Universidad de Oviedo

Programa de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

Dieta, microbiota y salud intestinal: Impacto de los xenobióticos generados durante el procesado de los alimentos

Sergio Ruiz Saavedra

Tesis Doctoral

Oviedo, 2024

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Este trabajo ha sido realizado en el Instituto de Productos Lácteos de Asturias (IPLA-CSIC) y en la Universidad de Oviedo

IPLA

CSIC
Consejo Superior de Investigaciones Científicas

Universidad de Oviedo

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: "Dieta, microbiota y salud intestinal: Impacto de los xenobióticos generados durante el procesado de los alimentos"	Inglés: "Diet, microbiota and intestinal health: Impact of xenobiotics generated during food processing"

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Programa de Doctorado: Doctorado en Ingeniería Química, Ambiental y Bioalimentaria	
Órgano responsable: Centro Internacional de Postgrado	

RESUMEN (en español)

La dieta juega un papel clave en el mantenimiento del estado fisiológico del individuo. Los alimentos pueden ser fuente de compuestos xenobióticos formados en el procesado de los alimentos como los hidrocarburos aromáticos policíclicos, las aminas heterocíclicas, los N-nitrosocompuestos y la acrilamida. Se ha demostrado en los últimos años que la ingesta de algunos xenobióticos puede aumentar el riesgo de desarrollar ciertas patologías como el cáncer colorrectal (CCR). La microbiota intestinal puede contribuir a la bioactivación, transformación y eliminación de estos compuestos, modificando por tanto los niveles de toxicidad en el intestino y el estado fisiológico del individuo. Además, los xenobióticos pueden alterar la microbiota intestinal, aumentando el riesgo de disbiosis. Sin embargo, la información disponible sobre las modificaciones de la dieta, de la microbiota y del sistema inmunometabólico que ocurren en etapas tempranas de daño de la mucosa intestinal previas al desarrollo del CCR es escasa. Por ello, el Objetivo General de esta Tesis ha sido evaluar las asociaciones de la dieta y de los xenobióticos derivados del procesado de los alimentos con distintos tipos y niveles de daño en la mucosa intestinal y con cambios en parámetros inmunológicos, microbiológicos, metabólicos y de mutagenicidad fecal analizados en el contexto del CCR.

Para la consecución de este Objetivo se han reclutado voluntarios adultos procedentes del Principado de Asturias sobre los que los facultativos participantes en el estudio han realizado un diagnóstico clínico y un diagnóstico histopatológico para poder determinar el tipo y grado de lesión intestinal. Para la obtención de la información dietética se emplearon cuestionarios de frecuencia de consumo de alimentos que se diseñaron y validaron para recoger los hábitos alimentarios de los voluntarios y la información relativa al consumo de xenobióticos en la dieta. Mediante la recopilación bibliográfica de bases de datos de xenobióticos previamente desarrolladas por otros autores y de tablas de composición de alimentos se pudo cuantificar la ingesta dietética de xenobióticos, macronutrientes, micronutrientes y energía. A partir de muestras biológicas de heces y sangre se determinó la composición de la microbiota fecal, los niveles de mutagenicidad fecal, la actividad enzimática fecal y los niveles circulantes de marcadores inmunometabólicos séricos.

Los resultados obtenidos mostraron que la ingesta de hidrocarburos aromáticos policíclicos se asociaba con un mayor riesgo de pertenecer al grupo de individuos diagnosticados con pólipos intestinales. Además, este grupo de diagnóstico presentaba mayor consumo de etanol y menores niveles de adiponectina sérica que el grupo control. El análisis de la microbiota fecal reveló que el consumo de carnes rojas ≥50 g/día o la ingesta de hidrocarburos aromáticos policíclicos totales ≥0.75 µg/día se asociaban con un aumento de la abundancia relativa de la familia *Coriobacteriaceae* y una disminución de la familia *Bacteroidaceae* en el grupo control. La presencia de focos de criptas aberrantes en la mucosa intestinal del grupo de voluntarios diagnosticados con pólipos intestinales se asoció a mayores niveles del factor de necrosis tumoral alfa y a una mayor mutagenicidad fecal. La diferenciación entre pólipos de la vía serrada y la vía adenomatosa permitió observar que los cambios en el perfil microbiano eran diferentes según el tipo y el nivel de daño en la mucosa intestinal. La progresión en el grado de daño de la mucosa intestinal apuntó a una reorganización de grupos y consorcios microbianos involucrados en procesos fermentativos en el intestino, así como a un aumento en la actividad fecal del enzima α-glucosidasa. Por otro lado, la ingesta de nitritos y nitrosaminas procedentes de carnes procesadas se correlacionó positivamente con la concentración fecal de N-nitrosocompuestos hemo y N-nitrosocompuestos totales, aumentando la concentración de estos compuestos según el tipo y grado de daño de la mucosa intestinal y esta ingesta se asoció asimismo con una menor abundancia relativa de la familia microbiana *Bifidobacteriaceae* en el grupo control.

Los resultados de esta Tesis suponen una contribución relevante al conocimiento de las asociaciones entre los xenobióticos derivados del procesado de los alimentos, la microbiota intestinal y la presencia de daño en la mucosa intestinal. Aunque son necesarios estudios más profundos, los hallazgos que se presentan pueden servir para proponer dianas de intervención microbiológicas y/o dietéticas con el fin de revertir o minimizar el daño presente en etapas previas al desarrollo del CCR.

RESUMEN (en Inglés)

Diet plays a key role in maintaining the homeostasis of the individual. Food can be a source of xenobiotic compounds formed during food processing such as polycyclic aromatic hydrocarbons, heterocyclic amines, N-nitrosocompounds and acrylamide. In recent years it has been shown that the intake of some xenobiotics can increase the risk of developing certain pathologies such as colorectal cancer (CRC). The intestinal microbiota can contribute to the bioactivation, transformation and depletion of these compounds, thus modifying the toxicity levels in the intestine and the physiological state of the individual. In addition, xenobiotics can alter the gut microbiota, increasing the risk of suffering dysbiosis. However, little information is available on the shifts of diet, microbiota and immunometabolic system occurring in early stages of intestinal mucosal damage prior to the development of CRC. Therefore, the Main Objective of this Doctoral Thesis was to evaluate the associations of diet and xenobiotics derived from food processing with different types and degrees of intestinal mucosal damage and with changes in immunological, microbiological, metabolic, and faecal mutagenicity parameters analysed in the context of CRC.

To achieve this Objective, adult volunteers from the Principality of Asturias were recruited and

both a clinical diagnosis and a histopathological analysis were performed by the physicians participating in the study to determine the type and degree of intestinal lesion. To obtain the dietary information, specific food consumption frequency questionnaires were designed and validated to collect the dietary habits of the volunteers and the information on the consumption of xenobiotics from diet. Previously developed xenobiotic databases and food composition tables by other authors were reviewed and compiled to quantify the dietary intake of xenobiotics, macronutrients, micronutrients, and energy. The composition of the faecal microbiota, faecal mutagenicity levels, faecal enzymatic activity and circulating levels of serum immunometabolic markers were determined in biological samples of faeces and blood.

The results obtained showed that polycyclic aromatic hydrocarbon intake was associated with a higher risk of belonging to the group of individuals diagnosed with intestinal polyps. In addition, this diagnostic group showed higher ethanol consumption and lower serum adiponectin levels than the control group. Faecal microbiota analysis revealed that red meat consumption ≥50 g/day or total polycyclic aromatic hydrocarbon intake ≥0.75 µg/day were associated with an increase in the relative abundance of the *Coriobacteriaceae f*amily and a decrease in the *Bacteroidaceae* family in the control group. The presence of aberrant crypt foci on the intestinal mucosa of the group of volunteers diagnosed with intestinal polyps was associated with increased levels of tumour necrosis factor-alpha and increased faecal mutagenicity. Differentiation between polyps from the serrated or adenomatous pathway allowed us to observe that the changes in the microbial profile were different according to the type and level of intestinal mucosal damage. The progression in the degree of intestinal mucosal damage pointed to a reorganization of microbial groups and consortia involved in fermentative processes in the intestine, as well as an increase in the faecal activity of the enzyme α-glucosidase. On the other hand, the intake of nitrites and nitrosamines from processed meats was positively correlated with the faecal concentration of haem N-nitrosocompounds and total N-nitrosocompounds. The concentration of these compounds increased in accordance with the type and degree of intestinal mucosal damage, and the intake of nitrites and nitrosamines was also associated with a lower relative abundance of the microbial family *Bifidobacteriaceae* in the control group.

The results of this Thesis represent a relevant contribution to the knowledge of the associations between xenobiotics derived from food processing, intestinal microbiota and the presence of intestinal mucosal damage. Although further studies are needed, the findings presented here could serve to target microbiological and/or dietary intervention in order to reverse or minimize the damage occurring in early stages prior to the development of CRC.

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ÍNDICE

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LISTA DE ABREVIATURAS

CCR:	Cáncer colorrectal
FCA:	Foco de criptas aberrantes
OMS:	Organización Mundial de la Salud
APC:	Gen de la poliposis adenomatosa
P53:	Gen codificante para la proteína tumoral 53
KRAS:	Oncogén homólogo al Virus Kirsten de sarcoma de rata
SMAD4:	Gen codificante para "Mothers against decapentaplegic homolog 4"
PI3KCA:	Gen codificante para la subunidad catalítica alfa de la fosfatidilinositol 3-cinasa
MMR:	Genes codificantes para proteínas del sistema de reparación de errores del ADN
BRAF:	Gen codificante para la proteína B-Raf
hMLH1:	Gen codificante para mutL homólogo 1 humano
METs:	Equivalentes metabólicos
PAC:	Proyecto de Actualización Continua
ONUAA:	Organización de las Naciones Unidas para la Alimentación y la Agricultura
AESAN:	Agencia Española de Seguridad Alimentaria y Nutrición
RR:	Riesgo relativo
IC:	Intervalo de confianza
IMC:	Índice de masa corporal
CMM:	Estudio de la Carga Mundial de Morbilidad
FMIC:	Fondo Mundial para la Investigación del Cáncer
IAIC:	Instituto Americano para la Investigación del Cáncer
IARC:	Centro Internacional de Investigaciones sobre el Cáncer / "International Agency for Research on Cancer"
IID:	Índice Inflamatorio de la Dieta
IL:	Interleucina
PCR:	Proteína C reactiva
TNF-α:	Factor de necrosis tumoral alfa
IEID:	Índice Empírico de Inflamación de la Dieta
LDL:	Lipoproteína de baja densidad
LDLox:	Lipoproteína de baja densidad oxidada
HAPs:	Hidrocarburos aromáticos policíclicos
AHs:	Aminas heterocíclicas
NOCs:	N-nitrosocompuestos
B(a)P:	Benzo(a)pireno
DiB(a)A:	Dibenzo(a,h)antraceno
EFSA:	Autoridad Europea de Seguridad Alimentaria
MoE:	Margen de exposición
HAP4:	Combinación de Benzo(a)pireno, criseno, benzo(a)antraceno y benzo(b)fluoranteno
PhIP:	2-amino-1-metil-6-fenilimidazo(4,5-b)piridina
MeIQx:	2-amino-3,8-dimetilimidazo(4,5-f)quinoxalina
DiMeIQx:	2-amino-3,4,8-trimetilimidazo(4,5-f)quinoxalina
IQ:	2-amino-3-metilimidazo(4,5-f)quinolina

MeIQ:	2-amino-3,4-dimetilimidazo(4,5-f)quinolina
AαC:	2-amino-9-H-pirido(2,3-b)indol
Glu-P-1:	2-amino-6-metildipirido(1,2-a:3′,2′-d)imidazol
Trp-P-1:	3-amino-1,4-dimetill-5-H-pirido(4,3-b)indol
EPIC:	Estudio Prospectivo Europeo sobre Cáncer y Nutrición / "European Prospective Investigation into Cancer and Nutrition"
NAs:	Nitrosaminas
NANV:	Nitrosaminas no volátiles
NAV:	Nitrosaminas volátiles
NDMA:	N-nitrosodimetilamina
NMEA:	N-nitrosometiletilamina
NDEA:	N-nitrosodietilamina
NDPA:	N-nitrosodipropilamina
NDBA:	N-nitrosodibutilamina
NPIP:	N-nitrosopiperidina
NPYR:	N-nitrosopirrolidina
NPRO:	N-nitrosoprolina
E-249:	Nitrito de potasio
E-250:	Nitrito de sodio
E-251:	Nitrato de sodio
E-252:	Nitrato de potasio
CFCA:	Cuestionario de frecuencia de consumo de alimentos
CHARRED:	“Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease”
FDA:	Agencia de Alimentos y Medicamentos
DM:	Dieta Mediterránea
MI:	Microbiota intestinal
AGCCs:	Ácidos grasos de cadena corta
qPCR:	Reacción en cadena de la polimerasa cuantitativa y cualitativa
ARNr:	Gen del ARN ribosómico
BAL:	Bacterias del ácido láctico
IQ:	2-amino-3-metilimidazo[4,5-f]quinolina
-G:	Conjugado glucurónido (G)
HUCA:	Hospital Universitario de Asturias
CXCL10:	Ligando de quimiocina 10 con motivo C-X-C
IP-10:	Proteína 10 inducida por interferón gamma
AMPK	Proteína cinasa activada por AMP
PANCAKE	“Pilot Study for the Assessment of Nutrient intake and food Consumption Among Kids in Europe”
BDML10	Límite mínimo de confianza para la dosis de referencia
ADME	Absorción, distribución, metabolización y eliminación
ROC	“Receiver Operating Characteristic”
AUC	Área bajo la curva ROC

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Los resultados obtenidos mostraron que la ingesta de hidrocarburos aromáticos policíclicos se asociaba con un mayor riesgo de pertenecer al grupo de individuos diagnosticados con pólipos intestinales. Además, este grupo de diagnóstico presentaba mayor consumo de etanol y menores niveles de adiponectina sérica que el grupo control. El análisis de la microbiota fecal reveló que el consumo de carnes rojas ≥50 g/día o la ingesta de hidrocarburos aromáticos policíclicos totales ≥0.75 µg/día se asociaban con un aumento de la abundancia relativa de la familia *Coriobacteriaceae* y una disminución de la familia *Bacteroidaceae* en el grupo control. La presencia de focos de criptas aberrantes en la mucosa intestinal del grupo de voluntarios diagnosticados con pólipos intestinales se asoció a mayores niveles del factor de necrosis tumoral alfa y a una mayor mutagenicidad fecal. La diferenciación entre pólipos de la vía serrada y la vía adenomatosa permitió observar que los cambios en el perfil microbiano eran diferentes según el tipo y el nivel de daño en la mucosa intestinal. La progresión en el grado de daño de la mucosa intestinal apuntó a una reorganización de grupos y consorcios microbianos involucrados en procesos fermentativos en el intestino, así como a un aumento en la actividad fecal del enzima α-glucosidasa. Por otro lado, la ingesta de nitritos y nitrosaminas procedentes de carnes procesadas se correlacionó positivamente con la concentración fecal de N-nitrosocompuestos hemo y N-nitrosocompuestos totales, aumentando la concentración de estos compuestos según el tipo y grado de daño de la mucosa intestinal y esta ingesta se asoció asimismo con una menor abundancia relativa de la familia microbiana *Bifidobacteriaceae* en el grupo control.

Los resultados de esta Tesis suponen una contribución relevante al conocimiento de las asociaciones entre los xenobióticos derivados del procesado de los alimentos, la microbiota intestinal y la presencia de daño en la mucosa intestinal. Aunque son necesarios estudios más profundos, los hallazgos que se presentan pueden servir para proponer dianas de intervención microbiológicas y/o dietéticas con el fin de revertir o minimizar el daño presente en etapas previas al desarrollo del CCR.

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Diet plays a key role in maintaining the homeostasis of the individual. Food can be a source of xenobiotic compounds formed during food processing such as polycyclic aromatic hydrocarbons, heterocyclic amines, N-nitrosocompounds and acrylamide. In recent years it has been shown that the intake of some xenobiotics can increase the risk of developing certain pathologies such as colorectal cancer (CRC). The intestinal microbiota can contribute to the bioactivation, transformation and depletion of these compounds, thus modifying the toxicity levels in the intestine and the physiological state of the individual. In addition, xenobiotics can alter the gut microbiota, increasing the risk of suffering dysbiosis. However, little information is available on the shifts of diet, microbiota and immunometabolic system occurring in early stages of intestinal mucosal damage prior to the development of CRC. Therefore, the Main Objective of this Doctoral Thesis was to evaluate the associations of diet and xenobiotics derived from food processing with different types and degrees of intestinal mucosal damage and with changes in immunological, microbiological, metabolic, and faecal mutagenicity parameters analysed in the context of CRC.

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The results obtained showed that polycyclic aromatic hydrocarbon intake was associated with a higher risk of belonging to the group of individuals diagnosed with intestinal polyps. In addition, this diagnostic group showed higher ethanol consumption and lower serum adiponectin levels than the control group. Faecal microbiota analysis revealed that red meat consumption ≥50 g/day or total polycyclic aromatic hydrocarbon intake ≥0.75 µg/day were associated with an increase in the relative abundance of the *Coriobacteriaceae f*amily and a decrease in the *Bacteroidaceae* family in the control group. The presence of aberrant crypt foci on the intestinal mucosa of the group of volunteers diagnosed with intestinal polyps was associated with increased levels of tumour necrosis factor-alpha and increased faecal mutagenicity. Differentiation between polyps from the serrated or adenomatous pathway allowed us to observe that the changes in the microbial profile were different according to the type and level of intestinal mucosal damage. The progression in the degree of intestinal mucosal damage pointed to a reorganization of microbial groups and consortia involved in fermentative processes in the intestine, as well as an increase in the faecal activity of the enzyme α-glucosidase. On the other hand, the intake of nitrites and nitrosamines from processed meats was positively correlated with the faecal concentration of haem N-nitrosocompounds and total N-nitrosocompounds. The concentration of these compounds increased in accordance with the type and degree of intestinal mucosal damage, and the intake of nitrites and nitrosamines was also associated with a lower relative abundance of the microbial family *Bifidobacteriaceae* in the control group.

The results of this Thesis represent a relevant contribution to the knowledge of the associations between xenobiotics derived from food processing, intestinal microbiota and the presence of intestinal mucosal damage. Although further studies are needed, the findings presented here could serve to target microbiological and/or dietary intervention in order to reverse or minimize the damage occurring in early stages prior to the development of CRC.

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INTRODUCCIÓN

1. CARACTERÍSTICAS Y DESARROLLO DEL CÁNCER COLORRECTAL

El cáncer colorrectal (CCR) representa aproximadamente el 10% de todos los casos de cáncer diagnosticados a nivel mundial, posicionándose el segundo en términos de mortalidad y el tercero en incidencia [1,2]. Se ha registrado que en hombres el CCR es el tercer cáncer con mayor incidencia detrás del cáncer de pulmón y el de próstata, y el tercero en mortalidad detrás del cáncer de pulmón y el de hígado. En mujeres, el CCR se posiciona como el segundo cáncer en términos de incidencia, detrás de cáncer de mama, y el tercero en mortalidad, detrás del cáncer de mama y el de pulmón [1]. Si se mantiene la tendencia actual se estima que en el año 2040 se detectarán aproximadamente 3,2 millones de casos nuevos de CCR y 1,6 millones de muertes a nivel global [2]. En el Principado de Asturias, donde se enmarca la presente Tesis Doctoral, en el año 2019 se diagnosticaron 1.050 nuevos casos y 524 defunciones asociados a esta patología [3].

1.1. Vías de desarrollo de la carcinogénesis

Uno de los eventos más tempranos en el desarrollo de CCR es la alteración histológica de la mucosa intestinal mediante la formación de focos de criptas aberrantes (FCA). Observadas por primera vez por Bird y colaboradores en 1987 en mucosa colónica de ratones C57BL/6 y CF1 tratados con azoximetano, este tipo de lesiones se tiñen diferencialmente con azul de metileno debido a la presencia de un revestimiento epitelial anormalmente grueso en la mucosa colónica [4]. Posteriormente, se confirmó en colon humano que las FCA observadas macroscópicamente tenían un diámetro 3 veces mayor que las criptas normales y que además presentaban aperturas luminales atípicas [5]. Los FCA pueden ocurrir cuando el proceso es todavía reversible y pueden ser típicos, sin alteraciones celulares, o presentar hiperplasia o displasia. El uso de FCA como un posible biomarcador de riesgo de CCR ha mostrado resultados dispares, observándose diferencias en la predicción de desarrollo de la enfermedad en función de la localización colónica y de las características histopatológicas de la lesión [6–9]. Por ello, el análisis de FCA no se utiliza rutinariamente en la práctica clínica para la predicción de riesgo de CCR [7].

Las primeras alteraciones macroscópicas de la mucosa colorrectal suelen manifestarse en forma de pólipos intestinales, cuya incidencia aumenta con la edad. En un ensayo aleatorizado llevado a cabo en 12.000 personas europeas, con edades comprendidas entre los 55 y los 64 años y que habían sido sometidos a colonoscopia, el 48% presentaban pólipos [10]. En esta misma línea, un meta-análisis incluyendo 13.618 individuos adultos norteamericanos reveló que el 30,2% de ellos presentaba pólipos [11]. La mayoría de los pólipos intestinales suelen ser de naturaleza benigna y no acaban desembocando en CCR. Se ha estimado que desde la aparición de un pólipo intestinal en la mucosa colorrectal hasta el desarrollo final de un

adenocarcinoma pueden pasar entre 5 y 10 años, con una tasa de transformación a células malignas cercana al 5% [12–16]. En este sentido, la realización de una colonoscopia permite la correcta valoración de la lesión intestinal por parte del profesional clínico, la extirpación de pólipos de manera preventiva y la obtención de biopsias para su posterior examen histopatológico [17].

Debido a la disparidad en los criterios de diagnosis de CCR y a la controversia histórica sobre la clasificación y nomenclatura de los pólipos intestinales, en 2019 la Organización Mundial de la Salud (OMS) emitió la 5ª edición del informe sobre la clasificación de los tumores relativos al sistema digestivo, que permite la unificación de criterios para su aplicación a nivel mundial [18]. A nivel histopatológico, se pueden distinguir dos vías biológicas principales en las lesiones de la mucosa intestinal de origen esporádico (Figura 1).

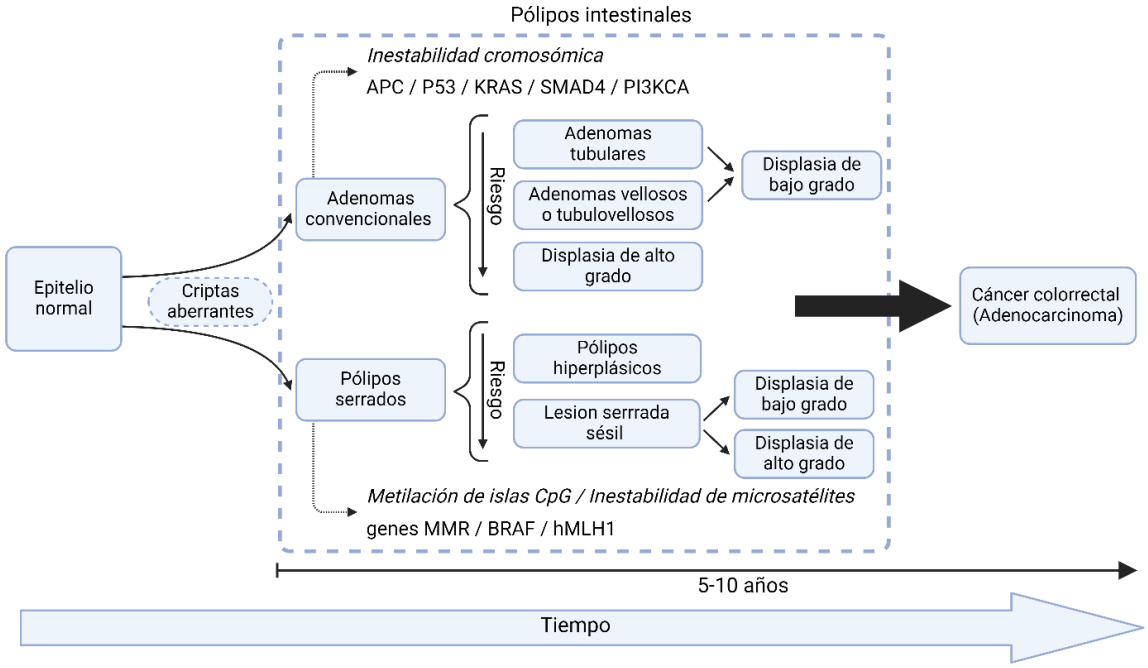

Figura 1. Esquema de las vías biológicas principales de desarrollo de cáncer colorrectal y sus características principales. *APC*, gen de la poliposis adenomatosa; *P53*, gen codificante para la proteína tumoral 53; *KRAS*, oncogén homólogo al Virus Kirsten de sarcoma de rata; *SMAD4*, gen codificante para "Mothers against decapentaplegic homolog 4"; *PI3KCA*, gen codificante para la subunidad catalítica alfa de la fosfatidilinositol 3-cinasa; *MMR*, Genes codificantes para proteínas del sistema de reparación de errores del ADN; *BRAF*, gen codificante para la proteína B-Raf; *hMLH1*, gen codificante para MutL homólogo 1 humano. Figura creada con Biorender.com y adaptada de Nguyen y colaboradores (2018) y Snover (2011) [19,20].

Aproximadamente el 80% de los casos de CCR se originan en células del epitelio intestinal productoras de moco y se desarrollan por la vía adenomatosa o vía de los adenomas convencionales [21]. En esta vía, la activación de proto-oncogenes como *KRAS*, de genes relacionados con el crecimiento celular como *PI3KCA* y la inactivación de genes que codifican proteínas supresoras de tumores como *P53* o *SMAD4* conducen a un fenotipo de inestabilidad

cromosómica con presencia de anomalías estructurales cromosómicas y reordenamientos cromosómicos [19,22,23]. Estos pólipos presentan lesiones displásicas de bajo o alto grado y estructuras tubulares y/o vellosas y pueden transformarse finalmente en adenocarcinomas [19,23]. Los adenocarcinomas malignos se extienden e invaden los tejidos cercanos, llegando a penetrar las cuatro capas histológicas intestinales (Figura 2) [24]. Posteriormente, los tumores pueden diseminarse a otros órganos y metastatizar por vía linfática o hematógena [24].

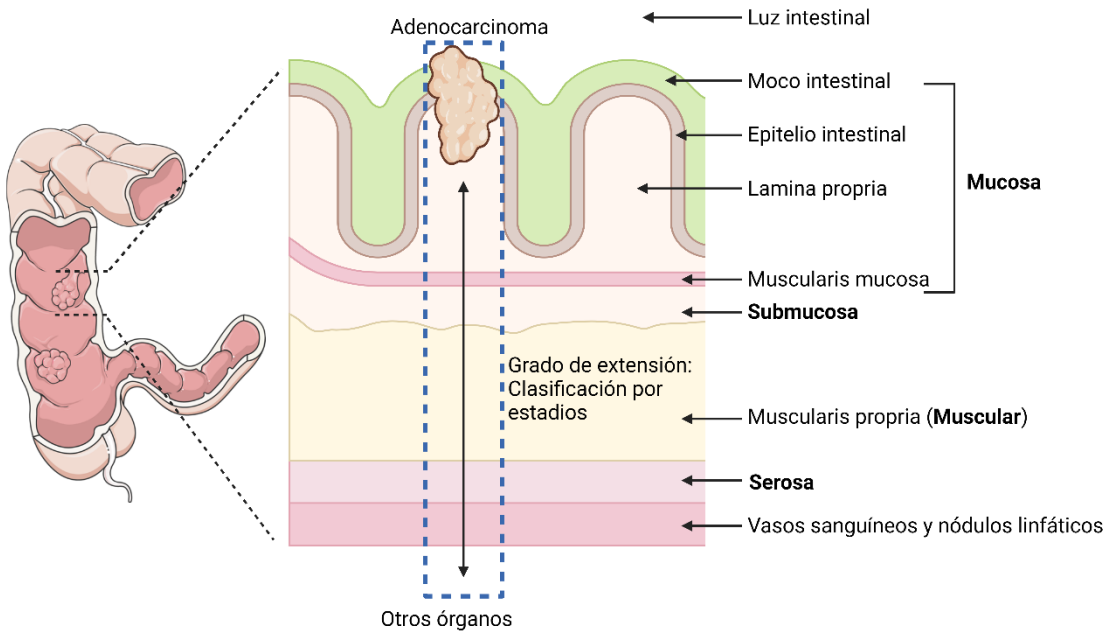

Figura 2. Esquema de la penetración del adenocarcinoma en las distintas capas histológicas del intestino grueso. En negrita se reflejan las cuatro capas histológicas intestinales. Dependiendo del grado de extensión y penetración del adenocarcinoma en estas capas, el tumor será catalogado con un grado de severidad y pronóstico concreto. Figura creada con Biorender.com y adaptada de Kasper y colaboradores (2017) [25].

Aproximadamente el 20% restante de los casos de CCR de origen esporádico se desarrollan por la vía serrada que muestra firmas endoscópicas, anatomopatológicas y moleculares distintas de los adenomas convencionales [21]. Habitualmente presentan forma de lesiones planas o sésiles de apariencia pálida, lo que dificulta su detección por parte del especialista [26]. Es frecuente encontrar un fenotipo de metilación de islas CpG acompañado en ocasiones de inestabilidad de microsatélites, que a nivel molecular se asocia con mutaciones en el gen *BRAF*, disfunción de genes implicados en la reparación de los errores de emparejamiento del ADN como *hMLH1*, e inestabilidad epigenética [20]. Los pólipos hiperplásicos son las alteraciones serradas que se asocian a un menor riesgo de convertirse en malignas, representando el 80% de los casos de esta vía [26–28]. El 20% restante lo representan las lesiones serradas sésiles, acompañadas o no de displasia, que muestran un mayor riesgo de convertirse en CCR, por lo que siempre se recomienda su extirpación quirúrgica [29,30].

1.2. Influencia de factores etiológicos en el desarrollo de cáncer colorrectal

Diversos factores etiológicos pueden influir en el riesgo de desarrollar CCR. Por ejemplo, se ha estimado que la incidencia y mortalidad es un 25 % menor en mujeres que en hombres, tanto en etapas tempranas como tardías de la enfermedad [21,31,32]. Además, la edad avanzada y la presencia de enfermedad inflamatoria intestinal, obesidad o diabetes se suelen asociar con una mayor incidencia de este tumor [33].

Aproximadamente un 10% de los casos totales de CCR se deben a síndromes hereditarios [34,35]. Entre ellos, destacan la poliposis adenomatosa familiar, en la que se hereda una copia mutada del gen de la *APC*, y el síndrome de Lynch o CCR no asociado a poliposis, en el cual existen mutaciones que conllevan a la aparición de mecanismos defectuosos de reparación del ADN [36,37]. Otro 25% de los casos totales de CCR se asocia a mutaciones en genes poco caracterizados que se suelen identificar por la presencia de antecedentes familiares de CCR [38,39]. En este sentido, los antecedentes en parientes de primer grado duplican el riesgo de desarrollar la enfermedad [40]. Aproximadamente el 65% restante de los casos totales de CCR se asocian a un origen esporádico [41].

Se estima que hasta un 50% de los casos de CCR esporádico se podrían evitar mediante la adopción de una dieta y un estilo de vida saludable [42,43]. Respecto al estilo de vida, algunos estudios han señalado que hábitos como el consumo de tabaco incrementan hasta un 38% el riesgo de desarrollar CCR por cada 40 cigarrillos al día o un 20% más de riesgo por fumar durante 40 años o más [44]. Otros hábitos relacionados con el estilo de vida, como una alta actividad física (aproximadamente >1,5 horas/semana o 2.400 equivalentes metabólicos (METs), están asociados a una disminución del 20% en el riesgo de desarrollar CCR en comparación con una baja o nula actividad física, según un análisis de 6 estudios y 8.396 casos llevado a cabo por el Proyecto de Actualización Continua (PAC), un programa global que analiza cómo la dieta, la nutrición y la actividad física afectan al riesgo de cáncer y a la supervivencia [45].

2. DIETA Y CÁNCER COLORRECTAL

La dieta es uno de los factores modificables que más pueden contribuir a la aparición del CCR [46]. Sin embargo, el hecho de que el desarrollo tumoral sea un proceso a largo plazo dificulta la identificación de relaciones causa-efecto. Además, los seres humanos están expuestos a una mezcla de sustancias a través del consumo de alimentos y evaluar su interacción mediante estudios *in vivo* resulta un gran reto. Por todo ello, algunos autores sugieren considerar los patrones dietéticos de los individuos en lugar de un enfoque basado en las relaciones entre alimentos aislados y el riesgo de la patología [47–50]. A nivel mundial, dos patrones dietéticos han recibido la atención de la comunidad científica y acumulan un grado sólido de evidencia en relación con la salud.

2.1. El patrón de dieta occidental o dieta Western

Los profundos cambios en la dieta y el estilo de vida que han tenido lugar de manera rápida desde el inicio del siglo XX no han permitido la fijación de adaptaciones genéticas en la población [51,52]. Muchos autores han sugerido que el aumento actual de la prevalencia de las llamadas "enfermedades de la civilización" podría ser el resultado de la discordancia entre un sistema gastrointestinal humano adaptado a una dieta del paleolítico y las dietas modernas. Entre estas últimas se encuentra el patrón de dieta occidental, generalizado en muchas partes del mundo y que se caracteriza por un elevado consumo de carne, productos cárnicos derivados, alimentos procesados, salsas y de alimentos ricos en grasas saturadas, azúcares simples y sal [53,54]. A pesar de que estas dietas occidentales presentan una alta densidad energética, son pobres en micronutrientes y se alejan de las recomendaciones de la Organización de las Naciones Unidas para la Alimentación y la Agricultura (ONUAA) y el Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) de un consumo de carnes rojas y procesadas por debajo de 350-500 g/semana, un umbral de 5 g/día para el consumo de sal y una ingesta superior a 400 g/día de hortalizas y verduras [52,53,55,56]. En términos generales, está ampliamente establecido que la adherencia al patrón de dieta occidental se relaciona con un mayor riesgo de CCR, siendo varios de los componentes de este patrón considerados factores de riesgo para el desarrollo de la enfermedad (Tabla 1) [57–60].

Como se observa en los datos presentados en la Tabla 1, el consumo de carne y derivados se incluye entre los factores que han acumulado una mayor evidencia científica en este contexto [61,62]. De hecho, la carne roja y la carne procesada han sido clasificadas por Centro Internacional de Investigaciones sobre el Cáncer (IARC, “International Agency for Research on Cancer”) como probable carcinógeno para humanos (grupo 2A) y carcinógeno para humanos (Grupo 1), respectivamente [63]. Esta información es de alta importancia teniendo en cuenta que, en la mayoría de los países desarrollados, como es el caso de España, el consumo de carne

procesada ha aumentado en los últimos años. Los datos publicados por el Ministerio de Agricultura de España mostraron que el consumo diario de carne *per cápita* a principios de 2023 era de aproximadamente 95 g, de los cuales 36 g eran carne de cerdo, vacuno, ovino o caprino, 28 g carne procesada y 31 g carne de pollo o conejo [64]. Actualmente, el mecanismo exacto por el cual el consumo de carne se relaciona con el cáncer se desconoce. Se ha propuesto que la desregulación de los fenómenos inflamatorios o el consumo de compuestos con potencial carcinogénico podrían ser algunas de las causas subyacentes a esta asociación [65].

Tabla 1. Factores del estilo de vida y de la dieta y los valores umbrales correspondientes asociados con un aumento o disminución del riesgo de desarrollar cáncer colorrectal.

Factores	Umbral	RR (IC)	Referencia
De riesgo:			
Carnes rojas	>50 g/día	1,08 (1,02-1,16)	CMM [66]
Carnes procesadas	>25 g/día	1,06 (1,02-1,10)	CMM [66]
Vino	>10 g/día	1,04 (1,01–1,08)	PAC/FMIC/IAIC [45]
Cerveza	>10 g/día	1,08 (1,05–1,11)	PAC/FMIC/IAIC [45]
Destilados	>10 g/día	1,08 (1,02–1,14)	PAC/FMIC/IAIC [45]
Etanol	>12 g/día	1,08 (1,03-1,12)	CMM [66]
Hierro hemo	>0,6 mg/día	1,09 (1,05–1,13)	PAC/FMIC/IAIC [45]
Tabaco	>40 cigarrillos/día	1,38	PAC/FMIC/IAIC [44]
Glucosa plasmática	Diabetes	1,527 (1,09-2,32)	CMM [66]
IMC	Por cada incremento de 5kg/m^2 a partir de 25kg/m^2	Hombres: 1,18 (1,15-1,21) Mujeres: 1,06 (1,03- 1,08)	CMM [66] CMM [66]
Protectores:			
Pescado	>100 g/día	0,89 (0,80-0,99)	PAC/FMIC/IAIC [45]
Leche	>120 g/día	0,95 (0,90-0,99)	CMM [66]
Cereales integrales	>50 g/día	0,93 (0,88-0,99)	CMM [66]
Frutas	>300 g/día	0,99 (0,98–0,99)	PAC/FMIC/IAIC [45]
Verduras	>300 g/día	0,96 (0,95–0,97)	PAC/FMIC/IAIC [45]
Fibra dietética	>10 g/día	0,91 (0,89-0,97)	CMM [66]
Calcio	>300 mg/día	0,93 (0,90-0,98)	CMM [66]
Vitamina C	>40 mg/día	0,94 (0,89–0,99)	PAC/FMIC/IAIC [45]
Vitamina D	>30 nmol/L	0,92 (0,85-1,00)	PAC/FMIC/IAIC [45]

RR, Riesgo relativo; IC, Intervalo de confianza; METs, Equivalentes metabólicos; IMC, Índice de masa corporal; CMM, Estudio de la Carga Mundial de Morbilidad; PAC/FMIC/IAIC, Proyecto de Actualización Continua/Fondo Mundial para la Investigación del Cáncer/Instituto Americano para la Investigación del Cáncer.

2.1.1. Mecanismos asociados a la carcinogenicidad de las carnes rojas y procesadas: Inflamación

Un consumo alto de carnes rojas y procesadas se ha asociado a una mayor adherencia a dietas proinflamatorias capaces de promover un estado de meta-inflamación, que está estrechamente relacionada con obesidad y el desarrollo de CCR [67–69]. Actualmente se puede valorar el posible impacto inflamatorio de la dieta gracias a la utilización de índices dietéticos que reúnen gran evidencia científica con relación al desarrollo CCR. Entre ellos destaca el Índice Inflamatorio de la Dieta (IID), que combina una selección de compuestos dietéticos, nutrientes y alimentos (dentro de los cuales están las carnes), a partir de su impacto sobre diversos parámetros inflamatorios como las interleucinas (IL)-1β, IL-4, IL-6, IL-10, la proteína C reactiva (PCR) y el factor de necrosis tumoral alfa (TNF-α) [70]. Un meta-análisis llevado a cabo en 2021 reveló que una mayor puntuación en el IID, es decir, un mayor potencial inflamatorio de la dieta, estaba asociada con el aumento de la incidencia de CCR de manera muy significativa (evidencia de clase II: $p < 10^{-6}$, >1.000 casos) [71]. Este resultado apoyaba las conclusiones previamente obtenidas en dos meta-análisis publicados en 2017 por Shivappa y colaboradores y Fan y colaboradores [72,73]. Además, el Índice Empírico de Inflamación de la Dieta" (IEID) también está basado en la puntuación del potencial inflamatorio de la dieta [74,75]. El IEID se ha desarrollado a partir de diversos análisis de regresión sobre valores de parámetros sanguíneos como la PCR, la IL-6, la adiponectina y el receptor 2 de TNF-α procedentes de muestras de plasma de voluntarios [75]. Los autores observaron que una mayor puntuación en este índice IEID (dieta con mayor potencial inflamatorio) estaba asociada con un mayor riesgo de desarrollar CCR con independencia del sexo, aunque aumentaba en hombres con obesidad o sobrepeso [76].

A nivel molecular, un posible mecanismo explicativo de estas asociaciones es la desregulación de múltiples biomarcadores de inflamación y el estado metabólico como la PCR, el TNF-α, las citocinas IL-6, IL-8, IL-10, o las adipocinas adiponectina, leptina y resistina, que pueden influir en la generación de un microambiente inflamatorio obesogénico capaz de promover la formación de tumores, la angiogénesis y la metástasis [77–82]. Se ha sugerido que el aumento de lipoproteína de baja densidad (LDL) en su forma oxidada (LDLox) derivado de un alto consumo de carnes también podría acabar promoviendo fenómenos inflamatorios mediante la activación del inflamasoma NLRP3 y la producción de IL-1β [83,84]. Además, las dietas proinflamatorias y la obesidad se suelen relacionar con la alteración de las funciones metabólicas del tejido adiposo blanco, pudiendo dar lugar a la producción y liberación de moléculas inflamatorias como TNF-α o IL-6 o adipocinas como la leptina o la resistina y a la inhibición de los procesos de autofagia [85,86]. En relación con la obesidad, la activación crónica por estrés del retículo endoplasmático, la activación del receptor de tipo Toll-4 derivada

de una mayor presencia de ácidos grasos o la activación del factor inducible por hipoxia 1 por deficiencia de oxígeno derivada del exceso de crecimiento de tejido adiposo se ha asociado a la generación de especies reactivas de oxígeno, la angiogénesis y la activación de vías inflamatorias [87–92]. A su vez, el consumo de carnes podría favorecer la alteración de vías clave de señalización inflamatoria frecuentemente alteradas en desarrollo de CCR, como la activación del receptor de TNF-α y del receptor de IL-1 que provocan la activación del factor nuclear-κB [93–95]. Todo este conjunto de factores derivado del consumo de carnes podría promover un estado inflamatorio, la generación de estrés oxidativo, daño en el ADN y alteraciones en la barrera intestinal, lo que aumentaría la exposición a microorganismos patógenos y compuestos con potencial dañino [96].

2.1.2. Mecanismos asociados a la carcinogenicidad de las carnes rojas y procesadas: Xenobióticos

Otro de los mecanismos propuestos para explicar la relación entre consumo de carnes y el desarrollo de CCR se basa en su contenido en compuestos xenobióticos. Bajo el término xenobiótico agrupan todos los compuestos químicos externos a un organismo, es decir, aquellos que no se producen de forma endógena o no se espera que estén presentes en el organismo [97]. El procesado de la carne mediante la adición de nitritos o los procesos de ahumado y el cocinado mediante métodos abrasivos y prolongados de cocinado como la barbacoa o la plancha favorecen la formación de algunos de estos compuestos [61,98]. En este sentido, según el último informe de consumo alimentario en España, los ciudadanos muestran una preferencia por el cocinado a la plancha de los alimentos, mientras que otras formas de cocinado como es el uso del horno se sitúan en 6º lugar de preferencia [99]. Los hidrocarburos aromáticos policíclicos (HAPs), las aminas heterocíclicas (AHs), los N-nitrosocompuestos (NOCs) y la acrilamida se encuentran entre los xenobióticos más estudiados en relación con su consumo a largo plazo y su potencial carcinogénico. Los xenobióticos de la dieta son capaces de impactar en la salud a través de diversos mecanismos, pudiendo provocar daño oxidativo y dar lugar a la formación de aductos de ADN y a roturas de roturas de cadena sencilla en el ADN, lo que se asocia con desregulación génica, generación de aberraciones cromosómicas y la parada del ciclo celular [100]. Además, la ingesta concomitante de otros compuestos como el hierro hemo, principalmente de la carne roja, puede favorecer la formación de xenobióticos y potenciar la generación de aldehídos con potencial citotóxico y genotóxico [101].

2.1.2.1. Hidrocarburos aromáticos policíclicos

Son compuestos químicos consistentes en dos o más anillos aromáticos simples unidos, de manera que solo presentan átomos de carbono e hidrógeno [102]. Se forman en la combustión incompleta y la pirólisis de materia orgánica a más de 400ºC, lo que permite la

fusión de anillos de benceno [103]. En general, se consideran compuestos lipofílicos con poca solubilidad en agua que suelen ser volátiles y presentan capacidad de ser absorbidos por partículas de materia orgánica, lo que facilita su ingesta y acumulación en los organismos [104].

Las principales vías de entrada en el organismo de los HAPs son el consumo de alimentos y aguas, aunque también pueden entrar por inhalación o a través de la piel [105,106]. En lo que respecta a alimentos, su concentración aumenta con el proceso de ahumado y tras el uso de métodos de cocinado abrasivos y de contacto directo con la fuente de calor, como pueden ser las barbacoas [107]. La presencia simultánea de moléculas lipídicas y proteicas en el alimento, como ocurre en el caso de las carnes rojas, potencia su formación [108]. De hecho, los niveles máximos de HAPs se han encontrado en alimentos ahumados y carnes a la parrilla aunque también pueden detectarse en grasas vegetales, verduras, hortalizas y cereales [109,110]. Dada la ubicuidad de los HAPs en la dieta y el ambiente, es muy difícil evaluar en qué medida la cantidad ingerida a través de los alimentos puede contribuir al desarrollo del cáncer [111].

El impacto en la salud de los HAPs ha sido revisado por la IARC, clasificando el Benzo(a)pireno (B(a)P) como agente carcinógeno para humanos (Grupo 1) y el Dibenzo(a,h)antraceno (DiB(a)A), el Dibenzo(a,l)pireno y el Ciclopenta(cd)pireno como probables carcinógenos (Grupo 2ª) (Figura 3) [112]. El resto de HAPs evaluados han sido catalogados como posibles carcinógenos (Grupo 2B) o no clasificables respecto a su carcinogenicidad para humanos (Grupo 3) [112]. La exposición a los HAPs ha sido evaluada por la Autoridad Europea de Seguridad Alimentaria (EFSA), considerando que las personas con una ingesta alta de estos compuestos presentan un margen de exposición (MoE) para el que no se puede descartar un riesgo para la salud [113,114]. La relación entre la ingesta de HAPs y el desarrollo de CCR no está totalmente probada. Varios autores han referido no encontrar evidencias de asociación entre la ingesta de B(a)P y el desarrollo de pólipos intestinales o CCR [115–118]. Sin embargo, en un estudio llevado a cabo por Sinha y colaboradores se notificó una asociación significativa entre la ingesta de B(a)P procedente del consumo de carne y un mayor riesgo de presentar adenoma colorrectal [119]. En este sentido, Miller y colaboradores también encontraron una asociación positiva entre la ingesta de B(a)P y el cáncer rectal [120]. Por otro lado, el registro de la ingesta dietética de un marcador resultante de la combinación de B(a)P, criseno, 26enzo(a)antraceno y 26enzo(b)fluoranteno denominado HAP4, reveló una asociación estadísticamente significativa entre el consumo de HAPs y el riesgo de mortalidad por cáncer de pulmón y tráquea, pero no de CCR [121].

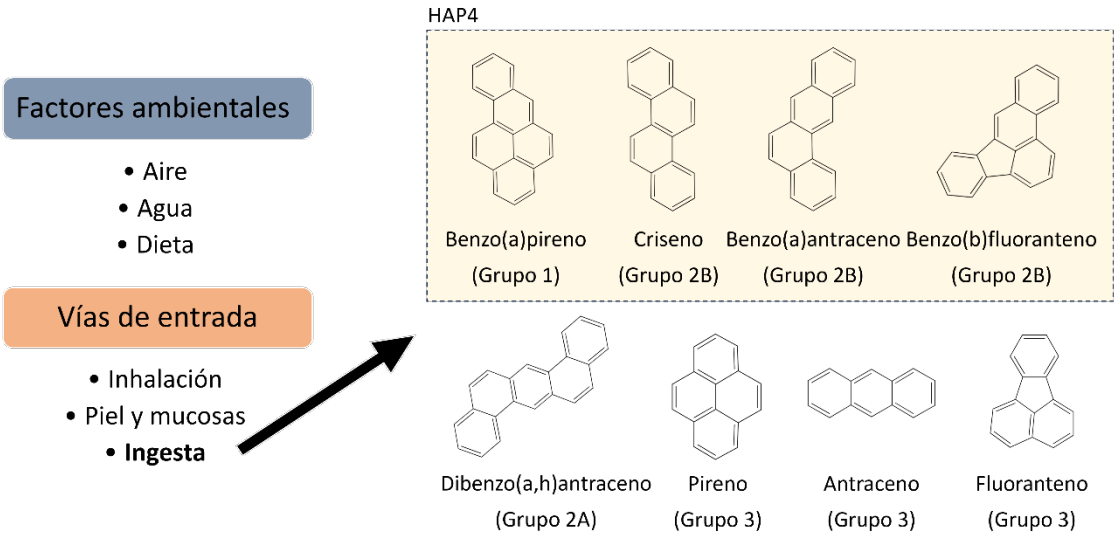

Figura 3. Vías de entrada de hidrocarburos policíclicos aromáticos en el organismo y clasificación de riesgo de los HAPs más habituales en la dieta. Adaptado de Patel y colaboradores (2020), Phillips y colaboradores (1999) y Palade y colaboradores (2023) [122–124]. HAP4, Combinación de Benzo(a)pireno, criseno, 27enzo(a)antraceno y 27enzo(b)fluoranteno. Figura creada parcialmente con MolView.

Los estudios llevados a cabo en España han situado la ingesta de HAPs totales entre 6,72 µg/día y 12,04 µg/día, muy alejada de la de otros países de la Unión Europea como Francia o Suecia donde la mediana de ingesta de HAP4 es de 0,104 µg/día y 0,27 µg/día, respectivamente [125–131]. Un informe de la OMS estimó que la media de ingesta de B(a)P en 18 países que incluían Australia, Brasil, Nueva Zelanda y Reino Unido, entre otros, se situaba entre los 0,0006 y los 2,04 µg/día, mientras que la ingesta media de DiB(a)A variaba entre 0,0046 y 0,76 µg/día [132]. En otras poblaciones del continente asiático como la pakistaní la exposición a B(a)P oscilaba de de 2,86 µg/día a 2,57 µg/día para hombres y mujeres, respectivamente y en China la exposición a B(a)P y HAP4 se ha estimado en 0,216 µg/día y 1,233 µg/día, respectivamente, considerando una persona promedio de 70 kg de peso [133,134].

2.1.2.2. Aminas heterocíclicas

Las AHs son un grupo de compuestos químicos caracterizados por presentar, como mínimo, un anillo aromático heterocíclico y un átomo de nitrógeno en su estructura [135]. Generalmente se suelen distinguir dos tipos de AHs, los aminoimidoazoazarenos y las aminocarbolinas [136]. Las primeras también se denominan AHs térmicas y se forman a temperaturas entre 100 ºC y 300 ºC, como producto de la reacción entre aminoácidos, hexosas (como azúcar reductor), creatina y/o creatinina presentes en tejido muscular de carnes y pescados [137]. Las aminocarbolinas se forman a más de 300 ºC por una reacción pirolítica y por tanto también se conocen como AHs pirolíticas [138]. Sin embargo, la vía de formación de

estos compuestos varía según los precursores y aún no se conoce con totalidad [139]. Más de 25 AHs han sido identificadas y clasificadas en las últimas décadas.

Las carnes rojas, procesadas y el pescado son las principales fuentes dietéticas de estos compuestos. Existen diversos factores como los métodos de cocinado abrasivos y un mayor grado de dorado que favorecen la formación de estos compuestos [140–143]. Junto a ellos, la presencia de grasa en el alimento se propone como un agente efectivo en la transmisión de calor y puede contribuir a la consecución de mayores temperaturas en el alimento en menos tiempo, dando lugar a radicales libres en un proceso de oxidación que favorece la formación de AHs [144,145]. Además, algunos compuestos formados a partir de lípidos pueden influir en la formación de AHs de otras formas. Por ejemplo, se considera que el compuesto carbonilo requerido para la formación de la AH térmica 2-amino-1-metil-6-fenilimidazo(4,5-b)piridina (PhIP) es un producto de la reacción de Maillard producida entre carbohidratos y fenilalanina, aunque se ha comprobado que en ciertas condiciones los aminoácidos pueden interaccionar con los productos de oxidación lipídica para contribuir a la formación de PhIP en ausencia de carbohidratos [141]. Por otro lado, se ha sugerido que la presencia de algunos compuestos antioxidantes de la dieta podría inhibir la formación de AHs [146].

La IARC ha catalogado algunas de las AHs que presentan mayor concentración tras el procesado y cocinado de la carne como los aminoimidoazoazarenos PhIP, 2-amino-3,8-dimetilimidazo(4,5-f)quinoxalina (MeIQx) y 2-amino-3,4,8-trimetilimidazo(4,5-f)quinoxalina (DiMeIQx) como posibles carcinógenos para humanos (grupo 2B) y ha recomendado disminuir su exposición dietética (Figura 4) [147].

Específicamente, algunas AHs muestran un índice de mutagenicidad 1.000 veces superior al del B(a)P [148]. Respecto al consumo de AHs y el desarrollo de CCR, los resultados son dispares. Por un lado, en el estudio con voluntarios del Registro Familiar de Cáncer Colorrectal de Estados Unidos y en el Estudio longitudinal de Cohortes Multiétnico llevado a cabo con más de 200.000 voluntarios residentes en California y Hawái no se observaron asociaciones significativas entre la ingesta de PhIP, MeIQx, DiMeIQx o AHs totales y el riesgo de desarrollar CCR [149,150]. Además, en dos cohortes norteamericanas tampoco se observó una asociación significativa entre el consumo de PhIP procedente de carnes rojas y el riesgo de CCR (*p*-valor: 0,06) [151]. Por el contrario, dos revisiones sistemáticas y meta-análisis independientes llevados a cabo por Martínez Góngora y colaboradores y Chiavarini y colaboradores identificaron un efecto dosis-respuesta en el riesgo de presentar adenomas colorrectales provocado por la ingesta de PhIP, MeIQx y DiMeIQx, siendo estos resultados similares a los obtenidos por otros autores y por el Estudio Prospectivo Europeo sobre Cáncer y Nutrición (EPIC, “European Prospective Investigation into Cancer and Nutrition”) [148,152–

154]. Otros estudios apuntan a una relación de la ingesta de MeIQx y DiMeIQx procedentes exclusivamente de carnes rojas con el riesgo de desarrollar CCR [118,155].

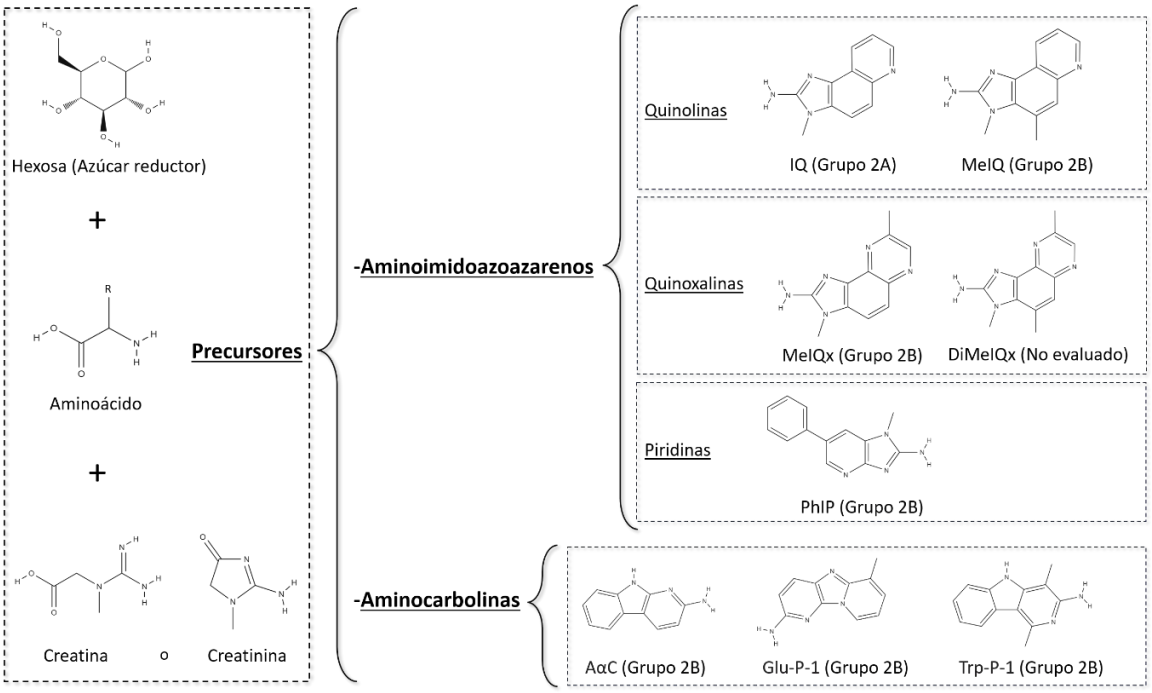

Figura 4. Representación de los precursores de Ahs habituales, de las Ahs más abundantes en la dieta y de su clasificación de riesgo según la IARC. Adaptado de Meurillon y colaboradores (2016) y Chen y colaboradores (2020) [156,157]. IQ, 2-amino-3-metilimidazo(4,5-f)quinolina; MeIQ, 2-amino-3,4-dimetilimidazo(4,5-f)quinolina; MeIQx, 2-amino-3,8-dimetilimidazo(4,5-f)quinoxalina; DiMeIQx, 2-amino-3,4,8-trimetilimidazo(4,-f)quinoxalina; PhIP, 2-amino-1-metil-6-fenilimidazo(4,5-b)piridina; AαC, 2-amino-9-H-pirido(2,3-b)indol; Glu-P-1, 2-amino-6-metildipirido(1,2-a:3′,2′-d)imidazol; Trp-P-1, 3-amino-1,4-dimetill-5-H-pirido(4,3-b)indol. Figura creada parcialmente con MolView.

En población europea se han comunicado valores medios de ingesta total de AHs en el rango de los 60 a los 700 ng/día [158–160]. La ingesta de AHs puede variar según el compuesto examinado. En un estudio del EPIC se observó que en individuos control la ingesta de consumos de PhIP, MeIQx y DiMeIQx era de 41 ng/día, 16,8 ng/día y 3 ng/día, mientras que en casos de pacientes diagnosticados con adenoma colorrectal era de 44,8 ng/día, 21,5 ng/día y 3,8 ng/día respectivamente [160]. Estudios norteamericanos han mostrado una ingesta media situada entre aproximadamente 70-230 ng/día, 20-75 ng/día y 1,5-4 ng/día de PhIP, MeIQx y DiMeIQx respectivamente, siendo menor la ingesta de estos compuestos en grupos control que en grupos de pacientes diagnosticados con adenomas colorrectales [119,150,155].

2.1.2.3. N-nitrosocompuestos

Los NOCs se caracterizan por presentar un grupo nitroso funcional unido a un átomo de nitrógeno [161]. Se pueden dividir en nitrosaminas (NAs) y nitrosamidas según presenten un grupo amina o amida [162]. Las nitrosamidas son inestables químicamente y acaban

descomponiéndose, mientras que las Nas muestran mayor estabilidad ambiental [163]. Este último grupo se puede subclasificar en nitrosaminas no volátiles (NANV) y las nitrosaminas volátiles (NAV) (Figura 5). Las NAV pueden formarse de manera exógena o endógena, siendo necesaria la presencia de aminas procedentes de aminoácidos libres o de proteínas y de agentes nitrosantes como el ácido nitroso derivado de la ingesta de nitritos [164].

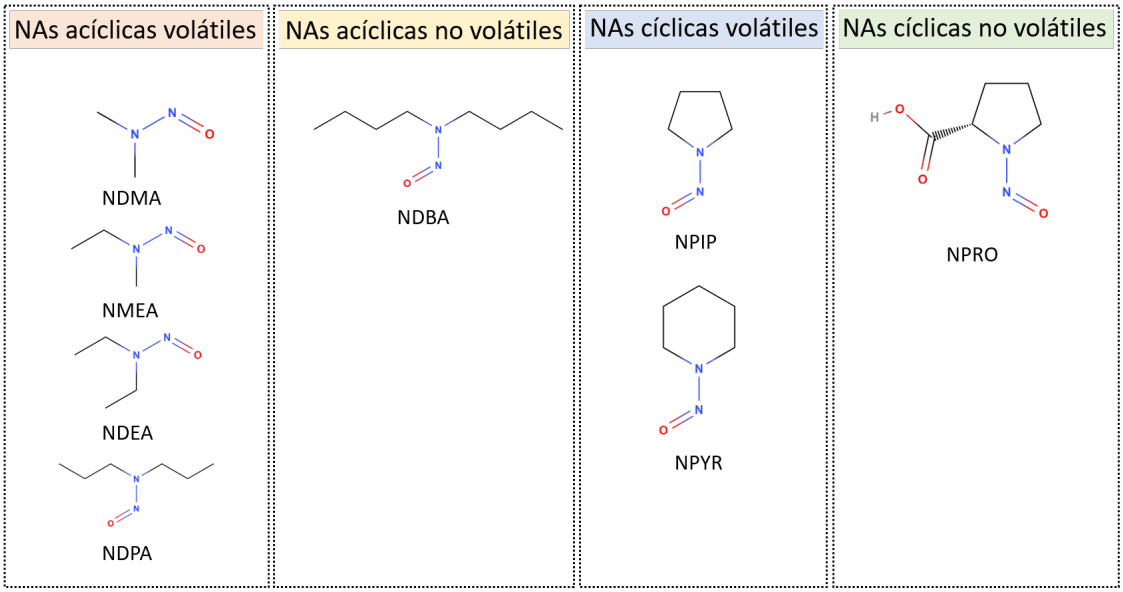

Figura 5. Clasificación y estructura química de las nitrosaminas procedentes de la dieta más relevantes según la EFSA [165]. NDMA, N-nitrosodimetilamina; NMEA, N-nitrosometiletilamina; NDEA, N-nitrosodietilamina; NDPA, N-nitrosodipropilamina; NDBA, N-nitrosodibutilamina; NPIP, N-nitrosopiperidina; NPYR, N-nitrosopirrolidina; NPRO; N-nitrosoprolina. Figura creada parcialmente con MolView.

La principal fuente exógena de NAs es la dieta, pero estos compuestos también pueden llegar al organismo a través del tabaco o diversos productos farmacéuticos [166]. Los alimentos que más contribuyen a la formación de NAs son las carnes rojas y procesadas, pescados, quesos y productos fermentados [167]. Estos alimentos suelen presentar mayores niveles de nitratos y nitritos debido en gran parte a la aplicación en ellos de conservantes alimentarios como el nitrito de potasio (E-249), nitrito de sodio (E-250), nitrato de sodio (E-251) y nitrato de potasio (E-252) con el fin de prolongar su vida útil y evitar contaminaciones microbiológicas o favorecer el proceso de maduración de la carne [168]. Diversos factores como la presencia de compuestos antioxidantes, la microbiota o la presencia de aminoácidos y grupos hemo pueden afectar a la formación de NAs en el alimento [169]. En este sentido, las carnes rojas y procesadas suelen ser ricas en proteína y en hierro hemo, cuya presencia también favorece la formación de NAs en el organismo. La formación de NAs endógenas comienza en la cavidad oral tras la ingesta del alimento, donde se generan moléculas de nitrito a partir de nitrato mediante procesos de

reducción catalizados por la microbiota oral y el grupo hemo [170]. Debido a la presencia de condiciones ácidas en el estómago, las moléculas de nitrito previamente generadas pueden transformarse a ácido nitroso, el cual se considera un agente nitrosante, capaz de reaccionar con aminas secundarias de la dieta generando NAs endógenas [169,171,172]. En el intestino, predominantemente en el intestino grueso, también pueden formarse NAs gracias a diversos factores entre los que se incluyen la presencia de aminas secundarias y agentes nitrosantes como el óxido nítrico, S-nitrosotioles, hierro hemo nitrosilado, nitratos y nitritos y también la acción de la microbiota presente [169,173–175].

Las NANV no han mostrado actividad biológica hasta la fecha, por lo que el interés de la comunidad científica se ha centrado en las NAV más abundantes en alimentos como es el caso de la N-nitrosodimetilamina (NDMA), la N-nitrosopiperidina (NPIP) y la N-nitrosopirrolidina (NPYR) [167,175,176]. Respecto a las NAVs, la IARC ha clasificado la NDMA como probable carcinógeno para humanos (grupo 2A) y la NPIP y NPYR como posibles carcinógenos para humanos (grupo 2B) [177]. La ingesta de NDMA, especialmente la procedente de carne procesada y cerveza, se ha asociado a un mayor riesgo de desarrollar CCR [178,179]. De manera similar, Seyyedsalehi y colaboradores realizaron en 2023 una revisión sistemática y meta-análisis que reveló una asociación entre el riesgo de desarrollar cáncer gastrointestinal y la ingesta de nitritos y NDMA [180].

La ingesta de NAs ha sido analizada en diversos proyectos en el contexto de la salud digestiva. Estudios realizados en población europea sitúan la ingesta de estos compuestos entre 52 y 280 ng/día, existiendo diferencias entre sexos y los distintos grupos de diagnóstico [179,181–183]. En otras poblaciones como la canadiense, el quintil de mayor consumo de NDMA era de 2.290 ng/día, mientras que en población china adulta la ingesta media de este compuesto fue de 251 ng/día [178,184]. La ingesta de otros compuestos como NPYR y NPYP se ha estimado sobre población alemana, arrojando valores de 11 ng/día y 15 ng/día respectivamente [183]. Según un informe de la EFSA, la exposición total a un panel de 10 NAs carcinogénicas presentes en comidas variaba entre 0 ng/día y 14.623 ng/día para una persona de 70 kg, dependiendo de los grupos de edad, escenarios y tipo de entrevista realizada [165].

2.1.2.4. Acrilamida

El compuesto prop-2-enamida, también conocido como acrilamida, es una amida insaturada con la fórmula C_3H_5NO. Cuando se encuentra en forma aislada, tiene apariencia sólida cristalina, blanca y es inodoro y soluble en agua, etanol, éter y cloroformo [185]. La principal vía de formación de acrilamida en alimentos es mediante la descarboxilación de la asparagina en presencia de azúcares reductores por la reacción de Maillard a temperaturas superiores a 120 ºC [186]. Se han descrito, además, otras vías minoritarias de formación de este

compuesto. La primera ocurre en presencia de aminoácidos como la glutamina, metionina o alanina [187]. La segunda tiene lugar con la participación de una molécula de acroleína generada a partir de gliceroles, azúcares o almidón de la dieta y puede involucrar la formación de ácido acrílico como intermediario, lo que podría explicar el rol potenciador de los aceites en la formación de este compuesto [185].

La acrilamida se puede encontrar de forma natural en los alimentos, pero además existen otras fuentes no dietéticas para su introducción en el organismo como son el tabaco, el uso de ciertos cosméticos o la cercanía a ciertas industrias como la del papel, el petróleo o los adhesivos [188]. En lo que respecta a la dieta, los alimentos ricos en carbohidratos como las patatas, los productos a base de cereales y el café son la principal fuente dietética de este compuesto [189]. Los métodos de cocinado que implican altas temperaturas y potencian el sabor y olor de los alimentos, como la fritura o el horneado, también promueven la formación de acrilamida, cuya concentración es proporcional directamente al tiempo de cocinado y al grado de tostado de los alimentos [190–192].

La acrilamida ha sido clasificada como probable carcinógeno para el ser humano (Grupo 2A) [193]. Actualmente no existe regulación sobre la ingesta máxima recomendada, aunque sí se ha señalado una ingesta diaria tolerable máxima de 2,6 µg por día y kg de peso corporal con el fin de evitar efectos carcinogénicos, sugiriendo limitar su consumo [194]. Sin embargo, las evidencias epidemiológicas existentes de su relación con el CCR no son concluyentes. En una revisión sistemática y meta-análisis llevado a cabo por Pelucchi y colaboradores se ha señalado una escasa relación entre la ingesta de acrilamida y el riesgo de la mayoría de canceres, con excepción de una modesta asociación con el cáncer de riñón [195]. Estos resultados fueron similares a los obtenidos en otra revisión sistemática de Virk-Baker y colaboradores [196]. Más recientemente, un estudio prospectivo llevado a cabo sobre población japonesa señaló de nuevo falta de asociación entre CCR y la ingesta de acrilamida en la población estudiada [197]. A este respecto, Riboldi y colaboradores señalaron que la posible causa de esta ausencia de asociación podía deberse a la falta de homogeneidad para el registro de la ingesta de acrilamida y las limitaciones de los estudios, lo que a su vez podría estar repercutiendo en la validez de los resultados obtenidos en los mismos [198].

A partir de 31 estudios sobre acrilamida y el riesgo de desarrollar cáncer llevados a cabo en diversos países como Italia, Suiza, Países Bajos, Japón, Finlandia o los Estados Unidos se ha estimado que su consumo medio es de 23 µg/día, estando los trabajos centrados en CCR en un rango de consumo de 6,8 µg/día a 36,1 µg/día [195,197,199–203].

2.1.2.5. El registro de los xenobióticos de la dieta

Como ocurre con muchos otros componentes de la dieta, el impacto de la ingesta de xenobióticos relacionados con el procesado de alimentos sobre la salud depende de la dosis de ingesta y de la frecuencia de exposición al agente o agentes tóxicos. A la hora de cuantificar la ingesta real de xenobióticos en una población, es esencial contar con un método que permita registrar su consumo con un cierto grado de precisión. Una de las principales dificultades en este punto es la selección del método más adecuado. Entre las herramientas más utilizadas para este fin se encuentran el cuestionario de frecuencia de consumo de alimentos (CFCA), los registros de alimentos de varios días y el recuerdo dietético de 24 horas. Con independencia de los errores sistemáticos y aleatorios inherentes a estos métodos, algunos factores como el periodo de tiempo cubierto por los cuestionarios dietéticos, el número de alimentos incluidos o la cuantificación de las porciones consumidas afectan a la calidad de la información recogida y, por tanto, a las conclusiones extraídas [204]. Es importante recordar que la exposición a largo plazo a estos compuestos xenobióticos es lo que podría tener un impacto negativo sobre la salud [205]. Por este motivo, se podría considerar más apropiado utilizar cuestionarios con capacidad para describir los hábitos dietéticos a largo plazo, como el CFCA [206]. Sin embargo, el CFCA tiene la desventaja de proporcionar información menos precisa sobre la ingesta de energía y nutrientes en comparación con los otros métodos mencionados anteriormente [207]. Debido a los mecanismos involucrados en la formación de xenobióticos de la dieta, para su correcta cuantificación es importante detallar de forma armonizada algunas características relacionadas con la preparación culinaria de los alimentos, como el tiempo de cocción, el método de procesado, la temperatura o el grado de dorado [98]. Se trata de una dificultad añadida importante ya que prolonga la duración de los cuestionarios de referencia, ampliando el número de elementos incluidos y aumentando la complejidad del análisis debido a que para cada uno de los alimentos encuestados debe considerarse el tipo de procesado, la duración y la temperatura de cocción.

La estimación de la ingesta de xenobióticos a partir de los alimentos de la dieta puede deducirse a partir de la información contenida en diversas bases de datos que recopilan información sobre la concentración de estos compuestos en función del método de conservación aplicado, el método de cocinado, la temperatura, el grado de dorado o la parte ingerida del alimento. Entre estos trabajos podemos destacar el libro titulado “Contenido de sustancias potencialmente cancerígenas en alimentos. Nitratos, nitritos, nitrosaminas, aminas heterocíclicas e hidrocarburos aromáticos policíclicos” desarrollado por el EPIC para población europea y la base de datos “Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease” (CHARRED) para población norteamericana [208,209]. El libro del EPIC recopila información obtenida de 139 referencias sobre el contenido por 100 g de alimento en HAPs,

AHs, NAs, nitritos y nitratos en más de 200 alimentos, mientras que la base de datos CHARRED se centra en proporcionar información de HAPs y AHs en relación con el cocinado de carnes. Además, existen otros trabajos, llevados a cabo por la EFSA y la Agencia de Alimentos y Medicamentos (FDA) para determinar niveles de nitratos y acrilamida en alimentos [210,211].

2.2. El patrón de dieta mediterránea

El modelo de dieta mediterránea (DM), originario de las poblaciones asentadas en la cuenca del Mar Mediterráneo, está caracterizado por un alto consumo de verduras, frutas, legumbres, aceite de oliva virgen extra, frutos secos, pescado, cereales integrales y una baja ingesta de azúcares simples, carnes rojas y procesadas y leche [212]. En general, evidencias científicas sólidas han indicado un papel protector de la DM frente al desarrollo de algunas patologías, incluido el CCR [213,214]. El efecto protector de la DM podría atribuirse, entre otros, a su alto contenido en ácidos grasos poliinsaturados y en compuestos bioactivos como son los polifenoles y la fibra dietética [215]. La ingesta moderada de vino con las comidas ha sido tradicionalmente un elemento representativo de la DM [212]. Sin embargo, actualmente se promueve un consumo moderado de alcohol y se han postulado varias hipótesis que apoyan la abstinencia de su consumo. En este sentido, se ha demostrado que el consumo de alcohol puede aumentar el riesgo de CCR, incluso a dosis bajas [66,216]. El etanol se metaboliza a acetaldehído, el cual si se acumula puede alterar la absorción de folato e interferir en los procesos de síntesis y reparación del ADN y en los sistemas antioxidantes del organismo [217,218].

Dentro de los compuestos bioactivos, los polifenoles se caracterizan por ser una familia de compuestos fitoquímicos conformados por uno o varios anillos fenólicos con estructuras complejas, normalmente glucosiladas, que habitualmente muestran una gran capacidad antioxidante [219]. Una vez ingeridos, los polifenoles glucoconjugados pueden ser desglucosilados por el conjunto de organismos que habitan en el intestino, es decir, por la microbiota intestinal (MI), lo que suele aumentar su biodisponibilidad y su actividad biológica [220]. Los polifenoles resultantes pueden ser absorbidos o metabolizados, dando lugar a otros compuestos fenólicos derivados [221]. Esta biotransformación de los compuestos fenólicos está sujeta a una gran variabilidad interindividual debido a las diferencias en la composición de la MI, lo que a su vez influye en el efecto final de los compuestos fenólicos sobre el hospedador [222]. De manera general, se ha descrito que los polifenoles son capaces de prevenir el daño al DNA causado por la presencia de radicales libres, de contribuir a la modulación de enzimas relacionadas con el estrés celular y de jugar un papel inmunomodulador asociado a la reducción de parámetros inflamatorios como IL-6, TNF-α o la PCR o al aumento de adiponectina [223–

226]. De hecho, en estudios clínicos y preclínicos se han observado resultados prometedores del uso combinado de polifenoles con terapias de tratamiento de CCR [227].

Las fibras se definen como polisacáridos u oligosacáridos de tres o más unidades monoméricas que no son digeridos ni absorbidos en el intestino [228,229]. Se ha evidenciado una relación inversa entre el consumo de fibra y el riesgo de desarrollar CCR [230,231]. Por un lado, las fibras dietéticas insolubles son capaces de acortar el tiempo de tránsito intestinal incrementando el volumen de las heces y secuestrando moléculas potencialmente tóxicas de origen dietético, lo que puede ayudar a disminuir la toxicidad del ambiente intestinal [222,232]. Por otro lado, en el colon, algunos microorganismos de la MI son capaces de utilizar las fibras como sustratos fermentables, produciendo ácidos grasos de cadena corta (AGCCs), principalmente acetato, butirato y propionato [233]. Estos compuestos pueden servir como fuentes de energía celular, interaccionar con las células epiteliales intestinales y con células inmunitarias de la lámina propia o en última instancia pasar a circulación sistémica para alcanzar otros tejidos [234]. Durante estas interacciones pueden mostrar actividad antiinflamatoria a través de receptores acoplados a proteínas G y mediar el aumento de la hiperacetilación de histonas, lo que promueve la apoptosis inmunomediada de las células cancerosas [235]. Más concretamente, a nivel intestinal los AGCCs participan en interacciones microbianas cruzadas, pudiendo promover efectos beneficiosos en la MI, provocando una bajada del pH luminal o inhibiendo el crecimiento de microorganismos patógenos [236,237]. Es de interés señalar la relación bidireccional en el intestino de la fibra y los polifenoles, concretamente para los compuestos que muestran actividades prebióticas y antibacterianas, lo que se podría traducir en cambios composicionales y metabólicos a nivel de la MI.

3. MICROBIOTA Y CÁNCER COLORRECTAL

La relación de la microbiota y la salud es estrecha como resultado de su coevolución, adaptación y codependencia con la fisiología del hospedador [238]. La interacción de los microorganismos que componen la microbiota humana con el hospedador ocurre principalmente a nivel de las mucosas. Una de las partes del cuerpo más densamente poblada por microorganismos es el tracto gastrointestinal [239,240]. La densidad microbiana aumenta progresivamente desde el estómago, donde las condiciones ácidas dificultan el crecimiento de microorganismos, hasta el intestino delgado y el intestino grueso, siendo esta última la zona del cuerpo humano más densamente poblada de microorganismos [241]. En el intestino delgado las condiciones fisiológicas favorecen el predominio de microorganismos aerotolerantes y anaerobios facultativos resistentes al ácido y la bilis, en concentraciones que varían entre 10 y 10^7 células por gramo de contenido intestinal [242]. En el intestino grueso se encuentran principalmente microorganismos anaerobios estrictos en concentraciones hasta 10^{11}-10^{12} células por gramo de contenido intestinal [243].

3.1. Métodos de análisis de la microbiota intestinal

La MI engloba al conjunto de microorganismos que habitan en el intestino grueso e incluye bacterias, arqueas, virus y hongos [244]. La MI comprende tanto a los organismos asociados a la mucosa del intestino como a los que se encuentran en el lumen intestinal por el que pasan los residuos resultantes de la digestión y donde tiene lugar la transformación de estos por parte de la MI y las enzimas intestinales. Finalmente, los restos no absorbidos serán excretados en las heces, junto con parte de la MI. La mayor parte de la evidencia científica existente hasta la fecha caracterizando la composición de la MI humana se ha llevado a cabo a partir de muestras de heces. La composición microbiana de las muestras de biopsia intestinal y de las muestras fecales puede ser diferente y el estudio de ambas puede ofrecer una visión de la MI complementaria. No obstante, la obtención de muestras fecales es una alternativa fácil y no invasiva, que permite realizar estudios comparativos entre individuos y poblaciones [245].

Las técnicas existentes para el estudio de la MI se pueden clasificar en dos grandes grupos: Las dependientes y las independientes de cultivo. Las primeras se basan en el cultivo de los microorganismos a estudiar utilizando medios generales y/o diferenciales con el objetivo de seleccionar poblaciones específicas, así como diferenciar y caracterizar los microorganismos según sus características metabólicas, bioquímicas o fisiológicas específicas [246]. Sin embargo, para el estudio de poblaciones microbianas complejas presentan el inconveniente de que la mayoría de las bacterias de la MI son anaerobias estrictas y presentan requerimientos específicos desconocidos, de manera que aproximadamente solo entre el 30-50% de la MI puede cultivarse en condiciones estándar de laboratorio [247,248]. Por otro lado, las técnicas

independientes de cultivo se basan en técnicas moleculares para la detección y cuantificación de los microorganismos, no requiriendo el cultivo de los mismos, con lo que permiten la caracterización de ecosistemas microbianos complejos [246]. Las primeras técnicas independientes de cultivo estaban dirigidas al estudio del ADN, incluyendo técnicas como la reacción en cadena de la polimerasa cualitativa y cuantitativa (qPCR), los microarrays filogenéticos o la hibridación fluorescente *in situ*, entre otras [249]. Posteriormente se han desarrollado metodologías basadas en la amplificación masiva del ADN total o la amplificación parcial de genes específicos (como el gen del ARN ribosómico (ARNr) 16S), la cual se ha convertido en una de las estrategias más utilizadas para el estudio de ecosistemas microbianos complejos y para la identificación de los microorganismos que habitan en dichos ecosistemas (metataxonomía microbiana) [250,251]. Esta técnica depende en gran medida del progresivo desarrollo de bases de datos específicas. A pesar de su elevado poder de procesamiento, presenta el inconveniente de que proporciona escasa información acerca de las propiedades funcionales de los microorganismos [252]. Entre las metodologías independientes de cultivo y de alto poder de procesamiento se incluyen todas las técnicas ómicas como la metagenómica, la metatranscriptomica, la proteómica o la metabolómica [246]. La metagenómica total (o "shotgun metagenomic sequencing") en la que se secuencian genomas completos, no requiriéndose la amplificación de un gen específico, permite el estudio de las comunidades de bacterias, hongos levaduras y virus dentro de un mismo proceso, así como la realización de estudios funcionales *in vitro*. Cuando la profundidad de secuenciación es alta, sirve también para identificar microorganismos poco frecuentes en la muestra. Sin embargo, todas estas técnicas requieren un complejo análisis computacional posterior [250]. Una de las técnicas independientes de cultivo que ha mostrado un uso creciente durante los últimos años es la culturómica. Esta se basa en el empleo de medios complejos y condiciones de cultivo diversas, siendo combinado con incubaciones prolongadas y el posterior uso de otras técnicas ómicas como la espectrometría de masas [253]. Otro enfoque posible dentro de la culturómica es el cultivo de microorganismos a partir del estudio de sus características funcionales detectadas "in vitro" mediante técnicas como la proteómica o la metagenómica [254]. Esta técnica tiene su origen en el estudio de la MI, permitiendo el aislamiento de nuevas especies y el posterior estudio de su papel en la salud y la enfermedad [255,256].

De esta manera, el empleo de enfoques globales e integrativos permite el estudio de la MI y su vinculación con el metabolismo del hospedador, la patogénesis de enfermedades y las predicciones de dianas terapéuticas [257].

3.2. Composición y funcionalidad de la microbiota intestinal

Gracias al desarrollo y aplicación de las técnicas ómicas de alto poder de procesamiento al estudio de la MI, se ha podido obsevar que en individuos adultos sanos la MI está dominada por los filos *Bacillota* y *Bacteroidota*, seguidos en términos de abundancia relativa por los filos *Actinobacteriota*, *Pseudomonadota* y *Verrucomicrobiota* [258]. Sin embargo, a niveles taxonómicos inferiores, la complejidad de la MI aumenta notablemente y difiere considerablemente en grupos diferentes de población. Todo ello hace muy difícil definir composición normal o saludable del MI a nivel poblacional [259].

El establecimiento de la MI comienza con el nacimiento y evoluciona notablemente en los primeros años de vida. Su interacción con el intestino, el órgano humano con mayor número de células inmunitarias, es esencial para el correcto desarrollo y mantenimiento de las funciones del sistema inmunitario [260,261]. En etapas posteriores, durante la vida adulta y en condiciones de salud, la MI contribuye al correcto mantenimiento del sistema digestivo y nervioso del huésped mediante el refuerzo de la integridad del epitelio y la barrera intestinal [262]. El tejido linfoide asociado a la mucosa intestinal expresa receptores específicos de reconocimiento que interaccionan con diferentes componentes de la pared celular bacteriana entre los que se incluyen el lipopolisacárido de la membrana externa de las bacterias Gram negativas, el peptidoglicano o los ácidos teicoicos, induciendo “tolerancia” cuando se trata de microorganismos comensales no patógenos o desencadenando una respuesta inflamatoria cuando se trata de patógenos, conduciendo todo ello a la correcta modulación de la defensa frente a patógenos [261,263]. La MI también contribuye a la transformación de nutrientes de la dieta. La fermentación en el colon por parte de la MI de los componentes no digeridos de la dieta, principalmente carbohidratos complejos y en menor medida proteínas, péptidos y lípidos conduce a la obtención de energía y a la producción de AGCC, que desempeñan notables funciones en la fisiología del hospedador y en el ecosistema microbiano intestinal. Finalmente, la MI contribuye a la transformación y excreción de multitud de metabolitos como ácidos biliares secundarios, aminoácidos, neurotransmisores y algunas vitaminas esenciales [240,264,265].

3.2.1. Metabolismo de compuestos tóxicos

La MI es capaz de modificar la toxicidad de algunos componentes de la dieta, que en función de su efecto pueden ser citotóxicos, genotóxicos, mutagénicos y/o carcinogénicos. La citotoxicidad es la capacidad de ciertas sustancias de provocar lesiones celulares, con efectos nocivos sobre el metabolismo, la estructura y/o la viabilidad de las células. La genotoxicidad es la capacidad de causar daños en el material genético celular y, más concretamente, la mutagenicidad es la capacidad de los agentes químicos, físicos o biológicos de alterar la

secuencia del ADN a través de eventos de mutación, modificando la expresión y la funcionalidad de los genes [266–268]. Todos los compuestos mutagénicos son genotóxicos, pero esto no siempre ocurre a la inversa, ya que los compuestos genotóxicos pueden también producir cambios epigenéticos o inestabilidad genómica sin afectar la secuencia de ADN. Tanto los agentes genotóxicos como mutagénicos pueden ser clasificados como compuestos carcinogénicos si son capaces de inducir el desarrollo de neoplasias malignas [269].

La modificación de la toxicidad de los compuestos de la dieta por parte de la MI puede ocurrir de diversas maneras. En primer lugar, la MI puede modular la absorción de estos compuestos alterando la permeabilidad intestinal o modificando el grosor del moco intestinal [270]. En segundo lugar, los microorganismos pueden unirse de manera directa a los compuestos tóxicos y facilitar su eliminación con las heces, como ocurre en el caso de algunas bacterias del ácido láctico (BAL) y otros microorganismos [271,272]. En tercer lugar, los microorganismos de la MI pueden metabolizar y disminuir la toxicidad de compuestos potencialmente tóxicos gracias a su amplio repertorio metabólico de procesos de hidrolisis, reducción, acetilación, metilación o adición de grupos funcionales [273,274]. Un ejemplo es la conjugación de las AHs MeIQx, PhIP y 2-amino-3-metilimidazo(4,5-f)quinolina (IQ) gracias a la acción de microorganismos renombrados como *Anaerobutyricum hallii*, *Limosilactobacillus reuteri* y *Furfurilactobacillus rossiae*, generando compuestos menos tóxicos y mutagénicos para las células de colon [275–277]. De manera opuesta, la MI también puede transformar compuestos en otros que muestran mayor toxicidad. En este sentido, la MI es capaz de transformar la AH IQ en el compuesto potencialmente mutagénico 7-hidroxi-IQ o también intervenir en la formación de NOCs tal y como se comentaba en apartados anteriores [278]. Por último, la MI puede interferir con los mecanismos de detoxificación de compuestos tóxicos del hospedador [98]. Resulta notable el papel de la MI en la circulación enterohepática de los xenobióticos de la dieta, como se detallará a continuación.

3.2.2. Detoxificación de compuestos xenobióticos

Tras ser ingeridos, los xenobióticos pasan por el tracto gastrointestinal donde pueden ser absorbidos de forma directa a través de la pared intestinal y transportados por la vena porta hepática (Figura 6). Una vez en el hígado, pueden ser transformados mediante enzimas de fase I de la superfamilia de enzimas del citocromo P450 a través de reacciones de oxidación, reducción o hidrólisis [279,280]. Los compuestos generados son conjugados con otras moléculas más polares para aumentar su solubilidad, gracias a los enzimas de fase II, predominantemente transferasas que son capaces glucuronidar, metilar o acetilar [281]. Estos compuestos transformados en el hígado pueden pasar a circulación sistémica o ser transportados de nuevo al intestino mediante la circulación enterohepática para su excreción (fase III) [282].

Sin embargo, en el intestino la MI puede reactivar estas moléculas mediante reacciones hidrolíticas como las llevadas a cabo por las β-glucuronidasas procedentes de enterobacterias, lo que podría aumentar el tiempo de permanencia, la exposición, así como modificar la reabsorción de los xenobióticos, contribuyendo al aumento de los efectos tóxicos [273,283,284]. Algunos conjugados glucurónidos (-G) son especialmente susceptibles de sufrir estas transformaciones, como es el caso del conjugado IQ-G o los compuestos N-OH-PhIP o PhIP-G [285–287].

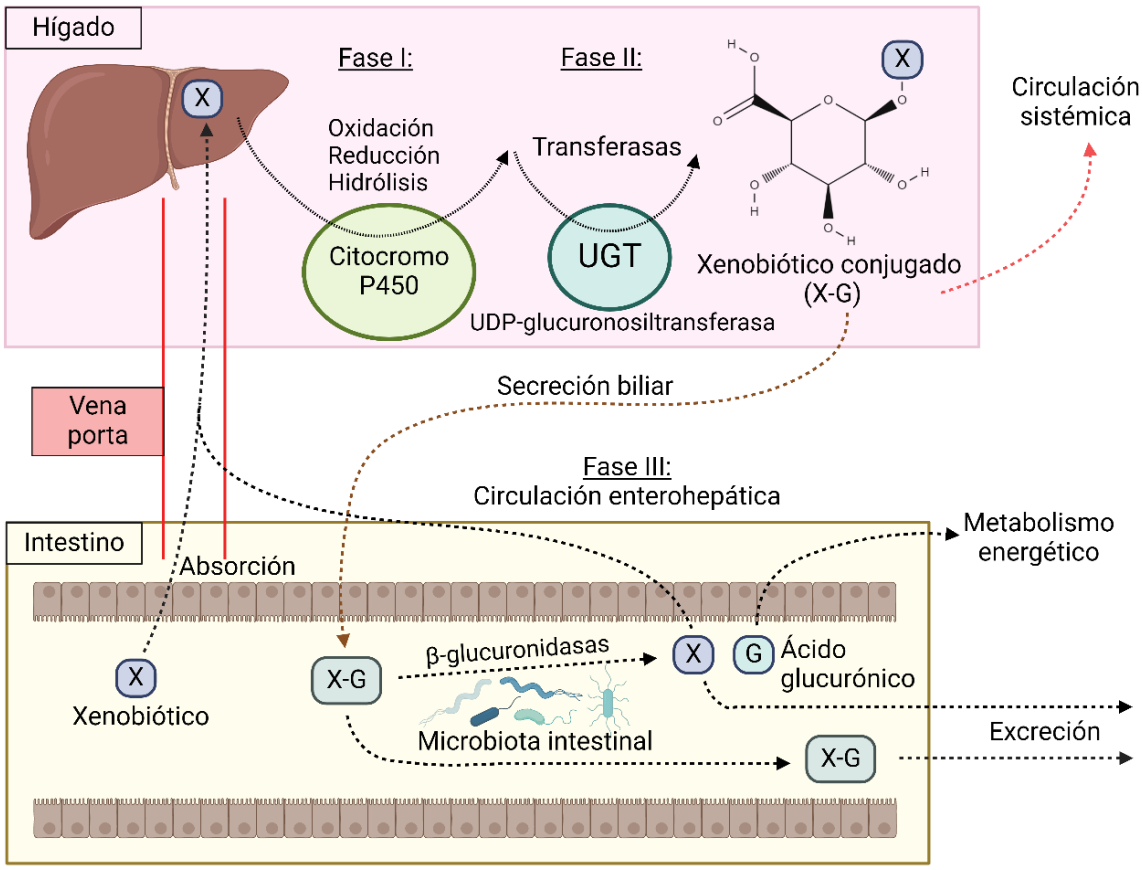

Figura 6. Circulación enterohepática de los xenobióticos de la dieta. Figura creada con Biorender.com y adaptada de Pellock y colaboradores (2017) [288].

Esta relación no es unidireccional, ya que la composición y funcionalidad de la MI también puede verse alterada por la ingesta de xenobióticos [274]. Por ejemplo, algunos subproductos resultantes de la metabolización de estos compuestos tóxicos pueden servir como fuente de carbono para el crecimiento de microorganismos [274]. Además, se ha observado que la exposición oral al HAP B(a)P induce un cambio en la composición de la MI en ratones, mientras que la suplementación con una mezcla de NAs a concentraciones entre 23 y 189 ng/L es capaz de modular la composición de la microbiota hacia un perfil relacionado con obesidad [289,290]. Por todo ello, la identificación de cambios en la microbiota asociados a la ingesta de compuestos tóxicos con potencial carcinogénico podría ser útil para elaborar directrices sobre el procesado de alimentos y facilitar recomendaciones dietéticas.

3.3. Disbiosis y enfermedad

Los cambios en la composición y funcionalidad de la MI pueden dar lugar a un estado de desequilibrio conocido como disbiosis intestinal. Este fenómeno se asocia con el establecimiento y progresión de diversas enfermedades, especialmente las enfermedades no transmisibles, como es el caso del CCR, y suele ir acompañado de una disminución de la diversidad microbiana [291–293]. También es habitual la sustitución de las comunidades comensales de la MI por especies patógenas oportunistas conforme se desarrolla la enfermedad. En CCR, se ha propuesto que ciertas bacterias intestinales denominadas "conductoras" podrían inducir la enfermedad mediante un daño progresivo al ADN del epitelio intestinal y desencadenar la tumorigénesis. A su vez, estas bacterias conductoras también podrían favorecer la proliferación de bacterias "pasajeras" al proporcionarles un entorno adecuado para su establecimiento. Este fenómeno se conoce como la teoría del "conductor-pasajero" [294]. Sin embargo, es difícil atribuir la causa de la disbiosis a uno o varios microorganismos y en muchas ocasiones resulta difícil determinar si los cambios de la MI son la causa o la consecuencia de una enfermedad [295]. La identificación de alteraciones clave de la MI es de gran interés por su potencial aplicación en la prevención y para la búsqueda de nuevos biomarcadores asociados a los distintos estadios y características de la enfermedad y su potencial influencia en dicha progresión.

3.4. Alteraciones de la microbiota intestinal en el desarrollo de cáncer colorrectal

De manera general, la prevalencia muestras fecales o de mucosa intestinal de ciertas bacterias como *Escherichia coli* (*pks+*), *Enterococcus faecalis*, *Streptococcus gallolyticus* (antes conocido como *Streptococcus bovis*), *Bacteroides fragilis*, *Fusobacterium nucleatum* o *Parvimonas micra*, con el riesgo de desarrollo de CCR. Esto se asocia parcialmente a la capacidad de algunos de estos microorganismos de producir toxinas con actividad genotóxica y citotóxica como la adhesina A, la colibactina o la fragilisina [98,296,297]. No obstante, se ha señalado una gran variabilidad en los grupos microbianos implicados en función de la vía de desarrollo de CCR [298].

Las alteraciones microbianas en la vía adenomatosa de la carcinogénesis son las que se han estudiado en mayor profundidad. Un trabajo llevado a cabo con pacientes que habían sido diagnosticados con pólipos adenomatosos mostró una disminución de la abundancia relativa del filo *Bacillota* y un aumento del filo *Fusobacteriota* en las muestras fecales de individuos con pólipos en comparación con las muestras controles sanos [299]. Esto concuerda con las observaciones de Hale y colaboradores, que observaron un aumento diferencial de la abundancia del filo *Bacteroidota* y de los géneros *Bilophila*, *Sutterella* y *Mogibacterium* en muestras fecales del grupo de individuos con pólipos adenomatosos y un aumento diferencial de

la abundancia de *Bacillota* y del género *Veillonella* en las heces del grupo de control [300]. Por el contrario, el estudio realizado por Shen y colaboradores en muestras de mucosa intestinal puso de manifiesto una menor abundancia de *Bacteroidota* y una mayor abundancia de *Pseudomonadota* en los casos de adenoma en comparación con el grupo de control. En este trabajo, a nivel taxonómico de género, los sujetos con adenomas intestinales mostraron una mayor abundancia de especies de los géneros *Dorea* y *Faecalibacterium* y menor proporción de especies de los géneros *Bacteroides* y *Coprococcus* que los controles [301]. Se ha observado además que varios miembros de la clase *Clostridia* y algunos géneros como *Cronobacter*, *Pseudomonas* y *Ruminococcus* pueden aparecer reducidos en las heces de individuos con adenomas convencionales en comparación con las heces de los controles [302–304]. Un estudio llevado a cabo por Yachida y colaboradores ha demostrado que varias especies pueden encontrarse alteradas en muestras fecales de sujetos con múltiples pólipos adenomatosos en comparación con las muestras de individuos sanos. Entre ellas se incluyen niveles enriquecidos de *Atopobium parvulum*, *Fusobacterium nucleatum* subsp. *nucleatum*, *Schaalia odontolytica*, *Phascolarctobacterium succinatutens*, *Selenomonas sputigena* y *Oleidesulfovibrio vietnamensis* y la disminución de *Bifidobacterium longum* subsp. *longum*, *Lachnospira multipara* y *Lachnospira eligens* [305]. Cambios similares se han hallado también en muestras de mucosa con pólipos adenomatosos en comparación con muestras de mucosa sana, con un aumento significativo de *Atopobium*, *Parvimonas* y *Fusobacterium* en las primeras [306]. En la mucosa asociada a adenomas convencionales, una revisión sistemática llevada a cabo por Aprile y colaboradores señalaba una mayor prevalencia de los filos *Pseudomonadota*, *Fusobacteriota* y *Bacteroidota* y una disminución de *Clostridium*, *Faecalibacterium*, *Bacteroides* y *Romboutsia* [307]. A medida que se producen transformaciones malignas, los pólipos adenomatosos pueden llegar a presentar un alto riesgo de desembocar en CCR. Se ha observado que las muestras fecales de individuos que presentan un alto riesgo de desarrollar CCR pueden mostrar una mayor abundancia relativa de *Erysipelotrichaceae* y del género *Blautia* que las muestras fecales de individuos control [308]. De hecho, este último género también se ha encontrado aumentado en muestras fecales de adenomas avanzados en otro estudio realizado por Xu y colaboradores, junto con una mayor abundancia de *Bifidobacterium*, *Dorea*, *Sutterella* y *Clostridium*, mientras que *Parabacteroides* y *Coprobacter* estaban enriquecidos en los controles [309]. Es interesante mencionar que la comparación de muestras fecales de individuos con adenomas frente a casos de CCR sugiere que *Porphyromonas endodontalis*, *Ruminococcus torques* y *Odoribacter splanchnicus* podrían jugar un papel clave en el desarrollo de la enfermedad [310].

En lo concerniente a la vía serrada, los estudios son más escasos. Diversos trabajos han señalado que la microbiota fecal de individuos diagnosticados con pólipos hiperplásicos presentaba mayor abundancia de los géneros *Prevotella* y *Anaerostipes* y menor abundancia del

orden *Enterobacterales* y del género *Coprobacillus* que la microbiota fecal de individuos sanos control [302,308]. Las heces de sujetos diagnosticados con lesiones serradas sésiles mostraron una menor abundancia de la clase *Erysipelotrichia* en comparación con las muestras de sujetos control [302]. Un estudio llevado a cabo por Yoon y colaboradores no encontró diferencias en las abundancias relativas en mucosa colorrectal entre muestras de individuos sanos e individuos diagnosticados con adenomas convencionales, con lesiones serradas sésiles o con CCR [311]. No obstante, se ha propuesto que los grupos *Blautia*, *Escherichia-Shigella*, *Streptococcus*, *Lactobacillus* y *Bacteroides* podrían contribuir a diferenciar entre CCR poco diferenciado y moderadamente diferenciado en las fases tempranas de CCR [312].

ANTECEDENTES Y OBJETIVOS

Durante la introducción de esta Tesis Doctoral se ha puesto de manifiesto el papel clave de la dieta en la salud humana. Mientras que la adherencia a patrones dietéticos como la dieta Mediterránea se ha relacionado con un mejor estado de salud y una modulación beneficiosa de la microbiota intestinal, principalmente atribuida a la ingesta de compuestos bioactivos como las fibras y los polifenoles, el patrón de dieta occidental se ha relacionado con una mayor incidencia de diversas enfermedades, entre ellas el cáncer colorrectal. Este patrón se caracteriza, entre otros, por un consumo elevado de alimentos grasos, procesados y ricos en hierro hemo entre los que se incluyen las carnes rojas y procesadas. Se ha sugerido que el impacto inflamatorio y la ingesta de compuestos xenobióticos derivados del procesado y cocinado de estos alimentos podrían ser algunas de las causas subyacentes de la asociación entre el patrón de dieta occidental y el cáncer colorrectal. Algunos de los compuestos xenobióticos más destacados son los hidrocarburos aromáticos policíclicos, las aminas heterocíclicas, los N-nitrosocompuestos y la acrilamida. Sin embargo, el registro de su ingesta es complejo y requiere el desarrollo de bases de datos y de métodos de recogida de la información específicos. La ingesta de xenobióticos procedentes de la dieta es capaz de modular la microbiota intestinal, siendo esta interacción bidireccional, ya que la microbiota intestinal también puede contribuir a la eliminación, bioactivación y/o prolongación de la vida media de dichos compuestos en el organismo.

El impacto en la sociedad derivado de la incidencia y mortalidad de cáncer colorrectal ha generado un gran interés por el estudio de la enfermedad. Si bien la información respecto a estadios avanzados de la enfermedad es amplia, habiéndose descrito cambios moleculares y microbiológicos relevantes, la información concerniente a etapas tempranas previas al desarrollo de cáncer colorrectal, en las que ya es posible detectar la presencia de alteraciones en la mucosa intestinal, es más escasa y variable. Una de las razones es la dificultad para unificar criterios a la hora de evaluar y clasificar el daño de la mucosa intestinal, lo que en los últimos años ha llevado a la Organización Mundial de la Salud a establecer guías y criterios para el diagnóstico de las lesiones intestinales. Existen diferencias histopatológicas claras entre las dos vías biológicas principales descritas en el desarrollo del cáncer colorrectal y se han sugerido patrones microbiológicos diferenciales a nivel intestinal. Estas diferencias podrían condicionar el riesgo de desarrollo de cáncer colorrectal, por lo que resultarían de gran interés en la prevención de la enfermedad. En este contexto de daño en la mucosa intestinal, la desregulación de diversos parámetros inmunológicos también se ha asociado con el futuro desarrollo de la enfermedad, sugiriendo alteraciones a nivel local y sistémico.

El cáncer colorrectal suele desarrollarse en etapas avanzadas de la vida, principalmente por la influencia de factores ambientales, y tiene una progresión lenta en comparación con otros tipos de cáncer. Estas características hacen posible proponer estrategias de prevención en etapas anteriores al desarrollo de la enfermedad (prevención primaria) o en sus inicios (prevención secundaria) a través de recomendaciones nutricionales y de hábitos de vida, que pueden incidir en la composición de la microbiota intestinal y en los niveles de algunos parámetros inmunológicos a nivel sistémico.

Por tanto, el **OBJETIVO GENERAL** de esta Tesis Doctoral ha sido:

Evaluar las asociaciones de la dieta y de los xenobióticos derivados del procesado de los alimentos con la presencia de daño en la mucosa intestinal y con cambios en parámetros inmunológicos, microbiológicos, metabólicos y de mutagenicidad fecal analizados en el contexto del cáncer colorrectal a partir de muestras biológicas procedentes de una población adulta con el fin de proponer dianas de intervención microbiológicas y/o dietéticas capaces de revertir el daño presente en etapas previas al desarrollo de la enfermedad.

Para alcanzar este objetivo general, se plantearon los siguientes objetivos parciales:

Objetivo 1: Estudiar las relaciones de la dieta con la mutagenicidad fecal y los niveles de parámetros inmunológicos séricos en función de la presencia de alteraciones de la mucosa intestinal relacionadas con el desarrollo de cáncer colorrectal.

1.1. Caracterización de la dieta y la mutagenicidad fecal en una población adulta en función del daño de la mucosa intestinal.
1.2. Evaluar la asociación entre parámetros inmunológicos y metabólicos séricos y la dieta en función del daño de la mucosa intestinal.

Objetivo 2: Caracterizar los perfiles microbianos y la actividad enzimática fecal en función de las diferentes vías biológicas de progresión de cáncer colorrectal.

2.1. Analizar diferencias en la composición de la microbiota fecal en función de la vía de desarrollo de la carcinogénesis.
2.2. Analizar las potenciales asociaciones de la actividad enzimática y la microbiota fecal en función de la vía de desarrollo de la carcinogénesis.

Objetivo 3: Estudio de la asociación de factores dietéticos protectores y de riesgo para el desarrollo de cáncer colorrectal con el perfil de la microbiota fecal.

Objetivo 4: Estudio de la relación entre la ingesta de nitratos, nitritos, nitrosaminas y sus fuentes dietéticas con la excreción fecal de N-nitrosocompuestos en función de las lesiones de la mucosa intestinal.

RESULTADOS

Objetivo 1:

Estudiar las relaciones de la dieta con la mutagenicidad fecal y los niveles de parámetros inmunológicos séricos en función de la presencia de alteraciones de la mucosa intestinal relacionadas con el desarrollo de cáncer colorrectal

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La dieta y el estilo de vida juegan un papel determinante en el desarrollo de alteraciones a nivel de la mucosa intestinal, como los pólipos intestinales y/o los focos de criptas aberrantes. En este sentido, los niveles de mutagenicidad fecal podrían ser indicativos de la presencia de un ambiente protumorigénico en el intestino. A su vez, se ha señalado que desequilibrios en los niveles de parámetros inmunológicos y metabólicos sistémicos también podrían contribuir al establecimiento de microambientes tumorales. El trabajo desarrollado en este Objetivo 1 se recoge en dos Artículos científicos que tratan de aportar información referente a los Objetivos 1.1. y 1.2. de esta Tesis Doctoral:

Artículo 1. Ruiz-Saavedra, S.; Zapico, A.; del Rey, C.G.; Gonzalez, C.; Suárez, A.; Díaz, Y.; de los Reyes-Gavilán, C.G.; González, S. Dietary Xenobiotics Derived from Food Processing: Association with Fecal Mutagenicity and Gut Mucosal Damage. *Nutrients* **2022**, *14*, 3482. doi: 10.3390/nu14173482

Artículo 2. González, C.; Ruiz Saavedra, S.; Gómez-Martín, M.; Zapico, A.; López-Suarez, P.; Suárez, A.; Suarez González, A.; del Rey, C.G.; Díaz, E.; Alonso, A.; et al. Immunometabolic Profile Associated with Progressive Damage of the Intestinal Mucosa in Adults Screened for Colorectal Cancer: Association with Diet. *International Journal of Molecular Sciences* **2023**, *24*, 16451. doi:10.3390/ijms242216451

En estos artículos se describe la recogida de la información dietética de los pacientes voluntarios del estudio. Para ello se diseñó específicamente un cuestionario de frecuencia de consumo de alimentos con preguntas sobre los métodos de cocinado y los hábitos de consumo que permitieron la cuantificación de la ingesta de xenobióticos procedentes del procesado de alimentos en la muestra de estudio. Los apartados de este cuestionario incluyeron preguntas adicionales sobre el tipo de carne y pescado consumido, el método de cocinado (ej: horno, a la plancha, a la barbacoa, etc.), las partes consumidas del alimento (ej: muslo de pollo cocinado y comido con piel, muslo cocinado con piel, pero comido sin ella, etc.) y el grado de cocinado (ej: poco hecho, medio hecho, etc.). Todos estos apartados se combinaron con material fotográfico para facilitar la estandarización de la información referida y para facilitar la cumplimentación del cuestionario por parte del paciente. Debido a las restricciones sociosanitarias impuestas como consecuencia de la pandemia por COVID-19, parte de los cuestionarios se adaptaron y fueron completados en formato online. Además, tras una exhaustiva búsqueda bibliográfica, se desarrolló una base de datos que recopilaba información sobre las concentraciones de compuestos xenobióticos tales como los hidrocarburos aromáticos policíclicos, las aminas

heterocíclicas, los N-nitrosocompuestos y la acrilamida por cada 100 gramos de alimento consumido en función del método de conservación y el método, tiempo y temperatura de cocinado. Todo esto se combinó con bases de datos nutricionales previamente desarrolladas y puestas a punto por el grupo de investigación y se realizó una evaluación dietética de cada voluntario, de especial relevancia para el desarrollo de los Objetivos 1, 3 y 4 de esta Tesis Doctoral. Todos los voluntarios del estudio fueron sometidos a una colonoscopia llevada a cabo por personal médico del Hospital Universitario de Asturias (HUCA) y del Hospital Carmen y Severo Ochoa mediante la cual se determinó el grupo de diagnóstico clínico al que pertenecía cada paciente. Durante este procedimiento también se obtuvo una muestra de biopsia intestinal, a excepción de los casos en los que no fue posible, analizada histológicamente por personal del Servicio de Anatomía Patológica del HUCA para detectar la presencia de focos de criptas aberrantes y las características de la mucosa. Esta información médica ha sido primordial en todos los Objetivos de esta Tesis Doctoral. Además, se obtuvieron muestras de sangre de los voluntarios del estudio en las que se determinaron los niveles circulantes de citocinas, quimiocinas y adipocinas junto a la capacidad antioxidante y el colesterol total en suero. Por otro lado, los pacientes facilitaron muestras de heces en el momento en el que acudían a consulta para la recogida de los resultados médicos. A partir de sobrenadantes fecales de estas muestras se determinó la mutagenicidad fecal mediante una modificación del test de Ames.

Los resultados obtenidos en Artículo 1 reflejaron una menor probabilidad de pertenecer al grupo de pacientes diagnosticados con pólipos intestinales en aquellos individuos con consumo alto de cereales integrales (>50 g/día). Por el contrario, los pacientes que mostraban una ingesta de etanol mayor a 11,62 g/día y de hidrocarburos aromáticos policíclicos totales mayor a 1,29 µg/día presentaban una mayor probabilidad de pertenecer al grupo de individuos diagnosticados con pólipos intestinales. Además, los pacientes pertenecientes al grupo "pólipos" que presentaban focos de criptas aberrantes en la mucosa intestinal mostraban mayores niveles de mutagenicidad fecal en comparación con los que no los presentaban. En el grupo de "pólipos", esta mutagenicidad fecal presentó una fuerte correlación positiva con la ingesta de aminas heterocíclicas como PhIP, MeIQx y DiMeIQx.

En los resultados recogidos en el Artículo 2 se observó que el daño progresivo en la mucosa intestinal estaba relacionado con niveles decrecientes de adiponectina. Además, las muestras del grupo con adenocarcinomas intestinales presentaban una reducción en los niveles de resistina y un incremento en los niveles del ligando de quimiocina 10 con motivo C-X-C (CXCL10), anteriormente conocido como proteína 10 inducida por interferón gamma (IP-10). La presencia de focos de criptas aberrantes en el grupo con pólipos intestinales se asoció con una mayor concentración del TNF-α, la cual mostró una correlación negativa con la ingesta de pectina soluble. También se observó que el consumo de carnes estaba inversamente correlacionado con los niveles de adiponectina, mientras que una mayor ingesta de vegetales se asociaba con mayores niveles de adiponectina y resistina.

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Dietary Xenobiotics Derived from Food Processing: Association with Fecal Mutagenicity and Gut Mucosal Damage

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Abstract: Whereas the mechanisms underlying the association of toxic dietary xenobiotics and cancer risk are not well established, it is plausible that dietary pattern may affect the colon environment by enhancing or reducing exposure to mutagens. This work aimed to investigate the association between xenobiotics intake and different stages of intestinal mucosal damage and colorectal cancer (CRC) screening and examine whether these associations may be mediated by altered intestinal mutagenicity. This was a case control study with 37 control subjects, 49 patients diagnosed with intestinal polyps, and 7 diagnosed with CRC. Lifestyle, dietary, and clinical information was registered after colonoscopy. For xenobiotics intake estimation the European Prospective Investigation into Cancer (EPIC) and the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) databases were used. The mutagenicity of fecal supernatants was assayed by the Ames test and light microscopy was used for the presence of aberrant crypt formation. Among all the potential carcinogens studied, the polyp group showed higher intakes of ethanol and dibenzo (a) anthracene (DiB(a)A). Besides, intakes between 0.75 and 1.29 µg/d of total polycyclic aromatic hydrocarbons (PAHs) were related with a higher risk of belonging to the polyp group. On the contrary, an intake of wholegrain cereals greater than 50 g/d was associated with a reduction in the relative risk of belonging to the polyp group. Heterocyclic amines (HAs) such as 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine (PhIP) were associated with an increased level of mutagenicity in polyps. This study is of great interest for the identification of possible therapeutic targets for the early prevention of colon cancer through diet.

Keywords: xenobiotics; colorectal cancer; fecal mutagenicity; food processing; potential carcinogens

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1. Introduction

Despite the progress that has been achieved in the early detection of colorectal cancer (CRC) in the last few years, this disease is one of the most frequently diagnosed and the second leading cause of death in Spain [1,2]. In addition to the genetic factors, age, or the presence of colon polyps, several epidemiological studies have also identified lifestyle factors either promoting or protecting against CRC. Of them, obesity, smoking habit, alcohol consumption, and diet are accepted risk factors for this pathology [3–5]. Through foods, humans are exposed to complex mixtures of substances that may cause, modulate, or prevent diseases. However, there is not enough conclusive scientific evidence on the effect

of dietary habits on the development of CRC. From all food groups, red and processed meats are considered the most scientifically proven CRC risk factors, being classified by the International Agency for Research on Cancer (IARC) as "carcinogenic" and "probably carcinogenic" to humans, respectively. In addition, diets with high content of sugar, animal products, and alcohol have been related to CRC development, contrary to whole grains, fruits, and vegetables, which have shown a protective effect [3]. In the general population, diet represents one of the major factors of exposure of the colonic epithelium to mutagenic and genotoxic compounds, being involved in both the initiation of cell transformation and tumor progression [6,7]. Nitrosamines (NA) (formed during the preservation process applied to some types of foods), heterocyclic amines (HAs), (derived from creatinine, amino acids, and sugars), and polycyclic aromatic hydrocarbons (PAHs) (formed from the incomplete combustion of organic compounds), are the major mutagenic/genotoxic compounds derived from food processing and have accumulated strong scientific evidence of their relationship with cancer in animal studies [8–11]. Among PAHs, a positive association has been observed between the intake of benzo(a)pyrene (B(a)P) through the consumption of meat mainly cooked on the grill or barbecue (daily intakes $\geq$ 2.7 ng/d) and the probability of developing rectal adenomas [12]. Regarding HAs, some carbolines have a mutagenicity index more than 1000 times higher than that of hydrocarbons such as B(a)P, which evidences their potential toxicity [13]. Despite some food components having genotoxic potential, others, such as some bioactive compounds derived from vegetable foodstuffs, have shown to inhibit various stages of the carcinogenic process [14–17]. For example, it has been shown that the intake of nitrates over 142.5 mg/d can increase the risk of CRC only when the daily intake of vitamin C is under 83.9 mg/d [18]. On the other hand, the intake of nitroso-dimethylamine (NDMA) $\geq$ 0.07 µg/d was associated with an increased risk of this pathology when daily doses of vitamin E were under recommendations [19]. In this regard, fiber consumption may have the potential to decrease CRC risk by means of increasing the colonic transit rate, diminishing the exposure of colonic epithelial cells to ingested carcinogens, or by promoting proliferation of some beneficial microorganisms such as some colonic butyrate producers [20–23]. Most dietary sources of fiber are also known to contain phenolic compounds such as flavonoids, phenolic acids, anthocyanins, or lignans, which have received extensive attention because of their chemoprotective actions in animal models and human epidemiology studies [24].

The process of progressive intestinal mucosa damage leading to CRC can take several years. Polypous endoscopic lesions, frequently accompanied by histological examination, are used in routine clinical practice to determine the intestinal mucosal damage and CRC stage. Hyperplastic polyps present low risk of evolving to neoplasia, whereas serrated (traditional and sessile serrated adenomas) and adenomatous (tubular, tubulovillous, villous) polyps, with a low or high grade of dysplasia, present a progressively augmented risk of adenocarcinoma development [25]. One of the earliest events in CRC is the formation of aberrant crypt foci (ACF) in the colonic mucosa (normal or typical without cell alterations or with hyperplasia or dysplasia), which occurs when the process is still reversible. However, the analysis of ACF is not a routine practice in the diagnosis and prognosis of CRC [26].

Scarce information is still available about the biological relevance of some dietary components produced during food processing or cooking on the mutagenicity and genotoxicity of the intestinal environment. Although the mechanisms to explain the relationship between toxic xenobiotics in diet and cancer risk are not well established yet, it is plausible that differences in the dietary habits affect the colonic environment by increasing or reducing the exposure to mutagens. Therefore, the aim of this work was to analyze the impact of xenobiotics intake as related to different stages of intestinal mucosa damage and CRC and to examine whether these associations may be mediated through modification of intestinal mutagenicity.

2. Materials and Methods

2.1. Study Design and Volunteers

This transversal analysis is part of the broader project "Effect of Diet and exposure to XEnobiotics generated during food processing on the genotoxic/cytotoxic capacity of the intestinal Microbiota" (MIXED).

The recruitment of volunteers and collection of human samples (faeces and biopsies of intestinal mucosa), anamnesis, and analytical controls of volunteers was carried out from October 2019 to December 2021 by the faculties of the Digestive Section from the Central University Hospital of Asturias (HUCA) and the Carmen and Severo Ochoa Hospital from Asturias, in the north of Spain. Volunteers were selected among patients who came to the hospital for consultation due to clinical symptoms or from those included in the colon cancer screening program in our region. Three groups of control patients (n = 37), patients diagnosed with intestinal polyps (n = 49), and patients diagnosed with CRC (n = 7) were recruited among adults submitted to a diagnostic colonoscopy (Figure 1). The following exclusion criteria were applied: age <40 or >75 years, treatment with omeprazole, antibiotics, corticoids, non-steroidal anti-inflammatory drugs, or specific cancer treatment at the time of the study or in the previous two months, previous surgery of the digestive system, autoimmunity, altered thyroid function, or history of diabetes or goiter. Patients were asked to provide a stool sample collected prior to the preparation for colonoscopy. A biopsy of intestinal mucosa was extracted during colonoscopy for examination of ACF at the Pathological Anatomy Section at HUCA.

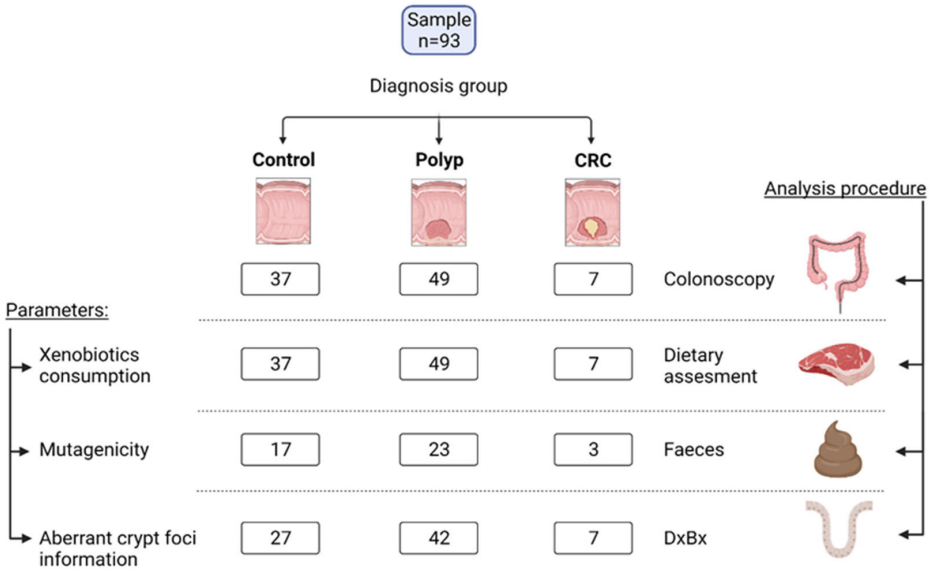

Figure 1. Graphical information on the study design and sample size. The left column information indicates the variables studied and the right column text indicates the methodology or the raw material used to study those variables. Each box in the central part of the figure shows the number of volunteers belonging to the different diagnosis group, with available data for each of the determinations. CRC, colorectal cancer; DxBx, pathological analysis.

Those individuals interested in participating were informed of the objectives of the study and signed an informed consent form. This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in Spanish legislation on bioethics. Directive 95/46/EC of the

European Parliament and the Council of October 1995, on the protection of individuals regarding the processing of personal data and on the free movement of such data, was strictly followed.

2.2. Nutritional Assessment

Dietary information was obtained from patients when they arrived for colonoscopy results at the medical consultation by means of a personalized interview conducted by trained interviewers. Exceptionally, as a result of the pandemic and COVID-19 restriction of visitors to hospitals in Spain, some of the surveys were conducted through online tools. For this purpose, a semi-quantitative food-frequency questionnaire (FFQ) was constructed with 155 items. In addition to food and culinary preparations, the specific type of food was recorded, as well as cooking methods and other related questions, when necessary. For each food, the frequency of intake and portion size were registered by means of a validated photograph album adapted from the Pilot Study for Assessment of Nutrient Intake and Food Consumption Among Kids in Europe (PANCAKE) [27]. A specific section about cooking habits (boiled, fried, grilled, baked/broiled, or barbecued) and the degree of cooking or toasting in the case of meats, fried potatoes, or toasted bread (undercooked, medium, well done, very well done) was included in the FFQ. To standardize this point, photographs of the different temperatures, in which the degree of browning increased progressively, were developed specifically for this study: low, medium, well done, and very well done were incorporated. Additionally, complementary questions such as which part of the food was consumed (breast or thigh in the case of chicken) or the possible consumption and/or cooking of the skin (cooking with skin and eating the skin; cooking with skin but not consuming it; and cooking without skin) were incorporated in order to improve the quality of the information. The intake of xenobiotics obtained from FFQ was previously validated by means of a 24 h food dietary recall [28].

The classification of the food into food groups was carried out according to the Centre for Higher Education in Nutrition and Dietetics (CESNID) criteria [29]. Food composition tables of CESNID [29] and the United States Department of Agriculture (USDA) [30] were used to transform food consumption into energy and macronutrient intake. The phenolic content of the foods was extracted from Phenol Explorer 3.6 [31] and fiber content from the tables by Marlett and Cheung [32].

2.3. Xenobiotics Derived from Food Processing

Based on food consumption per individual, cooking method, cooking time, and degree of browning, the nutritional analysis of the sample was carried out. For this purpose, information on the consumption of HAs, PAHs, nitrates, and nitrites was obtained mainly from the European Prospective Investigation into Cancer and Nutrition (EPIC) carcinogen database [33]. The EPIC database compiles information obtained from 139 references regarding the content per 100 g of food in nitrosamines, HAs, PAHs, nitrites, and nitrates in more than 200 food items. The food composition table is classified according to the preservation method, cooking method, degree of browning, and temperature [33]. HA and B(a)P information was completed with the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) [34], whereas in the case of nitrates European Food Safety Authority (EFSA) information was used [35]. Acrylamide content was provided by the U.S. Food and Drug Administration (FDA) composition tables [36] and other external reference sources were used for acrylamide [37–39], HAs [40], total PAHs [41], and nitrosamines [42–45].

2.4. Anthropometrical Determinations

Height (m) and weight (kg) were taken by standardized protocols [46]. Body mass index (BMI) was calculated using the formula weight/(height)^2. Subjects were classified into normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obese ($\geq$30.0 kg/m^2), based on the Spanish Society for the Study of Obesity (SEEDO) criteria [47].

2.5. Pathological Assessment

A total of 76 biopsies of colorectal mucosa fixed with 10% formaldehyde and paraffin-embedded were analyzed. Serial tissue sections were stained with hematoxylin–eosin and analyzed by light microscopy for the presence of ACF. Discordant diagnoses were reviewed to reach a consensus. Based on previously reported categorizations [48–51] histological findings were classified into 3 groups: normal–typical ACF (crypt with increased diameter only), hyperplastic ACF, and dysplastic ACF.

2.6. Fecal Samples and Mutagenicity

Fecal samples were collected in sterile plastic containers at hospitals participating in the study. Samples were frozen after deposition within a period not exceeding two hours and transported to the laboratory. Four grams of frozen samples were weighted, diluted 1/10, and homogenized with sterile PBS in a LabBlender 400 Stomacher (Seward Medical, London, UK) for 3 min at maximum speed. Samples were centrifuged for 15 min at 4 °C and 14,000 rpm and the obtained supernatants were separated from pellets and kept frozen at −20 °C until use.

The mutagenicity of fecal supernatants was assayed by the Ames test, without metabolic activation, against the strain *Salmonella enterica* serovar typhimurium TA100 using the 5051 Muta-ChromoPlate™ kit (EBPI, Mississauga, ON, USA) and following the manufacturer's instructions, with minor modifications. Briefly, fecal supernatants were thawed on ice, filtered through Amicon® Ultracel 3K filters (Merck Milipore Ltd., Cork, Ireland) at 16,000× *g* and 4 °C for 30 min, and serially diluted with sterile mili-Q water at 1/150-1/200-1/250, or 1/300-1/350-1/400. Fecal supernatant dilutions were combined with S. *typhimurium* TA 100 strain grown over 16 h at 37 °C in the sterile liquid medium provided by the manufacturer and the solution mix containing Davis–Mingoli salts, D-glucose, bromocresol purple, D-biotine, and L-histidine in the concentrations indicated by the manufacturer. Positive control (including sodium azide as a mutagen, grown bacteria, and solution mix), negative control (including only solution mix), and the appropriate series of dilutions of fecal supernatants (providing a variable number of positive revertant wells) were each added to 96-well microtiter plates containing 200 µL per well and incubated at 37 °C for 5 days. Reversion rates (RR) in fecal dilutions were calculated for conditions displaying the following criteria: less than 96 revertant wells per plate and more than 48 revertant wells in positive control. Considering the dilution factor, the level of mutagenicity was expressed as the mean of values corresponding to the three dilutions tested for each sample.

Given that fecal mutagenicity in our assays was determined by the frequency of reversion of the L-histidine auxotrophy present in the strain *S. Typhimurium* TA100, we first ruled out any interference of this amino acid, naturally present in fecal samples, with the mutagenicity assays performed. To this end, L-histidine levels of fecal supernatant dilutions tested were determined by ultra-high-performance liquid chromatography (UHPLC) using the method described by Redruello et al. [52] and adapted to fecal supernatants by Salazar et al. [53]. The highest level of histidine among our fecal dilutions was 2.53 µM, the rest of samples being below this value. Then, the mutagenicity of serial increasing concentrations of aqueous dilutions of L-histidine (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5 µM) was tested following the same procedure as indicated above. The Spearman correlation coefficient (see 4.7) for aqueous L-histidine concentrations and RR values in the mutagenicity reversion test (0.5833, 2.125, 2.5833, 1.75, 2.1666, 1.6666, 1.9166, 1.9166, 2.3333) was 0.226 (p-value = 0.559; not significant), allowing us to discard any interference of L-histidine with the mutagenicity assays of our fecal supernatants in the conditions described here.

2.7. Statistical Analyses

Results were analyzed using the IBM SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, USA) and RStudio software version 1.4.3. Goodness of fit to the normal distribution was checked by means of the Kolmogorov–Smirnov test. As normality of the

variables was not achieved, nonparametric tests were used. Overall, categorical variables were summarized as percentages and continuous ones as median and interquartile range (IQR = Q1–Q3). Fisher and Z tests and Kruskal–Wallis and Mann-Whitney U tests were performed for categorical and continuous variables, respectively (p-value < 0.05), with Bonferroni correction. Logistic regressions were calculated through categorical tertiles of consumption of each variable and adjusted by age and BMI. To more deeply explore the associations between fecal mutagenicity and dietary components, Spearman correlation analyses were conducted. A heatmap was generated using the RStudio software version 1.4.1103 package corrplot. GraphPad Prism 9 was used for graphical representations.

3. Results

General characteristics, anthropometric parameters, factors related to intestinal function, and anatomopathological diagnosis are presented in Table 1 for the three clinical endoscopic diagnosis groups: healthy controls, polyps, and CRC. In spite of no significant differences being found in the gender ratio between the polyp group and controls, CRC patients were exclusively males. Individuals in the polyp group had higher BMI and lower physical activity than controls.

Table 1. General description of the sample population according to diagnosis group.

	Control (*n* = 37)	**Polyps (*n* = 49)**	**CRC (*n* = 7)**
Male gender	17 (45.95) $_{a}$	30 (61.22) $_{a}$	7 (100.00) $_{b}$
Age (years)	60 (54–66) $_{a}$	63 (56–66) $_{a}$	63 (61–70) $_{a}$
Energy intake (kcal/d)	1974.87 (1492.99–2463.87) $_{a}$	1926.43 (1691.48–2675.80) $_{a}$	2070.43 (1915.76–2830.29) $_{a}$
BMI (kg/m^2)	25.70 (23.67–28.94) $_{a}$	27.56 (25.14–31.22) $_{b}$	24.68 (24.21–29.32) $_{a}$
CRC history (1st grade)	9 (24.32) $_{a}$	11 (22.45) $_{a}$	1 (14.29) $_{a}$
Physical activity (min/d)	75.00 (37.50–75.00) $_{a}$	50.00 (37.50–75.00) $_{a}$	90.00 (75.00–90.00) $_{b}$
Sleeping (hours/d)	7.00 (6.00–7.25) $_{a}$	7.00 (6.00–8.00) $_{a}$	7.00 (6.00–8.00) $_{a}$
Current smoker	6 (16.22) $_{a}$	13 (26.53) $_{a}$	1 (14.29) $_{a}$
Gastrointestinal functionality			
Deposition/week	8.50 (6.00–8.50) $_{a}$	7.00 (6.00–8.50) $_{a}$	8.50 (8.50–8.50) $_{a}$
Liquid feces	0 (0.00) $_{a}$	1 (2.04) $_{a}$	-
Soft feces	27 (72.97) $_{a}$	32 (65.31) $_{a}$	5 (71.43) $_{a}$
Hard feces	10 (27.03) $_{a}$	16 (32.65) $_{a}$	2 (28.57) $_{a}$
Pathological analysis (DxBx)			
HP	4 (10.81) $_{a}$	7 (14.29) $_{a}$	0 (0.00) $_{a}$
TA	0 (0.00) $_{a}$	22 (44.90) $_{b}$	-
TVA	0 (0.00) $_{a}$	5 (10.20) $_{b}$	-
SSA	0 (0.00) $_{a}$	1 (2.04) $_{a}$	-
HGD	0 (0.00) $_{a}$	6 (12.24) $_{b}$	-
AC	0 (0.00) $_{a}$	1 (2.04) $_{a}$	7 (100.00) $_{b}$
LSC	24 (64.86) $_{a}$	3 (6.12) $_{b}$	0 (0.00) $_{b}$
Not available	9 (24.32) $_{a}$	4 (8.16) $_{b}$	0 (0.00) $_{a}$

Values are presented as median (IQR = interquartile range = Q1–Q3) for continuous variables or number (%) for categorical ones. Values in the same row showing different subscripts present a statistically significant difference ($p \leq 0.05$) than control group. BMI, body mass index; CRC, colorectal cancer; DxBx, pathological analysis; HP, hyperplastic polyps; TA, tubular adenoma; TVA, tubulovillous adenoma; SSA, sessile serrated adenoma; HGD, high-grade dysplasia; AC, adenocarcinoma; LSC, less severe conditions.

Variations in the dietary intake and lifestyle factors related to CRC risk, according to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) [54], in polyp and CRC groups vs. control are presented in Figure 2. According to the results, the percentage of individuals in the polyp group consuming alcoholic beverages (>12 g/d) was higher than in the control (84% vs. 65%; p-value < 0.05). Differences were also found for the percentage of CRC patients consuming milk under 120 g/d when compared to the control group (0% vs. 43%). Although not significant, the CRC group showed the highest consumption of red and processed meats (>50 and >25 g/d, respectively).

On the other hand, the median intake of bioactive and potential carcinogenic compounds (xenobiotics and ethanol) in the total sample and polyp and CRC groups vs. control is presented in Table 2. The intake of ethanol and DiB(a)A was significantly increased in the polyps group compared to the control (8.13 vs. 1.88 and 0.07 vs. 0.04, respectively).

Table 2. Differences in the median intake of bioactive and carcinogenic compounds in the total sample and by diagnosis group.

Variables	Total Sample (n = 93)	Diagnosis Group		
		Control (n = 37)	Polyps (n = 49)	CRC (n = 7)
Bioactive				
Total fiber (g/d)	20.88 (14.77–25.15)	21.89 (14.77–26.87) $_{a}$	20.41 (15.22–23.75) $_{a}$	22.33 (13.80–29.55) $_{a}$
Insoluble fiber (g/d)	12.50 (8.66–15.08)	12.50 (8.66–16.57) $_{a}$	12.29 (9.25–14.64) $_{a}$	13.27 (8.04–17.70) $_{a}$
Soluble fiber (g/d)	2.40 (1.87–3.06)	2.62 (1.86–3.17) $_{a}$	2.32 (1.88–2.85) $_{a}$	2.77 (1.91–3.03) $_{a}$
Cellulose (g/d)	5.01 (3.62–6.39)	5.01 (3.50–6.40) $_{a}$	4.93 (3.64–6.24) $_{a}$	5.35 (2.97–8.02) $_{a}$
Insoluble hemicellulose (g/d)	3.88 (2.80–4.90)	4.02 (2.86–5.38) $_{a}$	3.63 (2.68–4.51) $_{a}$	4.01 (2.69–5.48) $_{a}$
Soluble hemicellulose (g/d)	1.65 (1.15–2.27)	1.77 (1.09–2.32) $_{a}$	1.57 (1.19–2.04) $_{a}$	1.90 (1.04–2.35) $_{a}$
Insoluble pectin (g/d)	1.34 (1.01–1.96)	1.53 (1.11–2.02) $_{a}$	1.29 (0.94–1.81) $_{a}$	1.58 (1.20–2.06) $_{a}$
Soluble pectin (g/d)	0.66 (0.51–0.89)	0.70 (0.55–0.92) $_{a}$	0.62 (0.45–0.88) $_{a}$	0.69 (0.58–1.29) $_{a}$
Klason lignin (g/d)	1.63 (1.22–2.26)	1.63 (1.30–2.26) $_{a}$	1.69 (1.22–2.11) $_{a}$	1.42 (1.13–2.44) $_{a}$
Total polyphenols (mg/d)	1482.46 (963.00–1951.48)	1509.00 (1074.04–1877.40) $_{a}$	1376.33 (904.37–1951.48) $_{a}$	1454.97 (1000.45–2051.10) $_{a}$
Flavonoids (mg/d)	128.09 (72.89–302.17)	136.93 (78.28–251.34) $_{a}$	122.87 (53.65–331.58) $_{a}$	174.05 (80.98–498.80) $_{a}$
Lignans (mg/d)	46.95 (26.29–74.22)	47.39 (28.61–85.26) $_{a}$	40.18 (22.28–60.34) $_{a}$	55.39 (30.43–92.01) $_{a}$
Other polyphenols (mg/d)	24.27 (15.19–42.26)	27.32 (16.37–45.15) $_{a}$	19.93 (14.86–35.08) $_{a}$	29.48 (19.32–60.48) $_{a}$
Phenolic acids (mg/d)	496.08 (211.63–836.53)	609.92 (222.81–958.37) $_{a}$	386.62 (188.52–781.00) $_{a}$	496.08 (262.91–1223.65) $_{a}$
Stilbenes (mg/d)	0.11 (0.04–0.76)	0.09 (0.04–0.36) $_{a}$	0.16 (0.03–1.87) $_{a}$	0.11 (0.04–2.10) $_{a}$
Carcinogens				
Ethanol (g/d)	2.18 (0.19–10.56)	1.88 (0.28–8.80) $_{a}$	8.13 (1.76–22.93) $_{b}$	6.02 (0.00–24.46) $_{a}$
Xenobiotics				
B(a)P (µg/d)	0.06 (0.04–0.08)	0.06 (0.05–0.08) $_{a}$	0.06 (0.04–0.08) $_{a}$	0.07 (0.03–0.08) $_{a}$
DiB(a)A (µg/d)	0.03 (0.01–0.10)	0.03 (0.00–0.04) $_{a}$	0.05 (0.01–0.15) $_{b}$	0.03 (0.00–0.32) $_{a}$
Total PAH (µg/d)	1.09 (0.66–1.44)	0.93 (0.58–1.44) $_{a}$	1.15 (0.75–1.43) $_{a}$	1.22 (1.07–1.46) $_{a}$
PhIP (ng/d)	82.56 (25.14–232.97)	77.78 (24.45–182.10) $_{a}$	82.64 (23.79–329.91) $_{a}$	83.12 (36.77–222.53) $_{a}$
DiMelQx (ng/d)	6.67 (3.29–14.72)	5.13 (3.00–13.83) $_{a}$	6.90 (3.47–16.83) $_{a}$	9.96 (4.53–18.22) $_{a}$
MelQx (ng/d)	23.50 (13.44–61.12)	22.15 (13.44–61.12) $_{a}$	23.24 (13.42–56.27) $_{a}$	25.27 (16.64–69.07) $_{a}$
MelQ (ng/d)	0.81 (0.00–1.68)	0.93 (0.34–1.82) $_{a}$	0.81 (0.00–1.30) $_{a}$	0.00 (0.00–2.16) $_{a}$
IQ (ng/d)	0.13 (0.00–0.27)	0.13 (0.00–0.25) $_{a}$	0.13 (0.00–0.27) $_{a}$	0.00 (0.00–0.17) $_{a}$
Total HAs (sum) (ng/d)	119.54 (53.34–315.45)	103.17 (46.23–269.12) $_{a}$	125.27 (53.95–381.99) $_{a}$	185.87 (83.32–245.86) $_{a}$
Nitrates (mg/d)	91.15 (55.92–140.60)	95.09 (65.41–140.60) $_{a}$	69.44 (55.74–113.17) $_{a}$	97.39 (54.94–186.96) $_{a}$
Nitrites (mg/d)	2.39 (1.52–4.17)	2.48 (1.74–4.34) $_{a}$	2.37 (1.31–4.17) $_{a}$	2.44 (1.73–2.62) $_{a}$
NDMA (µg/d)	0.16 (0.11–0.30)	0.16 (0.10–0.28) $_{a}$	0.16 (0.11–0.30) $_{a}$	0.17 (0.13–0.35) $_{a}$
NPIP (µg/d)	0.07 (0.04–0.11)	0.08 (0.05–0.11) $_{a}$	0.07 (0.03–0.11) $_{a}$	0.06 (0.04–0.08) $_{a}$
NPYR (µg/d)	0.11 (0.06–0.18)	0.12 (0.08–0.17) $_{a}$	0.10 (0.05–0.18) $_{a}$	0.09 (0.07–0.12) $_{a}$
Acrylamide (µg/d)	14.70 (8.66–24.20)	15.07 (8.66–25.11) $_{a}$	14.70 (8.06–21.42) $_{a}$	14.15 (13.29–36.22) $_{a}$

Values are presented as median (IQR = interquartile range = Q1–Q3). Values in the same row showing different subscripts display a statistically significant difference ($p \leq 0.05$) from the control group. AαC (amino-alpha-carboline) and Comb. (combined nitroso compounds) are removed from the analysis due to extremely low frequency of consumption. B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total PAHs, total polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; IQ, 2-amino-3-methylimidazo (4,5,f) quinoline; Total HA, total heterocyclic amines. NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

In order to evaluate the overall impact of GBD risk-related factors along with the intake of bioactive and carcinogenic compounds into the polyp risk, logistic regression analyses adjusted by age and BMI were conducted (Table 3). According to the results obtained, there was a higher risk of belonging to the polyps group for those subjects with an alcohol consumption greater than 48 g/d. The consumption of more than 60 g/d increased the risk of polyps by 3 (OR = 3.01; p-value = 0.020; data not shown). Regarding protective factors, the consumption of more than 50 g/d of whole grains led to an 83% decrease in the risk of being in the polyp group. On the other hand, xenobiotics such as Total PAH were associated with a three-fold increase in the risk of polyps.

Table 3. GBD risk-related factors, bioactive and carcinogen tertiles as predictors of polyp risk.

	N (%)	Mean ± SD	OR (95% CI)	*p*-Value
GBD factors				
BMI				
5 kg/m^2	93 (100)	27.24 ± 4.06	1.705 (0.975–2.980)	0.061
Alcoholic beverages (g/d)				
≤48.00	32 (37)	11.10 ± 14.11	–	–
>48.00	54 (63)	442.81 ± 559.93	2.539 (0.997–6.467)	0.051
Whole grains (g/d)				
≤50.00	78 (91)	5.47 ± 11.66	–	–
>50.00	8 (9)	130.69 ± 107.33	0.168 (0.029–0.966)	0.046 *
Bioactives				
Soluble pectin (g/d)				
≤0.57	32 (37)	0.43 ± 0.09	–	–
0.57–0.85	29 (34)	0.71 ± 0.08	0.357 (0.117–1.089)	0.070
≥0.85	25 (29)	1.33 ± 0.60	0.408 (0.125–1.327)	0.136
Flavonoids (mg/d)				
≤82.18	28 (33)	44.96 ± 22.29	–	–
82.18–251.34	30 (35)	152.20 ± 55.08	0.343 (0.112–1.052)	0.061
≥251.34	28 (33)	525.49 ± 323.63	1.099 (0.347–3.482)	0.872
Other polyphenols (mg/d)				
≤16.45	30 (35)	11.26 ± 4.56	–	–
16.45–32.15	28 (33)	23.91 ± 5.00	0.761 (0.249–2.324)	0.631
≥32.15	28 (33)	74.05 ± 52.77	0.358 (0.116–1.107)	0.074
Carinogens				
Ethanol (g/d)				
≤1.70	29 (34)	0.39 ± 0.56	–	–
1.70–11.62	28 (33)	5.33 ± 3.24	1.720 (0.575–5.148)	0.332
≥11.62	29 (34)	35.12 ± 25.93	3.542 (1.117–11.234)	0.032 *
DiB(a)A (µg/d)				
≤0.01	29 (34)	0.00 ± 0.00	–	–
0.01–0.07	28 (33)	0.04 ± 0.01	0.587 (0.191–1.803)	0.352
≥0.07	29 (34)	0.34 ± 0.33	3.100 (0.950–10.118)	0.061
Total PAH (µg/d)				
≤0.75	30 (35)	0.57 ± 0.14	–	–
0.75–1.29	27 (31)	1.07 ± 0.14	3.753 (1.154–12.204)	0.028 *
≥1.29	29 (34)	1.77 ± 0.37	1.530 (0.510–4.595)	0.448
Nitrates (mg/d)				
≤63.75	29 (34)	44.77 ± 13.95	–	–
63.75–106.65	28 (33)	85.25 ± 12.54	0.561 (0.182–1.729)	0.314
≥106.65	29 (34)	206.06 ± 102.67	0.371 (0.121–1.133)	0.082
Nitrites (mg/d)				
≤1.69	30 (35)	1.16 ± 0.38	–	–
1.69–3.34	26 (30)	2.45 ± 0.45	0.297 (0.094–0.944)	0.040 *
≥3.34	30 (35)	8.38 ± 13.07	0.515 (0.168–1.584)	0.247

The variables considered in this analysis were age, sex, risk of GBD-related factors (alcoholic beverages: 12, 24, 36, 48, 60, and 72 g/d; whole grains: 50, 100, and 150 g/d; milk: 60, 120, 180, and 240 g/d; red meat: 50, 100, 150, and 200 g/d; processed meat: 25, 50, 75, and 100 g/d; fiber: 10, 20, and 30 g/d; calcium: 300, 600, 900, and 1200 mg/d; physical activity: 2400, 3000, 3600, and 4200 METs/d; BMI: 5 kg/m^2) and tertiles of consumption of all bioactives and carcinogens. Only variables showing significant (*) p-value < 0.05 or proximal (p-value < 0.10) results in at least one category are shown. For each variable considered, the lowest tertile is considered as the reference group. Values are adjusted for BMI and age. BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; DiB(a)A, dibenzo (a) anthracene; GBD, Global Burden of Diseases, Injuries, and Risk Factors Study; MET; metabolic equivalent of task; OR, odds ratio.

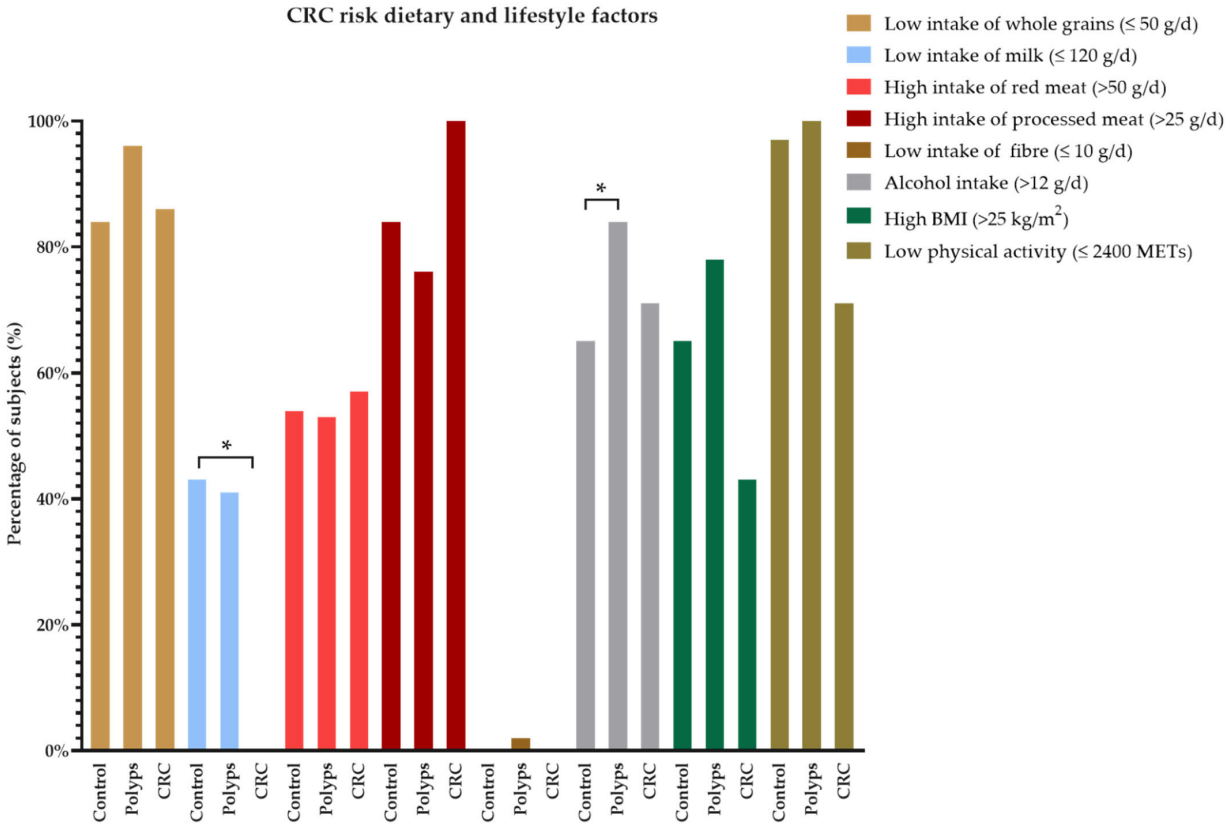

Figure 2. Analysis of the dietary and lifestyle significant risk factors for CRC according to GBD and clinical diagnosis group. The control group is used for comparison. BMI, body mass index; CRC, colorectal cancer; GBD, Global Burden of Diseases, Injuries, and Risk Factors Study; MET, metabolic equivalent of task. (*) p-value < 0.05.

Differences in fecal mutagenicity levels according to the clinical diagnosis group are presented in Figure 3a, with no significant differences found among groups. A significant increase in fecal mutagenicity was observed for those individuals from the polyp group presenting ACF (Figure 3b), and a trend to higher fecal mutagenicity, not reaching statistical significance, was also obtained for individuals presenting ACF in the other two groups (control and CRC) (Figure 3b). A higher occurrence of ACF with hyperplasia was observed in control and polyp groups compared to other types of ACF (with dysplasia or without cell alterations) (Figure 3c). In the CRC group, two patients presented ACF, either hyperplastic or dysplastic. The occurrence of ACF in intestinal mucosal samples was analyzed through histomorphological evaluation of colorectal mucosa sections. Two samples of normal (without cellular morphological alterations) (Figure 4A,B), hyperplastic (Figure 4C,D), and dysplastic (Figure 4E,F) ACF from different subjects are shown in Figure 4.

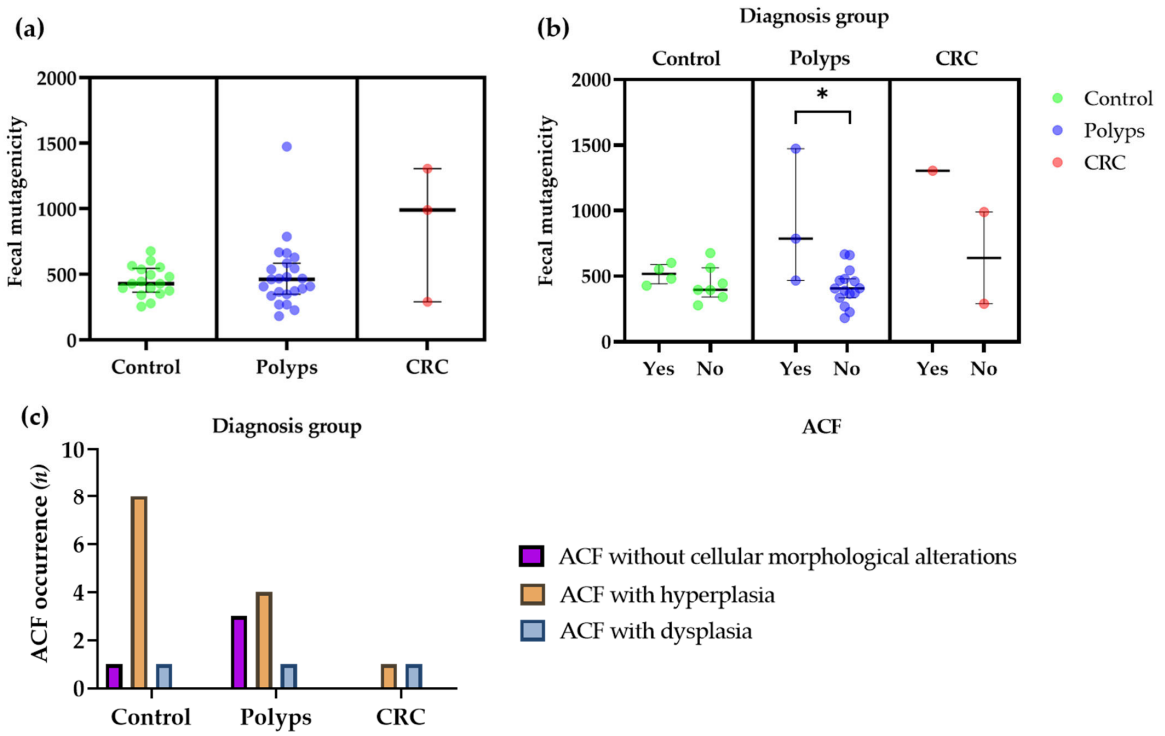

Figure 3. Dot plots comparing the mutagenicity of volunteers' fecal samples according to (**a**) the clinical endoscopic diagnosis group and (**b**) the ACF occurrence. The fecal mutagenicity for each volunteer is represented by colored circles. Wide horizontal lines indicate the median for each condition and error bars represent the interquartile range or the range for the CRC group. (*) Significant differences between groups ($p \leq 0.05$). (**c**) ACF occurrence and type (hyperplastic, dysplastic, or without cellular morphological alterations) in the sample for each clinical endoscopic diagnosis group (control, polyps, and CRC). Each bar represents the number of cases detected for each ACF category in each diagnosis group. ACF, aberrant crypt foci; CRC, colorectal cancer.

Correlations between the intake of the major food groups, dietary bioactive compounds (fibres and polyphenols), and dietary xenobiotics with fecal mutagenicity are presented in Figure 5. Intake of whole grains, soft drinks, potatoes, and tubers, as well as fiber and B(a)P intake, showed an inverse association with mutagenicity levels (Figure 5a–c). In contrast, in the polyp group, PhIP, MeIQ, MeIQx, DiMeIQx, and total HA were positively associated with the mutagenicity (Figure 5c). Mutagenicity mean levels were also higher for those individuals presenting higher BMI (>25 kg/m^2) (515.97 vs. 464.74, p-value < 0.05; data not shown). These differences were persistant for indivuals belonging to the control and polyp groups. We finally analyzed the main dietary sources of xenobiotics such as Total PAH and DiB(a)A, the carcinogens that, along with ethanol, displayed the highest risk for polyps in our study population (Figure 6a). Alcoholic beverages such as beer and wine were the main sources of DiB(a)A intake in the sample, whereas meats such as chicken, beef, and pork loin contributed to explaining almost 80% of the total HA intake (Figure 6b). Indeed, total HA values were greatly influenced by PhIP consumption from chicken breast (29% from total xenobiotics consumption), DiMeIQx from chicken breast (28%) and croquettes (26%), and MeIQx from pork loin (22%) and beef (18%). The processed meats of cured and cooked ham were the main dietary sources of nitrites (48% and 18%, respectively) and nitrosamines (Figure 6c), whereas nitrates were derived mainly from vegetables (Figure 6c) and acrylamide from cereal-derived products (potato, white bread, and cookies) (Figure 6d).

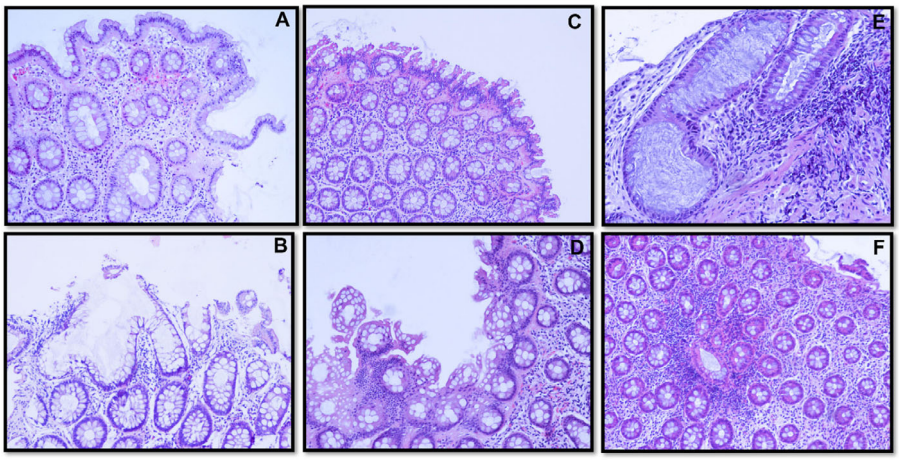

Figure 4. Photographs of histological sections showing ACF (H&E stain). Non-dysplasic distorted architecture (cells without morphological alterations) ((**A**,**B**), ×100). Hyperplastic with "serrated" lumen, "sawtooth" appearance ((**C**,**D**), ×100). Low-grade dysplasia with enlarged crypts, nuclear stratification, mucin depletion, elongated and hyperchromatic nuclei with loss of nuclear polarity and associated lymphocytic infiltrate ((**E**), ×200; (**F**), ×100). ACF, aberrant crypt foci; H&E, hematoxylin and eosin stain.

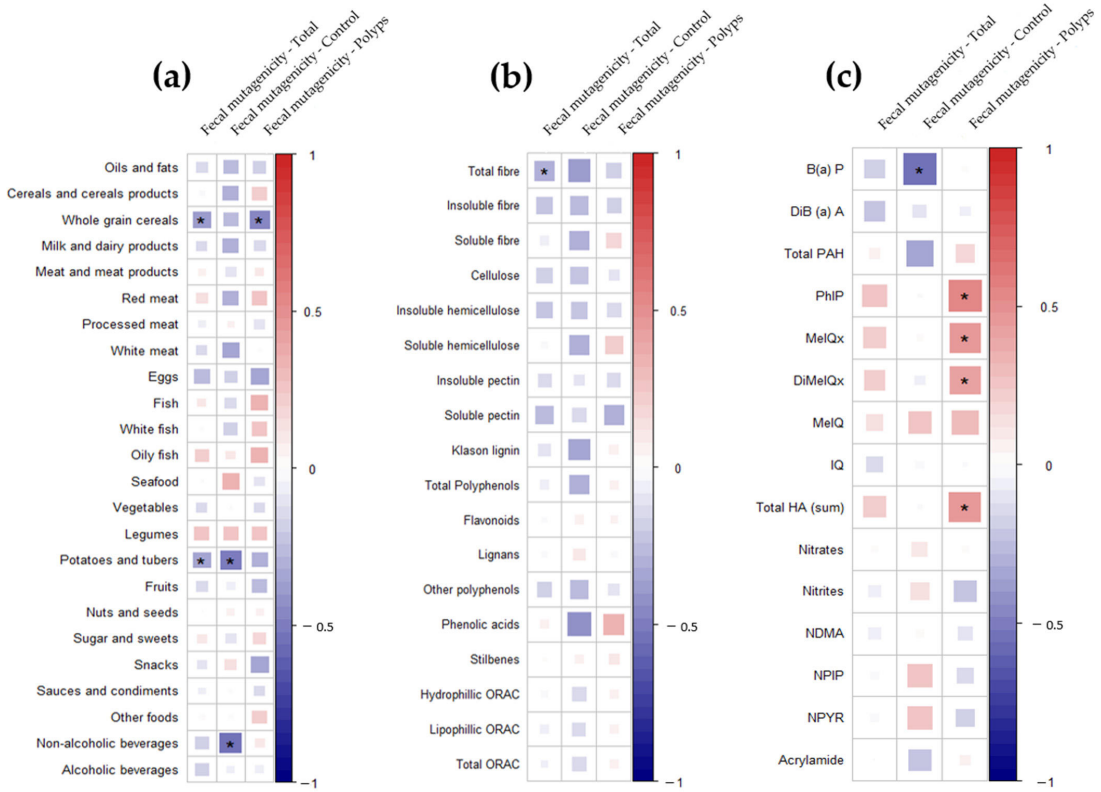

Figure 5. Heatmap defined by Spearman correlations between fecal mutagenicity and (**a**) food groups, (**b**) bioactive compounds, and (**c**) xenobiotics. Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association between fecal mutagenicity and the factors considered. (*) $p \leq 0.05$.

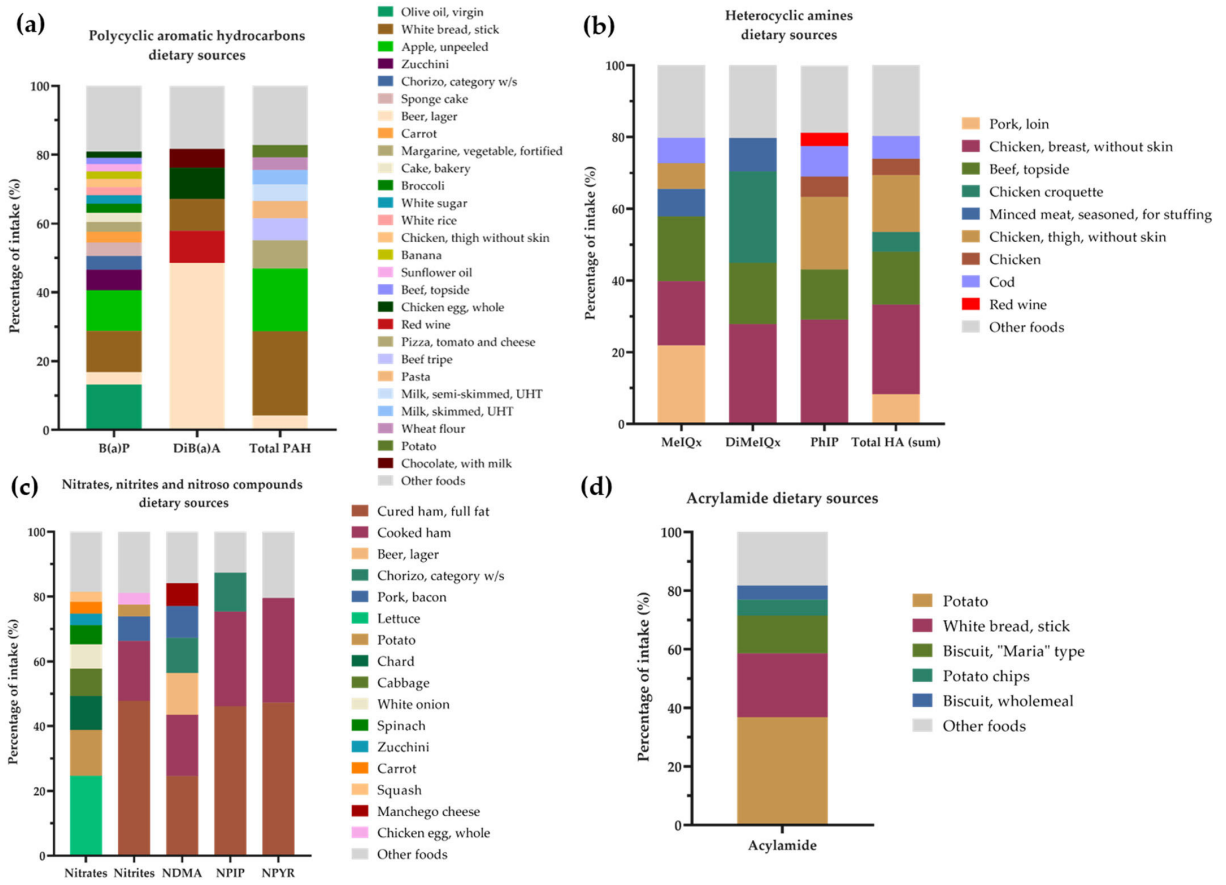

Figure 6. Major dietary sources of (**a**) polycyclic aromatic hydrocarbons; (**b**) heterocyclic amines; (**c**) nitrates, nitrites, and nitroso compounds; and (**d**) acrylamide in the sample. Only the most frequently consumed xenobiotics were considered. For each compound, food items accounting for at least 80% of total intake were included. B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; Total HAs, total heterocyclic amines; Total PAHs, total polycyclic aromatic hydrocarbons.

4. Discussion

There are controversial findings in the literature about the impact of environmental factors on the risk of CRC. It is generally assumed that diets with a high content of animal fat, alcohol, and processed meat and low in milk products, calcium, and whole grains increase the risk of CRCs, whereas those with a high presence of fruit and vegetables reduce it [55–58]. Thus, the detection in our study of a higher consumption of alcoholic beverages and lower consumption of whole grains on the risk of polyps support these hypotheses. Though cancer–food relationships appear to be very complex, several food components may be potentially genotoxic [59]. Given the scarce knowledge in the literature about the interactions between the different food-related genotoxic and protective factors, the observation of changes in mutagenicity levels associated with the dietary balance between the intake of substances with a possible pro-carcinogenic effect, such as dietary xenobiotics and the consumption of potentially beneficial components such as dietary fibers, may be the main novel contributions of this research work.

The intake of xenobiotics in our study sample was similar to that reported by other authors applying comparable methodology [28,60]. In this line, the intake of total HA in the

sample (especially DiMeIQx, MeIQx, and PhIP) fell into the range of the third quartile of intake of an EPIC study [61]. In addition, the intake of hydrocarbons such as B(a)P, DiB(a)A, and total PAHs showed similar values as previously described in Spain [28,40]. Regarding dietary sources, in general terms they were in consonance with other European countries. HA derived mainly from meats such as chicken, beef, and pork, whereas processed meats were the main dietary sources of nitrosamines and nitrites [28]. Carbohydrate-rich foods such as potato and bread were identified as the main dietary sources of acrylamide [28,62]. Finally, in our sample, PAH was the xenobiotics group with the highest variety of dietary sources, especially in the case of B(a)P, which mainly derived from vegetable foodstuffs such as olive oil, white bread, apple, and zucchini. Similar to other works, total PAHs were mainly provided by cereals and oils and fats [40,63], whereas the alcoholic beverage of lager beer was the major DiB(a)A foodstuff according to other studies [28].

Based on our results, we are not able to propose the existence of a dietary pattern associated with the presence of polyps or CRC. In line with several previous studies, a significant increase in the consumption of alcoholic drinks was observed in the polyp group respective to the controls [64,65]. In this regard, alcoholic beverage consumption in the sample, particularly beer, cider, and red wine, was around five times higher in the polyp group (data not shown). Elucidating the possible mechanisms behind this association, the eternal question is whether the observed effects are attributable to the ethanol content of these beverages or whether they could be protective based on their content in phenolic compounds. Although given the nature of the study we cannot establish causality, our data suggest that ethanol consumption above 12 g/d increases by 2.7 times the risk of being in the polyp group (p-value 0.05; data not shown). Consistent with this finding, the intake of stilbenes, mainly derived from red wine, was higher in the group of individuals with polyps, although the differences did not reach statistical significance. It may be of interest that although some meta-analyses have reported a 17% additional risk of CRC per 100 g/d of red meat consumption, we did not find this increased risk [66]. A possible explanation may be that even when the intake of meat and meat products in our study was within the range of the average consumption of America, Australia, New Zealand, Europe, and Russia [67], the proportion of red meat was considerably lower with respect to these other populations. In contrast to countries such as America, where red meat represents around 50% of the total meat consumed, in the present study the proportion was around 30% for both healthy individuals and those with polyps (data not shown). According to previous epidemiological evidence, in this work it was found that wholegrain cereals consumption greater than 50 g/d was associated with a reduction in the relative risk of belonging to the polyp group [68,69]. Several hypotheses have been put forward to explain the mechanisms that may connect whole grains to the risk of CRC. It is plausible that the high fiber content of these foods, together with the presence of several dietary compounds with antioxidant activity, may be some of the underlying causes for this connection. Certain components of cereal fiber exhibit some physiological effects, with a potential positive impact on CRC, including the ability to shorten intestinal transit time and the modulation of intestinal lipid and glucose absorption. In addition, some studies have highlighted the protective effect of high-fiber dietary patterns at the gut level by modulating the composition and activity of the intestinal microbiota [70,71].

The development of CRC is a long-term process in which a complex series of changes take place, culminating in the formation of a carcinoma. During this process, a healthy mucous membrane can suffer morphological transformations resulting in ACF formation and hyperplasia [72,73]. Due to the epidemiological and genetic association of ACF with initial intestinal lesions, they may be suggested as CRC biomarkers [26]. Although there is limited research on this subject, a greater ACF occurrence has been observed in people with BMI > 35 kg/m^2 and in those with a diet with a high intake of meat and low residues content [74,75]. In this complex scenario, we propose that an alteration in the balance between compounds with pro-carcinogenic activity and those with a protective role could favor the generation of a mutagenic environment at the colonic level, promoting the appearance of

ACF. To test this hypothesis, the intake of food groups, xenobiotics, and bioactive compounds was related to fecal mutagenicity levels through Spearman correlations. Total HA, DiMeIQx, MeIQx, and PhIP were positively associated with increased fecal mutagenicity, contrary to whole grains and other xenobiotics such as B(a)P (probably due to its mostly vegetable-derived dietary sources). Indeed, greater mean mutagenicity levels were found for higher BMI values. Furthermore, we also observed that the risk of developing a lesion identifying a precancerous stage was significantly higher in those subjects with polyps who had a higher dietary intake of pro-carcinogenic compounds such as ethanol. This is of great interest for the identification of possible therapeutic targets for the prevention of early colon cancer through diet.

Limitations of the study: Although relationships between diet and risk of polyp group membership were observed, it was not possible to establish causality in this association. Despite the high degree of detail carried out in the recording of dietary information, including the efforts made to quantify the degree of cooking of foods, this information is difficult to quantify accurately. It would be desirable in the future to use biological markers to validate the accuracy of the collection of dietary information.

5. Conclusions

This preliminary study points to ethanol and dibenzo(a)anthracene (DiB(a)A) intake as potential factors related to the increase in intestinal polyp risk and to the intake of whole-grain cereals above 50 g/d as a protecting factor. The intake of some of the heterocyclic amines under evaluation, such as 2-amino-1-methyl-6-phenylimidazo(4,5,b)pyridine (PhIP), was associated with a higher level of fecal mutagenicity in the polyp group. This study is of great interest for the generation of new hypotheses focused on designing nutritional strategies for early prevention of colon cancer.

Author Contributions: S.G. and C.G.d.l.R.-G. designed the study; A.S. and Y.D. recruited participants and carried out the colonoscopy and clinical diagnosis; S.R.-S. and A.Z. performed the nutritional assessment, statistical analyses, and experimental work; C.G.d.R. performed the pathological diagnosis and C.G. collected intestinal mucosal samples; S.G., S.R.-S., and A.Z. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in Spanish legislation on bioethics. Directive 95/46/EC of the European Parliament and the Council of 24 October 1995, on the protection of individuals regarding the processing of personal data and on the free movement of such data, was strictly followed.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Immunometabolic Profile Associated with Progressive Damage of the Intestinal Mucosa in Adults Screened for Colorectal Cancer: Association with Diet

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Abstract: Environmental factors such as diet and lifestyle have been shown to influence the development of some intestinal mucosal lesions that may be precursors of colorectal cancer (CRC). The presence of these alterations seems to be associated with misbalanced immunological parameter levels. However, it is still unclear as to which immunological parameters are altered in each phase of CRC development. In this work, we aimed to study the potential relationships of immunological and metabolic parameters with diet in a CRC-related lesion context. Dietary information was obtained using an annual semi-quantitative food-frequency questionnaire (FFQ) from 93 volunteers classified via colonoscopy examination according to the presence of intestinal polyps or adenocarcinoma. Cytokines, chemokines, and adipokines were determined from serum samples. We observed a reduction in adiponectin according to the damage to the mucosa, accompanied by an increase and decrease in C-X-C motif chemokine ligand 10 (CXCL10) and resistin, respectively, in CRC cases. The presence of aberrant crypt foci (ACF) in the polyp group was associated with higher tumor necrosis factor-alpha (TNF-α) concentrations. Vegetables were directly correlated with adiponectin and resistin levels, while the opposite occurred with red meat. A bioactive compound, soluble pectin, showed a negative association with TNF-α. Future dietary strategies could be developed to modulate specific immunological parameters in the context of CRC.

Keywords: immunological factors; adipokines; cytokines; chemokines; colorectal cancer; intestinal mucosa; diet; bioactive compounds; xenobiotics

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1. Introduction

According to GLOBOCAN data, about 1.9 million cases of colorectal cancer (CRC) occurred worldwide in 2020, this type of cancer ranking third in the global incidence of malignant tumors and second in mortality from cancer worldwide [1]. Despite improved survival and prognosis of CRC through primary prevention, the global number of new CRC diagnoses is expected to reach 3.2 million by 2040 [2]. The increasing incidence of this gastrointestinal pathology is mainly attributed to the exposure to different environmental risk factors resulting from lifestyle and dietary changes in Westernized countries [3]. In contrast to other cancers, in most cases, CRC precursor lesions develop slowly over years,

if not decades, following the initial transformation of a normal colorectal epithelium to an adenoma [4]. Aberrant crypt foci (ACF) commonly appear as the first microscopic precursor lesions of the intestinal mucosa, being frequently concurrent with the presence of adenomas and hyperplastic polyps [5]. The interaction between systemic inflammation and the local immune response has been demonstrated to be one of the key factors involved in the initiation, development, and progression of several tumor types, including colon cancer [6,7]. Although the factors triggering the development of these lesions are not known exactly, some authors suggest that certain dietary compounds may modulate the colonic environment by modifying inflammation- and oxidative-stress-related parameters and promoting mutagenicity at the local level [8]. Among them, some antioxidants provided by vegetable foodstuffs and beverages exhibit anti-inflammatory actions, while red meat, ethanol, and fats have shown the opposite effect [9]. Closely linked to diet, overweight and obesity were related with a 10% increase in the risk of CRC in a population-based cohort study of 5.24 million U.K. adults [10]. Although the exact mechanisms connecting cancer and obesity are unknown, some of the proposed ways include the regulation of obesity-associated adipokines such as leptin, adiponectin, and resistin; an increase in plasma insulin and glucose intolerance; and other proinflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [11–13]. On one hand, it has been postulated that an increase in fat in the adipocytes can lead to increased blood levels of resistin, leptin, adiponectin, IL-6, and TNF-α, thereby enhancing the rate of growth, progression, and metastasis of tumors [13]. Results from in vitro studies also demonstrate that leptin activates the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB/AKT)/mammalian target of rapamycin (mTOR) signaling pathway, enhancing the proliferation of human colon cancer cells [14]. On the other hand, several authors pointed to a protective role of circulating adiponectin against CRC based on its anti-inflammatory and anti-neoplastic properties. This suggests a possible usefulness of this adipokine as a marker of the different stages of tumor progression [15–20]. Lifestyle factors such as physical activity and smoking were associated with adiponectin levels in a Japanese sample [21]. While more scientific evidence is needed, these data open new avenues of interest in the study of diet-mediated colon cancer prevention. Based on this background, the aim of this study was to describe the immunological and metabolic profile associated with intestinal mucosal lesions and to assess the potential impact of diet on these biomarkers.

2. Results

A general description of the study sample is presented in Table 1. The percentage of female volunteers in the control group was slightly higher, while male gender predominated in the other groups. The numbers of those playing sports and engaging in walking activity were statistically higher in the CRC group compared to the control group. In order to test whether these differences were attributable to gender, the data were examined by gender within each group, with no significant differences observed. Changes in the concentration of immunological parameters such as circulating adipokines, cytokines, and chemokines according to the clinical classification of the volunteers are represented in Figure 1 (Supplementary Table S1). The results revealed low levels of adiponectin and resistin in patients with CRC, with adiponectin also decreased in the presence of polyps. Conversely, the interferon-gamma (IFN-γ)-inducible C-X-C motif chemokine ligand 10 (CXCL10) was increased in the CRC group compared with controls, but no significant differences between the study groups were detected in the levels of the other chemokines or cytokines analyzed. Nevertheless, several molecules displayed an elevated dispersion, mainly in the polyp group.

The presence or absence of ACF in the group of volunteers diagnosed with intestinal polyps was examined to analyze whether, in the presence of ACF, there were differences in immunological parameters (Table 2). We observed statistically significantly increased levels of TNF-α. Chemokine (C-C motif) ligand 2 (CCL-2) also showed an increased concentration in the presence of ACF, near statistical significance (p = 0.067). Moreover,

the TNF-α/Interleukin(IL)-10 ratio was also increased in patients with polyps and ACF ($p = 0.035$), thus supporting an inflammatory environment.

Table 1. General description of the sample according to clinical diagnosis groups.

Variable		Control (n = 37)	Polyps (n = 49)	CRC (n = 7)
Gender	Male	17 (45.9%)	30 (61.2%)	7 (100.0%)
	Female	20 (54.1%)	19 (38.8%)	0 (0.0%)
Age (years)		59 ± 9	61 ± 6	64 ± 5
BMI (kg/m^2)		26.17 ± 3.49	28.20 ± 4.41	26.25 ± 2.90
Energy intake (kcal/day)		2084.80 ± 759.64	2226.89 ± 848.08	2335.22 ± 540.20
Sport practice	Yes	9 (24.3%)	10 (20.4%)	4 (57.1%)
	No	28 (75.7%)	39 (79.6%)	3 (42.9%)
Sport activity (h/week)		1.04 ± 2.30	0.59 ± 1.43	4.29 ± 4.57 *
Walking activity (min/day)		57.77 ± 27.28	55.75 ± 29.52	83.57 ± 8.02 *
BMR (kcal/day)		1485.59 ± 230.60	1542.60 ± 253.91	1575.37 ± 126.01
Sleeping (h/day)		6.99 ± 0.92	6.82 ± 1.36	6.86 ± 0.90
Smoking habit	Current	6 (16.2%)	13 (26.5%)	1 (14.3%)
	Never	17 (45.9%)	21 (42.9%)	2 (28.6%)
	Former	14 (37.8%)	15 (30.6%)	4 (57.1%)

Values are shown as mean ± standard deviation (SD) for continuous variables or number (%) for categorical ones. (*) Statistically significant differences compared to control group ($p < 0.05$) found via t-test. BMI, body mass index; BMR, basal metabolic rate; CRC, colorectal cancer.

Table 2. Differences in immunological parameters according to aberrant crypt foci (ACF) presence in intestinal mucosa of volunteers diagnosed with intestinal polyps.

Parameter	ACF Presence	
	No	Yes
Antioxidant capacity (nM)	0.28 ± 0.05 (23)	0.33 ± 0.08 (6)
Total cholesterol (mg/dL)	230.20 ± 68.53 (22)	294.58 ± 71.28 (6)
Cytokines and chemokines		
IL-17 (pg/mL)	17.92 ± 28.90 (25)	16.43 ± 11.91 (6)
IL-6 (pg/mL)	26.85 ± 15.57 (25)	30.27 ± 20.32 (6)
IL-10 (pg/mL)	87.89 ± 21.61 (25)	89.35 ± 16.32 (6)
IL-12 (pg/mL)	43.06 ± 9.88 (25)	50.69 ± 18.99 (6)
IL-4 (pg/mL)	6.37 ± 5.86 (25)	12.55 ± 19.05 (6)
IFN-γ (pg/mL)	8.94 ± 7.56 (25)	7.13 ± 1.83 (6)
TGF-β (pg/mL)	109.35 ± 39.63 (25)	132.22 ± 42.51 (6)
IL-2 (pg/mL)	6.42 ± 2.55 (25)	15.54 ± 15.55 (6)
CXCL10 (pg/mL)	55.35 ± 31.44 (25)	51.93 ± 18.67 (6)
IL-1β (pg/mL)	37.22 ± 19.82 (25)	51.22 ± 44.89 (6)
TNF-α (pg/mL)	58.68 ± 27.47 (25)	131.24 ± 96.91 * (6)
CCL2 (pg/mL)	60.11 ± 47.50 (25)	171.69 ± 171.02 (6)
Adipokines		
Adiponectin (ng/mL)	163,146.13 ± 63,210.04 (24)	129,418.76 ± 57,864.85 (6)
Adipsin (ng/mL)	2153.22 ± 408.36 (24)	2284.82 ± 301.35 (6)
Leptin (pg/mL)	19.24 ± 7.70 (25)	21.27 ± 6.37 (6)
Resistin (ng/mL)	26.86 ± 20.71 (25)	23.72 ± 15.20 (6)

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. (*) Statistically significant differences between groups ($p < 0.05$). Differences were analyzed via non-parametric tests. ACF, aberrant crypt foci; IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumor necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

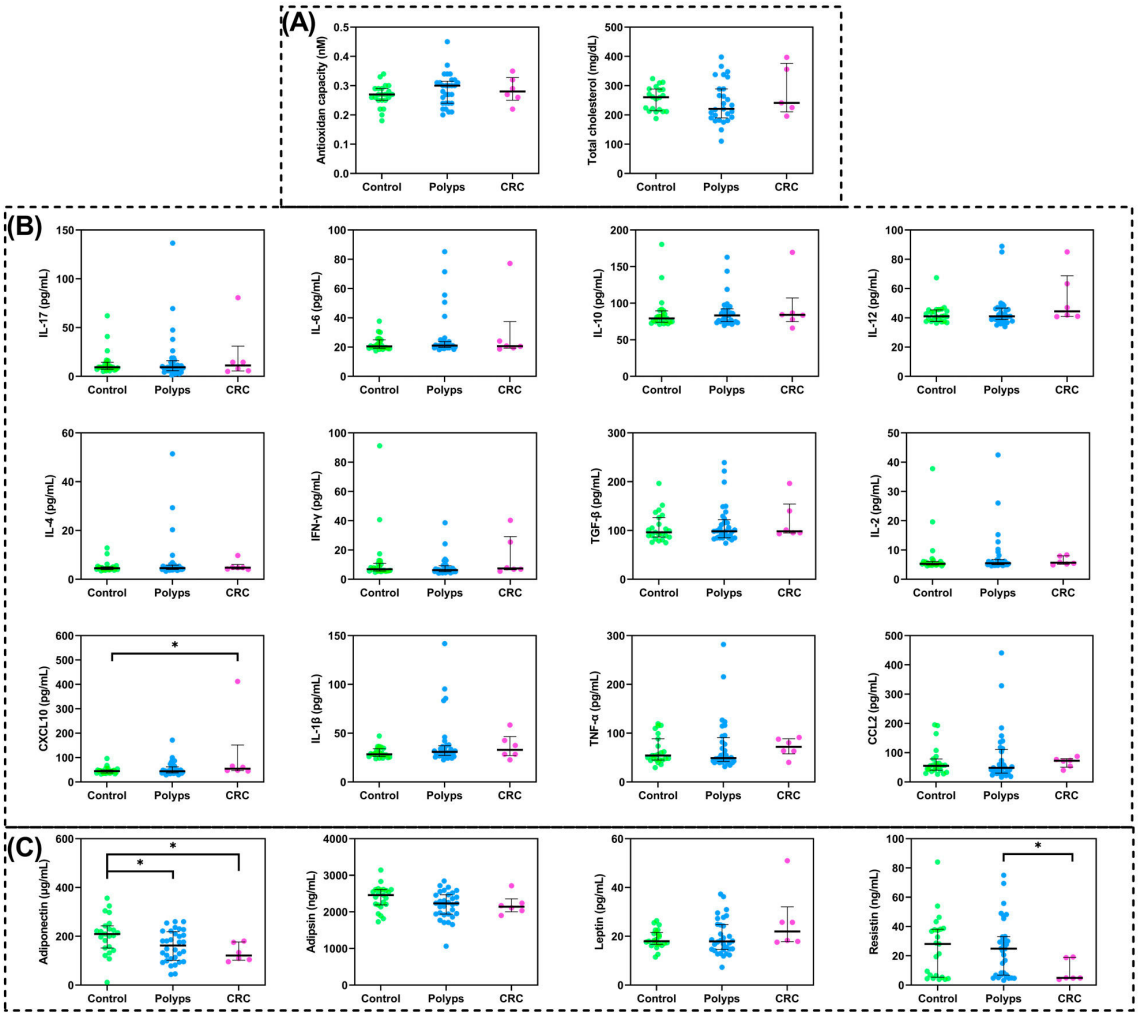

Figure 1. Differences in concentration of immunological parameters according to clinical diagnosis groups. (**A**) Antioxidant capacity and total cholesterol; (**B**) Cytokines and chemokines: IL-17, IL-6, IL-10, IL-12, IL-4, IFN-γ, TGF-β, IL-2, CXCL10, IL-1β, TNF-α, and CCL2; (**C**) Adipokines: Adiponectin, adipsin, leptin, and resistin. (*) Statistically significant differences between groups ($p < 0.05$) found via non-parametric tests. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumor necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

To explore the association in the sample between immunological parameters and the intake of food groups through diet, Spearman correlations were performed and heatmaps were plotted with the associations obtained (Figure 2). Among the immunological parameters, total cholesterol showed an inverse association with milk, dairy, and meat products, and more precisely with red meat. Red meat was also negatively correlated with resistin and IFN-γ. Both red and white meat were associated negatively with adiponectin, while vegetable intake showed the opposite tendency. Oils and fats were found to be directly correlated with the levels of leptin and inversely correlated with the levels of IFN-γ. Resistin showed a direct association with the consumption of fish, seafood, vegetables, and nuts and seeds, and an inverse association with the intake of cereal products, fruits, and red meat, as mentioned above. Alcoholic beverages were found to be positively correlated with IL-1β. The correlations of immunological parameters and food groups depending on the

diagnosis groups were also evaluated (Supplementary Figures S1–S3). It was found that in the control and polyp groups, the negative correlation between red meat and adiponectin was maintained. The consumption of food groups was analyzed according to clinical diagnosis groups, showing a statistically significant increase in the consumption of alcoholic beverages in the polyp group with respect to controls (Supplementary Table S2). The CRC group did not show significant differences, possibly due to the low number of samples with available dietary information. When the differences in the intake of food groups were evaluated according to ACF presence in the polyp group, the subgroup diagnosed with ACF showed increased intake of seafood (Supplementary Table S3).

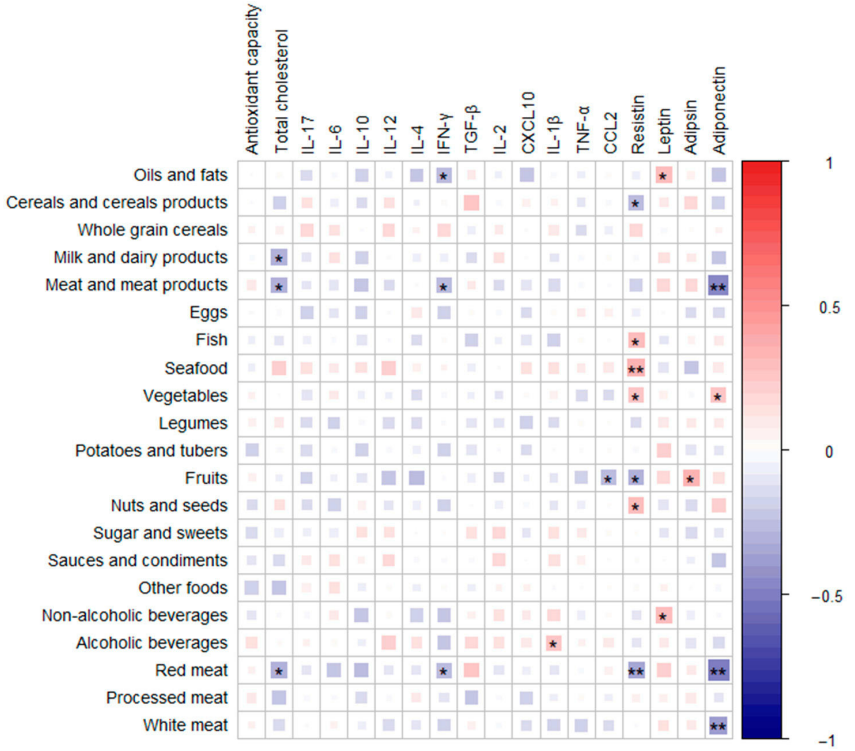

Figure 2. Heatmap defined by Spearman correlations between immunological parameters and food groups in the sample. Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association. (*) $p < 0.05$, (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumor necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

We looked deeper into the associations of immunological parameters and diet by analyzing the intake of their constituents, including macro- and micronutrients, bioactive compounds, and xenobiotic compounds (Figure 3). In concordance with the findings above, dietary proteins and lipids—more precisely, animal proteins and saturated, monounsaturated, and polyunsaturated fatty acids—were associated with decreased levels of adiponectin. The direction of this adiponectin association with dietary components was also maintained for vitamin B_2, while it was inverted with soluble pectin. When evaluating leptin, monounsaturated fatty acids from oils and fats and minerals such as iron, magnesium, selenium, and sodium showed positive correlations with this immunological parameter.

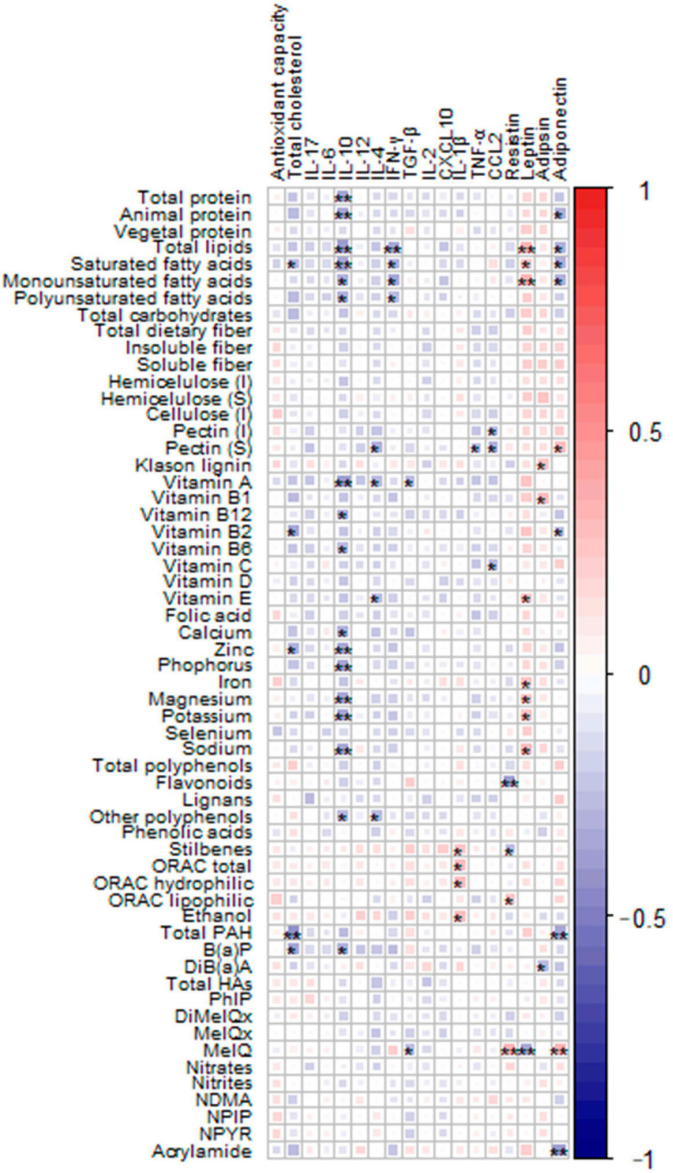

Figure 3. Heatmap defined by Spearman correlations between immunological parameters and dietary compounds in the sample. Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association. (*) $p < 0.05$, (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumor necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2; I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

Negative correlations were found with the xenobiotic 2-amino-3,4 dimethylimidazo (4,5,f) quinoline (MeIQ). Higher levels of resistin were associated with lower intakes of flavonoids and stilbenes. Soluble pectin displayed a similar negative association with both

TNF-α and chemokine CCL2, interestingly similar to what was found in the polyp group with ACF presence. Lipids were the unique compounds inversely associated with IFN-γ. We found that IL-10 was negatively correlated with total protein and animal protein; with lipids; with vitamins such as vitamin A, B12, or B6; with several minerals; and with the xenobiotic benzo(a)pyrene (B(a)P), a polycyclic aromatic hydrocarbon (PAH), the latter also being negatively correlated with cholesterol levels. The correlations of immunological parameters and dietary compounds depending on the diagnosis groups were also evaluated (Supplementary Figures S4–S6). The intake of all these dietary compounds was evaluated according to clinical diagnosis groups, revealing a statistically significant increase in the intake of ethanol and dibenzo (a) anthracene (DiB(a)A) in the group of volunteers diagnosed with intestinal polyps compared to the control group (Supplementary Table S4). Moreover, we found higher concentrations of vitamin A and calcium in the polyp group compared to the CRC group. The intake of dietary compounds was also evaluated according to ACF presence in the polyp group, not showing any significant difference (Supplementary Table S5).

3. Discussion

The present study provides further insight into the existing knowledge on the impact of diet on the immune system at different stages of CRC development. Our findings pointed to CXCL10, adiponectin, and resistin as potential markers involved in the progression of damage on the colorectal mucosa. The association of some dietary components with these immunological parameters may help in the design of dietary strategies aimed at the primary or secondary prevention of this pathology [22,23].

The decrease in serum adiponectin accompanied by increased levels of CXCL10 in subjects from the polyp and CRC groups as compared to volunteers from the control group is one of the main findings of the present study. Clinically, adiponectin is used as a biomarker in obesity-related diseases, and its level showed an inverse relationship with increasing central adiposity and type 2 diabetes [24,25]. There is strong evidence supporting the assertion that low levels of plasma adiponectin promote the suppression of AMP-activated protein kinase (AMPK) activity, activating the mTOR pathway, which is directly related to the proliferation of colorectal epithelial cells and colorectal carcinogenesis [26]. Our results are in accordance with previous ones showing a reduction in adiponectin in cancer models, although there is no consensus across the literature [16,17,27,28]. Previous studies showed that modulation of the adiponectin concentration is possible by adhering to Mediterranean dietary patterns [29]. In this regard, we found negative correlations of adiponectin with red and white meat. Interestingly, fiber supplementation seemed to be an effective therapeutic way of increasing adiponectin levels in blood [30]. Accordingly, a positive correlation of adiponectin with vegetables and soluble pectin was observed in this study. Moreover, in the studied sample, CXCL10 levels increased and resistin levels decreased in CRC compared to the control and polyp groups, respectively. CXCL10 is considered a pro-inflammatory chemokine associated with lipotoxicity, and its upregulation has been described in liver injury in murine models [31,32]. CXCL10 was proposed as mediator of CD4+ T cell trafficking, while in mice with high-fat diets, an increase in the expression of this cytokine occurs in muscle [33,34]. This chemokine can contribute to the heat in tumor areas, promoting its development [35]. Recently, CXCL10 has gained attention due to its role in the clinical outcome of patients infected with SARS-CoV-2 [36].

In our sample population, we did not find any significant correlation of CXCL10 with diet, although when the polyp group was evaluated, a negative correlation with oils and fats was found. Regarding resistin, several authors reported higher levels of this cytokine in different inflammation-related disorders such as atherosclerosis, chronic inflammatory bowel disease, chronic renal disease, systemic lupus erythematosus (SLE), or arthritis [37–39]. The expression of resistin seems to be increased in the presence of high concentrations of other pro-inflammatory cytokines [40]. However, in our sample, the levels of resistin were reduced in the CRC group, and we could not corroborate any

association with the pro-inflammatory cytokines IL17, IL6, IL12, or TNF-α. Recently, the European Prospective Investigation into Cancer and Nutrition (EPIC) study found no association between pre-diagnostic circulating resistin concentrations and the risk of CRC, suggesting the role of resistin as a possible marker of CRC instead of a risk factor [41]. In the present sample, several factors such as higher resistin levels in those of male gender (31.04 vs. 19.59 ng/mL), increased walking activity, or greater age in the CRC group may contribute to explaining why this group, composed exclusively of males, showed lower resistin levels compared to the control group, in accordance with a previous study enrolling Greek students that revealed increased resistin concentrations in healthy females compared to males [42]. Surprisingly, we found direct and inverse correlations of resistin with vegetables and red meat, respectively, probably related to the decreased consumption of vegetables in the CRC group, which also displayed lower resistin levels, or to the presence of any confounding factor we may be neglecting.

Moreover, when immunological parameters were evaluated in volunteers diagnosed with intestinal polyps and examined as a function of the presence or absence of ACF, TNF-α showed a statistically significant increase in subjects with ACF. Factors such as age, body mass index (BMI), and diet seem to affect the ACF number, which has been proposed to be utilized as a marker for the presence of lesions in the intestine [5,43]. TNF-α is a known inducer of NF-κB activity, secreted during the early phase of acute and chronic inflammatory diseases [44]. It is known that chronic inflammation leads to immune tolerance, thus promoting tumor formation and development [45]. Therefore, the modulation of inflammation in individuals with ACF could be a target for colon cancer prevention. In this sense, it has been reported that dietary components regulated the expression of TNF-α and other inflammatory cytokines and attenuated the progression of ACF in rat models [46]. In this regard, we previously described that the presence of ACF was accompanied by increased fecal mutagenicity in fecal samples of volunteers diagnosed with intestinal polyps [47]. The existence of several TNF inhibitors (e.g., infliximab, adalimumab, etc.) in the market and the current ongoing clinical trials highlight the clinical importance of this molecule in the treatment of diseases such as systemic rheumatic disease and inflammatory bowel disease [48]. TNF-α may also be downregulated by high adiponectin levels, thereby reducing TNF-α-associated inflammation, as suggested by several authors [49]. Furthermore, we found that the TNF-α/IL-10 ratio was increased in the presence of ACF. IL-10 shows opposite effects compared to TNF-α in inflammation, being mainly considered as an anti-inflammatory cytokine [50]. Given the opposite roles and the tightly regulated relationship between IL-10 and TNF-α levels, we consider the TNF-α/IL-10 ratio to be a better biomarker than any of the individual cytokines. This ratio has been found to be increased in infectious diseases and associated with poor health outcomes [50–52]. However, upregulation of IL-10 by either pharmacological (e.g., TNF-α blockage) or dietary interventions could be a promising target for individuals at risk. Many of the immunological parameters measured in this study are evaluated routinely by physicians. The existence of commercial panels makes the analysis of several parameters easy and affordable, and the information provided helps to associate abnormalities with a disease and its progression.

It is also important to note that in the population under study, the consumption of alcoholic beverages was higher in both the polyp group and the CRC group, with the intake of ethanol being statistically increased in the polyp group with respect to controls, as previously reported in individuals of this sample population [47]. Some authors found a clear association between high consumption of alcoholic beverages and mortality by CRC [53–56]. It is known that alcohol intake can contribute to initiating carcinogenic processes by destroying folate when the microbiota transforms ethanol into acetaldehyde in the colon. Subsequently, folate deficiency would cause chromosomal deterioration, inadequate incorporation of uracil into DNA, and other anomalies in DNA precursors, initiating tumor processes [57]. In the CRC group, the intake of red and processed meats and the consumption of alcoholic beverages was lower than that in the polyp group, which may be attributed to the possible existence of gastric symptoms accompanied by diet

self-moderation previous to the clinical diagnosis of the CRC. In terms of immunological and dietary parameters, in the present study, volunteers from the polyp group generally showed values between those for the control and CRC groups, as many variables followed a continuous trend across the different stages of development of the disease. Interventions focusing on the group of volunteers diagnosed with intestinal polyps could be a useful approach to avoid the development of CRC. Specifically, dietetic strategies to reduce the levels of certain inflammatory parameters could be of paramount interest. In this regard, higher adherence to Mediterranean dietary patterns seems to be the best protective factor against gastrointestinal pathologies, being capable of reducing inflammatory parameters related with CRC processes [58,59]. This diet, rich in fruits, legumes, vegetables, olive oil, herbs, and spices, involves high intake of fiber and of polyphenols such as apigenin, curcumin, epigallocatechin gallate, quercetin-rutine, and resveratrol [60,61]. Moreover, the intrinsic abundant intake of antioxidant molecules is associated with health-promoting properties [61]. The Mediterranean diet also contributes to modulating potentially toxic bile acids and the gut microbiota, frequently altered in CRC development [62]. Another potentially protective dietary habit could be related with a decrease in the consumption of dietary emulsifiers and additives associated with CRC risk [63]. Interestingly, nutraceuticals derived from Mediterranean diet products have gained attention and are being proposed as specific dietary components for personalized adjuvant therapies in the prevention of CRC, by either reducing inflammation or preserving a healthy microbiota in the intestine [58,64].

Among the limitations of the present study, results from the CRC group should be considered with caution due to the limited sample size. Surprisingly, the subjects in this group were more active than those in the polyp or control group. These findings may be influenced by the small sample size. It cannot be discarded that, as a consequence of the discomfort caused by the development of the disease, they may have attempted to improve their lifestyle. Moreover, the diverse molecular characteristics and pathways of the intestinal polyps should be considered when analyzing higher intragroup variability in those volunteers diagnosed with polyps.

4. Materials and Methods

4.1. Study Design and Volunteers

This transversal analysis is part of the broader project "Effect of Diet and exposure to XEnobiotics generated during food processing on the genotoxic/cytotoxic capacity of the intestinal Microbiota" (MIXED).

The recruitment of volunteers and collection of blood and tissue samples was carried out from October 2019 to December 2021 by the faculties of the Digestive Section of the Central University Hospital of Asturias (HUCA) and the Carmen and Severo Ochoa Hospital in Asturias, in the north of Spain. Volunteers were selected from among patients who came to the hospital for consultation due to clinical symptoms and among those included in the colon cancer screening program in our region. The exclusion criteria applied to subjects with age under 40 years or over 75 years, as well as those receiving omeprazole, antibiotics, corticoids, or non-steroidal anti-inflammatory drugs. Also, having specific cancer treatment at the time of the study or in the previous two months, previous surgery of the digestive system, autoimmunity, altered thyroid function, or history of diabetes or goiter were considered as exclusion criteria. Those individuals interested in participating were informed of the objectives of the study and signed an informed consent form. Prior to the preparation of the volunteers for colonoscopy, blood samples were taken by venipuncture at proposed intervals between 09.00 and 11.00 after an overnight fast. Blood samples were collected in separate tubes for serum and plasma, immediately kept on ice, and centrifuged at 1000× *g*, 15 °C. The resultant aliquots were immediately frozen at −80 °C until analyses. A biopsy of intestinal mucosa from volunteers diagnosed with intestinal polyps was extracted during colonoscopy for examination of ACF at the Pathology Department. In total, 93 participants were included in the study. Subjects were

classified into three clinical groups according to their colonoscopy results: control (n = 37), polyp presence (n = 49), and CRC (n = 7).

This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in Spanish legislation on bioethics. Directive 95/46/EC of the European Parliament and the Council of October 1995, on the protection of individuals regarding the processing of personal data, was strictly followed.

4.2. Nutritional Assessment

Dietary information was obtained from patients when they arrived for their colonoscopy results at a medical consultation by means of a personalized interview conducted by trained interviewers. Exceptionally, as a result of the pandemic and COVID-19 restrictions on visitors to hospitals in Spain, some of the surveys were conducted through online tools. For this purpose, a semi-quantitative food-frequency questionnaire (FFQ) was constructed with 155 items. In addition to food and culinary preparations, the specific type of food was recorded, as well as cooking methods and other related information, when necessary. Information relating to dietary assessment has been previously published [47]. The classification of the food into food groups was carried out according to the Centre for Higher Education in Nutrition and Dietetics (CESNID) criteria [65]. Food composition tables from CESNID and the United States Department of Agriculture (USDA) were used to transform food consumption into energy and macronutrient intake [65,66]. The phenolic content of the foods was extracted using Phenol Explorer 3.6, and the fiber content was taken from the tables by Marlett and Cheung [67,68]. Oxygen Radical Activity Capacity (ORAC) was calculated according to the article by Wu et al. [69]. During personalized interviews, sleeping hours and physical activity were recorded as the self-referred time per day for each in the last year, while information on smoking habits was obtained by asking about cigarette smoking throughout life. Basal metabolic rate (BMR) was calculated using the Harris and Benedict formula.

4.3. Anthropometrical Determinations

Height (m) and weight (kg) were taken via standardized protocols [70]. Body mass index was calculated using the formula weight/(height)^2. Subjects were classified into normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2), and obese (30.0 kg/m^2), based on the Spanish Society for the Study of Obesity (SEEDO) criteria [71].

4.4. Measurement of Immunological Parameters

To quantify the total antioxidant capacity of serum samples, a spectrophotometric method based on the ferric reducing antioxidant power (FRAP method) was performed using a commercial kit (Total antioxidant capacity (T-AOC) Assay Kit; ELK Biotechnology, Denver, CO, USA). Total cholesterol was analyzed enzymatically via the cholesterol oxidase e-p-aminophenazone (CHOD-PAP) method using a commercial kit (Total-Cholesterol Assay Kit; ELK Biotechnology, Denver, CO, USA). Circulating levels of cytokines, chemokines, and adipokines were quantified in serum samples using two pre-defined bead-based multiplex assays, following the protocol provided by the manufacturer and using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Serum samples were maintained at −80 °C until determinations. IL-4, IL-2, IP-10 (CXCL10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, and free active TFGβ1 levels were assessed using the *Human Essential Immune Response Panel* (LEGENDplex, BioLegend, San Diego, CA, USA). The detection limits were 0.97 pg/mL, 1.81 pg/mL, 1.28 pg/mL, 0.65 pg/mL, 0.88 pg/mL, 1.45 pg/mL, 2.02 pg/mL, 0.97 pg/mL, 0.77 pg/mL, 0.76 pg/mL, 0.77 pg/mL, and 3.10 pg/mL, respectively. For adiponectin, adipsin, leptin, and resistin determination,

the *Human Metabolic Panel 1* (LEGENDplex, BioLegend, San Diego, CA, USA) was used, with detection limits of 41.4 pg/mL, 5.4 pg/mL, 1.6 pg/mL, and 1.4 pg/mL, respectively.

4.5. Statistical Analyses

The results were analyzed using IBM SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, USA) and RStudio software version 1.4.3 (Posit Software, Boston, MA, USA). GraphPad Prism 9 (GraphPad Software, Boston, MA, USA) and RStudio software were used for graphical representations. Overall, categorical variables were summarized as the number and percentage, and continuous ones were summarized as the mean and standard deviation. Fisher tests and *t*-tests were performed for categorical and continuous variables regarding a general description of the sample, respectively (p-value < 0.05). The goodness of fit to a normal distribution was checked by means of the Kolmogorov–Smirnov test. As normality of the immunological and dietary variables was not achieved, Mann–Whitney U tests were performed to detect group differences (p-value < 0.05). Correlations were assessed using Spearman rank tests to explore the associations between immunological parameters and food groups and compounds. Heatmaps were generated using the "corrplot" R package version 0.92.

5. Conclusions

The variation in the levels of adipokines and chemokines with the progression of intestinal mucosal damage revealed the potential use of certain immune parameters as markers of the disease. Moreover, TNF-α concentrations were increased in the presence of ACF, supporting its pro-inflammatory role. The modulation of immunological parameters in patients by shifting to healthy dietary habits could be a non-invasive approach of great interest for future studies.

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MATERIAL SUPLEMENTARIO

Table S1. Differences in inflammation parameters according to clinical diagnosis groups.

Parameter	Control	Polyps	CRC
Antioxidant capacity (mM)	0.27 ± 0.04 (22)	0.29 ± 0.06 (29)	0.29 ± 0.05 (6)
Total cholesterol (mg/dL)	255.74 ± 40.79 (20)	243.43 ± 72.23 (27)	283.00 ± 87.98 (5)
Cytokines and chemokines			
IL-17 (pg/mL)	13.89 ± 13.14 (23)	17.13 ± 25.55 (33)	21.37 ± 29.32 (6)
IL-6 (pg/mL)	22.40 ± 4.90 (23)	27.08 ± 15.85 (33)	30.14 ± 23.11 (6)
IL-10 (pg/mL)	86.67 ± 24.59 (23)	87.12 ± 20.25 (33)	94.61 ± 37.39 (6)
IL-12 (pg/mL)	42.41 ± 6.43 (23)	44.32 ± 11.80 (33)	53.18 ± 17.82 (6)
IL-4 (pg/mL)	5.08 ± 2.20 (23)	7.42 ± 9.42 (33)	5.39 ± 2.16 (6)
IFN-γ (pg/mL)	12.91 ± 18.64 (23)	8.40 ± 6.67 (33)	15.45 ± 14.27 (6)
TGF-β (pg/mL)	105.84 ± 29.52 (23)	113.86 ± 40.17 (33)	120.23 ± 41.29 (6)
IL-2 (pg/mL)	7.61 ± 7.28 (23)	8.00 ± 7.47 (33)	6.28 ± 1.47 (6)
CXCL10 (pg/mL)	46.15 ± 13.84 (23)	54.20 ± 28.47 (33)	112.82 ± 146.79 * (6)
IL-1β (pg/mL)	30.03 ± 5.40 (23)	39.17 ± 25.47 (33)	36.29 ± 12.96 (6)
TNF-α (pg/mL)	64.63 ± 28.33 (23)	73.33 ± 53.34 (32)	71.06 ± 19.16 (6)
CCL2 (pg/mL)	69.74 ± 49.54 (23)	81.91 ± 90.37 (33)	67.09 ± 17.16 (6)
Adipokines			
Adiponectin (ng/mL)	204376.99 ± 76815.36 (23)	159034.48 ± 63473.78 * (32)	133043.19 ± 36979.07 * (6)
Adipsin (ng/mL)	2384.29 ± 337.36 (23)	2202.65 ± 400.04 (32)	2194.81 ± 278.95 (6)
Leptin (pg/mL)	18.91 ± 3.79 (23)	19.97 ± 7.30 (33)	26.02 ± 12.82 (6)
Resistin (ng/mL)	25.93 ± 20.78 (23)	25.01 ± 19.6 (33)	9.38 ± 7.46 $^{+}$ (6)

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. (*) Statistically significant differences compared to control group ($p < 0.05$). ($^{+}$) Statistically significant differences compared to polyps group ($p < 0.05$). Differences were analyzed by non-parametric tests. CRC, colorectal cancer; IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

Table S2. Food groups consumption according to clinical diagnosis groups.

Food group (g/day)	Control (*n* = 37)	Polyps (*n* = 49)	CRC (*n* = 3)
Oils and fats	22.81 ± 11.2	26.54 ± 21.52	25.41 ± 13.16
Cereals and cereals products	183.86 ± 124.31	183.69 ± 93.36	176.76 ± 95
Whole grain cereals	26.82 ± 67.53	9.78 ± 26.85	18.21 ± 44.97
Milk and dairy products	338.15 ± 227.7	372.08 ± 230.56	449.65 ± 122.45
Meat and meat products	170.06 ± 101.78	193.44 ± 132.03	172.72 ± 83.1
Eggs	40.54 ± 26.87	46.57 ± 25.18	38.16 ± 24.85
Fish	60.54 ± 43.62	55.53 ± 31.89	69.23 ± 51.88
Seafood	17.41 ± 18.23	17.39 ± 16.79	18.41 ± 17.91
Vegetables	262.07 ± 156.87	253.46 ± 164.55	245.91 ± 156.56
Legumes	35 ± 39.43	32.09 ± 28.59	35.03 ± 38.57
Potatoes and tubers	64.01 ± 54.73	67.2 ± 45.71	80.08 ± 60.78
Fruits	152.17 ± 117.17	137.87 ± 146.59	154.98 ± 41.64
Nuts and seeds	11.81 ± 13.51	9.56 ± 11.13	11.67 ± 12.58
Sugar and sweets	17.07 ± 20.82	15.97 ± 16.22	16.25 ± 14.06
Sauces and condiments	10.13 ± 7.44	11.59 ± 10.79	8.28 ± 2.34
Other foods	22.11 ± 46.74	21.17 ± 44.72	24.86 ± 42.7
Non-alcoholic beverages	1669.98 ± 493.18	1755.13 ± 807.48	1947.74 ± 584.16
Alcoholic beverages	159.2 ± 263.48	375.03 ± 593.13 *	206.18 ± 252.17
Red meat	62.33 ± 56.19	73.15 ± 60.66	68.71 ± 39.37
Processed meat	65.52 ± 50.87	71.19 ± 75.42	61.87 ± 55.3
White meat	45.76 ± 35.42	54.93 ± 37.68	44.68 ± 19.31

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. (*) Statistically significant differences compared to control group ($p < 0.05$). Differences were analyzed by non-parametric tests. CRC, colorectal cancer.

Table S3. Food groups consumption according to aberrant crypt foci (ACF) presence in intestinal mucosa of volunteers diagnosed with intestinal polyps.

Food group (g/day)	ACF presence	
	No (*n* = 34)	**Yes (*n* = 8)**
Oils and fats	23.68 ± 15.01	30.8 ± 18
Cereals and cereals products	186.65 ± 101.32	165.97 ± 69.58
Whole grain cereals	9.69 ± 26.94	6.23 ± 16.2
Milk and dairy products	392.49 ± 230.51	380.48 ± 271.04
Meat and meat products	171.12 ± 90.45	218.82 ± 133.09
Eggs	44.9 ± 22.64	46.34 ± 29.63
Fish	56.46 ± 34.19	45.6 ± 27.33
Seafood	14.33 ± 14	34.01 ± 23.94 *
Vegetables	261.26 ± 189.27	257.89 ± 104.16
Legumes	28.45 ± 21.13	34.04 ± 25.59
Potatoes and tubers	66.5 ± 45.71	78.17 ± 62.07
Fruits	154.68 ± 152.66	120.58 ± 164.17
Nuts and seeds	10.26 ± 11.2	9.56 ± 13.33
Sugar and sweets	15.94 ± 15.6	14.3 ± 10.88
Sauces and condiments	10.66 ± 7.66	13.33 ± 14.57
Other foods	21.47 ± 52	18.08 ± 24.4
Non-alcoholic beverages	1738.15 ± 859.69	1615.58 ± 527.71
Alcoholic beverages	375.15 ± 663.8	268.72 ± 219.55
Red meat	66.78 ± 57.87	79.14 ± 46.3
Processed meat	56.16 ± 44.53	85.39 ± 89.79
White meat	52.81 ± 36.12	63.43 ± 48.94

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. (*) Statistically significant differences between groups ($p < 0.05$). Differences were analyzed by non-parametric tests. ACF, aberrant crypt foci.

Table S4. Dietary compounds intake according to clinical diagnosis groups.

Compounds	Control (*n* = 37)	Polyps (*n* = 49)	CRC (*n* = 3)
Total protein (g/day)	104.73 ± 36.84	106.74 ± 35.65	115.9 ± 26.42
Animal protein (g/day)	74.63 ± 32.12	76.98 ± 31.61	84.6 ± 21.51
Vegetal protein (g/day)	28.24 ± 11.68	27.87 ± 10.95	28.01 ± 10.35
Total lipids (g/day)	92.1 ± 36.24	97.25 ± 44.31	102.09 ± 27.73
Saturated fatty acids (g/day)	29.95 ± 14.54	31.49 ± 15.48	36.46 ± 11.04
Monounsaturated fatty acids (g/day)	39.47 ± 15.35	41.78 ± 19.47	42.01 ± 14.5
Polyunsaturated fatty acids (g/day)	15.48 ± 6.66	16.2 ± 8.98	15.66 ± 5.35
Total carbohydrates (g/day)	196.01 ± 91.23	197.54 ± 67.96	219.28 ± 72.88
Total dietary fiber (g/day)	22.7 ± 9.36	20.77 ± 7.77	22.71 ± 7.74
Insoluble fiber (g/day)	13.82 ± 7.19	12.49 ± 5.19	13.57 ± 5.38
Soluble fiber (g/day)	2.79 ± 1.43	2.5 ± 1.04	2.69 ± 0.85
Hemicelulose (I) (g/day)	4.63 ± 3.19	3.9 ± 1.81	4.52 ± 2.18
Hemicelulose (S) (g/day)	1.87 ± 1.11	1.72 ± 0.78	1.79 ± 0.74
Cellulose (I) (g/day)	5.47 ± 2.68	5.12 ± 2.06	5.43 ± 2.15
Pectin (I) (g/day)	1.63 ± 0.78	1.48 ± 0.78	1.63 ± 0.46
Pectin (S) (g/day)	0.86 ± 0.57	0.72 ± 0.42	0.83 ± 0.33
Klason lignin (g/day)	1.95 ± 1.18	1.83 ± 0.9	1.84 ± 0.96
Vitamin A (µg R.E/day)	799.69 ± 398.26	777.12 ± 342.67	1088.42 ± 414.49 +
Vitamin B1 (mg/day)	1.82 ± 0.66	1.79 ± 0.88	1.79 ± 0.68
Vitamin B12 (µg/day)	7.98 ± 3.83	8.64 ± 3.38	10.35 ± 1.99
Vitamin B2 (mg/day)	1.85 ± 0.68	1.91 ± 0.64	2.06 ± 0.35
Vitamin B6 (mg/day)	2.43 ± 0.77	2.37 ± 0.76	2.43 ± 0.49
Vitamin C (mg/day)	153.1 ± 78.76	158.53 ± 98.62	156.93 ± 57.73
Vitamin D (µg/day)	4.82 ± 2.92	5.07 ± 3.6	5.81 ± 3.15
Vitamin E (mg E.T/day)	11.6 ± 5.06	12.14 ± 6.71	11.96 ± 3.79
Folic acid (µg/day)	338.89 ± 118.29	343.43 ± 135.02	354.06 ± 98.88
Calcium (mg/day)	997.3 ± 451.74	965.26 ± 347.48	1209.98 ± 214.49 +
Zinc (mg/day)	11.65 ± 4.61	11.47 ± 3.67	12.52 ± 2.94
Phophorus (mg/day)	1565.99 ± 560.66	1568.49 ± 455.02	1758.32 ± 240.64
Iron (mg/day)	15.19 ± 4.95	16.38 ± 6.17	15.87 ± 3.29
Magnesium (mg/day)	335.35 ± 109.79	333.13 ± 100.75	355.89 ± 55.55
Potassium (mg/day)	3598.93 ± 1019.16	3709.59 ± 1122.11	3891.69 ± 641.04
Selenium (µg/day)	4.1 ± 5.92	3.79 ± 8.08	8.72 ± 15.75
Sodium (mg/day)	3334.65 ± 1404.68	3393.44 ± 1685.51	3623.42 ± 1619.36
Total polyphenols (mg/day)	1578.42 ± 777.42	1615.74 ± 1015.52	1682.65 ± 885.68
Flavonoids (mg/day)	175.21 ± 120.2	286.85 ± 345.35	260.19 ± 219.78
Lignans (mg/day)	63.79 ± 46.72	58.12 ± 70.45	62.99 ± 31.56
Other polyphenols (mg/day)	39.27 ± 43.4	33.22 ± 38.42	40.85 ± 36.41
Phenolic acids (mg/day)	610.82 ± 425.24	499.23 ± 412	634.2 ± 464.98
Stilbenes (mg/day)	0.9 ± 2.43	3.03 ± 6.85	1.91 ± 3.76
ORAC total	5394.79 ± 3520.51	7063.27 ± 7951.55	5862.57 ± 3487.33
ORAC hydrophilic	5246.67 ± 3475.62	6945.9 ± 7928.78	5774.74 ± 3509.21
ORAC lipophilic	155.93 ± 122.86	124.53 ± 100.24	92.44 ± 50.44
Ethanol (g/day)	7.08 ± 10.48	18.72 ± 26.11 *	10.11 ± 11.15
Total PAH (µg/day)	1.08 ± 0.64	1.16 ± 0.49	1.24 ± 0.41
B(a)P (µg/day)	0.06 ± 0.03	0.07 ± 0.03	0.06 ± 0.03
DiB(a)A (µg/day)	0.09 ± 0.18	0.16 ± 0.28 *	0.15 ± 0.24
Total HAs (ng/day)	269.62 ± 441.18	559.94 ± 1568.05	175.42 ± 102.72
PhlP (ng/day)	199.39 ± 318.42	438.41 ± 1253.78	117.5 ± 93.43
DiMelQx (ng/day)	18.28 ± 40.36	36.6 ± 119.83	15.28 ± 16.58

Compounds	Control (n = 37)	Polyps (n = 49)	CRC (n = 3)
MelQx (ng/day)	50.35 ± 95.57	83.58 ± 209.91	41.65 ± 33.52
MelQ (ng/day)	1.43 ± 1.66	0.9 ± 1.06	0.83 ± 1.12
Nitrates (mg/day)	121.89 ± 103.85	105.13 ± 81.44	111.66 ± 72.04
Nitrites (mg/day)	3.3 ± 2.56	4.65 ± 10.77	3.3 ± 3.04
NDMA (μg/day)	0.2 ± 0.14	0.23 ± 0.22	0.22 ± 0.12
NPIP (μg/day)	0.1 ± 0.08	0.09 ± 0.1	0.09 ± 0.09
NPYR (μg/day)	0.16 ± 0.14	0.14 ± 0.16	0.14 ± 0.14
Acrylamide (μg/day)	16.23 ± 11.44	16.54 ± 10.94	22.81 ± 15.44

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. (*) Statistically significant differences compared to control group ($p < 0.05$). (+) Statistically significant differences compared to polyps group ($p < 0.05$). Differences were analyzed by non-parametric tests. CRC, colorectal cancer. I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

Table S5. Dietary compounds intake according to aberrant crypt foci (ACF) presence in intestinal mucosa of volunteers diagnosed with intestinal polyps.

Compounds	ACF presence	
	No (*n* = 34)	Yes (*n* = 8)
Total protein (g/day)	102.61 ± 30.5	109.61 ± 42.58
Animal protein (g/day)	72.7 ± 24.49	79.89 ± 40.28
Vegetal protein (g/day)	28.11 ± 11.71	27.5 ± 11.48
Total lipids (g/day)	91.97 ± 37.36	100.23 ± 43.22
Saturated fatty acids (g/day)	30.03 ± 13.05	29.67 ± 15.08
Monounsaturated fatty acids (g/day)	39.27 ± 16.94	47.07 ± 21.59
Polyunsaturated fatty acids (g/day)	15.2 ± 7.53	15.5 ± 6.61
Total carbohydrates (g/day)	198.33 ± 73.09	194.07 ± 50.95
Total dietary fiber (g/day)	21.5 ± 8.25	19.96 ± 8.49
Insoluble fiber (g/day)	12.93 ± 5.53	11.5 ± 5.17
Soluble fiber (g/day)	2.62 ± 1.12	2.23 ± 0.97
Hemicelulose (I) (g/day)	4.03 ± 1.94	3.48 ± 1.64
Hemicelulose (S) (g/day)	1.78 ± 0.82	1.53 ± 0.76
Cellulose (I) (g/day)	5.27 ± 2.1	4.86 ± 2.33
Pectin (I) (g/day)	1.57 ± 0.86	1.35 ± 0.63
Pectin (S) (g/day)	0.79 ± 0.46	0.6 ± 0.3
Klason lignin (g/day)	1.89 ± 0.97	1.69 ± 0.67
Vitamin A (μg R.E/day)	808.64 ± 358.28	629.3 ± 240.55
Vitamin B1 (mg/day)	1.68 ± 0.59	1.87 ± 0.93
Vitamin B12 (μg/day)	8.26 ± 3.26	9.74 ± 4.51
Vitamin B2 (mg/day)	1.91 ± 0.58	1.9 ± 0.84
Vitamin B6 (mg/day)	2.32 ± 0.65	2.43 ± 1.04
Vitamin C (mg/day)	164.71 ± 104.99	153.41 ± 110.94
Vitamin D (μg/day)	4.98 ± 3.55	3.61 ± 2.14
Vitamin E (mg E.T/day)	11.52 ± 5.2	11.25 ± 4.29
Folic acid (μg/day)	354.25 ± 144.24	334 ± 144.09
Calcium (mg/day)	1003.22 ± 350.52	889.54 ± 380.46
Zinc (mg/day)	11.2 ± 3.22	12.11 ± 5.12
Phophorus (mg/day)	1549.18 ± 426.31	1563.66 ± 528.49
Iron (mg/day)	15.98 ± 6.36	17.63 ± 6.42
Magnesium (mg/day)	334.62 ± 107.54	333.66 ± 94.47
Potassium (mg/day)	3704.77 ± 1183.21	3832.88 ± 1075.26
Selenium (μg/day)	3.97 ± 9.37	3.86 ± 4.77
Sodium (mg/day)	3119.58 ± 1363.89	3648.36 ± 2233.26
Total polyphenols (mg/day)	1646.85 ± 1114.45	1477.53 ± 878.06
Flavonoids (mg/day)	306.34 ± 361.08	258.43 ± 324.63
Lignans (mg/day)	63.32 ± 82.27	46.57 ± 38.3
Other polyphenols (mg/day)	37.1 ± 44.43	23.2 ± 12.21
Phenolic acids (mg/day)	489 ± 379.15	391.23 ± 379.95
Stilbenes (mg/day)	3.3 ± 7.75	2.76 ± 3.95
ORAC total	7560.47 ± 9068.59	6881.95 ± 4560.86
ORAC hydrophilic	7433.18 ± 9041.56	6757.92 ± 4559.61
ORAC lipophilic	134.19 ± 104.58	130.39 ± 115.42
Ethanol (g/day)	18.42 ± 28.41	14.81 ± 10.86
Total PAH (μg/day)	1.17 ± 0.53	1.12 ± 0.39
B(a)P (μg/day)	0.07 ± 0.02	0.05 ± 0.02
DiB(a)A (μg/day)	0.18 ± 0.31	0.07 ± 0.09
Total HAs (ng/day)	594.48 ± 1840.22	540.1 ± 864.75

Compounds	ACF presence	
	No (*n* = 34)	Yes (*n* = 8)
PhlP (ng/day)	456.46 ± 1461.76	467.7 ± 785.14
DiMelQx (ng/day)	43.92 ± 143.38	18.71 ± 23.75
MelQx (ng/day)	92.56 ± 248.2	52.66 ± 60.21
MelQ (ng/day)	0.98 ± 1.15	0.83 ± 0.89
Nitrates (mg/day)	109.05 ± 83.62	126.77 ± 98.05
Nitrites (mg/day)	4.7 ± 12.67	3.44 ± 3.07
NDMA (μg/day)	0.21 ± 0.17	0.23 ± 0.24
NPIP (μg/day)	0.07 ± 0.06	0.1 ± 0.1
NPYR (μg/day)	0.11 ± 0.08	0.16 ± 0.15
Acrylamide (μg/day)	16.85 ± 11.35	20.01 ± 12.33

Values are shown as mean ± standard deviation (SD) and number of volunteers with available information in parentheses. No statistical significant differences were found between groups. Differences were analyzed by non-parametric tests. ACF, aberrant crypt foci. I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

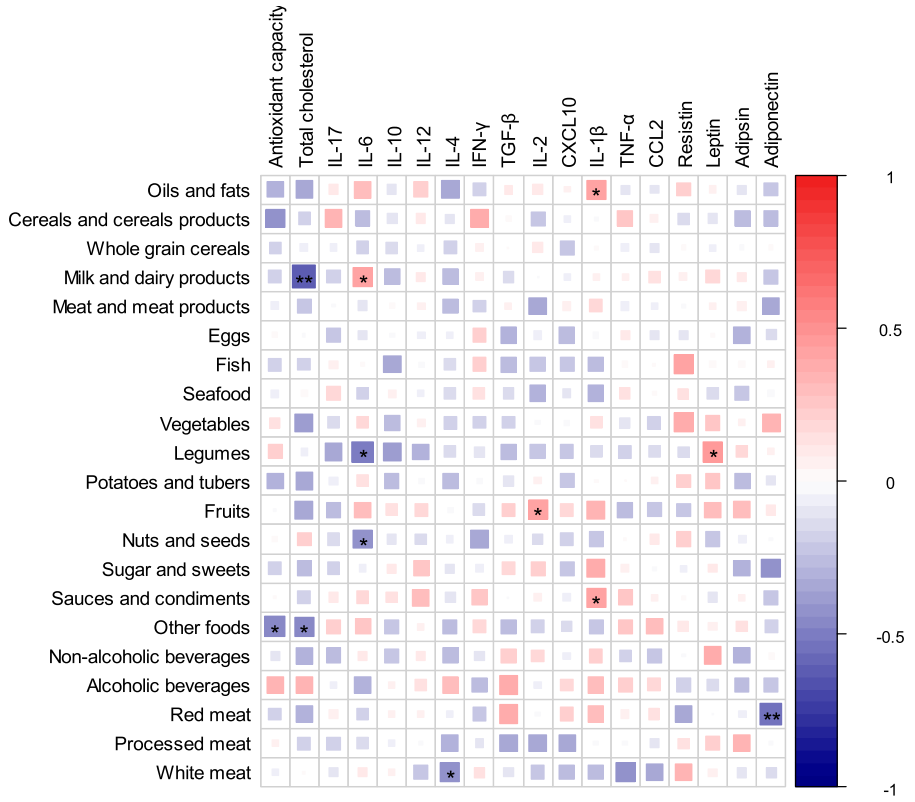

Figure S1. Heatmap defined by Spearman correlations between immunological parameters and food groups in the group of control volunteers. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

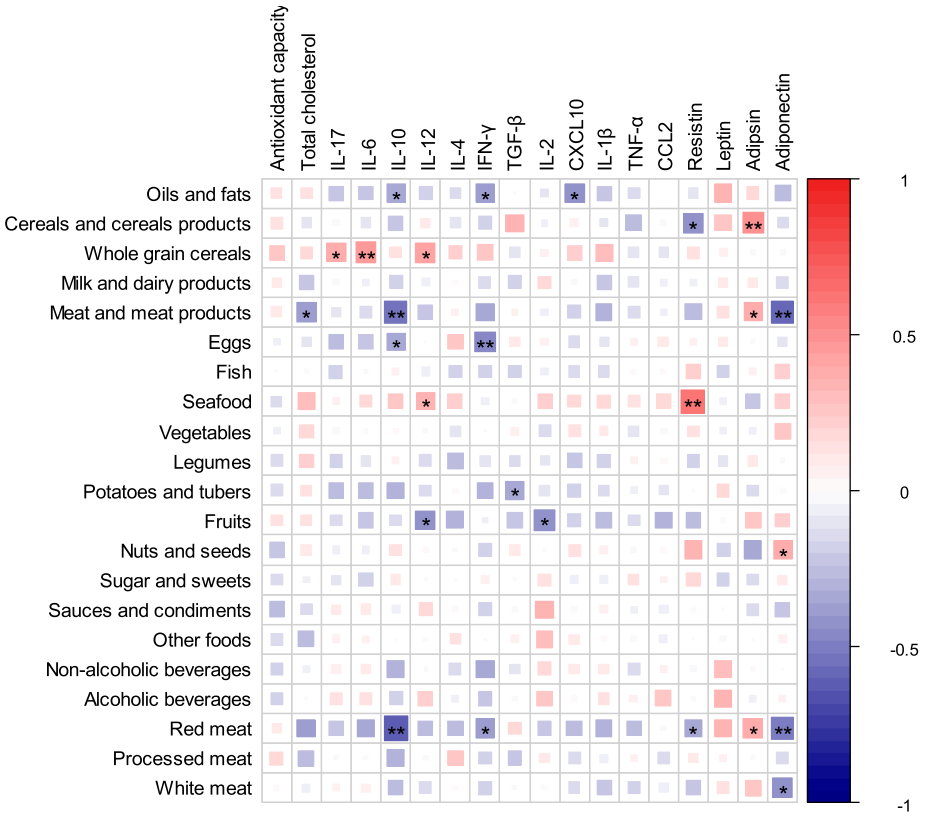

Figure S2. Heatmap defined by Spearman correlations between immunological parameters and food groups in the group of volunteers diagnosed with intestinal polyps. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

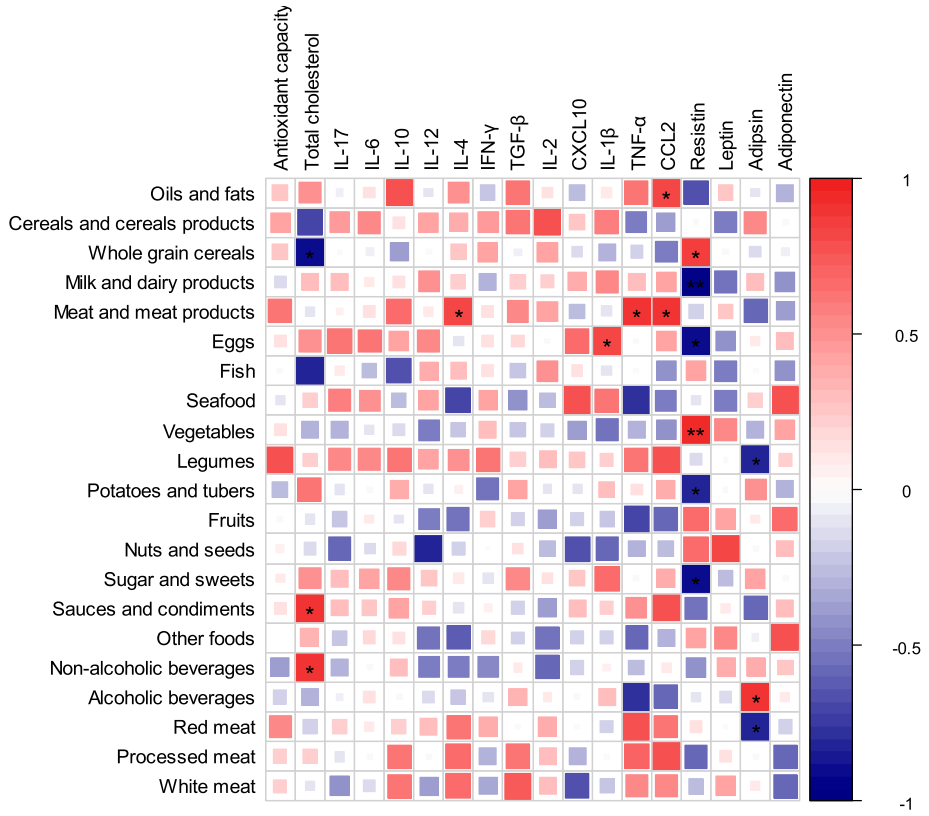

Figure S3. Heatmap defined by Spearman correlations between immunological parameters and food groups in the group of volunteers diagnosed with CRC. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2.

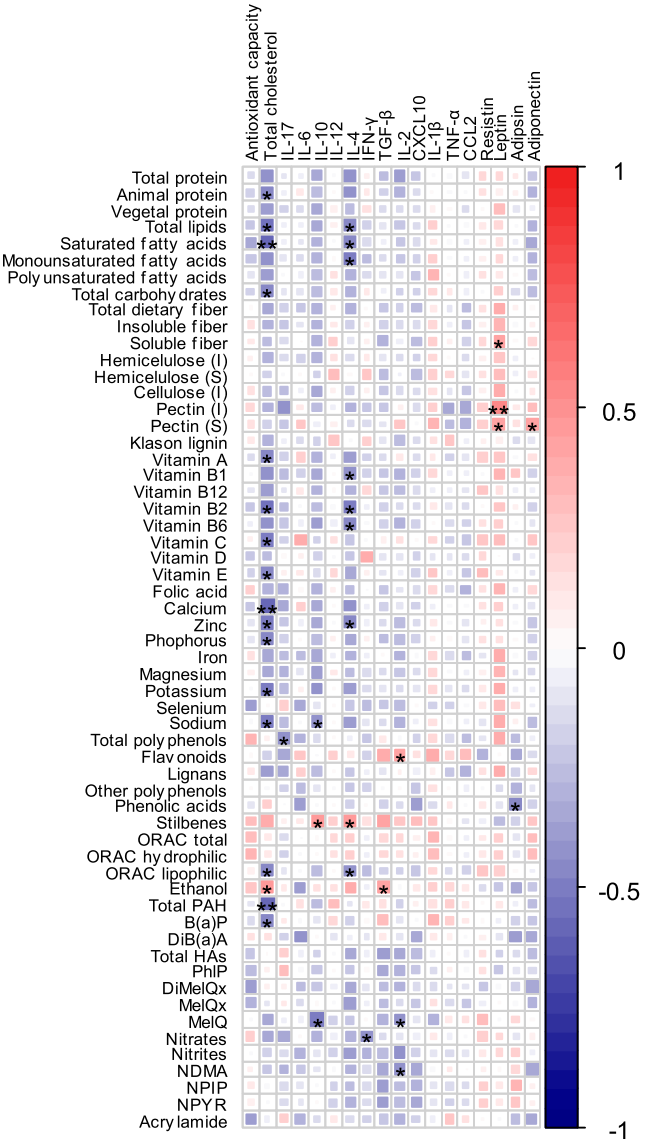

Figure S4. Heatmap defined by Spearman correlations between immunological parameters and dietary compounds in the group of control volunteers. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2; I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

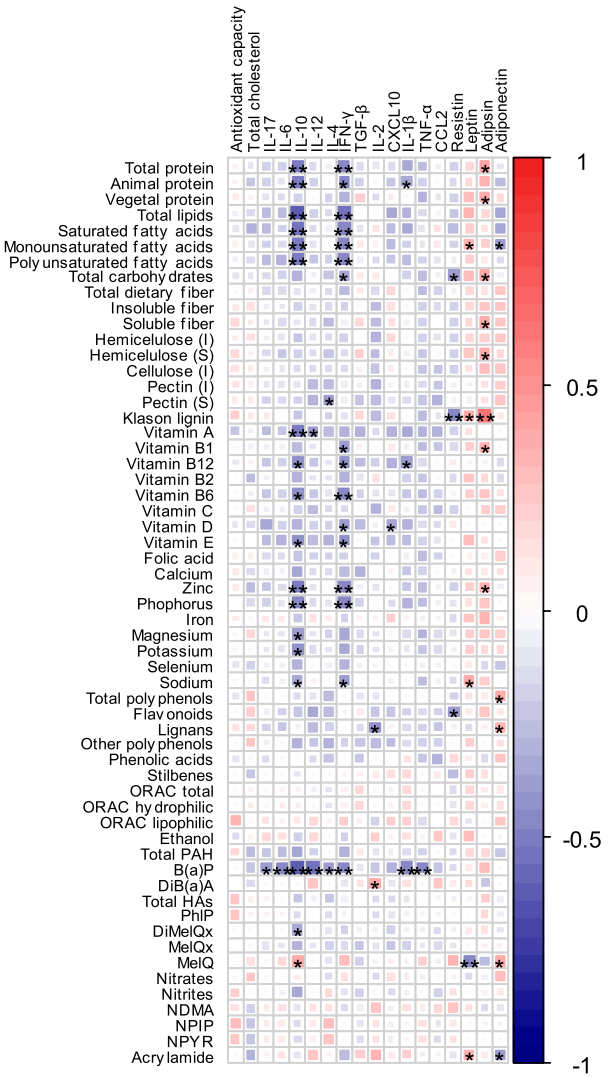

Figure S5. Heatmap defined by Spearman correlations between immunological parameters and dietary compounds in the group of volunteers diagnosed with intestinal polyps. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2; I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

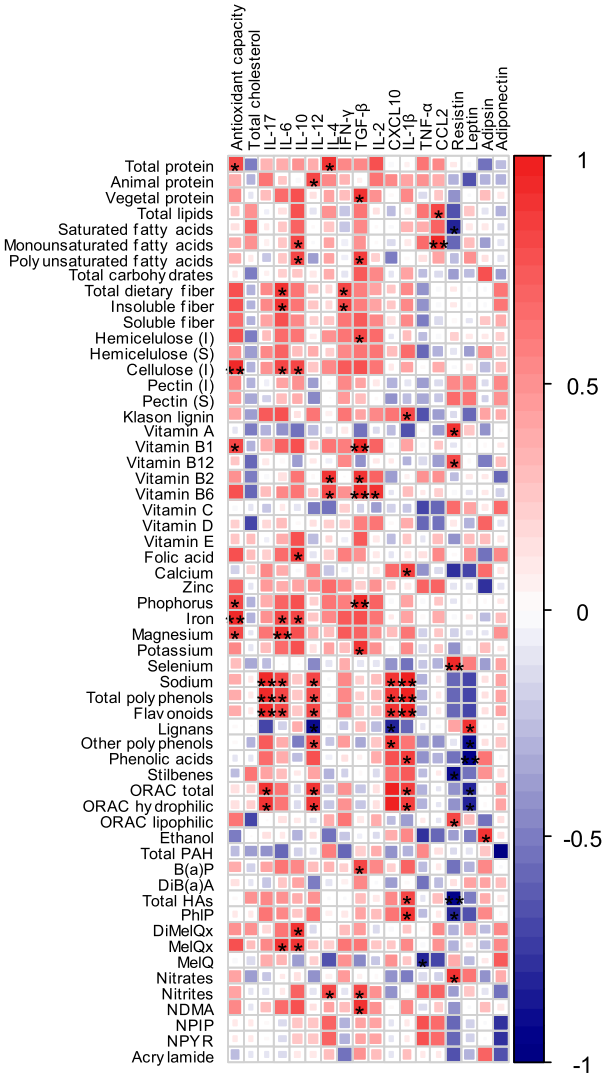

Figure S6. Heatmap defined by Spearman correlations between immunological parameters and dietary compounds in the group of volunteers diagnosed with CRC. Blue and red colors represent negative and positive associations, respectively. The colour intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.001$. IL, interleukin; IFN-γ, interferon-gamma; TGF-β, transforming growth factor-beta; CXCL10, C-X-C motif chemokine ligand 10; TNF-α, tumour necrosis factor-alpha; CCL2, chemokine (C-C motif) ligand 2; I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; Total PAHs, total polycyclic aromatic hydrocarbons; B(a)P, benzo (a) pyrene; DiB(a)A, dibenzo (a) anthracene; Total HA, total heterocyclic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; DiMeIQx, 2-amino-3,4,8 trimethylimidazo (4,5,f) quinoxaline; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,f) quinoline; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

Objetivo 2:

Caracterizar los perfiles microbianos y la actividad enzimática fecal en función de las diferentes vías biológicas de progresión de cáncer colorrectal

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La alteración de la composición y/o funcionalidad habitual de la microbiota intestinal se ha asociado a una mayor incidencia de diversas enfermedades intestinales, incluido el cáncer colorrectal. Estudios previos se han centrado en analizar la composición y alteraciones del perfil de la microbiota intestinal en casos de cáncer colorrectal mediante diversas técnicas analíticas. A pesar de ello, los cambios en la microbiota y el metaboloma que ocurren en las primeras etapas de daño de la mucosa intestinal, se conocen con bastante menos profundidad. Esta información es aún más escasa cuando se pretende diferenciar entre la vía biológica serrada y adenomatosa de la carcinogénesis y según el grado de displasia. Estos cambios tempranos resultan de gran importancia, pues podrían contribuir a determinar el riesgo de desarrollo futuro de la enfermedad. Para ahondar en el conocimiento en este campo, el trabajo desarrollado en el Objetivo 2 se incluyó en dos Artículos científicos que se corresponden, respectivamente, con los Objetivos 2.1. y 2.2. de esta Tesis Doctoral:

Artículo 3. Ruiz-Saavedra, S.; Arboleya, S.; Nogacka, A.M.; González del Rey, C.; Suárez, A.; Diaz, Y.; Gueimonde, M.; Salazar, N.; González, S.; de los Reyes-Gavilán, C.G. Commensal Fecal Microbiota Profiles Associated with Initial Stages of Intestinal Mucosa Damage: A pilot study. *Cancers* **2024**, *16*, 104. doi: 10.3390/cancers16010104

Artículo 4. Ruiz-Saavedra, S.; Salazar, N.; Suárez, A.; Diaz, Y.; del Rey, C. G.; González, S.; de los Reyes-Gavilán, C. G. Human Fecal Alpha-Glucosidase Activity and Its Relationship with Gut Microbiota Profiles and Early Stages of Intestinal Mucosa Damage. *Anaerobe* **2024**, *87*, 102853. doi: 10.1016/j.anaerobe.2024.102853

Los voluntarios del estudio se clasificaron en función del diagnóstico clínico y el análisis histopatológico de la mucosa intestinal, ya mencionados en el Objetivo 1. De esta manera se configuró un grupo de individuos control y otro con individuos que presentaban pólipos intestinales. En este último grupo se diferenció entre pacientes que presentaban alteraciones propias de la vía serrada, incluyéndose aquí los pólipos hiperplásicos, y pacientes con alteraciones pertenecientes a la vía adenomatosa, incluyéndose a los adenomas convencionales. Además, dentro de los adenomas convencionales se diferenció entre aquellos que presentaban displasia de bajo o de alto grado. A partir de muestras biológicas de heces se realizó un análisis de la microbiota fecal mediante la amplificación y secuenciación de la región V3-V4 del gen del ARN ribosómico 16S. Por otro lado, se determinó la concentración fecal de ácidos grasos de cadena corta mediante cromatografía de gases. Además, se examinaron las diferencias en las actividades enzimáticas intestinales entre grupos mediante un método semicuantitativo basado en el uso del sistema API ZYM ®.

Los resultados contenidos en el Artículo 3 mostraron que, pese a no observarse diferencias en la alfa y beta diversidad en las comparaciones intra e inter-grupos, se encontraron diferencias a nivel taxonómico de familia y de género entre los distintos grupos de estudio, afectando principalmente a los filos *Bacillota* y *Euryarchaeota*. Cabe destacar que *Ruminococcus_torques* (nivel taxonómico de género) se encontró aumentado en los grupos de pólipos hiperplásicos y adenomas convencionales con respecto al grupo control. A pesar de no obtenerse diferencias en las concentraciones de los ácidos grasos de cadena corta mayoritarios, los resultados parecen sugerir una reorganización de grupos y consorcios microbianos involucrados en procesos fermentativos en el intestino.

Los resultados que se presentan en el artículo 4 mostraron que la actividad de la enzima α-glucosidasa estaba significativamente aumentada en individuos diagnosticados con pólipos con respecto al grupo control y más específicamente, en sujetos que presentaban adenomas convencionales, y dentro de éstos, aquellos que presentaban displasia de alto grado. Una baja actividad α-glucosidasa en sobrenadantes fecales se asoció con una mayor abundancia relativa a nivel taxonómico de género de los grupos *Christensenellaceae_R-7* y *Oscillospiraceae_UCG-002*, lo que coincide con el aumento de la abundancia relativa de estos mismos grupos microbianos en la comparación del grupo control frente al grupo de adenomas convencionales.

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Commensal Fecal Microbiota Profiles Associated with Initial Stages of Intestinal Mucosa Damage: A Pilot Study

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Simple Summary: The high incidence and mortality of colorectal cancer (CRC) have influenced society to promote research in this field. Dysbiosis of the intestinal microbiota occurring in CRC has been extensively studied. However, microbial shifts occurring at the initial stages of mucosal alterations are less known. In this work, the fecal microbiota of volunteers diagnosed with intestinal polyps were compared with the microbial compositions of nonpathological control volunteers, thereby focusing on the nature of the hyperplastic polyps or conventional adenomas, as well as on the degree of dysplasia (low grade vs. high grade) in the last ones as indicators of colorectal cancer risk development. The findings provide insights into the microbiota changes occurring at the early stages of intestinal mucosal lesions. This work could set a starting point for further studies focusing on the influence of diet and lifestyle factors on the initial alterations of the intestinal mucosa and for the proposal of strategies for their prevention.

Abstract: Progressive intestinal mucosal damage occurs over years prior to colorectal cancer (CRC) development. The endoscopic screening of polyps and histopathological examination are used clinically to determine the risk and progression of mucosal lesions. We analyzed fecal microbiota compositions using 16S rRNA gene-based metataxonomic analyses and the levels of short-chain fatty acids (SCFAs) using gas chromatography in volunteers undergoing colonoscopy and histopathological analyses to determine the microbiota shifts occurring at the early stages of intestinal mucosa alterations. The results were compared between diagnosis groups (nonpathological controls and polyps), between samples from individuals with hyperplastic polyps or conventional adenomas, and between grades of dysplasia in conventional adenomas. Some microbial taxa from the *Bacillota* and *Euryarchaeota* phyla were the most affected when comparing the diagnosis and histopathological groups. Deeper microbiota alterations were found in the conventional adenomas than in the hyperplastic polyps. The *Ruminococcus torques* group was enriched in both the hyperplastic polyps and conventional adenomas, whereas the family *Eggerthellaceae* was enriched only in the hyperplastic polyps. The abundance of *Prevotellaceae*, *Oscillospiraceae*, *Methanobacteriaceae*, *Streptococcaceae*, *Christensenellaceae*, *Erysipelotrichaceae*, and *Clostridiaceae* shifted in conventional adenomas depending on the grade of dysplasia, without affecting the major SCFAs. Our results suggest a reorganization of microbial consortia involved in gut fermentative processes.

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1. Introduction

The progression of colorectal cancer (CRC) is a gradual process in which the intestinal mucosa damage generally occurs over years [1,2] and seems to be strongly linked to modifiable external factors, such as lifestyle and diet [3–6]. The detection of endoscopic polyp lesions, accompanied by the further histological examination of resected polyps or mucosa samples during colonoscopy, is used in routine clinical practice to determine the degree of mucosal damage and the risk of CRC-related lesions [7,8]. Most CRC cases (>80%) arise from the adenomatous pathway of genetic alterations related to phenotypic changes in the adenoma–carcinoma sequence. Conventional colorectal adenomas frequently carry dysplastic cells with low-grade dysplasia (LGD), which generally occurs in tubular adenomas (TAs), tubulovillous adenomas (TVAs), or with high-grade dysplasia (HGD). The alternative pathway is less frequent (<20%), originates from *BRAF* and *KRAS* gene mutations, and is linked to the progression of serrated lesions to carcinoma. The serrated lesions include hyperplastic polyps (HPs), as well as traditional serrated adenoma and sessile serrated lesions (SSLs) that normally contain cells with dysplasia [9]. Hyperplastic polyps generally present low risk of evolving to neoplasia, whereas LGD and HGD present a progressively augmented risk of adenocarcinoma development [7,10,11].

Changes occurring over time on the intestinal microbiota, which are partly driven by their interaction with dietary components, can influence host health [12–14]. Dietary components, such as complex carbohydrates (fibers) and, to a lesser extent, peptides and fats, reach the colon undigested and can be used as fermentable substrates by the colonic microbiota. The major microbial products of these fermentations are short-chain fatty acids (SCFAs), which mainly include acetate, propionate, and butyrate, that promote differential effects in host health [12,13]. In contrast, the ammonium, phenolic, and indolic compounds produced by the microbial colonic fermentation of aromatic amino acids, as well as hydrogen sulfide, have mutagenic, genotoxic, and cytotoxic potential regarding the intestinal mucosa, which could alter the microbiota [14]. Some intestinal microorganisms can produce and excrete toxic compounds as toxins and some protease-like factors, such as the colibactin produced by *Escherichia coli*, the cytotoxin A produced by *Helicobacter pylori*, an enterotoxin produced by *Bacteroides fragilis*, the adhesin A produced by *Fusobacterium nucleatum*, or the cysteine protease-like factor produced by *Shigella flexneri*, among others [15]. All these factors modify the intestinal environment and may contribute to the onset and progression of gut mucosal damage, as well as the further establishment of preneoplastic lesions.

Dysbiosis occurs in CRC and in preneoplastic stages. The "driver-passenger" theory tries to explain how the gut bacteria can induce carcinogenesis through progressive damage of the DNA in the gut epithelium ("drivers"), thereby modifying the gut environment to promote the proliferation of "passenger" bacteria [16]. Most studies have focused on the microbiota of CRC, whereas less information is available regarding shifts in the microbiota profiles in the initial intestinal mucosal lesions that develop prior to CRC [17,18]. This pilot study sheds light on the changes to the fecal microbiota associated with early intestinal mucosal lesions by considering the morphological alterations of intestinal polyps (hyperplastic and conventional adenomas) and the grade of dysplasia in the adenomatous pathway of carcinogenesis.

2. Materials and Methods

2.1. Study Design and Volunteers

Volunteers were randomly recruited by trained physicians from among those subjects who reached out to the Digestive Section at the Central University Hospital of Asturias (HUCA) or to the Carmen and Severo Ochoa Hospital in Asturias (North of Spain) for

consultation about clinical symptoms or as part of the CRC screening program in the region between October 2019 and December 2021. To enroll in the study, volunteers aged 40–79 years were first informed about the objectives and procedures and signed an informed consent to participate. Anamnesis was performed, and fecal samples were collected at the time of recruitment. Biopsies of the intestinal mucosa and polyps were resected during colonoscopy and examined at the Department of Anatomical Pathology of HUCA, as described elsewhere [19]. Patients reporting specific cancer treatment at the time of the study, treatment with medical drugs in the previous two months, immune-related diseases, or previous surgery of the digestive system were excluded. Individuals were classified according to the colonoscopy and histopathological examination. The group of "controls" included individuals with normal colonoscopy results and confirmation of the absence of mucosal lesions according to histopathological analysis, whereas the group of "polyps" included those individuals with alterations detected through colonoscopy results and confirmed by histopathological analyses. This group included individuals with either hyperplastic polyps or conventional adenomas as per morphological features detected by histopathology; conventional adenomas were subclassified depending on the grade of dysplasia. This project was evaluated and approved by the Regional Ethics Committee of the Clinical Research of Asturias (Ref. 163/19) and the Committee on Bioethics of CSIC (Ref. 174/2020). This study followed the fundamental principles of the Declaration of Helsinki, through the Council of Europe Convention on Human Rights and Biomedicine, and Spanish legislation on bioethics. Personal data protection was treated according to Directive 95/46/EC of the European Parliament and the Council of October 1995 on the Protection of Individuals.

2.2. Fecal Sample Processing

Instructions and material necessary for collecting fecal samples were provided to the volunteers. Briefly, patients deposited fresh fecal samples in sterile plastic containers and transported them to the hospitals participating in the study within a period not exceeding two hours after deposition. The samples were then frozen and transported to the laboratory. Frozen samples (4 g) were weighed, diluted 1/10, and homogenized with sterile phosphate-buffered saline (PBS) in a LabBlender 400 Stomacher (Seward Medical, London, UK) for 3 min at maximum speed. After 15 min of centrifugation at 4 °C and 14,000 rpm, the supernatants and pellets were separated and kept frozen at −20 °C until use.

2.3. DNA Extraction and Microbiota Metataxonomic Analyses

The fecal pellets obtained after dilution and homogenization were used to extract DNA. The Q protocol for DNA extraction defined by the International Human Microbiome Standards Consortium was applied using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) [20]. After extraction, a 260/280 ratio was determined using a Take3 Microvolume plate and Gen5 microplate reader (BioTek Instrument Inc., Winooski, VT, USA). DNA was frozen at −20 °C until analysis. Sequencing and annotation of the bacterial 16S rRNA genes were performed at Novogene Bioinformatics Technology Co., Ltd., Cambridge, UK. First, using specific primers connected with barcodes, the variable region V3–V4 of bacterial 16S rRNA genes was amplified using PCR protocol, and a DNA library was prepared. The Illumina NovaSeq 6000 platform was used to sequence the libraries. After obtaining each individual read, they were assigned to the samples using barcodes and merged using FLASH (version 1.2.7). QIIME (version 1.7.0) was used to obtain high-quality clean tags, thereby allowing the removal of low-quality sequences. The obtained tags were then compared with the reference SILVA 138 database, and chimeric sequences were removed using the UCHIME algorithm. To perform sequencing analysis, effective tags were utilized using Uparse software (Uparse V 7.0.1090). Sequences sharing ≥97% homology were assigned to the same OTUs, and OTU abundance was normalized. The representative sequence for each OTU was obtained against the SSU rRNA database of SILVA138 using QIIME (V 1.7.0) and the mothur method to annotate species at each taxonomic rank. Chao1 and Shannon

indices of alpha diversity were obtained using QIIME (V 1.7.0) and are represented using GraphPad Prism 9 software. PCoA of beta diversity was calculated based on Bray Curtis dissimilarity index and visualized using "vegan" and "ggplot2" packages of RStudio software version 1.4.3.

2.4. Fecal Short-Chain Fatty Acid Concentrations

Gas chromatography analyses were performed to determine the concentration of SCFAs, acetic, propionic, butyric, isobutyric, isovaleric, valeric, and caproic acids, in feces following a previously described procedure, with minor modifications [21]. Briefly, fecal supernatants were diluted with methanol, 20% v/v formic acid, and an internal standard to reach a dilution of 10/65. Next, the dilutions were centrifuged for 10 min at room temperature and 14,000 rpm to obtain the supernatants, which were transferred to suitable chromatography vials. To identify and quantify SCFAs, a chromatograph 6890N (Agilent Technologies Inc., Palo Alto, CA, USA) connected to a mass spectrometry detector (MS) 5973N (Agilent Technologies) and a flame ionization detector (FID) were used. The theoretical detection limit values were calculated for minor SCFAs and applied to samples that were not detectable.

2.5. Statistical Analyses

IBM SPSS software (version 25.0; IBM SPSS, Inc., Chicago, IL, USA) and RStudio software version 1.4.3 were used to analyze the data. GraphPad Prism 9 and RStudio software were used for graphical representations. Goodness of fit to the normal distribution was checked using the Kolmogorov–Smirnov test. As normality of the variables was not achieved, nonparametric tests were applied. Overall, categorical variables were presented as numbers and percentages, and continuous variables were presented as mean ± standard deviation values. Mann–Whitney U tests were performed for pairwise comparison of continuous variables (p-value < 0.05), whereas the categorical variable "gender" was analyzed by a chi-squared test.

Alpha and beta diversity indices were compared between diagnosis groups. A Mann–Whitney U test was applied to compare alpha diversity indices, whereas PCoA was performed on beta diversity using Bray–Curtis dissimilarity index. Relative microbial abundances were obtained from OTU data and analyzed using Mann–Whitney U tests to detect differences between groups at the taxonomic levels of phylum, family, and genus. The Benjamini–Hochberg procedure was applied for multiple test comparisons. LEfSe were conducted to estimate taxa by significantly discriminating the groups under study by using the Kruskal–Wallis rank sum test and a Wilcoxon test for pairwise comparison followed by a logarithmic linear discriminant analysis (LDA) to estimate the effect size at a threshold of 2.0, by using the LEfSe Galaxy web tool [22]. Only microbial families and genera with relative abundance of at least 1% in at least two samples were considered in the analysis. The differences in SCFA concentrations between the groups were compared using the Mann–Whitney test. Spearman correlations were performed to explore the associations between microbiota and SCFAs. Heatmaps were generated using "corrplot" R package. Microbial families significantly correlated with SCFAs were examined as predictors of fecal SCFAs using regression analyses, adjusting by diagnosis, and histopathological groups.

3. Results

3.1. General Characteristics of the Sample Population

The main anthropometric characteristics of volunteers from each diagnosis group and the main histopathological features of mucosal biopsies are shown in Table 1. Individuals in the control group did not present histological lesions of the intestinal mucosa, while volunteers with polyps displayed hyperplastic polyps or conventional adenomas. Individuals presenting conventional adenomas were more abundant than those with hyperplastic polyps (25 vs. 9 individuals). Conventional adenomas presented LGD (TA or TVA polyps) or HGD.

Table 1. General characteristics of the sample population according to the diagnosis after colonoscopy and histopathological analysis of intestinal mucosa resected samples during colonoscopy.

Variables	Control (n = 20)	Polyps (n = 34)
Male gender	6 (30.0%)	19 (55.9%)
Age (years)	60 ± 9	61 ± 6
BMI (kg/m^2) †	25.67 ± 3.81	27.87 ± 4.16
Histopathological analysis ††		
NP	20 (100.0%)	-
Hyperplastic polyps	-	9 (26.5%)
Conventional adenomas	-	25 (73.5%)
LGD	-	20
HGD	-	5

Values are presented in terms of n (percentage in the sample population) or mean ± standard deviation (SD). No significant differences ($p \geq 0.05$) were found for gender (chi-squared test), age, and BMI (Mann–Whitney U test) between diagnosis groups. † The number of volunteers with BMI available information was the following: control: n = 14; polyps: n = 27. †† Samples from individuals with more than one type of polyp were considered in the histopathological group of that of the highest risk. BMI: body mass index; NP: nonpathological histology; LGD: conventional adenomas with low-grade dysplasia; HGD: conventional adenomas with high-grade dysplasia.

3.2. Fecal Microbiota Profiles

Stool microbiota compositions were determined using partial 16S rRNA gene sequencing. The 54 fecal samples yielded 9585 OTUs. We compared the microbiota profiles according to diagnosis and histopathological groups (nonpathological controls and polyps: 20 and 34 samples, respectively) and analyzed the differences in microbiota between individuals presenting hyperplastic polyps (nine samples) or conventional adenomas (25 samples) within the group of polyps. We further analyzed the fecal microbiota of individuals carrying conventional adenomas with LGD (20 samples) or HGD (five samples).

3.2.1. Microbiota Comparison between Control and Polyps Groups

Figure 1 presents the main fecal microbiota profiles of the two groups: the control and polyps. The relative abundance of phyla in the control and polyps groups followed the order *Bacillota* (former *Firmicutes*) > *Actinomycetota* > *Bacteroidota* > *Pseudomonadota* (formerly *Proteobacteria*) > *Verrucomicrobiota* (Figure 1A). The family *Methanobacteriaceae* was significantly less abundant in the polyps group than in the control group (1.37% vs. 2.11%, respectively), and the same was observed for *Oscillospiraceae* and *Christensenellaceae*, which were more abundant in the control group than in the polyps group (Figure 1B) (3.08% vs. 2.18% and 1.89% vs. 1.26%, respectively). Alpha diversity, represented by Chao1 and Shannon indices (Figure 1C,D), did not differ significantly between the groups. Regarding beta diversity, principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity index using all OTU normalized relative abundances did not clearly separate the groups (Figure 1E). Linear discriminant analysis effect size (LEfSe) testing was conducted to identify the genera contributing the most to differentiating the control and polyps group. Differentially increased abundances of the *Ruminococcus_torques* group (family: *Lachnospiraceae*; order: *Lachnospirales*) and *Eubacterium_hallii* (family: *Lachnospiraceae*; order: *Lachnospirales*) in contrast to decreased abundances of *Ruminococcus* (family: *Ruminococcaceae*; order: *Oscillospirales*), g_*Clostridia_UCG-014*, the *Christensenellaceae R-7* group (family: *Christensenellaceae*; order: *Chistensenellales*), and *Methanobrevibacter* (family: *Methanobacteriaceae*; order: *Methanobacteriales*) were the most relevant differences found between the feces samples from individuals diagnosed with polyps with respect to the controls (Figure 1F). The cladogram generated from the LEfSe results (Figure 1 G) highlights the enrichment of the order *Coriobacteriales* and members from the family *Lachnospiraceae*, as well as the depletion of the families *Christensenellaceae*, *Oscillospiraceae*, f_*Clostridia_UCG-014*, and *Methanobacteriaceae* in the polyps group.

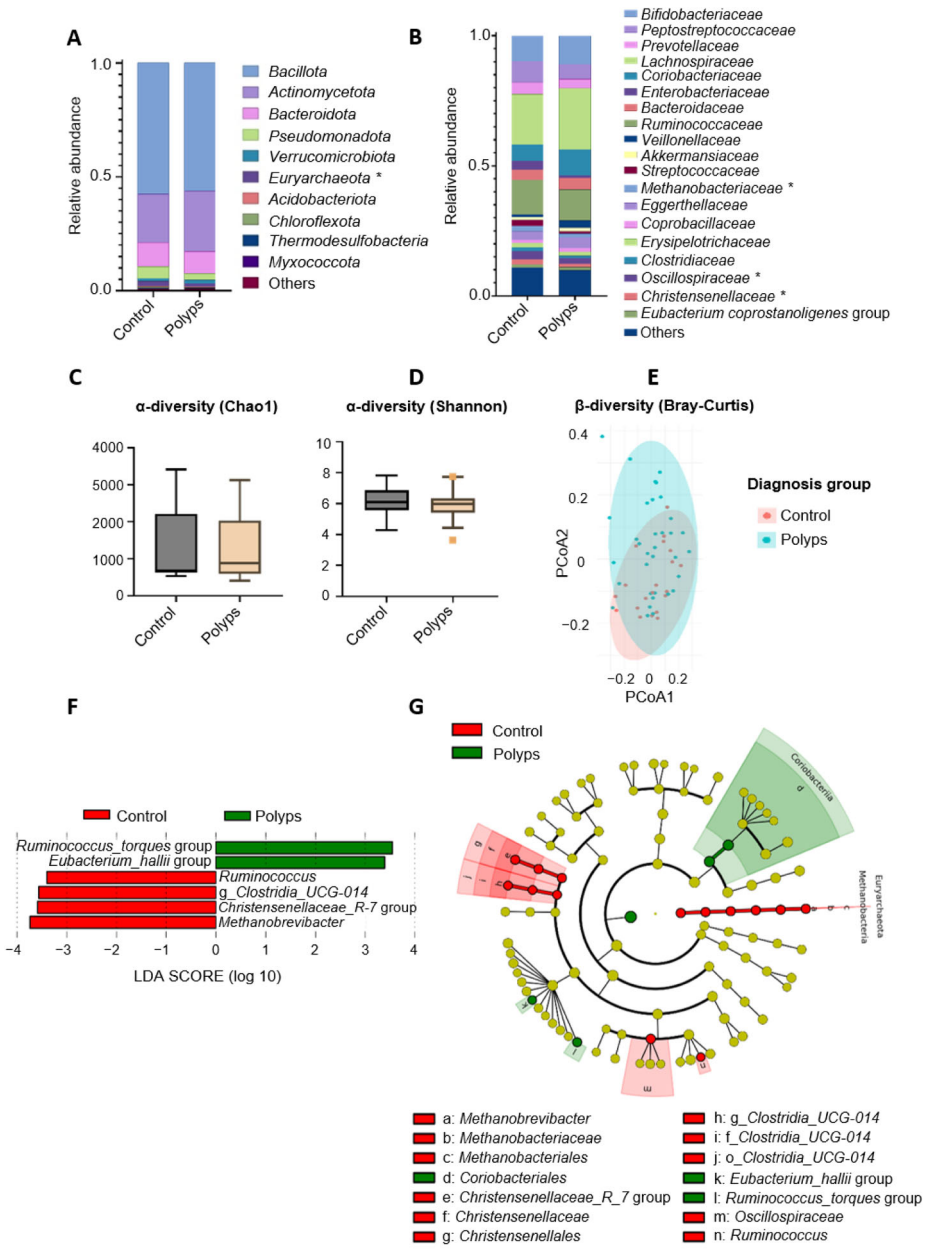

Figure 1. Differences in composition and taxonomic diversity of fecal microbiota between diagnosis groups (control and polyps). (**A**) Relative abundance at the phylum level (including top-10 most abundant). (**B**) Relative abundance of bacteria at the family level (including families showing relative abundance >1% in the sample population). *, Statistically significant differences between control and polyps groups (Mann–Withney U test adjusted by Benjamini–Hochberg; $p < 0.05$). Box plots of alpha diversity values: (**C**) Chao1 index and (**D**) Shannon index. Box elements show the median, upper, and lower quartiles. Dots represent values outside the IQR. (**E**) PCoA of beta diversity using Bray–Curtis dissimilarity index. (**F**) Bacterial taxa (at the genus taxonomic level) showing differential abundance according to linear effect size discriminant analysis (LEfSe) for the comparison of control vs. polyps groups. (**G**) Cladogram generated from LEfSe between control and polyps groups. Yellow circles represent bacterial taxa that show no significant differences between groups, and green and red circles represent taxa whose abundances were statistically differential between groups. For LEfSe, only genera with relative abundances higher than 1% in at least two samples were included.

3.2.2. Microbiota Comparison between Individuals with Hyperplastic Polyps and Conventional Adenomas

We compared the fecal microbiota of individuals with hyperplastic polyps and those with conventional adenomas (Table 1). Differences in the fecal microbiota profiles were observed at phylum and family taxonomic level (Figure 2A,B). Significantly ($p < 0.05$) lower abundances of the *Ruminococcaceae*, *Erysipelotrichaceae*, and *Clostridiaceae* families were found in fecal samples from individuals with conventional adenomas than in those presenting hyperplastic polyps (Figure 2B) (10.70% vs. 15.22%, 0.88% vs. 3.52% and 0.80% vs. 1.37%, respectively). LEfSe analysis (Figure 2C) evidenced that the genera *Holdemanella* (family: *Erysipelotrichaceae*; order: *Erysipelotrichales*), *Clostridium sensu_stricto 1* (family: *Clostridiaceae*; order: *Clostridiales*), and *Clostridium* sp. *CAG-352* (family *Ruminococcaceae*; order: *Oscilllospirales*) were enriched in the feces of individuals with hyperplastic polyps, which was in contrast to the higher comparative abundance of *Alistipes* (family *Rikenellaceae*; order: *Bacteroidales*) in the group of individuals with conventional adenomas. Cladograms generated from the LEfSe (Figure 2D) confirmed differential abundances for the upper taxa related to the genera mentioned above, including the *Erysipelotrichaceae*, *Clostridiaceae*, and *Ruminococcaceae* families, thereby also affecting the orders *Erysipelotrichales* and *Clostridiales*, as well as the *Bacillota* phylum.

3.2.3. Shifts in the Microbiota of Individuals with Hyperplastic Polyps

To identify changes that specifically affected the microbiota of individuals with hyperplastic polyps, we compared their fecal microbiota with that from the control group (Figure 3). LEfSe analysis at the genus level revealed a differentially higher abundance of *Ruminococcus_torques* in the feces of individuals with hyperplastic polyps as compared to the controls (Figure 3A). The corresponding cladogram also showed a differentially higher abundance of the family *Eggerthellaceae* and the order *Coriobacteriales* in the group with hyperplastic polyps (Figure 3B).

3.2.4. Shifts in the Microbiota of Individuals with Conventional Adenomas

The fecal microbiota of individuals diagnosed with conventional adenomas were compared with the microbiota of the control group. We further compared the microbiota from individuals without intestinal mucosal lesions and the microbiota from those presenting conventional adenomas with different grades of dysplasia (LGD and HGD). LEfSe analysis showed a differential enrichment of the *Ruminococcus_torques* and *Ruminococcus_gnavus* groups in patients with conventional adenomas (Figure 4A,B). In contrast, a differential depletion of the following taxa was also found in these individuals: genus *Methanobrevibacter*/family *Methanobacteriaceae*/order *Methanobacteriales*; genus *Holdemanella*/family *Erysipelotrichaceae*; genus *Christensenellaceae R-7* group/family *Christensenellaceae*/order *Christensenellales*; genus *Clostridia_UCG-014* and its related upper taxa; the *Clostridium_sensu_stricto_1*/family *Clostridiaceae*/order *Clostridiales*; genus *Ruminococcus*, *Oscillospiraceae_UCG-002*, and *Clostridium* sp. *CAG-352* within the order *Oscillospirales*; and genus *Romboutsia*/family *Peptostreptococcaceae*/order *Peptostreptococcales_Tissierellales*.

The relative abundance of the phylum *Euryarchaeota* decreased abruptly in the feces of individuals with conventional adenomas displaying HGD as compared to control samples (Table S1). A consistent decrease ($p < 0.05$) related to the increase in the grade of dysplasia was found for the families *Methanobacteriaceae* (2.11% in controls; 1.63% in LGD; 0.26% in HGD), *Christensenellaceae* (1.89% in controls; 1.11% in LGD; 1.00% in HGD), and *Erysipelotrichaceae* (1.75% in controls; 0.99% in LGD; 1.00% in HGD) (Figure 5). Notably, the families *Oscillospiraceae*, *Clostridiaceae*, and f_*Clostridia*_UCG-014 showed a significant decrease in samples from individuals with LGD conventional adenomas with respect to the control group (2.12% vs. 3.08 %, 0.72% vs. 1.47% and 0.70% vs. 1.05%, respectively), whereas *Prevotellaceae* and *Streptococcaceae* decreased significantly in samples from volunteers with HGD conventional adenomas (1.68% vs. 4.55% and 0.25% vs. 2.25%, respectively). Significant variations in some microbial genera from the families mentioned just

above as related to the grade of dysplastic lesions supported these results: these included *Clostridium_sensu_stricto_1* (*Clostridiaceae*), *Streptococcus* (*Streptococcaceae*), *Methanobrevibacter* (*Methanobacteriaceae*), *Holdemanella* (*Erysipelotrichaceae*), *Christensenellaceae_R-7 group* (*Christensenellaceae*), and genus *Clostridia_UCG-014* (Table S2).

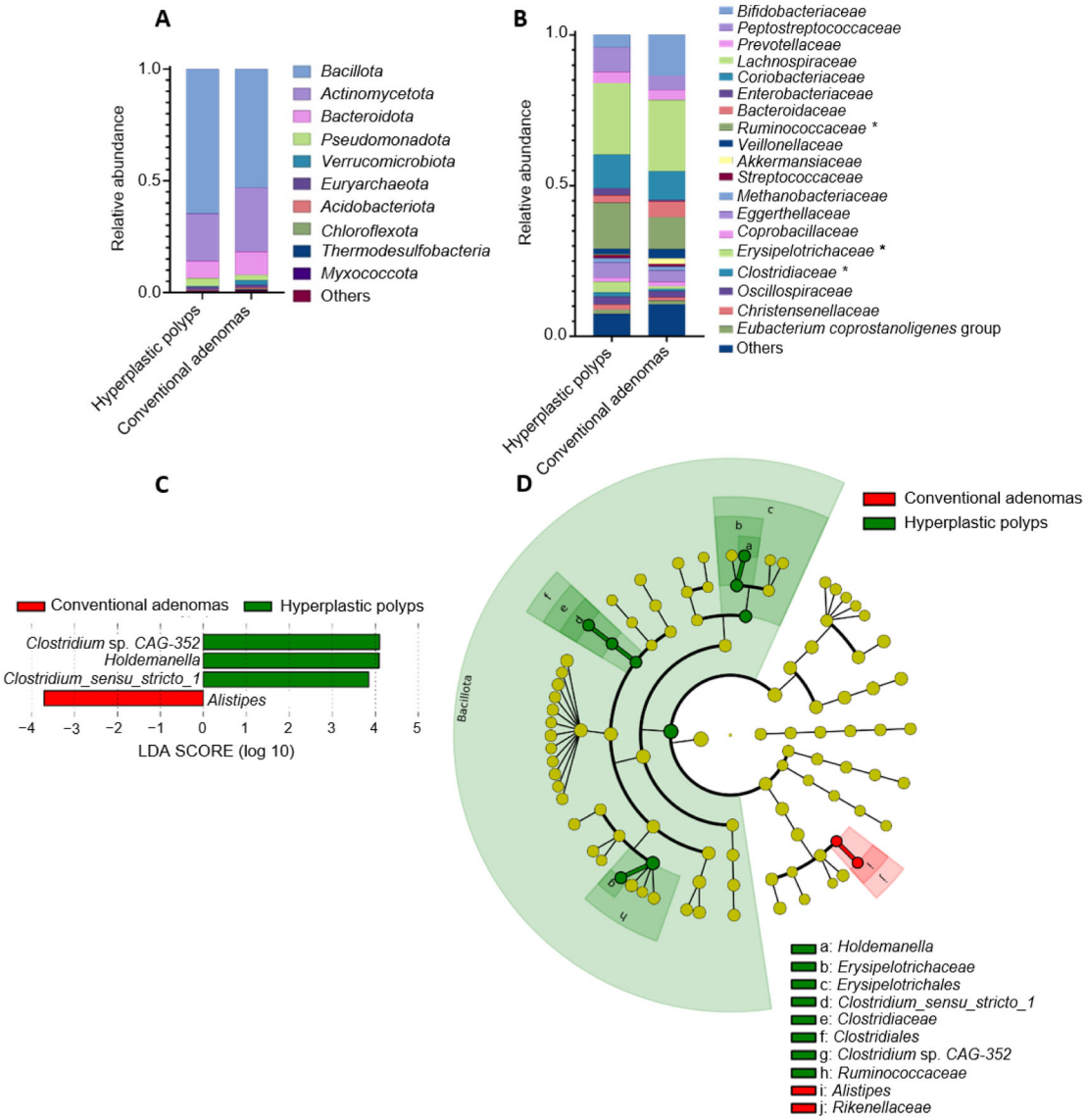

Figure 2. Differences in fecal microbiota compositions from individuals diagnosed with hyperplastic polyps or conventional adenomas. (**A**) Relative abundance of bacteria at the phylum level (including top-10 most abundant). (**B**) Relative abundance of bacteria at the family level (including those families showing relative abundance >1% in the sample). *, Statistically significant differences between samples from individuals with hyperplastic polyps or conventional adenomas (Mann–Whitney U test adjusted by Benjamini–Hochberg; $p < 0.05$). (**C**) Bacterial taxa (at the genus taxonomic level) showing differential abundance according to linear effect size discriminant analysis (LEfSe) for the comparison of hyperplastic polyps and conventional adenomas groups. (**D**) Cladogram generated from LEfSe between samples from individuals diagnosed with hyperplastic polyps or conventional adenomas. Yellow circles represent bacterial taxa that show no significant differences between groups, and green and red circles represent taxa whose abundances were significantly differential between groups.

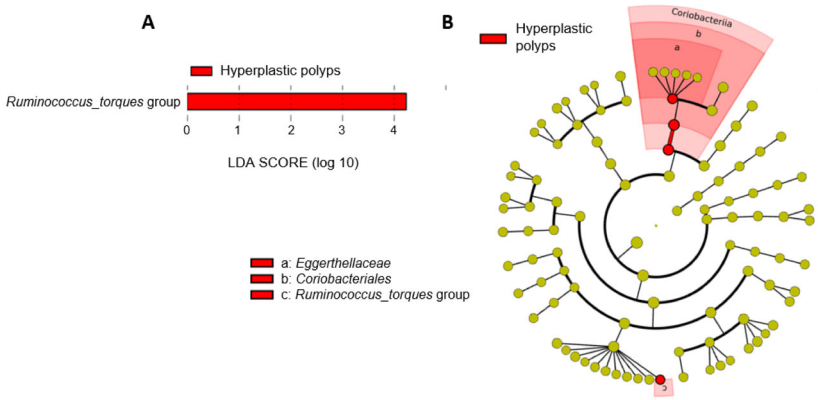

Figure 3. Differences in fecal microbiota compositions from individuals diagnosed with hyperplastic polyps compared to control samples. (**A**) Microbial taxa (at the genus taxonomic level) showing differential abundances according to linear effect size discriminant analysis (LEfSe) for the comparison of samples from individuals diagnosed with hyperplastic polyps and individuals from the control group. (**B**) Cladogram generated from LEfSe between samples from individuals with hyperplastic polyps and the control group. Yellow circles represent bacterial taxa showing no significant differences between groups, and red circles represent taxa that were significantly more abundant in the group with hyperplastic polyps.

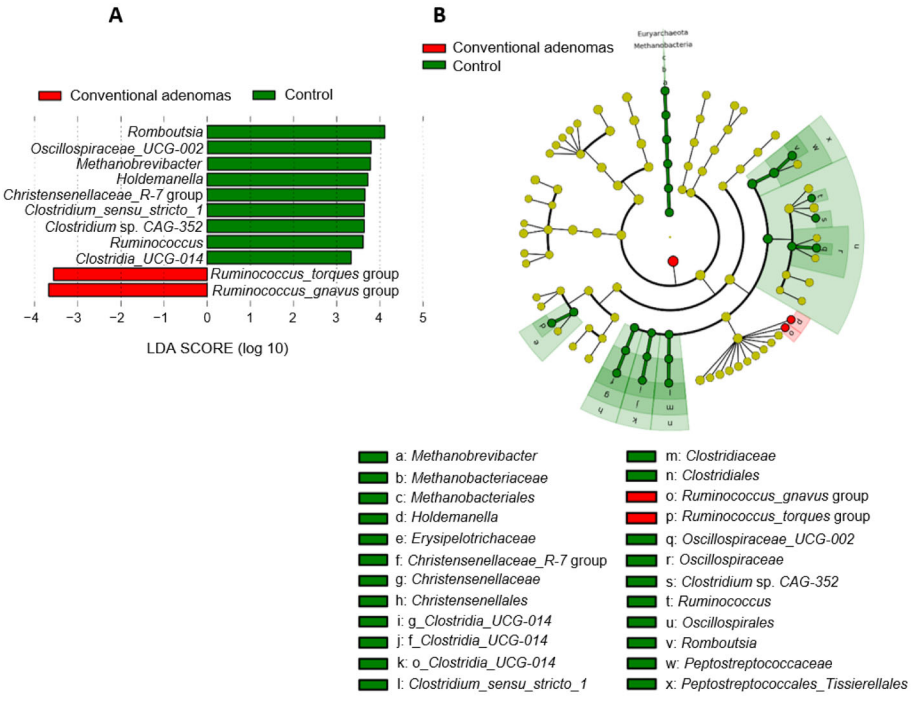

Figure 4. Differences in fecal microbiota compositions from individuals diagnosed with conventional adenomas compared with control samples. (**A**) Microbial taxa (at the genus taxonomic level) showing differential abundance according to linear effect size discriminant analysis (LEfSe) for the comparison of samples from individuals with conventional adenomas and individuals from the control group. (**B**) Cladogram generated from LEfSe between samples from individuals with conventional adenomas and the control group. Yellow circles represent bacterial taxa showing no significant differences between groups, and red circles represent taxa that were significantly more abundant in the group with conventional adenomas.

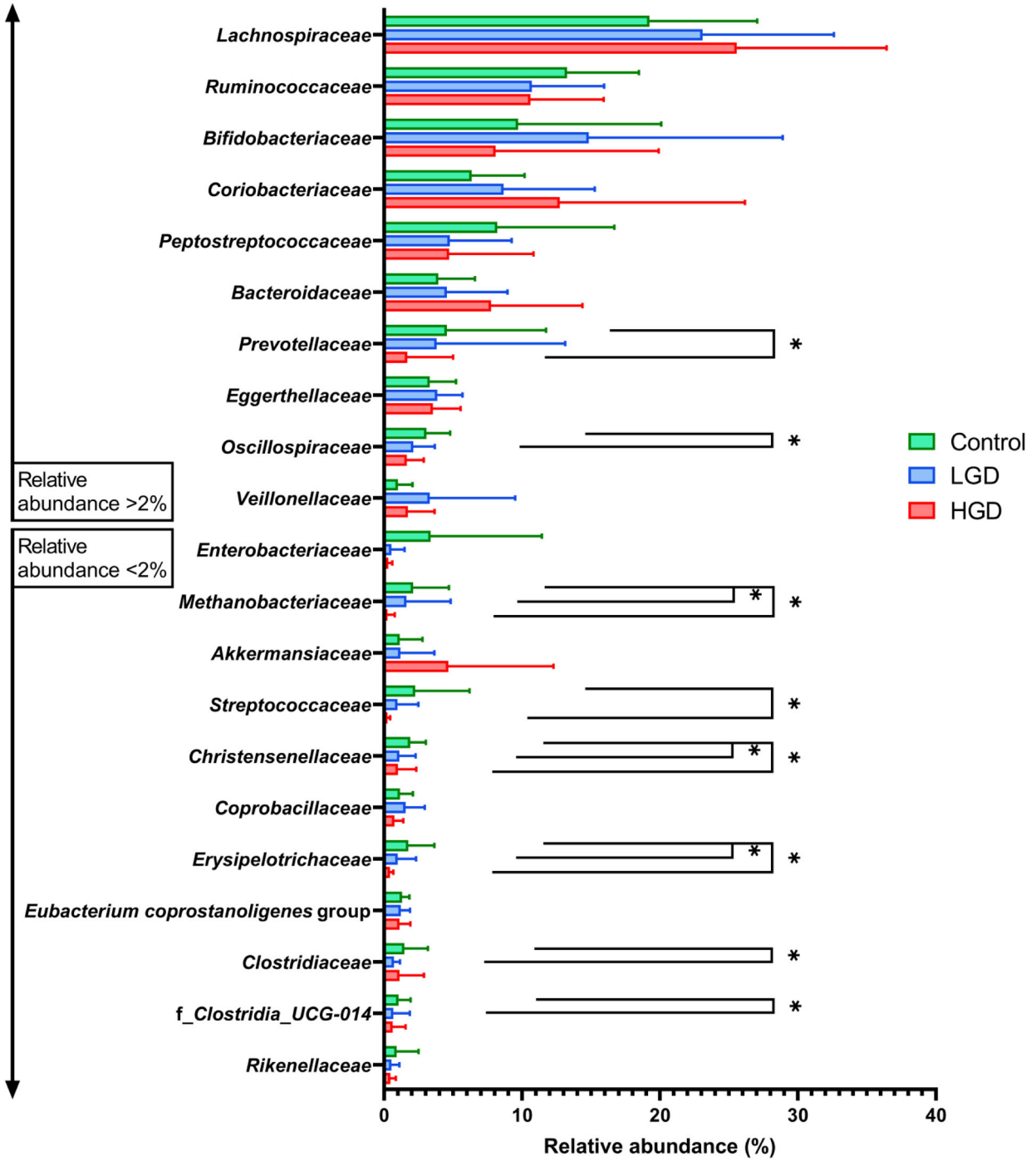

Figure 5. Relative abundances of the main microbial families according to the grade of dysplasia in individuals with conventional adenomas. (*) Statistically significant differences between LGD or HGD with respect to control are indicated by linked upper-black bars (Mann–Whitney U test adjusted by Benjamini–Hochberg; $p < 0.05$). LGD: low-grade dysplasia; HGD: high-grade dysplasia.

3.3. Fecal Short-Chain Fatty Acids

As expected, acetic acid was the major fecal SCFA, followed by propionic and butyric acid, in the two diagnosis groups: the control and polyps (Table S3). Isobutyric, isovaleric, valeric, and caproic acids were present in considerably low proportions. Decreased levels of caproic acid in the polyps group with respect to the control group was the only statistically significant difference found when the SCFA levels were compared according to diagnosis groups. Using histopathological examination as the classification criteria, significantly lower fecal levels of caproic acid were found in samples from individuals with hyperplastic polyps and conventional adenomas when compared with samples from the control group (Table 2).

Table 2. Fecal short-chain fatty acid (SCFA) concentrations (in mM) according to histopathological groups.

	Histopathological Groups		
Fecal SCFAs (mM)	**Control (*n* = 20)**	**Hyperplastic Polyps (*n* = 9)**	**Conventional Adenomas (*n* = 25)**
Acetic acid	47.16 ± 22.35	37.98 ± 23.88	49.86 ± 23.46
Propionic acid	13.65 ± 8.08	14.10 ± 7.94	15.92 ± 8.42
Butyric acid	13.16 ± 9.73	9.38 ± 6.04	11.48 ± 6.67
Isobutyric acid	0.99 ± 1.11	1.08 ± 1.29	0.96 ± 1.01
Isovaleric acid	2.15 ± 1.85	2.06 ± 1.21	2.18 ± 1.76
Valeric acid	2.38 ± 1.77	1.33 ± 0.37	1.67 ± 1.01
Caproic acid	0.80 ± 1.26	0.17 ± 0.35 *	0.23 ± 0.45 *

Values are presented in terms of mean ± standard deviation. (*) Statistically significant differences of hyperplastic polyps or conventional adenomas compared to control group (Mann–Whitney U test; $p < 0.05$). SCFAs: short chain fatty acids.

Correlations between the SCFAs and microbiota evidenced significant positive associations of the *Lachnospiraceae* family with the three major SCFAs and of the *Coprobacillaceae* family with propionic and butyric acid. Negative associations were found between *Oscillospiraceae*, *Christensenellaceae*, and *Rikenellaceae* and the major SCFAs (Figure 6). Significant positive associations were found for *Prevotellaceae*, *Oscillospiraceae*, and *Christensenellaceae* with caproic acid.

Therefore, to more precisely characterize those microbial families that are potential predictors of the fecal levels of SCFAs, thereby avoiding possible confounding factors, linear regression analyses were conducted through adjustment according to the diagnosis and histopathological groups for those associations displaying significant correlations in Figure 6 (Table 3). The *Oscillospiraceae* and *Rikenellaceae* families were negative predictors of acetic, propionic, and butyric acid in several classification groups, whereas *Coprobacillaceae* was a positive predictor of propionic acid in the control group and of propionic and butyric acid in the group with conventional adenomas. *Lachnospiraceae* was directly associated with acetic acid concentrations only in the control group. *Enterobacteriaceae* was directly associated with the fecal levels of isobutyric and isovaleric acid in several diagnosis and histopathological groups. The *Oscillospiraceae* family was associated with caproic acid in the group with conventional adenomas with HGD.

Table 3. Results obtained from linear regression analyses identifying bacterial families as predictors of SCFAs according to the diagnosis and histopathological groups of volunteers.

Diagnosis Group	Dependent Variable	Independent Variable	R^2	β	*p*
Control	Acetic acid	*Lachnospiraceae*	0.301	0.581	0.007
	Propionic acid	*Oscillospiraceae*	0.434	−0.623	0.002
		Coprobacillaceae	0.434	0.376	0.044
	Butyric acid	*Oscillospiraceae*	0.275	−0.560	0.010

Table 3. *Cont.*

Diagnosis Group	Dependent Variable	Independent Variable	R^2	β	*p*
Polyps	Acetic acid	*Oscillospiraceae*	0.240	−0.512	0.002
	Propionic acid	*Rikenellaceae*	0.232	−0.505	0.002
	Butyric acid	*Rikenellaceae*	0.145	−0.413	0.015
	Isobutyric acid	*Enterobacteriaceae*	0.267	0.537	0.001
	Isovaleric acid	*Lachnospiraceae*	0.115	−0.376	0.028
Hyperplastic polyps	Propionic acid	*Rikenellaceae*	0.388	−0.681	0.043
	Butyric acid	*Oscillospiraceae*	0.373	−0.672	0.047
	Isobutyric acid	*Enterobacteriaceae*	0.943	0.975	0.000
	Isovaleric acid	*Enterobacteriaceae*	0.835	0.925	0.000
Conventional adenomas	Acetic acid	*Rikenellaceae*	0.238	−0.519	0.008
	Propionic acid	*Christensenellaceae*	0.312	−0.423	0.022
		Coprobacillaceae	0.312	0.367	0.044
	Butyric acid	*Coprobacillaceae*	0.138	0.417	0.038
Low-grade dysplasia	Acetic acid	*Rikenellaceae*	0.221	−0.512	0.021
	Propionic acid	*Eubacterium coprostanoligenes* group	0.494	−0.544	0.04
		Christensenellaceae	0.494	−0.457	0.013
	Isobutyric acid	*Ruminococcaceae*	0.232	−0.522	0.018
High-grade dysplasia	Isobutyric acid	*Enterobacteriaceae*	0.706	0.883	0.047
	Caproic acid	*Oscillospiraceae*	0.739	0.897	0.039

Linear regression analyses were adjusted according to diagnosis and histopathological groups. Only the variables with $p < 0.05$ in each model are shown. R^2: coefficient of multiple determination; β: standardized regression coefficient.

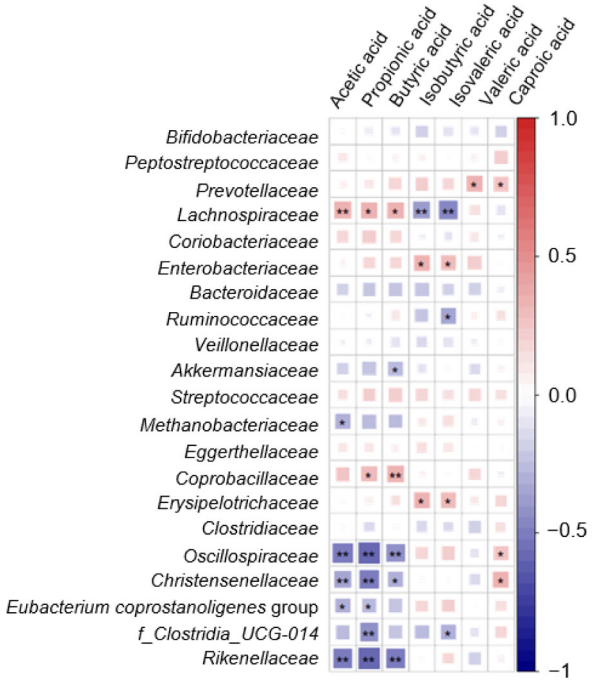

Figure 6. Heatmap defined by Spearman correlations in the sample population under study between most abundant microbial families (relative abundance ≥ 1%) and SCFAs. The intensity of red and blue colors represents the degree of positive and negative associations, respectively. (*) $p < 0.05$; (**) $p < 0.01$.

4. Discussion

Gut microbiota composition is considered as one of the main factors influencing the pathogenesis of CRC. Although alterations have been reported in the fecal microbiota compositions of patients with CRC, the initial shifts in the microbiota associated with early intestinal mucosa damage prior to CRC development are less known [23,24]. In this study, changes to the intestinal microbiota at the initial stages of intestinal mucosal damage were examined according to several criteria: diagnosis groups (control and polyps), morphological type of polyp (hyperplastic vs. conventional adenoma), and grade of dysplasia (low or high) in conventional adenomas.

In contrast to previous reports, no differences were found in the alpha and beta diversity of fecal microbiota between the diagnosis groups. This discrepancy could be due to the different stages of mucosal damage used for the analysis of the microbiota compositions among studies [25–27].

The results of the present work indicate that specific and different microbial taxa from the *Bacillota* and *Euryarchaeota* phyla were predominantly affected in the two diagnosis groups: the control and polyps. Genera determining differences between the control and polyps groups included *Ruminococcus*, g_*Clostridia_UCG-014*, the *Christensenellaceae_R-7* group, and *Methanobrevibacter*, which were less abundant in individuals with polyps, and the genera *Eubacterium_hallii* and *Ruminococcus_torques*, which were enriched in the polyps group. The altered abundances of these microorganisms and/or some of the directly related taxa have been reported previously with regard to CRC and preneoplastic stages in the same direction found in the present work [24–27]. The family *Christensenellaceae*, which was significantly less abundant in the polyps group, has been generally associated with a positive impact on human health, as well as some members of the butyrate-producing family *Ruminococcaceae*, which have a protective effect against the development of CRC that is linked to the consumption of a high-fiber diet and consequent microbial colonic fermentation [28–30]. Regarding *Oscillospiraceae*, which also decreased in the polyps group, previous studies have reported a higher abundance of this family in samples of healthy controls or in samples from individuals at the initial stages of CRC as compared to individuals with more advanced CRC [31,32]. Methanogenic archaea in the colon are mostly represented by the genus *Methanobrevibacter*. These microorganisms are functionally associated with the fermentation of dietary fibers through the use of hydrogen produced by saccharolytic bacteria as an electron donor for reduction reactions to produce methane. This contributes to maintain a beneficial circle that provides energy substrates to the host, enhances the epithelial barrier, and protects against intestinal pathogens [33]. However, the imbalance of methanogenic bacteria during dysbiosis, as evidenced in individuals with polyps in the present work, is associated with several dysimmune conditions and CRC [34]. Under normal conditions, *Eubacterium hallii* contributes to the formation of butyrate and propionate in the intestine and has also shown the ability to conjugate xenobiotic compounds such as the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), thereby contributing to their detoxification in the colon [35,36]. Moreover, *Eubacterium hallii* was included in a panel of 10 species that were used for differentiating adenomas among CRC patients [37].

Significant differences in the intestinal microbiota compositions have been obtained, thereby affecting some important taxa from the *Bacillota* (including the families *Erysipelotrichaceae*, *Clostridiaceae*, and *Ruminococcaceae*) and *Bacteroidota* phyla, between samples from individuals with hyperplastic polyps and those with conventional adenomas. Mori et al. recently reported alterations in the bacterial communities belonging to the *Firmicutes* phylum (renamed *Bacillota*) in patients with intestinal preneoplastic lesions, including samples from the "adenoma-carcinoma" sequence and hyperplastic polyps [38]. The *Erysipelotrichaceae* family has been considered as a possible indicator of the intermediate steps in the sequence of preneoplastic intestinal mucosal lesions in sporadic carcinogenesis, and it has been related to inflammatory processes of the gastrointestinal tract, as well as to host lipid metabolism [17,38,39]. Among the families whose abundances were found to significantly

reduced in individuals with conventional adenomas with respect to hyperplastic polyps, *Ruminococcaceae* is of special relevance, as it is the sole family accounting for a relative abundance over 10% [29].

The results obtained support recent studies pointing to some differences In the intestinal microbiota between the serrated and the conventional adenomatous pathways of carcinogenesis [17,40]. Microbial taxa were found to be more altered in conventional adenomas than in hyperplastic polyps. Remarkably, the *Ruminococcus_torques* group was the only shared taxon that was differentially enriched in samples from individuals with either hyperplastic polyps or conventional adenomas. Members of the *Ruminococcus torques* group have been positively associated with proinflammatory diets and, as also occurs with *Ruminococcus gnavus* (both were differentially enriched in the group with conventional adenomas), were found to be increased in several inflammatory processes and diseases [41–43]. *Ruminococcus torques* has been recently identified as a "driver" in the initiation and progression of the cancerous process [44].

When the fecal microbiota of individuals with LGD and HGD conventional adenomas were compared, a reduction in the relative abundances of the *Christensenellaceae*, *Methanobacteriaceae*, and *Erysipelotrichaceae* families was obtained as related to the increase in the grade of dysplasia. Mori et al. proposed the *Lachnospiraceae* and *Erysipelotrichaceae* families and members of the phylum *Actinomycetota* as markers of the gut microbiota of healthy individuals and those with low-risk intestinal polyps [38]. The family *Prevotellaceae* was also decreased in individuals with HGD conventional adenomas compared to the control samples. These microorganisms have been positively associated with anti-inflammatory diets characterized by a high consumption of vegetables and a reduced risk of CRC; however, they have also been found to be increased in the microbiota of individuals with CRC in several studies [45–47]. *Streptococcaceae*, which were also decreased in HGD individuals, are carbohydrate-fermentative and organic acid-producing microorganisms that are commensal inhabitants of the oral cavity and gut; they contribute to immune homeostasis, although some species are pathogenic and have been related to oral disease and CRC [48]. In the present study, the significant decrease in some relevant families of carbohydrate-fermentative microorganisms in the gut (*Christensenellaceae*, *Clostridiaceae*, *Erysipelotrichaceae*, *Streptococcaceae*, *and Prevotellaceae*), together with the decrease in methanogenic archaea, could reflect a progressive alteration of the gut microbial consortia participating in fermentative processes.

In short, the work performed evidenced deeper changes in the fecal microbiota compositions of individuals with conventional adenomas than in those with hyperplastic polyps. The increased abundance of the *Ruminococcus_torques* group was a differential signature of the microbiota compositions of individuals with polyps, with either conventional adenomas or hyperplastic polyps. The evolution of certain taxonomic groups was different for both types of alterations, and the decrease in specific microbial families as related to the grade of dysplasia in conventional adenomas was of relevance.

Despite the fact that some microbial families were associated with the fecal levels of SCFAs, no significant variations in the fecal concentrations of the major SCFAs were found, which suggests that although important shifts occur in the fermentative gut microbial consortia alongside the progression of intestinal mucosal lesions, they do not globally affect SCFA concentrations. Nevertheless, the relatively small sample size may be influencing the lack of significant differences in this regard. The decrease in the fecal levels of caproic acid in individuals with conventional adenomas with HGD was associated with the decrease in *Oscillospiraceae* amounts in this histopathological group. Caproic acid is mostly derived from diet and can participate in the enhancement of Th1 and Th17 cell differentiation in the immune system [49]. However, the reduced concentration of this compound in feces and the lack of sufficient data preclude our formulating any hypothesis. Alterations to the intestinal microbiota associated with intestinal mucosa damage could be also related to shifts in the concentrations of a variety of metabolites, other than SCFAs, such as those derived from the metabolism of dietary phenolic compounds, amino acids, lipids, nucleotides, and

xenobiotics. Metabolomic studies will help to identify the metabolic changes associated with altered microbiota profiles and damage of the intestinal mucosa.

We have recently reported altered levels of *Lachnospiraceae* and *Eggerthellaceae*, as well as of the *Muribaculaceae*, *Streptococcaceae* and *Eubacterium coprostanoligenes* groups, in a collective of socially vulnerable individuals associated with the consumption of 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx) and PhIP, which are two heterocyclic amines formed during food cooking [50]. Therefore, although a causality cannot be established, these preliminary data may suggest a possible relationship between initial alterations to the intestinal mucosa derived from toxic dietary compounds and gut intestinal microbiota profiles.

5. Conclusions

The comparison between control and polyps groups and the differences found examining the histopathological features, together with the grade of dysplasia in stages prior to CRC development, revealed important shifts in fecal microbiota profiles; some of the relevant taxa compositions that were altered are microorganisms that are normally involved in fermentative processes in the intestine.

The present work could provide a starting point for wider studies focused on the possible influence regarding modification of diet and lifestyle on the initial stages of intestinal alterations that could potentially trigger the CRC process and other diseases.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/cancers16010104/s1. Table S1: Differences in the relative abundances of microbial phyla in fecal samples from individuals with conventional adenomas according to the grade of dysplasia; Table S2: Differences in the relative abundances of microbial genera, ranked according to their taxonomic family membership, in fecal samples from individuals with conventional adenomas according to the grade of dysplasia; Table S3: Fecal short-chain fatty acids (SCFAs) concentrations (mM) according to diagnosis groups.

Author Contributions: Conceptualization: C.G.d.l.R.-G. and S.G.; Methodology: S.R.-S., A.S., C.G.d.R. and Y.D.; Validation: N.S. and S.A.; Formal analysis: S.R.-S., A.M.N. and S.A.; Investigation: S.R.-S., S.A., A.M.N., N.S., C.G.d.R., M.G., S.G. and C.G.d.l.R.-G.; Data curation: S.R.-S. and A.M.N.; Funding acquisition: C.G.d.l.R.-G. and S.G.; Writing—original draft: C.G.d.l.R.-G. and S.R.-S.; Writing—review and editing: S.R.-S., C.G.d.l.R.-G. and S.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in accordance with Spanish legislation on bioethics. Directive 95/46/EC of the European Parliament and the Council of 24 October 1995, regarding the protection of individuals with respect to the processing of personal data and the free movement of such data, was strictly followed.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during metagenomic sequencing of fecal DNA samples were deposited in the NCBI Sequence Read Archive PRJNA994445 (http://www.ncbi.nlm.nih.gov/bioproject/994445).

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

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MATERIAL SUPLEMENTARIO

Table S1. Differences in the relative abundance of microbial phyla in fecal samples from individuals with conventional adenomas according to the grade of dysplasia.

Microbial phyla (% abundance)	Control (*n*=20)	LGD (*n*=20)	HGD (*n*=5)
Bacillota	57.60 ± 11.86	53.48 ± 12.27	51.47 ± 20.63
Actinomycetota	21.23 ± 11.08	29.06 ± 14.44	27.09 ± 15.75
Bacteroidota	10.64 ± 7.55	10.19 ± 9.7	10.76 ± 5.84
Pseudomonadota	5.10 ± 9.10	2.42 ± 2.68	2.59 ± 2.70
Verrucomicrobiota	1.24 ± 1.72	1.3 ± 2.46	4.90 ± 7.46
Euryarchaeota	2.11 ± 2.59 a	1.63 ± 3.19 a,b	0.26 ± 0.52 b
Acidobacteriota	0.41 ± 0.78	0.33 ± 0.45	0.88 ± 1.23
Chloroflexota	0.38 ± 0.83	0.38 ± 0.53	0.36 ± 0.39
Thermodesulfobacteria	0.31 ± 0.60	0.26 ± 0.43	0.17 ± 0.19
Myxococcota	0.17 ± 0.31	0.12 ± 0.16	0.41 ± 0.55
Others	0.81 ± 1.43	0.83 ± 0.81	1.12 ± 1.14

Values are shown as mean ± standard deviation. Values in the same row showing different letters present a statistically significant difference of LGD or HGD with respect to the control group (U Mann Whitney test, adjusted by Benjamini-Hochberg; $p < 0.05$). LGD, low grade dysplasia; HGD, high grade dysplasia.

Table S2. Differences in the relative abundance of microbial genera, ranked according to their taxonomic family membership, in fecal samples from individuals with conventional adenomas according to the grade of dysplasia.

Microbial genera (% Abundance)	Control (*n*=20)	LGD (*n*=20)	HGD (*n*=5)
Bifidobacteriaceae:			
Bifidobacterium	9.71 ± 10.37	14.83 ± 14.05	8.09 ± 11.82
Prevotellaceae:			
Prevotella	3.14 ± 6.27	3.37 ± 9.07	1.61 ± 3.31
Coriobacteriaceae:			
Collinsella	6.33 ± 3.87	8.66 ± 6.61	12.73 ± 13.41
Peptostreptococcaceae:			
Intestinibacter	2.16 ± 3.65	1.53 ± 2.35	0.97 ± 1.58
Romboutsia	5.13 ± 4.64 a	2.73 ± 2.47 b	3.12 ± 3.65 a,b
Clostridiaceae:			
Clostridium_sensu_stricto_1	1.42 ± 1.72 a	0.68 ± 0.45 b	1.10 ± 1.76 a,b
Bacteroidaceae:			
Bacteroides	3.94 ± 2.65	4.56 ± 4.39	7.76 ± 6.62
Enterobacteriaceae:			
Escherichia_Shigella	3.04 ± 7.69	0.47 ± 0.97	0.24 ± 0.30
Veillonellaceae:			
Dialister	0.91 ± 1.02	3.22 ± 6.19	1.67 ± 1.84
Lachnospiraceae:			
Agathobacter	4.47 ± 3.27	6.04 ± 4.81	4.18 ± 4.10
Roseburia	1.24 ± 1.15	1.11 ± 1.06	1.11 ± 0.93
Blautia	2.36 ± 1.12	2.51 ± 0.93	2.72 ± 1.36
Fusicatenibacter	1.11 ± 1.13	1.18 ± 0.64	1.02 ± 0.76
Coprococcus	1.25 ± 0.94	1.10 ± 0.72	0.88 ± 0.59
Dorea	1.17 ± 0.54	1.55 ± 0.77	1.27 ± 0.96
Ruminococcus_gnavus group	0.27 ± 0.71 b	0.60 ± 0.89 b	1.38 ± 2.49 a,b
Ruminococcus_torques group	0.62 ± 0.37 b	0.99 ± 0.60 b	1.22 ± 1.52 a,b
Akkermansiaceae:			
Akkermansia	1.14 ± 1.65	1.19 ± 2.46	4.65 ± 7.62
Oscillospiraceae:			
Oscillospiraceae_UCG-002	1.64 ± 1.15	1.07 ± 1.00	0.66 ± 0.56
Streptococcaceae:			
Streptococcus	2.22 ± 3.87 a	0.98 ± 1.49 a,b	0.25 ± 0.20 b
Methanobacteriaceae:			
Methanobrevibacter	1.99 ± 2.50 a	1.60 ± 3.13 b	0.26 ± 0.52 b
Erysipelotrichaceae:			
Holdemanella	1.33 ± 1.89 a	0.68 ± 1.32 b	0.19 ± 0.23 a,b
Eggerthellaceae:			
Slackia	0.92 ± 1.12	0.65 ± 0.78	1.33 ± 1.50
Senegalimassilia	0.68 ± 0.60	1.06 ± 1.05	0.61 ± 1.11
Christensenellaceae:			
Christensenellaceae_R-7 group	1.86 ± 1.13 a	1.08 ± 1.16 b	0.98 ± 1.32 a,b
Ruminococcaceae:			
Subdoligranulum	3.60 ± 2.89	2.91 ± 2.16	3.39 ± 2.15
Faecalibacterium	5.87 ± 2.97	4.59 ± 3.12	5.12 ± 2.49
Clostridium sp. *CAG-352*	1.07 ± 2.60 a	0.62 ± 1.56 b	0.29 ± 0.34 a,b

Ruminococcus	0.54 ± 0.33 a	0.37 ± 0.3 b	0.33 ± 0.34 a,b
Rikenellaceae:			
Alistipes	0.34 ± 0.31	0.51 ± 0.55	0.45 ± 0.37
Eubacterium coprostanoligenes group:			
Eubacterium coprostanoligenes group	1.30 ± 0.54	1.22 ± 0.66	1.12 ± 0.79
Clostridia_UCG-014	1.05 ± 0.87 a	0.67 ± 1.19 b	0.63 ± 0.93 a,b

Values are shown as mean ± standard deviation. Values in the same row showing different letters present a statistically significant difference of LGD or HGD with respect to the control group (U Mann Whitney test, adjusted by Benjamini-Hochberg; $p < 0.05$). LGD, low grade dysplasia; HGD, high grade dysplasia.

Table S3. Fecal short chain fatty acids (SCFAs) concentrations (mM) according to diagnosis groups

	Diagnosis groups	
Fecal SCFAs (mM)	Control (*n*=20)	Polyps (*n*=34)
Acetic acid	47.16 ± 22.35	46.72 ± 23.81
Propionic acid	13.65 ± 8.08	15.44 ± 8.22
Butyric acid	13.16 ± 9.73	10.92 ± 6.49
Isobutyric acid	0.99 ± 1.11	0.99 ± 1.07
Isovaleric acid	2.15 ± 1.85	2.15 ± 1.62
Valeric acid	2.38 ± 1.77	1.58 ± 0.90
Caproic acid	0.80 ± 1.26	0.22 ± 0.42 *

Values are presented as mean ± standard deviation. (*) Statistically significant differences between groups (U Mann Whitney test; $p < 0.05$). SCFAs, short chain fatty acids.

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Anaerobes in the microbiome

Human fecal alpha-glucosidase activity and its relationship with gut microbiota profiles and early stages of intestinal mucosa damage

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ABSTRACT

Objectives: We investigated potential relationships among initial lesions of the intestinal mucosa, fecal enzymatic activities and microbiota profiles.
Methods: Fecal samples from 54 volunteers were collected after recruitment among individuals participating in a colorectal cancer (CRC) screening program in our region (Northern Spain) or attending for consultation due to clinical symptoms; intestinal mucosa samples were resected during colonoscopy. Enzymatic activities were determined in fecal supernatants by a semi-quantitative method. The fecal microbiota composition was determined by 16S rRNA gene-based sequencing. The results were compared between samples from clinical diagnosis groups (controls and polyps), according with the type of polyp (hyperplastic polyps or conventional adenomas) and considering the grade of dysplasia for conventional adenomas (low and high grade dysplasia).
Results: High levels of α-glucosidase activity were more frequent among samples from individuals diagnosed with intestinal polyps, reaching statistical significance for conventional adenomas and for low grade dysplasia adenomas when compared to controls. Regarding the microbiota profiles, higher abundance of *Christensenellaceae_R-7* group and *Oscillospiraceae_UCG-002* were found in fecal samples displaying low α-glucosidase activity as compared with those with higher activity as well as in controls with respect to conventional adenomas. A relationship was evidenced among intestinal mucosal lesions, gut glucosidase activities and intestinal microbiota profiles.
Conclusions: Our findings suggest a relationship among altered fecal α-glucosidase levels, the presence of intestinal mucosal lesions, which can be precursors of CRC, and shifts in defined microbial groups of the fecal microbiota.

1. Introduction

The incidence of colorectal cancer (CRC), the third most diagnosed globally, is increasing worldwide [1]. Frequently, this cancer initiates in the adult age, strongly linked to the accumulated influence of environmental factors [2]. In most cases, CRC follows a slow development with the progression of precursor lesions in the intestinal mucosa generally taking long time, even years, to become malignant. This allows their detection at initial stages of the mucosal damage in routine colonoscopy screenings [3]. In adults older than 40 years, it is common to find macroscopic alterations in the form of intestinal polyps during routine colonoscopy examinations [4]. These lesions can be of different types. Analyses of biopsies obtained from intestinal mucosa by trained physicians allow the detection of specific histopathological features within

Abbreviations: CRC, Colorectal cancer; T2DM, Type-2 diabetes mellitus; LGD, Conventional adenoma with low grade dysplasia; HGD, Conventional adenoma with high grade dysplasia; LEfSe, Linear discriminant analysis effect size; LDA, Logarithmic Linear Discriminant analysis; HUCA, Central University Hospital of Asturias.

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polyps [5]. Depending on the genetic nature and degree of the morphological changes and molecular alterations of the intestinal mucosa, polyps may be classified into the adenomatous pathway, which represents up to 80 % of cases, or in the serrated pathway, being around 20 % of cases [6,7]. The presence of these lesions in the intestinal mucosa is usually accompanied by microbiota alterations [8]. In this way, the gut microbiota is thought to play a key role in the development of CRC and is already altered in previous stages of intestinal mucosa damage [9–11]. The gut microbiota contributes to some metabolic processes occurring in the intestine in which glucosidases are of special relevance. Alpha-glucosidases can be produced and poured to the intestinal lumen by enterocytes but also by members of the intestinal microbiota [12]. Intestinal glucosidases are involved in the final stage of complex polysaccharides digestion and participate in the conversion of phytoestrogens into their aglycone forms [13,14]. However, misbalanced activity of intestinal glucosidases may be related with some health conditions as Type-2 diabetes mellitus (T2DM), some gastrointestinal pathologies or with obesity [15,16].

The use of commercial strips with different substrates applied to profile the enzymatic activity of fecal samples has been reported as a useful tool to detect intra and inter-individual intestinal differences [17–19]. To the best of our knowledge, the relationship between the intestinal enzymatic activities detected through the commercial API® ZYM system (bioMerieux, France) and the initial stages of the intestinal mucosal damage previous to CRC development has not been previously reported. Therefore, in the present work we performed a descriptive and preliminary study to deepen into the relationships among fecal enzymatic activities, the fecal microbiota profile and the status of the intestinal mucosa in individual without alterations and volunteers diagnosed with intestinal polyps, including those diagnosed with hyperplastic polyps and conventional adenomas with different grades of dysplasia.

2. Materials and methods

2.1. Study design and volunteers

Samples for this study (feces and biopsies of intestinal mucosa) were collected between October 2019 and December 2021 by physicians from the Digestive Service at the Central University Hospital of Asturias (HUCA) and Carmen and Severo Ochoa Hospital in Asturias (North of Spain). Sample's donors were selected among individuals from 40 to 79 years who participated in a CRC screening program in our Region or who attended for consultation due to nonspecific clinical gastrointestinal symptoms and that were submitted to colonoscopy. Objectives and procedures of the study were provided to the volunteers at the time of the first appointment and individuals interested in participating signed an informed consent. Exclusion criteria were:immune-related diseases, previous surgery of the digestive system, cancer history, or drug treatment in the previous two months.

Fecal samples were collected after recruitment, and biopsies of the intestinal mucosa and polyps were resected during colonoscopy. Fifty four volunteers for which both fecal samples and histopathological results were available were included in the study.

2.2. Histopathological classification

Biopsies were examined at the Department of Anatomical Pathology of HUCA, as described elsewhere [20]. Briefly, samples were fixed with 10 % formaldehyde and embedded in paraffin. Serial tissue sections of 3 μm thick were stained with hematoxylin-eosin and analyzed by light microscopy. Established histomorphological criteria by WHO were used for the classification of polyps lesions [21]. According to colonoscopy and histopathological analyses, samples from those volunteers with normal colonoscopy and absence of alterations on the intestinal mucosa (n = 20) were classified as non-pathological controls. In contrast, the polyps group (n = 34) included samples from volunteers with one or more intestinal polyps. Polyps were classified as hyperplastic (n = 9) (carrying cells with hyperplastic lesions) or conventional adenomas (n = 25) (carrying cells with dysplastic lesions). Conventional adenomas were further classified according to the grade of dysplasia: Low Grade Dysplasia (LGD) (n = 20) or High Grade Dysplasia (HGD) (n = 5). Samples from individuals displaying more than one type of polyps and mucosal lesions were included into the group or subgroup with the highest CRC risk.

2.3. Fecal samples processing

Instructions to collect fecal samples were given to the volunteers, consisting on the deposition of fresh fecal samples in sterile plastic containers and their transportation to the hospitals participating in the study within a period not exceeding 2 h after deposition. Samples were frozen at −20 °C in the hospital and transported to the laboratory for processing. Frozen samples (4 g) were weighed, diluted 1:10, and homogenized with sterile phosphate-buffered saline (PBS) in a LabBlender 400 Stomacher (Seward Medical, London, UK) for 3 min at maximum speed. After 15 min of centrifugation at 4 °C and 14000 rpm, supernatants and pellets were separated and kept frozen at −20 °C until use.

2.4. Enzymatic activities

The enzymatic activities of fecal supernatants were examined by using the semi-quantitative API® ZYM system (bioMérieux, France), following the manufacturer's instructions with minor modifications. Briefly, fecal supernatants were diluted 1:300 using sterile Milli-Q water and 65 μl of the suspension were inoculated into wells of the API® ZYM strip, containing 19 different substrates. One strip was used per sample. Strips were incubated at 37 °C for 4 h in an anaerobic chamber (MG500, Don Whitley Scientific, West York-Shire, UK, with an 80 % v/v N_2, 10 % v/v CO_2, and 10 % v/v H_2 atmosphere). After incubation, enzymatic reactions were revealed using ZYM A and ZYM B reagents and the color developed was assigned to a value on a scale of 0 (negative reaction) to 5 (maximum activity) according to the color chart scale provided by the manufacturer. Wells with color score ranging from 0 to 2 were codified as with "low" activity while wells with color ranging from 3 to 5 were codified as with "high" activity.

2.5. Microbial analyses

DNA extraction and microbial analyses were performed as described previously [22]. The fecal pellets obtained after dilution and homogenization were used to extract DNA. The Q protocol for DNA extraction defined by the International Human Microbiome Standards Consortium was applied using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK) [23]. After extraction, the 260/280 wavelength ratio was determined using a Take3 Micro-Volume plate in a Gen5 microplate reader (BioTek Instrument Inc., Winooski, VT, USA). DNA was kept frozen at −20 °C until analysis. The procedure of sequencing and annotation of the bacterial 16S rRNA genes were performed at Novogene Bioinformatics Technology Co., Ltd. First, the Variable region V3–V4 of bacterial 16S rRNA genes was amplified by PCR using specific primers (341F and 806R) connected with barcodes and the DNA library was prepared. The Illumina NovaSeq 6000 platform was used to sequence the libraries. After obtaining each individual read, they were assigned to the samples using barcodes and merged using FLASH (version 1.2.7). QIIME (version 1.7.0) was used to obtain high-quality clean tags, allowing the removal of low-quality sequences. The obtained tags were then compared with the reference SILVA 138 database, and chimeric sequences were removed using the UCHIME algorithm. To perform sequencing analysis, effective tags were utilized using Uparse software (Uparse V 7.0.1090). Sequences sharing ≥97 % homology were assigned to the same Operational Taxonomic Units (OTUs) and OTUs abundance was normalized.

To assign sequences at each taxonomic rank, the representative sequence for each OTU was performed against the SSUrRNA database of SILVA138 Database using QIIME (V 1.7.0) in Mothur method. Moreover, to obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (V 3.8.3) was used.

2.6. Statistical analyses

IBM SPSS software (version 25.0; IBM SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. GraphPad Prism 9 software (GraphPad Software, Boston, MA, USA) was used for graphical representations. Categorical variables were presented as numbers and percentages. Differences in proportions between diagnosis groups were analyzed by chi-square tests. Linear discriminant analysis effect size (LefSe) was conducted to estimate microbial taxa significantly discriminating the groups under study by using the LEfSe Galaxy web tool [24]. This analysis consisted of a Kruskal-Wallis sum-rank test and a Wilcoxon test for pairwise comparison followed by a Logarithmic Linear Discriminant analysis (LDA) to estimate the effect size at a threshold of 2.0 as indicated by Segata et al. [24]. Only microbial families and genera with relative abundance of minimum 1 % in at least two samples were considered in the analysis [22].

3. Results

The distribution of sample donors according to colonoscopy screening and histopathological examination of biopsies from the intestinal mucosa is presented in Table 1. From volunteers diagnosed with polyps, 73.53 % of the cases were classified into the conventional adenoma group, among which those presenting LGD were the most abundant, followed by HGD.

Enzymatic activities of fecal supernatants tested with the API® ZYM system showed some differences in the percentage of samples displaying high activity levels between the control and intestinal polyps groups. However, only differences for α-glucosidase reached statistical significance, as samples with high activity were more abundant in the polyps group (88.2 %) than in the control group (55 %) (Table 2). Then, we analyzed more in depth the profile of α-glucosidase activity according to the presence of hyperplastic polyps or conventional adenomas. Both subgroups showed higher percentage of samples with high α-glucosidase activity levels than the control group (Fig. 1A), these differences reaching statistical significance only for samples from the group of the conventional adenomas. Moreover, the analysis of fecal supernatants from individuals with conventional adenomas showing different grades of dysplasia evidenced higher percentage of samples with high α-glucosidase activity for all type of histopathological lesions when compared to the control group, but these differences reached statistical significance only for the comparison of LGD vs. control group (Fig. 1B). The rest of the enzymatic activities tested did not show statistical differences in any of the comparisons performed.

Table 1
Distribution of sample donors into groups of volunteers without intestinal mucosal lesions (Controls) or those presenting polyps, according to colonoscopy screening and histopathological tissue examination.

	Control (n = 20)	Polyps (n = 34)
NP	20 (100 %)	–
Hyperplastic polyps	–	9 (26.47 %)
Conventional adenomas	–	25 (73.53 %)
LGD	–	20
HGD	–	5

Values are presented as number (percentage in the sample population). Individuals with more than one type of polyp's lesion were considered into the histopathological group of highest CRC risk. NP, non-pathological; LGD, conventional adenomas with low grade dysplasia; HGD, conventional adenomas with high grade dysplasia.

Table 2
Number of fecal samples showing high enzymatic activity levels in the API® ZYM system according to samples from individuals without intestinal mucosal lesions and those presenting polyps.

Enzymes	Control (n = 20)	Polyps (n = 34)
Control	0 (0 %)	0 (0 %)
Alkaline phospatase	20 (100 %)	34 (100 %)
Esterase (C4)	1 (5 %)	1 (2.9 %)
Esterase lipase (C8)	0 (0 %)	1 (2.9 %)
Lipase (C14)	0 (0 %)	1 (2.9 %)
Leucine arylamidase	3 (15 %)	7 (20.6 %)
Valine arylamidase	1 (5 %)	3 (8.8 %)
Cystine arylamidase	0 (0 %)	0 (0 %)
Trypsin	0 (0 %)	0 (0 %)
α-Chymotrypsin	0 (0 %)	0 (0 %)
Acid phosphatase	11 (55 %)	20 (58.8 %)
Naphthol-AS-B1-phophohydrolase	11 (55 %)	22 (64.7 %)
α-Galactosidase	1 (5 %)	4 (11.8 %)
β-Galactosidase	9 (45 %)	16 (47.1 %)
β-Glucuronidase	3 (15 %)	5 (14.7 %)
α-Glucosidase	11 (55 %)	30 (88.2 %) *
β-Glucosidase	0 (0 %)	4 (11.8 %)
n-Acetyl-β-lucosaminidase	13 (65 %)	16 (47.1 %)
α-Mannosidase	0 (0 %)	0 (0 %)
α-Fucosidase	0 (0 %)	0 (0 %)

Values are presented as number (percentage in the sample population). (*) Statistically significant differences ($p < 0.05$) in the percentage of samples with high enzymatic activity between diagnosis groups obtained by Chi-square test.

A LefSe analysis was conducted to identify the main microorganisms contributing to differentiate fecal samples displaying high levels of α-glucosidase activity (≥3) and low levels of α-glucosidase activity (<3) (Fig. 2). Genera *Adlercreutzia*, *Erysipelotrichaceae_UCG-003* and *Blautia* were differentially more abundant in the group of high α-glucosidase activity, while *Enterorhabdus*, *Muribaculum*, *Rikenellaceae_RC9_gut* group, *Christensenellaceae_R_7* group, *Oscillospiraceae_NK4A214* group, *Oscillospiraceae_UCG-002*, *Oscillospiraceae_UCG-005* and *Dialister* were more abundant in the group of low α-glucosidase activity. This also involved differential abundances of upper taxa related with these genera; thus, higher differential abundance was obtained for the family *Christensenellaceae* (order *Christensenellales*), family *Muribaculaceae*, family *Veillonellaceae* (order *Veillonelales-Selenomonadales*), family *Oscillospiraceae* (order *Oscillospirales*) and also for the family *Prevotellaceae* in samples from individuals presenting lower α-glucosidase activity.

When comparing the relative abundance of genera differentially affected as a function of the levels of α-glucosidase activity and those that were differential in the comparison of the fecal microbiota from samples showing conventional adenomas vs. the control group analyzed in a previous work [22], we noticed some notable coincidences. Genera *Christensenellaceae_R-7* group and *Oscillospiraceae_UCG-002* were increased both in samples displaying low α-glucosidase levels and in samples from the control group (Fig. 3).

These results point to a possible relationship between gut glucosidase activities, the stage of intestinal mucosal lesions and microbiota profiles.

4. Discussion

Intestinal enzymatic activities play an important role in the digestion and other physiological processes [19]. Alterations of these activities may contribute to an impairment of intestinal homeostasis, with a potential health impact [25]. In the last few decades, some information related to intestinal changes occurring in precursor lesions in the intestinal mucosa that can potentially lead to CRC development has been brought to light [4]. However, the etiopathology of the disease and the previous stages of CRC development are not yet fully understood. In the present work, we followed an affordable approach by using commercial APY® ZYM strips to assess differences in enzymatic activities according to the stage and type of damage of the intestinal mucosa of adult volunteers.

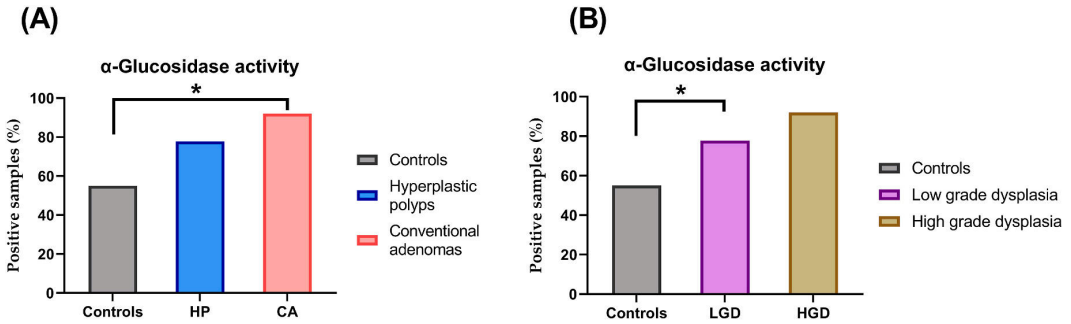

Fig. 1. Percentage of samples displaying high enzymatic activity in the API®-ZYM system according to the histopathological analysis of biopsies from intestinal mucosa. Differences in α-glucosidase with respect to the control group of A) hyperplastic polyps (HP) and conventional adenomas (CA), B) different grades of dysplasia in CA: LGD-low grade dysplasia; HGD-high grade dysplasia. (*) Statistically significant difference ($p < 0.05$) obtained by Chi-square test.

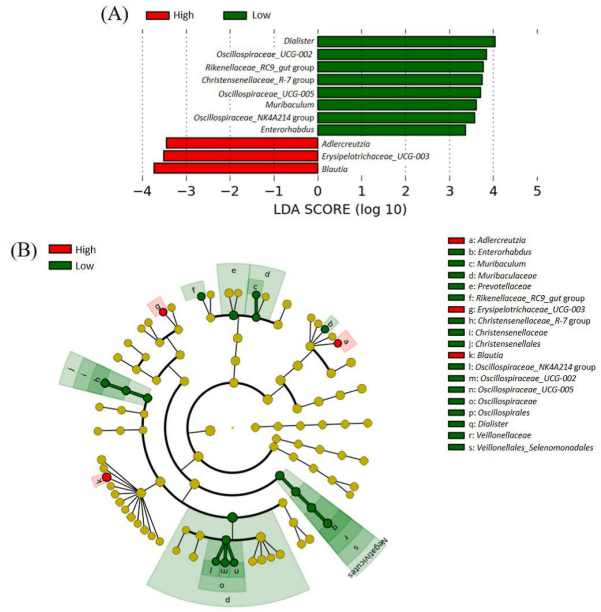

Fig. 2. Bacterial taxa at the taxonomic genus level showing differential abundance according to linear effect size discriminant analysis (LEfSe) for the comparison of A) α-glucosidase activity ≥3 (High) vs. α-glucosidase activity<3 (Low) groups. B) Cladogram generated from LEfSe analysis indicating differences in taxa between samples with high and low α-glucosidase levels. The dots indicate OTUs and each successive circle connected by lines is one step higher phylogenetically. Yellow circles represent bacterial taxa that show no significant differences between groups. Letters embedded in green and red circles represent taxa whose abundance is statistically differential between groups (genus > family > order > class). For LEfSe analyses only genera with relative abundance higher than 1 % in at least two samples were included. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The greater percentage of fecal samples with high levels of α-glucosidase activity found among volunteers diagnosed with intestinal polyps as compared to samples from individuals without intestinal mucosal lesions was the main feature evidenced in the present work when these two clinical diagnosis groups were compared. Moreover, differences with respect to controls in α-glucosidase activity occurred both for hyperplastic polyps and for conventional adenomas as well as for LGD and HGD within the conventional adenomas.

Inhibitors of intestinal α-glucosidase are among the most used hypoglycemic oral drugs for the treatment of T2DM [14]. Individuals suffering from T2DM and/or obesity have an augmented risk of developing CRC [26]. A meta-analysis performed by Zhao et al. showed that the use of α-glucosidase inhibitors was associated with a lower risk of developing CRC in these patients [15] that occurred in a dose-dependent manner for individuals administered with the α-glucosidase inhibitor acarbose [27]. Moreover, in a diabetes and obesity related CRC mice model, the use of the α-glucosidase inhibitor voglibose suppressed colorectal carcinogenesis [28]. Obesity, western diet, and high alcohol consumption are conditions and lifestyle risk factors common to diabetes and CRC [29]. The mechanisms of action suggested in the literature for the protective role of α-glucosidase inhibitors are related with the reduction of the inflammation by decreasing oxidative stress and insulin-like growth factor-1 activity, or by modulating the fecal excretion of primary bile acids such as cholic acid which shows toxic effects [28,30]. A possible hypothesis contributing to explain the reduction of CRC risk by α-glucosidase inhibitors could be related with the Warburg effect, related with the altered metabolism of glucose, taking place in cancerous cells. The Warburg effect is based on the observation that in most cancer cells the production of energy is not through the usual and energy-efficient tricarboxylic acid cycle and oxidative phosphorylation into the mitochondria as occurs in normal cells, but through a less efficient process for which high level of glucose uptake is necessary to enter the glycolysis that is followed by lactic acid formation, this process taking place in the cytosol and even in the presence of oxygen [31]. In this way, increased α-glucosidase activity will lead to more glucose available, that may be preferentially used by altered and potentially malignant cells in the intestinal mucosa. Intriguingly, in the present work we were analyzing samples from individuals without any carcinogenic process in which the α-glucosidase activity seems to be already high, which support the idea that enzymatic and metabolic alterations compatible with a cancerous process may begin very early, with the first changes of the intestinal mucosa and long before the initiation of the cancerous process. It has been reported that Wistar albino rats feed high fat diet and treated with N,N-dimethylhydrazine (DMH), a carcinogenesis inducing drug, displayed higher levels of α-glucosidase activity as compared to the control group fed a conventional diet and absence of treatment [32]; this suggests that diet could also contribute to the expression and modulation of different intestinal enzymes, and to the alterations of the intestinal mucosa.

We have analyzed the microorganisms of the fecal microbiota that contribute the most to differentiate samples with high and low α-glucosidase activity levels, independently on the type and degree of intestinal mucosa damage. In murine models, acarbose treatment was associated with the increased relative abundance of genera such as *Oscillospira, Desulfovibrio* and *Ruminococcus*, suggesting a potential impact of α-glucosidase activity on the gut microbiota [33]. Moreover,

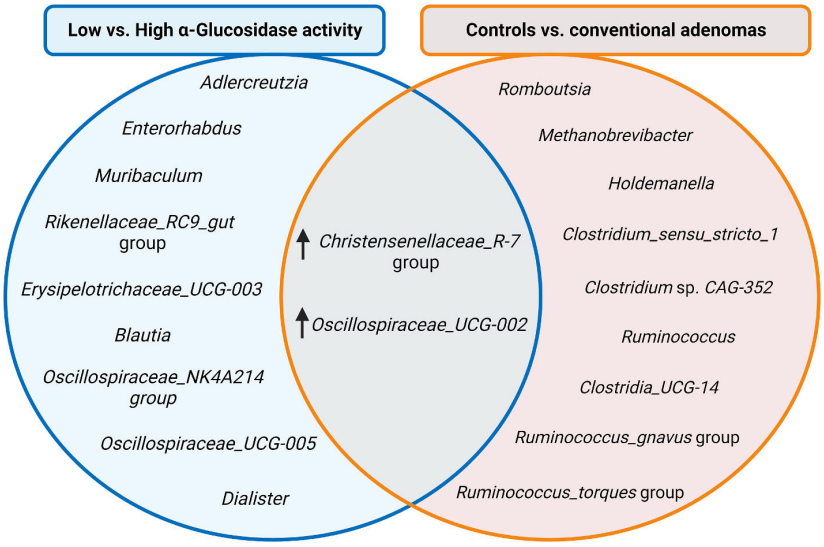

Fig. 3. Venn diagram representing differentially abundant bacterial taxa at the genus level obtained by linear effect size discriminant analysis (LEfSe) according to comparison groups. The blue circle represents bacterial genera differentially abundant resulting from the comparison of fecal samples displaying low vs. high α-glucosidase activity. The orange circle represents bacterial genera differentially abundant resulting from the comparison of fecal samples of volunteers without histopathological lesions in the intestinal mucosa vs. those diagnosed with conventional adenomas as indicated in a previous work [23]. The intersection represents bacterial genera that resulted differential by the two comparisons. Table S1 contains taxonomic upper levels of genera included in this Figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

some lactic acid bacteria have been proposed as probiotics to reduce the intestinal α-glucosidase activity, including strains as *Lacticaseibacillus casei* 2W, *Lacticaseibacillus rhamnosus* Z7, and *L. rhamnosus* LB1lac10 [34,35]. In the present work, we found differentially lower relative abundance of *Christensenellaceae_R-7* group and *Oscillospiraceae_UCG-002* in samples with high α-glucosidase levels; the abundance of these microorganisms was altered in the same way in the group of volunteers diagnosed with conventional adenomas, as we reported recently [22]. A previous study reported higher relative abundance of the *Christensenellaceae_R-7* group in control patients when compared with patients suffering inflammatory gastrointestinal diseases which is associated with augmented CRC risk [36]. However, opposite results showing an increment of *Christensenellaceae_R-7* group in adenoma samples and the association of *Oscillospiraceae_UCG-002* with abnormal health conditions such as altered mood, increased anxiety and memory loss, dizziness or anorexia have also been reported [37,38].

The anaerobic taxonomic families *Christensenellaceae* and *Oscillospiraceae* have been related with metabolic health, although the mechanistic relationship remains to be elucidated [39,40]. In the case of *Christensenellaceae*, these microorganisms seem to be responsive to diet, and its association with metabolic health may in part be due to its association with a diet rich in protein and fiber [39]. A direct association was found between incretins GLP-1 and GLP-2 and the *Oscillospiraceae* family, among other members of the intestinal microbiota, in patients with severe obesity and T2DM submitted to bariatric surgery. This was concomitant with a negative association with metabolic parameters related with glucose tolerance impairment and adiposity [40]. Our results suggest an interrelationship between intestinal mucosa damage, α-glucosidase activity and specific members of the gut microbiota. Based on the current still limited knowledge, the potential role of diet and general metabolic health as drivers of these associations should be considered.

A semi-quantitative standardized method for the screening of different enzymatic activities allowed identifying the intestinal α-glucosidase activity as a key feature connecting intestinal mucosal lesions precursors of CRC and the gut microbiota. Future studies focusing on the quantitative assessment of α-glucosidase, the characterization of encoding genes and the possible contribution of members of the intestinal microbiota to the intestinal activity could help to disentangle the role of α-glucosidase and its connection with the microbiota in the progression of intestinal mucosa lesions leading to CRC.

5. Conclusions

In this work, we report a relationship among altered fecal α-glucosidase levels, the presence of intestinal mucosal lesions which can be precursors of CRC and shifts in defined microbial groups of the fecal microbiota. Although a causality cannot be established, deeper studies to decipher the relationships among these parameters could contribute to the comprehension of the association between the intestinal microbiota and CRC and the influence of the intestinal environment in this binomial.

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Availability of data and materials

The datasets generated during metagenomic sequencing of fecal DNA samples were deposited in the NCBI Sequencing Read Archive PRJNA994445 (http://www.ncbi.nlm.nih.gov/bioproject/994445).

Ethics approval and consent to participate

This study is part of broader projects related with the effect of diet and dietary xenobiotics on intestinal mucosa and related gut microbiota profiles in the context of CRC (MIXED and MiToxicDiet). The study was approved by the Regional Ethics Committee of the Clinical Research of Asturias (Ref. 163/19), and Committee on Bioethics of CSIC (Ref. 174/2020). All participants provided informed consent before their inclusion in the study.

CRediT authorship contribution statement

Sergio Ruiz-Saavedra: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Nuria Salazar:** Writing – review & editing, Methodology, Investigation. **Adolfo Suárez:** Writing – review & editing, Resources. **Ylenia Diaz:** Writing – review & editing, Resources. **Carmen González del Rey:** Writing – review & editing, Supervision, Methodology. **Sonia González:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Clara G. de los Reyes-Gavilán:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during metagenomic sequencing of fecal DNA samples were deposited in the NCBI Sequencing Read Archive PRJNA994445 (http://www.ncbi.nlm.nih.gov/bioproject/994445).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2024.102853.

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MATERIAL SUPLEMENTARIO

Table S1. Taxonomic classification of genera (Table 3) displaying differences in α-glucosidase levels and/or between Controls and Conventional adenomas

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacillota	Negativicutes	Veillonellales-Selenomonadales	Veillonellaceae	Dialister
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculum
Bacteria	Bacillota	Clostridia	Lachnospirales	Lachnospiraceae	Blautia
Bacteria	Bacillota	Clostridia	Christensenellales	Christensenellaceae	Christensenellaceae_R-7 group
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut group
Bacteria	Bacillota	Clostridia	Oscillospirales	Oscillospiraceae	Oscillospiraceae_UCG-002
Bacteria	Bacillota	Bacilli	Erysipelotrichales	Erysipelatoclostridiaceae	Erysipelotrichaceae_UCG-003
Bacteria	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	Adlercreutzia
Bacteria	Actinobacteriota	Coriobacteriia	Coriobacteriales	Eggerthellaceae	Enterorhabdus
Bacteria	Bacillota	Clostridia	Oscillospirales	Oscillospiraceae	UCG-005
Bacteria	Bacillota	Clostridia	Oscillospirales	Oscillospiraceae	Oscillospiraceae_NK4A214 group
Bacteria	Bacillota	Clostridia	Peptostreptococcales-Tissierellales	Peptostreptococcaceae	Romboutsia
Bacteria	Bacillota	Clostridia	Oscillospirales	Ruminococcaceae	CAG-352
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Bacteria	Bacillota	Clostridia	Clostridiales	Clostridiaceae	Clostridium_sensu_stricto_1
Bacteria	Bacillota	Bacilli	Erysipelotrichales	Erysipelotrichaceae	Holdemanella
Bacteria	Bacillota	Clostridia	Lachnospirales	Lachnospiraceae	Ruminococcus_gnavus group
Bacteria	Bacillota	Clostridia	Clostridia_UCG-014	Clostridia_UCG-014	Clostridia_UCG-014
Bacteria	Bacillota	Clostridia	Lachnospirales	Lachnospiraceae	Ruminococcus_torques_group
Bacteria	Bacillota	Clostridia	Oscillospirales	Ruminococcaceae	Ruminococcus
Bacteria	Bacillota	Negativicutes	Veillonellales-Selenomonadales	Veillonellaceae	Dialister
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	Muribaculum
Bacteria	Bacillota	Clostridia	Lachnospirales	Lachnospiraceae	Blautia
Bacteria	Bacillota	Clostridia	Christensenellales	Christensenellaceae	Christensenellaceae_R-7 group
Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut group
Bacteria	Bacillota	Clostridia	Oscillospirales	Oscillospiraceae	Oscillospiraceae_UCG-002
Bacteria	Bacillota	Bacilli	Erysipelotrichales	Erysipelatoclostridiaceae	Erysipelotrichaceae_UCG-003

Objetivo 3:

Estudio de la asociación de factores dietéticos protectores y de riesgo para el desarrollo de cáncer colorrectal con el perfil de la microbiota fecal

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Diversos estudios epidemiológicos señalan que ciertos factores dietéticos pueden favorecer o proteger frente al desarrollo de cáncer colorrectal. Considerando la estrecha relación entre la microbiota intestinal y la dieta, y en base a los resultados obtenidos en los Objetivos 1 y 2, resultaba de interés evaluar la asociación de los factores dietéticos identificados en los trabajos previos con la microbiota intestinal, en función de la presencia de daño en la mucosa intestinal. Los resultados obtenidos se incluyeron en el artículo científico que se indica a continuación:

> **Artículo 5.** Ruiz-Saavedra, S.; González del Rey, C.; Suárez, A.; Diaz, Y.; Zapico, A.; Arboleya, S.; Salazar, N.; Gueimonde, M.; González, S.; de los Reyes-Gavilán, C.G. Associations of Dietary Factors and Xenobiotic Intake with Faecal Microbiota Composition According to the Presence of Intestinal Mucosa Damage. *Food & Function* **2023**, *14*, 9591-9605. doi: 10.1039/d3fo01356a

Los voluntarios del estudio se clasificaron en función del diagnóstico clínico como individuos controles o individuos con presencia de pólipos intestinales, según se mencionaba en el Objetivo 1. La abundancia relativa microbiana fecal se obtuvo según lo descrito en el Objetivo 2. Los factores dietéticos evaluados incluyeron alimentos y compuestos de la dieta, así como algunos xenobióticos. La ingesta individual de cada factor se pudo calcular a partir de la evaluación nutricional llevaba a cabo en el Objetivo 1. El punto de corte de ingesta para cada factor se estableció en base al valor mínimo de consumo que estudios epidemiológicos previos realizados por otros autores, asociaban directamente a un aumento o disminución en la incidencia de cáncer colorrectal.

Se observó que factores como el consumo de carnes rojas ≥50 g/día o la ingesta de hidrocarburos aromáticos policíclicos totales ≥0,75 µg/día se asociaban con un aumento de la abundancia relativa de la familia *Coriobacteriaceae* y una disminución de la familia *Bacteroidaceae* en el grupo control. Además, en este mismo grupo de diagnóstico, una elevada ingesta de aminas heterocíclicas como PhIP y MeIQx se relacionó con una menor abundancia relativa de *Akkermansiaceae* mientras que una alta ingesta de nitritos y NDMA se asoció con una menor abundancia relativa de *Bifidobacteriaceae*. En el grupo de pólipos, una ingesta ≥12 g/día de etanol se asoció con una abundancia relativa aumentada de la familia *Peptostreptococcaceae* y disminuida de la familia *Veillonellaceae*. Además, la ingesta de compuestos bioactivos como los polifenoles o la celulosa insoluble se identificaron como factores predictores de las variaciones en la abundancia relativa de ciertos grupos microbianos de la microbiota fecal.

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Associations of dietary factors and xenobiotic intake with faecal microbiota composition according to the presence of intestinal mucosa damage†

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Diet is a major modulator of gut microbiota, which plays a key role in the health status, including colorectal cancer (CRC) development. Several studies and meta-analyses have evidenced an association of certain dietary factors and xenobiotic intake with the incidence of CRC. Nevertheless, how these dietary factors impact the first stages of intestinal mucosa damage is still uncertain. This study aimed at exploring the associations of relevant dietary factors with the gut microbiota of control individuals and subjects diagnosed with intestinal polyps. A total of 60 volunteers were recruited, clinically classified according to colonoscopy criteria and interviewed using food frequency questionnaires (FFQs). The nutritional status of each volunteer was determined and the intake of dietary xenobiotics was quantified. The relative abundance of faecal microbiota taxonomic groups was obtained through 16S rRNA gene sequencing. The association of dietary factors and xenobiotics with faecal microbiota composition showed differences according to the clinical diagnosis group. Our results showed that the intake of red meat (≥50 g day^{-1}) and total polycyclic aromatic hydrocarbons (PAHs) (≥0.75 μg day^{-1}) was associated with a decreased abundance of the family Bacteroidaceae and an increased abundance of Coriobacteriaceae in control subjects. The intake of the heterocyclic amines 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) (≥40 ng day^{-1}) and 2-amino-3,8 dimethylimidazo(4,5,*f*) quinoxaline (MeIQx) (≥50 ng day^{-1}) was associated with a decreased abundance of Akkermansiaceae in the control diagnosis group. Moreover, *N*-nitroso compounds (NOCs), nitrites (≥1.69 mg day^{-1}) and *N*-nitrosodimethylamine (NDMA) (≥0.126 μg day^{-1}) were associated with a decreased abundance of Bifidobacteriaceae. The intake of ethanol (≥12 g day^{-1}) in the polyps group was associated with an increased abundance of Peptostreptococcaceae and a decreased abundance of Veillonellaceae. Moreover, linear regression analyses allowed us to identify ethanol, calcium, bioactive compounds such as flavonoids, stilbenes, cellulose, phenolic acids or total polyphenols, and dietary xenobiotics such as PhIP and MeIQx, the NOC *N*-nitrosopyrrolidine (NPYR) or the total PAHs as potential predictors of faecal microbiota group abundances. These results indicated that the consumption of milk, red meat, processed meat and ethanol and the intake of polyphenols, dietary PAHs, HAs and NOCs are associated with specific groups of the intestinal microbiota, depending on the clinical diagnosis group.

1. Introduction

Colorectal cancer (CRC) has been estimated to be responsible for more than 1.9 million new cancer cases and 935 000 deaths worldwide.[1] Despite the efforts made through screening programs, the incidence of CRC is still rising.[2] Besides genetic factors, environmental factors, with a special focus on diet, are proposed to account for at least 70% of CRC cases.[3,4] Several studies, including the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD), pointed to certain risk factors for CRC, such as the consumption of red meat, processed meat and ethanol, and also to protective ones such as

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the consumption of milk, fibre, polyphenols and calcium.[5–8] These findings were in accordance with previous data from the International Agency for Research on Cancer (IARC), the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR).[9,10] Therefore, diet as a whole becomes a key factor for CRC development. Apart from the traditional components, diet can be a source of ethanol and xenobiotic compounds such as heterocyclic amines (HAs), polycyclic aromatic hydrocarbons (PAHs), acrylamide and *N*-nitroso compounds (NOCs) largely formed during the processing and cooking of fish, meat and other foods at high temperature.[11] The final toxicity of these compounds results from their absorption and metabolic transformation, in which intestinal microbiota is involved. For example, the intestinal microbiota can modulate the absorption of xenobiotic compounds by altering the intestinal permeability or modifying the thickness of the intestinal mucus layer.[12] Moreover, gut microbes are also capable of transforming compounds, resulting in others with increased or decreased toxicity depending on the intestinal microbiota profile of the host.[13] Direct binding of microorganisms to toxic compounds and the excretion of the latter in faeces are also possible and may contribute to the decrease in the damage in the host.[14] Finally, conjugated molecules, ready to be excreted after their transformation by host phase II enzymes, can be reactivated by the gut microbiota as occurs with hydrolytic reactions carried out by β-glucuronidases.[15,16] Nevertheless, this relationship is not unidirectional, as the gut microbiota can also be altered by the intake of dietary xenobiotics. In humans, a shift from healthy diets to poor quality ones, such as the so-called "western" pattern diet, promotes a decrease in the consumption of bioactive compounds, tends to enrich the intake of potentially carcinogenic compounds and may also modify the composition of the gut microbiota.[8,17–19] In this sense, faecal samples represent a useful material to study changes occurring in the gut microbiota along disease development. The enrichment of species such as *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Escherichia coli* or *Streptococcus bovis* has been reported in faecal samples from individuals diagnosed with CRC. These microorganisms are associated with the promotion of tumorigenesis.[20] *Bacteroides*, *Prevotella*, *Porphyromonas*, *Enterococcus* or *Streptococcus* genera have also been found to be elevated in faecal samples from individuals diagnosed with CRC.[21–24] Precancerous states also exhibited alterations in the gut microbiota. Thus, when the samples of patients with adenoma polyps were compared with healthy individuals, a decrease in families Ruminococcaceae, Clostridiaceae, and Lachnospiraceae was found, while the classes Bacilli and Gammaproteobacteria (order Enterobacterales) were increased.[25] The hypothesis proposed in this work is that dietary factors can modulate the intestinal microbiota composition in different ways depending on the stage of mucosal damage in CRC development. To test it, major dietary components previously reported to be associated with CRC development were evaluated to assess their impact on the composition and activity of the faecal microbiota according to the clinical presence of mucosal damage in the form of intestinal polyps.

2. Materials and methods

2.1 Study design and volunteers

Data used in this study have been obtained in the context of the project "Effect of diet and exposure to xenobiotics generated during food processing on the genotoxic/cytotoxic capacity of the intestinal microbiota" (MIXED).

Physicians of the Digestive Service from the Central University Hospital of Asturias (HUCA) and the Carmen and Severo Ochoa Hospital, both located in Asturias, the northern region of Spain, have performed the recruitment of the sample population. Volunteers were randomly selected from those subjects who participated in a screening program of CRC and who fulfilled the inclusion criteria: age between 40 and 75 years, absence of cancer history, digestive pathology, immune-related diseases or drug treatment. Volunteers were recruited at the time of the first interview and scheduled for colonoscopy. Prior to enrolling, all the participants were informed about the objectives and procedures of the study. Once they agreed to participate, an informed consent form was signed. Human samples of faeces and anamnesis, and analytical data were also collected at the time of recruitment, as described elsewhere.[26] A total of 60 volunteers for which dietary information and faecal samples were available were included in the study. According to the screening colonoscopy results, volunteers were classified as controls not showing any alteration of the intestinal mucosa (n = 25), or polyps (n = 35) in the case of those volunteers showing the presence of one or more intestinal polyps in the colonic mucosa. This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures stated in the Declaration of Helsinki, the Oviedo Bioethics Convention, the Council of Europe Convention on Human Rights and Biomedicine, as well as in the Spanish legislation on bioethics were strictly followed.

2.2. Nutritional assessment and anthropometry

Dietary information was obtained from a validated semi-quantitative food-frequency questionnaire (FFQ) conducted by trained interviewers.[26,27] This survey was performed at the time of medical consultation for the results of colonoscopy. The FFQ was composed of 155 food items, and portion sizes were obtained using the Pilot Study for Assessment of Nutrient Intake and Food Consumption Among Kids in Europe (PANCAKE).[28] Moreover, the FFQ included specific questions regarding culinary preparations, type of food (*i.e.* chicken breast or thigh), consumption habits (*i.e.* thigh cooked and consumed with or without skin), cooking methods (*i.e.* boiled, fried, *etc.*) and degree of doneness (rare, medium, well done, very well done). The last question was accompanied by standardized pictures to illustrate the degree of cooking for meats,

fried potatoes and bread. The Higher Education in Nutrition and Dietetics (CESNID) criteria was followed for the classification of food into groups.[29] The nutritional information was obtained from the food composition tables of CESNID and the United States Department of Agriculture (USDA).[29,30] Energy and macronutrient intake data were completed with information about phenolic compounds and dietary fibre consumption, extracted from Phenol Explorer 3.6 and the studies by Marlett and Cheung.[31,32] Oxygen Radical Activity Capacity (ORAC) was calculated from the article by Wu *et al.*[33] After fulfilling the FFQ, height (m) and weight (kg) of each volunteer were determined and the BMI was calculated using the formula weight (kg)/height $(m)^2$. The criteria of the Spanish Society for the Study of Obesity (SEEDO) were applied to classify the sample into normal weight (18.5–24.9 kg m^{-2}), overweight (25.0–29.9 kg m^{-2}), and obese (≥30.0 kg m^{-2}). Dietary data generated in this study are available from authors under reasonable request.

2.3. Xenobiotic intake estimation

The content of HAs, PAHs, NOCs and acrylamide xenobiotics were obtained and compiled from the European Prospective Investigation into Cancer and Nutrition (EPIC) carcinogen database.[34] This information was completed with data from the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED), the European Food Safety Authority (EFSA), the U.S. Food Drug Administration (FDA) along with other references.[35–46] The content of these compounds was estimated by considering the preservation method, type of cooking, degree of browning and temperature.

2.4. Dietary factors

These xenobiotics together with certain dietary variables showing protective or hazardous roles in previous studies and meta-analyses with defined intake cut-off values were included in the analyses. From the GBD,[5] the selected variables and their cut-off values were as follows: milk ≥120 g day^{-1}, red meat ≥50 g day^{-1}, processed meat ≥25 g day^{-1}, ethanol ≥12 g day^{-1}, fibre ≥20 g day^{-1} and calcium ≥900 mg day^{-1}. From the Southern Community Cohort Study:[47] total polyphenols ≥650 mg day^{-1}. From the EPIC:[48] *N*-nitrosodimethylamine (NDMA) ≥0.125 μg day^{-1}. From the study conducted by Martínez Góngora *et al.*:[49] amino-1-methyl-6-phenylimidazo (4,5-*b*)pyridine (PhIP) ≥40 ng day^{-1} and 2-amino-3,8 dimethylimidazo (4,5,*f*) quinoxaline (MeIQx) ≥50 ng day^{-1}. Moreover, those dietary variables showing significant associations ($p < 0.05$) or closeness to significance ($p < 0.10$) with shifts in the risk of being in the group of volunteers diagnosed with polyps in a previous study of our research team conducted with the same sample of volunteers were also included:[26] soluble pectin ≥0.57 g day^{-1}, flavonoids ≥82.18 mg day^{-1}, other polyphenols ≥32.15 mg day^{-1}, nitrites ≥1.69 mg day^{-1}, dibenzo (a) anthracene (DiB(a)A) ≥0.07 μg day^{-1} and total PAH ≥0.75 μg day^{-1}.

2.5. Faecal samples' processing

A total of 4 g of frozen faecal samples were weighed, diluted 1/10 and homogenized with sterile PBS in a LabBlender 400 Stomacher (Seward Medical, London, UK) for 3 min at the maximum speed. After 15 min centrifugation at 4° C and 14 000 rpm, supernatants and pellets were separated and kept frozen at −20 °C until use.

2.6. Faecal short chain fatty acids

The concentration of the major short chain fatty acids (SCFAs) such as acetic, propionic, butyric as well as isobutyric, isovaleric, valeric and caproic acids in the faeces was determined through the procedure previously described, with some minor modifications.[50] Faecal supernatants were diluted with methanol and 20% v/v formic acid; an internal standard reaching a dilution of 10/65 was added to the sample. Then, preparations were centrifuged for 10 min at room temperature and 14 000 rpm and the obtained supernatants were transferred to suitable chromatography vials. A chromatograph 6890N (Agilent Technologies Inc., Palo Alto, CA, USA) connected to a mass spectrometry detector (MS) 5973N (Agilent Technologies) and a flame ionization detector (FID) was used for the identification and quantification of SCFAs. Theoretical detection limit values were calculated for less abundant SCFAs and applied to those samples under the limit of detection.

2.7. Faecal amino acids and biogenic amines

Amino acid and biogenic amine levels in faeces were determined in faecal supernatants using the method described by Redruello *et al.* and adapted to the faecal supernatants analyzed by Salazar *et al.*[51,52] Briefly, the faecal supernatants were filtered through 3 kDa centrifugal filters (Amicon Ultra-0.5, Merck KGaA, Germany) and derivatized using diethyl ethoxymethylenemalonate (DEEMM, Sigma-Aldrich, USA). Before injection, the samples were also filtered using 0.22 μm-pore diameter polytetrafluoroethylene membranes (VWR International, USA). An H-Class Acquity UPLC™ system (Waters, Milford, MA, USA) coupled to a photodiode array detector at 280 nm was used to quantify amino acids and biogenic amines. The detection limit values were applied when necessary.

2.8. DNA extraction and metataxonomic analyses

The pellets obtained after the dilution and homogenization of faeces were used to extract the DNA. Q protocol for DNA extraction defined by the International Human Microbiome Standards Consortium was applied using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Sussex, UK).[53] After extraction, the 260/280 ratio was quantified using a Take3 Micro-Volume plate and a Gen5 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). DNA was kept frozen at −20 °C until analysis. The whole procedure of bacterial 16S rRNA gene sequencing and annotation was conducted at Novogene Bioinformatics Technology Co., Ltd. First, the variable region V3–V4 of bacterial 16S rRNA genes was amplified by PCR using

specific primers (341F and 806R) connected with barcodes and the DNA library was prepared. Libraries were sequenced on an Illumina NovaSeq 6000 platform instrument. The obtained individual reads were assigned to samples using barcodes and merged using FLASH (V 1.2.7).[54] High quality clean tags were obtained by filtration using QIIME (V 1.7.0), allowing for the removal of low-quality sequences.[55,56] The tags were then compared with the reference SILVA 138 database and chimera sequences were removed using the UCHIME algorithm.[57,58] Effective tags were utilized by Uparse software (Uparse V 7.0.1090) to perform the sequencing analysis. Sequences sharing ≥97% homology were assigned to the same OTUs, and the OTU abundance was normalized. To annotate species at each taxonomic rank, the representative sequence for each OTU was determined against the SSUrRNA database of the SILVA138 database using QIIME (V 1.7.0) in the Mothur method.[59] Moreover, to obtain the phylogenetic relationship of all OTU representative sequences, MUSCLE (V 3.8.3) was used.[60] The ACE index of alpha diversity was obtained from QIIME (V 1.7.0). The datasets generated during metataxonomic sequencing of faecal DNA samples in the MIXED project were deposited in the NCBI Sequencing Read Archive PRJNA994445 (**https://www.ncbi.nlm.nih.gov/bioproject/994445**).

2.9. Statistical analyses

The data obtained were analysed using SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, USA), RStudio software version 1.4.3 and Galaxy web tool.[61] GraphPad Prism 9 and RStudio software were used for graphical representations. Goodness of fit to the normal distribution was checked by means of the Kolmogorov–Smirnov test. As normality of the metabolite variables (SCFAs, amino acids and biogenic amines) was not achieved, Mann–Whitney *U* tests were performed to detect group differences. Linear discriminant analysis effect size (LEfSe) analyses were conducted to differentiate at the family taxonomic level the microorganisms contributing to discriminate between study groups by using the Galaxy web tool.[61] These analyses consist of a Kruskal–Wallis sum-rank test and a Wilcoxon test for pairwise comparison, followed by a logarithmic linear discriminant analysis (LDA) to estimate the effect-size at a threshold of 2.0. To determine whether the different diagnosis groups could lead to a statistically significant influence on the relative abundance of microbiological families, a MANOVA analysis was performed. In this multivariate test, clinical diagnosis group levels were selected as the explanatory variables, while the most abundant bacterial families in the sample (relative abundance >1%) were introduced as the dependent variables. Abundance-based coverage estimator (ACE) index differences of alpha diversity were evaluated according to dietary consumption and diagnosis groups by the Mann–Whitney tests. To explore the associations between microbial families and metabolic and food group variables, Spearman correlation analyses were performed. Significant correlations were adjusted for multiple tests using the Benjamini–Hochberg procedure. Heatmaps were generated using the "pheatmap" R package. Redundancy analyses (RDA) of food groups and dietary compounds were performed using the "vegan" R package. To discard collinearity, bioactive and potentially carcinogenic compounds were examined as predictors of gut microbial families, faecal SCFAs and amino acids by regression analyses, adjusting by gender and BMI to reduce their potential confounding role on CRC as previously described.[62,63]

3. Results

General characteristics and anthropometric parameters of the sample are presented in Table 1 according to the clinical diagnosis groups. No significant differences were found for the variables considered between groups.

3.1. Differential microbiota profiles according to dietary intake

The relative abundance of the faecal microbiota in the samples at the family taxonomic level and metabolites is presented in Table S1† according to the clinical diagnosis groups. The faecal microbial composition of volunteers was then analysed according to dietary factors for each diagnosis group (Fig. 1). Given the high taxonomic complexity of the microbiota, we focused the present study to the family level, in order to not split results and maintain the main aim of the current work. When milk consumption was evaluated, control volunteers with an intake under 120 g day^{-1} showed lower abundances of Peptostreptococcaceae, Coriobacteriaceae and Erysipelotrichaceae and higher abundance of Bacteroidaceae (Fig. 1A) (Table S2†). A higher abundance of Akkermansiaceae was detected in the polyps group with milk intake above 120 g day^{-1}. A differentially increased abundance of Coriobacteriaceae and decreased abundance of Bacteroidaceae and Akkermansiaceae was observed in control volunteers consuming more than 50 g of red meat per day compared to those whose consumption of this food was lower (Fig. 1B) (Table S3†). On the other hand, the consumption of ≥25 g of processed meat per day was accompanied by a reduced abundance of Bifidobacteriaceae and by an increased abundance of Prevotellaceae and Erysipelatoclostridiaceae in the control group, while these families were also increased in the group diagnosed with polyps consuming high levels of processed meat (Fig. 1C) (Table S4†). When fibre consumption was ana-

Table 1 General characteristics of the study sample according to clinical diagnosis

	Control (n = 25)	Polyps (n = 35)
Male gender	12 (48.00%)	18 (51.40%)
Age (years)	58.08 ± 9.61	61.03 ± 6.31
Energy intake (kcal day^{-1})	2036.90 ± 752.13	2117.44 ± 850.13
BMI (kg m^{-2})	26.22 ± 3.88	27.70 ± 3.90

Values are shown as mean ± standard deviation for continuous variables or number and percentage (%) for the categorical ones. BMI, body mass index.

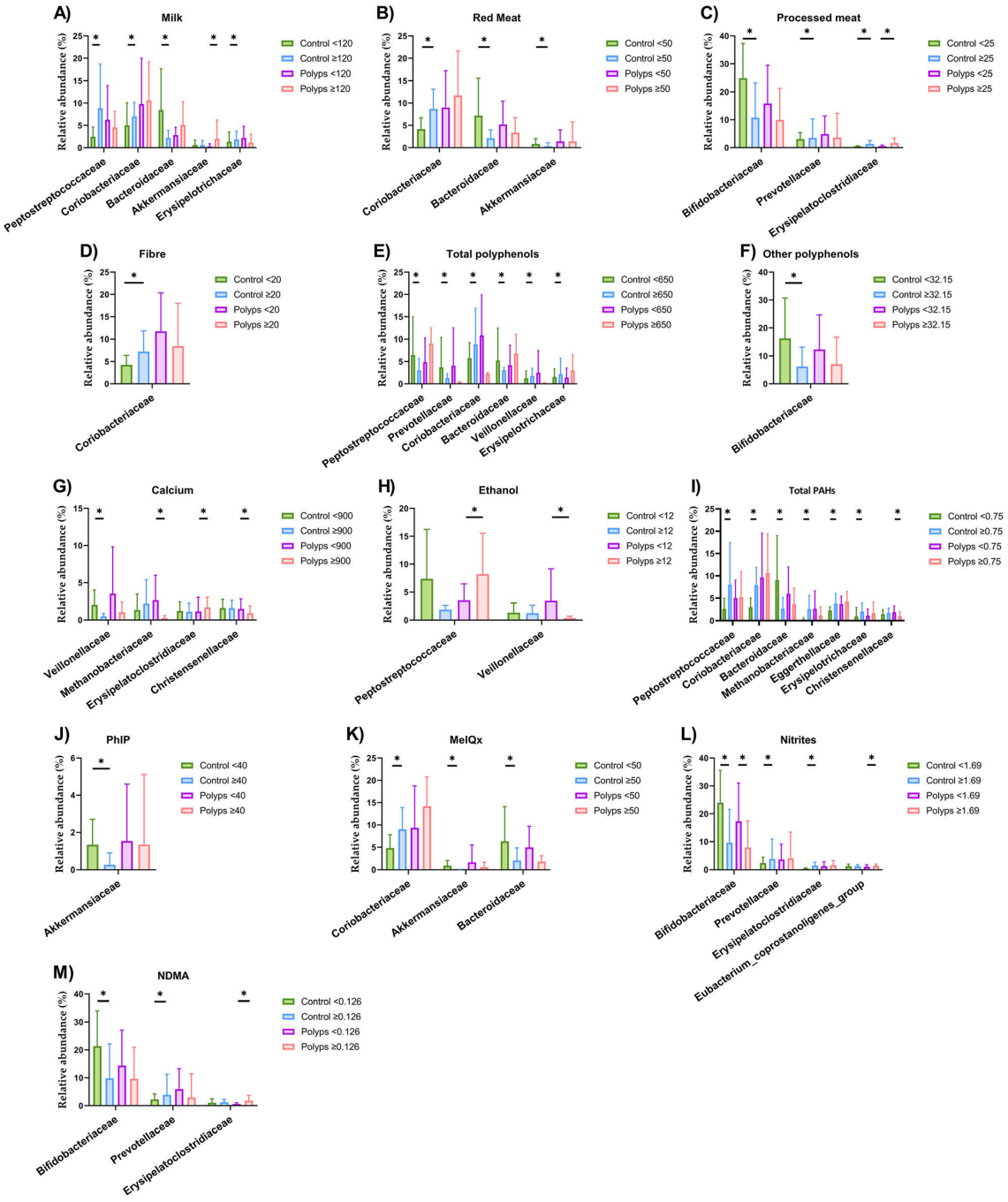

Fig. 1 Differential abundance according to linear effect size discriminant analysis (LEfSe) at the taxonomical family level in the faecal microbiota of volunteers according to the clinical diagnosis group and cut-off intake values for (A) milk (g day^{-1}), (B) red meat (g day^{-1}), (C) processed meat (g day^{-1}), (D) fibre (g day^{-1}), (E) total polyphenols (mg day^{-1}), (F) other polyphenols (mg day^{-1}), (G) calcium (mg day^{-1}), (H) ethanol (g day^{-1}), (I) total PAH (μg day^{-1}), (J) PhIP (ng day^{-1}), (K) MeIQx (ng day^{-1}), (L) nitrites (mg day^{-1}) and (M) NDMA (μg day^{-1}). Only families showing relative abundances higher than 1% in the sample are represented. Total PAHs, total polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,*f*) quinoxaline; NDMA, *N*-nitrosodimethylamine.

lysed, increased concentrations of isobutyric and isovaleric acids and Coriobacteriaceae relative abundance were found in the control volunteers consuming ≥20 g fibre per day (Fig. 1D) (Table S5†). Moreover, no differences were found in faecal microbiota abundances when soluble pectin intake was examined (Table S6†). Regarding total polyphenols, increased abundances of Coriobacteriaceae, Veillonellaceae and Erysipelotrichaceae and decreased abundances of Peptostreptococcaceae, Prevotellaceae and Bacteroidaceae were detected in the control group with intake of total polyphenols ≥650 mg day^{-1} (Fig. 1E) (Table S7†). Among them, the intake of flavonoids (≥82.18 mg day^{-1}) was not associated with any shift in microbial families (Table S8†). Furthermore, the control group with an intake of other polyphenols ≥32.15 mg day^{-1} showed a decreased abundance of Bifidobacteriaceae and higher concentrations of SCFAs such as acetic, butyric and propionic acids (Fig. 1F) (Table S9†).

A lower abundance of the family Veillonellaceae was detected in the control group with intake of calcium ≥900 mg day^{-1} (Fig. 1G) (Table S10†). Furthermore, the polyps group showing higher intake of calcium revealed an increase in the abundance of Erysipelatoclostridiaceae and in the concentrations of acetic and propionic acids, together with a decrease in the abundance of Methanobacteriaceae and Christensenellaceae. Regarding xenobiotics, in those subjects diagnosed with intestinal polyps, higher ethanol intake was concomitantly associated with a reduced abundance of Veillonellaceae and increased abundance of Peptostreptococcaceae (Fig. 1H) (Table S11†). No statistically significant differences were found according to DiB(a)A intake (Table S12†). When the dietary consumption of total PAHs was evaluated, many differences were revealed in microbial faecal abundances and SCFA concentrations (Fig. 1I) (Table S13†). The control volunteers with a total PAH intake of ≥0.75 μg day^{-1} displayed increased abundances of Peptostreptococcaceae, Coriobacteriaceae, Eggerthellaceae and Erysipelotrichaceae in contrast to the decreased abundance of Bacteroidaceae. The control group also showed higher concentrations of isobutyric and isovaleric acids together with total amino acids and protein amino acids in the faeces. In the polyps group, the total PAH intake of ≥0.75 μg day^{-1} was associated with a decreased abundance of Christensenellaceae and higher concentrations of acetic acid, propionic acid and biogenic amines. Control volunteers with PhIP intake higher than 40 ng day^{-1} revealed a decreased abundance of Akkermansiaceae (Fig. 1J) (Table S14†). Also, in the control diagnosis group, a decreased abundance of Bacteroidaceae and increased abundance of Erysipelatoclostridiaceae was detected when the intake of MeIQx was higher than 50 ng day^{-1} (Fig. 1K) (Table S15†). In both the control and polyps groups, the intake of ≥1.69 mg day^{-1} of nitrites was associated with a decreased abundance of Bifidobacteriaceae (Fig. 1L) (Table S16†). Moreover, in the control group, higher intake of nitrites was associated with an increased abundance of Prevotellaceae and Erysipelatoclostridiaceae, while in the polyps groups, this increase occurred in the *Eubacterium coprostanoligenes* group. When the intake of NDMA was evaluated, a reduction in Bifidobacteriaceae abundance accompanied by an increase in Prevotellaceae was observed for the control volunteers with consumption ≥0.126 μg day^{-1}, while in the polyps group, this intake was associated with an increased abundance of Erysipelatoclostridiaceae (Fig. 1M) (Table S17†).

A MANOVA analysis was conducted to determine whether the clinical diagnosis groups and microbial profiles were associated. The value observed for the multivariate significance test was not statistically significant (overall F statistic: 0.932, *p*-value: 0.551).

3.2. Microbiological richness according to dietary intake

To determine the differences in the alpha diversity according to the dietary intake in each diagnosis group, ACE index values were evaluated (Fig. 2). In the control group, richness decreased significantly with elevated consumption of red meat (≥50 g day^{-1}) and total PAH intake (≥0.75 μg day^{-1}). Moreover, in the subjects diagnosed with polyps, DiB(a)A intake ≥0.07 μg day^{-1} was associated with a lower ACE index.

3.3. Food groups, faecal metabolites and microbiota correlations

Exploration into the association of food groups with faecal microbiota at the taxonomic family level was conducted through Spearman correlation analyses and was represented by heatmaps (Fig. 3A).

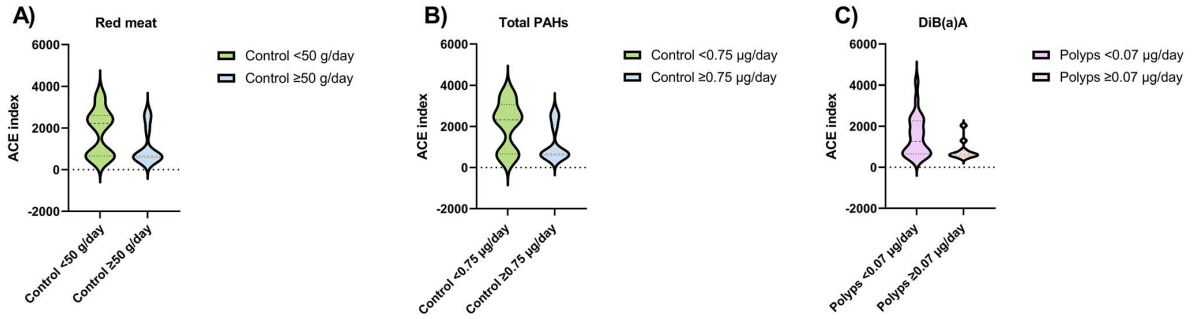

Fig. 2 Statistically significant differences in alpha diversity (ACE index) obtained from the faecal microbiota of volunteers according to clinical diagnosis group and cut-off intake values for (A) red meat (g day^{-1}), (B) total PAH (μg day^{-1}) and (C) DiB(a)A (μg day^{-1}). ACE, abundance-based coverage estimator; total PAHs, total polycyclic aromatic hydrocarbons; DiB(a)A, dibenzo (a) anthracene.

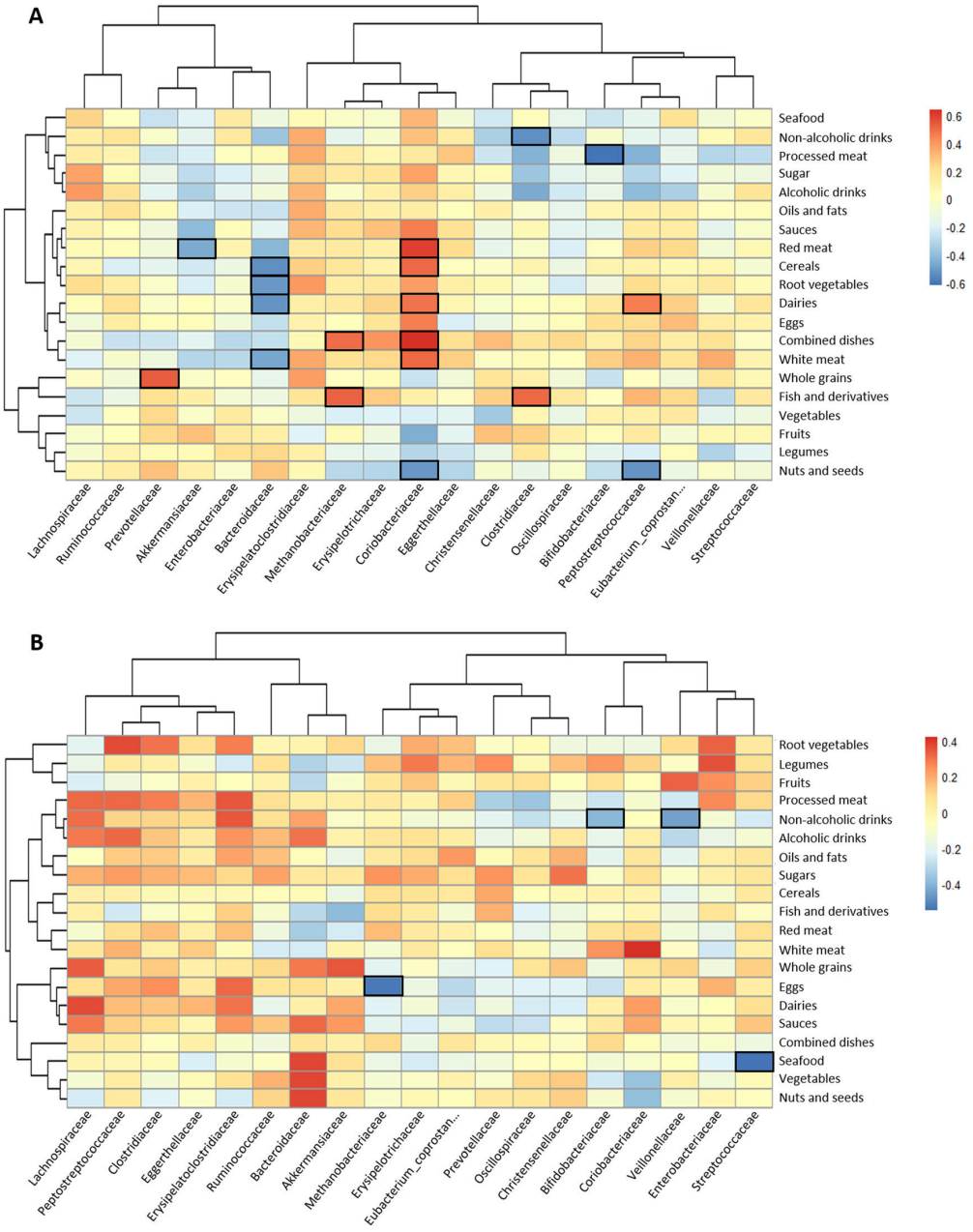

Fig. 3 Heatmaps defined by Spearman correlations between food groups and faecal microbiota at the taxonomic family level for the most abundant bacterial families (relative abundance ≥1%) for clinical diagnosis groups: (A) control or (B) polyps. Blue and red colours denote negative and positive associations, respectively. The intensity of the colour is proportional to the degree of association between variables. Bordered cells indicate a statistically significant association adjusted for multiple testing by the Benjamini–Hochberg procedure.

In the controls, the consumption of processed meat was negatively correlated with the relative abundance of Bifidobacteriaceae in the faeces (Fig. 3A), whereas red meat showed an inverse association with Akkermansiaceae and a direct association with Coriobacteriaceae. In contrast, the abundance of this last family was inversely correlated with nuts and seeds. Bacteroidaceae was inversely linked to the consumption of cereals, root vegetables and dairy products. Clostridiaceae family was negatively correlated with non-alcoholic drinks and positively correlated with fish and derivatives.

In polyps, only statistically significant inverse relationships were found: sea food with Streptococcaceae, eggs with Methanobacteriaceae, and non-alcoholic drinks with Veillonellaceae and Bifidobacteriacae (Fig. 3B). In contrast, abundant correlations were found for some food groups and faecal metabolites (Fig. 4). In the control group, branched chain amino acids isobutyric and isovaleric directly correlated with white meat and fish and derivatives (Fig. 4A). The former food group also correlated with total amino acids and ammonium. Non-alcoholic drinks were positively associated with most of the variables except for biogenic amines and caproic acid. On the other hand, in the group of volunteers diagnosed with polyps, red meat, dairy products and processed meat were positively correlated with biogenic amines (Fig. 4B). Moreover, red meat and dairy products correlated with acetic acid and propionic or butyric acid, respectively. Three major SCFAs (acetic, propionic and butyric) were associated directly with non-alcoholic drinks and fish and derivatives. The direction of the associations of drinks with ammonium and total amino acids shifted from positive in the controls to negative in the polyps group.

3.4. Linear regression analyses between dietary compounds and microbiological families

Food groups and their components were further examined to obtain the underlying basis of the previous findings about the associations between diet and microbiological families. However, RDA models searching for the best explanatory variables did not reveal statistically significant results (data not shown). Therefore, to identify potential dietary predictors of faecal microbiological families, linear regression analyses adjusted by gender and BMI were performed (Table 2). In the control group, NPYR, total PAHs, and total ORAC variables were identified as negative predictors of Bacteroidaceae, Bifidobacteriaceae and Christensenellaceae levels, respectively. Flavonoids were positive predictors of Lachnospiraceae and ethanol was found to be inversely associated with Clostridiaceae. While MeIQ was found as a direct predictor of Clostridiaceae, acrylamide and MeIQx were associated with the relative abundance of Coriobacteriaceae. Acrylamide was the main variable appearing in amino acid regression analysis. Other polyphenols were found to be the best predictors of the major SCFAs (acetic, propionic and butyric). In the polyps group, gender and BMI appeared as significant adjusting parameters when Prevotellaceae and Bacteroidaceae were analysed, respectively. Nitrites were positive predictors of Peptostreptococcaceae, and calcium – of Lachnospiraceae. The xenobiotics NDMA and PhIP directly predicted Erysipelatoclostridiaceae. A bioactive compound, insoluble cellulose, was positively associated with the abundance of Erysipelotrichaceae. Total ORAC was a positive predictor of Ruminococcaceae. Ethanol was inversely related to the total amino acid concentration in the faeces. Regarding SCFAs, acetic acid was significantly adjusted by gender, and nitrites and phenolic acid were found as positive predictors of butyric and caproic acids, respectively. To explore the correlation between the bacterial family Bifidobacteriaceae and SCFAs, Spearman correlations were conducted, and no statistically significant associations were obtained, not even according to the clinical diagnosis groups (Table S18†).

4. Discussion

Assessing the relationship between diet and intestinal mucosal health as related with faecal microbiota composition is a complex issue. This goal could be addressed by individual analysis of key dietary factors, as those analysed in this work. The evaluation of each dietary factor revealed significant shifts in gut microbial families with differences according to the clinical diagnosis group.

The findings of a lower abundance of Bifidobacteriaceae in the group of individuals with a higher intake of processed meat, nitrites, NDMA and other polyphenols are in line with the literature describing the association of plant-based diets with higher faecal excretion of SCFAs and increased *Bifidobacterium* abundance, in contrast to meat-based diets.[64] However, any statistically significant correlation between Bifidobacteriaceae and the SCFA concentration in faeces was found in the sample. The differences in the diet–microbiota binomial between the different study groups are probably one of the most interesting findings of the present work. In this sense, while in the polyps group, the abundance of Peptostreptococcaceae increased with an intake of ethanol $\geq$12 g day^{-1} and was positively predicted by nitrites, in the control group, Clostridiaceae abundance was reduced, when the intake of ethanol was above 12 g day^{-1}. In previous studies, Peptostreptococcaceae has been found to be increased in alcohol consumers in comparison with non-drinkers, and in the faecal samples of CRC patients.[65,66] It is important to remark that in our sample the mean ethanol intake was higher in the polyps group than in the control group (15.8 g day^{-1} *vs.* 6.8 g day^{-1}), although this difference was not significant. These findings could help to support the possible relationship between alcohol intake and the increased risk of intestinal polyps development.[26] In the control subjects, volunteers with intake of total polyphenols $\geq$650 mg day^{-1} and or calcium intake <900 mg day^{-1} revealed an increased abundance of Veillonellaceae. A decreased abundance of the genus *Veillonella*, a member of the family Veillonellaceae, has been previously associated with gut mucosal damage in humans.[67] We also detected lower levels of Christensenellaceae in the faecal microbiota of individuals belonging to the polyps group, which showed a total PAH intake $\geq$0.75 μg day^{-1}. The main route of exposure to PAHs for the human body is food and smoking.[68] In this regard, the presence of PAHs in food and their consequent direct accession by gut microbiota members could cause reduction of the abundance of certain bacteria such as Christensenellaceae, whose presence has been related with a positive impact on human health.[69] We found that a higher intake of calcium in the polyps group was associated with a reduced abundance of Methanobacteriaceae

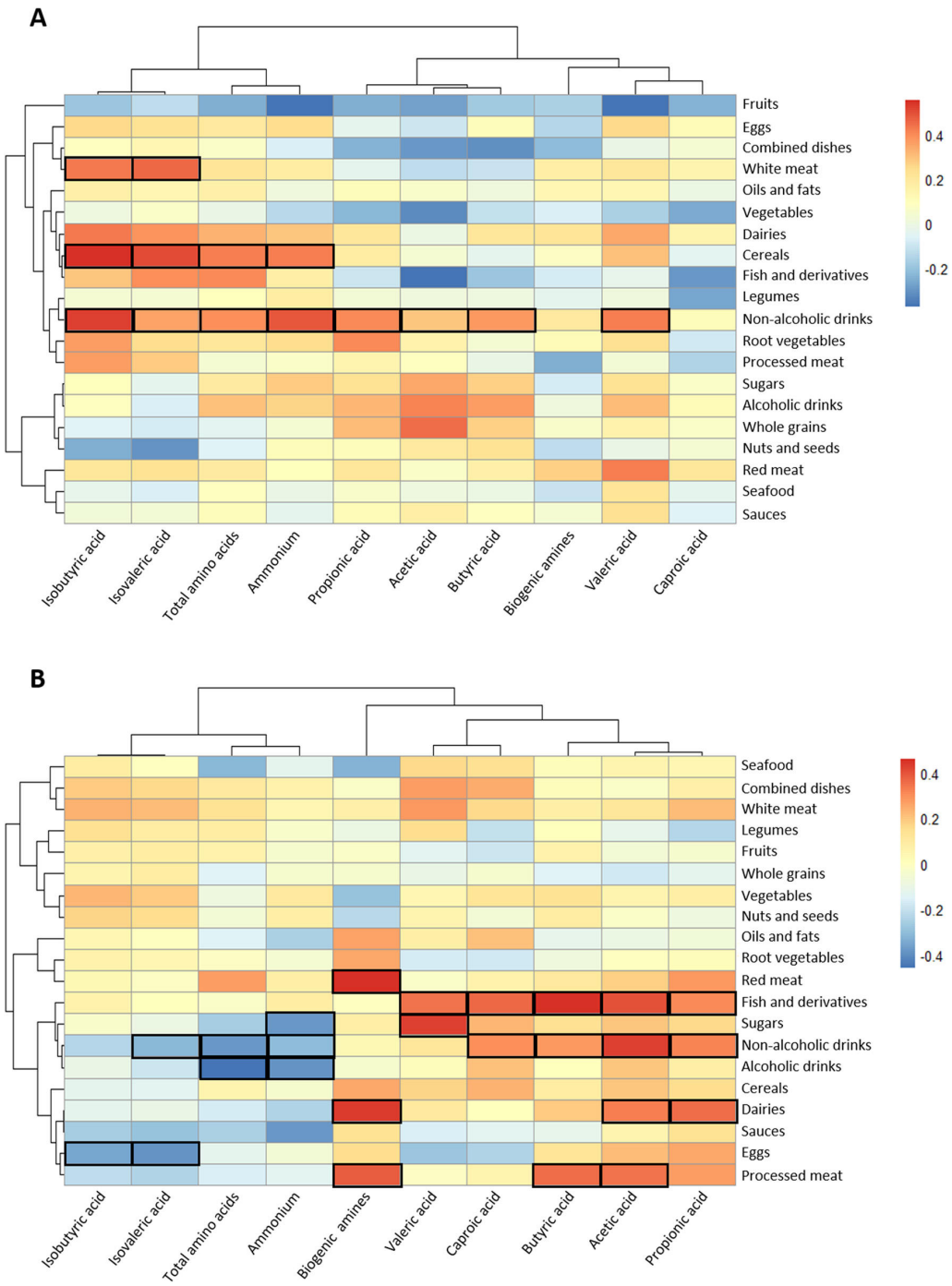

Fig. 4 Heatmaps defined by Spearman correlations between food groups and faecal metabolites (SCFAs) and amino acids for the clinical diagnosis groups: (A) control or (B) polyps. Blue and red colours denote negative and positive associations, respectively. The intensity of the colour is proportional to the degree of association between variables. Bordered cells indicate a statistically significant association adjusted for multiple testing by the Benjamini–Hochberg procedure.

Table 2 Results obtained from linear regression analyses identifying ORAC, bioactive and potential carcinogenic compounds as predictors of faecal microbiota relative abundances, faecal amino acids and SCFAs in each clinical diagnosis group of volunteers

Diagnosis group	Dependent variable	Independent variable	R^2	β	p
Control	Bifidobacteriaceae	NPYR	0.168	−0.450	0.024
	Lachnospiraceae	Flavonoids	0.244	0.525	0.007
	Coriobacteriaceae	Acrylamide	0.672	0.533	0.001
		MeIQx	0.672	0.462	0.002
	Bacteroidaceae	Total PAHs	0.140	−0.419	0.037
	Clostridiaceae	MeIQ	0.544	0.620	0.001
		Ethanol	0.544	−0.317	0.037
	Oscillospiraceae	MeIQ	0.291	0.566	0.003
	Christensenellaceae	Total ORAC	0.142	−0.422	0.036
	Eubacterium coprostanoligenes group	MeIQ	0.282	0.559	0.004
	Total amino acids	Acrylamide	0.263	0.542	0.005
	Protein amino acids	Acrylamide	0.254	0.534	0.006
	Ammonium	Acrylamide	0.345	0.573	0.002
		Total polyphenols	0.345	0.350	0.047
	Acetic acid	Other polyphenols	0.371	0.511	0.005
		MeIQ	0.371	−0.407	0.020
	Propionic acid	Other polyphenols	0.192	0.475	0.016
	Butyric acid	Stilbenes	0.424	0.487	0.005
		Other polyphenols	0.424	0.421	0.013
	Isobutyric acid	Acrylamide	0.322	0.592	0.002
	Isovaleric acid	Calcium	0.212	0.495	0.012
	Valeric acid	Acrylamide	0.532	0.743	0.001
Polyps	Peptostreptococcaceae	Nitrites	0.109	0.368	0.030
	Prevotellaceae	Gender	0.098	0.353	0.038
	Lachnospiraceae	Calcium	0.102	0.358	0.035
	Bacteroidaceae	BMI	0.288	0.445	0.005
		Lipophilic ORAC	0.288	−0.313	0.039
	Ruminococcaceae	Total ORAC	0.311	0.575	0.001
	Erysipelatoclostridiaceae	NDMA	0.363	0.466	0.002
		PhIP	0.363	0.358	0.015
	Erysipelotrichaceae	Cellulose (I)	0.114	0.374	0.027
	Oscillospiraceae	Total PAHs	0.122	−0.384	0.023
	Total amino acids	Ethanol	0.097	−0.351	0.038
	Protein amino acids	Ethanol	0.097	−0.351	0.039
	Ammonium	Ethanol	0.098	−0.353	0.037
	Acetic acid	Gender	0.135	−0.400	0.017
	Butyric acid	Nitrites	0.178	0.450	0.007
	Caproic acid	Phenolic acids	0.163	0.433	0.009

Linear regression analyses were adjusted by gender and BMI. Only the variables with $p < 0.05$ in each model are shown. R^2, coefficient of multiple determination; β, standardized regression coefficient; BMI, body mass index; *I*, insoluble; B(a)P, benzo (a) pyrene; total PAHs, total polycyclic aromatic hydrocarbons; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine; MeIQx, 2-amino-3,8 dimethylimidazo (4,5,*f*) quinoxaline; MeIQ, 2-amino-3,4 dimethylimidazo (4,5,*f*) quinoline; NDMA, *N*-nitrosodimethylamine; NPYR, *N*-nitrosopyrrolidine; ORAC, oxygen radical absorbance capacity.

and Christensenellaceae families and also higher concentrations of acetic acid and propionic acid. The metabolism of calcium has been previously bidirectionally associated with shifts in the gut microbiota, with SCFAs playing key roles in these processes.[70,71] In this sense, SCFAs are capable of lowering pH in the intestinal environment, reducing calcium–phosphorus complexes and improving dietary calcium release and absorption.[72,73] An increased abundance of Coriobacteriaceae was detected in the control individuals with an intake of ≥120 g day^{-1} milk, ≥50 g day^{-1} red meat, ≥20 g day^{-1} fibre, ≥650 mg day^{-1} total polyphenols, ≥0.75 μg day^{-1} total PAH or ≥50 ng day^{-1} MeIQx. Indeed, in our sample, Coriobacteriaceae was negatively associated with nuts and seeds and positively associated with dairy products, cereals, and red and white meat in the controls. The HA MeIQx, which can be generated during meat cooking, was revealed as a direct predictor of Coriobacteriaceae abundance in the faeces from control volunteers. Members of the Coriobacteriaceae family have been found to be increased in individuals with some pro-inflammatory diseases and obesity, and in individuals with low fibre consumption.[74] Interestingly, some species belonging to the family Coriobacteriaceae could be involved in polyphenol metabolism, which could partly contribute to explain our results. However, the potential role of Coriobacteriaceae in human health is still poorly understood.[75] This study evidenced that the abundance of Bacteroidaceae in faeces was negatively correlated with the intake of white meat, dairy products, root vegetables and cereals in the control group. In our sample, the main root vegetables consumed were potatoes, which can be prepared using various cooking methods, such as frying, with different impacts on the data obtained. Previous studies have detected a higher proportion of

Bacteroidaceae in individuals consuming enriched plant diets.[76] Moreover, Bacteroidaceae was negatively predicted by total PAHs in the controls and lipophilic ORAC in the polyps group. When Akkermansiaceae was evaluated, the control group volunteers with an intake ≥50 g day^{-1} red meat, ≥40 ng day^{-1} PhIP and ≥50 ng day^{-1} MeIQx showed a decreased abundance of this family. There is reportedly evidence of the regulatory and beneficial effects in studies about *Akkermansia muciniphila*, a mucin-degrading microorganism.[77,78] Furthermore, in the polyps group, an increase in the faecal abundance of Erysipelatoclostridiaceae was detected when the consumption of processed meat, calcium, nitrites and NDMA was over the cut-off points. In this diagnosis group, this family was also positively predicted by NDMA and PhIP. There is scarce bibliography about the possible interaction between the family Erysipelatoclostridiaceae and diet, as this group was usually combined at the taxonomic level with the Erysipelotrichaceae family, in the order Erysipelotrichales, which makes it difficult to extract confident hypotheses and conclusions. The Erysipelatoclostridiaceae family has been linked with class I obesity (BMI between 30 and 34.9), but no previous associations with intestinal damage have been described.[79] In our study, we detected an increased abundance of members from the *Eubacterium coprostanoligenes* group in individuals from the polyps group when the intake of nitrites was ≥1.60 mg day^{-1}. This family has been identified in the mucosal samples of adenomatous polyps patients, hypothesizing a harmful role.[80] In our sample, the mean consumption of processed meat, a dietary source of nitrites, was higher in the polyps group compared to the control group (78 g day^{-1} *vs.* 61 g day^{-1}). These values were high as compared with the reported global mean intake of 17 g of processed meat per day reported in a survey across 185 countries around the world.[81] Also, the GBD study established that the intake of processed meat over 25 g day^{-1} was associated with an increased relative risk of being diagnosed with CRC in 1.06 (1.02–1.10), a threshold that is clearly surpassed in our sample.[5] The consumption of vegetables was lower in the polyps group than in the control group (256 g day^{-1} *vs.* 279 g day^{-1}), and again, higher in our sample than the estimated global mean intake (208.8 g day^{-1}).[82] This could suggest that although the consumption of food groups with harmful potential remains elevated in our sample, their impact on health may be reduced through higher intake of bioactive compounds naturally present in vegetables. These distinct dietary features may also contribute to explain the differences found in the microbiota profiles. Whether all these shifts on the microbiota are causes or consequences or are concomitant to the gut mucosal damage is still uncertain and remains to be determined.

In the present work, we assessed the relationships of bioactive and xenobiotic compounds together with microbiological families, by performing linear regressions. Proceeding in this way, we observed that in the control group, the variable "other polyphenols", which includes compounds such as curcuminoids, hydroxybenzaldehydes, phenolic terpenes or tyrosols, predicted faecal concentrations of the major SCFAs (acetic, propionic and butyric), whereas total polyphenols predicted the concentration of ammonium in the faeces. In this direction, recent studies have suggested the role of some polyphenols as prebiotic substrates, contributing to explain the positive relationship between some types of polyphenols and faecal levels of SCFAs.[83] On the other hand, we hypothesize that the higher faecal levels of ammonium predicted by the intake of total polyphenols in the controls could be mediated by red meat consumption through the metabolism of members from the family Coriobacteriaceae, among others. Other possible hypotheses for the relationship between the intake of phenolic compounds and ammonium is that some phenolic compounds (*i.e.* tannins) are capable of trapping amino acid metabolites such as ammonia.[84] Therefore, increased ammonium concentrations in faeces could be linked to phenolic molecules. Although the directionality of the results could not be established due to the observational nature of our study, this work links the consumption of some groups of foods and xenobiotics with specific microorganisms of the intestinal microbiota. Moreover, along the study, opposite tendencies have been observed in the abundances of certain microorganisms according to the diagnosis group. Although not reaching statistical significance in some cases, these results support the hypothesis that the impact of the diet could be different depending on the diagnosis group. In this sense, the observation of a higher impact on the microbiota of the control group than that of the polyps groups for certain dietary components could be related to the intestinal anatomopathological homogeneity in the control group with regard to higher heterogeneity in the polyps group. Despite the absence of a significant association of clinical diagnosis groups and the relative abundance of microbial families obtained by MANOVA tests, our results suggest that once mucosal damage has been established, the microbiota of volunteers showing intestinal polyps may be less susceptible to variations as compared to that of the controls.

Despite our findings, the present study also showed some limitations. The relative abundances of Methanobacteriaceae obtained in this study should be considered with caution as the primers used for amplification were more optimized for bacteria than for archaea. Moreover, the metataxonomic analyses by 16S RNA gene sequencing did not provide precise information about the microbial species present in the samples. Furthermore, as foods are consumed together as a part of a diet, it is possible that we are losing the effect of interactions among dietary components.

5. Conclusions

The gut microbiota composition in different intestinal mucosa damage statuses has been associated with the consumption of some foods and dietary components. Further studies elucidating the mechanisms by which these compounds are involved in the alteration of the intestinal microbiota could be helpful to develop dietary recommendations in order to reduce the

intake of dietary xenobiotics for preventing intestinal mucosal damage and further development of CRC.

Author contributions

Conceptualization: SG and CGRG. Medical resources: AS, YD, and CGR. Investigation: SRS, NS, SA, AZ, MG, CGRG and SG. Methodology: SRS and AZ. Formal analysis: SRS and SG. Funding acquisition: CGRG and SG. Writing – original draft: SRS. Writing, review and editing: SRS, SG and CGRG. All authors contributed to manuscript revision, and read and approved the submitted version.

Conflicts of interest

There are no conflicts of interest to declare.

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MATERIAL SUPLEMENTARIO

Table S1. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to clinical diagnosis group**.**

	Control (*n*=25)	Polyps (*n*=35)
Microbial families (% Abundance)		
Bifidobacteriaceae	13.05 ± 13.25	11.13 ± 11.83
Peptostreptococcaceae	6.04 ± 8.06	5.12 ± 5.30
Prevotellaceae	3.42 ± 6.29	3.86 ± 8.22
Lachnospiraceae	19.82 ± 7.29	21.98 ± 8.99
Coriobacteriaceae	6.14 ± 4.13	10.31 ± 9.05
Enterobacteriaceae	2.98 ± 5.09	1.08 ± 2.78
Bacteroidaceae	4.95 ± 6.83	4.32 ± 4.45
Ruminococcaceae	12.43 ± 4.27	11.95 ± 5.40
Veillonellaceae	1.30 ± 1.63	2.39 ± 4.83
Akkermansiaceae	0.59 ± 1.04	1.41 ± 3.50
Streptococcaceae	1.63 ± 3.31	0.87 ± 1.30
Methanobacteriaceae	1.75 ± 2.67	1.57 ± 2.73
Eggerthellaceae	3.25 ± 1.95	4.04 ± 2.13
Erysipelatoclostridiaceae	1.17 ± 1.14	1.40 ± 1.66
Erysipelotrichaceae	1.63 ± 1.97	1.50 ± 2.18
Clostridiaceae	0.78 ± 0.48	1.01 ± 0.86
Oscillospiraceae	2.64 ± 1.51	2.22 ± 1.60
Christensenellaceae	1.61 ± 1.07	1.23 ± 1.21
Eubacterium_coprostanoligenes_group	1.21 ± 0.58	1.22 ± 0.69
Others	13.59 ± 11.16	11.40 ± 9.56
SCFAs (mM)		
Acetic acid	45.97 ± 20.25	50.00 ± 28.23
Butyric acid	13.13 ± 8.72	11.75 ± 7.64
Propionic acid	13.23 ± 6.98	16.12 ± 8.94
Isobutyric acid	0.92 ± 1.04	0.92 ± 1.07
Isovaleric acid	2.01 ± 1.71	2.00 ± 1.52
Valeric acid	2.13 ± 1.56	1.55 ± 1.08
Caproic acid	0.85 ± 1.02	0.27 ± 0.55
Amino acids (mM)		
Total amino acids	48.55 ± 24.78	49.13 ± 22.44
Protein amino acids	47.46 ± 23.82	47.86 ± 21.43
Biogenic amines	0.94 ± 1.40	1.12 ± 2.03
Ammonium	28.51 ± 17.22	29.36 ± 17.36

Values are presented as mean ± standard deviation. SCFAs, short chain fatty acids.

Table S2. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to milk consumption (g/day) category in each diagnosis group**.**

	Control		Polyps	
	Milk<120 (*n*=11)	Milk≥120 (*n*=14)	Milk<120 (*n*=12)	Milk≥120 (*n*=23)
Microbial families (% Abundance)				
Bifidobacteriaceae	9.03 ± 11.98	16.21 ± 13.75	12.52 ± 14.58	10.40 ± 10.42
Peptostreptococcaceae	2.48 ± 2.16	8.85 ± 9.86 *	6.26 ± 7.66	4.53 ± 3.62
Prevotellaceae	2.71 ± 4.67	3.98 ± 7.45	3.82 ± 6.71	3.88 ± 9.05
Lachnospiraceae	21.51 ± 4.96	18.5 ± 8.65	18.33 ± 5.74	23.88 ± 9.86
Coriobacteriaceae	5.02 ± 5.06	7.02 ± 3.14 *	9.79 ± 10.21	10.59 ± 8.61
Enterobacteriaceae	3.85 ± 5.45	2.30 ± 4.88	2.14 ± 4.63	0.52 ± 0.56
Bacteroidaceae	8.46 ± 9.17	2.19 ± 1.69 *	2.84 ± 1.74	5.09 ± 5.22
Ruminococcaceae	12.18 ± 3.08	12.63 ± 5.12	11.73 ± 3.69	12.06 ± 6.19
Veillonellaceae	1.39 ± 2.15	1.23 ± 1.17	2.81 ± 5.46	2.17 ± 4.59
Akkermansiaceae	0.61 ± 1.13	0.58 ± 1.02	0.30 ± 0.58	1.98 ± 4.21 *
Streptococcaceae	2.07 ± 4.70	1.29 ± 1.73	0.75 ± 0.68	0.94 ± 1.54
Methanobacteriaceae	1.70 ± 3.15	1.8 ± 2.35	2.76 ± 3.72	0.94 ± 1.84
Eggerthellaceae	3.34 ± 2.45	3.18 ± 1.56	4.44 ± 2.26	3.83 ± 2.09
Erysipelatoclostridiaceae	0.94 ± 1.19	1.35 ± 1.11	1.24 ± 2.26	1.48 ± 1.30
Erysipelotrichaceae	1.35 ± 2.18	1.85 ± 1.84 *	2.20 ± 2.60	1.14 ± 1.89
Clostridiaceae	0.65 ± 0.41	0.89 ± 0.52	0.94 ± 0.80	1.05 ± 0.90
Oscillospiraceae	2.34 ± 1.04	2.87 ± 1.80	2.29 ± 1.45	2.19 ± 1.70
Christensenellaceae	1.77 ± 1.32	1.48 ± 0.86	1.25 ± 0.73	1.22 ± 1.41
Eubacterium_coprostanoligenes_group	1.10 ± 0.53	1.30 ± 0.62	1.48 ± 0.59	1.08 ± 0.72
Others	17.50 ± 12.50	10.51 ± 9.31	12.12 ± 9.28	11.03 ± 9.89
SCFAs (mM)				
Acetic acid	50.77 ± 21.68	42.20 ± 18.98	44.33 ± 24.44	52.95 ± 30.10
Butyric acid	14.44 ± 9.79	12.10 ± 8.02	11.44 ± 6.65	11.91 ± 8.25
Propionic acid	12.64 ± 5.4	13.69 ± 8.18	13.89 ± 8.84	17.29 ± 8.95
Isobutyric acid	0.61 ± 0.70	1.16 ± 1.22	1.08 ± 1.26	0.85 ± 0.97
Isovaleric acid	1.48 ± 1.14	2.43 ± 1.99	2.14 ± 1.32	1.92 ± 1.64
Valeric acid	1.79 ± 1.02	2.39 ± 1.88	1.74 ± 1.3	1.44 ± 0.96
Caproic acid	1.08 ± 1.28	0.67 ± 0.77	0.62 ± 0.83	0.09 ± 0.15
Amino acids (mM)				
Total amino acids	42.14 ± 19.91	53.58 ± 27.70	48.31 ± 16.52	49.56 ± 25.33
Protein amino acids	41.10 ± 19.50	52.46 ± 26.34	47.46 ± 15.66	48.06 ± 24.24
Biogenic amines	0.91 ± 1.39	0.97 ± 1.47	0.67 ± 1.34	1.35 ± 2.31
Ammonium	26.32 ± 15.23	30.23 ± 19.02	29.3 ± 13.25	29.39 ± 19.44

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. SCFAs, short chain fatty acids.

Table S3. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to red meat consumption (g/day) category in each diagnosis group**.**

	Control		Polyps	
	R.M<50 (*n*=14)	R.M≥50 (*n*=11)	R.M<50 (*n*=18)	R.M≥50 (*n*=17)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.37 ± 12.26	13.92 ± 14.98	10.37 ± 10.25	11.93 ± 13.59
Peptostreptococcaceae	5.45 ± 8.60	6.80 ± 7.66	4.76 ± 4.07	5.51 ± 6.47
Prevotellaceae	2.43 ± 4.17	4.68 ± 8.32	5.4 ± 10.52	2.22 ± 4.53
Lachnospiraceae	19.10 ± 5.03	20.74 ± 9.64	23.46 ± 9.24	20.42 ± 8.70
Coriobacteriaceae	4.15 ± 2.56	8.67 ± 4.46 *	9.02 ± 8.18	11.69 ± 9.95
Enterobacteriaceae	2.66 ± 4.89	3.40 ± 5.54	0.58 ± 0.49	1.60 ± 3.95
Bacteroidaceae	7.17 ± 8.45	2.12 ± 1.88 *	5.22 ± 5.21	3.36 ± 3.36
Ruminococcaceae	12.22 ± 3.63	12.71 ± 5.14	12.80 ± 4.74	11.04 ± 6.04
Veillonellaceae	1.10 ± 1.26	1.56 ± 2.05	2.25 ± 4.17	2.54 ± 5.58
Akkermansiaceae	0.82 ± 1.18	0.31 ± 0.80 *	1.40 ± 2.56	1.41 ± 4.36
Streptococcaceae	2.36 ± 4.3	0.72 ± 0.82	0.83 ± 1.49	0.92 ± 1.12
Methanobacteriaceae	1.65 ± 2.95	1.89 ± 2.42	0.88 ± 2.19	2.29 ± 3.10
Eggerthellaceae	3.31 ± 2.37	3.18 ± 1.37	4.04 ± 1.77	4.04 ± 2.52
Erysipelatoclostridiaceae	0.87 ± 0.92	1.55 ± 1.31	0.96 ± 0.69	1.87 ± 2.22
Erysipelotrichaceae	1.43 ± 2.04	1.89 ± 1.93	1.26 ± 1.69	1.77 ± 2.64
Clostridiaceae	0.75 ± 0.48	0.83 ± 0.51	1.01 ± 1.01	1.01 ± 0.69
Oscillospiraceae	2.85 ± 1.50	2.37 ± 1.55	2.38 ± 1.50	2.06 ± 1.73
Christensenellaceae	1.62 ± 0.97	1.60 ± 1.24	1.18 ± 0.94	1.29 ± 1.47
Eubacterium_coprostanoligenes_group	1.11 ± 0.68	1.34 ± 0.40	1.29 ± 0.54	1.13 ± 0.84
Others	16.50 ± 11.89	9.75 ± 9.30	10.92 ± 7.14	11.91 ± 11.81
SCFAs (mM)				
Acetic acid	44.44 ± 20.51	47.91 ± 20.72	44.66 ± 21.14	55.65 ± 33.95
Butyric acid	12.00 ± 9.30	14.56 ± 8.13	10.44 ± 4.83	13.13 ± 9.76
Propionic acid	11.52 ± 5.46	15.40 ± 8.29	13.89 ± 6.98	18.49 ± 10.31
Isobutyric acid	0.66 ± 0.63	1.24 ± 1.38	0.91 ± 0.90	0.94 ± 1.24
Isovaleric acid	1.60 ± 0.97	2.54 ± 2.28	2.06 ± 1.47	1.93 ± 1.62
Valeric acid	1.48 ± 0.58	2.95 ± 2.02 $^{+}$	1.61 ± 0.92	1.48 ± 1.25
Caproic acid	0.62 ± 0.86	1.14 ± 1.18	0.25 ± 0.59	0.29 ± 0.52
Amino acids (mM)				
Total amino acids	43.00 ± 20.40	55.60 ± 28.90	44.18 ± 21.34	54.37 ± 23.02
Protein amino acids	42.21 ± 20.08	54.13 ± 27.39	43.64 ± 20.75	52.32 ± 21.86
Biogenic amines	0.65 ± 1.05	1.32 ± 1.74	0.40 ± 0.77	1.88 ± 2.64
Ammonium	24.74 ± 11.90	33.31 ± 21.96	28.37 ± 17.11	30.41 ± 18.08

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis group. R.M, red meat; SCFAs, short chain fatty acids.

Table S4. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to processed meat consumption (g/day) category in each diagnosis group**.**

	Control		Polyps	
	P.M<25 (*n*=4)	P.M≥25 (*n*=21)	P.M<25 (*n*=7)	P.M≥25 (*n*=28)
Microbial families (% Abundance)				
Bifidobacteriaceae	24.94 ± 12.35	10.79 ± 12.41 *	15.83 ± 13.65	9.95 ± 11.31
Peptostreptococcaceae	9.97 ± 11.73	5.30 ± 7.33	4.15 ± 3.22	5.37 ± 5.73
Prevotellaceae	3.09 ± 2.30	3.48 ± 6.83 *	4.87 ± 6.52	3.60 ± 8.68
Lachnospiraceae	15.11 ± 5.77	20.72 ± 7.31	20.14 ± 10.08	22.44 ± 8.83
Coriobacteriaceae	4.81 ± 1.67	6.39 ± 4.43	9.79 ± 7.36	10.44 ± 9.54
Enterobacteriaceae	2.62 ± 4.75	3.06 ± 5.26	0.43 ± 0.50	1.24 ± 3.09
Bacteroidaceae	2.91 ± 1.01	5.34 ± 7.41	2.57 ± 1.83	4.76 ± 4.81
Ruminococcaceae	9.99 ± 3.07	12.90 ± 4.36	10.23 ± 3.46	12.38 ± 5.76
Veillonellaceae	1.63 ± 1.36	1.24 ± 1.70	3.98 ± 8.17	1.99 ± 3.71
Akkermansiaceae	0.62 ± 1.00	0.59 ± 1.07	0.78 ± 1.32	1.56 ± 3.86
Streptococcaceae	5.12 ± 7.22	0.97 ± 1.57	0.58 ± 0.42	0.95 ± 1.44
Methanobacteriaceae	0.53 ± 0.46	1.99 ± 2.86	1.11 ± 2.32	1.68 ± 2.84
Eggerthellaceae	2.77 ± 0.77	3.34 ± 2.11	4.78 ± 2.65	3.85 ± 2.00
Erysipelatoclostridiaceae	0.46 ± 0.20	1.30 ± 1.20 *	0.52 ± 0.39	1.62 ± 1.79 *
Erysipelotrichaceae	0.91 ± 0.83	1.77 ± 2.10	1.65 ± 1.93	1.47 ± 2.27
Clostridiaceae	0.75 ± 0.41	0.79 ± 0.50	0.90 ± 0.80	1.04 ± 0.88
Oscillospiraceae	2.46 ± 0.50	2.68 ± 1.64	2.83 ± 1.62	2.07 ± 1.59
Christensenellaceae	1.54 ± 0.29	1.62 ± 1.17	1.86 ± 1.70	1.07 ± 1.03
Eubacterium_coprostanoligenes_group	1.05 ± 0.26	1.24 ± 0.62	0.94 ± 0.36	1.29 ± 0.74
Others	8.75 ± 4.64	14.51 ± 11.86	12.08 ± 7.76	11.23 ± 10.08
SCFAs (mM)				
Acetic acid	40.38 ± 30.09	47.03 ± 18.67	45.19 ± 17.95	51.20 ± 30.4
Butyric acid	13.92 ± 13.92	12.98 ± 7.88	11.65 ± 5.37	11.77 ± 8.19
Propionic acid	10.77 ± 8.13	13.70 ± 6.86	15.25 ± 7.90	16.34 ± 9.30
Isobutyric acid	0.43 ± 0.19	1.01 ± 1.12	1.34 ± 1.04	0.82 ± 1.06
Isovaleric acid	1.30 ± 0.40	2.15 ± 1.83	2.77 ± 1.74	1.80 ± 1.43
Valeric acid	1.59 ± 0.73	2.23 ± 1.67	1.87 ± 0.80	1.47 ± 1.14
Caproic acid	0.87 ± 0.89	0.84 ± 1.07	0.13 ± 0.17	0.30 ± 0.61
Amino acids (mM)				
Total amino acids	46.90 ± 10.80	48.86 ± 26.81	60.01 ± 30.34	46.41 ± 19.78
Protein amino acids	45.70 ± 9.64	47.79 ± 25.81	59.36 ± 29.56	44.98 ± 18.48
Biogenic amines	0.98 ± 1.64	0.94 ± 1.40	0.49 ± 0.80	1.28 ± 2.22
Ammonium	25.34 ± 10.42	29.11 ± 18.36	41.14 ± 26.43	26.42 ± 13.38

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. P.M, processed meat; SCFAs, short chain fatty acids.

Table S5. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to fibre consumption (g/day) category in each diagnosis group**.**

	Control		Polyps	
	Fibre<20 (*n*=9)	Fibre≥20 (*n*=16)	Fibre<20 (*n*=20)	Fibre≥20 (*n*=15)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.41 ± 15.75	13.41 ± 12.17	11.12 ± 11.22	11.13 ± 13.01
Peptostreptococcaceae	3.16 ± 2.12	7.67 ± 9.69	4.83 ± 4.09	5.52 ± 6.73
Prevotellaceae	3.24 ± 4.88	3.52 ± 7.11	1.84 ± 4.03	6.54 ± 11.34
Lachnospiraceae	20.93 ± 5.58	19.20 ± 8.20	24.32 ± 9.78	18.86 ± 6.93
Coriobacteriaceae	4.23 ± 2.16	7.21 ± 4.63 *	11.72 ± 8.62	8.43 ± 9.55
Enterobacteriaceae	3.76 ± 5.85	2.55 ± 4.75	0.58 ± 0.65	1.74 ± 4.17
Bacteroidaceae	8.15 ± 9.86	3.15 ± 3.64	3.73 ± 3.96	5.10 ± 5.05
Ruminococcaceae	12.93 ± 4.79	12.15 ± 4.08	11.4 ± 4.25	12.67 ± 6.75
Veillonellaceae	0.78 ± 0.71	1.59 ± 1.94	1.84 ± 3.95	3.12 ± 5.88
Akkermansiaceae	0.80 ± 1.32	0.48 ± 0.88	1.25 ± 2.45	1.61 ± 4.63
Streptococcaceae	2.62 ± 5.10	1.08 ± 1.66	0.82 ± 1.03	0.95 ± 1.64
Methanobacteriaceae	1.87 ± 3.08	1.69 ± 2.52	1.74 ± 3.14	1.34 ± 2.14
Eggerthellaceae	3.13 ± 2.60	3.32 ± 1.58	4.35 ± 1.93	3.62 ± 2.38
Erysipelatoclostridiaceae	0.86 ± 0.88	1.34 ± 1.26	1.61 ± 2.05	1.12 ± 0.93
Erysipelotrichaceae	1.82 ± 2.33	1.52 ± 1.80	1.03 ± 1.46	2.14 ± 2.81
Clostridiaceae	0.70 ± 0.34	0.83 ± 0.55	1.18 ± 0.99	0.79 ± 0.60
Oscillospiraceae	2.58 ± 1.40	2.68 ± 1.62	2.38 ± 1.94	2.01 ± 1.01
Christensenellaceae	1.96 ± 1.38	1.41 ± 0.84	1.34 ± 1.32	1.08 ± 1.07
Eubacterium_coprostanoligenes_group	1.03 ± 0.43	1.32 ± 0.64	1.14 ± 0.66	1.32 ± 0.74
Others	13.04 ± 12.13	13.90 ± 10.99	11.77 ± 7.49	10.92 ± 12.06
SCFAs (mM)				
Acetic acid	51.37 ± 25.88	42.93 ± 16.47	46.87 ± 27.55	54.17 ± 29.54
Butyric acid	14.45 ± 11.19	12.39 ± 7.31	10.39 ± 6.73	13.56 ± 8.62
Propionic acid	14.23 ± 9.15	12.67 ± 5.69	15.30 ± 9.55	17.22 ± 8.24
Isobutyric acid	0.35 ± 0.28	1.23 ± 1.19 $^{+}$	0.65 ± 0.68	1.29 ± 1.37
Isovaleric acid	1.12 ± 0.59	2.51 ± 1.93 $^{+}$	1.67 ± 1.09	2.43 ± 1.91
Valeric acid	1.68 ± 0.86	2.37 ± 1.83	1.41 ± 1.13	1.73 ± 1.02
Caproic acid	1.11 ± 1.00	0.70 ± 1.04	0.28 ± 0.65	0.26 ± 0.41
Amino acids (mM)				
Total amino acids	38.51 ± 19.94	54.19 ± 26	46.43 ± 22.34	52.73 ± 22.83
Protein amino acids	37.50 ± 19.46	53.05 ± 24.75	44.93 ± 20.96	51.76 ± 22.17
Biogenic amines	0.84 ± 1.21	1.00 ± 1.54	1.35 ± 2.52	0.81 ± 1.12
Ammonium	19.83 ± 10.19	33.39 ± 18.66	28.40 ± 17.63	30.64 ± 17.51

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. SCFAs, short chain fatty acids.

Table S6. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to soluble pectin intake (g/day) category in each diagnosis group**.**

	Control		Polyps	
	S.P.<0.57 (*n*=9)	S.P.≥0.57 (*n*=16)	S.P.<0.57 (*n*=17)	S.P.≥0.57 (*n*=18)
Microbial families (% Abundance)				
Bifidobacteriaceae	10.37 ± 7.17	14.56 ± 15.7	10.08 ± 10.20	12.12 ± 13.42
Peptostreptococcaceae	4.70 ± 4.41	6.80 ± 9.59	5.98 ± 6.81	4.32 ± 3.34
Prevotellaceae	4.17 ± 8.47	3.00 ± 4.95	2.10 ± 4.54	5.52 ± 10.48
Lachnospiraceae	20.04 ± 8.15	19.7 ± 7.04	25.02 ± 9.17	19.11 ± 8.02
Coriobacteriaceae	6.23 ± 4.41	6.08 ± 4.11	11.04 ± 7.44	9.63 ± 10.51
Enterobacteriaceae	3.76 ± 5.91	2.55 ± 4.72	1.49 ± 3.88	0.69 ± 1.00
Bacteroidaceae	6.93 ± 10.12	3.84 ± 4.06	4.09 ± 3.57	4.54 ± 5.24
Ruminococcaceae	11.54 ± 4.02	12.94 ± 4.44	11.10 ± 4.49	12.74 ± 6.17
Veillonellaceae	1.20 ± 1.23	1.36 ± 1.86	0.90 ± 1.10	3.80 ± 6.42
Akkermansiaceae	0.50 ± 1.12	0.65 ± 1.03	1.77 ± 4.35	1.06 ± 2.53
Streptococcaceae	2.31 ± 5.06	1.26 ± 1.86	0.75 ± 1.55	0.99 ± 1.05
Methanobacteriaceae	1.30 ± 1.49	2.01 ± 3.17	1.19 ± 2.03	1.93 ± 3.27
Eggerthellaceae	3.41 ± 1.79	3.16 ± 2.09	4.33 ± 2.21	3.76 ± 2.08
Erysipelatoclostridiaceae	0.98 ± 0.82	1.27 ± 1.30	1.50 ± 2.00	1.31 ± 1.32
Erysipelotrichaceae	2.17 ± 2.62	1.33 ± 1.50	1.91 ± 2.70	1.13 ± 1.53
Clostridiaceae	0.66 ± 0.38	0.85 ± 0.53	1.12 ± 1.03	0.90 ± 0.67
Oscillospiraceae	2.85 ± 1.65	2.52 ± 1.47	1.86 ± 1.38	2.57 ± 1.75
Christensenellaceae	1.70 ± 0.99	1.55 ± 1.15	1.03 ± 1.15	1.42 ± 1.26
Eubacterium_coprostanoligenes_group	1.03 ± 0.47	1.31 ± 0.62	1.18 ± 0.70	1.25 ± 0.70
Others	14.15 ± 11.91	13.27 ± 11.11	11.58 ± 9.33	11.23 ± 10.04
SCFAs (mM)				
Acetic acid	53.17 ± 21.53	41.92 ± 18.97	54.18 ± 25.66	46.05 ± 30.66
Butyric acid	14.16 ± 11.44	12.55 ± 7.14	12.50 ± 7.45	11.04 ± 7.96
Propionic acid	13.39 ± 5.86	13.14 ± 7.72	17.37 ± 7.42	14.95 ± 10.24
Isobutyric acid	0.96 ± 1.47	0.89 ± 0.77	1.08 ± 1.32	0.78 ± 0.76
Isovaleric acid	2.13 ± 2.46	1.94 ± 1.19	2.12 ± 1.75	1.87 ± 1.31
Valeric acid	2.48 ± 2.28	1.93 ± 1.01	1.85 ± 1.26	1.26 ± 0.82
Caproic acid	10 ± 1.06	0.76 ± 1.03	0.40 ± 0.72	0.14 ± 0.28
Amino acids (mM)				
Total amino acids	42.88 ± 24.88	51.73 ± 24.95	49.89 ± 25.29	48.42 ± 20.10
Protein amino acids	41.39 ± 23.48	50.87 ± 24.06	48.76 ± 24.22	47.00 ± 19.12
Biogenic amines	1.35 ± 1.73	0.71 ± 1.18	0.96 ± 1.34	1.27 ± 2.56
Ammonium	27.19 ± 15.39	29.25 ± 18.61	29.89 ± 18.07	28.86 ± 17.17

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. S.P , soluble pectin; SCFAs, short chain fatty acids.

Table S7. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to total polyphenols intake (mg/day) category in each diagnosis group**.**

	Control		Polyps	
	T.P.<650 (*n*=2)	T.P.≥650 (*n*=23)	T.P.<650 (*n*=4)	T.P.≥650 (*n*=31)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.33 ± 12.69	18.32 ± 19.19	11.64 ± 12	2.71 ± 0.24
Peptostreptococcaceae	6.46 ± 8.49	3.02 ± 2.68 *	4.89 ± 5.34	9 ± 3.52
Prevotellaceae	3.71 ± 6.66	1.30 ± 0.99 *	4.07 ± 8.43	0.41 ± 0.11
Lachnospiraceae	19.65 ± 7.34	21.11 ± 8.3	21.39 ± 8.91	31.73 ± 0.7
Coriobacteriaceae	5.76 ± 3.49	8.86 ± 8.02 *	10.8 ± 9.10	2.32 ± 0.25
Enterobacteriaceae	3.05 ± 5.29	2.49 ± 4.08	1.11 ± 2.86	0.62 ± 0.02
Bacteroidaceae	5.21 ± 7.26	3.03 ± 0.67 *	4.17 ± 4.48	6.79 ± 4.25
Ruminococcaceae	12.28 ± 4.30	13.56 ± 4.68	11.2 ± 4.55	24.29 ± 2.87
Veillonellaceae	1.24 ± 1.65	1.74 ± 1.78 *	2.52 ± 4.95	0.23 ± 0.03
Akkermansiaceae	0.56 ± 1.06	0.86 ± 1.09	1.47 ± 3.59	0.27 ± 0.30
Streptococcaceae	1.82 ± 3.50	0.27 ± 0.25	0.87 ± 1.34	0.91 ± 0.11
Methanobacteriaceae	1.94 ± 2.80	0.41 ± 0.42	1.64 ± 2.79	0.43 ± 0.15
Eggerthellaceae	3.23 ± 2.00	3.41 ± 1.98	3.98 ± 2.18	4.97 ± 0.83
Erysipelatoclostridiaceae	1.27 ± 1.18	0.44 ± 0.09	1.39 ± 1.71	1.64 ± 0.84
Erysipelotrichaceae	1.56 ± 1.78	2.17 ± 3.57 *	1.41 ± 2.13	2.99 ± 3.54
Clostridiaceae	0.83 ± 0.48	0.46 ± 0.37	0.99 ± 0.86	1.28 ± 0.89
Oscillospiraceae	2.63 ± 1.59	2.73 ± 0.85	2.23 ± 1.65	2.03 ± 0.47
Christensenellaceae	1.64 ± 1.10	1.4 ± 0.98	1.22 ± 1.24	1.4 ± 0.34
Eubacterium_coprostanoligenes_group	1.15 ± 0.57	1.69 ± 0.41	1.2 ± 0.70	1.49 ± 0.63
Others	31.23 ± 0.53	12.05 ± 10.26	9.04 ± 9.10	11.71 ± 9.72
SCFAs (mM)				
Acetic acid	44.99 ± 19.46	53.15 ± 29.21	49.79 ± 28.94	53.32 ± 16.16
Butyric acid	12.61 ± 8.52	16.91 ± 11.27	11.63 ± 7.83	13.71 ± 3.63
Propionic acid	13.55 ± 7.19	10.88 ± 5.66	16.23 ± 9.2	14.32 ± 0.95
Isobutyric acid	0.91 ± 1.06	0.98 ± 1.17	0.96 ± 1.08	0.28 ± 0.39
Isovaleric acid	2.00 ± 1.75	2.05 ± 1.69	2.06 ± 1.54	0.92 ± 0.75
Valeric acid	2.08 ± 1.59	2.49 ± 1.64	1.56 ± 1.11	1.31 ± 0.02
Caproic acid	0.66 ± 0.80	2.2 ± 1.67	0.28 ± 0.56	0.01 ± 0.00
Amino acids (mM)				
Total amino acids	49.21 ± 25.84	43.71 ± 17.95	49.8 ± 22.85	38.16 ± 12.82
Protein amino acids	48.14 ± 24.89	42.46 ± 16.13	48.48 ± 21.81	37.57 ± 13.34
Biogenic amines	0.91 ± 1.38	1.21 ± 1.86	1.16 ± 2.09	0.46 ± 0.48
Ammonium	27.91 ± 17.69	32.93 ± 15.49	30.28 ± 17.43	14.23 ± 5.56

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. T.P., total polyphenols; SCFAs, short chain fatty acids.

Table S8. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to flavonoids intake (mg/day) category in each diagnosis group**.**

	Control		Polyps	
	Flavonoids<82.18 (*n*=9)	Flavonoids≥82.18 (*n*=16)	Flavonoids<82.18 (*n*=15)	Flavonoids≥82.18 (*n*=20)
Microbial families (% Abundance)				
Bifidobacteriaceae	14.14 ± 16.08	12.43 ± 11.9	11.57 ± 10.18	10.79 ± 13.19
Peptostreptococcaceae	4.11 ± 4.54	7.13 ± 9.46	5.50 ± 6.88	4.84 ± 3.91
Prevotellaceae	5.25 ± 9.30	2.39 ± 3.75	3.34 ± 5.88	4.25 ± 9.76
Lachnospiraceae	15.74 ± 3.98	22.12 ± 7.80	22.21 ± 10.99	21.81 ± 7.45
Coriobacteriaceae	5.50 ± 4.35	6.49 ± 4.10	13.39 ± 9.53	8.01 ± 8.16
Enterobacteriaceae	4.14 ± 7.55	2.33 ± 3.12	1.49 ± 4.15	0.77 ± 0.96
Bacteroidaceae	7.04 ± 10.13	3.78 ± 3.98	4.18 ± 3.85	4.43 ± 4.95
Ruminococcaceae	10.35 ± 2.86	13.61 ± 4.55	10.32 ± 5.12	13.16 ± 5.42
Veillonellaceae	1.32 ± 1.51	1.29 ± 1.75	2.44 ± 5.61	2.35 ± 4.31
Akkermansiaceae	0.47 ± 1.13	0.66 ± 1.02	1.87 ± 4.64	1.05 ± 2.39
Streptococcaceae	0.63 ± 0.88	2.20 ± 4.03	0.39 ± 0.41	1.23 ± 1.61
Methanobacteriaceae	1.24 ± 1.53	2.05 ± 3.15	0.77 ± 1.60	2.17 ± 3.25
Eggerthellaceae	3.38 ± 1.94	3.18 ± 2.02	4.22 ± 2.42	3.90 ± 1.95
Erysipelatoclostridiaceae	1.24 ± 0.98	1.13 ± 1.25	1.81 ± 2.17	1.09 ± 1.13
Erysipelotrichaceae	2.04 ± 2.36	1.40 ± 1.75	1.38 ± 2.59	1.60 ± 1.88
Clostridiaceae	0.80 ± 0.47	0.77 ± 0.50	1.04 ± 1.10	0.98 ± 0.65
Oscillospiraceae	2.74 ± 1.64	2.59 ± 1.49	1.82 ± 1.37	2.52 ± 1.72
Christensenellaceae	1.37 ± 1.06	1.74 ± 1.09	0.72 ± 0.48	1.61 ± 1.44
Eubacterium_coprostanoligenes_group	1.04 ± 0.50	1.31 ± 0.61	1.02 ± 0.76	1.36 ± 0.63
Others	17.46 ± 13.43	11.41 ± 9.44	10.51 ± 9.06	12.07 ± 10.10
SCFAs (mM)				
Acetic acid	43.17 ± 12.70	47.54 ± 23.72	51.58 ± 28.04	48.80 ± 29.04
Butyric acid	11.19 ± 7.56	14.22 ± 9.37	12.12 ± 8.02	11.47 ± 7.54
Propionic acid	12.37 ± 4.93	13.71 ± 8.01	17.59 ± 8.21	15.03 ± 9.50
Isobutyric acid	1.25 ± 1.48	0.73 ± 0.68	1.12 ± 1.34	0.78 ± 0.81
Isovaleric acid	2.68 ± 2.43	1.63 ± 1.04	2.21 ± 1.73	1.84 ± 1.37
Valeric acid	2.38 ± 2.19	1.98 ± 1.14	1.74 ± 1.24	1.40 ± 0.95
Caproic acid	0.89 ± 0.94	0.82 ± 1.10	0.30 ± 0.56	0.25 ± 0.56
Amino acids (mM)				
Total amino acids	56.11 ± 34.96	44.29 ± 16.65	55.61 ± 26.03	44.27 ± 18.55
Protein amino acids	54.62 ± 33.74	43.43 ± 15.89	54.00 ± 25.21	43.25 ± 17.37
Biogenic amines	1.33 ± 1.79	0.72 ± 1.14	1.41 ± 1.95	0.90 ± 2.11
Ammonium	34.31 ± 24.25	25.25 ± 11.36	34.16 ± 20.09	25.76 ± 14.48

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. SCFAs, short chain fatty acids.

Table S9. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to other polyphenols intake (mg/day) category in each diagnosis group**.**

	Control		Polyps	
	O.P.<32.15 (*n*=17)	O.P.≥32.15 (*n*=8)	O.P.<32.15 (*n*=27)	O.P.≥32.15 (*n*=8)
Microbial families (% Abundance)				
Bifidobacteriaceae	16.28 ± 14.39	6.17 ± 7.00 *	12.34 ± 12.31	7.04 ± 9.64
Peptostreptococcaceae	6.54 ± 9.35	4.98 ± 4.62	4.72 ± 5.36	6.49 ± 5.22
Prevotellaceae	1.07 ± 0.89	8.41 ± 9.60	2.50 ± 4.83	8.45 ± 14.5
Lachnospiraceae	18.87 ± 7.36	21.85 ± 7.16	21.45 ± 8.83	23.76 ± 9.91
Coriobacteriaceae	6.36 ± 4.18	5.66 ± 4.27	10.63 ± 8.77	9.23 ± 10.49
Enterobacteriaceae	3.12 ± 5.76	2.70 ± 3.59	1.26 ± 3.15	0.46 ± 0.49
Bacteroidaceae	5.46 ± 7.60	3.87 ± 5.09	4.34 ± 4.78	4.27 ± 3.37
Ruminococcaceae	11.26 ± 3.27	14.92 ± 5.25	11.12 ± 4.98	14.72 ± 6.21
Veillonellaceae	1.43 ± 1.94	1.03 ± 0.65	2.86 ± 5.41	0.79 ± 0.85
Akkermansiaceae	0.81 ± 1.21	0.14 ± 0.11	1.66 ± 3.94	0.54 ± 0.70
Streptococcaceae	1.14 ± 1.83	2.69 ± 5.30	0.92 ± 1.46	0.73 ± 0.57
Methanobacteriaceae	2.11 ± 3.05	0.99 ± 1.51	1.77 ± 2.95	0.89 ± 1.75
Eggerthellaceae	3.41 ± 2.25	2.92 ± 1.14	4.05 ± 2.21	3.99 ± 2.00
Erysipelatoclostridiaceae	1.10 ± 1.29	1.30 ± 0.78	1.43 ± 1.87	1.3 ± 0.69
Erysipelotrichaceae	1.53 ± 1.78	1.86 ± 2.43	1.28 ± 2.09	2.25 ± 2.47
Clostridiaceae	0.85 ± 0.55	0.64 ± 0.26	0.91 ± 0.65	1.36 ± 1.35
Oscillospiraceae	2.75 ± 1.50	2.40 ± 1.62	2.20 ± 1.76	2.29 ± 0.96
Christensenellaceae	1.70 ± 1.23	1.42 ± 0.64	1.10 ± 1.16	1.68 ± 1.35
Eubacterium_coprostanoligenes_group	1.25 ± 0.66	1.12 ± 0.36	1.19 ± 0.74	1.29 ± 0.57
Others	12.96 ± 10.20	14.93 ± 13.66	12.27 ± 10.54	8.47 ± 4.33
SCFAs (mM)				
Acetic acid	37.13 ± 14.42	64.75 ± 18.37 $^{+}$	51.62 ± 30.02	44.51 ± 21.89
Butyric acid	9.23 ± 5.36	21.40 ± 8.95 $^{+}$	12.13 ± 8.39	10.46 ± 4.41
Propionic acid	10.95 ± 5.27	18.08 ± 7.99 $^{+}$	17.20 ± 9.66	12.49 ± 4.64
Isobutyric acid	0.83 ± 0.75	1.10 ± 1.55	0.98 ± 1.18	0.75 ± 0.53
Isovaleric acid	1.90 ± 1.11	2.24 ± 2.66	2.07 ± 1.68	1.74 ± 0.84
Valeric acid	1.84 ± 1.19	2.74 ± 2.13	1.60 ± 1.18	1.35 ± 0.66
Caproic acid	0.72 ± 1.04	1.12 ± 1.00	0.31 ± 0.59	0.15 ± 0.39
Amino acids (mM)				
Total amino acids	49.29 ± 23.68	46.96 ± 28.64	52.49 ± 24.19	37.80 ± 9.13
Protein amino acids	48.27 ± 22.83	45.73 ± 27.36	50.93 ± 23.13	37.47 ± 9.22
Biogenic amines	0.86 ± 1.19	1.12 ± 1.86	1.39 ± 2.25	0.19 ± 0.26
Ammonium	27.53 ± 17.01	30.59 ± 18.65	31.74 ± 18.75	21.33 ± 7.85

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. O.T., other polyphenols; SCFAs, short chain fatty acids.

Table S10. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to calcium intake (mg/day) category in each diagnosis group**.**

	Control		Polyps	
	Ca<900 (*n*=13)	Ca≥900 (*n*=12)	Ca<900 (*n*=19)	Ca≥900 (*n*=16)
Microbial families (% Abundance)				
Bifidobacteriaceae	13.69 ± 15.62	12.35 ± 10.75	13.00 ± 12.41	8.90 ± 11.08
Peptostreptococcaceae	3.67 ± 3.40	8.61 ± 10.74	3.57 ± 3.13	6.97 ± 6.74
Prevotellaceae	4.49 ± 7.70	2.26 ± 4.35	4.03 ± 6.19	3.65 ± 10.35
Lachnospiraceae	19.23 ± 6.53	20.46 ± 8.27	20.46 ± 8.07	23.78 ± 9.92
Coriobacteriaceae	4.84 ± 2.76	7.54 ± 4.97	11.00 ± 8.71	9.50 ± 9.65
Enterobacteriaceae	4.36 ± 6.53	1.50 ± 2.32	0.72 ± 1.01	1.50 ± 3.99
Bacteroidaceae	6.39 ± 9.06	3.39 ± 2.68	2.88 ± 2.61	6.03 ± 5.56
Ruminococcaceae	11.92 ± 4.56	12.99 ± 4.04	12.11 ± 4.09	11.75 ± 6.78
Veillonellaceae	2.04 ± 1.99	0.50 ± 0.38 *	3.53 ± 6.29	1.03 ± 1.36
Akkermansiaceae	0.54 ± 1.04	0.66 ± 1.08	0.53 ± 0.92	2.44 ± 4.96
Streptococcaceae	1.60 ± 4.23	1.67 ± 2.10	0.81 ± 0.99	0.94 ± 1.64
Methanobacteriaceae	1.34 ± 2.13	2.21 ± 3.19	2.66 ± 3.35	0.27 ± 0.33 *
Eggerthellaceae	3.00 ± 1.55	3.52 ± 2.35	4.21 ± 2.24	3.84 ± 2.06
Erysipelatoclostridiaceae	1.22 ± 1.19	1.11 ± 1.13	1.14 ± 1.89	1.70 ± 1.34 *
Erysipelotrichaceae	1.95 ± 2.36	1.28 ± 1.45	1.59 ± 2.14	1.40 ± 2.30
Clostridiaceae	0.76 ± 0.47	0.81 ± 0.52	0.86 ± 0.61	1.19 ± 1.07
Oscillospiraceae	2.58 ± 1.48	2.70 ± 1.61	2.64 ± 1.49	1.73 ± 1.63
Christensenellaceae	1.61 ± 1.16	1.61 ± 1.03	1.49 ± 1.34	0.92 ± 0.98 *
Eubacterium_coprostanoligenes_group	1.11 ± 0.47	1.32 ± 0.68	1.44 ± 0.64	0.96 ± 0.69
Others	13.66 ± 11.50	13.50 ± 11.29	11.33 ± 8.05	11.49 ± 11.37
SCFAs (mM)				
Acetic acid	51.94 ± 21.55	39.49 ± 17.31	38.88 ± 18.19	63.2 ± 32.66 $^{+}$
Butyric acid	13.70 ± 9.82	12.51 ± 7.75	9.73 ± 5.61	14.15 ± 9.13
Propionic acid	13.70 ± 7.99	12.71 ± 6.00	13.27 ± 7.56	19.51 ± 9.48 $^{+}$
Isobutyric acid	0.53 ± 0.59	1.33 ± 1.28 $^{+}$	0.92 ± 0.87	0.93 ± 1.29
Isovaleric acid	1.38 ± 1.09	2.69 ± 2.02 $^{+}$	2.15 ± 1.35	1.81 ± 1.74
Valeric acid	1.78 ± 0.86	2.50 ± 2.06	1.69 ± 1.14	1.37 ± 1.01
Caproic acid	0.98 ± 0.88	0.70 ± 1.19	0.36 ± 0.69	0.16 ± 0.30
Amino acids (mM)				
Total amino acids	41.54 ± 26.83	56.14 ± 20.83	53.19 ± 24.02	44.31 ± 20.10
Protein amino acids	40.48 ± 25.87	55.01 ± 19.7	52.17 ± 23.24	42.73 ± 18.48
Biogenic amines	0.90 ± 1.32	0.99 ± 1.55	0.83 ± 1.35	1.46 ± 2.64
Ammonium	25.16 ± 18.68	32.14 ± 15.44	31.66 ± 19.29	26.63 ± 14.89

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. Ca, Calcium; SCFAs, short chain fatty acids.

Table S11. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to ethanol intake (g/day) category in each diagnosis group**.**

	Control		Polyps	
	Ethanol<12 (*n*=19)	Ethanol≥12 (*n*=6)	Ethanol<12 (*n*=23)	Ethanol≥12 (*n*=12)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.56 ± 11.74	14.60 ± 18.49	13.86 ± 13.51	5.88 ± 4.69
Peptostreptococcaceae	7.36 ± 8.88	1.87 ± 0.79	3.52 ± 2.96	8.21 ± 7.33 *
Prevotellaceae	3.16 ± 6.50	4.22 ± 6.07	3.34 ± 5.81	4.84 ± 11.83
Lachnospiraceae	18.89 ± 6.57	22.79 ± 9.25	20.23 ± 8.92	25.34 ± 8.47
Coriobacteriaceae	5.94 ± 4.39	6.77 ± 3.42	11.62 ± 9.98	7.82 ± 6.59
Enterobacteriaceae	3.08 ± 5.47	2.69 ± 4.04	0.67 ± 0.91	1.87 ± 4.60
Bacteroidaceae	5.73 ± 7.67	2.47 ± 1.63	4.11 ± 4.83	4.73 ± 3.76
Ruminococcaceae	12.42 ± 4.25	12.47 ± 4.71	11.05 ± 5.33	13.66 ± 5.34
Veillonellaceae	1.32 ± 1.73	1.24 ± 1.43	3.44 ± 5.72	0.38 ± 0.33 *
Akkermansiaceae	0.75 ± 1.16	0.11 ± 0.10	1.95 ± 4.22	0.36 ± 0.60
Streptococcaceae	1.23 ± 1.78	2.92 ± 6.22	0.95 ± 1.53	0.73 ± 0.73
Methanobacteriaceae	2.10 ± 2.94	0.65 ± 1.08	1.35 ± 2.61	1.99 ± 3.00
Eggerthellaceae	3.32 ± 1.99	3.04 ± 2.01	3.85 ± 2.22	4.41 ± 2.00
Erysipelatoclostridiaceae	1.12 ± 1.24	1.31 ± 0.81	1.19 ± 1.34	1.80 ± 2.16
Erysipelotrichaceae	1.55 ± 1.83	1.89 ± 2.53	1.20 ± 1.92	2.09 ± 2.61
Clostridiaceae	0.89 ± 0.51	0.46 ± 0.15	0.80 ± 0.62	1.41 ± 1.11
Oscillospiraceae	2.88 ± 1.62	1.88 ± 0.80	2.34 ± 1.73	2.00 ± 1.35
Christensenellaceae	1.79 ± 1.15	1.04 ± 0.52	1.13 ± 1.27	1.42 ± 1.11
Eubacterium_coprostanoligenes_group	1.29 ± 0.64	0.96 ± 0.23	1.16 ± 0.73	1.32 ± 0.63
Others	12.62 ± 10.11	16.63 ± 14.68	12.26 ± 10.63	9.75 ± 7.22
SCFAs (mM)				
Acetic acid	43.06 ± 21.08	55.17 ± 15.31	48.59 ± 30.67	52.69 ± 23.88
Butyric acid	11.64 ± 7.99	17.83 ± 10.03	11.78 ± 8.36	11.68 ± 6.39
Propionic acid	12.38 ± 7.46	15.91 ± 4.70	16.44 ± 9.58	15.51 ± 7.93
Isobutyric acid	0.97 ± 1.18	0.74 ± 0.43	0.93 ± 0.94	0.92 ± 1.32
Isovaleric acid	2.13 ± 1.91	1.63 ± 0.77	2.13 ± 1.56	1.75 ± 1.49
Valeric acid	2.02 ± 1.69	2.46 ± 1.12	1.50 ± 0.99	1.64 ± 1.29
Caproic acid	0.83 ± 1.07	0.91 ± 0.97	0.13 ± 0.29	0.53 ± 0.81
Amino acids (mM)				
Total amino acids	45.64 ± 26.59	57.77 ± 16.41	54.89 ± 23.22	38.09 ± 16.62 $^{+}$
Protein amino acids	44.55 ± 25.41	56.67 ± 16.3	53.39 ± 22.31	37.25 ± 15.41 $^{+}$
Biogenic amines	0.95 ± 1.46	0.91 ± 1.33	1.33 ± 2.32	0.72 ± 1.33
Ammonium	26.37 ± 18.49	35.28 ± 10.98 $^{+}$	33.18 ± 18.67	22.04 ± 12.04

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. SCFAs, short chain fatty acids.

Table S12. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to dibenzo (a) anthracene intake (µg/day) category in each diagnosis group**.**

	Control		Polyps	
	DiB(a)A<0.07 (*n*=19)	DiB(a)A≥0.07 (*n*=6)	DiB(a)A<0.07 (*n*=24)	DiB(a)A≥0.07 (*n*=11)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.45 ± 11.76	14.94 ± 18.41	11.92 ± 13.44	9.39 ± 7.46
Peptostreptococcaceae	7.17 ± 8.95	2.49 ± 1.91	4.50 ± 4.09	6.49 ± 7.36
Prevotellaceae	3.38 ± 6.53	3.54 ± 6.03	3.25 ± 5.69	5.17 ± 12.35
Lachnospiraceae	18.64 ± 6.36	23.56 ± 9.33	21.47 ± 9.42	23.09 ± 8.28
Coriobacteriaceae	5.88 ± 4.40	6.95 ± 3.33	10.81 ± 9.80	9.24 ± 7.45
Enterobacteriaceae	3.59 ± 5.62	1.07 ± 2.15	0.69 ± 0.90	1.92 ± 4.82
Bacteroidaceae	5.68 ± 7.69	2.63 ± 1.67	3.24 ± 3.45	6.69 ± 5.55
Ruminococcaceae	12.01 ± 3.97	13.79 ± 5.26	11.64 ± 5.69	12.61 ± 4.91
Veillonellaceae	1.31 ± 1.73	1.27 ± 1.44	2.94 ± 5.71	1.19 ± 1.42
Akkermansiaceae	0.74 ± 1.16	0.13 ± 0.13	1.06 ± 2.28	2.16 ± 5.36
Streptococcaceae	2.02 ± 3.74	0.41 ± 0.16	1.00 ± 1.520	0.59 ± 0.57
Methanobacteriaceae	2.07 ± 2.96	0.74 ± 1.06	1.74 ± 3.07	1.18 ± 1.81
Eggerthellaceae	3.34 ± 1.99	2.99 ± 2.01	4.11 ± 2.09	3.89 ± 2.33
Erysipelatoclostridiaceae	1.12 ± 1.24	1.33 ± 0.78	1.27 ± 1.33	1.69 ± 2.28
Erysipelotrichaceae	1.23 ± 1.44	2.89 ± 2.92	1.37 ± 1.96	1.80 ± 2.69
Clostridiaceae	0.87 ± 0.52	0.53 ± 0.22	1.03 ± 0.92	0.97 ± 0.73
Oscillospiraceae	2.79 ± 1.63	2.16 ± 1.02	2.40 ± 1.82	1.83 ± 0.89
Christensenellaceae	1.74 ± 1.14	1.18 ± 0.76	1.23 ± 1.24	1.24 ± 1.20
Eubacterium_coprostanoligenes_group	1.26 ± 0.64	1.05 ± 0.31	1.21 ± 0.71	1.22 ± 0.68
Others	12.71 ± 10.04	16.36 ± 14.95	13.13 ± 10.52	7.64 ± 5.79
SCFAs (mM)				
Acetic acid	42.85 ± 20.64	55.85 ± 16.73	51.3 ± 30.77	47.15 ± 22.78
Butyric acid	12.02 ± 8.85	16.63 ± 8.00	12.37 ± 8.19	10.38 ± 6.42
Propionic acid	12.71 ± 7.68	14.87 ± 4.10	15.83 ± 9.27	16.76 ± 8.54
Isobutyric acid	0.97 ± 1.18	0.73 ± 0.43	0.82 ± 0.95	1.15 ± 1.30
Isovaleric acid	2.13 ± 1.91	1.64 ± 0.75	1.95 ± 1.60	2.11 ± 1.41
Valeric acid	2.02 ± 1.69	2.45 ± 1.13	1.43 ± 0.99	1.80 ± 1.28
Caproic acid	0.81 ± 1.05	0.95 ± 1.03	0.21 ± 0.53	0.40 ± 0.60
Amino acids (mM)				
Total amino acids	46.87 ± 26.54	53.85 ± 19.16	52.59 ± 24.44	41.57 ± 15.72
Protein amino acids	45.60 ± 25.33	53.33 ± 18.94	51.14 ± 23.51	40.68 ± 14.45
Biogenic amines	1.12 ± 1.55	0.38 ± 0.50	1.29 ± 2.28	0.75 ± 1.38
Ammonium	27.29 ± 18.68	32.36 ± 11.95	32.21 ± 18.92	23.14 ± 11.77

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. DiB(a)A, dibenzo (a) anthracene; SCFAs, short chain fatty acids.

Table S13. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to total polycyclic aromatic hydrocarbons intake (µg/day) category in each diagnosis group**.**

	Control		Polyps	
	PAHs<0.75 (*n*=9)	PAHs≥0.75 (*n*=16)	PAHs<0.75 (*n*=10)	PAHs≥0.75 (*n*=25)
Microbial families (% Abundance)				
Bifidobacteriaceae	9.95 ± 13.19	14.79 ± 13.38	11.83 ± 10.43	10.85 ± 12.54
Peptostreptococcaceae	2.61 ± 2.36	7.97 ± 9.49 *	5.01 ± 4.01	5.17 ± 5.82
Prevotellaceae	4.75 ± 6.37	2.67 ± 6.33	2.76 ± 5.51	4.30 ± 9.15
Lachnospiraceae	21.03 ± 5.60	19.14 ± 8.18	19.31 ± 7.24	23.05 ± 9.52
Coriobacteriaceae	3.02 ± 1.99	7.89 ± 4.01 *	9.62 ± 9.95	10.59 ± 8.86
Enterobacteriaceae	4.25 ± 5.78	2.27 ± 4.71	0.47 ± 0.45	1.32 ± 3.26
Bacteroidaceae	9.01 ± 9.99	2.67 ± 2.45 *	5.97 ± 6.01	3.66 ± 3.59
Ruminococcaceae	13.25 ± 4.65	11.98 ± 4.12	12.91 ± 2.56	11.56 ± 6.20
Veillonellaceae	1.09 ± 1.18	1.42 ± 1.87	3.16 ± 5.42	2.08 ± 4.66
Akkermansiaceae	0.73 ± 1.22	0.52 ± 0.96	1.77 ± 3.28	1.26 ± 3.63
Streptococcaceae	2.12 ± 5.08	1.36 ± 1.89	1.21 ± 1.23	0.74 ± 1.33
Methanobacteriaceae	0.29 ± 0.39	2.57 ± 3.06 *	2.65 ± 3.96	1.13 ± 1.99
Eggerthellaceae	2.27 ± 0.83	3.80 ± 2.20 *	3.70 ± 1.72	4.18 ± 2.30
Erysipelatoclostridiaceae	0.77 ± 0.55	1.39 ± 1.33	0.68 ± 0.34	1.69 ± 1.89
Erysipelotrichaceae	0.93 ± 2.03	2.02 ± 1.88 *	1.11 ± 1.53	1.66 ± 2.40
Clostridiaceae	0.61 ± 0.32	0.88 ± 0.53	0.84 ± 0.69	1.08 ± 0.92
Oscillospiraceae	2.30 ± 1.20	2.83 ± 1.67	3.05 ± 1.78	1.89 ± 1.43
Christensenellaceae	1.43 ± 1.06	1.71 ± 1.10	1.88 ± 1.43	0.97 ± 1.02 *
Eubacterium_coprostanoligenes_group	1.03 ± 0.42	1.32 ± 0.64	1.43 ± 0.64	1.13 ± 0.71
Others	18.56 ± 13.26	10.79 ± 9.08	10.64 ± 6.39	11.71 ± 10.67
SCFAs (mM)				
Acetic acid	56.72 ± 23.11	39.92 ± 16.21	33.27 ± 15.84	56.69 ± 29.52 $^{+}$
Butyric acid	16.39 ± 10.45	11.29 ± 7.32	8.29 ± 4.21	13.13 ± 8.31
Propionic acid	14.92 ± 9.36	12.27 ± 5.34	11.19 ± 7.04	18.10 ± 8.96 $^{+}$
Isobutyric acid	0.39 ± 0.42	1.21 ± 1.18 $^{+}$	0.85 ± 0.41	0.95 ± 1.24
Isovaleric acid	1.10 ± 0.79	2.52 ± 1.88 $^{+}$	2.08 ± 0.53	1.96 ± 1.78
Valeric acid	1.69 ± 0.76	2.37 ± 1.85	1.36 ± 0.67	1.62 ± 1.21
Caproic acid	0.94 ± 0.97	0.79 ± 1.08	0.18 ± 0.36	0.30 ± 0.61
Amino acids (mM)				
Total amino acids	35.93 ± 21.01	55.64 ± 24.45 $^{+}$	42.78 ± 10.79	51.67 ± 25.42
Protein amino acids	35.07 ± 20.76	54.43 ± 23.11 $^{+}$	42.15 ± 10.86	50.14 ± 24.24
Biogenic amines	0.75 ± 1.23	1.05 ± 1.52	0.48 ± 1.26	1.37 ± 2.24 $^{+}$
Ammonium	23.58 ± 15.02	31.28 ± 18.20	25.78 ± 11.89	30.79 ± 19.14

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. PAHs, polycyclic aromatic hydrocarbons; SCFAs, short chain fatty acids.

Table S14. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to 2-amino-1-methyl-6-phenylimidazo intake (ng/day) category in each diagnosis group**.**

	Control		Polyps	
	PhIP<40 (*n*=8)	PhIP≥40 (*n*=17)	PhIP<40 (*n*=12)	PhIP≥40 (*n*=23)
Microbial families (% Abundance)				
Bifidobacteriaceae	14.27 ± 14.15	12.48 ± 13.21	9.80 ± 9.33	11.82 ± 13.1
Peptostreptococcaceae	6.52 ± 11.28	5.82 ± 6.46	6.60 ± 7.23	4.36 ± 3.94
Prevotellaceae	1.45 ± 1.96	4.34 ± 7.41	4.00 ± 11.73	3.78 ± 5.97
Lachnospiraceae	17.29 ± 5.57	21.02 ± 7.83	21.71 ± 10.40	22.12 ± 8.41
Coriobacteriaceae	4.38 ± 3.02	6.96 ± 4.40	8.38 ± 6.93	11.32 ± 9.97
Enterobacteriaceae	4.28 ± 6.10	2.38 ± 4.62	1.82 ± 4.59	0.69 ± 0.97
Bacteroidaceae	4.81 ± 4.98	5.02 ± 7.69	5.22 ± 4.66	3.85 ± 4.36
Ruminococcaceae	11.32 ± 3.82	12.96 ± 4.47	11.20 ± 3.56	12.34 ± 6.19
Veillonellaceae	1.40 ± 1.53	1.25 ± 1.72	4.40 ± 7.53	1.34 ± 2.08
Akkermansiaceae	1.33 ± 1.37	0.25 ± 0.64 *	1.53 ± 3.07	1.34 ± 3.77
Streptococcaceae	2.83 ± 5.51	1.07 ± 1.45	1.05 ± 1.17	0.78 ± 1.39
Methanobacteriaceae	1.52 ± 2.68	1.87 ± 2.75	1.72 ± 3.66	1.49 ± 2.18
Eggerthellaceae	2.88 ± 1.97	3.43 ± 1.98	4.57 ± 2.26	3.76 ± 2.06
Erysipelatoclostridiaceae	1.04 ± 1.18	1.23 ± 1.15	0.83 ± 0.68	1.70 ± 1.94
Erysipelotrichaceae	1.10 ± 1.80	1.88 ± 2.04	2.03 ± 2.41	1.23 ± 2.06
Clostridiaceae	0.81 ± 0.60	0.77 ± 0.43	1.04 ± 0.71	1.00 ± 0.94
Oscillospiraceae	3.15 ± 1.80	2.40 ± 1.35	2.75 ± 1.82	1.95 ± 1.44
Christensenellaceae	1.61 ± 0.93	1.61 ± 1.16	1.35 ± 1.07	1.17 ± 1.29
Eubacterium_coprostanoligenes_group	1.22 ± 0.83	1.21 ± 0.45	1.43 ± 0.60	1.10 ± 0.73
Others	16.79 ± 11.36	12.08 ± 11.09	8.58 ± 6.23	12.88 ± 10.74
SCFAs (mM)				
Acetic acid	42.1 ± 20.44	47.79 ± 20.52	38.67 ± 25.22	55.90 ± 28.41 $^{+}$
Butyric acid	11.37 ± 10.36	13.96 ± 8.06	9.76 ± 6.32	12.79 ± 8.18
Propionic acid	10.63 ± 6.20	14.45 ± 7.16	14.11 ± 9.35	17.18 ± 8.73
Isobutyric acid	0.75 ± 0.73	0.99 ± 1.18	1.30 ± 1.35	0.73 ± 0.86
Isovaleric acid	1.73 ± 1.11	2.14 ± 1.94	2.59 ± 1.65	1.69 ± 1.39
Valeric acid	1.40 ± 0.51	2.47 ± 1.78	1.38 ± 0.75	1.63 ± 1.23
Caproic acid	0.43 ± 0.69	1.04 ± 1.11	0.13 ± 0.30	0.34 ± 0.64
Amino acids (mM)				
Total amino acids	43.87 ± 19.56	50.75 ± 27.16	49.51 ± 24.55	48.93 ± 21.84
Protein amino acids	42.95 ± 18.68	49.58 ± 26.14	48.42 ± 24.09	47.56 ± 20.49
Biogenic amines	0.77 ± 1.21	1.03 ± 1.52	0.95 ± 1.34	1.21 ± 2.34
Ammonium	26.72 ± 8.19	29.35 ± 20.32	32.1 ± 22.09	27.93 ± 14.67

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. PhIP, 2-amino-1-methyl-6-phenylimidazo (4,5,b) pyridine; SCFAs, short chain fatty acids.

Table S15. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to 2-amino-3,8 dimethylimidazo intake (ng/day) category in each diagnosis group**.**

	Control		Polyps	
	MeIQx<50 (*n*=17)	MeIQx≥50 (*n*=8)	MeIQx<50 (*n*=28)	MeIQx≥50 (*n*=7)
Microbial families (% Abundance)				
Bifidobacteriaceae	12.96 ± 11.64	13.24 ± 17.08	12.23 ± 12.86	6.73 ± 4.59
Peptostreptococcaceae	6.31 ± 9.45	5.49 ± 4.28	5.11 ± 5.67	5.18 ± 3.84
Prevotellaceae	2.28 ± 3.77	5.84 ± 9.66	4.37 ± 9.05	1.81 ± 2.96
Lachnospiraceae	19.20 ± 6.94	21.14 ± 8.30	20.75 ± 8.55	26.91 ± 9.66
Coriobacteriaceae	4.80 ± 3.00	8.98 ± 4.93	9.35 ± 9.41	14.18 ± 6.59
Enterobacteriaceae	3.59 ± 5.86	1.71 ± 2.74	1.14 ± 3.09	0.85 ± 0.82
Bacteroidaceae	6.33 ± 7.76	2.02 ± 2.85 *	4.96 ± 4.74	1.78 ± 1.31
Ruminococcaceae	11.97 ± 3.71	13.41 ± 5.41	11.20 ± 5.35	14.92 ± 4.86
Veillonellaceae	1.10 ± 1.34	1.74 ± 2.17	2.88 ± 5.30	0.41 ± 0.29
Akkermansiaceae	0.84 ± 1.20	0.07 ± 0.05 *	1.62 ± 3.86	0.53 ± 1.12
Streptococcaceae	2.05 ± 3.94	0.74 ± 0.95	0.88 ± 1.30	0.86 ± 1.44
Methanobacteriaceae	1.73 ± 2.80	1.80 ± 2.56	1.49 ± 2.90	1.88 ± 2.03
Eggerthellaceae	3.26 ± 2.19	3.23 ± 1.46	4.04 ± 2.21	4.05 ± 1.98
Erysipelatoclostridiaceae	0.84 ± 0.86	1.86 ± 1.40 *	1.13 ± 1.16	2.47 ± 2.83
Erysipelotrichaceae	1.38 ± 1.85	2.18 ± 2.23	1.17 ± 1.80	2.84 ± 3.13
Clostridiaceae	0.83 ± 0.52	0.69 ± 0.41	0.95 ± 0.90	1.27 ± 0.64
Oscillospiraceae	2.77 ± 1.49	2.37 ± 1.64	2.32 ± 1.74	1.83 ± 0.76
Christensenellaceae	1.65 ± 1.08	1.52 ± 1.12	1.28 ± 1.34	1.02 ± 0.40
Eubacterium_coprostanoligenes_group	1.15 ± 0.64	1.34 ± 0.43	1.16 ± 0.65	1.45 ± 0.87
Others	14.98 ± 11.93	10.63 ± 9.35	11.99 ± 9.97	9.05 ± 7.91
SCFAs (mM)				
Acetic acid	42.43 ± 21.09	53.49 ± 17.15	48.16 ± 28.19	57.33 ± 29.36
Butyric acid	11.46 ± 8.86	16.67 ± 7.78	10.97 ± 7.35	14.84 ± 8.60
Propionic acid	11.78 ± 5.65	16.30 ± 8.84	15.63 ± 9.40	18.11 ± 7.00
Isobutyric acid	0.75 ± 0.68	1.27 ± 1.58	0.97 ± 1.13	0.75 ± 0.83
Isovaleric acid	1.74 ± 1.09	2.59 ± 2.59	2.08 ± 1.59	1.68 ± 1.25
Valeric acid	1.68 ± 1.02	3.08 ± 2.12 $^{+}$	1.40 ± 0.93	2.12 ± 1.50
Caproic acid	0.48 ± 0.75	1.63 ± 1.13 $^{+}$	0.17 ± 0.32	0.66 ± 1.01
Amino acids (mM)				
Total amino acids	47.92 ± 24.28	49.88 ± 27.48	48.85 ± 21.97	50.25 ± 26.08
Protein amino acids	47.09 ± 23.41	48.24 ± 26.29	47.71 ± 21.04	48.44 ± 24.70
Biogenic amines	0.68 ± 1.17	1.50 ± 1.77	1.00 ± 2.08	1.59 ± 1.92
Ammonium	28.41 ± 17.48	28.72 ± 17.83	30.44 ± 18.18	25.03 ± 13.87

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. ($^{+}$) U Mann-Whitney statistically significant difference ($p < 0.05$) in metabolite between the two levels of consumption in the diagnosis groups. MeIQx, 2-amino-3,8 dimethylimidazo (4,5,f) quinoxaline; SCFAs, short chain fatty acids.

Table S16. Microbial abundance and faecal SCFAs, amino acids, biogenic amines and ammonium concentrations according to nitrites intake (mg/day) category in each diagnosis group**.**

	Control		Polyps	
	Nitrites<1.69 (*n*=6)	Nitrites≥1.69 (*n*=19)	Nitrites<1.69 (*n*=12)	Nitrites≥1.69 (*n*=23)
Microbial families (% Abundance)				
Bifidobacteriaceae	23.98 ± 11.58	9.60 ± 12.01 *	17.28 ± 13.77	7.92 ± 9.50 *
Peptostreptococcaceae	12.94 ± 13.91	3.87 ± 3.48	3.32 ± 2.66	6.06 ± 6.10
Prevotellaceae	2.32 ± 2.14	3.77 ± 7.14 *	3.59 ± 5.51	3.99 ± 9.45
Lachnospiraceae	16.26 ± 6.26	20.95 ± 7.37	20.60 ± 9.06	22.70 ± 9.07
Coriobacteriaceae	5.44 ± 2.39	6.36 ± 4.58	11.32 ± 6.79	9.79 ± 10.13
Enterobacteriaceae	1.91 ± 3.84	3.32 ± 5.47	0.34 ± 0.41	1.46 ± 3.38
Bacteroidaceae	2.33 ± 1.20	5.78 ± 7.67	4.00 ± 3.80	4.49 ± 4.82
Ruminococcaceae	10.74 ± 4.32	12.97 ± 4.22	10.25 ± 5.21	12.83 ± 5.40
Veillonellaceae	1.36 ± 1.14	1.28 ± 1.79	2.95 ± 6.21	2.09 ± 4.06
Akkermansiaceae	0.70 ± 0.93	0.56 ± 1.10	2.08 ± 5.14	1.05 ± 2.30
Streptococcaceae	4.40 ± 5.96	0.76 ± 1.18	0.76 ± 0.73	0.93 ± 1.53
Methanobacteriaceae	0.39 ± 0.42	2.18 ± 2.94	1.51 ± 2.43	1.60 ± 2.92
Eggerthellaceae	2.49 ± 0.75	3.49 ± 2.16	3.99 ± 2.55	4.07 ± 1.95
Erysipelatoclostridiaceae	0.45 ± 0.20	1.40 ± 1.22 *	1.21 ± 1.57	1.50 ± 1.74
Erysipelotrichaceae	0.91 ± 0.76	1.86 ± 2.18	1.71 ± 2.54	1.40 ± 2.02
Clostridiaceae	0.96 ± 0.64	0.73 ± 0.43	0.81 ± 0.67	1.11 ± 0.93
Oscillospiraceae	2.58 ± 1.40	2.66 ± 1.58	2.26 ± 1.55	2.20 ± 1.66
Christensenellaceae	1.36 ± 0.37	1.69 ± 1.21	1.60 ± 1.71	1.04 ± 0.82
Eubacterium_coprostanoligenes_group	1.23 ± 0.74	1.21 ± 0.54	0.97 ± 0.77	1.34 ± 0.63 *
Others	7.27 ± 4.41	15.58 ± 11.97	9.44 ± 6.99	12.42 ± 10.66
SCFAs (mM)				
Acetic acid	39.41 ± 23.77	48.04 ± 19.25	44.50 ± 21.73	52.86 ± 31.15
Butyric acid	12.44 ± 11.28	13.34 ± 8.12	9.69 ± 5.20	12.82 ± 8.56
Propionic acid	11.14 ± 7.74	13.89 ± 6.81	15.85 ± 9.80	16.27 ± 8.68
Isobutyric acid	0.75 ± 0.80	0.97 ± 1.12	1.01 ± 0.89	0.88 ± 1.16
Isovaleric acid	1.70 ± 0.98	2.11 ± 1.89	2.27 ± 1.47	1.85 ± 1.56
Valeric acid	1.60 ± 0.62	2.29 ± 1.74	1.48 ± 0.84	1.58 ± 1.20
Caproic acid	0.63 ± 0.79	0.92 ± 1.10	0.08 ± 0.14	0.37 ± 0.66
Amino acids (mM)				
Total amino acids	50.00 ± 19.31	48.09 ± 26.73	58.82 ± 28.18	44.07 ± 17.42
Protein amino acids	48.86 ± 18.42	47.01 ± 25.72	57.34 ± 26.80	42.91 ± 16.62
Biogenic amines	0.93 ± 1.38	0.95 ± 1.45	1.29 ± 2.48	1.03 ± 1.81
Ammonium	25.66 ± 8.86	29.41 ± 19.23	35.82 ± 22.69	25.99 ± 13.17

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. SCFAs, short chain fatty acids.

Table S17. Differences in microbial abundance and faecal SCFAs and amino acids concentrations according to N-nitrosodimethylamine intake (μg/day) category in each diagnosis group**.**

	Control		Polyps	
	NDMA<0.126 (*n*=7)	NDMA≥0.126 (*n*=18)	NDMA<0.126 (*n*=11)	NDMA≥0.126 (*n*=24)
Microbial families (% Abundance)				
Bifidobacteriaceae	21.36 ± 12.65	9.82 ± 12.32 *	14.39 ± 12.74	9.63 ± 11.36
Peptostreptococcaceae	11.88 ± 13.01	3.77 ± 3.55	3.16 ± 2.85	6.02 ± 5.95
Prevotellaceae	2.25 ± 1.96	3.87 ± 7.33 *	5.92 ± 7.37	2.91 ± 8.57
Lachnospiraceae	17.90 ± 7.18	20.57 ± 7.39	17.85 ± 6.8	23.87 ± 9.34
Coriobacteriaceae	6.05 ± 2.72	6.17 ± 4.63	11.89 ± 9.91	9.59 ± 8.75
Enterobacteriaceae	1.67 ± 3.56	3.50 ± 5.58	0.40 ± 0.48	1.39 ± 3.32
Bacteroidaceae	2.08 ± 1.28	6.07 ± 7.79	3.83 ± 3.42	4.55 ± 4.90
Ruminococcaceae	10.71 ± 3.95	13.10 ± 4.30	10.61 ± 5.02	12.56 ± 5.57
Veillonellaceae	2.16 ± 2.35	0.97 ± 1.18	3.29 ± 6.43	1.97 ± 3.99
Akkermansiaceae	0.60 ± 0.88	0.59 ± 1.12	2.26 ± 5.36	1.01 ± 2.26
Streptococcaceae	3.81 ± 5.66	0.79 ± 1.21	0.54 ± 0.54	1.02 ± 1.52
Methanobacteriaceae	1.33 ± 2.51	1.92 ± 2.78	1.20 ± 2.28	1.73 ± 2.94
Eggerthellaceae	2.58 ± 0.72	3.51 ± 2.22	3.92 ± 2.57	4.10 ± 1.96
Erysipelatoclostridiaceae	1.00 ± 1.49	1.23 ± 1.01	0.57 ± 0.46	1.78 ± 1.88 *
Erysipelotrichaceae	1.40 ± 1.49	1.72 ± 2.15	1.19 ± 1.64	1.65 ± 2.41
Clostridiaceae	1.03 ± 0.61	0.69 ± 0.41	0.66 ± 0.56	1.17 ± 0.93
Oscillospiraceae	2.44 ± 1.33	2.72 ± 1.61	2.86 ± 1.67	1.93 ± 1.51
Christensenellaceae	1.73 ± 1.03	1.56 ± 1.11	1.76 ± 1.71	0.99 ± 0.83
Eubacterium_coprostanoligenes_group	1.24 ± 0.67	1.2 ± 0.56	1.03 ± 0.55	1.30 ± 0.75
Others	6.78 ± 4.23	16.23 ± 11.97	12.66 ± 8.99	10.82 ± 9.94
SCFAs (mM)				
Acetic acid	43.09 ± 23.78	47.09 ± 19.35	38.22 ± 16.13	55.39 ± 31.11
Butyric acid	12.48 ± 10.30	13.38 ± 8.36	9.30 ± 5.50	12.87 ± 8.31
Propionic acid	12.12 ± 7.53	13.66 ± 6.93	13.05 ± 7.19	17.53 ± 9.43
Isobutyric acid	0.70 ± 0.74	1.00 ± 1.15	1.07 ± 0.93	0.86 ± 1.13
Isovaleric acid	1.61 ± 0.92	2.17 ± 1.93	2.38 ± 1.49	1.82 ± 1.54
Valeric acid	1.69 ± 0.61	2.30 ± 1.79	1.65 ± 0.82	1.50 ± 1.19
Caproic acid	0.65 ± 0.72	0.92 ± 1.13	0.22 ± 0.38	0.29 ± 0.62
Amino acids (mM)				
Total amino acids	49.60 ± 17.66	48.14 ± 27.50	53.37 ± 25.19	47.18 ± 21.36
Protein amino acids	48.55 ± 16.83	47.03 ± 26.46	52.83 ± 24.60	45.57 ± 19.97
Biogenic amines	0.83 ± 1.28	0.99 ± 1.48	0.38 ± 0.64	1.45 ± 2.36
Ammonium	24.99 ± 8.27	29.88 ± 19.68	34.77 ± 22.45	26.88 ± 14.34

Values are presented as mean ± standard deviation. (*) Differential microbial family abundance according to linear effect size discriminant analysis (LEfSe) at a threshold of 2.0 for the comparison of samples from each category of consumption in diagnosis groups. No significant differences were found by U Mann-Whitney analysis ($p < 0.05$) of metabolites between the two consumption levels. NDMA, N-nitrosodimethylamine; SCFAs, short chain fatty acids.

Table S18. Spearman correlation between the relative abundance of Bifidobacteriaceae and faecal SCFAs concentration according to clinical diagnosis group**.**

	Bifidobacteriaceae	
SCFAs (mM)	ρ	p-value
Control		
Acetic acid	-0.208	0.319
Butyric acid	-0.087	0.679
Propionic acid	-0.219	0.293
Isobutyric acid	-0.001	0.997
Isovaleric acid	0.037	0.861
Valeric acid	0.124	0.556
Caproic acid	0.247	0.233
Polyps		
Acetic acid	-0.053	0.764
Butyric acid	-0.104	0.554
Propionic acid	0.018	0.916
Isobutyric acid	0.234	0.176
Isovaleric acid	0.247	0.152
Valeric acid	0.079	0.651
Caproic acid	-0.293	0.088

Correlation values are presented as Spearman's rank correlation coefficient (ρ) and its associated p-value.

Objetivo 4:

Estudio de la relación entre la ingesta de nitratos, nitritos, nitrosaminas y sus fuentes dietéticas con la excreción fecal de N-nitrosocompuestos en función de las lesiones de la mucosa intestinal

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Entre los xenobióticos de la dieta se encuentran los N-nitrosocompuestos, que pueden tener origen exógeno si son ingeridos con la dieta, o endógenos si se forman en el organismo a partir de los precursores presentes en la dieta. Las nitrosaminas conforman la mayor parte de los N-nitrosocompuestos exógenos y algunas han mostrado potencial carcinogénico. Por otro lado, la ingesta de precursores como el nitrato o el nitrito puede contribuir a la formación endógena de N-nitrosocompuestos. Por tanto, resulta de gran interés conocer la relación entre los N-nitrosocompuestos y sus precursores ingeridos con la dieta, así como la concentración final de N-nitrosocompuestos en el organismo y su posible relación con el estado de la mucosa intestinal. Esto serviría para poder formular indicaciones dietéticas específicas con el objetivo de modular el impacto de los xenobióticos en el organismo, a fin de prevenir el desarrollo de cáncer colorrectal. Los resultados obtenidos se han organizado en el siguiente manuscrito, actualmente en proceso de revisión.

Manuscrito 1. Ruiz-Saavedra, S.; Pietilä, T.K.; Zapico, A.; de los Reyes-Gavilán, C.G.; Pajari, A.-M.; González, S. Dietary Nitrosamines from Processed Meat Intake as Drivers of the Fecal Excretion of Nitrosocompounds. *Journal of Agricultural and Food Chemistry.* Enviado para su publicación.

Los voluntarios del estudio se clasificaron según los grupos de análisis histopatológico mencionados en el Objetivo 1: Control, pólipos hiperplásicos, adenomas convencionales o adenocarcinomas. A partir de la evaluación nutricional realizada en objetivos anteriores, se determinó la ingesta de N-nitrosocompuestos de origen exógeno y precursores de N-nitrosocompuestos endógenos. Además, se determinó la concentración de N-nitrosocompuestos hemo y N-nitrosocompuestos totales en heces. Se analizó la relación entre N-nitrosocompuestos endógenos y exógenos, así como con la dieta y la mutagenicidad fecal en función del daño de la mucosa intestinal.

Los resultados obtenidos corroboran que la ingesta de carnes procesadas es la principal fuente dietética de la ingesta de nitritos y nitrosaminas, siendo el chorizo un predictor de la concentración fecal de N-nitrosocompuestos hemo y N-nitrosocompuestos totales en la muestra poblacional estudiada. Además, los resultados reflejan que las concentraciones fecales de N-nitrosocompuestos hemo y N-nitrosocompuestos totales son mayores en el grupo con adenocarcinomas que en el grupo control, presentando estas concentraciones una fuerte correlación positiva con la ingesta dietética de las nitrosaminas NDMA, NPIP y NPYR. A pesar de no observarse diferencias significativas en la concentración fecal de los N-nitrosocompuestos según los niveles de mutagenicidad fecal, la concentración de estos N-nitrosocompuestos tiende ser más alta en el grupo de voluntarios con mayor mutagenicidad fecal.

Tittle: Dietary nitrosamines from processed meat intake as drivers of the fecal excretion of nitrosocompounds

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Abstract: The main exogenous source of potentially carcinogenic nitrosamines (NAs) is diet. However, several factors can affect endogenous N-nitroso compounds (NOCs) formation in the organism, and therefore the potential damage of the intestinal mucosa at stages previous to CRC development. To address this issue, a total of 49 volunteers were recruited and classified according to histopathology analyses of intestinal biopsy samples. Lifestyle and dietary information was registered after colonoscopy. The mutagenicity of fecal supernatants was assayed by a modified Ames test. Fecal heme-derived NOCs and total NOCs concentrations were determined analytically by selective de-nitrosation and chemiluminescence-based detection. Our results revealed the consumption of processed meats as the main source of dietary nitrites and NAs, and specifically, the Spanish sausage "chorizo" as a predictor of the fecal concentration of heme-derived and total NOCs. Furthermore, increased fecal NOCs concentrations were found as the severity of colonic mucosal damage increased from the control to the adenocarcinoma group, being these concentrations strongly correlated with the intake of the NAs N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolidine (NPYR). Higher fecal NOCs concentrations were also noted in higher fecal mutagenicity samples. These results could contribute to a better understanding of the modulation of diet and derived xenobiotics and their impact on the intestinal environment as related to colonic mucosa damage.

Keywords: Food processing, Nitrosamines, N-nitroso compounds, Intestinal mucosa lesions, hyperplastic polyps, conventional adenomas, fecal mutagenicity.

1. Introduction

The consumption of red and processed meat has been assessed by the World Cancer Research Fund (WCRF) as a risk factor for colorectal cancer (CRC) in humans.[1] The presence of heme iron, the thermal formation of different carcinogens during cooking, the generation of lipid and protein oxidation products, and the formation of exogenous N-nitroso compounds (NOCs) in cured meats or endogenous NOCs in the gastrointestinal tract are considered among the possible mechanisms underlying this association.[1] Exogenous NOCs are mainly divided into nitrosamides and nitrosamines (NAs). Nitrosamides are chemically unstable and eventually decompose.[2] Therefore, the most abundant NOCs found in foods are NAs such as N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolidine (NPYR).[3] In this regard, the European Food Safety Authority (EFSA) has recently issued a technical report on the risks to human health associated with the intake of NAs in food.[4] Although unprocessed and uncooked meat may contain traces of NAs, these compounds have been detected at higher concentrations in cured meats, smoked fish, cheese, preserved vegetables, beer, and human milk, highlighting the importance of cooking and processing methods in the final concentrations.[2] Previous studies in animal and cell models have proposed that some NAs may be linked to a higher risk of CRC through the increase of fecal genotoxicity and the formation of highly reactive diazonium ions, which can generate DNA-adducts.[5–7] Furthermore, the excess of protein fermentation in the intestine is associated with higher levels of amines and other compounds potentially toxic to the gut mucosa such as heme iron, which induces the generation of free radicals in the colon.[8,9] Most of the available research in humans comes from epidemiological studies in which the assessment of NAs exposure is limited. The food frequency questionnaire (FFQ), a commonly employed method for intake assessment, estimates long-term dietary intake and enables the comparison among individuals in a population.[10] However, it lacks precision and the ability to reflect the exposure to other environmental sources of these compounds such as tobacco, water or cosmetic products.[11] Moreover, predicted endogenous concentrations of NOCs have been calculated to be 100 times higher than the estimated dietary intakes.[12] In the gastrointestinal tract, dietary components such as red meat, protein and NOCs precursors such as nitrates, nitrites and heme iron can contribute to further nitrosation processes and therefore to endogenous formation of nitrosyl heme (heme NOCs), S-nitrosothiols (SNOs) and NAs by acid catalyzed, intestinal cell or microbiota mediated

pathways, being finally excreted in feces as total (apparent) NOCs.[12–16] However, there are no studies that have analyzed the correlation between the consumption of these dietary sources and the fecal NOCs concentration obtained by analytical determinations in the context of CRC. Based on this evidence, a more comprehensive approach is needed to lay the groundwork for a better understanding of the complex associations between diet and cancer in the long-term.

In the present study we therefore investigated the impact of diet and dietary xenobiotics on fecal NOCs concentrations and their association with fecal mutagenicity in a sample population of adults at different stages of intestinal mucosa damage in the progression to CRC, after biopsy examination.

2. Subjects and Methods

Study Design and Volunteers

This study is part of broader projects related to the effect of diet and dietary xenobiotics on intestinal mucosa and related gut microbiota profiles in the context of CRC (MIXED and MiToxicDiet). The recruitment of volunteers was carried out from October 2019 to December 2021 by the facultatives of the Digestive Service from the Central University Hospital of Asturias (HUCA) and the Carmen and Severo Ochoa Hospital in Cangas de Narcea, Asturias, Spain. Volunteers were selected among individuals enrolled in a colon cancer screening program. Inclusion criteria were being between 40-79 years old and not referring the intake of omeprazole, antibiotics, corticoids, or non-steroidal anti-inflammatory drugs. Also, having specific cancer treatment at the time of the study or in the previous two months, previous surgery of the digestive system, autoimmunity, altered thyroid function, or history of diabetes or goiter were exclusion criteria. Those individuals interested in participating were informed of the objectives of the study and signed an informed consent form. A total of 49 subjects were included in the study. Patients were asked to provide a stool sample collected prior to the preparation for colonoscopy. During procedure, a biopsy for the removal of tissue samples was performed. Biopsies were examined at the Department of Anatomical Pathology of HUCA, as described elsewhere.[17] After biopsy examination patients were classified into four histopathology groups in order of increasing risk of CRC development: non-pathological control (NP) (n=18), hyperplastic polyps (HP) (n=10), conventional adenomas (CA) (n=18) and adenocarcinomas (AC) (n=3).

This project was evaluated and approved by the Regional Ethics Committee of Clinical Research of Asturias (Ref. 163/19) and by the Committee on Bioethics of CSIC (Ref. 174/2020). The procedures were performed in accordance with the fundamental principles set out in the Declaration of Helsinki, the Oviedo Bioethics Convention, and the Council of Europe Convention on Human Rights and Biomedicine, as well as in Spanish legislation on bioethics. Directive 95/46/EC of the European Parliament and the Council of October 1995, on the protection of individuals regarding the processing of personal data was strictly followed.

Nutritional Assessment

Dietary information was obtained from volunteers through a personalized interview conducted by a trained interviewer when they were informed about the colonoscopy results at the medical consultation. Exceptionally, as a result of COVID-19 restriction of visitors to hospitals in Spain during the pandemic, some of the surveys were conducted through online tools. For this purpose, a 155-item semi-quantitative FFQ previously developed by the research group and validated for the estimation of dietary xenobiotics intake was used.[18] In addition to food and culinary preparations, the specific type of food was recorded, as well as cooking methods and other related questions as the degree of doneness, when necessary. Information relative to dietary assessment for the estimation of the dietary intake of xenobiotics has been previously published.[17] The classification of foods into food groups was carried out according to the Centre for Higher Education in Nutrition and Dietetics (CESNID) criteria.[19] Food composition tables of CESNID were used to transform food consumption into energy, macro- and micro-nutrient intake.[19] The content of nitrates, nitrites and the exogenous NAs NDMA, NPIP and NPYR was estimated using the European Prospective Investigation into Cancer and Nutrition (EPIC) Potential Carcinogen Database and other databases previously indicated.[17,20] The phenolic content of the foods was extracted from Phenol Explorer 3.6 and fiber content from the tables by Marlett and Cheung.[21,22] Oxygen Radical Activity Capacity (ORAC) was calculated based on the article by Wu et al.[23] For each dietary compound of interest, the food sources with at least 5% of contribution in each case were considered. Heme iron intake was calculated by assuming that heme iron was 40% of total iron contained in all meats, fish and eggs, as proposed by Monsen and Balintfy.[24] Dietary information was obtained for 36 volunteers from NP (n=11), HP (n=8), CA (n=14) and AC (n=3) groups.

General Characteristics

During personalized interviews, sleeping hours and physical activity were recorded as the average self-referred time per day for each individual considering the last year, while number of depositions were recorded as the self-referred times during a normal week. Information on smoking habit was obtained by asking about cigarette smoking throughout life. The anthropometrical parameters height (m) and weight (kg) were taken by standardized protocols.[25] Body mass index (BMI) was calculated using the formula weight/(height)^2.

Measurement of Fecal Total NOCs and Heme NOCs

Fecal homogenates were prepared by diluting samples with ultrapure Milli Q water (1:5, Millipore, Rios30) and homogenized with T-18 Digital Ultra Turrax (IKA, Germany). Total NOCs and heme NOCs were analyzed from the fecal homogenates using selective de-nitrosation and chemiluminescence-based detection by Ecomedics CLD 88 Exhalyzer (Eco Medics, Switzerland) equipped with a custom-made liquid purge vessel and a NaOH (1 mol/L, kept at 4°C) trap as modified from previous studies.[26,27] Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Merck Life Science).

Preservation solution (250 mg of N-ethylmaleimide (M = 125 g/mol), and 78 mg of DTPA (M = 393 g/mol) in 20 ml of Milli-Q water) was used to prevent artificial nitrosation via binding of metal iron and alkylating free thiol groups, whereas acid sulphanilamide (SA) solution (5 g/100ml in 1 M hydrogen chloride (HCl)) was used to remove nitrite.[28] Use of the selective de-nitrosation enables the indirect determination of heme NOCs. Therefore, mercury (II) and ferricyanide stable nitroso compounds (non Heme) were determined using aqueous $HgCl_2$ (10mM $HgCl_2$, M = 271.5 g/mol) and $K_3Fe(CN)_6$ (10 mM $K_3Fe(CN)_6$, M = 368.35 g/mol).

Sample incubated with preservation and SA solution with or without $HgCl_2$ and $K_3Fe(CN)_6$ was injected into the purge vessel containing 15 mL of reducing agent triiodine mixture (1g potassium iodine (M = 166.0 g/mol) and 0.65 g iodine (M = 253.81 g/mol, Acros Organics, UK), 70 ml glacial acetic acid (WWR Chemicals, France), 20 ml Milli-Q water, and antifoam solution (A6707) kept at 60 °C that releases nitrogen oxide (NO) from NAs, SNOs, alkyl nitrites, and iron nitrosyl compounds. System helium gas flow (110 ml/min, adjusted 0 ± 5.0 mbar) mixes the sample and transfers the released NO to the CLD 88 via a condenser, the chemical trap and a 0.20 μm polypropylene filter.

The signal (sampled at 0.05 Hz) was analyzed by the instrument software (PowerChrom, eDAQ, Australia). Calibration curves of known standards of aqueous sodium nitrate ($NaNO_2$) were used for quantification by comparing the area under the curve to the area of known standards. Heme NOCs were determined by subtracting the values of mercury (II) and ferricyanide stable compounds from the total NOC. Concentrations were obtained for volunteers from NP=15, HP=9, CA=14 and AC=3 groups and expressed as picomoles per milligram of fecal sample (pmol/mg).

Fecal Mutagenicity

The mutagenicity of fecal supernatants was assayed by the Ames test without metabolic activation against the strain *Salmonella enterica* serovar Typhimurium TA100. The 5051 Muta-ChromoPlateTM kit (EBPI, Ontario, USA) was used. Filtered fecal supernatant dilutions were mixed with the bacteria grown over 16 h at 37ºC in the sterile medium provided by the manufacturer and the solution mix containing Davis-Mingoli salts, D-glucose, bromocresol purple, D-biotine, and L-histidine as indicated by the manufacturer. Positive control (including sodium azide as a mutagen, grown bacteria and solution mix), negative control (including only solution mix) and the appropriate series of dilutions of fecal supernatants were added to 96-well microtiter plates containing 200 µl per well, and incubated at 37ºC for 5 days. Reversion rates (RR) were calculated for conditions where less than 96 revertant wells per plate and more than 48 revertant wells in positive control were obtained. Considering the dilution factor, the levels of mutagenicity were expressed as the mean of values corresponding to the three dilutions tested per sample and were arbitrarily classified as “low” [180-299] (n=6), “medium” [300-599] (n=27) or “high” [600-1474] (n=8) mutagenicity. The interference with L-histidine during the mutagenicity assay was ruled out as indicated in Ruiz-Saavedra et al.[17] Fecal mutagenicity values were obtained for 41 volunteers from NP (n=15), HP (n=9) CA (n=14) and AC (n=3) groups.

Statistical Analyses

Results were analyzed using the IBM SPSS software version 25.0 (IBM SPSS, Inc., Chicago, IL, SA). GraphPad Prism 9, RStudio version 1.4.3 and BioRender software were used for graphical representations. Overall, categorical variables were summarized as number and percentage and continuous ones as mean and standard deviation. Fisher tests were performed for categorical variables (p-value < 0.05). For continuous variables, the

goodness of fit to a normal distribution was checked by means of the Kolmogorov–Smirnov test. When normality of variables was achieved, *T*-tests were performed; otherwise, Mann-Whitney U tests were applied (p-value < 0.05). Spearman correlations were carried out to explore the associations between the intake of food groups, foods, dietary compounds, and the fecal NOCs concentration. Heatmaps were generated using "corrplot" R package. According to WCRF, a cut-off intake value of 50 g of processed meat per day was selected to analyze differences in fecal NOCs concentration.[1] The relationship between fecal NOCs concentrations and fecal mutagenicity was evaluated performing simple linear regressions.

3. Results

General characteristics of the sample population

The general characteristics of the human study sample according to post-colonoscopy histopathological diagnosis and nutritional assessment are presented in Table 1. The general sample is mostly composed of women (58%), the age average was 61 years old, with a mean BMI of 26.25 kg/m^2, which indicates overweight; most of the volunteers were non-smokers (56%). Out of the total sample, 31% were NP in comparison with 22% into the HP group, 39% displaying CA and 8% diagnosed with AC. The intake of ethanol was significantly higher in the CA group in comparison with the NP group (16.23 g/day *vs.* 2.93 g/day). No statistically significant differences were found for the rest of the variables analyzed between NP and HP, CA or AC groups.

Dietary sources of nitrates, nitrites and exogenous NOCs (NAs) and its association with fecal NOCs

The major foods contributing to the intake of nitrates, nitrites, and the NAs NDMA, NPIP and NPYR in the total sample are shown in Figure 1. While the intake of nitrates in the sample is explained by the consumption of roots and vegetables (mainly 27% lettuce, 11% potato, 11% chard, 8% white onion, 8% spinach, 6% cabbage and 5% squash), nitrites and NAs mainly derive from the consumption of processed meats (such as bacon, ham, or chorizo). Furthermore, a 12% of the intake of NDMA is provided by beer. No differences in the intake of nitrates, nitrites and the different NAs according to histopathology groups were detected (Table 2). A Spearman correlation analysis was conducted to further explore the association between the intake of the major dietary sources of nitrates, nitrites and NAs and the fecal concentration of NOCs (Table 3). From

the assessed foods, chard and spinach were negatively correlated with the heme NOCs, contrary to other foods, including potato, bacon, cured ham, chorizo and blood sausage that were positively correlated with the fecal total NOCs and with heme NOCs. A stepwise regression analysis was performed to determine which of these food sources correlating with fecal total and heme NOCs concentration were predictors of these variables. "Chorizo, category w/s" was revealed as one of the food sources predicting the levels of fecal total and heme NOCs (Table 4).

Table 1. General description of the study sample according to the histopathological classification.

		WS (*n*=36)	**NP (*n*=11)**	**HP (*n*=8)**	**CA (*n*=14)**	**AC (*n*=3)**
Gender	Male	15 (41.7%)	3 (27.3%)	3 (37.5%)	6 (42.9%)	3 (100.0%)
	Female	21 (58.3%)	8 (72.7%)	5 (62.5%)	8 (57.1%)	0 (0.0%)
Age (years)		61 ± 7	59 ± 9	59 ± 7	62 ± 6	64 ± 3
Weight (kg)		73.51 ± 14.49	71.14 ± 15.93	74.63 ± 13.62	74.57 ± 16.23	74.33 ± 1.15
Height (cm)		166.74 ± 9.79	165.18 ± 12.37	169.25 ± 7.23	164.75 ± 9.24	175.00 ± 2.65
BMI (kg/m^2)		26.25 ± 3.95	25.66 ± 4.04	25.97 ± 4.21	27.30 ± 4.18	24.28 ± 0.37
Energy intake (kcal/day)		1993.30 ± 812.16	1830.12 ± 779.93	1979.17 ± 1005.41	2103.66 ± 837.09	2114.34 ± 393.01
Ethanol (g/day)		11.40 ± 20.38	2.93 ± 5.69	17.49 ± 30.16	16.23 ± 21.79 *	3.68 ± 6.38
Smoking habit	Current	4 (11.1%)	0 (0.0%)	2 (25.0%)	1 (7.1%)	1 (33.3%)
	Never	20 (55.6%)	7 (63.6%)	5 (62.5%)	7 (50.0%)	1 (33.3%)
	Former	12 (33.3%)	4 (31.3%)	1 (12.5%)	6 (42.9%)	1 (33.3%)
Sleeping (hours/day)		7.11 ± 1.05	7.10 ± 0.74	7.25 ± 1.28	7.07 ± 1.21	7.00 ± 1.00
Physical activity (min/day)		57.29 ± 25.62	59.32 ± 21.33	64.69 ± 23.35	45.54 ± 27.35	85.00 ± 8.66
Depositions (times/week)		6.85 ± 2.26	6.91 ± 2.19	6.13 ± 2.74	7.04 ± 2.29	7.67 ± 1.44

Only individuals with information on fecal NOCs concentration and diet are included in the table. Values are presented as mean ± SD for continuous variables or number (%) for categorical ones. (*) Statistically significant differences compared to NP group ($p < 0.05$) found with Mann-Whitney U test. NOCs, N-nitroso compounds; BMI, body mass index; WS, whole sample; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenomas; AC, adenocarcinomas.

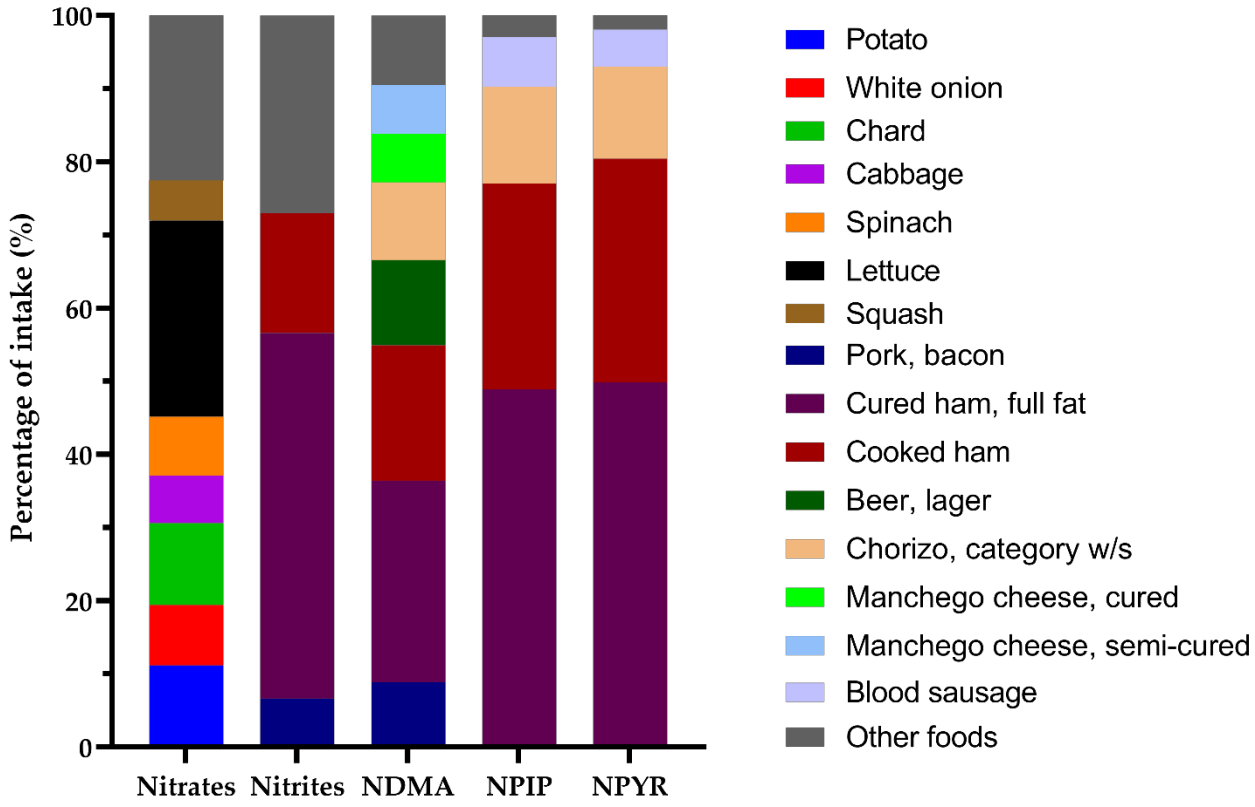

Figure 1. Major dietary sources of nitrates, nitrites, and NAs in the sample population. NAs, nitrosamines; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine; W/s, without specifying.

Table 2. Dietary intakes of nitrates, nitrites, and NAs according to histopathology groups**.**

	WS (*n*=36)	**NP (*n*=11)**	**HP (*n*=8)**	**CA (*n*=14)**	**AC (*n*=3)**
Nitrates (mg/day)	127.02 ± 117.03	171.86 ± 166.81	88.3 ± 47.57	112.04 ± 101.29	135.8 ± 84.88
Nitrites (mg/day)	3.13 ± 2.32	2.91 ± 1.75	3.02 ± 3.05	3.57 ± 2.58	2.23 ± 0.44
NDMA (µg/day)	0.20 ± 0.17	0.14 ± 0.1	0.23 ± 0.25	0.23 ± 0.17	0.17 ± 0.06
NPIP (µg/day)	0.09 ± 0.07	0.08 ± 0.05	0.09 ± 0.09	0.10 ± 0.07	0.06 ± 0.02
NPYR (µg/day)	0.14 ± 0.10	0.13 ± 0.09	0.13 ± 0.14	0.16 ± 0.11	0.10 ± 0.03

Values are presented as mean ± SD. No statistically significant differences were found ($p < 0.05$) with Mann-Whitney U test. NAs, nitrosamines; WS, whole sample; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenomas; AC, adenocarcinomas; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

Table 3. Spearman correlations between the intake of the major food sources of nitrates, nitrites and NAs and the concentration of fecal NOCs in the sample population.

Food	Intake (g/day)	Compound	Rho (Spearman)	*p* value
Potato	52.89 ± 30.41	Total NOCs	0.431	0.009
		Heme NOCs	0.437	0.008
Chard	14.99 ± 42.45	Total NOCs	-0.274	0.106
		Heme NOCs	-0.354	0.034
Spinach	9.46 ± 17.62	Total NOCs	-0.247	0.146
		Heme NOCs	-0.358	0.032
Pork, bacon	2.95 ± 6.52	Total NOCs	0.524	0.001
		Heme NOCs	0.517	0.001
Cured ham, full fat	26.19 ± 25.43	Total NOCs	0.364	0.029
		Heme NOCs	0.392	0.018
Chorizo, category w/s	11.99 ± 17.18	Total NOCs	0.468	0.004
		Heme NOCs	0.491	0.002
Blood sausage	3.91 ± 9.42	Total NOCs	0.433	0.008
		Heme NOCs	0.419	0.011

Intake values are presented as mean ± SD. Only dietary sources showing statistically significant Spearman correlation p-value < 0.05 in at least one fecal NOC variable are shown. NAs, nitrosamines; NOCs, N-nitroso compounds; W/s, without specifying.

Table 4. Results obtained from splitwise regression analyses identifying food sources of nitrates, nitrites and NAs predictors of fecal NOCs concentration.

Dependent variable	Independent variables	R^2	β	*p* value	*Included* *
Total NOCs	Potato	0.152	0.317	0.052	No
	Pork, bacon	0.152	-0.201	0.205	No
	Cured ham, full fat	0.152	-0.250	0.165	No
	Chorizo, category w/s	0.152	0.420	0.011	Yes
	Blood sausage	0.152	0.192	0.254	No
Heme NOCs	Potato	0.156	0.273	0.095	No
	Chard	0.156	-0.100	0.539	No
	Spinach	0.156	-0.205	0.205	No
	Pork, bacon	0.156	-0.227	0.149	No
	Cured ham, full fat	0.156	0.271	0.130	No
	Chorizo, category w/s	0.156	0.424	0.010	Yes
	Blood sausage	0.156	0.167	0.319	No

Only the variables with p < 0.05 in previous correlation analyses are included in the model. * Independent variables showing p < 0.05 are finally included in the splitwise regression. NAs, nitrosamines; NOCs, N-nitroso compounds; R^2, coefficient of multiple determination; β, standardized regression coefficient.

Fecal NOCs (total and heme NOCs) concentrations according to histopathology groups

The differences in fecal NOCs concentration according to histopathology groups are shown in Figure 2. Our data showed an increase in the fecal concentration of total NOCs and heme NOCs as the grade of intestinal mucosa lesion increases from NP to AC, obtaining statistically significant differences between these two extreme groups (NP and AC) for total NOCs (6.50 *vs.* 20.69 pmol/mg of feces, p=0.006) and heme NOCs (4.40 *vs.* 16.80, p=0.017). Although this trend was maintained in each histopathology group, differences were not statistically significant (data not shown).

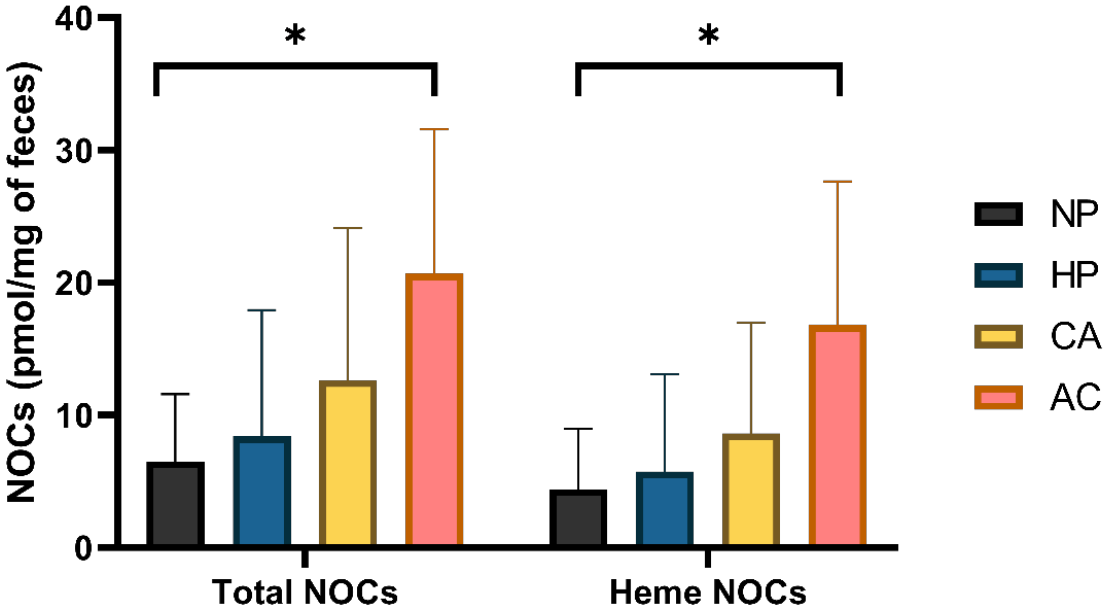

Figure 2. Differences in fecal NOCs concentration according to histopathology groups. Bars represent the mean concentration and vertical lines the standard deviation within each histopathology group. (*) Statistically significant differences were obtained between histopathology groups (Mann-Whitney U test, p <0.05). NOCs, N-nitroso compounds; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenomas; AC, adenocarcinoma.

Statistically significant higher fecal NOCs concentrations were also observed in volunteers consuming >50 g/day of processed meat in comparison with volunteers consuming <50 g/day, with total NOCs concentrations of 12.78 and 7.39 pmol/mg of feces respectively (p=0.044) and heme NOCs concentrations of 9.62 and 4.58 respectively (p=0.017). A closer examination of associations between fecal NOCs concentrations and dietary intake revealed inter-group differences (Figure 3). The whole sample presents positive associations between the intake of processed meats, red meat,

cereal products and potatoes with heme NOC concentrations. The same correlations as for the whole sample appeared at higher intensity in the NP as compared with the HP group. Specifically, the NP group displayed significant positive association of fish consumption with total NOCs and heme NOCs whereas the same was true for vegetables in the HP group (Figure 3 A). In contrast, negative correlations of seafood and snacks with fecal NOCs were observed in the CA group; this group maintained the same positive association found in the other groups for the intake of processed meat and fecal NOCs although these only reached statistical significance for the intake of pork and bacon. In Figure 3 B the associations found between dietary compounds and fecal NOCs are depicted. Whereas the whole sample presented negative correlations for the intake of phenolic acids and total polyphenols with fecal heme NOCs concentration, the dietary NDMA, NPIP or NPYR were positively correlated with total and heme fecal NOCs. Regarding the different histopathological groups, inverse associations were found for the NP group between the intake of nitrates and flavonoids with fecal heme NOCs concentrations whereas positive associations were found between the intake of total protein and micronutrients such as vitamin B12, vitamin D, phosphorus and selenium with fecal total and heme NOCs. No significant associations were found in the case of the CA group. Heme iron derived from meats, fish and eggs intake was positively correlated with total and heme fecal NOCs concentrations in the whole sample ($r=0.364$ and $r=0.374$, respectively) and NP group ($r=0.645$ and $r=0.745$, respectively) but not in HP and CA groups.

Relationships among fecal NOCs and fecal mutagenicity

The concentration of fecal NOCs was evaluated according to the fecal mutagenicity group (Table 5). No statistically significant differences were obtained in the concentration of these compounds as a function of mutagenicity levels although higher total and heme NOCs were observed in the high mutagenicity group. The relationship was further examined by linear regression analysis (Figure 4). When the whole sample was considered, the fecal concentrations of total NOCs ($R^2=0.117$, $p<0.05$) and heme NOCs ($R^2=0.167$, $p<0.05$) increased as fecal mutagenicity increased. The NP group presented the slightest positive association between fecal NOCs and mutagenicity in comparison with the rest of the groups. In the case of the HP group, a significant association between fecal heme NOCs and mutagenicity was found ($R^2=0.453$, $p<0.05$).

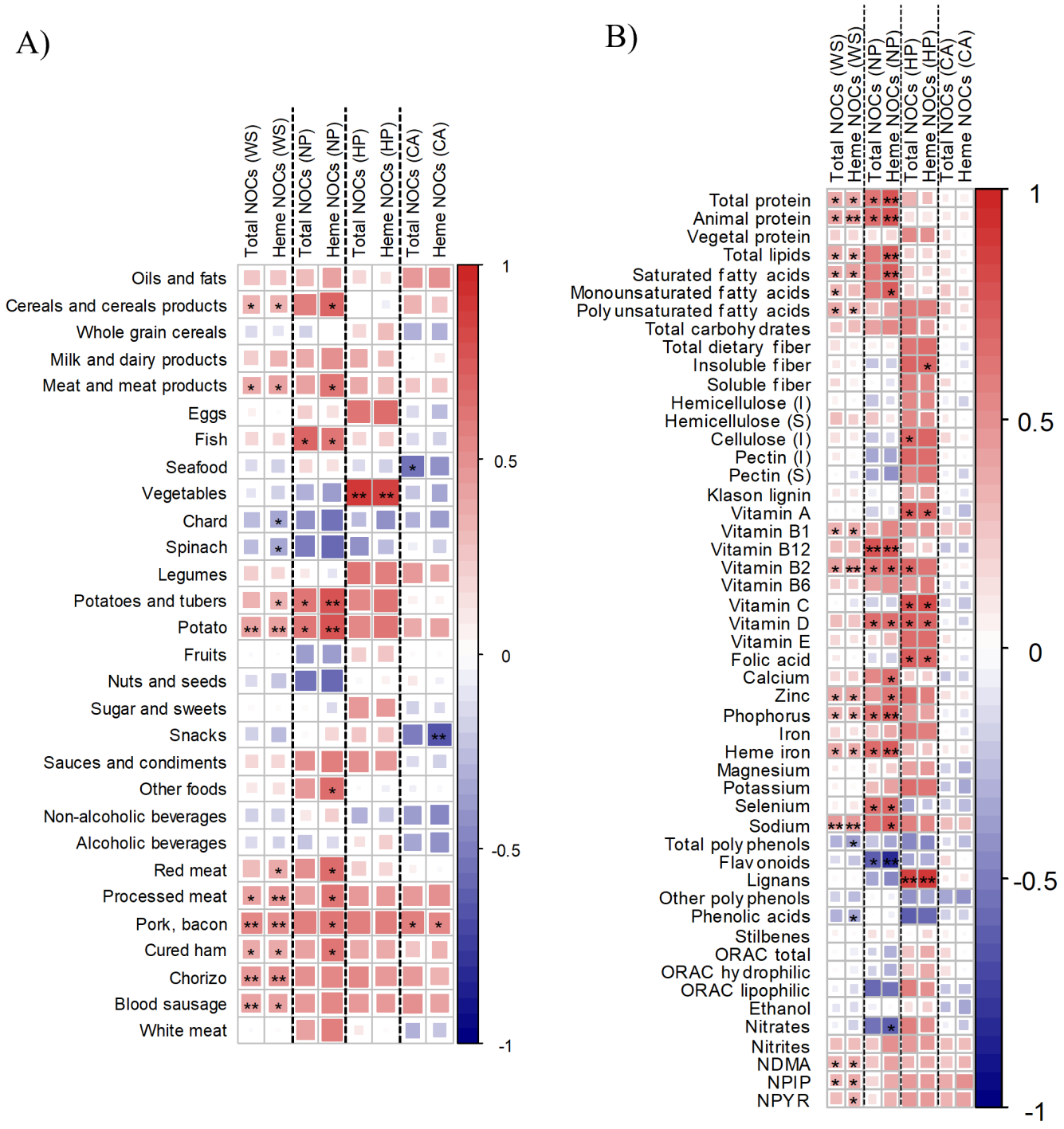

Figure 3. Heatmaps defined by Spearman correlations according to histopathology groups between fecal NOCs and A) food groups and foods or B) dietary compounds. Blue and red colors represent negative and positive associations, respectively. The color intensity is proportional to the degree of association. (*) $p < 0.05$ (**) $p < 0.01$. NOCs, N-nitroso compounds; WS, whole sample; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenoma; AC group was not included in the analysis as a result of the limited sample size (n=3); I, insoluble; S, soluble; ORAC, oxygen radical absorbance capacity; NDMA, N-nitrosodimethylamine; NPIP, N-nitrosopiperidine; NPYR, N-nitrosopyrrolidine.

Table 5. Fecal NOCs concentration according to fecal mutagenicity groups.

(pmol/mg of feces)	**Low mutagenicity (0-300) (n=6)**	**Medium mutagenicity (>300-600) (n=27)**	**High mutagenicity (>600) (n=8)**
Total NOCs	11.05 ± 7.76	9.44 ± 9.76	15.85 ± 12.68
Heme NOCs	7.81 ± 6.29	6.27 ± 7.78	12.52 ± 10.70

Values are presented as mean ± SD. No statistically significant differences were found ($p < 0.05$) with Mann-Whitney test. NOCs, N-nitroso compounds.

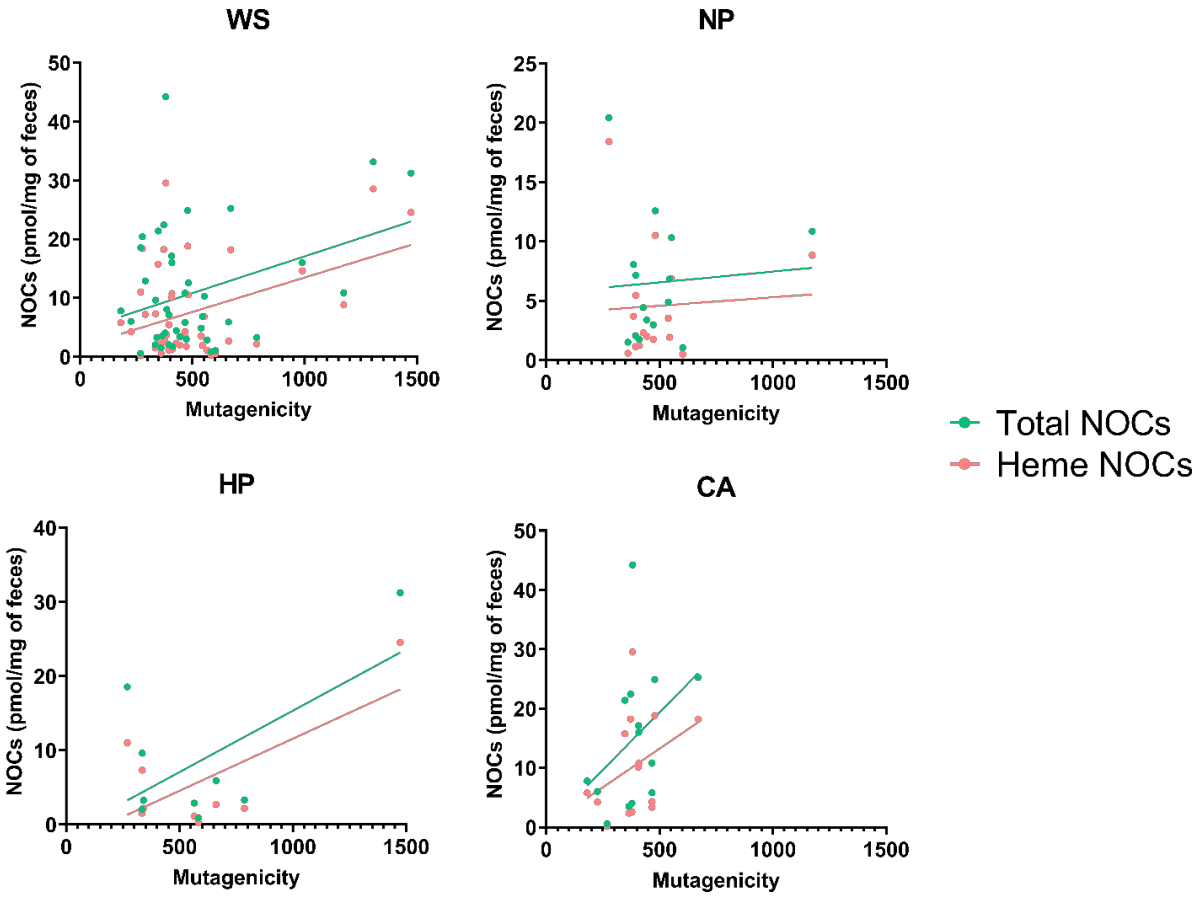

Figure 4. Linear regressions of fecal NOCs depending on the fecal mutagenicity values and according to histopathology groups. NOCs, N-nitroso compounds; WS, whole sample; NP, non-pathological controls; HP, hyperplastic polyps; CA, conventional adenomas**;** AC, adenocarcinomas.

4. Discussion

To the best of our knowledge, this is the first study investigating the contribution of diet and its components, with special emphasis on NAs derived from food processing, on endogenous NOCs formation in humans in the context of CRC. In this work, greater concentrations of fecal total NOCs and heme NOCs were found as the severity of colonic mucosa damage increased. Among the proposed mechanisms to explain the association

between CRC risk and higher red and processed meat consumption, is the endogenous formation of possible carcinogenic NOCs through the gastrointestinal tract.[14,15,29,30] The fecal concentration of NOCs depends on many factors such as the intake of exogenous NOCs, nitrate and nitrites that could act as nitrosating agents, amines and amides that could be transformed into secondary amines, heme iron from food and the host microbiota (Figure 5).[13,31,32]

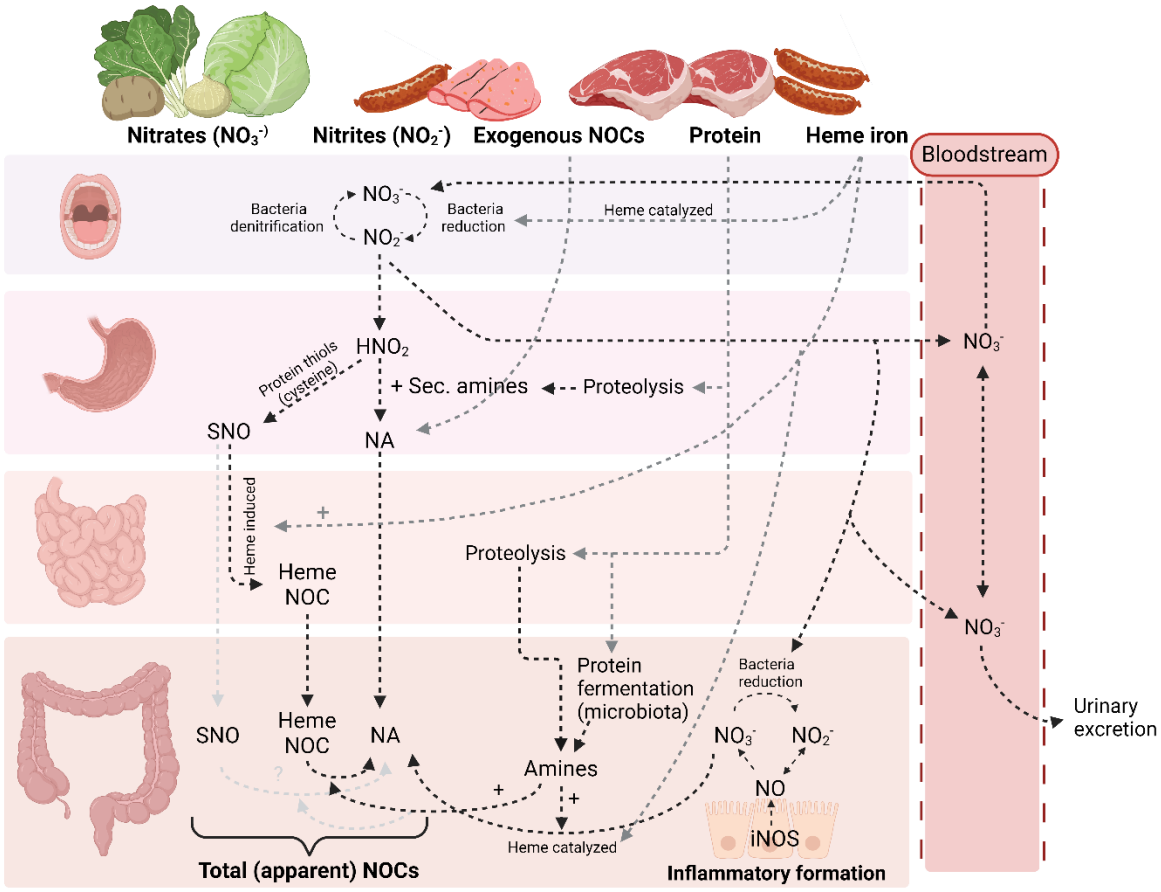

Figure 5. Schematic representation of the mechanisms and dietary factors affecting endogenous NOCs formation and excretion to stool. Grey arrows indicate dietary sources of precursors and inducers of NOCs formation and black arrows indicate endogenous NOCs formation. NOCs, N-nitroso compounds; NO_3^-, nitrate ion; NO_2-, nitrite ion; HNO_2, nitrous acid; Sec, secondary; NAs, nitrosamines; SNOs, S-nitrosothiols; Heme NOCs, nitrosyl heme; NO, nitrogen monoxide; iNOS, inducible NO synthase.

Approximately 7% of dietary nitrates can be reduced to nitrite by bacterial nitrate reductase in the oral cavity, for which the heme group acts as an electron donor favoring the catalytic formation of nitrite.[33] Once in the stomach, nitrite is transformed to nitrous acid due to the low pH conditions and may lead to the formation of NAs and SNOs after reaction with secondary amines and thiol groups obtained from proteolysis of protein

food.[13,34] SNOs facilitate NO release and the nitrosylation of heme iron from meat sources in the small intestine.[35] Nitrosating agents formed from NO produced by inducible NO synthase (iNOS) in colonocytes together with nitrate, nitrite, heme NOCs, SNOs and intestinal microbiota could contribute to the further formation of endogenous NOCs in this location.[13,36,37] Many NAs are carcinogenic compounds, causing damage through DNA alteration.[38] In addition to increasing the activity of nitrosating agents, the heme group can contribute to DNA damage by increasing lipid peroxidation and generation of cytotoxic and genotoxic aldehyde.[39–41]

Similar to what occurs in most Westernized societies, the average consumption of red (58.27 g/day) and processed meat (64.83 g/day) in the study sample was above the WCRF recommendations for a maximum of 500 g per week (approximately 71 g/day of combined red and processed meat).[1] In addition, the mean intake of processed meat in the sample of the study was above the risk dose for CRC (50 g/day)[1]. Moreover, volunteers showing processed meat intakes >50 g/day presented higher fecal concentrations of total and heme NOCs. Our findings point to processed meats rich in nitrites and NAs, in particular bacon, full fat cured ham, chorizo (a Spanish cured sausage) and blood sausage, as the main dietary sources of NOCs associated with fecal total NOCs and heme NOCs. Interestingly, potato intake was found to be correlated with fecal NOCs, probably because they are often consumed with meat, suggesting a confounding effect as previously reported by other authors.[42] Among all food sources, chorizo was a predictor of the levels of fecal total NOCs. It is also noteworthy that the intake of phenolic acids and total polyphenols were negatively correlated with fecal heme NOCs, suggesting an inhibitory effect of these plant-derived bioactives on endogenous NOC formation.

Case-control studies are often influenced by selection, recall and reporting biases when dietary intake assessment is done after diagnosis. However, since intestinal polyps are often asymptomatic, it is less likely that patients have altered their dietary intake before the visit to the hospital. The methodology used for the registration of the dietary intake and the further databases employed for the conversion into different dietary compounds with carcinogenic and bioactive effect, have allowed us to obtain a high degree of detail on dietary intake information. However, differences in the intake of nitrates, nitrites, NDMA, NPIP and NPYR among the few studies available in the literature are to be expected due to the different methods of dietary analysis used in each case. In this regard, Holtrop G et al. determined using semi-quantitative data from different food categories

that diet composition was associated with endogenous formation of NOCs in a sample population of obese men. For that purpose, they used the McCance and Widdowsons' tables instead of using a detailed estimation of the intake of each individual food item.[43] The use of dietary history questionnaires reported lower mean intakes of nitrites (0.99 *vs*. 3.13 mg/day in our study) and NDMA (0.114 µg/day *vs*. 0.198 µg/day in our study) on a Spanish population as compared to the intakes obtained nearly 30 years ago in a Finnish sample population (5.30 mg/day of nitrite and 0.05 µg/day of NDMA).[44,45] Regarding the intake of NPYR and NPIP, previous studies are scarce. In our sample, we detected an intake of 0.138 µg/day and 0.088 µg/day of NPYR and NPIP, respectively. Dietary estimations from German nutritional surveys set NPYR and NPIP exposure to 0.011 µg/day and 0.015 µg/day, respectively, but this study did not detail the meat consumption levels, which could have helped to better understand the differences.[46]

One of the main objectives of this work was to determine whether the estimation of dietary nitrates, nitrites and NAs could be representative and could display a meaningful relationship with the concentrations of NOCs excreted. The whole sample presented non-significant correlations between the intake of nitrates and nitrites, respectively, and fecal NOCs concentration. However, the intake of NDMA, NPIP, NPYR and heme iron from meats, eggs and fish was significantly and positively correlated with fecal total and heme NOCs. Although the statistical significance of these associations was not maintained within the groups established according to the damage of the colorectal mucosa, probably due to the limited sample size and the dispersion of the data, similar trends were noted in the case of the food dietary sources of these compounds. Specifically, heme NOCs were positively and significantly associated with the intake of bacon and cured ham in the NP and with bacon in the CA group, which also showed increased consumption of ethanol.

In a previous study by other authors, high NOCs concentrations were associated with longer transit time and lower fecal weight, suggesting more efficient microbiota mediated formation and accumulation of these compounds in feces.[47] In contrast, higher intake of dietary fiber can increase stool volume and shorten transit time, leading to lower NOCs concentration and decreasing their interactions with the colon mucosa. In this study it was not possible to calculate daily excretion of NOCs. Therefore, the concentrations reported here could be influenced by confounding factors such as fecal volume and transit time.

Although with these data we cannot establish causality between the intake of red and processed meats, nitrates, nitrites and NAs derived from food processing with the

endogenous formation of NOCs, it seems clear from our results that these factors are associated. Therefore, the next question is whether this increased excretion of NOCs is associated with increased fecal toxicity. Some previous work addressing this issue had found an increase in fecal water genotoxicity after red meat consumption during an intervention study although non-significant increments of NOCs fecal levels were observed.[48] In our study, the group of high fecal mutagenicity showed the highest total and heme fecal NOCs concentrations and according to the histopathology group, strong associations between fecal heme NOCs and fecal mutagenicity were only notable in the case of the HP group.

In the present work no differences were found in the intake of nitrites and dietary NOCs between the histopathology groups, but we observed that these compounds were mainly derived from the consumption of processed meats and were positively correlated with fecal total and heme NOCs concentrations. Increased fecal NOCs concentrations were noted among individuals consuming higher amounts of processed meat than recommended by WCRF, as the severity of colonic mucosal damage increased from NP to AC, and in higher fecal mutagenicity samples. The study of the associations among dietary components and endogenous NOCs considering the different intestinal environments may help to understand their impact in colonic mucosal damage and in the progression of certain diseases as CRC.

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DISCUSIÓN GENERAL

1. CONSIDERACIONES INICIALES

La presencia de compuestos potencialmente tóxicos como los xenobióticos en alimentos plantea un problema de salud en la población mundial. Agencias de seguridad alimentaria de carácter internacional como la EFSA o la IARC junto con diversos estudios epidemiológicos han evaluado cómo la ingesta de estos compuestos a través de la dieta se asocia con un mayor riesgo de desarrollar ciertas patologías como el CCR mediante la formación de aductos de ADN y el daño oxidativo, entre otros [100,112–114,119,121,147,148,152–154,165,180,195,196]. De esta manera, la acumulación de estos daños podría tener consecuencias negativas a nivel celular a largo plazo, favoreciendo la proliferación de células con alteraciones de carácter maligno [313]. La ingesta de algunos compuestos xenobióticos como los HAPs, las AHs o los NOCs procede mayoritariamente del procesado y cocinado de la carne y los productos cárnicos, y se proponen como una de las causas subyacentes para explicar la carcinogenicidad asociada al consumo de estos alimentos [45]. Uno de los problemas que se plantean en el estudio del impacto de estos compuestos en la salud es la ubicuidad de algunos de ellos, su formación endógena a partir de precursores, como ocurre en el caso de los NOCs, o el bajo grado de conocimiento sobre su presencia y concentración en categorías específicas de alimentos, lo que dificulta el estudio de su asociación con la salud [111,169]. En este sentido, los resultados presentados en esta Tesis Doctoral evidencian un incremento en la ingesta de algunos de estos xenobióticos en función del daño progresivo en la mucosa intestinal. Debido al papel inmunomodulador de la MI en el organismo y su capacidad para transformar diversos xenobióticos contribuyendo a su eliminación o a su permanencia en el organismo, el estudio de la MI en este contexto supone un factor clave a evaluar en esta asociación. Al inicio de la presente Tesis Doctoral los estudios existentes sobre los cambios que tienen lugar en la MI por la presencia de daño en la mucosa intestinal eran escasos [302,308,311]. Si bien este conocimiento ha aumentado en los últimos años, aún sigue siendo necesario un mayor grado de evidencia sobre los cambios que ocurren en la MI de acuerdo con las alteraciones tempranas de la mucosa, tanto en la vía adenomatosa de la carcinogénesis intestinal como especialmente en la vía serrada [314,315].

En este contexto, a partir de un enfoque multidisciplinar, nuestro trabajo es el primero en describir cómo la ingesta diferencial de compuestos xenobióticos de la dieta se asocia con cambios tanto en la MI como en algunos parámetros del ambiente intestinal como la mutagenicidad o la actividad enzimática fecal, incluyendo parámetros séricos relacionados con el estado inmunológico y metabólico sistémico, en función del tipo y nivel de daño la mucosa intestinal. Las evidencias generadas refuerzan el conocimiento existente acerca del papel de dichos parámetros en la salud intestinal, desvelando umbrales de estos compuestos xenobióticos con potencial interés de cara a la prevención del CCR (Figura 7). Por último, la identificación de

la MI como posible mediador de la asociación entre los xenobióticos y el daño intestinal abre nuevas posibilidades terapéuticas y vías de investigación para el futuro.

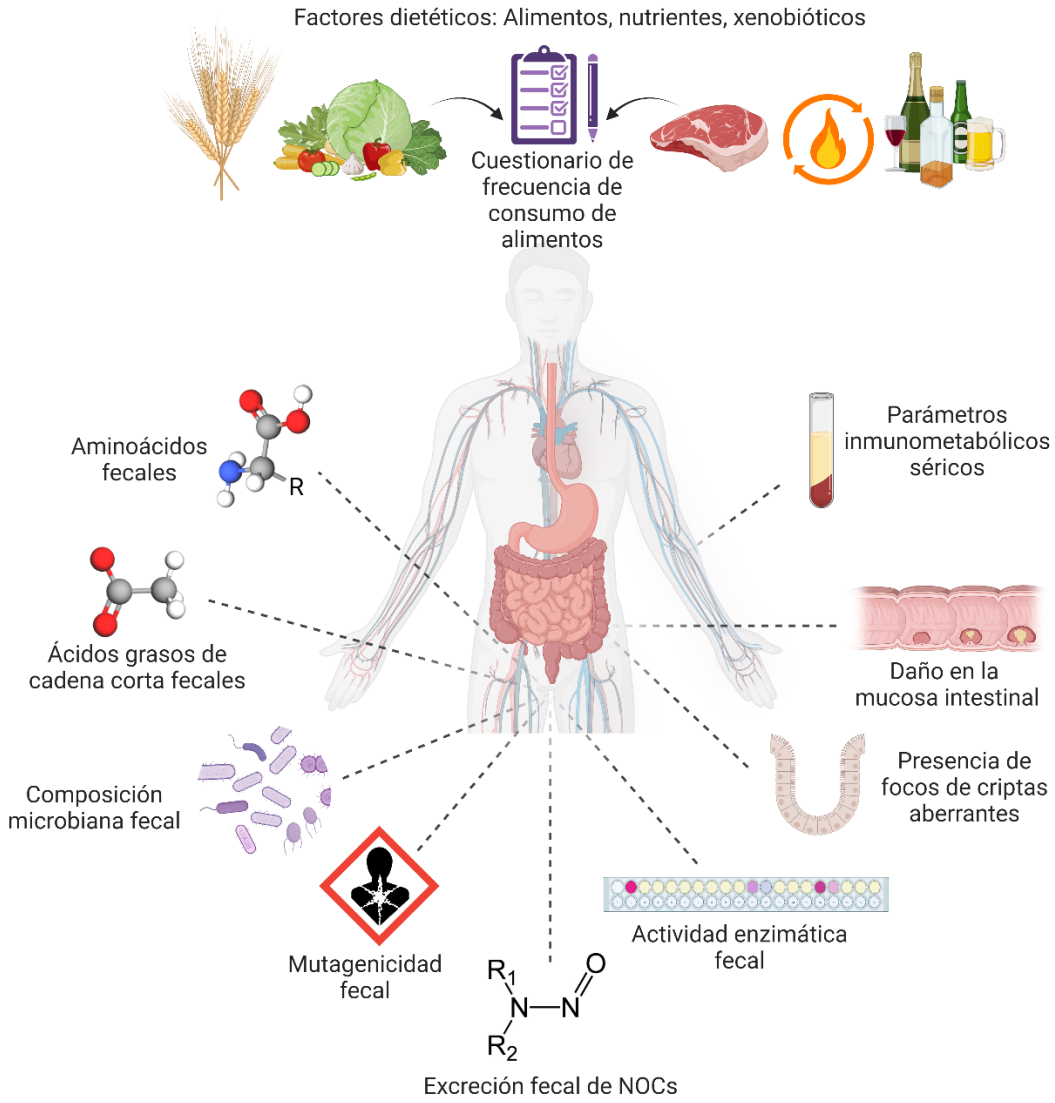

Figura 7. Esquema de los parámetros evaluados y analizados en este estudio.

2. INDICADORES DE ALTERACIONES EN LA MUCOSA INTESTINAL

2.1. El daño macroscópico en la mucosa intestinal y las vías biológicas de la carcinogénesis

A lo largo de esta Tesis Doctoral, todos los análisis e interpretación de resultados se han llevado a cabo utilizando como criterio de clasificación los datos de diagnóstico clínico y/o análisis histopatológico aportados por los profesionales médicos del Servicio Digestivo del HUCA, del Hospital Carmen y Severo Ochoa y del Servicio de Anatomía Patológica del HUCA. La muestra de este trabajo está compuesta por individuos clasificados como controles, pólipos y CCR según el diagnóstico clínico posterior al examen endoscópico. Los voluntarios clasificados como control mostraron ausencia de daño significativo en el examen histopatológico de la mucosa intestinal (Figura 8).

Dentro del grupo de voluntarios con pólipos intestinales, el análisis histopatológico permitió diferenciar entre los desarrollados por la vía adenomatosa (displasias de bajo grado como adenomas vellosos y tubulovellosos y displasias de alto grado) o la vía serrada de la carcinogénesis (pólipos hiperplásicos y lesiones serradas sésiles). Mediante la histopatología también se pudo corroborar el diagnóstico de adenocarcinoma. En general, la clasificación de los voluntarios por grupos de diagnóstico clínico y la consideración del análisis histopatológico ha contribuido a aumentar la uniformidad de los grupos de estudio y ha facilitado la obtención de resultados más robustos. Además, las resecciones de mucosa han permitido diferenciar la presencia o ausencia de FCA, uno de los primeros cambios morfológicos que suelen observarse en la progresión del daño de la mucosa intestinal, así como el tipo de lesión dentro de la cripta (sin cambios morfológicos, con cambios hiperplásicos o con cambios displásicos).

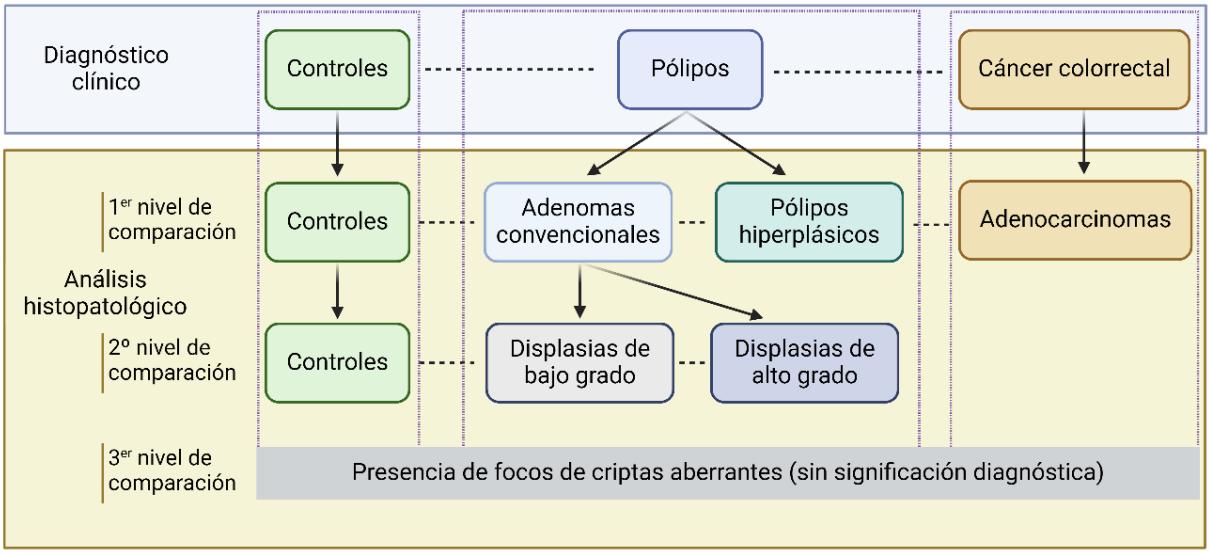

Figura 8. Niveles de comparación en el estudio de acuerdo con los diferentes grupos de diagnóstico clínico y de análisis histopatológico.

2.2. La presencia de focos de criptas aberrantes

A pesar de no utilizarse como criterio de diagnóstico en la práctica clínica para determinar el riesgo de CCR, para este trabajo se analizaron los FCA ya que pueden reflejar un ambiente intestinal alterado. Tal y como se había descrito previamente, hemos observado que el uso de FCA como biomarcador del riesgo de desarrollo de la enfermedad puede ser impreciso ya que estas lesiones se detectan tanto en presencia como en ausencia de pólipos intestinales [6,7]. Concretamente, un 37% de la muestra control tenía FCA, en comparación con un 20% en la muestra diagnosticada con pólipos intestinales, sin llegar a observar diferencias estadísticamente significativas en el porcentaje de presencia de FCA entre grupos de diagnóstico clínico. Sin embargo, cuando se compararon individuos con ausencia de FCA con aquellos con FCA dentro del grupo de voluntarios diagnosticados con pólipos intestinales sí se detectaron cambios significativos en varios parámetros que serán discutidos posteriormente en este apartado de la Tesis.

3. LA MICROBIOTA INTESTINAL Y SU RELACIÓN CON EL DAÑO EN LA MUCOSA INTESTINAL

El estado de la mucosa intestinal puede verse afectado por los diversos factores que interactúan con ella. Uno de los que tienen mayor relevancia en la salud es la MI. El intestino está poblado por millones de microorganismos, y la disbiosis intestinal se asocia a procesos patológicos [291,292]. Analizar la composición y funcionalidad de la MI puede resultar de gran interés en la prevención, diagnóstico y tratamiento de enfermedades como el CCR. Sin embargo, aún se requiere un mayor grado de conocimiento sobre los cambios que tienen lugar en la MI en las alteraciones tempranas de la mucosa colorrectal. Para aportar conocimiento al respecto, en nuestro estudio hemos analizado la composición microbiana fecal de los voluntarios del estudio a través de la secuenciación del gen del ARNr 16S. Este análisis ha revelado diferencias en la abundancia relativa a nivel taxonómico de filo, familia y género entre los grupos de diagnóstico clínico y de diagnóstico histopatológico estudiados, sin llegar a detectar diferencias significativas en la alfa y beta diversidad (Artículo 3). Estudios metataxonómicos de muestras fecales llevados a cabo por otros grupos de investigación han mostrado niveles similares de diversidad y riqueza microbiana en individuos control e individuos diagnosticados con pólipos hiperplásicos, adenomas (bajo o alto riesgo) o CCR [303,308]. De manera similar, estudios metagenómicos previos no han revelado diferencias en la diversidad y riqueza microbiana en muestras fecales de individuos diagnosticados con CCR respecto a las muestras control [304]. Sin embargo, estudios de mucosa colorrectal reflejan una mayor alfa diversidad en individuos con adenomas o lesiones de la vía serrada frente a individuos control [301,311,316]. Estas diferencias entre estudios señalan la existencia de cierto grado de variabilidad en los resultados en función del material biológico utilizado.

Además, en cada grupo de diagnóstico hemos observado diferencias en la microbiota fecal de acuerdo a los umbrales de ingesta de factores dietéticos protectores o de riesgo en el desarrollo de CCR definidos en estudios previos (Artículo 5) [66,152,179,317]. Considerando los grupos de diagnóstico clínico se observó que la abundancia relativa de familias como *Methanobacteriaceae*, *Christensenellaceae* y *Oscillospiraceae* eran menores en el grupo de individuos diagnosticados con pólipos intestinales que en el grupo control. Respecto a los grupos de análisis histopatológico se observó que, en comparación con el grupo control, en los adenomas convencionales propios de la vía adenomatosa de la carcinogénesis las diferencias en la microbiota fecal eran más numerosas que en los pólipos hiperplásicos de la vía serrada. Se comprobó que el único cambio en la microbiota fecal similar en pólipos de las vías adenomatosa y serrada respecto al grupo control era la mayor abundancia relativa del grupo *Ruminococcus_torques* (nivel taxonómico de género). Este género se ha identificado como una bacteria “conductora” en la iniciación y progresión del proceso tumoral [310]. Curiosamente, a

este mismo nivel taxonómico, la disminución observada en la abundancia relativa de*Christensenellaceae_R-7* y de *Oscillospiraceae_UCG-002* en el grupo de adenomas convencionales frente al grupo control era similar a la disminución detectada en la comparación de la microbiota fecal de las muestras que presentaban una alta actividad α-glucosidasa frente a aquellas que presentaban una baja actividad enzimática (Artículo 4).

En general, los cambios observados en nuestros análisis sugieren una reorganización del consorcio microbiano involucrado en procesos fermentativos en el intestino de acuerdo con la presencia de lesiones intestinales de la vía adenomatosa y del grado de displasia mostrado. Estos cambios afectan a familias como *Prevotellaceae*, *Oscillospiraceae*, *Streptococcaceae*, *Christensenellaceae*, *Erysipelotrichaceae* y *Clostridiaceae* (Artículo 3). Concretamente, la abundancia relativa de algunas arqueas metanogénicas como la familia *Methanobacteriaceae*

también se vio modificada. Los miembros de esta familia son capaces de utilizar el hidrógeno producido por otras bacterias fermentadoras de carbohidratos como donador de electrones para llevar a cabo reacciones de reducción que acaban dando lugar a la producción de metano, lo que contribuye al mantenimiento de la homeostasis intestinal [318]. Por ello, su alteración podría conllevar al establecimiento de un ambiente intestinal alterado [319].

3.1. La producción de ácidos grasos de cadena corta por la microbiota intestinal

La MI es capaz de producir y transformar multitud de metabolitos. Entre ellos se encuentran los AGCC mayoritarios, exclusivamente producidos por la MI, cuyos efectos beneficiosos para el organismo ha sido ampliamente descritos [235]. En este trabajo hemos analizado la concentración fecal de estos compuestos a partir de sobrenadantes de muestras fecales con el fin de conocer las posibles variaciones en sus niveles en función de la presencia de daño intestinal. Aun siendo capaces de detectar cambios en la microbiota fecal de acuerdo con la presencia de lesiones en la mucosa intestinal y de asociar ciertas familias microbianas con los niveles fecales de AGCC, no hemos detectado variaciones significativas en la concentración fecal de los distintos AGCC en la comparación entre los distintos grupos de diagnóstico histopatológico, a excepción del ácido caproico (Artículo 3). En este sentido, un meta-análisis reciente señaló que en la comparación de individuos con bajo riesgo de desarrollar CCR frente a aquellos que presentaban un alto riesgo las concentraciones de acetato, propionato y butirato no presentaban diferencias significativas cuando eran analizadas por separado. Sin embargo, cuando los niveles de estos compuestos se analizaron de forma conjunta, se observó una reducción en las muestras de individuos con alto riesgo de desarrollar CCR. Además, el análisis combinado de estos AGCC también reflejó concentraciones significativamente menores en individuos con CCR frente a individuos control [320]

De acuerdo con nuestros resultados, pese a producirse cambios importantes en los consorcios microbianos según las lesiones de la mucosa intestinal, estos no parecen afectar globalmente a las concentraciones de AGCC. Una explicación posible es que el grado de daño en la mucosa que hemos analizado en este trabajo puede no tener la magnitud suficiente como para producir un cambio significativo en el perfil de AGCC, a diferencia del cambio que podría ocurrir en fases más avanzadas como el adenocarcinoma, donde se ha descrito que el butirato puede inducir la muerte celular programada de células cancerígenas [321–323]. La ausencia de diferencias significativas entre los distintos grupos de diagnóstico clínico en el consumo de fibra dietética que posteriormente es utilizada en procesos fermentativos por la MI produciendo AGCCs también contribuir a explicar la ausencia de cambios en el perfil de estos compuestos [324]. Además, es posible que el limitado número de muestras analizadas y la desviación observada en las concentraciones fecales de AGCC dificulten la obtención de resultados significativos. Tampoco podemos descartar que los cambios en grupos taxonómicos microbianos que ocurren de acuerdo con el daño intestinal se compensen en lo que respecta a la producción de AGCC, de manera que aun cambiando la composición microbiana fecal estos metabolitos se mantengan.

4. PARÁMETROS CONSIDERADOS PARA LA ESTIMACIÓN DEL AMBIENTE INTESTINAL

4.1. Mutagenicidad fecal

Uno de los factores considerados en este trabajo que podrían mostrar relación con la homeostasis intestinal es la mutagenicidad. Parece lógico pensar que la presencia de niveles altos de mutagenicidad en el lumen intestinal podría influir directamente en el riesgo de desarrollar lesiones en la mucosa intestinal al favorecer el daño en el ADN de células epiteliales colónicas, o también reflejar un ambiente intestinal alterado. Para evaluar este ambiente mutagénico se puede analizar la presencia de compuestos específicos previamente descritos o emplear metodologías basadas en evaluar el ambiente intestinal de forma más integrativa. Basados en el último enfoque, en nuestro trabajo hemos puesto a punto una modificación del test de Ames en sobrenadantes fecales que ha permitido analizar la mutagenicidad fecal en las muestras biológicas recogidas. El test de Ames, está basado en el crecimiento de una cepa de *Salmonella entérica* serovar typhimurium auxótrofa para la histidina que en ausencia de este aminoácido sólo será capaz de crecer si revierte la auxotrofía mediante una mutación puntual en este gen, por lo que un mayor índice de reversión indica mayor mutagenicidad del medio [325]. Mediante la dilución de muestras de sobrenadantes fecales hemos conseguido cumplir los criterios de validez de la prueba y a la vez evitar la interferencia del aminoácido histidina naturalmente presente en las heces en los resultados de la prueba, disminuyendo la presencia de falsos positivos [326–329]. Nuestros resultados muestran diferencias en los niveles de mutagenicidad fecal en función de la presencia de FCA en el grupo de voluntarios diagnosticados con pólipos intestinales, así como una asociación de la mutagenicidad fecal con distintos componentes de la dieta y con la excreción de compuestos potencialmente carcinogénicos en función de los grupos resultantes del análisis histopatológico (Artículo 1 y Manuscrito 1). Cabe destacar que en un estudio previo llevado a cabo por otros autores no se detectaron diferencias en los niveles de mutagenicidad fecal entre muestras de individuos control y muestras de pacientes con CCR [330]. A este respecto, nuestro trabajo ha sido primero en describir diferencias en la mutagenicidad fecal asociadas a la presencia de alteraciones intestinales previas al desarrollo de CCR.

4.2. Aminoácidos fecales

Además de la histidina previamente referida, en el ambiente intestinal es posible encontrar, entre otros, aminoácidos proteicos, no proteicos, amonio y aminas biógenas. Los aminoácidos del ambiente intestinal pueden tener un origen endógeno, siendo producidos y liberados por las células del organismo y la MI, o exógeno en forma de aminoácidos libres o proteínas de la dieta que posteriormente son hidrolizadas en el tracto gastrointestinal [331]. Los

aminoácidos resultan claves en la reposición proteica de las células y en condiciones normales pueden mejorar las funciones de la barrera intestinal e incluso promover la expresión de citocinas antinflamatorias y neurotransmisores [332,333]. Por otro lado, el amonio tiene una función de mantenimiento del pH en el organismo, que resulta clave para evitar un estado de acidosis metabólica [334]. Sin embargo, cuando su homeostasis se encuentra alterada, el amonio libre puede actuar como un agente citotóxico [335]. El papel de las aminas en la salud está más discutido, aunque existe evidencia sobre su relación con el desarrollo de diversas patologías y también se consideran precursoras de NOCs endógenos [165,336].

Para valorar la relación de los aminoácidos, amonio y aminas biógenas con el daño en la mucosa intestinal, en nuestro trabajo hemos analizado la concentración de estos compuestos en las muestras biológicas fecales de los voluntarios del estudio. Nuestros resultados no mostraron diferencias significativas en la concentración fecal de estos compuestos en la comparación entre el grupo control y el grupo de individuos diagnosticados clínicamente con pólipos intestinales (Artículo 5). Sin embargo, sí fue posible asociar la ingesta de etanol con una reducción en la concentración fecal de aminoácidos proteicos y amonio en el grupo de individuos diagnosticados con pólipos intestinales. La ingesta de etanol se ha relacionado previamente con el metabolismo de las proteínas y estudios llevados a cabo en ratas han mostrado que el etanol es capaz de alterar la estructura del proteasoma y por ende inhibir la degradación de proteínas, contribuyendo a su acumulación [337,338]. Además, el grupo de individuos con pólipos mostró una correlación positiva entre la ingesta de carnes rojas y procesadas y la excreción de aminas biógenas. Se ha descrito que las carnes y especialmente las carnes procesadas poseen condiciones particulares que favorecen la formación de aminas biógenas [339].

4.3. Actividad enzimática fecal

Multitud de metabolitos presentes en el intestino son transformados por la acción de enzimas de origen endógeno o microbiano allí presentes. Se ha descrito que en CCR los perfiles enzimáticos pueden verse alterados, contribuyendo así a la modulación del ambiente intestinal [340]. Para comprobar las posibles alteraciones de este perfil que ocurren en etapas tempranas del daño intestinal en nuestro trabajo quisimos evaluar la actividad enzimática fecal empleando el sistema semicuantitativo API ZYM ® [341,342]. Hemos encontrado diferencias significativas en la actividad α-glucosidasa tanto entre individuos control e individuos con pólipos intestinales (diagnóstico clínico), como entre muestras control y aquellas que procedían de individuos con distinto grados de daño intestinal (análisis histopatológico) (Artículo 4). Este enzima es capaz de hidrolizar polisacáridos complejos liberando monómeros de glucosa. Previamente se ha observado que el uso de inhibidores del enzima α-glucosidasa con el fin provocar una disminución en los niveles de glucosa postprandial en personas con diabetes mellitus parece

tener un efecto protector frente al desarrollo de CCR [343]. Una explicación posible a esta asociación podría estar relacionada con el efecto Warburg. Este se basa en la observación de que la mayoría de las células cancerosas realizan un gran consumo de glucosa debido a que la producción de su energía ocurre mayoritariamente a través de un proceso poco eficiente de glucólisis seguido por la formación de ácido láctico en el citosol, a diferencia de las células normales que obtienen mayoritariamente su energía a través del ciclo del ácido tricarboxílico y la fosforilación oxidativa que ocurre en las mitocondrias [344]. No obstante, hay que tener en cuenta que hemos detectado estos mayores niveles de actividad α-glucosidasa en estadios previos al CCR. Por ello, planteamos la hipótesis de que, una mayor actividad de este enzima podría favorecer mayores concentraciones de glucosa libre, siendo utilizadas de forma preferente por células proliferativas que empiezan a manifestar alteraciones en varias de sus rutas metabólicas.

Entre las posibles vías de modulación externa de la actividad α-glucosidasa se encuentran la mencionada administración de inhibidores o la dieta. Se ha sugerido que situaciones de ayuno o la adherencia a dietas cetogénicas reducidas en glúcidos pueden proteger frente al desarrollo de CCR. Este efecto se conseguiría mediante una restricción del acceso a glucosa libre por parte de células con gran potencial maligno, mientras que las células normales del cuerpo podrían utilizar cuerpos cetónicos como el β-hidroxibutirato como fuente de energía, actuando estos cuerpos cetónicos a altas concentraciones como inhibidores del crecimiento tumoral [345,346]. Sin embargo, esta última cuestión ha generado gran controversia y continúa en debate, ya que los estudios en humanos son escasos. La MI es otro de los factores que puede modular la actividad α-glucosidasa, por ejemplo, mediante la síntesis y liberación al lumen intestinal del enzima α-glucosidasa de origen microbiano o incluso de proteasas extracelulares capaces de degradar y disminuir la actividad α-glucosidasa.

5. IMPACTO EN PARÁMETROS INMUNOMETABÓLICOS SISTÉMICOS DE LA PRESENCIA DE DAÑO EN LA MUCOSA INTESTINAL

Además de mostrar un impacto en el ambiente intestinal, la presencia de estímulos dañinos en el intestino puede iniciar una respuesta inflamatoria caracterizada por la liberación de moléculas proinflamatorias como IL-1β, IL-6 o TNF-α que en última instancia pueden aparecer con niveles alterados en el torrente sanguíneo [347]. Por otra parte, algunas condiciones multifactoriales como la obesidad pueden contribuir a la alteración de los niveles de moléculas circulantes como las adipocinas, citocinas o quimiocinas y promover microambientes intestinales proinflamatorios y por tanto el establecimiento y progresión del daño intestinal [348]. Considerando el papel en la salud que pueden presentar los diferentes parámetros inmunometabólicos, resulta interesante conocer su asociación con la presencia de alteraciones en la mucosa intestinal.

Para su evaluación en este trabajo se partió de muestras de suero sanguíneo de los voluntarios del estudio. Se pudo comprobar que la presencia de pólipos intestinales y de adenocarcinomas se asociaba con menores niveles de algunas adipocinas como la adiponectina o la resistina y con mayores niveles de quimiocinas como CXCL10 respecto al grupo control (Artículo 2). La adiponectina ha sido propuesta como un marcador de la progresión de CCR debido a su efecto antiinflamatorio y antineoplásico llevado a cabo mediante la supresión de la actividad de la proteína cinasa activada por AMP (AMPK) que activa la vía mTOR [349–354]. Sin embargo, los niveles de resistina se han visto aumentados en casos de CCR y bajo la presencia de concentraciones altas de otras citocinas proinflamatorias, un suceso que no hemos podido corroborar en nuestro estudio y que hemos atribuido a una diferencia en el porcentaje de hombres que componían el grupo de individuos con adenocarcinomas, los cuales mostraban mayores niveles de resistina que las mujeres [355]. Un estudio llevado a cabo por el proyecto EPIC ha sugerido el papel de la resistina como un posible marcador de CCR ya desarrollado, en lugar de ser considerado como un factor de riesgo [356]. La quimiocina CXCL10 es una molécula inducible por la presencia de interferón que a su vez puede inducir otras moléculas proinflamatorias como el TNF-α o la interleucina IL-1β [357,358]. Niveles aumentados de CXCL10 se han asociado a fenómenos de lipotoxicidad en modelos murinos y al calentamiento de las zonas tumorales favoreciendo el desarrollo tumoral [358,359]. Además, la presencia de FCA en el grupo con pólipos intestinales se asoció con una mayor concentración del TNF-α y con una mayor mutagenicidad fecal (Artículos 1 y 2). El TNF-α es uno de los parámetros inflamatorios empleados en la elaboración del IID y se considera una molécula proinflamatoria con gran importancia clínica, capaz de inducir la actividad NF-κB, la cual se ha asociado a enfermedades inflamatorias crónicas y al desarrollo tumoral si la presencia de niveles elevados se mantiene a lo largo del tiempo [70,360,361]. Se sabe que ciertos componentes de la dieta

pueden regular la expresión de TNF-α y atenuar la progresión de FCA en ratones, mientras que el seguimiento de dietas reducidas en fibra y ricas en carne roja favorece la formación de un mayor número de FCA en el intestino [362,363]. En la bibliografía se ha descrito a la interleucina IL-10 como antiinflamatoria y estrechamente relacionada al TNF-α, aunque con efectos opuestos. Por ello, se ha propuesto la ratio TNF-α/IL-10 como biomarcador de salud [364–366]. En nuestro trabajo hemos analizado la ratio TNF-α/IL-10 (Artículo 2), comprobando que estaba aumentada en individuos con pólipos intestinales y presencia de FCA frente a individuos con pólipos, pero sin FCA. En base a estos resultados, sería de interés la realización de estudios nutricionales orientados que favorezcan la modulación de los niveles de TNF-α y la reducción de la mutagenicidad fecal. Estos resultados sugieren que la progresión del daño intestinal se asocia con una modificación en el perfil inmunometabólico hacia un perfil más proinflamatorio, siendo este perfil potencialmente modulable a través de la dieta. Además, resulta de interés la realización de estudios nutricionales orientados que favorezcan la modulación de los niveles de TNF-α y la reducción de la mutagenicidad fecal.

6. LA RELACIÓN DE LA DIETA CON EL DAÑO DE LA MUCOSA INTESTINAL, LA MICROBIOTA INTESTINAL, EL AMBIENTE INTESTINAL Y LOS PARÁMETROS INMUNOMETABÓLICOS

Durante la Introducción de esta Tesis Doctoral se ha señalado la importancia de la dieta en la salud humana. Por ello, resulta de gran interés profundizar en su estudio y en las asociaciones que esta presenta con los distintos parámetros de homeostasis intestinal y sistémica.

6.1. El registro de la dieta y de la ingesta de xenobióticos

Uno de los problemas que se presentaban al inicio de nuestro trabajo para estudiar la dieta era la ausencia en la literatura de métodos armonizados a la hora de registrar la exposición dietética a xenobióticos [367]. Teniendo en cuenta que el proceso de cocinado es un factor clave en su formación, resultaba crucial desarrollar cuestionarios que permitieran recoger estos datos con la mayor precisión posible [368]. Durante el desarrollo de esta Tesis Doctoral se ha diseñado, armonizado y validado un CFCA con especial hincapié en el registro de los hábitos de cocinado y de la dieta, que se ha revelado como una herramienta útil para cuantificar la ingesta dietética de xenobióticos dietéticos [369]. Para conseguir registrar adecuadamente la dieta, este CFCA incluía 155 ítems, preguntas específicas sobre las recetas empleadas y un álbum fotográfico previamente validado en el estudio "Pilot Study for the Assessment of Nutrient intake and food Consumption Among Kids in Europe (PANCAKE)" mediante el cual los entrevistados también podían seleccionar el tamaño de ración habitualmente ingerida y la frecuencia de consumo [370]. En el registro dietético, la subjetividad y el grado de interpretación del entrevistado pueden resultar un problema. Para solventar este factor, el cuestionario presentaba un módulo que incluía preguntas sobre la metodología de cocinado empleada (a la plancha, frito, a la barbacoa, etc.) y el grado de cocinado o de tostado (poco hecho, hecho, etc.) para las carnes, las patatas fritas y el pan tostado, todo ello acompañado de material fotográfico específico para facilitar y uniformizar el registro de los hábitos de cocinado y consumo (Figura 9). Los datos recogidos en esta Tesis Doctoral provienen de entrevistas en formato presencial, y entrevistas en formato online debido a la situación de emergencia sociosanitaria por la COVID-19.

Otra de las limitaciones encontradas al inicio de nuestro trabajo estaba en el paso de conversión de los datos recogidos en el CFCA al valor final de ingesta de cada compuesto. Habíamos observado que las tablas y bases de composición alimentaria disponibles que recogían la concentración real de los compuestos xenobióticos en alimentos eran independientes unas de otras, de manera que incluían diferentes metodologías y contenido de alimentos. Para solventar este inconveniente y realizar un cálculo armonizado en nuestro estudio, realizamos

una búsqueda bibliográfica exhaustiva con el fin desarrollar una base de datos unificada y variada que contemplase la presencia de HAPs, AHs, nitratos, nitritos, NOCs y acrilamida en alimentos. El primer paso fue integrar la información procedente del estudio EPIC y de la herramienta CHARRED sobre el contenido de HAPs, AHs, nitratos, nitritos y NOCs en alimentos [208,209]. A continuación, estos datos se complementaron con información proporcionada por la EFSA y la FDA sobre los niveles de nitratos y acrilamida en alimentos y con información adicional referida por diversos autores[125,210,211,371–377]. De esta manera, se pudo lograr un cálculo más detallado y preciso de la ingesta de xenobióticos en la muestra de estudio.

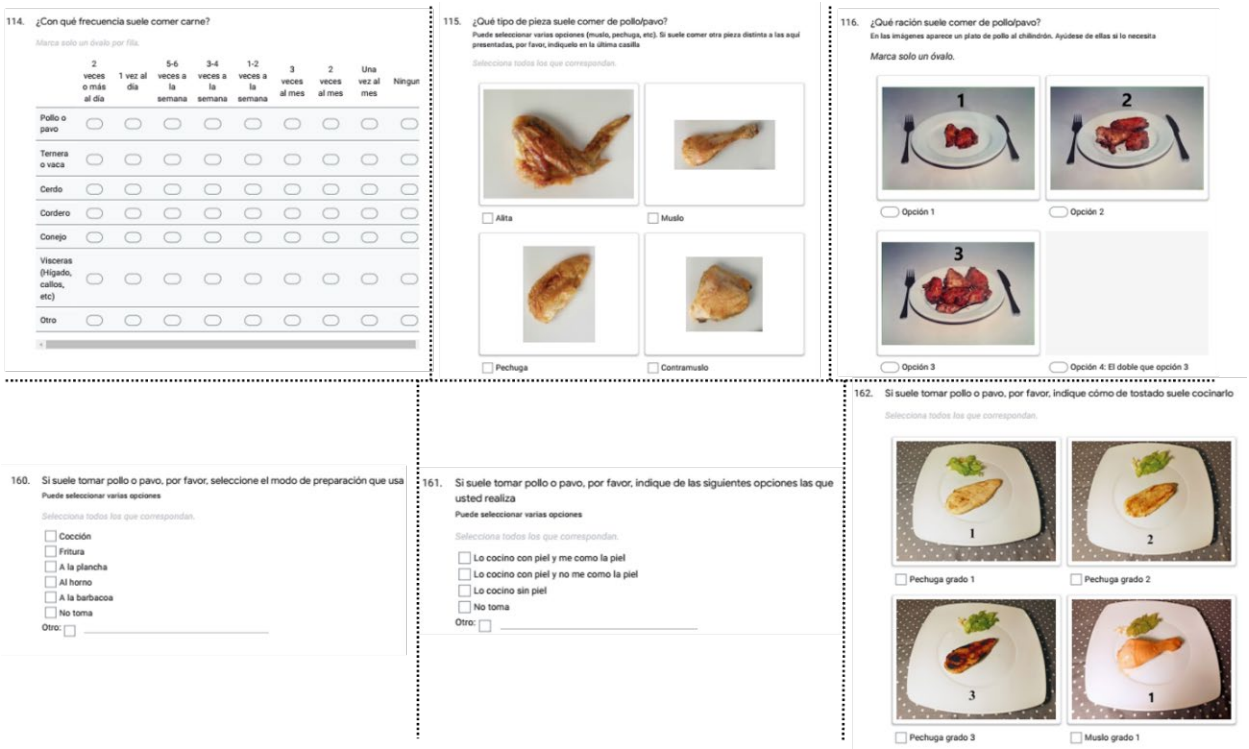

Figura 9. Ejemplo de la metodología empleada en el CFCA para el registro de la información dietética y de la ingesta de xenobióticos. En la imagen cada cuadro muestra algunas de las preguntas empleadas para registrar el consumo de pollo/pavo.

El siguiente paso llevado a cabo en la validación de la base desarrollada fue conocer si los valores de ingesta de xenobióticos obtenidos en la muestra de estudio presentaban semejanza o se alejaban de lo descrito en la bibliografía. En nuestros resultados hemos observado una ingesta media de HAPs totales de 1,09 µg/día (Artículo 1), un valor inferior al detectado en otros estudios llevados a cabo sobre diferentes poblaciones europea y que se sitúa dentro del rango de exposición dietética reflejado por un estudio de la OMS [129,132]. Esta Organización también ha indicado la dosis mínima de HAPs que presenta un 10% de probabilidad de causar una mayor incidencia de tumores u otros efectos adversos potenciales, conocido como límite mínimo de confianza para la dosis de referencia (BMDL10). Este valor se calculó utilizando el

B(a)P como marcador de exposición a 13 PAHs genotóxicas y carcinogénicas y se estableció en 100 mg por kg de peso corporal al día, muy por encima de lo observado en nuestra muestra de estudio [132].

Respecto a la ingesta AHs, la suma de los valores obtenidos para el PhIP, DiMeIQx, MeIQx, MeIQ e IQ reflejó una ingesta media de 119 ng/día (Artículo 1). Estos valores son un poco superiores a los detectados en estudios del EPIC e inferiores a los detectados empleando las bases del software CHARRED, lo que muestra cómo nuestra base combina datos de ambas bases de composición [119,150,160]. Meta-análisis previos han señalado que ingestas mayores o iguales a 40 ng de PhIP y/o 50 ng de MeIQx al día estaban relacionadas con una mayor incidencia de adenomas colorrectales [152]. En este sentido, los valores de ingesta medios detectados para PhIP y MeIQx en nuestra muestra fueron de 82 ng/día y 23 ng/día, respectivamente, lo que podría ser un factor de riesgo a largo plazo frente al que convendría diseñar estrategias dietéticas para su reducción.

También quisimos comprobar los niveles de ingesta de nitrito, que a diferencia del nitrato cuyas principales fuentes dietéticas eran alimentos de origen vegetal, en la muestra de estudio provenía de las carnes procesadas (Artículo 1 y Manuscrito 1). Actualmente la ingesta diaria admisible de nitrito se sitúa en 0,06 mg/kg peso corporal al día, un valor cercano a la ingesta media de 2,39 mg/día observada en nuestra muestra [378]. Por otro lado, la evaluación de las NAs reveló ingestas medias de 0,16 µg/día de NDMA, 0,07 µg/día de NPIP y 0,11 µg/día de NPYR (Artículo 1). Estos valores se situaban dentro de los valores referidos por la EFSA sobre la exposición a un panel de 10 NAs [165]. Sin embargo, la ingesta media de NDMA en nuestra muestra se situaba por encima de los 0,125 µg/día que el estudio EPIC señalaba en relación con un mayor riesgo de desarrollar cáncer, lo que refuerza el interés en reducir la ingesta de estos compuestos [179].

Por último, se evaluó también la acrilamida. Los valores de ingesta encontrados estaban en torno a los 15 µg/día, muy por debajo del BMDL10 de 0.17 mg/kg de peso corporal/día referido para este compuesto [379]. Considerando este hallazgo en el contexto de la evidencia científica existente en relación con la acrilamida y la salud, decidimos descartar la ingesta de este compuesto como un riesgo a considerar [195,196].

Una de las cuestiones que más interés generan sobre el impacto de la ingesta de xenobióticos en la salud humana es que aun conociendo la cantidad real de xenobióticos ingerida, desconocemos la fracción que llega al intestino, las transformaciones químicas o microbianas que ocurren y los compuestos que se excretan finalmente. Un aspecto novedoso de nuestro trabajo es que hemos asociado los datos de ingesta de xenobióticos obtenidos mediante encuestas nutricionales con datos analíticos en muestras biológicas, comparando y observando

relaciones entre los niveles de ingesta de NOCs registrados en los CFCA con las determinaciones cromatográficas de la presencia de estos compuestos en heces (Manuscrito 1).

6.2. Perfil nutricional asociado a lesiones de la mucosa intestinal

Conociendo el papel clave de la dieta en la salud, nos propusimos cómo la ingesta de los distintos de alimentos y los macronutrientes, micronutrientes y compuestos dietéticos derivados de ellos se asociaban con el grado y tipo de daño en la mucosa intestinal. Esto nos permitió detectar algunas diferencias significativas en las comparaciones entre grupos de diagnóstico clínico y análisis histopatológico. Entre los resultados más destacados se observa una mayor ingesta de etanol en el grupo de pacientes diagnosticados con pólipos intestinales, con independencia de la vía biológica de la lesión intestinal (Artículo 1 y Manuscrito 1). Nuestros datos revelan que una ingesta de etanol >11,62 g/día se asocia a un riesgo 3,54 mayor de pertenecer al grupo de individuos diagnosticados con pólipos intestinales (Artículo 1). Esta cifra se encuentra muy cercana a la establecida por el CMM, que indica que una ingesta de 12 g/día se asocia con un mayor riesgo de desarrollar CCR de manera significativa (RR:1,08; IC: 1,03-1,12) [66], y en la línea de estudios previos realizados por otros autores que han constatado un aumento de la prevalencia de pólipos intestinales con la ingesta de alcohol, prevalencia que se incrementa con consumos mayores a 8 g/día de etanol [380,381].

Otro de los factores que también se ha relacionado de forma directa con la probabilidad de pertenecer al grupo de individuos diagnosticados con pólipos intestinales es la ingesta de HAPs totales, siendo sus principales fuentes dietéticas productos diversos como el pan, la pasta, la manzana o la leche (Artículo 1). Concretamente, este grupo de diagnóstico mostró una mayor ingesta del HAP DiB(a)A que el grupo control, principalmente debido a un mayor consumo de cerveza. La ubicuidad de los HAPs y la inherente variabilidad de las matrices alimentarias es uno de los problemas a la hora de determinar analíticamente las concentraciones de estos compuestos en los alimentos [382]. A pesar del desarrollo en las últimas décadas de metodologías de extracción, identificación y cuantificación de HAPs, no se ha logrado una estandarización de su determinación, lo cual dificulta la comparabilidad de los estudios existentes [383,384].

De manera opuesta a lo indicado para los xenobióticos, hemos podido observar que el consumo de cereales integrales estaba asociado con un menor riesgo de pertenecer al grupo de voluntarios diagnosticados con pólipos intestinales (Artículo 1). Estos resultados están en consonancia con meta-análisis previos realizados por otros autores en los que se ha concluido que el consumo de cereales integrales podría reducir el riesgo de CCR desde un 3% por el consumo de 15 g/día de estos alimentos hasta un 17% por el consumo de 90 g/día [385,386]. Sin embargo, en nuestro estudio los voluntarios con un consumo de cereales integrales >50 g/día

sólo constituían un 9% de la muestra, lo que limita en cierta medida la fortaleza de nuestras conclusiones. Entre los mecanismos que más evidencia acumulan para explicar el papel protector para la salud de los cereales integrales se encuentran el alto contenido en fibra y la presencia de compuestos polifenólicos atrapados dentro de la matriz de fibra dietética, que pueden tener actividad antioxidante. Las fibras también pueden acortar el tiempo de tránsito intestinal, regular la absorción de lípidos y glucosa a nivel intestinal y modular la MI de manera beneficiosa promoviendo la producción de AGCC [387,388]. Se ha comprobado que los polifenoles, en concreto flavonoides como la epicatequina, el galato de epigalocatequina, el galato de epicatequina y la epigalocatequina son capaces de inhibir la formación de aductos de ADN similares a los generados por la ingesta de carcinógenos [389,390].

En base a estos resultados, nuestra hipótesis fue que los compuestos que acabamos de mencionar podrían estar relacionados específicamente con la composición de la MI y también con los diferentes parámetros relacionados con la homeostasis intestinal y sistémica evaluados en este trabajo. Tras llevar a cabo los correspondientes análisis, a continuación se comentan y discuten aquellos alimentos, componentes de los mismos y compuestos xenobióticos para los que hemos obtenido evidencias en el presente estudio de su asociación con alteraciones de la MI y/o parámetros inmunometabólicos en función del daño de la mucosa intestinal, así como la posible relación con factores del ambiente intestinal como la mutagenicidad fecal y las actividades enzimáticas fecales.

6.3. Relación de la ingesta de etanol con la microbiota intestinal y los parámetros inmunometabólicos

Previamente se ha indicado que el consumo de etanol estaba aumentado en el grupo de individuos diagnosticados con pólipos intestinales. Este consumo además se relacionó con mayores niveles de IL-1β (Artículo 2), uno de los parámetros empleados en la elaboración del IID [70]. La producción de IL-1β se encuentra estrechamente relacionada con el inflamasoma NLRP3, previamente asociada a la presencia de cristales de colesterol a nivel celular [84]. En este sentido, estudios llevados a cabo sobre células mononucleares de sangre periférica humana, líneas celulares de macrófagos de ratón y animales de experimentación apuntan a que la exposición crónica a etanol es capaz de activar el inflamasoma NLRP3 mediante la producción de óxido nítrico y la inducción del enzima óxido nítrico sintasa [391–393]. Resultaba por tanto lógico pensar que este consumo de etanol y la inflamación derivada podría tener un efecto en la composición de la MI. Evaluando la muestra de estudio al completo, hemos observado que el consumo de etanol es un factor predictor de la reducción en la abundancia relativa de la familia *Clostridiaceae* (Artículo 5). Esta familia presenta mayor abundancia relativa en individuos control y en pólipos hiperplásicos que en adenomas convencionales, y más específicamente en

la comparación del grupo control con los adenomas convencionales con displasia de bajo grado (Artículo 3). Enfocándonos en el grupo de voluntarios diagnosticados con pólipos intestinales se observó que el consumo de ≥12 g/día de etanol se encontraba asociado con variaciones en la MI y concretamente con un aumento en la abundancia relativa de la familia *Peptostreptococcaceae* (Artículo 5). Se ha observado un aumento de esta familia de bacterias en bebedores habituales de alcohol y en muestras fecales de CCR [394,395], pudiendo ser el aumento de estos microorganismos a lo largo del tiempo un indicador potencial del incremento de riesgo de desarrollar CCR. En base a estos resultados y de acuerdo con otros autores, se sugiere que el consumo de etanol ≥12 g/día podría mostrar un impacto potencial negativo en la salud. Para hacerse una idea de la cantidad de bebidas con contenido alcohólico a la que equivale esta ingesta de etanol, podemos indicar que el consumo de una lata de cerveza rubia con un 5% de graduación alcohólica supondría una ingesta de 15 g de etanol, mientras que tomar una copa de vino tinto de aproximadamente 100 mL equivaldría a 10 g de etanol. Por ello, considerando las evidencias científicas acumuladas, desde el sector sanitario se promueve la abstención en el consumo alcohol como una de las medidas para reducir la incidencia de CCR [216].

6.4. Relación de la ingesta de carnes rojas con la microbiota intestinal y los parámetros inmunometabólicos

Tal y como se comentaba en la Introducción de esta Tesis Doctoral, la ingesta de carnes rojas y procesadas se ha relacionado con un incremento en el riesgo de desarrollar CCR, por lo que resultaba de interés profundizar en las relaciones que presentaba el consumo de carnes con los distintos parámetros analizados en este trabajo [42,66]. Nuestros datos revelaron que un mayor consumo de carnes se asociaba con una disminución en el nivel de adiponectina y que este parámetro además mostraba una reducción en sus niveles de acuerdo con la presencia de pólipos intestinales y/o CCR (Artículo 2). También se observó que la ingesta de ≥50 g de carne roja al día estaba asociada con una disminución en la α-diversidad de la microbiota fecal de los voluntarios del estudio, acompañada de un aumento en la abundancia relativa de la familia *Coriobacteriaceae* y una disminución de las familias *Bacteroidaceae* y *Akkermansiaceae* en el grupo de individuos control (Artículo 5). En este sentido, se ha descrito que el efecto del consumo de carne en la MI suele tener mayor repercusión en niveles taxonómicos bajos, de género y especie, que en la diversidad global de la comunidad [396]. A nivel taxonómico de familia, el rol de *Coriobacteriaceae* en la salud humana no se conoce con exactitud, aunque su abundancia parece estar asociada a un bajo consumo de fibra, procesos inflamatorios y obesidad [221,397]. Por el contrario, también se ha sugerido una relación negativa de la abundancia de *Coriobacteriaceae* con un índice inflamatorio elaborado a partir de cuatro variables fisiológicas relacionadas con la inflamación asociada a obesidad [398]. Por otro lado, algunos autores indican un aumento de la familia *Bacteroidaceae* en individuos con una dieta rica en productos

vegetales [399]. Respecto a la familia *Akkermansiaceae*, actualmente está conformada por el género *Akkermansia* a su vez constituido por 4 especies, siendo *Akkermansia muciniphila* la primera en ser descrita y la que más evidencia acumula sobre su potencial antiinflamatorio [400]. Se ha comprobado en ensayos de intervención dietética sobre individuos adultos con sobrepeso y obesos que la abundancia de *Akkermansia* está inversamente relacionada con biomarcadores de disfunción metabólica [401]. En estudios previos se ha relacionado el sobrepeso y la obesidad con un incremento del 10% en el riesgo de desarrollar CCR, teniendo una repercusión mayor la presencia de grasa visceral o abdominal que la grasa subcutánea [402,403]. Otros autores apuntan a que la incidencia de estos factores podría ser mayor de lo epidemiológicamente mostrado, ya que existe un sesgo en el registro del IMC provocado por la pérdida de peso de muchos pacientes de manera previa al diagnóstico del CCR [404]. En este trabajo el grupo de individuos diagnosticados con pólipos intestinales presentaban sobrepeso (IMC en torno a 27 kg/m^2), un factor que puede resultar una diana de actuación para la prevención del CCR.

6.5. Relación de la ingesta de carnes procesadas, nitritos y nitrosaminas con la microbiota intestinal

Mientras que la carne roja ha sido clasificada como probablemente carcinogénica para humanos (Grupo 2A) por la IARC, la carne procesada ha sido clasificada como carcinogénica para humanos (Grupo 1) [63]. Se ha propuesto que el impacto de las carnes procesadas en la salud humana se debe al contenido en grasa, hierro hemo o aditivos precursores de NOCs como E-249, E-250, E-251 y E-252 [45]. Otro factor a tener en cuenta por su posible papel en esta asociación es la MI. Analizando este factor en nuestro trabajo, hemos visto que un consumo de carne procesada ≥25 g/día se asociaba con un aumento de la abundancia relativa de las familias *Prevotellaceae* y *Erysipelatoclostridiaceae* y con una disminución de la familia *Bifidobacteriaceae* en el grupo de individuos control (Artículo 5). Previamente, teniendo en cuenta los grupos de análisis histopatológico, habíamos observado la disminución de la abundancia relativa de la familia *Prevotellaceae* en individuos con adenomas convencionales que mostraban displasia de alto grado en comparación con individuos control (Artículo 3). Sin embargo, otros autores han observado un aumento en la abundancia de esta familia en individuos con CCR [405,406].

Los aditivos que se han mencionado y que habitualmente están presentes en carnes procesadas representan una fuente dietética de nitrito. Se ha señalado que ingestas mayores de nitrito y nitrato procedentes de fuentes animales pueden dar lugar a mayores niveles de N-óxido de trimetilamina, un metabolito potencialmente tóxico derivado de la actividad de la MI que se ha relacionado con el riesgo de desarrollo de CCR [407–409]. Analizando la relación de la

ingesta de nitritos con la composición de la MI dentro de cada grupo de diagnóstico clínico de la muestra de estudio, hemos encontrado relación entre la ingesta de nitritos superior a 1,69 mg/día y una menor abundancia relativa de *Bifidobacteriaceae* en el grupo control y en el grupo de individuos con pólipos intestinales (Artículo 5), en consonancia con lo observado para el consumo de carnes procesada. Otros compuestos que pueden aparecer en carnes procesadas son las NAs. En nuestro trabajo, la ingesta de las NAs NPYR y NDMA se asoció de forma inversa con la abundancia relativa de esta familia de microorganismos en el grupo control. La disminución observada de la familia *Bifidobacteriaceae* asociada al consumo de carnes procesadas y sus derivados podría estar relacionada con su alto contenido en grasas saturadas [410]. De hecho, el género *Bifidobacterium,* se ha encontrado aumentado en dietas vegetarianas bajas en grasas saturadas y se ha relacionado con una función protectora de la barrera intestinal [410–412]. Además, algunas BAL y miembros de la familia *Bifidobacteriaceae* han mostrado la capacidad de unir y absorber compuestos tóxicos a nivel intestinal, reduciendo su bioaccesibilidad y favoreciendo su eliminación con las heces [271,413–415]. Por ende, hipotetizamos que altas ingestas continuadas de NAs podrían provocar una disminución de la abundancia relativa de esta familia en la MI a largo plazo.

6.6. Ingesta de N-nitrosocompuestos y sus precursores y su influencia en la formación de N-nitrosocompuestos endógenos

Aunque las NAs representen los principales NOCs de la dieta, estos últimos compuestos también pueden tener un origen endógeno. A partir del consumo de nitratos, nitritos, proteína, hierro hemo o NAs exógenas se pueden generar agentes nitrosantes y otras moléculas reactivas como las aminas secundarias que a lo largo del tracto gastrointestinal pueden reaccionar entre sí dando lugar a la formación de NOCs endógenos (NOCs derivados de hemo y NOCs totales) [164,169]. Por ello, en este trabajo también hemos estudiado la excreción fecal de NOCs endógenos. En base a esto, hemos podido comprobar que las carnes procesadas son las principales fuentes dietéticas de NAs y de su precursor nitrito en la muestra (Manuscrito 1). También hemos correlacionado el consumo de estas fuentes dietéticas con la concentración fecal de NOCs derivados del grupo hemo y NOCs totales. Además, de acuerdo con los grupos de análisis histopatológico hemos relacionado de forma directa la concentración fecal de NOCs derivados de hemo y NOCs totales con el grado de daño de la mucosa intestinal. El chorizo, un embutido que se consume solo o como parte de guisos y otras preparaciones culinarias y está ampliamente distribuido en nuestro país, se reveló en nuestro estudio como un predictor de las concentraciones fecales de NOCs en la muestra de estudio. Un informe llevado a cabo en España en 2012 situó el consumo aproximado de chorizo en 3 g/persona y día, una cifra similar a los 2,76 g/día de consumo registrados en población asturiana por un estudio reciente, lo que equivaldría a una rodaja de este alimento; sin embargo, en nuestro estudio el consumo de

chorizo observado fue superior, con una ingesta media de 11 g/persona y día [416,417]. Se ha demostrado que la producción de ciertos alimentos cárnicos siguiendo buenas prácticas de fabricación logra reducir su contenido en NAs [418–420]. En base a estos resultados, sería interesante promover la vigilancia de los niveles de estos compuestos en alimentos a nivel industrial y comercial y la reducción en el consumo de carnes procesadas con el fin de minimizar el impacto que los nitritos y algunas NAs potencialmente carcinogénicas pueden tener sobre la salud humana [180]. Resulta importante considerar que los nitratos, nitritos, proteína, hierro hemo o NAs exógenas previamente mencionados son capaces de modular la MI, que también juega un papel clave en la formación de NOCs endógenos [164,169]. Por ello, descifrar las relaciones de la MI con la formación de NOCs endógenos se plantea como un objetivo de gran interés para abordar a corto plazo.

La relevancia de la ingesta de compuestos potencialmente perjudiciales para la salud como son los xenobióticos de la dieta ha sido debatida por el profesor Bruce Ames en el contexto de los pesticidas. Junto a sus colaboradores, señaló que en Estados Unidos la ingesta de compuestos con potencial tóxico formados en la naturaleza es 10.000 veces mayor que la exposición a tóxicos pesticidas sintéticos, remarcando que la ingesta de pesticidas no resultaba un peligro al ser prácticamente insignificante [421]. Este llamativo debate parece invitar al relajamiento en la regulación alimentaria sin valorar la magnitud del efecto a largo plazo de la exposición a xenobióticos. La valoración de la toxicidad de estos compuestos no debe limitarse únicamente a la observación aislada mediante estudios *in vitro* con cultivos celulares o *in vivo* con modelos animales, sino que es necesario considerarla junto con los procesos de absorción, distribución, metabolización y eliminación (procesos ADME), que conllevan la bioactivación de parte de estos xenobióticos tras su paso por el intestino y su interacción con la MI [422]. Para poder evaluar el potencial impacto final en la salud de los compuestos presentes en las heces se ha propuesto la valoración del potencial mutagénico y genotóxico [423]. Siendo este un parámetro evaluado en este trabajo, hemos podido observar que, de acuerdo con los grupos de análisis histopatológico, el grupo de voluntarios diagnosticados con pólipos hiperplásicos presentaban una asociación significativa entre la mutagenicidad fecal y la concentración fecal de NOCs derivados del grupo hemo (Manuscrito 1). Esta misma tendencia se observó también para los NOCs totales en el grupo de adenomas convencionales. Sin embargo, no se alcanzó en este caso la significación estadística, posiblemente debido al bajo tamaño muestral.

6.7. Relación de la ingesta de aminas heterocíclicas con la microbiota intestinal y la mutagenicidad fecal

Algunos de los xenobióticos ingeridos con la dieta que más evidencia han acumulado en relación con la salud y que se han clasificado como posibles carcinógenos para humanos por la

IARC son las AHs PhIP, MeIQx y DiMeIQx, que además han mostrado mayor capacidad mutagénica que otros xenobióticos como los HAPs [148,152]. Por ello, quisimos comprobar la relación de la ingesta de los diferentes xenobióticos de la dieta con los niveles detectados de mutagenicidad fecal en la muestra de estudio. En este sentido, hemos observado una fuerte correlación entre la ingesta de PhIP, MeIQx y DiMeIQx con la mutagenicidad fecal en el grupo de pacientes diagnosticados con pólipos intestinales (Artículo 1). Sin embargo, en las comparaciones entre los distintos grupos de diagnóstico clínico o de análisis histopatológico no hemos encontrado diferencias significativas en los niveles de ingesta de estos compuestos. Tras esto, quisimos comprobar si los valores umbral de ingesta de estos compuestos previamente relacionados con el desarrollo de CCR estaban asociados a cambios en la composición microbiana fecal dentro de cada grupo de diagnóstico clínico. De esta manera, observamos que a nivel microbiológico los cambios observados por la ingesta de PhIP ≥40 ng/día y MeIQx ≥50 ng/día resultaron similares a los observados para un consumo de carnes rojas ≥50 g/día, con un aumento en la abundancia relativa de la familia *Coriobacteriaceae* y una disminución de las familias *Bacteroidaceae* y *Akkermansiaceae* en el grupo de individuos control (Artículo 5). No se detectaron cambios en la familia *Lactobacillaceae*, pese a que algunos miembros de esta familia son capaces de conjugar AHs disminuyendo así su toxicidad [275–277].

Se ha sugerido que el tipo de carne podría condicionar la cantidad y el tipo de AHs formados durante el cocinado y procesado del alimento en base a parámetros como el pH, el nivel de humedad, el contenido graso y el contenido proteico de la pieza de carne [424]. Por ejemplo, un aumento en el pH puede incrementar el tostado de los alimentos al favorecer la reacción de Maillard debido a la mayor presencia de especies reactivas como grupos amino desprotonados y azúcares reductores [425–427]. La reacción de Maillard se ve favorecida por temperaturas altas y tiempos largos de cocinado, aunque también puede ocurrir a temperaturas bajas durante el almacenado de productos, siendo en este caso la naturaleza y composición de la matriz alimentaria la que influye en el grado de reactividad [426,428,429]. Con el fin de reducir la formación de AHs en el procesado de los alimentos se han propuesto varias estrategias, siendo una de ellas el marinado de las carnes. Las especias presentes en el marinado pueden ejercer de barrera y evitar el contacto directo del alimento con la fuente de calor, dependiendo su eficacia de la composición de especias y el tiempo de marinado [209]. Otra estrategia es cambiar el modo de consumo de las carnes. Por ejemplo, se ha sugerido cocinar el pollo con piel y desechar ésta antes de su consumo. La piel del pollo tiene un alto contenido en grasa que durante el cocinado del alimento sufre una exposición directa a la fuente de calor, lo cual favorece la formación de AHs. De esta manera, se propicia una mayor concentración de AHs en la piel, que va a ser posteriormente eliminada mientras el resto del alimento queda cocinado y presenta concentraciones mucho menores de AHs [424].

Recientemente se han desarrollado herramientas basadas en la minería de datos y métodos iterativos capaces de predecir la biotransformación que ocurre en el organismo de multitud de AHs para así evaluar su impacto en la salud. En uno de estos trabajos, publicado recientemente por otros autores, se ha podido comprobar que de los metabolitos predichos a nivel computacional procedentes de la ingesta de AHs, la mitad ya habían sido determinados analíticamente con anterioridad, mostrando por tanto el modelo un buen nivel de predicción. Además, la genotoxicidad predicha en dicho modelo computacional está próxima a la determinada experimentalmente [430]. Es importante considerar que algunas de las moléculas consideradas xenobióticos ingeridas con la dieta no muestran potencial carcinogénico, pero combinadas con otros xenobióticos carcinogénicos pueden potenciar su toxicidad mediante un efecto sinérgico, aumentando su efecto potencial en el organismo [431]. En este sentido, nuestros resultados sugieren una relación entre la mutagenicidad fecal, el consumo de xenobióticos como AHs y los NOCs endógenos.

6.8. Relación de la ingesta de hidrocarburos policíclicos aromáticos con la microbiota intestinal, los parámetros inmunometabólicos y la actividad enzimática

Otro de los xenobióticos evaluados en este trabajo ha sido la ingesta de HAPs. En concreto, cuando se ha analizado la relación entre estos compuestos y los niveles circulantes de los parámetros inmunometabólicos estudiados, los resultados han mostrado que la ingesta de HAPs totales estaba asociada inversamente con los niveles de adiponectina en suero, de manera similar a como ocurría con el consumo de carnes rojas y procesadas (Artículo 2). Además, en relación con la composición de la MI, durante este trabajo hemos podido comprobar que ingestas superiores a 0,75 µg/día de HAPs totales se asocian entre otros con una disminución de la abundancia relativa de la familia *Christensenellaceae* en el grupo de pólipos (Artículo 5). Previamente otros autores han asociado positivamente la familia *Christensenellaceae* con el estado de salud, considerando algunos miembros de esta familia como potenciales candidatos probióticos [432,433]. De hecho, en la comparación entre los grupos de análisis histopatológico habíamos observado que la abundancia relativa de la familia *Christensenellaceae* y en concreto del grupo *Christensellaceae_R-7* (nivel taxonómico de género) era mayor en el grupo de individuos control que en el grupo que presentaba adenomas convencionales con bajo y alto grado de displasia (Artículo 3). Por otro lado, cuando hemos considerado los niveles de actividad α-glucosidasa, hemos observado que el grupo *Christensellaceae_R-7* era más abundante en los individuos con baja actividad enzimática respecto a los de alta actividad (Artículo 4). Por todo lo descrito, la relación entre la familia *Christensenellaceae*, el tipo de daño presenten en la mucosa intestinal, la ingesta de HAPs, la actividad α-glucosidasa y los niveles circulantes de adiponectina resulta un punto de partida interesante para profundizar en el papel metabólico que esta familia podría ejercer en la prevención del daño intestinal.

6.9. Relación de la ingesta de cereales integrales con la microbiota intestinal y los ácidos grasos de cadena corta

Previamente hemos destacado que el consumo de cereales reducía el riesgo de presentar de daño en la mucosa intestinal (Artículo 1). Algunos estudios apuntan a que se podrían reducir los niveles de mutagenicidad fecal mediante el consumo de fibra alimentaria, mientras que otros no han sido capaces de corroborar una relación entre la dieta y la mutagenicidad fecal [434–438]. Concretamente, en nuestro trabajo hemos observado que un mayor consumo de cereales integrales se asociaba con una menor mutagenicidad fecal en toda la muestra estudiada y particularmente en el grupo de voluntarios con pólipos intestinales (Artículo 1). Sin embargo, no se han observado asociaciones del consumo de cereales integrales con los distintos parámetros séricos evaluados (Artículo 2). Sí se ha detectado una correlación directa entre el consumo de cereales integrales y una mayor abundancia relativa de la familia *Prevotellaceae* en el grupo control (Artículo 5). Previamente se había comentado la disminución de *Prevotellaceae* ante la presencia de un mayor grado de daño de la mucosa intestinal (Artículo 3). De manera general, la abundancia de *Prevotellaceae* se asocia a una alta adherencia a dietas antiinflamatorias como la DM [439]. La familia *Prevotellaceae* es capaz de producir acetato en el intestino, y este compuesto se ha relacionado con la secreción de hormonas en el tracto digestivo como el péptido-1 similar al glucagón y el péptido YY y por tanto con una reducción en el apetito y el nivel de citocinas proinflamatorias [440]. Otro de los AGCC producidos por la MI asociados al consumo de fibra y a la reducción en la formación de aductos de ADN es el butirato, que puede promover un incremento de la apoptosis en células expuestas a carcinógenos genotóxicos y que promueve la expresión de la enzima Glutatión S-transferasa [441,442]. En este sentido, es posible que la producción de AGCC beneficiosos como el acetato por miembros de la MI como *Prevotellaceae* se vea aumentada por el consumo de cereales integrales.

7. LIMITACIONES DEL ESTUDIO

A lo largo de esta Discusión se han destacado los principales hallazgos del estudio y se han señalado las fortalezas que este presenta. Sin embargo, durante el desarrollo de este trabajo también se han detectado algunas debilidades que serán comentadas a continuación.

7.1. Asimetría en el reclutamiento de voluntarios y en la obtención de muestras biológicas

La situación de emergencia sociosanitaria provocada por la pandemia de COVID-19 ha limitado el reclutamiento de voluntarios para este estudio, particularmente de aquellos diagnosticados con adenocarcinomas, lo que tuvo como consecuencia el reclutamiento de un bajo número de individuos en este grupo. Por este motivo, en algunos de los trabajos incluidos en esta Tesis Doctoral, el análisis estadístico se ha visto limitado, así como la fortaleza de las conclusiones obtenidas para el grupo de adenocarcinoma. Sería deseable aumentar el número de voluntarios incluidos en el grupo de diagnosis de adenocarcinomas en futuros estudios con el fin de poder obtener conclusiones más robustas en los estadios avanzados de daño de la mucosa intestinal e inicio del CCR. Además, la heterogeneidad en la obtención de muestras ha imposibilitado determinar algunos parámetros sobre la totalidad de la muestra objeto de estudio, conformando un tamaño muestral diferente en los distintos trabajos recogidos en la presente Tesis Doctoral. Con el fin de mejorar la comprensión de este escrito se ha elaborado una tabla para indicar la disponibilidad de información detallada de acuerdo con el voluntario y los parámetros analizados (Anexo 1).

7.2. Cuantificación de la mutagenicidad fecal

En nuestro trabajo hemos evaluado la mutagenicidad intestinal mediante el análisis de los niveles de mutagenicidad fecal. En este sentido, el uso de muestras biológicas complejas permite un análisis integrativo que refleje lo que realmente sucede en el organismo, aunque puede presentar algunos impedimentos metodológicos [171]. Nosotros hemos conseguido detectar cambios significativos en los niveles de mutagenicidad entre grupos de diagnóstico y de histopatología mediante una adaptación del test de Ames a sobrenadantes fecales. Sin embargo, al ser necesaria la dilución de las muestras para evitar la interferencia de la histidina presente en heces, existe cierto riesgo de infraestimar la capacidad mutagénica de las heces. Otro de los inconvenientes del método empleado es su falta de estandarización. Al tratarse de un método semicuantitativo es posible diferenciar entre muestras de un mismo estudio, pero no existen valores ni escala de referencia, lo que dificulta la comparación con otros estudios. Además, la relevancia clínica del análisis de la mutagenicidad fecal todavía no se ha establecido por la falta de estudios en humanos y la variabilidad de los datos epidemiológicos analizados [423]. Existen otros métodos capaces de analizar la mutagenicidad como el Chromotest SOS, basado en un cambio colorimétrico debido a la síntesis y secreción de la enzima beta-galactosidasa tras la

activación del promotor SOS producido por la presencia de daño en el ADN [443]. Otras metodologías disponibles se centran en evaluar parámetros como la citotoxicidad o genotoxicidad para medir los cambios estructurales que ocurren en el ADN o la cromatina. Entre estos métodos destaca el test del Cometa. Por ejemplo, se ha evaluado el efecto de una intervención dietética basada en el consumo de 300g/día de carne roja durante 7 días, observando mayor genotoxicidad fecal sin llegar a repercutir en la formación endógena de NOCs [444]. En la misma línea, otros autores indicaron que la genotoxicidad y el daño en el ADN provocados por una mayor ingesta de carne roja era independiente de la formación endógena de NOCs [445]. Otros estudios han mostrado como algunas AHs pueden incrementar la genotoxicidad fecal y la actividad enzimática fecal β-glucuronidasa en niños, adultos y ancianos [446]. En conclusión, sería aconsejable la combinación de varias de estas metodologías descritas para poder estudiar la genotoxicidad, la mutagenicidad y la citotoxicidad del ambiente intestinal de forma más integrativa y precisa.

7.3. Cuantificación del ácido caproico

Cuando se analizaron las diferencias en la concentración fecal de AGCC en función de los grupos de análisis histopatológico, uno de los hallazgos más llamativos fue la ausencia de diferencias significativas excepto en el caso del ácido caproico. El ácido caproico es un AGCC minoritario que procede principalmente de la dieta y se ha descrito su papel en la diferenciación de células Th1 y Th17 en el sistema inmunitario [447]. Habitualmente su concentración en heces es muy reducida, lo que provoca que muchas veces esté por debajo del límite de detección de la técnica empleada para su análisis e imposibilita una adecuada interpretación del significado biológico de los resultados.

8. PERSPECTIVAS FUTURAS

Teniendo en cuenta las fortalezas y limitaciones del estudio que se han mencionado previamente, surgen diferentes ideas de cara al futuro con el fin de cubrir las debilidades detectadas, complementar el trabajo realizado y aumentar el conocimiento de la relación de los parámetros estudiados con el desarrollo del daño de la mucosa intestinal que se comentan a continuación

8.1. Integración de los hallazgos del estudio con datos sobre el metaboloma fecal

Si bien en este trabajo hemos podido comprobar una reorganización de MI comensal según el grado de daño de la mucosa intestinal (Artículo 3), el diseño caso-control transversal de este estudio no permite discernir si estos cambios en la MI son causa o consecuencia de la progresión de la enfermedad. La composición y la funcionalidad de la MI presentan una gran variabilidad inter-individual y guardan relación directa no solo con el metabolismo endógeno del hospedador sino también con la dieta [448]. En la dieta se ingieren multitud de compuestos alimentarios, algunos de los cuales pueden alcanzar en diversa proporción el intestino grueso. Una vez allí, la MI puede contribuir a la producción, transformación y/o eliminación de los compuestos presentes, modificando por tanto el metaboloma fecal [449–451]. Es posible que entre dos dietas distintas no se detecten cambios en la diversidad y composición microbiana, pero que sí existan alteraciones en la funcionalidad de la MI por cambios en los perfiles de expresión genética y en los metabolitos asociados [452]. Una técnica útil para profundizar en los cambios que pueden estar ocurriendo a nivel intestinal y arrojar más información al respecto es la metabolómica fecal, abordando el estudio del conjunto de metabolitos presentes en las heces. De manera reciente se ha generado un interés por desarrollar paneles de microorganismos y metabolitos biomarcadores cuyos niveles puedan emplearse en la detección y pronóstico de la enfermedad [453–455]. Se ha descrito la alteración de diversos metabolitos fecales como ácidos biliares secundarios, aminas biógenas, aminoácidos ramificados y aromáticos, ácidos grasos poliinsaturados y otros compuestos en heces de pacientes con adenomas o CCR [305,456–458]. En algunos estudios se ha sugerido que la combinación de marcadores microbianos como *Fusobacterium*, *Parvimonas* y *Staphylococcus* y marcadores metabolómicos como ésteres de colesterol y esfingolípidos puede ser útil como herramienta de diagnóstico de adenomas avanzados y CCR, reflejando un poder predictivo mayor que el test de sangre oculta en heces [459]. Un parámetro que permite medir la capacidad de discriminación de estos marcadores es el análisis del área bajo la curva ROC (“Receiver Operating Characteristic”), también conocido como AUC, en el cual un valor de 1 refleja una capacidad discriminatoria diagnóstica del 100%. A partir de estudios metagenómicos de muestras fecales se ha llegado a combinar un total de 29 especies microbianas, 24 metabolitos y 16 genes diferencialmente expresados para discriminar los casos de carcinoma intramucoso de los controles sanos con un AUC de 0,78. Por otro lado,

empleando 14 especies microbiana y 2 metabolitos para diferenciar adenomas de muestras control se ha señalado un AUC de 0,88 [305,458], mientras que la combinación de 5 metabolitos y 4 especies aumentadas en muestras fecales de CCR ha permitido discriminar el grupo de pacientes con adenomas y el grupo control con un AUC de 0,90 [460]. Sin embargo, a pesar de las evidencias que muestran asociaciones significativas entre metabolitos fecales y miembros específicos de la MI en el contexto de CCR, los estudios en las fases iniciales de daño de la mucosa intestinal siguen siendo escasos [456,458,461,462]. En este sentido, el estudio integrativo de la composición microbiana fecal, el metaboloma fecal y la dieta mediante el uso de herramientas de inteligencia artificial como el aprendizaje automático ("Machine Learning"), el aprendizaje profundo ("Deep Learning") u otros métodos de computación avanzados podría resultar de gran interés para entender cómo el microbioma se relaciona con los cambios tempranos de la mucosa intestinal en el contexto del CCR [463–466].

8.2. Caracterización de la actividad α-glucosidasa fecal

Aunque el empleo del sistema semi-cuantitativo API ZYM se ha mostrado útil para llevar a cabo una aproximación inicial sobre los perfiles enzimáticos fecales de acuerdo con el tipo y grado de daño de la mucosa intestinal, de cara a futuros estudios resultaría de gran interés un estudio cuantitativo de la actividad enzimática α-glucosidasa. La adaptación de los protocolos de purificación del/los enzimas con actividad α-glucosidasa a muestras biológicas de heces o mucosa intestinal permitirían su caracterización a nivel bioquímico y molecular, así como la cuantificación de los niveles de actividad de las proteínas purificadas y la especificidad de sustrato [467,468]. No obstante, quizá sea más interesante centrar los esfuerzos futuros en conocer primero la fuente principal de la actividad α-glucosidasa, bien procedente de la microbiota o de las células del hospedador en cada uno de los grupos de diagnóstico y comprobar si la contribución de cada fuente varía en función del daño de la mucosa intestinal. Una vez identificada esta fuente se podrían secuenciar los genes codificantes de este enzima, complementando así los hallazgos bioquímicos que se puedan obtener a partir de la purificación y caracterización bioquímica del enzima.

8.3. Realización de estudios in vitro e in vivo para corroborar los datos obtenidos

A lo largo de esta Tesis Doctoral hemos puestos en evidencia diversas asociaciones novedosas entre la dieta y los distintos parámetros estudiados que podrían ser corroborados experimentalmente en modelos de estudio con el fin de confirmar los resultados obtenidos para algunos alimentos o compuestos dietéticos. Entre los posibles modelos de estudio se encuentran los ensayos *in vitro* con líneas celulares humanas. Los modelos *in vitro* presentan una gran reproducibilidad, menor coste y necesitan menos permisos éticos que los que se requieren para modelos *in vivo*. Las líneas celulares de adenocarcinoma de colon humano Caco-2 y HT29, han

sido ampliamente utilizadas para el estudio del epitelio intestinal [469,470]. A pesar de que la expansión de técnicas como el co-cultivo para mimetizar el ambiente intestinal ha permitido mejorar la calidad de los resultados obtenidos en los ensayos *in vitro*, las condiciones fisiológicas complejas existentes en el sistema digestivo, la interacción con otros sistemas como el inmunitario y la interacción con la microbiota son difíciles de recrear, lo que podría reducir la fiabilidad de los resultados obtenidos *in vitro* [471]. Otro modelo disponible es el estudio *ex vivo*, en el que a partir de tejidos humanos o animales se pueden realizar pruebas experimentales en el laboratorio más verosímiles que las pruebas *in vivo*. Entre las principales desventajas se encuentran el coste, la naturaleza invasiva del método y la dificultad de prolongar la duración y funcionalidad de la función intestinal original [472]. Existen algunos modelos más complejos como los organoides, que pretenden mimetizar el carácter tridimensional existente en el ambiente intestinal incluyendo células polarizadas y altamente diferenciadas con características propias de un tejido sano obtenidas a partir de células madre pluripotentes o de la reprogramación de células madre adultas [473]. Sin embargo, la polarización de las células y la necesidad de inyectar componentes a ensayar dificultan el empleo de esta metodología [472]. De forma más reciente se han desarrollado microdispositivos que están compuestos por un sistema de cultivo celular que mediante la aplicación de tecnologías de microfluidos y microingeniería simula el microambiente y los aspectos funcionales clave de tejido u órganos, evitando el sobrecrecimiento y contaminación de la microbiota comensal simulada [474]. Uno de los problemas que surgen en este tipo de dispositivos es el aumento en la absorción de compuestos lipofílicos debido a la hidrofobicidad de los materiales empleados en su construcción [474]. Además, la estabilidad y el reducido volumen que aceptan los microchips limita la productividad del modelo, describiéndose una gran variabilidad entre diferentes laboratorios, por lo que sería deseable una unificación de la tecnología mediante la fabricación de microdispositvos a gran escala [471]. Por último, también es posible utilizar modelos *in vivo*. Posiblemente los roedores murinos son los más utilizados en estos modelos, aunque también se emplean especies de cánidos, cerdos y monos entre otros [475]. Los modelos *in vivo* representan con un grado más alto de fidelidad que los modelos anteriores la microestructura del intestino humano y resultan valiosos como modelos preclínicos primarios, aunque no simulan totalmente el intestino humano. No obstante, su uso está sujeto a las lógicas limitaciones éticas. Además, la composición y funcionalidad de la MI observada en estos modelos animales puede diferir notablemente de la MI humana. Por todo lo expuesto, a la hora de plantear un estudio experimental sería necesario evaluar detenidamente cual es el objetivo final y valorar el posible uso de varios modelos para así adaptar el diseño al método más adecuado

8.4. Armonización de los cuestionarios de frecuencia de consumo alimentarios y los valores analíticos en heces

Una de las tareas pendientes en la comunidad científica relativa al conocimiento de los xenobióticos es ampliar las tablas existentes de concentración de estos compuestos en alimentos mediante la realización de nuevos estudios analíticos que empleen metodologías de análisis unificadas y armonizadas. En concreto, esta ampliación podría hacerse profundizando en los alimentos frecuentemente consumidos en cada sociedad. De esta manera, el registro de los xenobióticos de la dieta sería más exacto y verosímil, adaptándose a la realidad de la dieta que se observa en nuestro país y contribuyendo a la optimización de los CFCAs.

Una de las fortalezas de este trabajo es que hemos relacionado por primera vez la ingesta de NAs en la dieta con la concentración excretada de NOCs obtenida de manera analítica. Mediante esta asociación hemos conseguido demostrar que el método empleado para registrar y cuantificar los xenobióticos de la dieta tiene validez experimental. Pese a no ser un método completamente preciso, el error inherente al método está armonizado, de manera que permite la comparación entre los diferentes grupos de estudio. De cara al futuro, resultaría de gran interés poder estimar el grado de asociación de las estimaciones del CFCA empleado con la excreción determinada analíticamente de otros xenobióticos de la dieta como los PAHs o las AHs. En base a estos resultados, se podría modificar la metodología de registro desarrollada con el fin de mejorar la precisión en el análisis de las ingestas dietéticas.

En resumen, los trabajos de la presente Tesis Doctoral han permitido estudiar el papel de la dieta en la salud y las asociaciones existentes con la MI y diversos parámetros del ambiente intestinal e inmunometabólicos de acuerdo con la presencia de alteraciones de la mucosa intestinal previas al desarrollo de CCR en una población adulta. Aunque quedan muchas cuestiones por resolver, nuestros resultados ponen de manifiesto que recomendaciones dietéticas dirigidas a una población diana con el objetivo de regular la ingesta de xenobióticos generados en el procesado de los alimentos podrían reducir el riesgo de desarrollo de la enfermedad.

CONCLUSIONES

Primera. El cuestionario de frecuencia de consumo de alimentos diseñado junto con las herramientas desarrolladas para el cálculo de la ingesta de compuestos xenobióticos en este estudio han demostrado su utilidad para la estimación de la ingesta de estos compuestos en población adulta. Además, mediante la asociación de la ingesta de NAs en la dieta con la concentración excretada de NOCs obtenida de manera analítica se ha demostrado que el método empleado para registrar y cuantificar los xenobióticos de la dieta tiene validez experimental. En la muestra de estudio se han identificado valores de ingesta medios de etanol, HAPs totales, PhIP y NDMA que podrían aumentar el riesgo de desarrollar alteraciones en la mucosa intestinal, realzando el interés de diseñar estrategias nutricionales enfocadas en disminuir la ingesta de xenobióticos.

Segunda. Los niveles de mutagenicidad fecal se correlacionaron de forma directa con la ingesta de AHs. Además, estos niveles fueron más altos en muestras de individuos diagnosticados con pólipos intestinales que presentaban FCA, los cuales también mostraban mayores valores de la ratio TNF-α/IL-10 sérica, indicando un estado proinflamatorio. La presencia de pólipos intestinales y el consumo de carnes rojas se asoció a menores niveles séricos de adipocinas con efecto antiinflamatorio como la adiponectina, mientras que los niveles de IL-1β, relacionados con procesos inflamatorios, aumentaron con la ingesta de etanol. Estos resultados evidencian la asociación entre la dieta, el perfil inmunometabólico y la presencia de alteraciones en la mucosa intestinal.

Tercera. La secuenciación del gen del ARNr 16S permitió caracterizar el perfil microbiano en las heces de los voluntarios del estudio y detectar diferencias en la composición microbiana fecal en función de la presencia de daño intestinal y de las vías biológicas de desarrollo de la carcinogénesis colorrectal. Los pólipos hiperplásicos, dentro de la vía serrada de la carcinogénesis, mostraron menos alteraciones de la MI que los adenomas convencionales propios de la vía adenomatosa. Se observó una disminución en la abundancia relativa de familias involucradas en procesos fermentativos como *Methanobacteriaceae*, *Christensenellaceae, Oscillospiraceae* o *Prevotellaceae* en la vía adenomatosa. En ambas vías biológicas se detectó una mayor abundancia relativa del grupo *Ruminococcus_torques*, lo que refuerza la asociación de este grupo microbiano con alteraciones tempranas de la mucosa intestinal.

Cuarta. El análisis del perfil enzimático fecal permitió detectar un mayor porcentaje de muestras con actividad α-glucosidasa alta de acuerdo con la progresión en el daño de la mucosa intestinal, sugiriendo una posible relación de esta actividad enzimática con la alteración del ambiente intestinal. La abundancia relativa de los grupos microbianos *Christensellaceae_R-7* y Oscillospiraceae_UCG-002 fue menor en muestras de voluntarios que mostraban alta actividad α-glucosidasa, lo que coincidía con la menor abundancia de estos mismos grupos microbianos en

las muestras de individuos con adenomas convencionales respecto al grupo control. Estos resultados sugieren una relación entre los niveles intestinales de actividad α-glucosidasa, cambios en grupos específicos de la MI y la presencia de lesiones de la mucosa intestinal.

Quinta. El estudio de la composición de la microbiota fecal en función de los umbrales de ingesta de factores dietéticos asociados a un mayor riesgo en el desarrollo de CCR y del estado de la mucosa intestinal permitió asociar la ingesta de nitritos y NDMA con una disminución en la abundancia relativa de la familia *Bifidobacteriaceae*, mientras que la ingesta de otros xenobióticos como las AHs PhIP y MeIQx se relacionó con una mayor abundancia de *Coriobacteriaceae* y menor de *Akkermansiaceae* y *Bacteroidaceae*, sugiriendo una posible influencia de dichos xenobióticos sobre grupos específicos de la MI

Sexta. Se ha observado que la excreción de N-nitrosocompuestos de origen endógeno aumenta con el grado de daño de la mucosa intestinal, se relaciona directamente con los niveles de mutagenicidad fecal y está influida por la dieta, evidenciando que la disminución en el consumo de carnes procesadas podría ser una diana de intervención para disminuir la exposición a estos compuestos en el ambiente intestinal.

Séptima. La asociación de la ingesta de compuestos xenobióticos con cambios en la abundancia fecal relativa de ciertos grupos microbianos, con parámetros del ambiente intestinal como la mutagenicidad o la actividad enzimática fecal y con cambios en los niveles en suero de diversos parámetros inmunometabólicos ha permitido ampliar el conocimiento del papel de estos compuestos en etapas tempranas de daño en la mucosa intestinal en el contexto del CCR. Los resultados obtenidos han reforzado el concepto de la MI como un posible mediador de estas asociaciones y han permitido definir umbrales de ingesta con potencial interés de cara a la prevención del CCR. Corroborar los datos obtenidos a través de estudios *in vitro* e *in vivo* o mediante el análisis del metaboloma fecal podría contribuir a profundizar en estos hallazgos.

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ANEXOS

ANEXO I. Reclutamiento de voluntarios en el estudio

Código de voluntario	Diagnóstico clínico	Análisis histopatológico	Dieta	Mutagenicidad fecal	Suero sanguíneo	Microbiota fecal	Actividad enzimática fecal	Análisis NOCs fecales
1	Sí	Sí	Sí	No	Sí	No	No	No
2	Sí	Sí	Sí	No	Sí	No	No	No
3	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
4	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
5	Sí	Sí	Sí	No	Si*	Sí	Sí	No
6	Sí	No	Sí	No	No	Sí	Sí	No
7	Sí	No	Sí	Sí	No	Sí	Sí	Sí
8	Sí	Sí	No	Sí	No	Sí	Sí	Sí
9	Sí	Sí	Sí	No	Sí	No	No	No
10	Sí	Sí	No	Sí	No	Sí	Sí	No
11	Sí	No	Sí	Sí	No	Sí	Sí	No
12	Sí	No	Sí	No	No	Sí	Sí	No
13	Sí	Sí	Sí	No	No	No	No	No
14	Sí	No	Sí	No	No	No	No	No
15	Sí	No	Sí	Sí	No	Sí	Sí	No
16	Sí	No	Sí	Sí	No	Sí	Sí	Sí
17	Sí	No	Sí	Sí	No	Sí	Sí	No
18	Sí	Sí	Sí	No	Sí	Sí	Sí	No
19	Sí	Sí	Sí	No	Sí	No	No	No
20	Sí	Sí	Sí	No	Sí	No	No	No
21	Sí	Sí	Sí	No	Sí	No	No	No
22	Sí	Sí	Sí	No	Sí	No	No	No
23	Sí	Sí	Sí	No	Sí	No	No	No

*= Existencia de al menos un valor perdido en alguna variable

Código de voluntario	Diagnóstico clínico	Análisis histopatológico	Dieta	Mutagenicidad fecal	Suero sanguíneo	Microbiota fecal	Actividad enzimática fecal	Análisis NOCs fecales
24	Sí	Sí	Sí	Sí	Sí	Sí	Sí	No
25	Sí	Sí	Sí	No	Sí*	No	No	No
26	Sí	Sí	Sí	No	Sí	No	No	No
27	Sí	Sí	Sí	No	Sí*	Sí	Sí	Sí
28	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
29	Sí	Sí	Sí	No	Sí	No	No	No
30	Sí	Sí	Sí	No	Sí*	No	No	No
31	Sí	Sí	Sí	No	Sí*	No	No	No
32	Sí	Sí	Sí	No	Sí	Sí	Sí	No
33	Sí	Sí	Sí	No	Sí	Sí	Sí	Sí
34	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
35	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
36	Sí	Sí	Sí	No	Sí	Sí	Sí	No
37	Sí	Sí	Sí	No	Sí	No	No	No
38	Sí	Sí	Sí	No	Sí	No	No	No
39	Sí	Sí	Sí	No	Sí*	No	No	No
40	Sí	Sí	Sí	No	Sí*	No	No	No
41	Sí	Sí	Sí	No	Sí*	No	No	No
42	Sí	Sí	Sí	No	Sí	No	No	No
43	Sí	Sí	Sí	Sí	Sí	Sí	Sí	No
44	Sí	Sí	Sí	No	Sí	No	No	No
45	Sí	Sí	Sí	No	Sí	No	No	No
46	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí

Código de voluntario	Diagnóstico clínico	Análisis histopatológico	Dieta	Mutagenicidad fecal	Suero sanguíneo	Microbiota fecal	Actividad enzimática fecal	Análisis NOCs fecales
47	Sí	Sí	Sí	No	Sí	No	No	No
48	Sí	Sí	Sí	No	Sí	Sí	Sí	Sí
49	Sí	Sí	Sí	No	Sí	Sí	No	No
50	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
51	Sí	Sí	Sí	No	Sí	No	No	No
52	Sí	Sí	Sí	Sí	Sí*	Sí	Sí	Sí
53	Sí	Sí	Sí	No	Sí	No	No	No
54	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
55	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
56	Sí	Sí	Sí	Sí	Sí	Sí	Sí	No
57	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
58	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
59	Sí	Sí	Sí	No	Sí	Sí	Sí	No
60	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
61	Sí	No	Sí	Sí	No	Sí	Sí	Sí
62	Sí	Sí	No	Sí	No	Sí	Sí	Sí
63	Sí	Sí	No	Sí	No	Sí	Sí	Sí
64	Sí	Sí	No	Sí	No	Sí	Sí	Sí
65	Sí	Sí	Sí	No	No	Sí	Sí	No
66	Sí	Sí	No	No	No	Sí	Sí	Sí
67	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
68	Sí	Sí	Sí	No	Sí	No	No	No
69	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí

Código de voluntario	Diagnóstico clínico	Análisis histopatológico	Dieta	Mutagenicidad fecal	Suero sanguíneo	Microbiota fecal	Actividad enzimática fecal	Análisis NOCs fecales
70	Sí	Sí	No	Sí	No	Sí	Sí	No
71	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
72	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
73	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
74	Sí	No	No	Sí	No	Sí	Sí	No
75	Sí	Sí	Sí	No	No	Sí	Sí	Sí
76	Sí	Sí	No	Sí	No	Sí	Sí	Sí
77	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
78	Sí	Sí	Sí	No	Sí	No	No	No
79	Sí	No	Sí	No	No	Sí	Sí	No
80	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
81	Sí	Sí	Sí	Sí	Sí*	Sí	Sí	Sí
82	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
83	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
84	Sí	No	Sí	Sí	No	Sí	Sí	Sí
85	Sí	Sí	No	No	No	Sí	Sí	Sí
86	Sí	Sí	Sí	No	No	Sí	Sí	Sí
87	Sí	Sí	No	Sí	No	Sí	Sí	Sí
88	Sí	Sí	No	Sí	No	Sí	Sí	Sí
89	Sí	Sí	Sí	No	Sí	No	No	No
90	Sí	Sí	Sí	Sí	Sí	Sí	Sí	Sí
91	Sí	Sí	Sí	No	Sí	Sí	Sí	Sí
92	Sí	Sí	No	Sí	No	Sí	Sí	Sí

Código de voluntario	Diagnóstico clínico	Análisis histopatológico	Dieta	Mutagenicidad fecal	Suero sanguíneo	Microbiota fecal	Actividad enzimática fecal	Análisis NOCs fecales
93	Sí	Sí	Sí	No	No	Sí	Sí	No
94	Sí	Sí	No	Sí	No	Sí	Sí	Sí
95	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
96	Sí	Sí	Sí	No	No	Sí	Sí	No
97	Sí	Sí	Sí	No	No	Sí	Sí	No
98	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
99	Sí	No	Sí	Sí	No	Sí	Sí	No
100	Sí	Sí	Sí	No	Sí	No	No	No
101	Sí	No	Sí	Sí	No	Sí	Sí	Sí
102	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
103	Sí	Sí	No	Sí	No	Sí	Sí	Sí
104	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
105	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
106	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
107	Sí	Sí	No	Sí	No	Sí	Sí	Sí
108	Sí	Sí	Sí	Sí	No	Sí	Sí	Sí
109	Sí	Sí	Sí	No	Sí	Sí	Sí	No

Sí	109	95	93	57	52	79	78	55
No	0	14	16	52	47	30	31	54

ANEXO II. Informe sobre la calidad de las publicaciones científicas recogidas en la Tesis

La información sobre la calidad de los artículos que componen esta memoria de Tesis Doctoral ha sido recogida de la "*Web of Science*s" (www.recursoscientificos.fecyt.es). Se han recopilado los siguientes parámetros para cada artículo: el **área SCI** a la que se encuentra asociada la revista; el **factor de impacto** de la revista correspondiente al año de publicación del artículo o en caso de los artículos más recientes, a los últimos datos publicados por "*Journal Impact Factor*" (año 2022); el **cuartil (Q)** de la revista dentro de cada área y las citas, número de veces que ha sido citado cada artículo obtenidas a través de "*Scopus*" hasta el momento de la escritura de la Tesis.

- **Artículo 1.** Ruiz-Saavedra, S.; Zapico, A.; del Rey, C.G.; Gonzalez, C.; Suárez, A.; Díaz, Y.; de los Reyes-Gavilán, C.G.; González, S. Dietary xenobiotics derived from food processing: Association with fecal mutagenicity and gut mucosal damage. *Nutrients* **2022**, *14*, 3482. doi: 10.3390/nu14173482

Área SCI	Factor de impacto	Q	Citas
"Nutrition & Dietetics"	5.9	Q1 (17/88)	6

- **Artículo 2.** González, C.; Ruiz Saavedra, S.; Gómez-Martín, M.; Zapico, A.; López-Suarez, P.; Suárez, A.; Suarez González, A.; del Rey, C.G.; Díaz, E.; Alonso, A.; et al. Immunometabolic profile associated with progressive damage of the intestinal mucosa in adults screened for colorectal cancer: Association with diet. *International Journal of Molecular Sciences* **2023**, *24*, 16451. doi:10.3390/ijms242216451

Área SCI	Factor de impacto	Q	Citas
"Biochemistry & Molecular Biology	5.6	Q1 (66/285)	0

- **Artículo 3.** Ruiz-Saavedra, S.; Arboleya, S.; Nogacka, A.M.; González del Rey, C.; Suárez, A.; Diaz, Y.; Gueimonde, M.; Salazar, N.; González, S.; de los Reyes-Gavilán, C.G. Commensal fecal microbiota profiles associated with initial stages of intestinal mucosa damage: A pilot study. *Cancers* **2024**, *16*, 104. doi: 10.3390/cancers16010104

Área SCI	Factor de impacto	Q	Citas
"Oncology"	5.2	Q2 (72/241)	0

- **Artículo 4.** Ruiz-Saavedra, S.; Salazar, N.; Suárez, A.; Diaz, Y.; del Rey, C. G.; González, S.; de los Reyes-Gavilán, C. G. Human fecal alpha-glucosidase activity and its relationship with gut microbiota profiles and early stages of intestinal mucosa damage. *Anaerobe* **2024**, *87*, 102853. doi: 10.1016/j.anaerobe.2024.102853

Área SCI	Factor de impacto	Q	Citas
"Microbiology"	2.3	Q4 (112/135)	0

- **Artículo 5.** Ruiz-Saavedra, S.; González del Rey, C.; Suárez, A.; Diaz, Y.; Zapico, A.; Arboleya, S.; Salazar, N.; Gueimonde, M.; González, S.; de los Reyes-Gavilán, C.G. Associations of dietary factors and xenobiotic intake with faecal microbiota composition according to the presence of intestinal mucosa damage. *Food & Function* **2023**, *14*, 9591-9605. doi: 10.1039/d3fo01356a

Área SCI	Factor de impacto	Q	Citas
"Biochemistry & Molecular Biology	6.1	Q1 (59/285)	1