use + tannin group consists of participants who used the app frequently and were supplemented with tannins. Baseline stool samples were taken (T0) and then sample collection was repeated every 6 weeks (T1 and T2).

The DADA2 (v1.20.0) package as implemented in R (v4.1.0) was employed for sequence read processing and forward and reverse merging as well as clustering into ASVs [114]. Filtering and trimming parameters were as follows: maxN=0, maxEE=c(5,5), truncQ=0, trimLeft=c(17,21), truncLen=c(270,230), and rm.phix=TRUE. A minimum overlap of 15 nucleotides and a maximum mismatch of 1 were required for read merging. Reads were aligned against the human genome (GRCh38.p14) using Bowtie2 (v2.4.4) [150] and matches were discarded. Samples sequenced in different sequencing runs were processed separately and joined using the "mergeSequenceTables" function from DADA2.

Taxonomic identification was assigned to ASVs using DADA2 and the SILVA v.138.1 reference database [151]. The MegaBLAST tool from BLAST v(2.12.0) was further used for those ASVs identified only at genus level with DADA2, requiring at least 97% identity for species-level assignment and a minimum difference of 2% between the first- and second-best matches. ASVs with a total number of counts lower than 10 were removed. Count tables at different taxonomic levels were created by summing the count of the same taxonomic group.

2.3 Statistical analysis

Alpha and beta diversity indices (Shannon, Chao1 and Bray-Curtis) were computed using the vegan package (v2.5-2) [152] in the R platform. Differences in alpha-diversity were tested using the Wilcoxon rank-sum test. The Bray-Curtis dissimilarity index was used to quantify the overall dissimilarity between two microbial communities and was employed in PERMANOVA and PCoA. PERMANOVA was performed using the adonis function from Vegan with 999 permutations, considering as significant those with p value ≤ 0.05. The degree of compositional change through time for each individual (compositional dynamics) was calculated within each use group and compared between the two groups by means of a Wilcoxon test. The BH procedure was used for false discovery rate control in all applicable statistical tests. Only significant comparisons with a p-value or q-value ≤ 0.05 are illustrated.

ANCOM-BC2 was used to identify differentially abundant taxa among samples with BH correction for multiple testing and multiple pairwise comparisons. The comparisons with q value ≤ 0.05 have been described as significant[238].

3 Results

During the 12-week study, 107 participants provided fecal samples at three time points. The 16S rRNA amplicon gene analysis of these samples was then performed.

A total of 321 samples were sequenced, resulting in a large data set of 34,341,883 reads. The average sequencing output per sample was 106,984 (±46,143) reads. After applying filtering, a total of 23,836,978 reads were taxonomically classified to the genus level. On average, each individual sample had 74,258 (±30,495) classified reads. The taxonomic classification of the reads revealed the presence of 15 phyla, 89 families, 295 genera, and 308 species within the dataset.

3.1 Effect of app use on the gut microbiota

To assess the impact of mobile application use on the microbiota, a comparative analysis between the participants who had a high use of the app (high app use group) and those who had low use (low app use group) was conducted. As illustrated in **Supplementary Material IV-1**, no statistically significant differences were observed in the alpha diversity (measured by the Shannon diversity index and the Chao1 richness estimator) between the two groups over the different time points, with neither the activity nor the engagement app-usage classifications. However, **Figure IV-3** shows Chao1 differences between T0 and T1 in the Low app use group with the activity classification and in both groups with the engagement classification. The results suggest that the high use of the application did not have a significant global impact on the gut microbiota diversity.

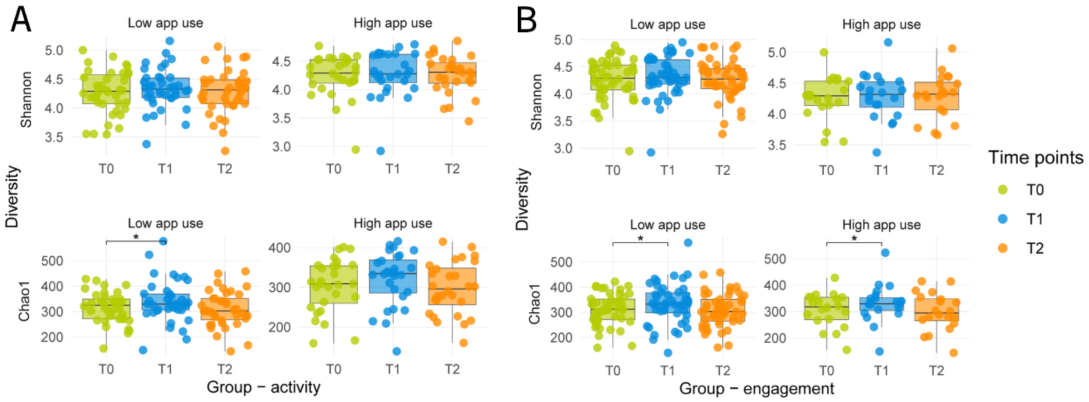


Figure IV-3. Shannon diversity index and Chao1 richness of the microbiota of participants with low and high app use. A) Alpha diversity of the activity classification groups over the time course of the trial. B) Alpha diversity of the engagement classification groups over the time course of the trial. Wilcoxon signed-rank tests were performed for mean comparisons, with significant comparisons (q value ≤0.05) marked as *, and BH was used for multiple testing correction.

Similarly, regarding beta diversity, the overall composition of the microbiota within each group and between groups did not show significant variations (PERMANOVA > 0.05) over the course of the study period in either the activity or the engagement usage classifications (data not shown). Additionally, the compositional dynamics within each individual over time showed no significant differences between the low app use group and the high app use group (**Supplementary Material IV-2**). This indicates a stable microbiota composition within each group and comparable dynamics between the low app use and high app use group throughout the study.

To evaluate whether the use of the mobile i-Diet app had a specific impact on any taxon by altering its abundance, a differential abundance analysis was performed using the ANCOM-BC2 method. We compared the microbiota from the low and high app use participant groups at the same point for each of the three time points. The analysis showed a significant difference in the abundance of the *Eubacterium nodatum* group, showing an increased abundance (q value = 0.025) in the high app use group at T2 for the engagement classification. We also studied the variation in taxon abundances over the course of the study for each group. This analysis showed several significant results, illustrated in **Figure IV-4** at genus level and in **Supplementary Material IV-3** at species level. Some of these results were found in both app usage classification methods in the same groups, such as the increased abundance of *Ruminococcus* and *Coprococcus* (*C. Comes*) with time in the high app use groups. Some unique differences were observed with high app use in only one of the classification methods, such as the increase of *Romboutsia*, *Lachnoclostridium*, and Lachnospiraceae ND3007 group according to the activity classification, and the increase of *Agathobacter*, *Collinsella*, *Actinomyces*, along with the decrease of *Gemella*, *Eubacterium fissicatena* group, and *Desulfovibrio* in the engagement classification.

3.2 Effect of tannin supplementation on the gut microbiota

The effect of the supplementation with tannin pills on the gut microbiota was also investigated. As explained above, only participants who highly used the mobile app and took tannin pills were included for analysis.

We first examined the alpha diversity within the microbiota of individuals in the high app use + tannin group along the three trial timepoints (**Figure IV-5**). A significant increase in the Chao1 index was found between T0 and T1 for the engagement classification. Higher, but not-significantly, Shannon values were also found in T1 and T2.

Additionally, there were no changes in alpha diversity when comparing the high app use + tannin group with the other groups at the same temporal

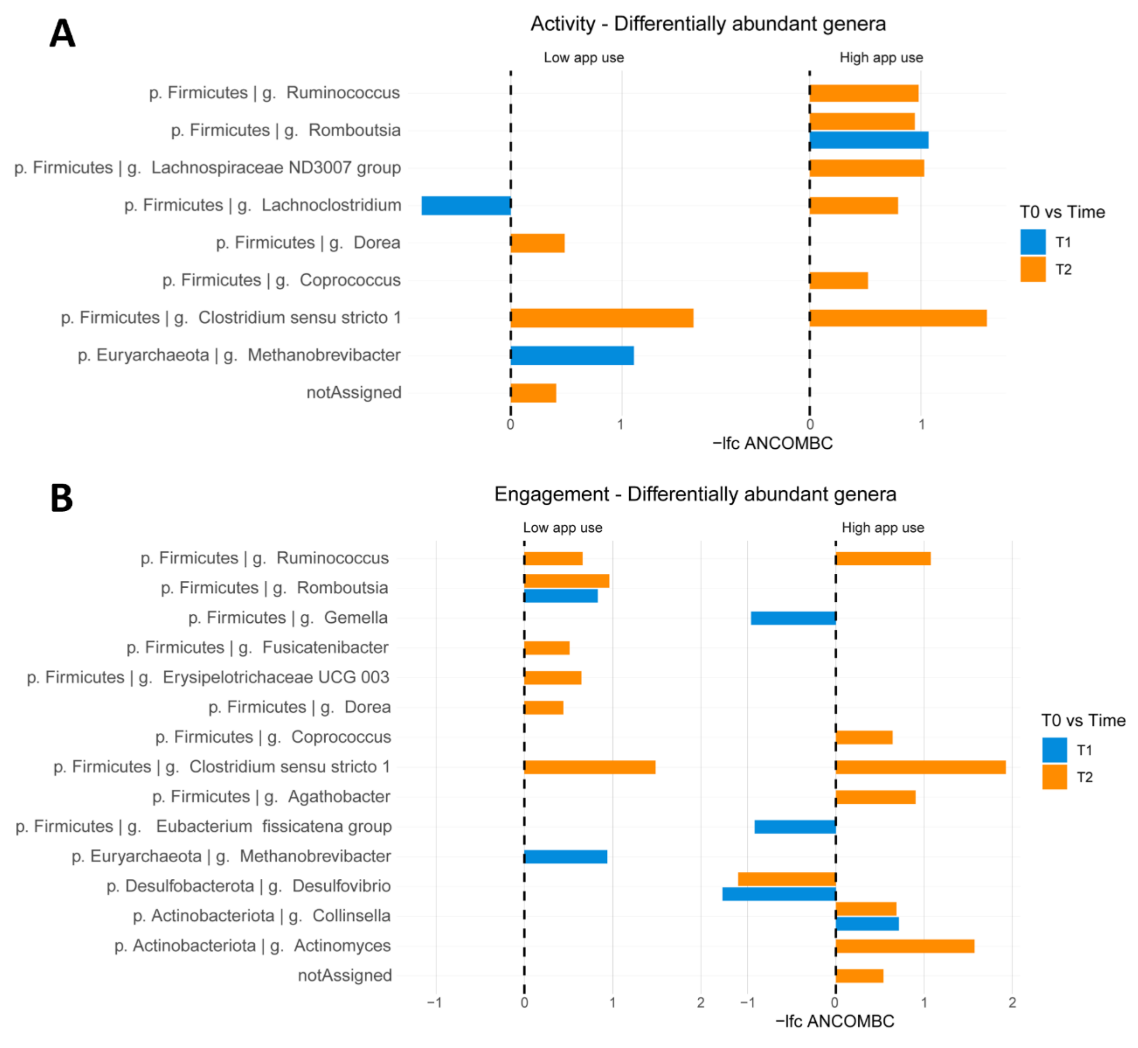


Figure IV-4. Differentially abundant genera at initial and middle/final time points in the two usage groups. A) Differentially abundant genera of groups classified according to app activity. B) Differentially abundant genera of groups classified according to app engagement. Only genera detected as differentially abundant according to ANCOM-BC2 are illustrated. The bars in the figures depict the - log-fold change (-lfc) values derived from ANCOM-BC2. Consequently, positive values signify an increase in abundance at the specified time compared to T0, while negative values indicate a decrease in abundance.

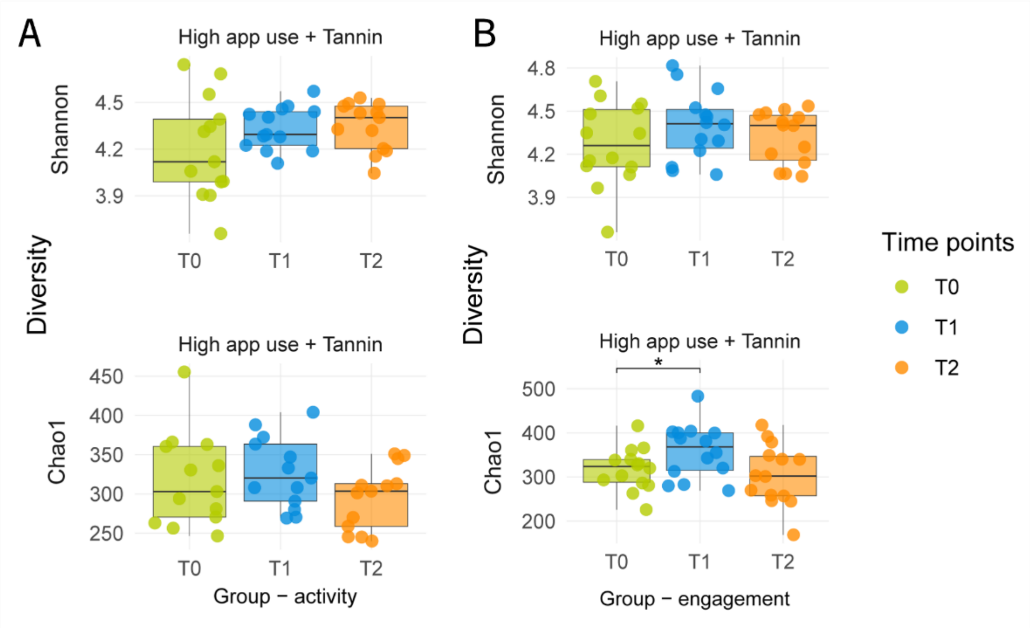


Figure IV-5. Shannon diversity index and Chao1 richness of the microbiota of participants with high app use that were supplemented with tannins. A) Alpha diversity of the activity classification groups over the time course of the trial. B) Alpha diversity of the engagement classification groups over the time course of the trial. Wilcoxon signed-rank tests were performed for mean comparisons, with significant comparisons (q value ≤0.05) marked as *, and BH was used for multiple testing correction.

point, neither for the activity nor for the engagement classifications (**Supplementary Material IV-1**).

Similarly, the analysis of beta diversity showed no statistically significant variations (PERMANOVA > 0.05) in the composition of the microbiota within the high app use + tannin group each group or between this and the other intervention groups throughout the study period (data not shown). Additionally, the compositional dynamics within individuals, showed no significant differences between the low app use, high app use, and high app use + tannin groups (**Supplementary Material IV-4**). These findings were also consistent across both the activity and engagement app usage classifications. These results suggest a stable microbiota composition within all groups, with comparable overall dynamics in the tannin-supplemented group and the other groups along the trial.

The ANCOM-BC2 method was used to evaluate the potential effects of the tannins in modulating the abundance of specific taxa. Initially, we compared the participants with high app use + tannin with the other two groups at the different time points at which the samples were collected. No significant abundance differences were found between the groups at baseline (q value $>$ 0.05) for either app usage classification. However, at temporal points T1 and T2, some differences were observed with the engagement app usage classification. Specifically, an increase in *Butyricimonas* in the high app use + tannin group was observed at T1 compared to both the low and high app use groups (with q values of 0.037 and 0.006, respectively). Furthermore, a slight increase in *Succinatimonas* at T2 was observed in the high app use + tannin group compared to the low app use group (q value = 0.0036), with a non-significant increase compared to the high app use group (q value = 0.073).

When examining the abundance changes throughout the course of the trial for each group, numerous differences were identified, many of which were absent in the low and high app groups, suggesting the implication of tannins. These differences are illustrated in **Figure IV-6** at the genus level or in **Supplementary Material IV-4** at the species level. Some noteworthy observations in the group supplemented with tannins include a decrease in the abundance of Lachnospiraceae GCA 900066575 and *Bilophila* (*B. wadsworthia*) at T1, as well as an increase in the abundance of *Collinsella* at T2, which was found in both app usage classification's groups. Also, there were some unique and significant differences within each app usage classification. With the classification by activity, we detected increases in *Ruminococcus* UBA1819 and

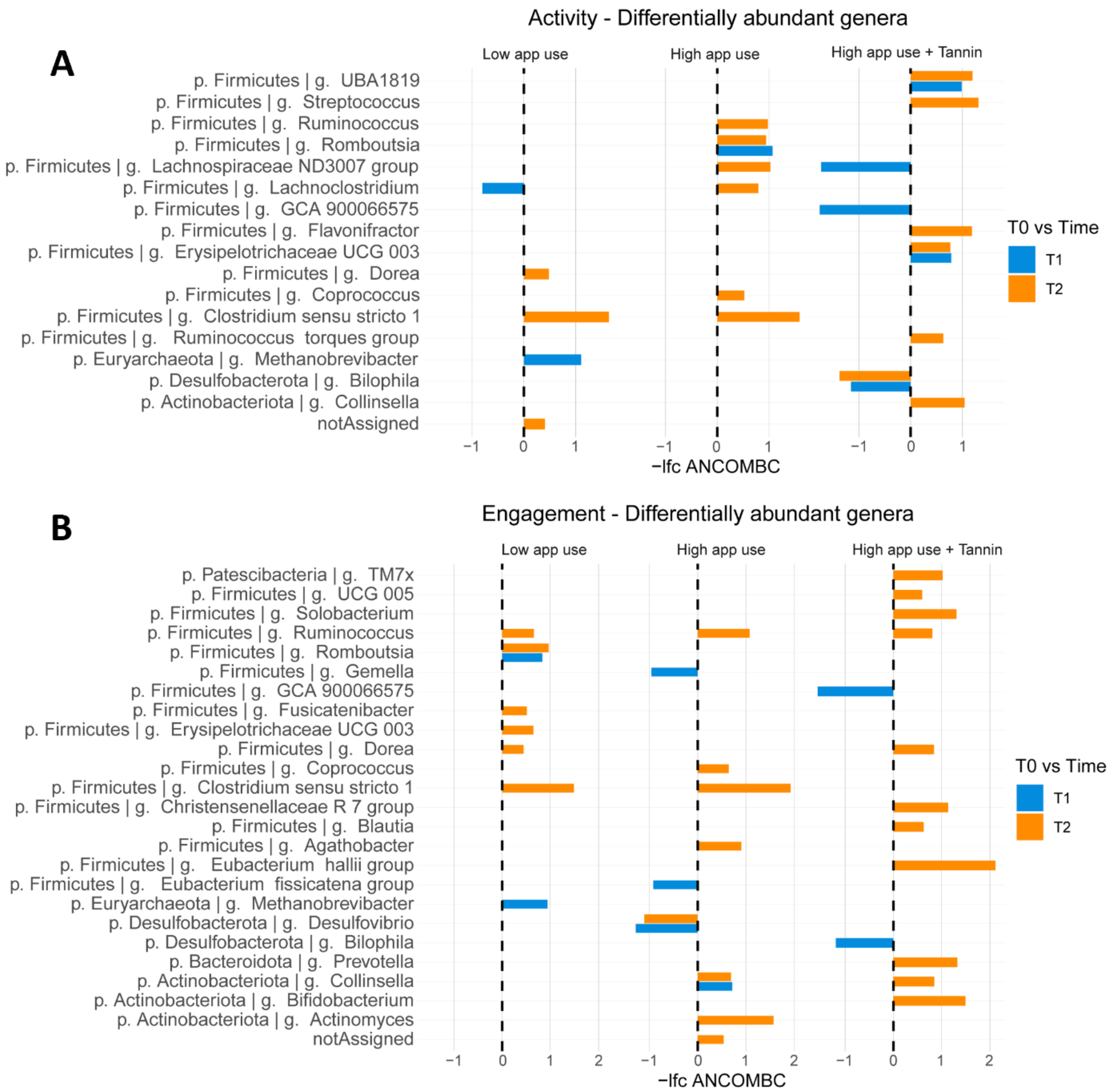


Figure IV-6. Differentially abundant genera at initial and middle/final time point in the two usage groups. A) Differentially abundant genera of groups classified according to app activity. B) Differentially abundant genera of groups classified according to app engagement. Only genera detected as differentially abundant according to ANCOM-BC2 are illustrated. The bars in the figures depict the - log-fold change (-lfc) values derived from ANCOM-BC2. Consequently, positive values indicate an abundance increase in the specified time compared to T0, while negative values indicate an abundance decrease.

Erysipelotrichaceae UCG 003 at both time points, as well as a reduction of Lachnospiraceae ND3007 group. Also, *Flavonifactor* (*F. plautii*), *Ruminococcus torques* group, or *Roseburia hominis* were increased at T2 compared with the baseline. In the engagement usage classification, a higher number of differentially abundant genera were found, with increases in *Solobacterium* (*S. moorei*), *Ruminococcus*, *Dorea*, *Blautia* (*B. massiliensis*), *Eubacterium hallii* group, *Prevotella*, and *Bifidobacterium*.

4 Discussion

For many years, nutritional studies have obtained generic conclusions about healthy eating habits and patterns for the population or stratified groups depending on pathologies, age, sex, etc. However, the rise of nutrigenomics has driven the field towards precision nutrition, tailoring these recommendations more individually. The great expansion of technologies such as smartphones has made it feasible to bring personalized nutrition regimes to individuals in an easy way, making this option more scalable and sustainable over time. In addition, it is possible to perform such personalized recommendations in real time, adjusting them according to how the recorded parameters evolve.

Nevertheless, this type of nutritional intervention faces multiple challenges such as participant recruitment, retention throughout the trial and, especially, ensuring strong adherence to the app's recommendations and consistent dietary self-monitoring [239,240]. In our case, the intervention works through a mobile app that generates personalized recommendations. The app was used to record the participant's changes in meals, physical activity, weight, habits, etc. This not only allowed the provision of the personalized diets through the app but also allowed for real-time modification based on the evaluation of these new parameters, recalculating the future diets. In addition, this technology also collects data on app usage by participants, which can be helpful in determining who is using the app, as we expect a correlation between app use and the following of the dietary recommendations. This analysis is complex due to the numerous parameters involved. Therefore, we opted to stratify the participants into different groups according to two different app usage metrics, developed by our colleagues, that we named “Activity” and “Engagement” and to compare the results obtained by these two different methods. Participants were grouped according to each classification, with less than 25% of users being shared in the high app usage groups of both classifications. The activity classification method had a higher number of participants with high app usage, while the engagement method was more restrictive in this classification. This highlights the challenge of classifying users based on their app usage behavior. Some participants strictly follow the app's indications and do not modify their meals or daily intake, while others modify their meals and interact with the app more frequently. However, these participants will not be classified in the same way, as our usage classifications depend on recorded interactions with the app. This renders our analyses of the impact of the app conservative, in the sense that some participants may have followed the app’s dietary recommendations in spite of being classified in low use groups. Despite the lower number of participants determined to have high app usage according to the engagement classification method, this group exhibited more differences throughout the trial than the others. This is likely due to the high commitment of these individuals to app usage and recommendations. However, studies with larger sample sizes may minimize differences depending on the classification method used.

This study aims to assess the impact of the dietary personalized recommendations generated through i-Diet on the gut microbiota of the participants. The effects of such intervention in other ambits have been analyzed by other colleagues, studying clinical parameters, food intake, habits, etc. within the European project that frames this study.

As described in previous studies on dietary interventions, detecting alpha diversity differences among the groups can be challenging due to various factors such as short intervention periods, high inter-individual variability, or a low number of participants. In our intervention, the increases in gut microbiota richness at T1 in both high and low app use groups suggest a rapid effect of recommendations based on the Mediterranean diet on this parameter. Nevertheless, this effect appears to be transient, as it is no longer detected at T2, suggesting a high resilience in the microbiota of healthy individuals.

Given the low likelihood of detecting global diversity changes, researchers often focus on evaluating intervention effects on the gut microbiota by examining the differentially abundant microbes found among the groups, as highlighted in the meta-analysis about dietary interventions using fiber reported in [72]. In our intervention, according to both app usage classifications, the high app use groups had a higher abundance of *Ruminococcus* and *Coprococcus comes*, two well-studied bacteria associated with SCFA production, particularly butyrate [241,242]. *Ruminococcus* has been linked to regular fruit and vegetable intake, and its role in the degradation of complex carbohydrates of plant origins and butyrate production has been extensively described. In addition, it has been associated with a lower BMI, improved lipids profile and lower risk of cardiovascular diseases [53,242,243]. Moreover, the groups with high app use were linked with a decrease in *Gemella* and *Desulfovibrio*. These genera have been described as potentially harmful due to their association with obesity and a proinflammatory action [196,244–246].

Tannins are polyphenols with antioxidant and anti-inflammatory activity that have proven useful in helping against chronic diseases [63,247]. Our group has previously studied the effect of tannins on the gut microbiota [64,231,232]. Here, our findings showed a significant increase in richness in the tannin-supplemented group. However, richness returns to values similar to the initial ones at the end of the trial, as also observed in the participants not supplemented with tannins. Additionally, a non-significant trend of increased Shannon diversity is observed with tannins that was not observed in the other groups, in accordance with what has been previously described. Thus, a larger sample size may be necessary to reach a significant conclusion [248].

Tannin supplementation resulted, in both app usage classification groups, in a decrease in the abundance of *Bilophila wadsworthia*, a pathobiont associated with high fat diets and inflammatory bowel disorders [54,249–251]. An increase of *Collinsella* was also found in both classifications. Although *Collinsella* also augmented in the high app use without tannins, this was only for the engagement classification and with a lower lfc values, suggesting that tannin supplementation further favored the *Collinsella* increase. This would be in accordance with the described capacity of this genus to degrade polyphenols. The increase of *Collinsella* by the action of tannins has been previously reported and correlated with the production of SCFAs[231]. The capacity of butyrate production has also been described for some species of this genus [252,253].

We also observed bacteria that varied in abundance as an effect of tannins only with one of the methods of classification of the use of the app. For instance, when the groups were classified by activity, we detected increases with time in the tannin group in the abundances of bacteria described as beneficial (*Roseburia hominis*) and as potentially harmful (*Flavonifactor*), as well as others such as *Ruminococcus torques* group for which the literature has not reached a consensus on their role [55,165,254–257]. Similarly, when classifying the participants by engagement, the tannin supplementation group showed increases with time of healthy bacteria, such as *Bifidobacterium*, *Ruminococcus*, and *Eubacterium hallii*, but also of bacteria associated with pathologies such as *Solobacterium moorei* and *Dorea* [220,258–261]. The engagement classification also showed higher *Succinatimonas* and *Butyricimonas* abundances in the tannin supplementation group compared with the other groups. These bacteria are known for their ability to produce SCFAs such as acetate, succinate, and butyrate. This makes them potentially beneficial to health and studies have reported lower *Butyricimonas* presence in patients with IBS or hypertension [251,262–264].

Overall, most of the bacteria increased with tannins are known SCFA producers and are considered beneficial to health. Although some of the bacteria increased by tannins have been associated with negative effects, the trend toward increased diversity and the promotion of bacteria with SCFAs production abilities suggest that tannins mainly exert beneficial health effects. Among other benefits, it has also been suggested that tannins might help to reduce the side effects of the Western diet, such as the occurrence of chronic low-grade inflammation, through regulation of the immune response [265].

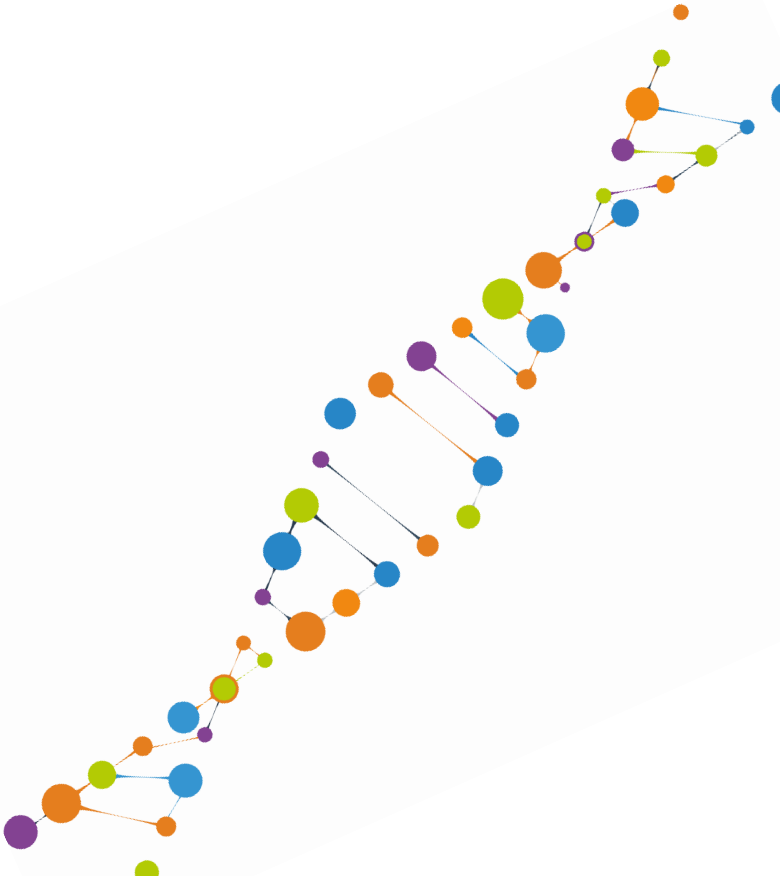
Dietary interventions present inherent challenges that must be carefully addressed. These include the significant inter-individual variability in gut microbiota composition among participants. Addressing this requires using appropriate statistical methods that may consider this variability and the inclusion of large numbers of participants. Additionally, monitoring and evaluating the adherence of the participants to the intervention recommendations is challenging. Here we show that classification of participants according to different app usage criteria influences substantially the evaluation of intervention results. Overall, the engagement-based classification revealed more changes with time in the high use groups as well as more differences between high and low use groups, suggesting that its stricter requirements for high use definition resulted in a better stratification of participants, potentially more reflective of their adherence to the app's dietary recommendations.

Furthermore, evaluating whether the participants' microbiota evolves towards a healthier state throughout the trial is especially complex, particularly as many of the effects or associations observed in the literature are species or strain dependent. It is necessary to develop new methods to determine this, despite the complexity involved due to the large number of factors at play. New large-scale studies with updated and standardized methodology, in which participants are comprehensively evaluated to collect detailed information about their diet, clinical and health parameters, genetics and gut microbiota, could greatly contribute to elucidate the interactions between diet, microbiota and host.

5 Conclusions

1. The classification of participants according to app usage is crucial to identify the intervention effects but may substantially influence the results.
2. The engagement-based classification was more useful for revealing changes associated with app use.
3. The high use of the app resulted in the modulation of the gut microbiota and promoted the increase of beneficial bacteria such as *Ruminococcus* and *Coprococcus comes.*
4. The supplementation with tannins shifted the gut microbiota, mainly decreasing the abundance of *Bilophila wadsworthia* and increasing that of SCFA-producing bacteria.

General discussion



General discussion

Throughout this thesis, we have explored the capacity of foods to modulate and shape the gut microbiota. Each food has a unique nutritional profile in which nutrients interact among them, and they also interact jointly with the gut microbiota. These interactions can promote certain microbes, improving their growth through the consumption of these nutrients, or inhibit others, thereby shifting the composition of the gut microbiota. As observational or interventional studies cannot evaluate the effects of single foods on the gut microbiota, it is necessary to further investigate the specific effects of each food through *in vitro* analyses and this study contribute to shed light on this matter.

Although there is considerable variability among individuals, we have identified common changes induced by specific foods. *In vitro* fermentations have allowed us to observe the short-term effects of food on the fermentative microbiota. For instance, we observed that animal-based foods, particularly those that are rich in fat, significantly impacted on a gut microbiota adapted to ferment complex carbohydrates that are not digestible by the host enzymes.

Furthermore, some of these effects have been identified in both children and adults, as illustrated by *Anaerostipes*, which increased its abundance in response to animal and vegetable fats in healthy children and adults. As reported in numerous studies, the gut microbiota composition of individuals with different health conditions such as obesity, celiac disease and allergy differs from that of healthy individuals [28,34,145]. However, the presence of *Alistipes* has decreased in the fermentative microbiota of obese, celiac and allergic children with the influence of animal-based foods. These findings have the potential to improve personalized diets that consider the gut microbiota and its essential role in health, as they showcase how to modulate the abundance of particular bacteria through specific foods. This is particularly relevant for children, where we have observed that the diversity of the gut microbiota is lower than that of adults and its composition is more susceptible to changes induced by food. Moreover, there is evidence that changes in the microbiota at this stage of development may have consequences later in life[10,212]. Understanding the different microbiota responses to foods from individuals with various pathologies can facilitate tailoring diets for patients with such conditions, promoting that their microbiota composition evolves toward a healthier one.

Likewise, cooking methods, which can alter the effects of foods on gut microbiota composition, should be taken into account when creating these personalized diets. Furthermore, each food is differently affected by the cooking methods used, as they have different nutrients in different alimentary matrices that may have different responses[104]. Nevertheless, we have found interesting general results, such as including the observation that the frying process has the greatest effect on the intestinal bacteria, perhaps due to the fact that fried foods have a higher fat content, and that fat, as we have previously mentioned, causes significant shifts in microbiota composition.

However, it is also important to note that most of the observed differences are dependent on the individual or study groups, due to the aforementioned large variability among individuals and the specific characteristics of the microbiota in different age or disease groups. This highlights the need for further studies evaluating the effects of foods in larger numbers of individuals, including participants from different locations to capture as much diversity as possible.

Based on the obtained results, there are several unanswered questions that require further investigation. It would be beneficial to analyze the complete metagenome and metatranscriptome produced in these food fermentations in order to evaluate not only the evolution of the composition of the gut microbiota, but also its functionality, as well as being able to get higher taxonomic resolution. These approaches would allow us to observe whether the microbiota functionality is maintained or whether it adapts rapidly to the nutrients with which it interacts. Additionally, other parameters were measured by our colleagues in the fermentation assays, including SCFAs and antioxidant capacity. Ongoing integration of these data with the microbiota composition will provide us in the near future with valuable information to obtain a global picture of the gut microbiota response to food.

Methods such as the *in vitro* fermentations used in the present work have several limitations to overcome, such as the fact that the physiological conditions of the gastrointestinal tract are not exactly replicated, that certain bacteria are unable to grow in these systems, and other biases that may occur during fermentation. However, they are very useful to study the effect of foods on the microbiota by eliminating complexities that are difficult to account for and allowing a large number of experiments to create better models to predict the response of the microbiota to foods. It would be valuable to perform extended *in vitro* fermentation studies using more complex systems, to validate the results under conditions that are more physiologically relevant. However, this would come at the cost of scalability as fewer individuals and foods would be tested due to the extended duration and costs of such experiments.

With the rise of personalized medicine and nutrition, there has been an increase in studies based on nutritional interventions that tailor diets according to goals, energy needs, genetic predisposition, and pathologies. In the intervention evaluated in this thesis within the framework of the Stance4Health project, we have observed mainly beneficial alterations in the composition of the gut microbiota of participants through dietary recommendations aimed at achieving a healthier diet. Moreover, during the nutritional intervention, a significant amount of data and measurements (such as food frequency, intestinal habits, or body weight) were collected from the participants. The ongoing integration of this information will in the near future allow us to identify which factors were most associated with the shifts of the gut microbiota. Additionally, the app's registry allows for a detailed report of the nutrients present in the participants' ingested food. This information will also be integrated with the taxonomic profile of the microbiota to create more accurate models of how the microbiota changes in response to specific nutrients, taking into account all studied factors and parameters.

Maintaining a healthy diet, such as the Mediterranean diet on which i-Diet recommendations were based in the Stance4Health intervention, is usually associated with improvements in the composition of the microbiota and the metabolites it produces. However, as each individual's microbiota is different and therefore responds differently to different foods and cooking methods, new strategies that incorporate this variability are required in order to allow for improved dietary recommendations based on each individual's particular microbiota.

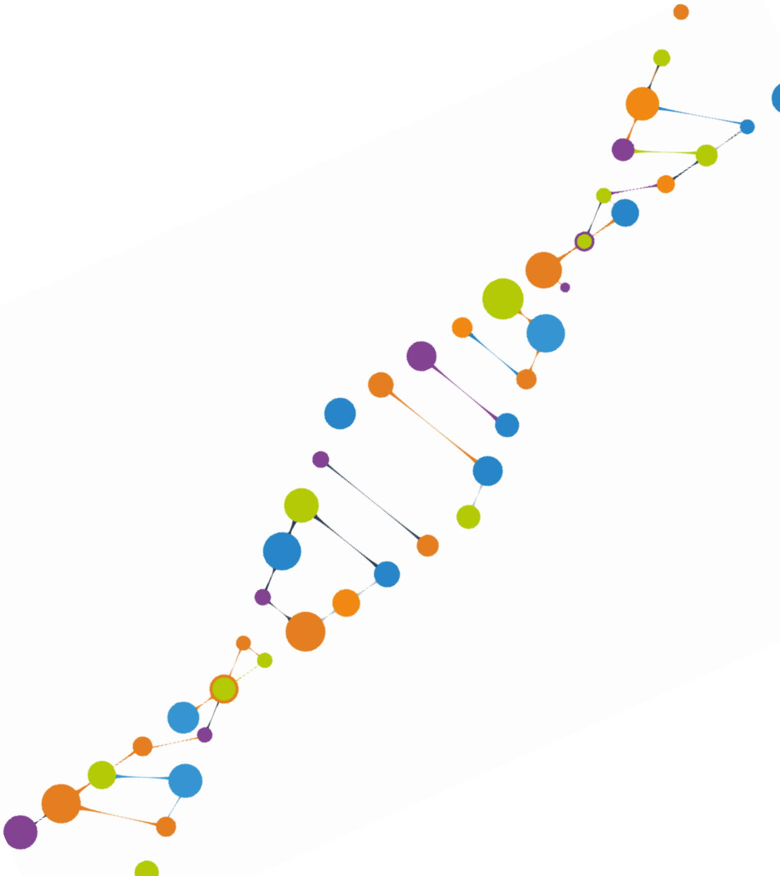
Current advances in the understanding of the gut microbiota metabolism, including those achieved in the Stance4Health project[266,267], open up the possibility of adjusting the diet to better utilize the specific metabolic capabilities of an individual's gut microbiota. Future interventions will implement and evaluate an i-Diet app module (GM module) that will incorporate such metabolic information to tailor dietary recommendations.

The module has already been developed and is based on the Stance4Health project's extended reconstruction of dietary metabolism in the human gut microbiota (AGREDA) [236,266]. The AGREDA network establishes relevant metabolic interactions between dietary compounds and the gut microbiota, allowing the prediction of the metabolites produced by the app users' gut microbiota when consuming specific foods. These predictions are translated into a score that enables the ranking of foods according to the microbial metabolites that will be produced, with the highest scores going to those that result in metabolite levels within healthy ranges. This ranking then influences the food recommendation process in the app. Thus, the application, complemented by the GM module, will prioritize recommendations that favor optimal combinations of ingredients for the microbiota while minimizing the less favorable ones [236]. This approach moves us forward in precision nutrition by integrating individual needs and microbiota capabilities.

The implementation of the nutritional intervention described in this thesis has provided us with very valuable information that can be used in future interventions such as the one that will evaluate the GM module. For instance, learning about the participants' engagement with the intervention and how to manage the associated variables will help achieve a better implementation and evaluation of the intervention's success.

Finally, it is worth noting that strategies such as the one implemented in GM are just starting to be developed and will require much further work to optimally address the many complexities involved, including the large amount of data required for accuracy. An important challenge will be to develop strategies that can take into account the dynamics of the microbiota as nutrients reach it and as it evolves through time, therefore changing its response to food.

Conclusions

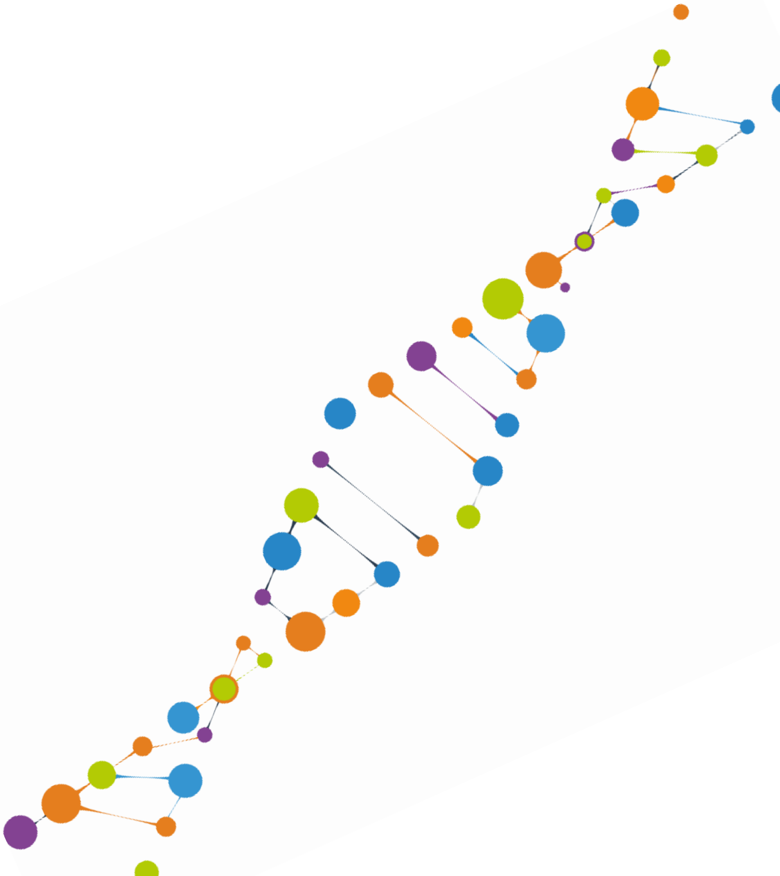


Conclusions

1. The fermentative gut microbiota of adults and children, despite inter-individual variability, shows several common changes due to the effect of various foods.
2. Animal-based foods and animal and vegetable fats significantly impact the fermentative microbiota of both adults and children.
3. Many food-induced microbiota changes are individual-specific or restricted to either adults or children, especially those related to the method of cooking.
4. Cooking methods are capable of influencing the effect that foods have on the fermentative microbiota, and vary according to the food, with frying having the greatest impact.
5. The fermentative microbiota of children shows greater food-mediated differences than that of adults, indicating greater malleability through diet.
6. Children afflicted with pathologies associated with, or resulting from, dietary disturbances exhibit discernible differences in the composition of their gut microbiota, which directly influence their response to dietary intakes.
7. The specific effects of individual foods and cooking methods on the gut microbiota should be considered for the elaboration of personalized diets to modulate the gut microbiota in a targeted way.
8. In a personalized dietary intervention involving use of a dietary app the commitment of the participants to high app usage is a crucial factor for intervention success and should be taken into account to identify the real effects of the intervention.

9. The metrics employed to assess app usage can substantially alter the evaluation of intervention results, with engagement-based metrics focusing on the number of menu modifications performed by the users seeming more adequate for establishing high app use.
10. High use of the i-Diet mobile app likely reflects a higher adherence to the personalized diet recommendations, resulting in the modulation of the gut microbiota with increase of beneficial bacteria such as *Ruminococcus* and *Coprococcus comes.*
11. Supplementation with tannins shifts the gut microbiota, increasing the abundance of beneficial SCFA-producing bacteria and decreasing that of pathobionts.

References



References

1. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al. The NIH Human Microbiome Project. Genome Res. 19:2317–23 doi:10.1101/gr.096651.109.

2. Davies J. In a map for human life, count the microbes, too. Science. 291:2316–2316 doi:10.1126/SCIENCE.291.5512.2316B.

3. Ehrlich SD. MetaHIT: The European Union Project on Metagenomics of the Human Intestinal Tract. Metagenomics of the Human Body. New York, NY: Springer New York; p. 307–16 doi:10.1007/978-1-4419-7089-3_15.

4. Sanz Y, Romaní-Perez M, Benítez-Páez A, Portune KJ, Brigidi P, Rampelli S, et al. Towards microbiome-informed dietary recommendations for promoting metabolic and mental health: Opinion papers of the MyNewGut project. Clinical Nutrition. 37:2191–7 doi:10.1016/j.clnu.2018.07.007.

5. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012 489:7415. 489:220–30 doi:10.1038/nature11550.

6. Oren A, Garrity GM. Valid publication of the names of forty-two phyla of prokaryotes. Int J Syst Evol Microbiol. 71 doi:10.1099/ijsem.0.005056.

7. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Cellular and Molecular Life Sciences. 76:473–93 doi:10.1007/s00018-018-2943-4.

8. D'Amelio P, Sassi F. Gut Microbiota, Immune System, and Bone. Calcif Tissue Int. 102:415–25 doi:10.1007/s00223-017-0331-y.

9. Góralczyk-Bińkowska A, Szmajda-Krygier D, Kozłowska E. The Microbiota–Gut–Brain Axis in Psychiatric Disorders. Int J Mol Sci. 23:11245 doi:10.3390/ijms231911245.

10. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiology and Molecular Biology Reviews. 81 doi:10.1128/MMBR.00036-17.

11. Biasucci G, Rubini M, Riboni S, Morelli L, Bessi E, Retetangos C. Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev. 86:13–5 doi:10.1016/j.earlhumdev.2010.01.004.

12. Del Chierico F, Vernocchi P, Petrucca A, Paci P, Fuentes S, Praticò G, et al. Phylogenetic and Metabolic Tracking of Gut Microbiota during Perinatal Development. PLoS One. 10:e0137347 doi:10.1371/journal.pone.0137347.

13. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proceedings of the National Academy of Sciences. 107:11971–5 doi:10.1073/pnas.1002601107.

14. Bäckhed F, Roswall J, Peng Y, Feng Q, Jia H, Kovatcheva-Datchary P, et al. Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life. Cell Host Microbe. 17:690–703 doi:10.1016/j.chom.2015.04.004.

15. O'Sullivan A, Farver M, Smilowitz JT. Article Commentary: The Influence of Early Infant-Feeding Practices on the Intestinal Microbiome and Body Composition in Infants. Nutr Metab Insights. 8s1:NMI.S29530 doi:10.4137/NMI.S29530.

16. Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human gut microbiome viewed across age and geography. Nature. 486:222–7 doi:10.1038/NATURE11053.

17. Radjabzadeh D, Boer CG, Beth SA, van der Wal P, Kiefte-De Jong JC, Jansen MAE, et al. Diversity, compositional and functional differences between gut microbiota of children and adults. Sci Rep. 10:1040 doi:10.1038/s41598-020-57734-z.

18. Hollister EB, Riehle K, Luna RA, Weidler EM, Rubio-Gonzales M, Mistretta T-A, et al. Structure and function of the healthy pre-adolescent pediatric gut microbiome. Microbiome. 3:36 doi:10.1186/s40168-015-0101-x.

19. Derrien M, Alvarez A-S, de Vos WM. The Gut Microbiota in the First Decade of Life. Trends Microbiol. 27:997–1010 doi:10.1016/j.tim.2019.08.001.

20. Weiss GA, Hennet T. Mechanisms and consequences of intestinal dysbiosis. Cellular and Molecular Life Sciences. 74:2959–77 doi:10.1007/s00018-017-2509-x.

21. Wlodarska M, Kostic AD, Xavier RJ. An Integrative View of Microbiome-Host Interactions in Inflammatory Bowel Diseases. Cell Host Microbe. 17:577–91 doi:10.1016/j.chom.2015.04.008.

22. Gérard P. Gut microbiota and obesity. Cell Mol Life Sci. 73:147–62 doi:10.1007/S00018-015-2061-5.

23. Knip M, Siljander H. The role of the intestinal microbiota in type 1 diabetes mellitus. Nat Rev Endocrinol. 12:154–67 doi:10.1038/nrendo.2015.218.

24. Tremlett H, Bauer KC, Appel-Cresswell S, Finlay BB, Waubant E. The gut microbiome in human neurological disease: A review. Ann Neurol. 81:369–82 doi:10.1002/ana.24901.

25. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, et al. Transmission of Atherosclerosis Susceptibility with Gut Microbial Transplantation. Journal of Biological Chemistry. 290:5647–60 doi:10.1074/jbc.M114.618249.

26. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444:1027–31 doi:10.1038/NATURE05414.

27. Denou E, Marcinko K, Surette MG, Steinberg GR, Schertzer JD. High-intensity exercise training increases the diversity and metabolic capacity of the mouse distal gut microbiota during diet-induced obesity. American Journal of Physiology-Endocrinology and Metabolism. 310:E982–93 doi:10.1152/ajpendo.00537.2015.

28. Bäckhed F, Ding H, Wang T, Hooper L V., Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences. 101:15718–23 doi:10.1073/pnas.0407076101.

29. Chen Y, Zhou J, Wang L. Role and Mechanism of Gut Microbiota in Human Disease. Front Cell Infect Microbiol. 11 doi:10.3389/fcimb.2021.625913.

30. Caruso R, Lo BC, Núñez G. Host-microbiota interactions in inflammatory bowel disease. Nat Rev Immunol. 20:411–26 doi:10.1038/S41577-019-0268-7.

31. Schaubeck M, Clavel T, Calasan J, Lagkouvardos I, Haange SB, Jehmlich N, et al. Dysbiotic gut microbiota causes transmissible Crohn's disease-like ileitis independent of failure in antimicrobial defence. Gut. 65:225–37 doi:10.1136/gutjnl-2015-309333.

32. De Palma G, Nadal I, Collado MC, Sanz Y. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. British Journal of Nutrition. 102:1154–60 doi:10.1017/S0007114509371767.

33. De Palma G, Capilla A, Nova E, Castillejo G, Varea V, Pozo T, et al. Influence of Milk-Feeding Type and Genetic Risk of Developing Coeliac Disease on Intestinal Microbiota of Infants: The PROFICEL Study. PLoS One. 7:e30791 doi:10.1371/journal.pone.0030791.

34. Thompson-Chagoyan OC, Vieites JM, Maldonado J, Edwards C, Gil A. Changes in faecal microbiota of infants with cow's milk protein allergy - a Spanish

prospective case-control 6-month follow-up study. Pediatric Allergy and Immunology. 21:e394–400 doi:10.1111/j.1399-3038.2009.00961.x.

35. Moriki D, Francino MP, Koumpagioti D, Boutopoulou B, Rufián-Henares JÁ, Priftis KN, et al. The Role of the Gut Microbiome in Cow's Milk Allergy: A Clinical Approach. Nutrients. 14:4537 doi:10.3390/nu14214537.

36. Fischbach MA. Microbiome: Focus on Causation and Mechanism. Cell. 174:785–90 doi:10.1016/j.cell.2018.07.038.

37. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. Science (1979). 336:489–93 doi:10.1126/science.1219328.

38. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. Clinical & Experimental Allergy. 44:842–50 doi:10.1111/cea.12253.

39. Dzidic M, Abrahamsson TR, Artacho A, Björkstén B, Collado MC, Mira A, et al. Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. Journal of Allergy and Clinical Immunology. 139:1017-1025.e14 doi:10.1016/j.jaci.2016.06.047.

40. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. ISME J. 1:56–66 doi:10.1038/ismej.2007.3.

41. Leeming ER, Johnson AJ, Spector TD, Roy CIL. Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. Nutrients. 11 doi:10.3390/NU11122862.

42. Taylor BC, Lejzerowicz F, Poirel M, Shaffer JP, Jiang L, Aksenov A, et al. Consumption of Fermented Foods Is Associated with Systematic Differences in the Gut Microbiome and Metabolome. mSystems. 5 doi:10.1128/MSYSTEMS.00901-19.

43. Cunningham M, Azcarate-Peril MA, Barnard A, Benoit V, Grimaldi R, Guyonnet D, et al. Shaping the Future of Probiotics and Prebiotics. Trends Microbiol. 29:667–85 doi:10.1016/j.tim.2021.01.003.

44. Wieërs G, Belkhir L, Enaud R, Leclercq S, Philippart de Foy JM, Dequenne I, et al. How Probiotics Affect the Microbiota. Front Cell Infect Microbiol. 9:454 doi:10.3389/FCIMB.2019.00454.

45. Lagoumintzis G, Patrinos GP. Triangulating nutrigenomics, metabolomics and microbiomics toward personalized nutrition and healthy living. Hum Genomics. 17:109 doi:10.1186/s40246-023-00561-w.

46. Delmée E, Cani PD, Gual G, Knauf C, Burcelin R, Maton N, et al. Relation between colonic proglucagon expression and metabolic response to oligofructose in high fat diet-fed mice. Life Sci. 79:1007–13 doi:10.1016/j.lfs.2006.05.013.

47. Donati Zeppa S, Agostini D, Ferrini F, Gervasi M, Barbieri E, Bartolacci A, et al. Interventions on Gut Microbiota for Healthy Aging. Cells. 12:34 doi:10.3390/CELLS12010034.

48. Yadav MK, Kumari I, Singh B, Sharma KK, Tiwari SK. Probiotics, prebiotics and synbiotics: Safe options for next-generation therapeutics. Appl Microbiol Biotechnol. 106:505–21 doi:10.1007/s00253-021-11646-8.

49. Morishima S, Aoi W, Kawamura A, Kawase T, Takagi T, Naito Y, et al. Intensive, prolonged exercise seemingly causes gut dysbiosis in female endurance runners. J Clin Biochem Nutr. 68:253 doi:10.3164/JCBN.20-131.

50. Gubert C, Kong G, Renoir T, Hannan AJ. Exercise, diet and stress as modulators of gut microbiota: Implications for neurodegenerative diseases. Neurobiol Dis. 134 doi:10.1016/J.NBD.2019.104621.

51. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med. 368:407–15 doi:10.1056/NEJMOA1205037.

52. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. Nature. 555:210–5 doi:10.1038/NATURE25973.

53. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes. 3:289–306 doi:10.4161/gmic.19897.

54. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 505:559–63 doi:10.1038/nature12820.

55. Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, et al. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut. 63:1275–83 doi:10.1136/gutjnl-2013-304833.

56. Ley RE, Bä Ckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. PNAS. doi:10.1073/pnas.0504978102.

57. Lv Z, Wang Y, Yang T, Zhan X, Li Z, Hu H, et al. Vitamin A deficiency impacts the structural segregation of gut microbiota in children with persistent diarrhea. J Clin Biochem Nutr. 59:113–21 doi:10.3164/jcbn.15-148.

58. Bielik V, Kolisek M. Bioaccessibility and Bioavailability of Minerals in Relation to a Healthy Gut Microbiome. Int J Mol Sci. 22 doi:10.3390/IJMS22136803.

59. Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components [Internet]. Eur J Nutr. Eur J Nutr; doi:10.1007/s00394-017-1445-8.

60. Barone M, D'Amico F, Brigidi P, Turroni S. Gut microbiome–micronutrient interaction: The key to controlling the bioavailability of minerals and vitamins? Biofactors. 48:307 doi:10.1002/BIOF.1835.

61. Molino S, Fernández-Miyakawa M, Giovando S, Rufián-Henares JÁ. Study of antioxidant capacity and metabolization of quebracho and chestnut tannins through in vitro gastrointestinal digestion-fermentation. J Funct Foods. 49:188–95 doi:10.1016/J.JFF.2018.07.056.

62. Cardona F, Andrés-Lacueva C, Tulipani S, Tinahones FJ, Queipo-Ortuño MI. Benefits of polyphenols on gut microbiota and implications in human health. J Nutr Biochem. 24:1415–22 doi:10.1016/j.jnutbio.2013.05.001.

63. Koch W. Dietary Polyphenols—Important Non-Nutrients in the Prevention of Chronic Noncommunicable Diseases. A Systematic Review. Nutrients. 11 doi:10.3390/NU11051039.

64. Molino S, Lerma-Aguilera A, Jiménez-Hernández N, Gosalbes MJ, Rufián-Henares JÁ, Francino MP. Enrichment of Food With Tannin Extracts Promotes Healthy Changes in the Human Gut Microbiota. Front Microbiol. 12:570 doi:10.3389/fmicb.2021.625782.

65. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y-Y, Keilbaugh SA, et al. Linking Long-Term Dietary Patterns with Gut Microbial Enterotypes. Science (1979). 334:105–8 doi:10.1126/science.1208344.

66. Johnson AJ, Vangay P, Al-Ghalith GA, Hillmann BM, Ward TL, Shields-Cutler RR, et al. Daily Sampling Reveals Personalized Diet-Microbiome Associations in Humans. Cell Host Microbe. 25:789-802.e5 doi:10.1016/J.CHOM.2019.05.005.

67. McDonald D, Hyde E, Debelius JW, Morton JT, Gonzalez A, Ackermann G, et al. American Gut: an Open Platform for Citizen Science Microbiome Research. mSystems. 3 doi:10.1128/MSYSTEMS.00031-18.

68. Sanz Y. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult humans. Gut Microbes. 1:135–7 doi:10.4161/gmic.1.3.11868.

69. Hill P, Muir JG, Gibson PR. Controversies and Recent Developments of the Low-FODMAP Diet. Gastroenterol Hepatol (N Y). 13:36–45 .

70. Reddel S, Putignani L, Del Chierico F. The Impact of Low-FODMAPs, Gluten-Free, and Ketogenic Diets on Gut Microbiota Modulation in Pathological Conditions. Nutrients. 11:373 doi:10.3390/nu11020373.

71. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 488:178–84 doi:10.1038/NATURE11319.

72. So D, Whelan K, Rossi M, Morrison M, Holtmann G, Kelly JT, et al. Dietary fiber intervention on gut microbiota composition in healthy adults: a systematic review and meta-analysis. Am J Clin Nutr. 107:965–83 doi:10.1093/AJCN/NQY041.

73. Berding K, Holscher HD, Arthur AE, Donovan SM. Fecal microbiome composition and stability in 4- to 8-year old children is associated with dietary patterns and nutrient intake. J Nutr Biochem. 56:165–74 doi:10.1016/J.JNUTBIO.2018.01.002.

74. Chassaing B, Vijay-Kumar M, Gewirtz AT. How diet can impact gut microbiota to promote or endanger health. Curr Opin Gastroenterol. 33:417–21 doi:10.1097/MOG.0000000000000401.

75. Zinöcker M, Lindseth I. The Western Diet–Microbiome-Host Interaction and Its Role in Metabolic Disease. Nutrients. 10:365 doi:10.3390/nu10030365.

76. Herman DR, Rhoades N, Mercado J, Argueta P, Lopez U, Flores GE. Dietary habits of two to nine-year-old American children are associated with gut microbiome composition. J Acad Nutr Diet. 120:517 doi:10.1016/J.JAND.2019.07.024.

77. Haro C, García-Carpintero S, Rangel-Zúñiga OA, Alcalá-Díaz JF, Landa BB, Clemente JC, et al. Consumption of Two Healthy Dietary Patterns Restored Microbiota Dysbiosis in Obese Patients with Metabolic Dysfunction. Mol Nutr Food Res. 61:1700300 doi:10.1002/mnfr.201700300.

78. Smits SA, Leach J, Sonnenburg ED, Gonzalez CG, Lichtman JS, Reid G, et al. Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. Science (1979). 357:802–6 doi:10.1126/science.aan4834.

79. Healey GR, Murphy R, Brough L, Butts CA, Coad J. Interindividual variability in gut microbiota and host response to dietary interventions. Nutr Rev. 75:1059–80 doi:10.1093/nutrit/nux062.

80. Tuohy KM, Finlay RK, Wynne AG, Gibson GR. A Human Volunteer Study on the Prebiotic Effects of HP-Inulin—Faecal Bacteria Enumerated Using Fluorescent In Situ Hybridisation (FISH). Anaerobe. 7:113–8 doi:10.1006/ANAE.2001.0368.

81. Reuter JA, Spacek D V., Snyder MP. High-Throughput Sequencing Technologies. Mol Cell. 58:586 doi:10.1016/J.MOLCEL.2015.05.004.

82. Bharti R, Grimm DG. Current challenges and best-practice protocols for microbiome analysis. Brief Bioinform. 22:178–93 doi:10.1093/bib/bbz155.

83. Pérez-Burillo S, Molino S, Navajas-Porras B, Valverde-Moya ÁJ, Hinojosa-Nogueira D, López-Maldonado A, et al. An in vitro batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality. Nat Protoc. 16:3186–209 doi:10.1038/s41596-021-00537-x.

84. Wang M, Wichienchot S, He X, Fu X, Huang Q, Zhang B. In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. Trends Food Sci Technol. 88:1–9 doi:10.1016/j.tifs.2019.03.005.

85. Ludwig IA, Paz de Peña M, Concepción C, Alan C. Catabolism of coffee chlorogenic acids by human colonic microbiota. BioFactors. 39:623–32 doi:10.1002/biof.1124.

86. Moon JS, Li L, Bang J, Han NS. Application of in vitro gut fermentation models to food components: A review. Food Sci Biotechnol. 25:1–7 doi:10.1007/s10068-016-0091-x.

87. Van de Wiele T, Van den Abbeele P, Ossieur W, Possemiers S, Marzorati M. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The Impact of Food Bioactives on Health. Cham: Springer International Publishing; p. 305–17 doi:10.1007/978-3-319-16104-4_27.

88. Agans R, Gordon A, Kramer DL, Perez-Burillo S, Rufián-Henares JA, Paliy O. Dietary Fatty Acids Sustain the Growth of the Human Gut Microbiota. Appl Environ Microbiol. 84 doi:10.1128/AEM.01525-18.

89. Molly K, Vande Woestyne M, Verstraete W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl Microbiol Biotechnol. 39:254–8 doi:10.1007/BF00228615.

90. Venema K, Van Den Abbeele P. Experimental models of the gut microbiome. Best Pract Res Clin Gastroenterol. 27:115–26 doi:10.1016/J.BPG.2013.03.002.

91. Wiese M, Khakimov B, Nielsen S, Sørensen H, van den Berg F, Nielsen DS. CoMiniGut—a small volume in vitro colon model for the screening of gut microbial fermentation processes. PeerJ. 6:e4268 doi:10.7717/peerj.4268.

92. Li C, Zhang X. Current in Vitro and Animal Models for Understanding Foods: Human Gut–Microbiota Interactions. J Agric Food Chem. 70:12733–45 doi:10.1021/acs.jafc.2c04238.

93. Hansen LBS, Roager HM, Søndertoft NB, Gøbel RJ, Kristensen M, Vallès-Colomer M, et al. A low-gluten diet induces changes in the intestinal microbiome of healthy Danish adults. Nature Communications 2018 9:1. 9:1–13 doi:10.1038/s41467-018-07019-x.

94. Wolters M, Ahrens J, Romaní-Pérez M, Watkins C, Sanz Y, Benítez-Páez A, et al. Dietary fat, the gut microbiota, and metabolic health – A systematic review conducted within the MyNewGut project. Clinical Nutrition. 38:2504–20 doi:10.1016/J.CLNU.2018.12.024.

95. Xu Z, Geng J, Zhang S, Zhang K, Yang L, Li J, et al. A Mobile-Based Intervention for Dietary Behavior and Physical Activity Change in Individuals at High Risk for Type 2 Diabetes Mellitus: Randomized Controlled Trial. JMIR Mhealth Uhealth. 8:e19869 doi:10.2196/19869.

96. Feng J, Liu H, Mai S, Su J, Sun J, Zhou J, et al. Protocol of a parallel, randomized controlled trial on the effects of a novel personalized nutrition approach by artificial intelligence in real world scenario. BMC Public Health. 23:1700 doi:10.1186/s12889-023-16434-9.

97. Qiu P, Ishimoto T, Fu L, Zhang J, Zhang Z, Liu Y. The Gut Microbiota in Inflammatory Bowel Disease. Front Cell Infect Microbiol. 12 doi:10.3389/fcimb.2022.733992.

98. Nicolucci AC, Hume MP, Martínez I, Mayengbam S, Walter J, Reimer RA. Prebiotics Reduce Body Fat and Alter Intestinal Microbiota in Children Who Are

Overweight or With Obesity. Gastroenterology. 153:711–22 doi:10.1053/j.gastro.2017.05.055.

99. Martín R, Miquel S, Benevides L, Bridonneau C, Robert V, Hudault S, et al. Functional Characterization of Novel Faecalibacterium prausnitzii Strains Isolated from Healthy Volunteers: A Step Forward in the Use of F. prausnitzii as a Next-Generation Probiotic. Front Microbiol. 8 doi:10.3389/FMICB.2017.01226.

100. Khoruts A, Staley C, Sadowsky MJ. Faecal microbiota transplantation for Clostridioides difficile: mechanisms and pharmacology. Nat Rev Gastroenterol Hepatol. 18:67–80 doi:10.1038/s41575-020-0350-4.

101. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nature Reviews Microbiology 2020 19:1. 19:55–71 doi:10.1038/s41579-020-0433-9.

102. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, et al. Role of gut microbiota in type 2 diabetes pathophysiology. EBioMedicine. 51:102590 doi:10.1016/j.ebiom.2019.11.051.

103. Chen Y, Chang SKC, Zhang Y, Hsu CY, Nannapaneni R. Gut microbiota and short chain fatty acid composition as affected by legume type and processing methods as assessed by simulated in vitro digestion assays. Food Chem. 312 doi:10.1016/j.foodchem.2019.126040.

104. Pérez-Burillo S, Pastoriza S, Jiménez-Hernández N, D'Auria G, Francino MP, Rufián-Henares JA. Effect of Food Thermal Processing on the Composition of the Gut Microbiota. J Agric Food Chem. 66:11500–9 doi:10.1021/acs.jafc.8b04077.

105. Carmody RN, Bisanz JE, Bowen BP, Maurice CF, Lyalina S, Louie KB, et al. Cooking shapes the structure and function of the gut microbiome. Nat Microbiol. 4:2052–63 doi:10.1038/s41564-019-0569-4.

106. Cattivelli A, Nissen L, Casciano F, Tagliazucchi D, Gianotti A. Impact of cooking methods of red-skinned onion on metabolic transformation of phenolic compounds and gut microbiota changes. Food Funct. 14:3509–25 doi:10.1039/D3FO00085K.

107. Nissen L, Cattivelli A, Casciano F, Gianotti A, Tagliazucchi D. Roasting and frying modulate the phenolic profile of dark purple eggplant and differently change the colon microbiota and phenolic metabolites after in vitro digestion and fermentation in a gut model. Food Research International. 160:111702 doi:10.1016/j.foodres.2022.111702.

108. Shen Q, Chen YA, Tuohy KM. A comparative in vitro investigation into the effects of cooked meats on the human faecal microbiota. Anaerobe. 16:572–7 doi:10.1016/j.anaerobe.2010.09.007.

109. Nissen L, Casciano F, Gianotti A. Intestinal fermentation in vitro models to study food-induced gut microbiota shift: an updated review. FEMS Microbiol Lett. 367:97 doi:10.1093/femsle/fnaa097.

110. Lerma-Aguilera AM, Pérez-Burillo S, Navajas-Porras B, León ED, Ruíz-Pérez S, Pastoriza S, et al. Effects of different foods and cooking methods on the gut microbiota: an in vitro approach. Front Microbiol. 14 doi:10.3389/FMICB.2023.1334623.

111. Caradonna F, Consiglio O, Luparello C, Gentile C. Science and Healthy Meals in the World: Nutritional Epigenomics and Nutrigenetics of the Mediterranean Diet. Nutrients. 12:1748 doi:10.3390/nu12061748.

112. Ramírez-Anaya JDP, Samaniego-Sánchez C, Castañeda-Saucedo MC, Villalón-Mir M, De La Serrana HLG. Phenols and the antioxidant capacity of Mediterranean vegetables prepared with extra virgin olive oil using different domestic cooking techniques. Food Chem. 188:430–8 doi:10.1016/j.foodchem.2015.04.124.

113. Pérez-Burillo S, Rufián-Henares JA, Pastoriza S. Towards an improved global antioxidant response method (GAR+): Physiological-resembling in vitro digestion-fermentation method. Food Chem. 239:1253–62 doi:10.1016/j.foodchem.2017.07.024.

114. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 13:581–3 doi:10.1038/nmeth.3869.

115. Salonen A, Lahti L, Salojärvi J, Holtrop G, Korpela K, Duncan SH, et al. Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. ISME Journal. 8:2218–30 doi:10.1038/ismej.2014.63.

116. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, et al. High-Fat Diet Determines the Composition of the Murine Gut Microbiome Independently of Obesity. Gastroenterology. 137:1716 doi:10.1053/j.gastro.2009.08.042.

117. Wan Y, Wang F, Yuan J, Li J, Jiang D, Zhang J, et al. Effects of dietary fat on gut microbiota and faecal metabolites, and their relationship with cardiometabolic risk factors: a 6-month randomised controlled-feeding trial. Gut. 68:1417–29 doi:10.1136/GUTJNL-2018-317609.

118. Ozato N, Saito S, Yamaguchi T, Katashima M, Tokuda I, Sawada K, et al. Blautia genus associated with visceral fat accumulation in adults 20–76 years of age. NPJ Biofilms Microbiomes. 5 doi:10.1038/s41522-019-0101-x.

119. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS One. 7 doi:10.1371/JOURNAL.PONE.0039743.

120. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: A case-control study. BMC Med. 11 doi:10.1186/1741-7015-11-46.

121. Jo JK, Seo SH, Park SE, Kim HW, Kim EJ, Kim JS, et al. Gut microbiome and metabolome profiles associated with high-fat diet in mice. Metabolites. 11 doi:10.3390/metabo11080482.

122. Wang B, Kong Q, Li X, Zhao J, Zhang H, Chen W, et al. A High-Fat Diet Increases Gut Microbiota Biodiversity and Energy Expenditure Due to Nutrient Difference. Nutrients. 12:3197 doi:10.3390/nu12103197.

123. De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut. 65:1812–21 doi:10.1136/gutjnl-2015-309957.

124. Cai YY, Huang FQ, Lao X, Lu Y, Gao X, Alolga RN, et al. Integrated metagenomics identifies a crucial role for trimethylamine-producing Lachnoclostridium in promoting atherosclerosis. NPJ Biofilms Microbiomes. 8:1–12 doi:10.1038/s41522-022-00273-4.

125. Aslam H, Marx W, Rocks T, Loughman A, Chandrasekaran V, Ruusunen A, et al. The effects of dairy and dairy derivatives on the gut microbiota: a systematic literature review. Gut Microbes. 12:1799533 doi:10.1080/19490976.2020.1799533.

126. Smith-Brown P, Morrison M, Krause L, Davies PSW. Dairy and plant based food intakes are associated with altered faecal microbiota in 2 to 3 year old Australian children. Sci Rep. 6:1–8 doi:10.1038/srep32385.

127. Woting A, Pfeiffer N, Loh G, Klaus S, Blaut M. Clostridium ramosum promotes High-Fat diet-induced obesity in Gnotobiotic Mouse Models. mBio. 5 doi:10.1128/mBio.01530-14.

128. Navajas-Porras B, Pérez-Burillo S, Valverde-Moya ÁJ, Hinojosa-Nogueira D, Pastoriza S, Rufián-Henares JÁ. Effect of cooking methods on the antioxidant capacity

of plant foods submitted to in vitro digestion–fermentation. Antioxidants. 9:1–23 doi:10.3390/antiox9121312.

129. Miglio C, Chiavaro E, Visconti A, Fogliano V, Pellegrini N. Effects of Different Cooking Methods on Nutritional and Physicochemical Characteristics of Selected Vegetables. J Agric Food Chem. 56:139–47 doi:10.1021/JF072304B.

130. Poirier S, Bize A, Bureau C, Bouchez T, Chapleur O. Community shifts within anaerobic digestion microbiota facing phenol inhibition: Towards early warning microbial indicators? Water Res. 100:296–305 doi:10.1016/j.watres.2016.05.041.

131. Monlau F, Sambusiti C, Barakat A, Quéméneur M, Trably E, Steyer JP, et al. Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review. Biotechnol Adv. 32:934–51 doi:10.1016/j.biotechadv.2014.04.007.

132. Rajakaruna S, Pérez-Burillo S, Kramer DL, Rufián-Henares JÁ, Paliy O. Dietary Melanoidins from Biscuits and Bread Crust Alter the Structure and Short-Chain Fatty Acid Production of Human Gut Microbiota. Microorganisms. 10 doi:10.3390/microorganisms10071268.

133. Maldonado-Mateus LY, Perez-Burillo S, Lerma-Aguilera A, Hinojosa-Nogueira D, Ruíz-Pérez S, Gosalbes MJ, et al. Effect of roasting conditions on cocoa bioactivity and gut microbiota modulation. Food Funct. 12:9680–92 doi:10.1039/D1FO01155C.

134. Hu Q, Niu Y, Yang Y, Mao Q, Lu Y, Ran H, et al. Polydextrose Alleviates Adipose Tissue Inflammation and Modulates the Gut Microbiota in High-Fat Diet-Fed Mice. Front Pharmacol. 12 doi:10.3389/fphar.2021.795483.

135. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the Genetic Basis of Fibrolytic Specialization by Lachnospiraceae and Ruminococcaceae in Diverse Gut Communities. Diversity (Basel). 5:627–40 doi:10.3390/d5030627.

136. Jandhyala SM. Role of the normal gut microbiota. World J Gastroenterol. 21:8787 doi:10.3748/wjg.v21.i29.8787.

137. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proceedings of the Nutrition Society. 62:67–72 doi:10.1079/PNS2002207.

138. Sonnenburg JL, Bäckhed F. Diet–microbiota interactions as moderators of human metabolism. Nature. 535:56–64 doi:10.1038/nature18846.

139. Anhê FF, Roy D, Pilon G, Dudonné S, Matamoros S, Varin T V, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. Gut. 64:872–83 doi:10.1136/gutjnl-2014-307142.

140. Tavella T, Rampelli S, Guidarelli G, Bazzocchi A, Gasperini C, Pujos-Guillot E, et al. Elevated gut microbiome abundance of Christensenellaceae, Porphyromonadaceae and Rikenellaceae is associated with reduced visceral adipose tissue and healthier metabolic profile in Italian elderly. Gut Microbes. 13:1–19 doi:10.1080/19490976.2021.1880221.

141. Aranaz P, Romo-Hualde A, Zabala M, Navarro-Herrera D, Ruiz de Galarreta M, Gil AG, et al. Freeze-dried strawberry and blueberry attenuates diet-induced obesity and insulin resistance in rats by inhibiting adipogenesis and lipogenesis. Food Funct. 8:3999–4013 doi:10.1039/C7FO00996H.

142. Cuevas-Sierra A, Ramos-Lopez O, Riezu-Boj JI, Milagro FI, Martinez JA. Diet, Gut Microbiota, and Obesity: Links with Host Genetics and Epigenetics and Potential Applications. Advances in Nutrition. 10:S17–30 doi:10.1093/advances/nmy078.

143. Caio G, Volta U, Sapone A, Leffler DA, De Giorgio R, Catassi C, et al. Celiac disease: a comprehensive current review. BMC Med. 17:142 doi:10.1186/s12916-019-1380-z.

144. Sellitto M, Bai G, Serena G, Fricke WF, Sturgeon C, Gajer P, et al. Proof of Concept of Microbiome-Metabolome Analysis and Delayed Gluten Exposure on Celiac Disease Autoimmunity in Genetically At-Risk Infants. PLoS One. 7:e33387 doi:10.1371/journal.pone.0033387.

145. Olivares M, Neef A, Castillejo G, Palma G De, Varea V, Capilla A, et al. The HLA-DQ2 genotype selects for early intestinal microbiota composition in infants at high risk of developing coeliac disease. Gut. 64:406–17 doi:10.1136/gutjnl-2014-306931.

146. Caio G, Lungaro L, Segata N, Guarino M, Zoli G, Volta U, et al. Effect of Gluten-Free Diet on Gut Microbiota Composition in Patients with Celiac Disease and Non-Celiac Gluten/Wheat Sensitivity. Nutrients. 12:1832 doi:10.3390/nu12061832.

147. Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. Journal of Allergy and Clinical Immunology. 133:291-307.e5 doi:10.1016/j.jaci.2013.11.020.

148. Spolidoro GCI, Amera YT, Ali MM, Nyassi S, Lisik D, Ioannidou A, et al. Frequency of food allergy in Europe: An updated systematic review and meta-analysis. Allergy. 78:351 doi:10.1111/ALL.15560.

149. Azad MB, Konya T, Guttman DS, Field CJ, Sears MR, HayGlass KT, et al. Infant gut microbiota and food sensitization: associations in the first year of life. Clinical & Experimental Allergy. 45:632–43 doi:10.1111/cea.12487.

150. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 9:357–9 doi:10.1038/nmeth.1923.

151. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–6 doi:10.1093/nar/gks1219.

152. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package "vegan" Title Community Ecology Package Version 2.5-7. R. p. 1–286 .

153. Simonyté Sjödin K, Hammarström M -L., Rydén P, Sjödin A, Hernell O, Engstrand L, et al. Temporal and long-term gut microbiota variation in allergic disease: A prospective study from infancy to school age. Allergy. 74:176–85 doi:10.1111/all.13485.

154. Ou G, Hedberg M, Hörstedt P, Baranov V, Forsberg G, Drobni M, et al. Proximal Small Intestinal Microbiota and Identification of Rod-Shaped Bacteria Associated With Childhood Celiac Disease. American Journal of Gastroenterology. 104:3058–67 doi:10.1038/ajg.2009.524.

155. Indiani CMDSP, Rizzardi KF, Castelo PM, Ferraz LFC, Darrieux M, Parisotto TM. Childhood Obesity and Firmicutes/Bacteroidetes Ratio in the Gut Microbiota: A Systematic Review. Childhood Obesity. Mary Ann Liebert Inc.; p. 501–9 doi:10.1089/chi.2018.0040.

156. Parker BJ, Wearsch PA, Veloo ACM, Rodriguez-Palacios A. The Genus Alistipes: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health. Front Immunol. 11 doi:10.3389/fimmu.2020.00906.

157. Park S, Zhang T, Yue Y, Wu X. Effects of Bile Acid Modulation by Dietary Fat, Cholecystectomy, and Bile Acid Sequestrant on Energy, Glucose, and Lipid Metabolism and Gut Microbiota in Mice. Int J Mol Sci. 23 doi:10.3390/IJMS23115935.

158. Weiss GA, Chassard C, Hennet T. Selective proliferation of intestinal Barnesiella under fucosyllactose supplementation in mice. British Journal of Nutrition. 111:1602–10 doi:10.1017/S0007114513004200.

159. Hiippala K, Barreto G, Burrello C, Diaz-Basabe A, Suutarinen M, Kainulainen V, et al. Novel Odoribacter splanchnicus Strain and Its Outer Membrane Vesicles Exert Immunoregulatory Effects in vitro. Front Microbiol. 11:575455 doi:10.3389/fmicb.2020.575455.

160. Uchino Y, Goto Y, Konishi Y, Tanabe K, Toda H, Wada M, et al. Colorectal Cancer Patients Have Four Specific Bacterial Species in Oral and Gut Microbiota in Common—A Metagenomic Comparison with Healthy Subjects. Cancers (Basel). 13:3332 doi:10.3390/cancers13133332.

161. Yu J, Feng Q, Wong SH, Zhang D, Liang Q yi, Qin Y, et al. Metagenomic analysis of faecal microbiome as a tool towards targeted non-invasive biomarkers for colorectal cancer. Gut. 66:70–8 doi:10.1136/gutjnl-2015-309800.

162. Nogal A, Louca P, Zhang X, Wells PM, Steves CJ, Spector TD, et al. Circulating Levels of the Short-Chain Fatty Acid Acetate Mediate the Effect of the Gut Microbiome on Visceral Fat. Front Microbiol. 12 doi:10.3389/fmicb.2021.711359.

163. Lopez-Siles M, Duncan SH, Garcia-Gil LJ, Martinez-Medina M. Faecalibacterium prausnitzii: from microbiology to diagnostics and prognostics. ISME J. 11:841–52 doi:10.1038/ismej.2016.176.

164. Maioli TU, Borras-Nogues E, Torres L, Barbosa SC, Martins VD, Langella P, et al. Possible Benefits of Faecalibacterium prausnitzii for Obesity-Associated Gut Disorders. Front Pharmacol. 12 doi:10.3389/fphar.2021.740636.

165. Kaiyrlykyzy A, Kozhakhmetov S, Babenko D, Zholdasbekova G, Alzhanova D, Olzhayev F, et al. Study of gut microbiota alterations in Alzheimer's dementia patients from Kazakhstan. Sci Rep. 12:15115 doi:10.1038/s41598-022-19393-0.

166. Hillman ET, Kozik AJ, Hooker CA, Burnett JL, Heo Y, Kiesel VA, et al. Comparative genomics of the genus Roseburia reveals divergent biosynthetic pathways that may influence colonic competition among species. Microb Genom. 6 doi:10.1099/mgen.0.000399.

167. Wu X, Pan S, Luo W, Shen Z, Meng X, Xiao M, et al. Roseburia intestinalis-derived flagellin ameliorates colitis by targeting miR-223-3p-mediated activation of NLRP3 inflammasome and pyroptosis. Mol Med Rep. doi:10.3892/mmr.2020.11351.

168. Barandouzi ZA, Starkweather AR, Henderson WA, Gyamfi A, Cong XS. Altered Composition of Gut Microbiota in Depression: A Systematic Review. Front Psychiatry. 11 doi:10.3389/fpsyt.2020.00541.

169. Wang S, Zhang R, Li X, Gao Y, Dai N, Wei Y, et al. Relationship between maternal–infant gut microbiota and infant food allergy. Front Microbiol. 13 doi:10.3389/fmicb.2022.933152.

170. Liang S, Sin ZY, Yu J, Zhao S, Xi Z, Bruzzone R, et al. Multi-cohort analysis of depression-associated gut bacteria sheds insight on bacterial biomarkers across populations. Cellular and Molecular Life Sciences. 80:9 doi:10.1007/s00018-022-04650-2.

171. Hidalgo-Cantabrana C, Delgado S, Ruiz L, Ruas-Madiedo P, Sánchez B, Margolles A. Bifidobacteria and Their Health-Promoting Effects. Microbiol Spectr. 5 doi:10.1128/microbiolspec.BAD-0010-2016.

172. Shi S, Zhang Q, Sang Y, Ge S, Wang Q, Wang R, et al. Probiotic Bifidobacterium longum BB68S Improves Cognitive Functions in Healthy Older Adults: A Randomized, Double-Blind, Placebo-Controlled Trial. Nutrients. 15:51 doi:10.3390/nu15010051.

173. Bui TPN, Mannerås-Holm L, Puschmann R, Wu H, Troise AD, Nijsse B, et al. Conversion of dietary inositol into propionate and acetate by commensal Anaerostipes associates with host health. Nat Commun. 12:4798 doi:10.1038/s41467-021-25081-w.

174. Wopereis H, Sim K, Shaw A, Warner JO, Knol J, Kroll JS. Intestinal microbiota in infants at high risk for allergy: Effects of prebiotics and role in eczema development. Journal of Allergy and Clinical Immunology. 141:1334-1342.e5 doi:10.1016/j.jaci.2017.05.054.

175. Salli K, Lehtinen MJ, Tiihonen K, Ouwehand AC. Xylitol's Health Benefits beyond Dental Health: A Comprehensive Review. Nutrients. 11:1813 doi:10.3390/nu11081813.

176. Álvarez-Arraño V, Martín-Peláez S. Effects of Probiotics and Synbiotics on Weight Loss in Subjects with Overweight or Obesity: A Systematic Review. Nutrients. 13:3627 doi:10.3390/nu13103627.

177. Song W, Song C, Li L, Wang T, Hu J, Zhu L, et al. Lactobacillus alleviated obesity induced by high-fat diet in mice. J Food Sci. 86:5439–51 doi:10.1111/1750-3841.15971.

178. Teixeira CG, Fusieger A, Milião GL, Martins E, Drider D, Nero LA, et al. Weissella: An Emerging Bacterium with Promising Health Benefits. Probiotics Antimicrob Proteins. 13:915–25 doi:10.1007/s12602-021-09751-1.

179. Ahmed S, Singh S, Singh V, Roberts KD, Zaidi A, Rodriguez-Palacios A. The Weissella Genus: Clinically Treatable Bacteria with Antimicrobial/Probiotic Effects on Inflammation and Cancer. Microorganisms. 10:2427 doi:10.3390/microorganisms10122427.

180. Lin H, An Y, Tang H, Wang Y. Alterations of Bile Acids and Gut Microbiota in Obesity Induced by High Fat Diet in Rat Model. J Agric Food Chem. 67:3624–32 doi:10.1021/acs.jafc.9b00249.

181. Nie K, Ma K, Luo W, Shen Z, Yang Z, Xiao M, et al. Roseburia intestinalis: A Beneficial Gut Organism From the Discoveries in Genus and Species. Front Cell Infect Microbiol. 11 doi:10.3389/fcimb.2021.757718.

182. Leonard MM, Valitutti F, Karathia H, Pujolassos M, Kenyon V, Fanelli B, et al. Microbiome signatures of progression toward celiac disease onset in at-risk children in a longitudinal prospective cohort study. Proceedings of the National Academy of Sciences. 118 doi:10.1073/pnas.2020322118.

183. Wang Y, Wang C, Huang J, Xie M, Li X, Fu L. Butyricicoccus plays a key role in mediating the antagonism between probiotic and antibiotic on food allergy. Food Agric Immunol. 30:446–61 doi:10.1080/09540105.2019.1594704.

184. Crost EH, Coletto E, Bell A, Juge N. Ruminococcus gnavus : friend or foe for human health. FEMS Microbiol Rev. 47 doi:10.1093/femsre/fuad014.

185. Bolte LA, Vich Vila A, Imhann F, Collij V, Gacesa R, Peters V, et al. Long-term dietary patterns are associated with pro-inflammatory and anti-inflammatory features of the gut microbiome. Gut. 70:1287–98 doi:10.1136/gutjnl-2020-322670.

186. Shi T, Feng Y, Liu W, Liu H, Li T, Wang M, et al. Characteristics of gut microbiota and fecal metabolomes in patients with celiac disease in Northwest China. Front Microbiol. 13 doi:10.3389/fmicb.2022.1020977.

187. Qian L, Gao R, Hong L, Pan C, Li H, Huang J, et al. Association analysis of dietary habits with gut microbiota of a native Chinese community. Exp Ther Med. doi:10.3892/etm.2018.6249.

188. Muralidharan J, Moreno-Indias I, Bulló M, Lopez JV, Corella D, Castañer O, et al. Effect on gut microbiota of a 1-y lifestyle intervention with Mediterranean diet

compared with energy-reduced Mediterranean diet and physical activity promotion: PREDIMED-Plus Study. Am J Clin Nutr. 114:1148–58 doi:10.1093/ajcn/nqab150.

189. Kong C, Gao R, Yan X, Huang L, Qin H. Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. Nutrition. 60:175–84 doi:10.1016/j.nut.2018.10.002.

190. Li J, Zou C, Liu Y. Amelioration of Ovalbumin-Induced Food Allergy in Mice by Targeted Rectal and Colonic Delivery of Cyanidin-3-O-Glucoside. Foods. 11:1542 doi:10.3390/foods11111542.

191. Wang S, Wei Y, Liu L, Li Z. Association Between Breastmilk Microbiota and Food Allergy in Infants. Front Cell Infect Microbiol. 11 doi:10.3389/fcimb.2021.770913.

192. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, et al. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J. 10:742–50 doi:10.1038/ismej.2015.151.

193. Khan MT, Duncan SH, Stams AJM, van Dijl JM, Flint HJ, Harmsen HJM. The gut anaerobe Faecalibacterium prausnitzii uses an extracellular electron shuttle to grow at oxic–anoxic interphases. ISME J. 6:1578–85 doi:10.1038/ismej.2012.5.

194. Mehrotra I, Serena G, Cetinbas M, Kenyon V, Martin VM, Harshman SG, et al. Characterization of the blood microbiota in children with Celiac disease. Curr Res Microb Sci. 2:100069 doi:10.1016/j.crmicr.2021.100069.

195. Hu J, Guo P, Mao R, Ren Z, Wen J, Yang Q, et al. Gut Microbiota Signature of Obese Adults Across Different Classifications. Diabetes Metab Syndr Obes. Volume 15:3933–47 doi:10.2147/DMSO.S387523.

196. Martini G, Ciardiello D, Dallio M, Famiglietti V, Esposito L, Corte CM Della, et al. Gut microbiota correlates with antitumor activity in patients with mCRC and NSCLC treated with cetuximab plus avelumab. Int J Cancer. 151:473–80 doi:10.1002/ijc.34033.

197. Aagaard K, Ma J, Antony KM, Ganu R, Petrosino J, Versalovic J. The Placenta Harbors a Unique Microbiome. Sci Transl Med. 6 doi:10.1126/scitranslmed.3008599.

198. DiGiulio DB. Diversity of microbes in amniotic fluid. Semin Fetal Neonatal Med. 17:2–11 doi:10.1016/j.siny.2011.10.001.

199. Kashtanova DA, Popenko AS, Tkacheva ON, Tyakht AB, Alexeev DG, Boytsov SA. Association between the gut microbiota and diet: Fetal life, early childhood, and further life. Nutrition. 32:620–7 doi:10.1016/j.nut.2015.12.037.

200. Canani RB, Di Costanzo M, Leone L, Bedogni G, Brambilla P, Cianfarani S, et al. Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev. 24:198–205 doi:10.1017/S0954422411000102.

201. Bergström A, Skov TH, Bahl MI, Roager HM, Christensen LB, Ejlerskov KT, et al. Establishment of intestinal microbiota during early life: a longitudinal, explorative study of a large cohort of Danish infants. Appl Environ Microbiol. 80:2889–900 doi:10.1128/AEM.00342-14.

202. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The Long-Term Stability of the Human Gut Microbiota. Science (1979). 341 doi:10.1126/science.1237439.

203. Rajilić-Stojanović M, Heilig HGHJ, Tims S, Zoetendal EG, de Vos WM. Long-term monitoring of the human intestinal microbiota composition. Environ Microbiol. 15:1146–59 doi:10.1111/1462-2920.12023.

204. Nakayama J, Yamamoto A, Palermo-Conde LA, Higashi K, Sonomoto K, Tan J, et al. Impact of Westernized Diet on Gut Microbiota in Children on Leyte Island. Front Microbiol. 8 doi:10.3389/fmicb.2017.00197.

205. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences. 107:14691–6 doi:10.1073/pnas.1005963107.

206. Korpela K, Salonen A, Virta LJ, Kumpu M, Kekkonen RA, de Vos WM. Lactobacillus rhamnosus GG Intake Modifies Preschool Children's Intestinal Microbiota, Alleviates Penicillin-Associated Changes, and Reduces Antibiotic Use. Mistry N, editor. PLoS One. 11:e0154012 doi:10.1371/journal.pone.0154012.

207. Ruggles K V, Wang J, Volkova A, Contreras M, Noya-Alarcon O, Lander O, et al. Changes in the Gut Microbiota of Urban Subjects during an Immersion in the Traditional Diet and Lifestyle of a Rainforest Village. mSphere. 3 doi:10.1128/mSphere.00193-18.

208. Burns AM, Zitt MA, Rowe CC, Langkamp-Henken B, Mai V, Nieves C, et al. Diet quality improves for parents and children when almonds are incorporated into

their daily diet: a randomized, crossover study. Nutr Res. 36:80–9 doi:10.1016/J.NUTRES.2015.11.004.

209. Lahti L, Salonen A, Kekkonen RA, Salojärvi J, Jalanka-Tuovinen J, Palva A, et al. Associations between the human intestinal microbiota, Lactobacillus rhamnosus GG and serum lipids indicated by integrated analysis of high-throughput profiling data. PeerJ. 1:e32 doi:10.7717/peerj.32.

210. Soldi S, Vasileiadis S, Lohner S, Uggeri F, Puglisi E, Molinari P, et al. Prebiotic supplementation over a cold season and during antibiotic treatment specifically modulates the gut microbiota composition of 3-6 year-old children. Benef Microbes. 10:253–63 doi:10.3920/BM2018.0116.

211. Stewart CJ, Ajami NJ, O'Brien JL, Hutchinson DS, Smith DP, Wong MC, et al. Temporal development of the gut microbiome in early childhood from the TEDDY study. Nature. 562:583–8 doi:10.1038/s41586-018-0617-x.

212. Bernstein CN, Burchill C, Targownik LE, Singh H, Roos LL. Events Within the First Year of Life, but Not the Neonatal Period, Affect Risk for Later Development of Inflammatory Bowel Diseases. Gastroenterology. 156:2190-2197.e10 doi:10.1053/j.gastro.2019.02.004.

213. Edwards CA, Xie C, Garcia AL. Dietary fibre and health in children and adolescents. Proceedings of the Nutrition Society. 74:292–302 doi:10.1017/S0029665115002335.

214. Endo A, Tanno H, Kadowaki R, Fujii T, Tochio T. Extracellular fructooligosaccharide degradation in Anaerostipes hadrus for co-metabolism with non-fructooligosaccharide utilizers. Biochem Biophys Res Commun. 613:81–6 doi:10.1016/j.bbrc.2022.04.134.

215. Arrieta M-C, Stiemsma LT, Dimitriu PA, Thorson L, Russell S, Yurist-Doutsch S, et al. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Sci Transl Med. 7 doi:10.1126/scitranslmed.aab2271.

216. Cornick NA, Stanton TB. Lachnospira. Bergey's Manual of Systematics of Archaea and Bacteria. Wiley; p. 1–6 doi:10.1002/9781118960608.gbm00647.

217. Leisner JJ, Laursen BG, Prévost H, Drider D, Dalgaard P. Carnobacterium: positive and negative effects in the environment and in foods. FEMS Microbiol Rev. 31:592–613 doi:10.1111/j.1574-6976.2007.00080.x.

218. Zhang P, Gänzle M, Yang X. Complementary Antibacterial Effects of Bacteriocins and Organic Acids as Revealed by Comparative Analysis of *Carnobacterium* spp. from Meat. Appl Environ Microbiol. 85 doi:10.1128/AEM.01227-19.

219. Evangelista AG, Danielski GM, Corrêa JAF, Cavalari CM de A, Souza IR, Luciano FB, et al. Carnobacterium as a bioprotective and potential probiotic culture to improve food quality, food safety, and human health – a scoping review. Crit Rev Food Sci Nutr. 63:6946–59 doi:10.1080/10408398.2022.2038079.

220. Tomova A, Bukovsky I, Rembert E, Yonas W, Alwarith J, Barnard ND, et al. The Effects of Vegetarian and Vegan Diets on Gut Microbiota. Front Nutr. 6 doi:10.3389/fnut.2019.00047.

221. Cena H, Calder PC. Defining a Healthy Diet: Evidence for the Role of Contemporary Dietary Patterns in Health and Disease. Nutrients. 12 doi:10.3390/NU12020334.

222. Fabozzi G, Verdone G, Allori M, Cimadomo D, Tatone C, Stuppia L, et al. Personalized Nutrition in the Management of Female Infertility: New Insights on Chronic Low-Grade Inflammation. Nutrients. 14:1918 doi:10.3390/nu14091918.

223. Mitchelson KAJ, Ní Chathail MB, Roche HM. Systems biology approaches to inform precision nutrition. Proceedings of the Nutrition Society. 82:208–18 doi:10.1017/S0029665123002732.

224. Shyam S, Lee KX, Tan ASW, Khoo TA, Harikrishnan S, Lalani SA, et al. Effect of Personalized Nutrition on Dietary, Physical Activity, and Health Outcomes: A Systematic Review of Randomized Trials. Nutrients. 14 doi:10.3390/NU14194104/S1.

225. Moore JB. From personalised nutrition to precision medicine: the rise of consumer genomics and digital health. Proc Nutr Soc. 79:300–10 doi:10.1017/S0029665120006977.

226. de Toro-Martín J, Arsenault B, Després J-P, Vohl M-C. Precision Nutrition: A Review of Personalized Nutritional Approaches for the Prevention and Management of Metabolic Syndrome. Nutrients. 9:913 doi:10.3390/nu9080913.

227. Lampe JW, Navarro SL, Hullar MAJ, Shojaie A. Inter-individual differences in response to dietary intervention: integrating omics platforms towards personalised dietary recommendations. Proceedings of the Nutrition Society. 72:207–18 doi:10.1017/S0029665113000025.

228. Chumpitazi BP, Cope JL, Hollister EB, Tsai CM, McMeans AR, Luna RA, et al. Randomised clinical trial: gut microbiome biomarkers are associated with clinical response to a low <scp>FODMAP</scp> diet in children with the irritable bowel syndrome. Aliment Pharmacol Ther. 42:418–27 doi:10.1111/apt.13286.

229. Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, et al. Personalized Nutrition by Prediction of Glycemic Responses. Cell. 163:1079–94 doi:10.1016/j.cell.2015.11.001.

230. Vega-López S, Ausman LM, Griffith JL, Lichtenstein AH. Interindividual Variability and Intra-Individual Reproducibility of Glycemic Index Values for Commercial White Bread. Diabetes Care. 30:1412–7 doi:10.2337/dc06-1598.

231. Molino S, Lerma-Aguilera A, Gómez-Mascaraque LG, Rufián-Henares JÁ, Francino MP. Evaluation of Tannin-Delivery Approaches for Gut Microbiota Modulation: Comparison of Pectin-Based Microcapsules and Unencapsulated Extracts. J Agric Food Chem. 71:13988–99 doi:10.1021/ACS.JAFC.3C02949.

232. Molino S, Lerma-Aguilera A, Jiménez-Hernández N, Rufián Henares JÁ, Francino MP. Evaluation of the Effects of a Short Supplementation With Tannins on the Gut Microbiota of Healthy Subjects. Front Microbiol. 13:848611 doi:10.3389/FMICB.2022.848611.

233. Kolodziejczyk AA, Zheng D, Elinav E. Diet–microbiota interactions and personalized nutrition. Nat Rev Microbiol. 17:742–53 doi:10.1038/s41579-019-0256-8.

234. Payne JE, Turk MT, Kalarchian MA, Pellegrini CA. Defining Adherence to Dietary Self-Monitoring Using a Mobile App: A Narrative Review. J Acad Nutr Diet. 118:2094–119 doi:10.1016/J.JAND.2018.05.011.

235. Hinojosa-Nogueira D, Pérez-Burillo S, Navajas-Porras B, Ortiz-Viso B, de la Cueva SP, Lauria F, et al. Development of an Unified Food Composition Database for the European Project "Stance4Health." Nutrients. 13:4206 doi:10.3390/nu13124206.

236. Hinojosa-Nogueira D, Ortiz-Viso B, Navajas-Porras B, Pérez-Burillo S, González-Vigil V, de la Cueva SP, et al. Stance4Health Nutritional APP: A Path to Personalized Smart Nutrition. Nutrients. 15:276 doi:10.3390/nu15020276.

237. Dello Russo M, Russo P, Rufián-Henares JÁ, Hinojosa-Nogueira D, Pérez-Burillo S, de la Cueva SP, et al. The Stance4Health Project: Evaluating a Smart Personalised Nutrition Service for Gut Microbiota Modulation in Normal- and Overweight

Adults and Children with Obesity, Gluten-Related Disorders or Allergy/Intolerance to Cow's Milk. Foods. 11:1480 doi:10.3390/foods11101480.

238. Lin H, Peddada S Das. Analysis of compositions of microbiomes with bias correction. Nat Commun. 11 doi:10.1038/s41467-020-17041-7.

239. Bonvicini L, Pingani I, Venturelli F, Patrignani N, Bassi MC, Broccoli S, et al. Effectiveness of mobile health interventions targeting parents to prevent and treat childhood Obesity: Systematic review. Prev Med Rep. 29:101940 doi:10.1016/J.PMEDR.2022.101940.

240. Kankanhalli A, Shin J, Oh H. Mobile-Based Interventions for Dietary Behavior Change and Health Outcomes: Scoping Review. JMIR Mhealth Uhealth. 7:e11312 doi:10.2196/11312.

241. Notting F, Pirovano W, Sybesma W, Kort R. The butyrate-producing and spore-forming bacterial genus Coprococcus as a potential biomarker for neurological disorders. Gut Microbiome. 4:e16 doi:10.1017/gmb.2023.14.

242. Klimenko NS, Tyakht A V., Popenko AS, Vasiliev AS, Altukhov IA, Ischenko DS, et al. Microbiome Responses to an Uncontrolled Short-Term Diet Intervention in the Frame of the Citizen Science Project. Nutrients. 10 doi:10.3390/NU10050576.

243. Lakshmanan AP, Al Zaidan S, Bangarusamy DK, Al-Shamari S, Elhag W, Terranegra A. Increased Relative Abundance of Ruminoccocus Is Associated With Reduced Cardiovascular Risk in an Obese Population. Front Nutr. 9:849005 doi:10.3389/fnut.2022.849005.

244. Lubomski M, Xu X, Holmes AJ, Muller S, Yang JYH, Davis RL, et al. The Gut Microbiome in Parkinson's Disease: A Longitudinal Study of the Impacts on Disease Progression and the Use of Device-Assisted Therapies. Front Aging Neurosci. 14 doi:10.3389/fnagi.2022.875261.

245. Loubinoux J, Bronowicki J-P, Pereira IAC, Mougenel J-L, Faou AE. Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. FEMS Microbiol Ecol. 40:107–12 doi:10.1111/j.1574-6941.2002.tb00942.x.

246. Palmas V, Pisanu S, Madau V, Casula E, Deledda A, Cusano R, et al. Gut microbiota markers associated with obesity and overweight in Italian adults. Sci Rep. 11:5532 doi:10.1038/s41598-021-84928-w.

247. Molino S, Dossena M, Buonocore D, Ferrari F, Venturini L, Ricevuti G, et al. Polyphenols in dementia: From molecular basis to clinical trials. Life Sci. 161:69–77 doi:10.1016/j.lfs.2016.07.021.

248. Molino S, Lerma-Aguilera A, Jiménez-Hernández N, Rufián Henares JÁ, Francino MP. Evaluation of the Effects of a Short Supplementation With Tannins on the Gut Microbiota of Healthy Subjects. Front Microbiol. 0:1141 doi:10.3389/FMICB.2022.848611.

249. Verhaar BJH, Hendriksen HMA, de Leeuw FA, Doorduijn AS, van Leeuwenstijn M, Teunissen CE, et al. Gut Microbiota Composition Is Related to AD Pathology. Front Immunol. 12 doi:10.3389/fimmu.2021.794519.

250. Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10−/− mice. Nature. 487:104–8 doi:10.1038/nature11225.

251. Li Y, Fu R, Li R, Zeng J, Liu T, Li X, et al. Causality of gut microbiome and hypertension: A bidirectional mendelian randomization study. Front Cardiovasc Med. 10 doi:10.3389/fcvm.2023.1167346.

252. Qin P, Zou Y, Dai Y, Luo G, Zhang X, Xiao L. Characterization a Novel Butyric Acid-Producing Bacterium Collinsella aerofaciens Subsp. Shenzhenensis Subsp. Nov. Microorganisms 2019, Vol 7, Page 78. 7:78 doi:10.3390/MICROORGANISMS7030078.

253. Mayta-Apaza AC, Pottgen E, De Bodt J, Papp N, Marasini D, Howard L, et al. Impact of tart cherries polyphenols on the human gut microbiota and phenolic metabolites in vitro and in vivo. J Nutr Biochem. 59:160–72 doi:10.1016/J.JNUTBIO.2018.04.001.

254. Kuffa P, Pickard JM, Campbell A, Yamashita M, Schaus SR, Martens EC, et al. Fiber-deficient diet inhibits colitis through the regulation of the niche and metabolism of a gut pathobiont. Cell Host Microbe. 31:2007-2022.e12 doi:10.1016/j.chom.2023.10.016.

255. Zhang L, Zi L, Kuang T, Wang K, Qiu Z, Wu Z, et al. Investigating causal associations among gut microbiota, metabolites, and liver diseases: a Mendelian randomization study. Front Endocrinol (Lausanne). 14 doi:10.3389/fendo.2023.1159148.

256. Liu B, Ye D, Yang H, Song J, Sun X, He Z, et al. Assessing the relationship between gut microbiota and irritable bowel syndrome: a two-sample Mendelian randomization analysis. BMC Gastroenterol. 23:150 doi:10.1186/s12876-023-02791-7.

257. Song L, Sun Q, Zheng H, Zhang Y, Wang Y, Liu S, et al. Roseburia hominis Alleviates Neuroinflammation via Short-Chain Fatty Acids through Histone Deacetylase Inhibition. Mol Nutr Food Res. 66 doi:10.1002/mnfr.202200164.

258. Sabo CM, Dumitrascu DL. Microbiota and the irritable bowel syndrome. Minerva Gastroenterology. 67 doi:10.23736/S2724-5985.21.02923-5.

259. Sárvári KP, Sántha D, Kovács R, Körmöndi S, Pető Z, Vereb T, et al. Six cases of Solobacterium moorei isolated alone or in mixed culture in Hungary and comparison with previously published cases. Anaerobe. 65:102241 doi:10.1016/j.anaerobe.2020.102241.

260. Mukherjee A, Lordan C, Ross RP, Cotter PD. Gut microbes from the phylogenetically diverse genus Eubacterium and their various contributions to gut health. Gut Microbes. 12:1802866 doi:10.1080/19490976.2020.1802866.

261. Turroni F, van Sinderen D, Ventura M. Genomics and ecological overview of the genus Bifidobacterium. Int J Food Microbiol. 149:37–44 doi:10.1016/j.ijfoodmicro.2010.12.010.

262. Su Q, Tun HM, Liu Q, Yeoh YK, Mak JWY, Chan FK, et al. Gut microbiome signatures reflect different subtypes of irritable bowel syndrome. Gut Microbes. 15 doi:10.1080/19490976.2022.2157697.

263. Sakamoto M, Takagaki A, Matsumoto K, Kato Y, Goto K, Benno Y. Butyricimonas synergistica gen. nov., sp. nov. and Butyricimonas virosa sp. nov., butyric acid-producing bacteria in the family "Porphyromonadaceae" isolated from rat faeces. Int J Syst Evol Microbiol. 59:1748–53 doi:10.1099/ijs.0.007674-0.

264. Morotomi M, Nagai F, Watanabe Y, Tanaka R. Succinatimonas hippei gen. nov., sp. nov., isolated from human faeces. Int J Syst Evol Microbiol. 60:1788–93 doi:10.1099/ijs.0.015958-0.

265. Kopp W. How Western Diet And Lifestyle Drive The Pandemic Of Obesity And Civilization Diseases. Diabetes Metab Syndr Obes. Volume 12:2221–36 doi:10.2147/DMSO.S216791.

266. Blasco T, Pérez-Burillo S, Balzerani F, Hinojosa-Nogueira D, Lerma-Aguilera A, Pastoriza S, et al. An extended reconstruction of human gut microbiota

metabolism of dietary compounds. Nat Commun. 12:1–12 doi:10.1038/s41467-021-25056-x.

267. Balzerani F, Hinojosa-Nogueira D, Cendoya X, Blasco T, Pérez-Burillo S, Apaolaza I, et al. Prediction of degradation pathways of phenolic compounds in the human gut microbiota through enzyme promiscuity methods. npj Systems Biology and Applications 2022 8:1. 8:1–9 doi:10.1038/s41540-022-00234-9.

List of abbreviations

ANCOM – Analysis of the composition of microbiomes

ANCOM-BC2 – Analysis of composition of microbiome with Bias correction

ASVs – Amplicon Sequence Variants

BH – Benjamini-Hochberg

BMI – Body mass index

CCA – Canonical Correspondence Analysis

DA – Differentially Abundant

FDR – False Discovery Rate

FODMAP – Fermentable Oligo-di-monosaccharides and polyols

GDPR – General Data Protection Regulation

IBD – Inflammatory bowel disease

ICSP – International committee on systematics of prokaryotes

lfc – log fold change

MetaHIT – Metagenomics of the human intestinal tract

NGS – Next generation sequencing

PCoA – Principal coordinates Analysis

PERMANOVA – Permutational multivariate analysis of variance

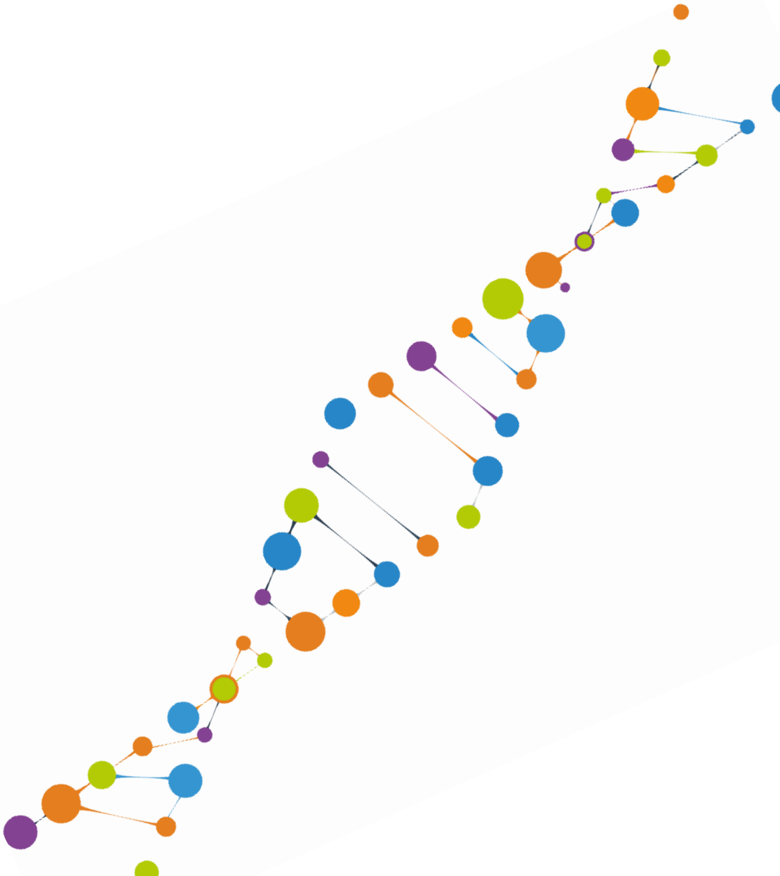
SCFAs – short-chain fatty acids

SHIME – Simulator of the Human Intestinal Microbial Ecosystem

TMA – trimethylamine

TMAO – trimethylamine N-oxide

Appendix



Appendix

1. Figure index

2. Supplementary Material

All the supplementary material are available at:

https://drive.google.com/drive/folders/1nWXxMC2UC1fvNHO7HKIXKkUhfpn3nauG?usp=sharing

Also available by scanning the following QR code:

Supp. Mat. I-1. Heatmap of relative abundance differences at genus level between food categories. Y axis food categories have higher (orange), or lower (green) abundance of a given genus against X axis food categories. The ANCOM method was used for comparisons with the BH procedure for false discovery rate control. Significant comparisons ($q < 0.05$) in all the individuals are represented.

Supp. Mat. I-2. Heatmap of relative abundance differences at species level between food categories. Y axis food categories have higher (orange), or lower (green) abundance of a given species against X axis food categories. The ANCOM method was used for comparisons with the BH procedure for false discovery rate control. Significant comparisons ($q < 0.05$) in all the individuals are represented.

Supp. Mat. I-3. Heatmap of relative abundance differences at genus level between foods. Y axis foods have higher (orange), or lower (green) abundance of a given genus against X axis foods. The ANCOM method was used for comparisons with the BH procedure for false discovery rate control. Significant comparisons ($q < 0.05$) in all the individuals are represented.

Supp. Mat. I-4. Heatmap of relative abundance differences at species level between foods. Y axis foods have higher (orange), or lower (green) abundance of a given species against X axis foods. The ANCOM method was used for comparisons with the BH procedure for false discovery rate control. Significant comparisons ($q < 0.05$) in all the individuals are represented.

Supp. Mat. I-5. Fold-changes in relative abundance of bacterial genera between cooking methods per food category. Fold-changes are represented as log2(FC). Significant comparisons ($q < 0.05$) according to ANCOM for each individual are represented. Cooking methods are represented in different colors and the individuals in which a given comparison is significant are indicated on the bars.

Supp. Mat. I-6. Fold-changes in relative abundance of bacterial species between cooking methods per food category. Fold-changes are represented as log2(FC). Significant comparisons ($q < 0.05$) according to ANCOM for each individual are represented. Cooking methods are shown in different colors and the individuals in which a given comparison is significant are indicated on the bars.

Supp. Mat. II-1. Differentially abundant species when comparing animal- and plant-based foods with water. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing animal-/plant-based foods vs. water.

Supp. Mat. II-2. Set of illustration of differentially abundant species when comparing food categories against water of Lean and A) obese, B) celiac and C) allergic children. Each page represents a different group of children in which food categories in the x axis are compared against water and species are represented in the y axis. Results are arranged by group in the x axis. Only significant differences (q value ≤0.05) are illustrated as dots, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing cooking methods vs water.

Supp. Mat. II-3. Set of illustration about differentially abundant genera when comparing cooking methods within each food category against water of all groups of children. Each page represents a food category in which cooking methods at x axis are compared against water and genera are represented at y axis. Results are arranged by group in x axis. Only significant differences (q value ≤0.05) are illustrated as dots, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing cooking methods vs water.

Supp. Mat. III-1 Differentially abundant species when comparing animal- and plant-based foods with water. Results are arranged by group in the x axis. Dots reflect significant differences, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing animal-/plant-based foods vs. water.

Supp. Mat. III-2 Set of illustration representing differentially abundant genera when comparing cooking methods within each food category against water. Each page represents a food category in which cooking methods at the x axis are compared against water and genera are represented at the y axis. Results are arranged by group in the x axis. Only significant differences (q value ≤0.05) are illustrated as dots, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing cooking methods vs water.

Supp. Mat. III-3. Set of illustration representing differentially abundant genera when comparing cooking methods within each food against water. Each page represents a food in which cooking methods at the x axis are compared against water and genera are represented at the y axis. Results are arranged by group in the x axis. Only significant differences (q value ≤0.05) are illustrated as dots, representing the absolute value of lfc by size. Green and orange color indicate negative/positive lfc when comparing cooking methods vs water.

Supp. Mat. IV-1. Shannon diversity index and Chao1 richness of the microbiota of all participant groups (low app use, high app use and high app use + tannin). A) Alpha diversity comparison of the activity classification groups per time point. B) Alpha diversity comparison of the engagement classification groups per time point. No significant differences (p value ≤0.05 or q value ≤0.05) were found in any of the comparisons through the Wilcoxon-test, paired when applicable, with BH as a method for multiple testing correction.

Supp. Mat. IV-2. Beta diversity analysis of the gut microbiota of high and low app use groups. Dissimilarity between the initial and the other timepoints of each participant's microbiota by group for each app usage classification, A) activity and D) engagement. No significant differences according to Wilcoxon-test. Principal Coordinates Analysis (PCoA) with PERMANOVA test for low and high app use groups at T0 and T2 for the activity classification (B and C) and engagement classification (E and F).

Supp. Mat. IV-3. Differentially abundant species at initial and middle/final time points in the two usage groups. A) Differentially abundant species of groups classified according to app activity. B) Differentially abundant species of groups classified according to app engagement. Only species detected as differentially abundant according to ANCOM-BC2 are illustrated. The bars in the figures depict the - log-fold change (-lfc) values derived from ANCOM-BC2. Consequently, positive values signify an increase in abundance at the specified time compared to T0, while negative values indicate a decrease in abundance.

Supp. Mat. IV-4. Beta diversity analysis of the gut microbiota of high app use + tannin, high app use and low app use groups. It illustrates compositional dynamics by showing the dissimilarity between the initial and the other timepoints for each participant's microbiota, by group and according to app usage classifications by A) activity and D) engagement. No significant differences according to Wilcoxon-test. PCoA with PERMANOVA test for low and high app use groups at T0 and T2 for the activity classification (B and C) and engagement classification (E and F).

Supp. Mat. IV-5. Differentially abundant species at initial and middle/final time points in the different groups, including the tannin supplemented group. A) Differentially abundant species of groups classified according to app activity. B) Differentially abundant species of groups classified according to app engagement. Only species detected as differentially abundant according to ANCOM-BC2 are illustrated. The bars in the figures depict the - log-fold change (-lfc) values derived from ANCOM-BC2. Consequently, positive values indicate an abundance increase in the specified time compared to T0, while negative values indicate an abundance decrease.

3. Publications

The scientific publications that are related to this thesis and in which I have taken part are as follows:

- **Lerma-Aguilera, A. M.**, Pérez-Burillo, S., Navajas-Porras, B., León, E. D., Ruíz-Pérez, S., Pastoriza, S., Jiménez-Hernández, N., Cämmerer, B. M., Rufián-Henares, J. Á., Gosalbes, M. J., & Francino, M. P. (2024). Effects of different foods and cooking methods on the gut microbiota: an *in vitro* approach. *Frontiers in microbiology*, *14*, 1334623. https://doi.org/10.3389/fmicb.2023.1334623
- Molino, S., **Lerma-Aguilera, A.**, Gómez-Mascaraque, L. G., Rufián-Henares, J. Á., & Francino, M. P. (2023). Evaluation of Tannin-Delivery Approaches for Gut Microbiota Modulation: Comparison of Pectin-Based Microcapsules and Unencapsulated Extracts. *Journal of agricultural and food chemistry*, *71*(38), 13988–13999. https://doi.org/10.1021/acs.jafc.3c02949
- Molino, S., **Lerma-Aguilera, A.**, Jiménez-Hernández, N., Rufián Henares, J. Á., & Francino, M. P. (2022). Evaluation of the Effects of a Short Supplementation with Tannins on the Gut Microbiota of Healthy Subjects. *Frontiers in microbiology*, *13*, 848611. https://doi.org/10.3389/fmicb.2022.848611
- Blasco, T., Pérez-Burillo, S., Balzerani, F., Hinojosa-Nogueira, D., **Lerma-Aguilera**, A., Pastoriza, S., Cendoya, X., Rubio, Á., Gosalbes, M. J., Jiménez-Hernández, N., Pilar Francino, M., Apaolaza, I., Rufián-Henares, J. Á., & Planes, F. J. (2021). An extended reconstruction of human gut microbiota metabolism of dietary compounds. *Nature communications*, *12*(1), 4728. https://doi.org/10.1038/s41467-021-25056-x
- Maldonado-Mateus, L. Y., Perez-Burillo, S., **Lerma-Aguilera, A.**, Hinojosa-Nogueira, D., Ruíz-Pérez, S., Gosalbes, M. J., Francino, M. P.,

Rufián-Henares, J. Á., & Pastoriza de la Cueva, S. (2021). Effect of roasting conditions on cocoa bioactivity and gut microbiota modulation. *Food & function*, *12*(20), 9680–9692. https://doi.org/10.1039/d1fo01155c

- Molino, S., **Lerma-Aguilera, A.**, Jiménez-Hernández, N., Gosalbes, M. J., Rufián-Henares, J. Á., & Francino, M. P. (2021). Enrichment of Food with Tannin Extracts Promotes Healthy Changes in the Human Gut Microbiota. *Frontiers in microbiology*, *12*, 625782. https://doi.org/10.3389/fmicb.2021.625782

Other scientific publications not related with this thesis and in which I have taken part are as follows:

- de la Fuente, L., Arzalluz-Luque, Á., Tardáguila, M., Del Risco, H., Martí, C., Tarazona, S., Salguero, P., Scott, R., **Lerma, A.**, Alastrue-Agudo, A., Bonilla, P., Newman, J. R. B., Kosugi, S., McIntyre, L. M., Moreno-Manzano, V., & Conesa, A. (2020). tappAS: a comprehensive computational framework for the analysis of the functional impact of differential splicing. *Genome biology*, *21*(1), 119. https://doi.org/10.1186/s13059-020-02028-w

Funding

The research leading to these results received funding from the European Research Commission (Research Executive Agency) under the research project Stance4Health (Grant Contract No. 816303)