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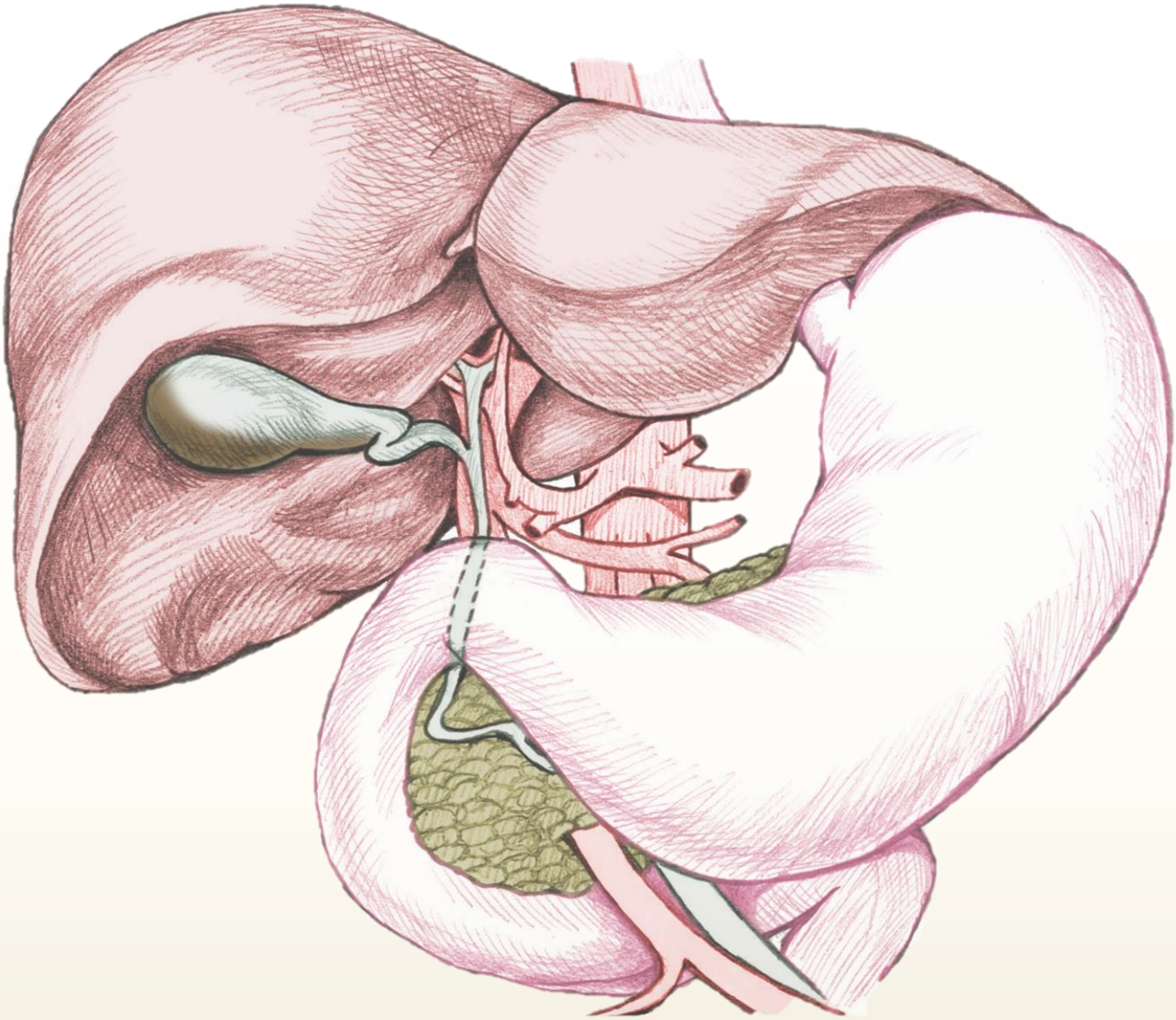
PROGRAMA DE INGENIERÍA QUÍMICA, AMBIENTAL Y BIOALIMENTARIA

Ecología y funcionalidad de la microbiota biliar humana. Relación con la dieta y patología biliar.



Natalia Molinero García

Tesis Doctoral
Oviedo, 2020

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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Español/Otro Idioma: ECOLOGÍA Y FUNCIONALIDAD DE LA MICROBIOTA BILIAR HUMANA.RELACIÓN CON LA DIETA Y PATOLOGÍA BILIAR	Inglés: ECOLOGY AND FUNCTIONALITY OF THE HUMAN BILIARY MICROBIOTA. RELATIONSHIP WITH DIET AND BILIARY PATHOLOGY

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RESUMEN (en español)

La microbiota del sistema gastrointestinal humano y su relación con diferentes estados fisiológicos ha sido objeto de numerosos estudios durante las últimas décadas. Sin embargo, la microbiota de la bilis y de la vesícula biliar apenas ha sido estudiada, debido fundamentalmente a la falta de protocolos adecuados y a la dificultad de tomar muestras de este ambiente. En la actualidad existen algunos trabajos que se han enfocado en analizar la microbiota biliar de animales o de pacientes con alguna patología biliar, sin embargo, hasta el inicio de esta Tesis Doctoral, no se ha realizado ningún estudio del microbioma biliar mediante el empleo de técnicas multi-ómicas en pacientes sin patología hepatobiliar.

Teniendo en cuenta lo anterior, el objetivo general de esta Tesis Doctoral ha sido caracterizar y estudiar en detalle el microbioma de la bilis humana, así como analizar su posible relación con la dieta y con la presencia de cálculos biliares o colelitiasis (la patología biliar más frecuente). Para ello, se optimizo un protocolo de extracción de ADN de bilis, y posteriormente, a través de metagenómica filogenética, se analizaron los perfiles microbianos biliares de un grupo de 13 sujetos donantes de hígado sin patologías hepatobiliares, considerado grupo control, y de un grupo de 14 pacientes con colelitiasis. Los resultados mostraron por primera vez la presencia de un ecosistema microbiano biliar, fundamentalmente compuesto por miembros de los filos Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria. La comparación de los perfiles microbianos característicos de ambos grupos mostró diferencias significativas en la abundancia relativa de algunos taxones, destacando miembros de los géneros *Bacteroides* y *Escherichia-Shigella*, que presentaron mayor abundancia relativa en la bilis de los pacientes con colelitiasis. El análisis mediante metagenómica funcional de la microbiota biliar y su comparación con el perfil funcional de la microbiota intestinal reveló diferencias significativas en la abundancia relativa de actividades relacionadas con el metabolismo del colesterol y sales biliares, presentando estas actividades una mayor frecuencia en el microbioma biliar. Finalmente, el análisis metabonómico por resonancia magnética nuclear de la bilis del grupo control y el grupo con colelitiasis mostró perfiles metabólicos distintos.

Por otro lado, la comparación de la ingesta de distintos componentes de la dieta y de los niveles de los principales parámetros sanguíneos entre el grupo de pacientes con colelitiasis y un grupo de 13 individuos sanos mostró diferencias significativas, destacando un menor consumo de vegetales y mayores niveles de triglicéridos en sangre en los pacientes con colelitiasis. El análisis de la posible relación entre los componentes de la dieta y la microbiota biliar en el grupo con colelitiasis reveló la presencia de asociaciones estadísticas entre ciertos micro- y macronutrientes y algunos taxones, destacando una asociación negativa entre el consumo de fibra insoluble y la abundancia relativa de miembros del género *Bacteroides*.

El cultivo de muestras de bilis humana permitió aislar dos cepas del orden *Clostridiales*: IPLA60001, perteneciente a la especie *Ruminococcus gauvreauii*; e IPLA60002, que mostró una baja identidad con las secuencias del gen ARNr16S disponibles en las bases de datos, siendo la especie más cercana *Ruminococcus bromii*. IPLA60002 mostró características particulares, entre las que destaca una mayor resistencia a sales biliares y un fenotipo de autolisis. Los resultados del análisis genómico y filogenético permitieron proponer esta cepa como perteneciente a una nueva especie, denominada *Ruminocoides biliarensis*. Finalmente, el análisis bioquímico, genómico y transcriptómico de las cepas IPLA60001 e IPLA60002 en

mono y co-cultivo, llevado a cabo con el fin de estudiar la posible relación metabólica entre las mismas, mostró una relación de alimentación cruzada a nivel del formato y otros nutrientes, revelando una posible cooperación o relación de simbiosis que les podría permitir subsistir en el complejo ecosistema biliar.

RESUMEN (en Inglés)

The human gastrointestinal microbiota and its relationship with different physiological states has been the subject of numerous studies during the last decades. However, bile and gallbladder microbiota has scarcely been studied, mainly due to the lack of adequate protocols and the difficulty to take samples from this environment. Nowadays, few works were focused on analyzing the biliary microbiota of animals or patients with some biliary pathology. However, at the beginning of this Doctoral Thesis, no study of the biliary microbiome has been performed through the use of multi-omic techniques in patients without hepatobiliary pathologies.

Accordingly, the general objective of this Doctoral Thesis was to study and characterize in detail the human bile microbiome, as well as its possible relationship with the diet and with the presence of gallstones or cholelithiasis (most prevalent biliary pathology). First of all, a bile DNA extraction protocol was optimized. Subsequently, through phylogenetic metagenomics, the biliary microbial profiles of a group of 13 liver donors without hepatobiliary pathologies (control group) and of a group of 14 patients with cholelithiasis were analyzed. The results showed the presence of a biliary microbial ecosystem, mainly composed by members of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla. Comparison of the microbial profiles of both groups showed significant differences in the relative abundance of some taxa, including members of *Bacteroides* and *Escherichia-Shigella* genera, who showed higher relative abundance in the bile of cholelithiasis group. Analysis by functional metagenomics of the biliary microbiota and its comparison with the functional profile of the intestinal microbiota showed significant differences in the relative abundance of some activities related to the metabolism of cholesterol and bile salts, presenting these activities a higher frequency in the biliary microbiome. Finally, metabonomic analysis of the bile of the control group and the cholelithiasis group by nuclear magnetic resonance showed different metabolic profiles.

On the other hand, comparison of different component's intake through diet and levels of the main blood parameters between the group of patients with cholelithiasis and a group of 13 healthy individuals showed significant differences. Remarkably, a lower consumption of vegetables and higher blood triglyceride levels were detected in patients with cholelithiasis. Possible relationship analysis between diet components and the biliary microbiota in the cholelithiasis group revealed the presence of statistical associations between some micro- and macronutrients and some taxa, standing out a negative association between insoluble fiber consumption and the relative abundance of members of *Bacteroides* genus.

The culture of human bile samples allowed the isolation of two strains of *Clostridiales* order: IPLA60001, belonging to *Ruminococcus gauvreauii* species; and IPLA60002, which showed a low identity with the available RNAr16S gene sequences in the databases, being the closest species *Ruminococcus bromii*. IPLA60002 showed particular characteristics, among which there is a higher resistance to bile salts and an autolysis phenotype. The results of the genomic and phylogenetic analysis allowed us to propose this strain as a new species, named *Ruminocoides biliarensis*. Finally, in order to study the possible metabolic relationship between IPLA60001 and IPLA60002, biochemical, genomic and transcriptomic analyses of the strains were carried out in mono and co-culture. A cross-feeding relationship involving formate and other nutrients was detected, revealing a possible cooperation or synergistic relationship that could allow them to survive in the biliary ecosystem.

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Lista de abreviaturas / Abbreviations list

ADN: Ácido desoxirribonucleico

ADNc: ADN complementario

AGCC: Ácidos grasos de cadena corta

ARN: Ácido ribonucleico

ARNr 16S: ARN ribosomal 16S

BAAT: Ácido biliar-CoA:amino ácido N-aciltransferasas

BACS: Ácido biliar:CoA sintetasas

BAL: Bacterias del ácido láctico

CCK: Colecistoquinina

CESNID: Centro de Enseñanza Superior de Nutrición Humana y Dietética

CLI: Células linfoides innatas

Da: Dalton

ERCP: "Endoscopic Retrograde Cholangiopancreatography". Colangiopancreatografía retrógrada endoscópica

GABA: Ácido gamma-amino butírico

HDL: Lipoproteína de alta densidad

HMP: "Human Microbiome Project". Proyecto del microbioma humano

IgA: Inmunoglobulina A

Il-1β: Interleucina 1β

LPS: Lipopolisacárido

MATE: "Multi-antimicrobial Extrusión". Transportadores de extrusión de antimicrobianos

MDR: "Multidrug Resistance". Bombas de expulsión de compuestos citotóxicos

MetaHIT: "Metagenomics of the Human Intestinal Tract". Proyecto europeo de metagenómica del tracto intestinal humano

MS: Espectrometría de masas

NF-κβ: Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas

NGS: "Next Generation Sequencing". Tecnologías de secuenciación de nueva generación

OTU: Unidad operativa taxonómica

Pb: Pares de bases

PCR: Reacción en cadena de la polimerasa

qPCR: PCR cuantitativa

RMN: Resonancia magnética nuclear

SEM: Microscopía electrónica de barrido

SMR: “Small Multidrug Resistance”. Transportadores de resistencia a pequeños fármacos

TGI: Tracto gastrointestinal

TLRs: Receptores Toll-like

TNF-α: Factor de necrosis tumoral α

UCI: Unidad de cuidados intensivos

VIH: Virus Inmunodeficiencia Humana

VLDL: Lipoproteína de baja densidad

Resumen

Summary

RESUMEN

La microbiota del sistema gastrointestinal humano y su relación con diferentes estados fisiológicos ha sido objeto de numerosos estudios durante las últimas décadas. Sin embargo, la microbiota de la bilis y de la vesícula biliar apenas ha sido estudiada, debido fundamentalmente a la falta de protocolos adecuados y a la dificultad de tomar muestras de este ambiente. En la actualidad existen algunos trabajos que se han enfocado en analizar la microbiota biliar de animales o de pacientes con alguna patología biliar, sin embargo, hasta el inicio de esta Tesis Doctoral, no se ha realizado ningún estudio del microbioma biliar mediante el empleo de técnicas multi-ómicas en pacientes sin patología hepatobiliar.

Teniendo en cuenta lo anterior, el objetivo general de esta Tesis Doctoral ha sido caracterizar y estudiar en detalle el microbioma de la bilis humana, así como analizar su posible relación con la dieta y con la presencia de cálculos biliares o colelitiasis (la patología biliar más frecuente). Para ello, se optimizo un protocolo de extracción de ADN de bilis, y posteriormente, a través de metagenómica filogenética, se analizaron los perfiles microbianos biliares de un grupo de 13 sujetos donantes de hígado sin patologías hepatobiliares, considerado grupo control, y de un grupo de 14 pacientes con colelitiasis. Los resultados mostraron por primera vez la presencia de un ecosistema microbiano biliar, fundamentalmente compuesto por miembros de los filos Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria. La comparación de los perfiles microbianos característicos de ambos grupos mostró diferencias significativas en la abundancia relativa de algunos taxones, destacando miembros de los géneros

Bacteroides y *Escherichia-Shigella*, que presentaron mayor abundancia relativa en la bilis de los pacientes con colelitiasis. El análisis mediante metagenómica funcional de la microbiota biliar y su comparación con el perfil funcional de la microbiota intestinal reveló diferencias significativas en la abundancia relativa de actividades relacionadas con el metabolismo del colesterol y sales biliares, presentando estas actividades una mayor frecuencia en el microbioma biliar. Finalmente, el análisis metabonómico por resonancia magnética nuclear de la bilis del grupo control y el grupo con colelitiasis mostró perfiles metabólicos distintos.

Por otro lado, la comparación de la ingesta de distintos componentes de la dieta y de los niveles de los principales parámetros sanguíneos entre el grupo de pacientes con colelitiasis y un grupo de 13 individuos sanos mostró diferencias significativas, destacando un menor consumo de vegetales y mayores niveles de triglicéridos en sangre en los pacientes con colelitiasis. El análisis de la posible relación entre los componentes de la dieta y la microbiota biliar en el grupo con colelitiasis reveló la presencia de asociaciones estadísticas entre ciertos micro- y macronutrientes y algunos taxones, destacando una asociación negativa entre el consumo de fibra insoluble y la abundancia relativa de miembros del género *Bacteroides*.

El cultivo de muestras de bilis humana permitió aislar dos cepas del orden *Clostridiales*: IPLA60001, perteneciente a la especie *Ruminococcus gauvreauii*; e IPLA60002, que mostró una baja identidad con las secuencias del gen ARNr16S disponibles en las bases de datos, siendo la especie más cercana *Ruminococcus bromii*. IPLA60002 mostró características particulares, entre las que destaca una mayor resistencia a sales biliares y un fenotipo de autolisis. Los resultados del análisis genómico y filogenético permitieron proponer esta cepa como perteneciente a una nueva especie,

denominada *Ruminocoides biliarensis*. Finalmente, el análisis bioquímico, genómico y transcriptómico de las cepas IPLA60001 e IPLA60002 en mono y co-cultivo, llevado a cabo con el fin de estudiar la posible relación metabólica entre las mismas, mostró una relación de alimentación cruzada a nivel del formato y otros nutrientes, revelando una posible cooperación o relación de simbiosis que les podría permitir subsistir en el complejo ecosistema biliar.

SUMMARY

The human gastrointestinal microbiota and its relationship with different physiological states has been the subject of numerous studies during the last decades. However, bile and gallbladder microbiota has scarcely been studied, mainly due to the lack of adequate protocols and the difficulty to take samples from this environment. Nowadays, few works were focused on analyzing the biliary microbiota of animals or patients with some biliary pathology. However, at the beginning of this Doctoral Thesis, no study of the biliary microbiome has been performed through the use of multi-omic techniques in patients without hepatobiliary pathologies.

Accordingly, the general objective of this Doctoral Thesis was to study and characterize in detail the human bile microbiome, as well as its possible relationship with the diet and with the presence of gallstones or cholelithiasis (most prevalent biliary pathology). First of all, a bile DNA extraction protocol was optimized. Subsequently, through phylogenetic metagenomics, the biliary microbial profiles of a group of 13 liver donors without hepatobiliary pathologies (control group) and of a group of 14 patients with cholelithiasis were analyzed. The results showed the presence of a biliary microbial ecosystem, mainly composed by members of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla. Comparison of the microbial profiles of both groups showed significant differences in the relative abundance of some taxa, including members of *Bacteroides* and *Escherichia-Shigella* genera, who showed higher relative abundance in the bile of cholelithiasis group. Analysis by functional metagenomics of the biliary microbiota and its comparison with the functional profile of the intestinal microbiota

showed significant differences in the relative abundance of some activities related to the metabolism of cholesterol and bile salts, presenting these activities a higher frequency in the biliary microbiome. Finally, metabonomic analysis of the bile of the control group and the cholelithiasis group by nuclear magnetic resonance showed different metabolic profiles.

On the other hand, comparison of different component's intake through diet and levels of the main blood parameters between the group of patients with cholelithiasis and a group of 13 healthy individuals showed significant differences. Remarkably, a lower consumption of vegetables and higher blood triglyceride levels were detected in patients with cholelithiasis. Possible relationship analysis between diet components and the biliary microbiota in the cholelithiasis group revealed the presence of statistical associations between some micro- and macronutrients and some taxa, standing out a negative association between insoluble fiber consumption and the relative abundance of members of *Bacteroides* genus.

The culture of human bile samples allowed the isolation of two strains of *Clostridiales* order: IPLA60001, belonging to *Ruminococcus gauvreauii* species; and IPLA60002, which showed a low identity with the available RNAr16S gene sequences in the databases, being the closest species *Ruminococcus bromii*. IPLA60002 showed particular characteristics, among which there is a higher resistance to bile salts and an autolysis phenotype. The results of the genomic and phylogenetic analysis allowed us to propose this strain as a new species, named *Ruminocoides biliarensis*. Finally, in order to study the possible metabolic relationship between IPLA60001 and IPLA60002, biochemical, genomic and transcriptomic analyses of the strains were carried out in mono and co-culture. A cross-feeding relationship involving formate and other nutrients

was detected, revealing a possible cooperation or synergistic relationship that could allow them to survive in the biliary ecosystem.

Introducción

Introduction

INTRODUCCIÓN

1.- El microbioma humano

1.1.- Aspectos generales

El cuerpo humano está formado por cerca de 30 billones de células, cohabitando con bacterias y arqueas, virus, protozoos y hongos principalmente, que han coevolucionado con nuestra especie (Sender *et al.*, 2016). Estas comunidades microbianas diversas que residen en distintos nichos ecológicos de nuestro cuerpo, son definidas en su conjunto como microbiota (Lederberg and McCray, 2001). La microbiota, incluyendo sus genes y genomas, y las condiciones del ambiente en el que se encuentra son lo que denominamos el microbioma (Marchesi and Ravel, 2015). Las alteraciones en el equilibrio y las proporciones relativas de los miembros de estas comunidades microbianas se denomina disbiosis.

Se conoce la existencia de una microbiota humana prácticamente desde los inicios de la microbiología. Los primeros estudios llevados a cabo por Leewenhoek en 1681 describieron la presencia de "pequeños animales" vivos presentes en muestras de heces, a los que denominó animáculos (Parker, 1965). Más tarde, Escherich en 1885 describió la presencia de la bacteria *Bacterium coli commune*, denominada posteriormente *Escherichia coli,* en la microbiota o flora (término usado por aquel entonces) intestinal de niños sanos y niños afectados con diarrea (Dunne *et al.*, 2017). En las siguientes décadas, numerosos trabajos se enfocaron en aislar y describir los microorganismos más representativos del tracto gastrointestinal (TGI) humano. A día de hoy se sabe que las técnicas de cultivo empleadas en aquella época permitían el aislamiento de un pequeño porcentaje de los microorganismos presentes (Finegold,

1969), debido a los estrictos requerimientos nutricionales y de anaerobiosis estricta que necesitan la mayor parte de los microorganismos intestinales.

El conocimiento acerca de la microbiota humana comenzó a crecer exponencialmente cuando surgieron las técnicas moleculares y posteriormente las tecnologías de secuenciación de nueva generación ("Next Generation Sequencing", NGS). Estas permitieron profundizar en el estudio de la microbiota presente en los distintos nichos del cuerpo humano, estudiar su diversidad, y comparar las comunidades microbianas presentes en distintas localizaciones, y entre distintos individuos y grupos poblacionales. El gran avance en el conocimiento que tenemos sobre el microbioma humano se produce a partir del año 2008, en el que aparecieron los proyectos MetaHIT ("Metagenomics of the Human Intestinal Tract", proyecto europeo de Metagenómica del Tracto Intestinal Humano), cuyo objetivo era secuenciar los genomas microbianos de muestras fecales de individuos enfermedad inflamatoria intestinal y obesidad e individuos sanos; y el proyecto HMP ("Human Microbiome Project", Proyecto del Microbioma Humano), cuya finalidad iba dirigida al estudio de las comunidades microbianas presentes en el cuerpo humano, así como analizar su función y su relación con la salud y enfermedad (Hiergeist *et al.*, 2015).

1.2.- Microbiota intestinal

El estudio del microbioma humano se ha centrado sobre todo en conocer en profundidad las comunidades microbianas presentes en el TGI y sus funciones, debido fundamentalmente al fácil acceso a muestras de heces y a la necesidad de comprender el papel de la microbiota intestinal en la salud humana. El TGI está poblado por comunidades microbianas organizadas y altamente especializadas, entre las que

encontramos bacterias, arqueas, hongos, protozoos y virus (Eckburg *et al.*, 2005). El microbioma intestinal, es un ecosistema complejo formado por varios cientos de especies distintas, que presentan una gran diversidad dentro del individuo y entre individuos. La mayoría de los miembros de la microbiota intestinal son bacterias, que pertenecen fundamentalmente a cuatro filos: Bacteroidetes, Firmicutes, Actinobacteria y Proteobacteria (Kim *et al.*, 2017), aunque de forma minoritaria también se encuentran miembros de los filos Synergistetes, Fusobacteria y Verrucomicrobia (Eckburg *et al.*, 2005). Los hongos y arqueas suponen una pequeña fracción de la microbiota intestinal; mientras que los virus, cuyo conjunto conforma el viroma intestinal, parecen estar presentes en niveles similares a los de sus huéspedes bacterianos (Ogilvie and Jones, 2015).

La microbiota intestinal de un individuo adulto sano está formada mayoritariamente por especies pertenecientes a los filos Firmicutes y Bacteroidetes. Dentro del este último, *Prevotella* y *Bacteroides* son los géneros más comunes, caracterizados por su capacidad de digerir polisacáridos complejos, produciendo la liberación de AGCC como acetato, propionato y butirato, relacionados con la regulación del crecimiento de las células del epitelio intestinal, y la diferenciación y estimulación del sistema inmune (Kim *et al.*, 2017). El filo Firmicutes se compone fundamentalmente por miembros de las clases *Bacilli* y *Clostridia*. La primera de ellas alberga miembros de los géneros *Lactobacillus*, *Enterococcus* y *Streptococcus*, todos ellas pertenecientes al grupo de bacterias del ácido láctico (BAL). La clase *Clostridia* se divide en 19 grupos o clusters, de los que Clostridium I, IV, XI, XIVa y XVI son los más frecuentes (Ohashi and Fujisawa, 2019). La mayoría de miembros del filo Firmicutes que residen en el TGI pertenecen a los clusters *Clostridium* XIVa y IV, albergando especies de las familias

Lachnospiraceae y *Ruminococcaceae*, entre otros. Representantes de estas familias han sido relacionadas con varias funciones beneficiosas, como la producción de butirato o la inducción de células T reguladoras (Kim *et al.*, 2017). Sin embargo, miembros del cluster I, como *Clostridium perfringens*; y del XI, al que pertenece *Clostridioides difficile*, provocan patologías de distinta gravedad (Hull and Beck, 2004).

Las condiciones en el TGI de un individuo no son constantes, de forma que las comunidades microbianas varían de acuerdo con las regiones anatómicas. Estas regiones varían en términos de fisiología, pH y tensión de oxígeno, flujo del contenido alimentario (rápido de la boca al ciego, más lento después), disponibilidad de sustratos y secreciones del hospedador (Flint *et al.*, 2012). El intestino delgado proporciona un entorno hostil debido a las altas concentraciones de sales biliares, mientras que el intestino grueso alberga la mayor comunidad microbiana en términos numéricos y de diversidad. En la Fig. 1 se representan los grupos bacterianos más abundantes en el TGI, así como la concentración microbiana aproximada en cada zona por gramo de contenido intestinal (Konturek *et al.*, 2015).

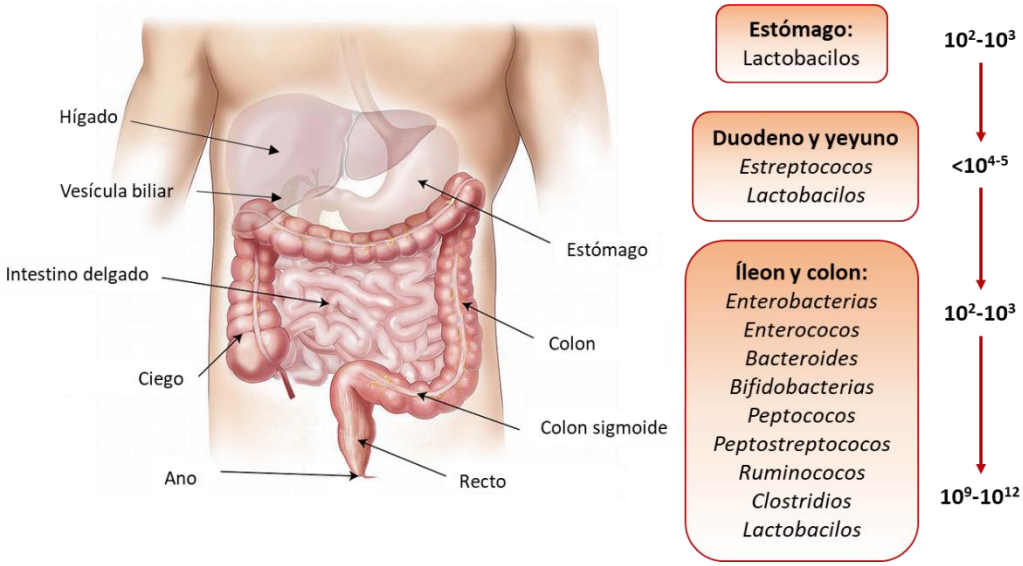

Fig. 1. Composición de la microbiota a lo largo del TGI. Se indican los grupos bacterianos más abundantes en cada zona y la concentración microbiana por gramo de contenido. Adaptado de Konturek et al., 2015.

1.3.- La microbiota intestinal y relación con la salud y la dieta

La microbiota se instala desde los primeros momentos de vida, y tras el nacimiento se desarrolla un ecosistema rico y dinámico que sufre cambios importantes durante la vida del hospedador. Estos cambios surgen en respuesta a una variedad de factores que incluyen entre otros la edad (Yatsunenko *et al.*, 2012), la dieta (Flint *et al.*, 2017), y los factores del medio ambiente. La microbiota intestinal y el hospedador mantienen una relación estrecha, de interacción continua, de forma que pequeñas variaciones pueden afectar considerablemente al equilibrio que mantienen. Las comunidades microbianas presentes en el TGI presentan una serie de funciones:

(i) Tienen un papel crucial en el mantenimiento y regulación de la homeostasis y la integridad de la barrera intestinal.

(ii) Ejercen un efecto protector a través de la exclusión competitiva y la producción de moléculas antimicrobianas.

(iii) Desempeñan un papel fundamental en el desarrollo y maduración del sistema inmunitario, desde el nacimiento, y participa en su regulación y mantenimiento en la etapa adulta. De forma indirecta actúan a través de la estimulación de células mieloides, células linfoides innatas (CLI), y mediante la inducción de respuesta adaptativa de linfocitos T y B (Ubeda *et al.*, 2017); y de forma directa actúa a través de la inducción de la producción de Inmunoglobulina A (IgA) (Macpherson *et al.*, 2018).

(iv) Participan en el desarrollo neuronal y en la estimulación de angiogénesis intestinal (Eckburg *et al.*, 2005; Barko *et al.*, 2018).

(v) Poseen actividades enzimáticas y metabólicas que no están presentes en el hospedador, siendo capaces de degradar sustratos y producir metabolitos fundamentales para el estado de salud. Entre estos productos de fermentación destacan

los AGCC acetato, lactato, propionato y butirato (Rowland *et al.*, 2018). El butirato es la principal fuente de energía de los colonocitos, y tiene efecto antiinflamatorio y epigenético (Valdes *et al.*, 2018). El acetato es el AGCC más abundante, es un metabolito esencial para el crecimiento de otras bacterias (Duncan *et al.*, 2004), y participa en el metabolismo del colesterol y la lipogénesis (Frost *et al.*, 2014). Por otro lado, la microbiota participa en la degradación de proteínas (Clayton *et al.*, 2009); tiene la capacidad de producir moléculas que actúan como neurotransmisores, como el ácido gamma-amino butírico o GABA (Mayer *et al.*, 2015); y es capaz de producir vitaminas, como la vitamina K y las vitaminas del grupo B (Hill, 1997). Finalmente, la microbiota intestinal es capaz de metabolizar las sales biliares, cuyos productos influyen significativamente en muchos procesos metabólicos y fisiológicos del hospedador (Winston and Theriot, 2019). En este sentido, además, algunos miembros de la microbiota metabolizan el colesterol de forma que éste es menos accesible para ser absorbido en el intestino, hecho que se relaciona con una posible capacidad de disminuir los niveles de colesterol en sangre (Pereira and Gibson, 2002; Zanotti *et al.*, 2015).

Las funciones mencionadas son fundamentales para mantener la homeostasis en el hospedador. Sin embargo, la microbiota intestinal puede verse afectada durante la vida por diversos factores como se mencionó anteriormente. Estos factores pueden alterar la microbiota y desencadenar un desequilibrio o disbiosis, con importantes consecuencias en la salud. Uno de los factores que influyen en la microbiota intestinal es la dieta. Existe una clara evidencia de que alteraciones en la dieta durante periodos largos de tiempo generan cambios en la composición de la microbiota intestinal (Johnson *et al.*, 2019). En este sentido, se ha descrito que una dieta rica en grasas de origen animal se asocia con un aumento de la abundancia relativa de miembros de los

filos Proteobacteria, Actinobacteria y Firmicutes, y una disminución de Bacteroidetes, además de una disminución significativa en la diversidad general de la microbiota intestinal (Turnbaugh *et al.*, 2009; Zhang and Yang, 2016). Esta disbiosis puede generar una desregulación del equilibrio de la respuesta inmune, alteraciones en el balance energético, y promover mecanismos proinflamatorios, induciendo patologías metabólicas como la obesidad (Baothman *et al.*, 2016; Valdes *et al.*, 2018). Por el contrario, se ha demostrado que una dieta rica en carbohidratos de origen vegetal y baja en grasas favorece el crecimiento de algunos microorganismos beneficiosos y reduce la población de enteropatógenos (Gibson, 2008). Esto se traduce en menores niveles de Firmicutes y Enterobacterias y en mayores niveles de Bacteroidetes (Turnbaugh *et al.*, 2009; Sánchez-Tapia *et al.*, 2019), capaces de metabolizar los polisacáridos de origen vegetal, y favoreciendo el crecimiento de bacterias productoras de butirato (Mahowald *et al.*, 2009). Por ejemplo, uno de los componentes asociados a una dieta alta en fibra, el almidón resistente, promueve el crecimiento de *Ruminococcus bromii* y *Eubacterium rectale*. Los productos de degradación del almidón resistente estimulan el crecimiento de otros grupos bacterianos que no están involucrados en su degradación primaria, principalmente productores de butirato, que parece ser el metabolito responsable de los beneficios para la salud del metabolismo del almidón resistente (Vital *et al.*, 2018). Por otro lado, el consumo de grandes cantidades de carbohidratos de origen vegetal se relaciona con niveles elevados *Prevotella* y *Xylanibacter* (De Filippo *et al.*, 2010), que degradan celulosa y xilanos, y están asociados con un aumento de los AGCC fecales.

1.4.- La microbiota de otras localizaciones

Las tecnologías NGS han permitido conocer la microbiota de varias localizaciones del cuerpo humano. En la actualidad, se han estudiado las microbiotas presentes en la cavidad oral, tracto respiratorio superior, piel, y vagina, entre otros; así como su relación con la presencia de algunas patologías. Actualmente sabemos que existen microbiotas en prácticamente todas las cavidades mucosas del cuerpo humano (Smith and Ravel, 2017; Proctor and Relman, 2017; Gilbert *et al.*, 2018). En la última década también se han estudiado lugares de difícil acceso o considerados durante mucho tiempo estériles, como el útero, cuya microbiota varía en función de la localización (cérvix, endometrio y trompas de Falopio) y la fase del ciclo hormonal (Chen *et al.*, 2017); el estómago (Delgado *et al.*, 2013); la leche materna, donde diversos estudios han demostrado la importancia de la microbiota mamaria, en la prevención de mastitis infecciosas y en la colonización del intestino del neonato (Ruiz *et al.*, 2019); o la placenta, donde las técnicas de NGS han permitido revelar que ésta alberga una microbiota propia aún en ausencia de infección histológica intrauterina (Aagaard *et al.*, 2014; Chen and Gur, 2019), aunque existen trabajos que describen lo contrario (de Goffau *et al.*, 2019).

1.4.1.- La microbiota biliar

Las comunidades microbianas presentes en la vesícula biliar y la bilis apenas han sido estudiadas hasta el momento, debido fundamentalmente al difícil acceso a las muestras biológicas. No obstante, en la última década, se han realizado algunos estudios en animales, como el conejo y el cerdo, describiéndose la presencia de cuatro filos mayoritarios: Firmicutes, Proteobacteria, Bacteroidetes y Actinobacteria; y una importante diversidad microbiana que puede variar a lo largo de la vida del animal (Dias

et al., 2014; Jiménez *et al.*, 2014; Xing *et al.*, 2019). En humanos, los estudios realizados hasta el inicio de esta Tesis Doctoral se habían llevado a cabo en sujetos con patologías biliares, principalmente colelitiasis (presencia de cálculos en la vesícula biliar), debido a la evidente dificultad para tomar muestras de bilis de individuos sanos. En estos sujetos, las muestras se toman directamente de la vesícula biliar durante la cirugía para eliminar los cálculos biliares, o mediante colangiopancreatografía retrógrada endoscópica ("Endoscopic Retrograde Cholangiopancreatography", ERCP) en la que se toma la bilis del conducto colédoco. Los filos mayoritarios descritos en la bilis humana son Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, y Actinobacteria; y las familias con mayor abundancia relativa: *Prevotellaceae*, *Streptococcaceae*, *Veillonellaceae*, *Fusobacteriaceae*, *Enterobacteriaceae* y *Pasteurellaceae* (Shen *et al.*, 2015; Ye *et al.*, 2016; Pereira *et al.*, 2017). Por otro lado, se ha detectado la presencia de especies particulares que actúan fundamentalmente como patógenos, como *Salmonella y Listeria*, conocidas por colonizar la vesícula biliar (Crawford *et al.*, 2008; Crawford *et al.*, 2010; Dowd *et al.*, 2011), *Shigella*, *Klebsiella* y *Escherichia coli* (Ye *et al.*, 2016; Di Carlo *et al.*, 2019). La mayoría de estos estudios se han centrado en estudiar la relación entre la presencia de este tipo de microorganismos y la aparición de patologías (Pereira *et al.*, 2017; Wang *et al.*, 2018). Sin embargo, hasta el momento no se había caracterizado la microbiota biliar de individuos sin patología hepatobiliar. Por ello, en esta tesis se ha querido abordar el estudio microbiológico de la bilis humana, para poder determinar los grupos bacterianos presentes en condiciones de salud vs. enfermedad y determinar la influencia de la dieta en la composición de la microbiota biliar.

2.- Métodos de estudio del microbioma humano. Aplicación de las -ómicas

Las primeras técnicas utilizadas para el estudio de la microbiota se basaban fundamentalmente en llevar a cabo recuentos bajo el microscopio y en el uso de técnicas convencionales o clásicas de cultivo. Sin embargo, mediante estas metodologías se estimó que desconocíamos aproximadamente un 80% de las bacterias presentes en el intestino humano (Finegold, 1969), considerándolas incultivables durante mucho tiempo. La aplicación de técnicas basadas en la reacción en cadena de la polimerasa (PCR) supuso una primera revolución en el conocimiento de la microbiota. Entre ellas, la amplificación y secuenciación del gen ARNr 16S (Weisburg *et al.*, 1991) y, posteriormente, las técnicas de PCR cuantitativa (qPCR), permitieron la determinación de los perfiles microbianos y la cuantificación de los microorganismos de interés.

Durante los últimos tiempos, la mejora en las técnicas de cultivo, así como el desarrollo de la tecnología NGS, han llevado el conocimiento sobre el microbioma humano a otro nivel. A continuación, se describen algunos de los métodos más utilizados en el estudio del microbioma humano en la actualidad (Fig. 2).

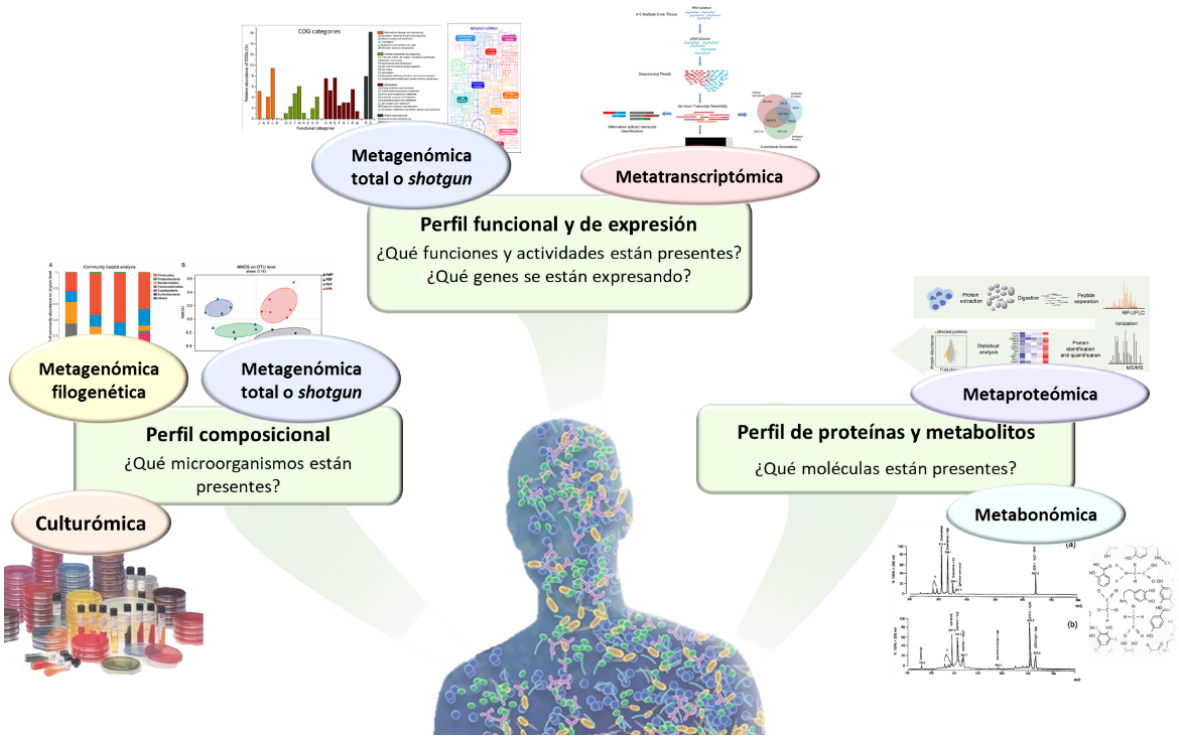

Figura 2.- Algunos métodos de estudio del microbioma humano. Las tecnicas “ómicas” caracterizan y cuantifican grupos completos de moléculas biológicas en una célula u organismo, y analizan sus funciones y relaciones. El sufijo "ómicas" se agrega para describir el uso de estas técnicas para analizar el genoma (genómica), moléculas de ARN (transcriptómica), examinar las proteínas (proteómica) y los metabolitos (metabolómica) presentes en una célula. El prefijo “meta” se utiliza cuando las “ómicas” abordan el estudio de sistemas o ecosistemas complejos, como el caso de las poblaciones de la microbiota.

2.1. Culturómica

En los últimos años han comenzado a desarrollarse nuevos métodos para facilitar el cultivo en el laboratorio de microorganismos utilizando las técnicas convencionales, iniciándose así el renacimiento del cultivo en microbiología (Lagier *et al.*, 2018). Los primeros estudios se basaron en mimetizar las condiciones del entorno natural de los microorganismos (Rappé *et al.*, 2002; Bollmann *et al.*, 2007), permitiendo el cultivo de especies consideradas incultivables hasta (Kaeberlein *et al.*, 2002). Por otro lado, el desarrollo de equipos y técnicas de cultivo específicos para el manejo en el laboratorio

de microorganismos anaerobios estrictos ha permitido aislar e identificar microorganismos intestinales que podrían ser claves para la salud del hospedador (Martín *et al.*, 2017).

El diseño de múltiples condiciones de cultivo, combinado con el uso de técnicas de identificación bacteriana es lo que se conoce como culturómica (Lagier *et al.*, 2018). El empleo de esta aproximación permite solucionar en parte algunos de los problemas asociados al uso de otras tecnologías "ómicas", como las basadas en la detección de ácidos nucleicos (que se abordarán posteriormente) puesto que permite la discriminación entre microorganismos vivos y ADN circulante (Lagier *et al.*, 2018). Además, la culturómica permite el aislamiento e identificación de un gran número de microorganismos que tienen secuencias de ADN no asignadas previamente en las bases de datos (Lagier *et al.*, 2016).

En los últimos años, se han desarrollado las NGS, que han supuesto una verdadera revolución en el estudio del microbioma humano. Entre ellas destaca la metagenómica, metatranscriptómica y metabonómica.

2.2.- Metagenómica

La metagenómica puede definirse como la aplicación de la secuenciación masiva (NGS) y el posterior análisis bioinformático de secuencias de ADN para estudiar una fracción representativa de los genomas presentes en una muestra biológica (Garza and Dutilh, 2015). El término fue descrito por primera vez en 1998 (Handelsman *et al.*, 1998) y la aplicación de este tipo de técnicas supuso una revolución en el estudio de las comunidades microbianas de distintos hábitats. El objetivo es el estudio y caracterización de los genomas presentes en una comunidad, a través de la

secuenciación del ADN extraído de una muestra. Para ello, es necesario un primer paso de extracción del ADN total presente en la muestra a analizar, que puede amplificarse por PCR utilizando cebadores específicos de marcadores filogenéticos, como el gen que codifica el ARNr 16S, y posteriormente secuenciarse los amplicones obtenidos (metagenómica filogenética); o bien puede secuenciarse directamente la totalidad del ADN presente en la muestra (metagenómica total o *shotgun*). Las lecturas obtenidas, mediante distintas herramientas computacionales, se analizan y comparan con la información depositada en las distintas bases de datos.

La parte de la metagenómica centrada en el estudio del gen ARNr 16S se define como *metagenómica filogenética* o *metataxonómica*. El estudio este marcador taxonómico, ha permitido identificar el perfil de microorganismos presentes en una determinada comunidad basándose en la similitud de secuencia con las secuencias depositadas en las bases de datos, y agrupar estos microorganismos en un contexto filogenético. Para ello, mediante programas bioinformáticos como el QIIME o el RDP Classifier (Wang *et al.*, 2007; Caporaso *et al.*, 2010) las lecturas obtenidas tras la secuenciación se analizan de forma que las secuencias con un valor de similitud determinado son asignadas a una unidad taxonómica (unidad operativa taxonómica (OTU), o variantes de secuencia exacta). Gracias a esta asignación, podemos calcular distintos índices ecológicos que nos aportan información sobre la diversidad alpha, es decir, la diversidad y riqueza microbiana dentro de una comunidad; y la diversidad beta, es decir, la diversidad microbiana presente en distintas comunidades (Calle, 2019). Posteriormente se lleva a cabo la asignación taxonómica comparando las secuencias de las unidades taxonómicas con las depositadas en bases de datos como SILVA (Quast *et al.*, 2013), Greengenes o RefSeq, entre otras, dándonos la oportunidad de caracterizar a

nivel taxonómico y filogenético las principales poblaciones microbianas presentes en la microbiota.

La metagenómica filogenética es una técnica relativamente rápida, que permite estimar la biodiversidad presente en una comunidad y ha revelado nuevas especies e incluso filos completos que hasta el momento no se conocían (Garza and Dutilh, 2015). Sin embargo, esta técnica únicamente nos proporciona información acerca del perfil microbiano, es decir, de quién está presente. En la actualidad, existen herramientas bioinformáticas que permiten llevar a cabo inferencia funcional a partir de datos obtenidos mediante metagenómica filogenética, como el PICRUSt, que permite predecir la composición funcional de una comunidad microbiana utilizando una base de datos de genomas de referencia (Langille *et al.*, 2013). La *metagenómica total* o funcional por "*shotgun*", tiene como finalidad la secuenciación no dirigida de los todos los genes/genomas microbianos presentes en una muestra (Quince et al., 2017). Esta técnica genera un perfil genético más amplio y complejo, aportando información no solo de la composición taxonómica, sino también del perfil funcional característico de una comunidad microbiana, permitiéndonos conocer las potenciales actividades que presentan los miembros de la microbiota. Conocer el perfil de actividades presentes en la microbiota abre la posibilidad de detectar secuencias pertenecientes a nuevos genes y actividades de interés.

Los sistemas de secuenciación de nueva generación como Illumina o Ion Torrent han ido reduciendo de forma significativa el coste y aumentando el rendimiento. En la actualidad, las denominadas tecnologías de secuenciación de tercera generación, como Pacific Bioscience y Oxford Nanopore, suponen una alternativa más eficaz para algunas aplicaciones. A pesar de que estas tecnologías han supuesto un gran avance en el

estudio del microbioma humano, existen ciertas limitaciones, como la presencia de secuencias que no podemos asignar a ningún microorganismo descrito en las bases de datos (Thomas and Segata, 2019).

2.3.- Metatranscriptómica

El transcriptoma es el conjunto de todos los transcritos de ARN presentes en un tipo de célula en un momento determinado. El estudio de los transcritos de ARN presentes en una muestra compleja, o lo que es lo mismo, todos los genes que están expresándose en una comunidad microbiana en el momento de la toma de muestra son lo que denominamos metatranscriptómica. Gracias a esta metodología podemos identificar aquellos genes que están expresándose, y detectar expresión diferencial en distintas poblaciones, o en respuesta a diferentes tratamientos. Estas técnicas han permitido estudiar la expresión génica en las comunidades microbianas del intestino (Franzosa *et al.*, 2014), analizar las diferencias en los patrones de expresión, tanto en el hospedador como en la microbiota, provocados por variaciones en la dieta (Marlow *et al.*, 2013), el uso de antibióticos (Maurice *et al.*, 2013) o la presencia de diversas enfermedades (Haberman *et al.*, 2014; Schirmer *et al.*, 2018; Lloyd-Price *et al.*, 2019).

Existen dos enfoques a la hora de estudiar el metatranscriptoma, que requieren un paso previo de conversión del ARN extraído de la muestra a ADNc. El primero, basado en técnicas de hibridación de los ácidos nucleicos, implica la incubación y el uso de microarrays. Los transcritos presentes se convierten en ADNc, y posteriormente hibridan con la secuencia de sus correspondientes sondas específicas. La cuantificación de cada transcrito se determina mediante la intensidad de señal de fluorescencia detectada en cada ubicación del microarray. Este enfoque es rápido y relativamente

económico, sin embargo, requiere un conocimiento previo del genoma o los genomas a analizar. Además, la comparación entre los niveles de expresión en diferentes experimentos es a menudo difícil y puede requerir métodos de normalización complejos (Lowe *et al.*, 2017). El otro enfoque, denominado RNA-seq, utiliza las tecnologías NGS combinadas con métodos computacionales para detectar y cuantificar los transcritos presentes en una muestra (Lowe *et al.*, 2017). Dependiendo del tipo de ARN a analizar, es necesario un paso de enriquecimiento selectivo, que en el caso del ARNm, se basa en el uso de kits de eliminación de ARN ribosómico, o el enriquecimiento de secuencias con colas poli(A). Posteriormente, los transcritos se convierten en ADNc, son normalmente fragmentados antes de llevar a cabo la secuenciación, y pueden ser o no amplificados por PCR para enriquecer la muestra. La secuenciación puede llevarse a cabo en una sola dirección ("single-end") o en ambas direcciones ("pair-end"), produciendo esta última alineamientos o ensamblajes más fiables y robustos (Ozsolak and Milos, 2011). Las lecturas obtenidas se anotan utilizando genomas de referencia, utilizando para ello herramientas bioinformáticas como Bowtie2 (Langmead and Salzberg, 2012); o bien mediante algoritmos de ensamblaje *de novo* si no existe una referencia disponible, para reconstruir los transcritos de ARNm originales a través de programas como Velvet (Zerbino and Birney, 2008). Finalmente, mediante distintas herramientas bioinformáticas como FeatureCounts (Liao *et al.*, 2014), se lleva a cabo la cuantificación y el análisis de la expresión diferencial. En la actualidad, se dispone de programas que permiten el análisis completo, desde el alineamiento de las secuencias hasta el análisis estadístico de los patrones de expresión (López-Fernández *et al.*, 2019). El RNA-seq necesita una cantidad de ARN mucho menor en comparación con la necesaria para la

tecnología de microarrays (Hashimshony *et al.*, 2012) y no necesita un conocimiento previo de los genomas a estudio (Wang *et al.*, 2009).

2.4.- Metaproteómica

La metaproteómica se define como el estudio a gran escala de todas las proteínas presentes en una comunidad microbiana en un momento determinado (Schneider and Riedel, 2010). El análisis comienza con la extracción y purificación de las proteínas presentes en una muestra, que se digieren enzimáticamente para obtener péptidos más pequeños. Estos péptidos se separan, generalmente por cromatografía, y finalmente se analizan mediante espectrometría de masas (MS). La identificación final de proteínas se obtiene comparando las distintas masas obtenidas con la información presente en las bases de datos (Issa Isaac *et al.*, 2019).

La metaproteómica es una técnica cuyo uso en el estudio del microbioma humano ha aumentado de forma exponencial en la última década. Los primeros estudios se basaron en la descripción del metaproteoma del intestino humano (Klaassens *et al.*, 2007; Verberkmoes *et al.*, 2009; Kolmeder *et al.*, 2012), y posteriormente se analizó la interacción microbiota-hospedador en distintas localizaciones del intestino (Li *et al.*, 2011) y los cambios relacionados con la presencia de patologías intestinales (Presley *et al.*, 2012; Erickson *et al.*, 2012; Debyser *et al.*, 2019). El uso combinado de información metagenómica y metaproteómica ha permitido comprobar la relación entre la abundancia de diferentes taxones y la abundancia de proteínas identificadas de los mismos grupos de microorganismos (Kolmeder *et al.*, 2012); así como detectar actividades importantes a nivel clínico, que podrían utilizarse como biomarcadores (Wilmes *et al.*, 2015). Sin embargo, estos estudios generan información muy compleja

de analizar (Zhang *et al.*, 2018), necesitándose potentes algoritmos para el análisis bioinformático, así como bases de datos actualizadas que permitan relacionar secuencias con espectros de masas.

2.5.- Metabonómica

La metabolómica es la técnica utilizada para el estudio del metaboloma, es decir, el conjunto de metabolitos presentes en cualquier microorganismo o tejido individual. Cuando el estudio se aplica a sistemas complejos, por ejemplo muestras en las que más de un tipo celular contribuyen al conjunto de metabolitos (como la microbiota), hablamos entonces de metabonómica (Marchesi and Ravel, 2015). Esta técnica se ha convertido en un enfoque poderoso que ha sido adoptado para el diagnóstico clínico (Patti *et al.*, 2012).

Las dos técnicas más aplicadas al estudio del metaboloma son la resonancia magnética nuclear (RMN) y la MS. La RMN produce datos espectrales que permiten cuantificar la concentración y además caracterizar la estructura química de los metabolitos (Alonso *et al.*, 2015). Es una técnica altamente selectiva y reproducible, requiere menos preparación de la muestra y produce espectros que se correlacionan directa y linealmente con la concentración del compuesto, pero tiene una sensibilidad relativamente baja por lo que solo somos capaces de detectar los metabolitos más abundantes (Turi *et al.*, 2018). Por otro lado, la MS nos proporciona datos espectrales en forma de una relación masa/carga (m/z) y una intensidad relativa del compuesto. En este caso, es necesario ionizar la muestra biológica previamente, y los compuestos ionizados resultantes de cada molécula generarán diferentes patrones de picos que definen la huella digital de la molécula original. En metabonómica, la MS está

generalmente precedida por una etapa de separación, que puede llevarse a cabo mediante cromatografía de gases o cromatografía líquida, que reduce la alta complejidad de la muestra biológica y permite el análisis de diferentes conjuntos de moléculas (Alonso *et al.*, 2015).

A pesar de que el campo de la metabolómica y metabonómica ha progresado notablemente en la última década, el desafío actual es ser capaces de interpretar la información obtenida e integrar estos datos bioquímicos complejos con los obtenidos mediante metagenómica, metatranscriptómica y metaproteómica, para obtener una visión global y comprender los mecanismos y relaciones presentes en el microbioma humano.

3.- El sistema hepatobiliar

El sistema hepatobiliar está formado por el hígado, los conductos biliares intrahepáticos (canalículos biliares y conductos biliares), los conductos biliares extrahepáticos (el conducto hepático común, el conducto cístico y el conducto colédoco) y la vesícula biliar, y es el encargado de la producción, almacenamiento y transporte de la bilis. A continuación, se describen los distintos componentes, así como sus funciones.

3.1.- Anatomía de la vesícula biliar y los conductos biliares

La vesícula biliar es el órgano que almacena la bilis producida en el hígado. Presenta un tamaño aproximado de 10 cm de longitud y una capacidad de unos 30-50 mL, y se sitúa en la cara inferior del hígado, presentando cuatro partes: el fondo, que corresponde con la parte inferior; el cuerpo; el infundíbulo y el cuello, que conecta con el conducto cístico (Keplinger and Bloomston, 2014; Mahadevan, 2014) (Fig. 3).

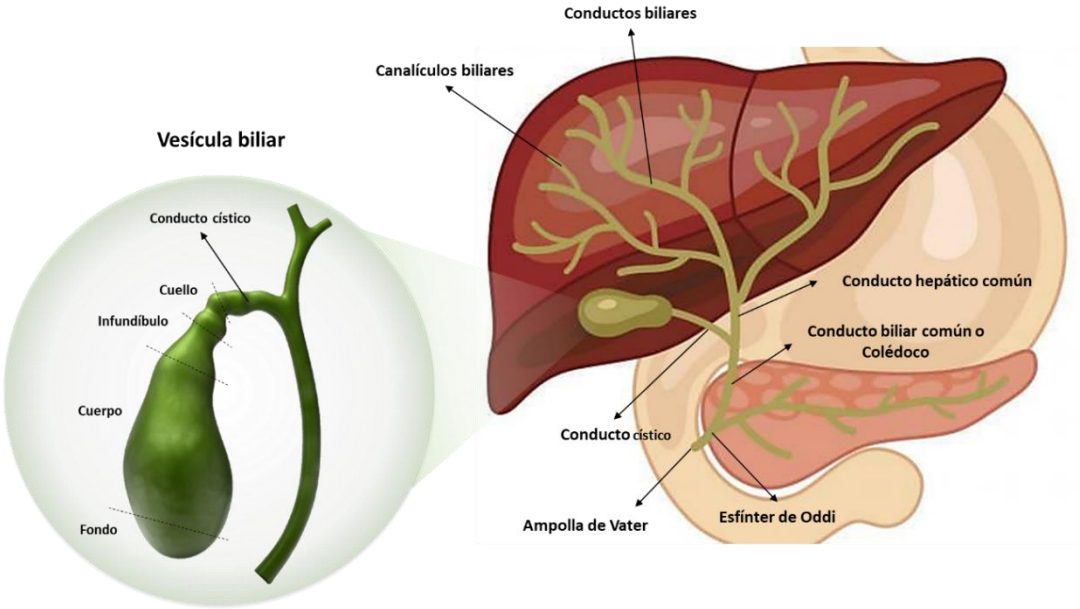

Fig. 3. Partes del sistema hepatobiliar. La imagen muestra la localización anatómica de la vesícula biliar y los conductos intrahepáticos: canalículos biliares (túbulos delgados situados entre los hepatocitos adyacentes que posteriormente se unen para formar los conductos biliares) y conductos biliares; y los conductos extrahepáticos (conducto hepático común, conducto cístico y conducto biliar común o colédoco), así como las partes en las que se divide la vesícula biliar (fondo, cuerpo, infundíbulo y cuello).

El conducto cístico está formado en su interior por una mucosa que contiene una serie de válvulas espirales que actúan como barrera, denominadas válvulas de Heister, y une la vesícula biliar con el conducto hepático común, dando lugar al conducto biliar común o conducto colédoco. Éste, posteriormente, se une con el conducto pancreático dando lugar al esfínter de Oddi, para alcanzar el duodeno finalmente a través de la ampolla de Vater (Behar, 2013).

A nivel histológico, podemos encontrar 3 capas claramente diferenciadas en la vesícula biliar:

- La capa mucosa, capa interna de la vesícula biliar y que está en contacto directo con la bilis. Está formada por un epitelio que forma pliegues o invaginaciones, y la lámina

propia. El epitelio participa en la concentración de la bilis. Y está formado por células caliciformes de tipo mucoso, encargadas de la producción de mucina.

- Capa muscular: formada por capas de músculo liso relativamente finas. Está inervada por el nervio vago y el nervio esplácnico. La secreción de hormonas gastrointestinales, como la colecistoquinina (CCK), producen a través de estos nervios la contracción del músculo liso, lo que genera una contracción de la vesícula biliar, facilitando la secreción de la bilis por el conducto cístico y su posterior salida al duodeno (Behar, 2013).

- Capa serosa: es la capa más externa de la vesícula biliar y proviene del peritoneo.

3.2.- La bilis: composición, secreción y funciones

La bilis es un fluido acuoso complejo, que se produce en el hígado, concretamente en los hepatocitos. Estos producen bilis a través de la secreción principalmente de bilirrubina conjugada, sales biliares, colesterol, fosfolípidos, proteínas, iones y agua en los canalículos biliares (Fig. 3). Posteriormente, esta secreción puede modificarse en los colangiocitos (las células que forman el epitelio de los conductos biliares) a través de procesos absorbentes y secretores regulados por hormonas que diluyen y alcalinizan la bilis. Finalmente, la bilis producida (aproximadamente 600 mL al día) llega a través de los conductos biliares al conducto hepático común, donde puede ir directamente al intestino por el conducto biliar común, o ser almacenada en la vesícula biliar (Boyer, 2013; Hundt *et al.*, 2019).

3.2.1.- Composición

La bilis se compone principalmente de un 95% de agua, además de sales biliares y colesterol, y biliverdina y bilirrubina, derivados de la degradación de la hemoglobina,

que dan el color típico a la bilis. Por otro lado, también podemos encontrar fosfolípidos (lecitina principalmente), aminoácidos, esteroides, enzimas, porfirinas, vitaminas y metales pesados, así como medicamentos exógenos, xenobióticos u otras toxinas ambientales (Boyer, 2013). El colesterol y las sales biliares son dos de los componentes más importantes de la bilis. El colesterol, es un lípido terpenoide con un esqueleto de carbono formado por cuatro anillos alicíclicos fusionados, componente esencial de las membranas celulares de mamíferos, además de ser precursor de hormonas esteroideas, la vitamina D y de las sales biliares primarias (García *et al.*, 2012). A parte de la producción en el hígado, el colesterol puede venir de la dieta y de la síntesis en tejidos extrahepáticos, y se almacena en el hígado hasta que se libera en forma de lipoproteínas de alta densidad (HDL) o lipoproteínas de baja densidad (VLDL), en forma de colesterol libre secretado con la bilis o en forma de sales biliares.

Las sales biliares se producen a partir de colesterol, mediante la vía el citocromo P450, y el proceso completo implica la actuación de 17 enzimas distintas localizadas en diferentes partes del hepatocito. Estas enzimas modifican los anillos y la cadena lateral del colesterol para dar lugar a los ácidos biliares primarios: el ácido cólico y el quenodesoxicólico (Chiang, 2013). La conversión puede producirse de dos formas: la ruta clásica, que se inicia por una colesterol 7-alpha-monooxigenasa, que gracias a la acción de una esterol 12-alpha-hidroxilasa y posteriormente una esterol 27-hidroxilasa produce ácido cólico y ácido quenodesoxicólico; o la ruta alternativa, que se inicia por una esterol 27-hidroxilasa y produce ácido quenodesoxicólico gracias a una 25-hidroxicolesterol 7alpha-hidroxasa (Jia *et al.*, 2018). Estas sales biliares primarias posteriormente se conjugan con glicina y taurina gracias a la acción de ácido biliar:CoA sintetasas (BACS) y ácido biliar-CoA:amino ácido N-aciltransferasas (BAAT), aumentando

así su solubilidad en medio acuoso. Las sales biliares presentes en la bilis son fundamentalmente: taurocolato, glicocolato, tauroquenodesoxicolato y glicoquenodesoxicolato. En el intestino, debido a la acción de la microbiota, estas sales biliares son modificadas dando lugar a las sales biliares secundarias.

3.2.2.- Secreción y circulación enterohepática

Durante el período digestivo, la actividad motora de la vesícula biliar está influenciada por las tres fases del proceso digestivo: cefálica, antral y duodenal. La fase cefálica se inicia mediante un estímulo (visual, olfativo, etc.) que activa el sistema nervioso central, y se estima que aproximadamente un 40% de la bilis presente en la vesícula biliar es secretada durante esta fase. La segunda fase comienza cuando el alimento alcanza el estómago, fenómeno que desencadena un reflejo antral de la vesícula biliar mediado por el nervio vago. Finalmente, durante la fase duodenal, la vesícula biliar libera la mayor parte de su contenido, inducido por la liberación de CCK en el duodeno y el yeyuno proximal. La CCK produce la contracción de la vesícula biliar y la relajación del esfínter de Oddi, lo que hace que se vierta la bilis y el jugo pancreático en el duodeno (Behar, 2013).

La mayoría de las sales biliares (95%) y el colesterol se reabsorben en el intestino delgado y vuelven al hígado mediante el proceso denominado circulación enterohepática. En el caso de las sales biliares, la reabsorción se produce fundamentalmente en el mediante transporte activo o procesos de difusión pasiva; mientras que en el caso del colesterol, éste se une a triglicéridos y lipoproteínas para generar complejos transportables llamados quilomicrones (Gérard, 2013). En ambos casos, las sales biliares y el colesterol, además de otros compuestos como la bilirrubina,

pasan a la vena porta para ser reconducidos hacia el hígado, donde los hepatocitos los reutilizan secretándolos de nuevo en la bilis. Aquella pequeña fracción de sales biliares, colesterol, bilirrubina, así como de los productos derivados del metabolismo de estos compuestos por parte de la microbiota intestinal, que no es reabsorbida en el intestino, es finalmente excretada en las heces.

3.2.3.- Funciones

La bilis cumple una serie de funciones fundamentales para el organismo. En primer lugar, la bilis es la principal ruta excretora de sustancias lipófilas potencialmente dañinas de origen exógeno, así como otros sustratos endógenos como la bilirrubina y las sales biliares cuyos pesos moleculares son mayores de 300-500 Da y no se filtran o excretan fácilmente por el riñón. Además, es la principal ruta de eliminación del colesterol. Por otro lado, las sales biliares actúan emulsionando las grasas que consumimos en la dieta, el colesterol y ciertas vitaminas, haciendo estos compuestos más solubles y facilitando de este modo su absorción en el intestino. Además, las sales biliares estimulan la secreción de agua en el intestino, lo que puede ayudar en el avance del contenido intestinal. La bilis también actúa protegiendo el TGI de microorganismos patógenos a través de la estimulación del sistema inmunitario innato en el intestino como la secreción de IgA, y contiene hormonas y feromonas que contribuyen al crecimiento y desarrollo del intestino en algunas especies (Boyer, 2013). El correcto desempeño de las funciones de la bilis, así como su producción y secreción, es fundamental para la salud.

3.3.- Principales patologías biliares

3.3.1.- Colelitiasis

La colelitiasis, o también denominada litiasis biliar, se caracteriza por la presencia de cálculos en la vesícula biliar o las vías biliares y constituye una de las patologías más comunes del sistema gastrointestinal, de forma que en los países desarrollados alrededor del 10% de los adultos y el 30% de los individuos mayores de 65 años tienen cálculos biliares, aunque en general son asintomáticos. Su incidencia se ve afectada por factores como la etnia, el género y la edad; además de otros como la ingesta de una dieta rica en grasas o la obesidad. Los cálculos o piedras generalmente se producen por la precipitación y cristalización del colesterol presente en la bilis. Normalmente existe un equilibrio entre la biosíntesis de colesterol en el hígado y la absorción de colesterol y sales biliares en el intestino, sin embargo, bajo ciertas condiciones fisiopatológicas, puede producirse una hipersecreción de colesterol en la bilis. Como resultado, la sobresaturación de colesterol provoca la precipitación del mismo, normalmente acompañado por otros componentes de la bilis, generando cálculos que pueden ser de varios tipos en función de su composición, su morfología y de dónde estén localizados (Wang *et al.*, 2013). A pesar de que en muchos de los casos la colelitiasis es asintomática, cuando se presentan síntomas en la mayoría de los casos la colecistectomía (cirugía realizada para extirpar la vesícula biliar) es el único tratamiento posible. Las complicaciones más graves de esta patología abarcan la colecistitis o inflamación de la vesícula biliar, la obstrucción de las vías biliares o también denominado coledocolitiasis, y la inflamación de las vías biliares o la colangitis.

3.3.2.- Colecistitis y colangitis

La colecistitis aguda se trata de una inflamación de la mucosa de la vesícula biliar. Es la complicación más frecuente de la colelitiasis, de hecho, más del 95% de los pacientes con colecistitis aguda presenta colelitiasis. En este caso, denominado colecistitis litiásica o calculosa, uno o varios cálculos quedan retenidos en el conducto cístico y lo obstruyen de forma persistente, no permitiendo la salida de la bilis. Esto aumenta la presión intraluminal, que junto con la bilis sobresaturada de colesterol, desencadena una respuesta inflamatoria aguda (Indar and Beckingham, 2002) que puede, en los casos más graves, provocar isquemia, necrosis y perforación de la vesícula. Cuando la colecistitis aguda no está provocada por la presencia de cálculos, denominada colecistitis aguda alitiásica o acalculosa, la inflamación se presenta como respuesta a una infección, normalmente provocada por *E. coli*, *Enterococcus faecalis y* distintas especies de *Klebsiella* (Indar and Beckingham, 2002); en respuesta a ciertos tipos de virus, como citomegalovirus, el virus de la Inmunodeficiencia Humana (VIH), y los virus de la Hepatitis A y B; o en respuesta a una cirugía mayor, fármacos, traumatismos o tumores (Cárdenas Quirós, 2018). En ocasiones, los episodios de inflamación aguda se repiten, provocando un engrosamiento de la vesícula biliar y desencadenando la pérdida de función de la misma. En este caso, la patología pasa a denominarse colecistitis crónica, condición que normalmente requiere la extirpación de la vesícula. Finalmente, otra patología es la colangitis aguda, que se genera cuando la inflamación se produce en las vías biliares (conducto hepático y colédoco). Del mismo modo que la colecistitis, la colelitiasis es la causa más frecuente de la colangitis aguda (85%).

4.- Interacciones entre la bilis y la microbiota intestinal

La microbiota intestinal está en contacto, en mayor o menor concentración (dependiendo de la localización en el intestino) con la bilis y por lo tanto con los componentes que en ella se encuentran. Existe una relación compleja entre las sales biliares y la microbiota intestinal: las sales biliares, por su naturaleza detergente, presentan un alto potencial antimicrobiano, jugando un importante papel en la homeostasis intestinal controlando la composición de la microbiota; por otro lado, el metabolismo por parte de la microbiota intestinal desempeña un papel fundamental en la composición del conjunto de sales biliares presentes en el intestino.

4.1.- Efectos tóxicos de la bilis sobre la microbiota intestinal

El efecto tóxico de las sales biliares sobre la microbiota parece ser consecuencia de múltiples factores. En primer lugar, las sales biliares son moléculas anfipáticas de superficie activa, y su actividad detergente daña las membranas celulares (Urdaneta and Casadesús, 2017), altera la integridad y la permeabilidad de la membrana, y en algunos casos incluso termina lisando la célula (Noh and Gilliland, 1993; Fujisawa and Mori, 1996; Leverrier *et al.*, 2003). Existen varios factores que influyen en la gravedad de la alteración de la membrana:

(i) La concentración de sales biliares: una alta concentración de sales biliares puede desestabilizar rápidamente la membrana, a través de la disolución de los lípidos y la disociación de las proteínas que la conforman, pudiendo provocar la salida rápida de material de interior de la bacteria y la muerte celular (Coleman *et al.*, 1980). Concentraciones bajas de sales biliares ejercen un efecto más sutil en la fluidez y permeabilidad de la membrana, mediante la alteración de las proteínas de membrana,

el incremento del flujo transmembrana de cationes divalentes (Noh and Gilliland, 1993; Fujisawa and Mori, 1996) y cambios en la hidrofobicidad de la superficie celular (Gómez Zavaglia *y cols.*, 2002).

(ii) El tipo de sales biliares y su estructura: la unión de las sales biliares a los lípidos de membrana está relacionada con su hidrofobicidad (Begley *et al.*, 2005). Las sales biliares conjugadas generalmente están completamente ionizadas a valores de pH fisiológicos, y permanecen en el ambiente externo de la bicapa a menos que haya un sistema de transporte disponible. Por el contrario, las sales biliares no conjugadas cruzan mediante difusión pasiva la membrana y acceden al interior celular (Hofmann *y cols.*, 2001).

(iii) La estructura y composición de la membrana celular: la presencia de cambios en la carga, hidrofobicidad y la fluidez de membrana, así como alteraciones en los lipopolisacáridos y la composición de ácidos grasos pueden aumentar la susceptibilidad a sales biliares (Urdaneta and Casadesús, 2017).

Otro de los efectos que ejercen las sales biliares es el daño en el ADN. Cuando las sales biliares logran entrar en el interior celular, inducen la formación de estructuras secundarias en el ARN y activan sistemas de reparación de nucleótidos (Prieto *et al.*, 2004), sistemas de reparación de ADN asociados a la respuesta SOS, y proteínas de respuesta a daño oxidativo (Kandell and Bernstein, 1991). La acción detergente de las sales biliares también puede alterar la conformación de las proteínas, provocando fallos en su plegamiento o su desnaturalización e induciendo proteínas que participan en el correcto plegamiento de las proteínas (Flahaut *y cols.*, 1996; Schmidt y Zink, 2000).

Las sales biliares también pueden generar radicales libres, ocasionando estrés oxidativo, e induciendo proteínas de respuesta a estrés oxidativo (Bernstein *et al.*, 1999; Leverrier *et al.*, 2003). Finalmente, otro posible efecto tóxico sobre las bacterias se

relaciona con la disociación de las sales biliares en el interior celular, que puede generar estrés ácido o efectos osmóticos debido a la generación de iones libres (Begley *et al.*, 2005).

4.2.- Mecanismos de resistencia frente a sales biliares

Las células bacterianas han desarrollado diversos sistemas y mecanismos de resistencia que les han permitido sobrevivir y colonizar las distintas localizaciones del TGI. En primer lugar, la estructura y composición de la membrana contribuye a la resistencia a sales biliares. En Gram-negativos, se ha descrito que la pérdida del antígeno O en el LPS produce una disminución de la resistencia a sales biliares, mientras que, la presencia de cadenas muy largas aumentan la resistencia (Gunn, 2000; Crawford *et al.*, 2012). En microorganismos Gram-positivos, variaciones en la estructura de la membrana favorecen la resistencia a la presencia de sales biliares, como un cambio en la composición de lípidos a través de un aumento en la producción de proteínas involucradas en el metabolismo de ácidos grasos (Sánchez *et al.*, 2007); o una mayor proporción en la membrana de ácidos grasos saturados (Ruiz *et al.*, 2007). Otro mecanismo de defensa frente a sales biliares es la producción de exopolisacáridos (EPS). Los EPS son polímeros de carbohidratos, de distinta composición y de mayor o menor pero molecular, que son sintetizados por las bacterias y que pueden excretarse al exterior celular o quedarse anclados a la membrana. La producción de estos polímeros es una característica extendida entre las bacterias intestinales (Ruas-Madiedo *et al.*, 2007; Ruas-Madiedo *et al.*, 2009; Ruiz *et al.*, 2013).

En caso de que las sales biliares entren en el interior celular, es necesario la presencia de sistemas que permitan la salida activa de sales biliares (Urdaneta and

Casadesús, 2017). Dentro de estos sistemas, encontramos las bombas de expulsión de compuestos citotóxicos ("Multidrug Resistance", MDR), que son sistemas de transporte transmembrana que permiten la salida de la célula de distintos compuestos tóxicos como sales biliares, detergentes y antibióticos. Se han descrito tanto en bacterias Gram-positivas como Gram-negativas, y están ampliamente distribuidos en los miembros de la microbiota intestinal (Paulsen, 2003; Horáčková *et al.*, 2018). Su función ha sido particularmente estudiada en relación con la resistencia a antibióticos, sin embargo, se sabe que la activación de este tipo de transportadores es la responsable del fenotipo de resistencia a sales biliares en algunos miembros de la microbiota intestinal o en patógenos (Thanassi *et al.*, 1997; Nikaido *et al.*, 2008; Ruiz *et al.*, 2013; Baucheron *et al.*, 2014). Otro de los mecanismos que contribuyen a la resistencia a sales biliares es la activación de enzimas de reparación de daño en el ADN. Como se comentó en el apartado anterior, las sales biliares activan enzimas de reparación de nucleótidos, sistemas de reparación de ADN asociados a la respuesta SOS, y proteínas de respuesta a daño oxidativo. La activación de estos sistemas parece ser fundamental para poder reparar los daños en el ADN producidos por las sales biliares y por lo tanto para la supervivencia en presencia de las mismas (Prieto *et al.*, 2004; Urdaneta and Casadesús, 2017).

Finalmente, como se ha comentado con anterioridad, las sales biliares generan estrés celular y estrés oxidativo, por lo que sistemas de respuesta a estrés celular; como oxidoreductasas (Leverrier *et al.*, 2003), proteínas de respuesta a choque térmico y peroxiredoxinas (Hernández *y cols.*, 2012), se activan en presencia de sales biliares. A pesar de que su función e implicación exacta aún se desconoce, existe una gran variedad de genes activados en respuesta a estrés por la presencia de sales biliares, hecho que

apoya la hipótesis de que esta respuesta múltiple a las condiciones de estrés contribuye al fenotipo de resistencia.

4.3.- Metabolismo microbiano de sales biliares y colesterol. Implicaciones en la salud

A continuación, se describen las actividades metabólicas frente a sales biliares y colesterol más frecuentes en la microbiota intestinal que están resumidas en la revisión publicada en *Frontiers in Physiology* y que constituye el primer artículo de esta Tesis Doctoral. En esta revisión se detalla también como la microbiota intestinal influye en el perfil de sales biliares en el intestino, siendo esta relación y su equilibrio fundamental para el mantenimiento de un estado de salud en el hospedador.

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Intestinal Bacteria Interplay With Bile and Cholesterol Metabolism: Implications on Host Physiology

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Bile is a biological fluid synthesized in the liver, mainly constituted by bile acids and cholesterol, which functions as a biological detergent that emulsifies and solubilizes lipids, thereby playing an essential role in fat digestion. Besides, bile acids are important signaling molecules that regulate key functions at intestinal and systemic levels in the human body, affecting glucose and lipid metabolism, and immune homeostasis. Apart from this, due to their amphipathic nature, bile acids are toxic for bacterial cells and, thus, exert a strong selective pressure on the microbial populations inhabiting the human gut, decisively shaping the microbial profiles of our gut microbiota, which has been recognized as a metabolic organ playing a pivotal role in host health. Remarkably, bacteria in our gut also display a range of enzymatic activities capable of acting on bile acids and, to a lesser extent, cholesterol. These activities can have a direct impact on host physiology as they influence the composition of the intestinal and circulating bile acid pool in the host, affecting bile homeostasis. Given that bile acids are important signaling molecules in the human body, changes in the microbiota-residing bile biotransformation ability can significantly impact host physiology and health status. Elucidating ways to fine-tune microbiota-bile acids-host interplay are promising strategies to act on bile and cholesterol-related disorders. This manuscript summarizes the current knowledge on bile and cholesterol metabolism by intestinal bacteria, as well as its influence on host physiology, identifying knowledge gaps and opportunities to guide further advances in the field.

Keywords: gut microbiota, bile acids, cholesterol, gut microbiota-host interplay, bile signaling

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INTRODUCTION

The human gastrointestinal tract (GIT) is colonized by a vast array of microbes which dynamically interact with dietary and host-derived molecules in the intestinal lumen, significantly contributing to host physiology. Indeed, several animal and human studies have demonstrated that specific gut microbiota configurations contribute to inflammatory and metabolic diseases (Wu et al., 2015), although the precise molecular mechanisms behind the microbiota-host interactions impacting host health remain largely unknown. Cholesterol and bile acids (BAs) are important signaling molecules that, apart from exerting digestive functions, regulate multiple physiological processes

in the host (Hegyi et al., 2018). Besides, the interaction of cholesterol and BAs with gut bacteria has been known for decades, although the role of these interactions in host health, and the possibility to modulate them through targeting the gut microbiota composition to improve human health, have only started to be recently explored.

Bile acids are synthesized in hepatocytes from cholesterol and conjugated to glycine and taurine before being secreted into the small intestine with the bile flow, which plays a major role in fat emulsification and absorption. Bile composition depends on the diet and intrinsic characteristics of the individuals, but usually contains over 50% BAs, over 20% fatty acids and cholesterol, and lower amounts of other molecules such as bilirubin or phospholipids (Farina et al., 2009). During its gastrointestinal transit, most BAs and cholesterol are reabsorbed in the distal small intestine, though a significant proportion evades this process, being excreted with feces (Islam et al., 2011).

Bile acids and cholesterol reaching the large intestine dynamically interact with our gut microbes. Indeed, BAs strongly compromise bacterial survival in the GIT, thus gut microbes must have developed mechanisms to counteract bile toxicity (Ruiz et al., 2013). Besides, gut microbial communities are capable of chemically modifying cholesterol and BAs, transformations that impact the gut microbiota and the BAs pool and, consequently, the signaling mechanisms they mediate. Accordingly, changes in this gut microbiota-bile axis are now acknowledged to have decisive implications in human health (Long et al., 2017).

The present minireview examines the current knowledge on the enzymatic activities of intestinal bacteria over BAs and cholesterol, and their implications in human physiology, with a particular emphasis on their impact on gastrointestinal disorders and aging-associated decline. Opportunities and limitations to translate this body of knowledge into novel microbiome-based applications for some of these diseases are also discussed.

CHOLESTEROL METABOLISM BY INTESTINAL BACTERIA

Cholesterol is a terpenoid lipid with a carbon skeleton formed by four fused alicyclic rings. It is an essential component of the mammalian cell membranes and precursor of steroid hormones, vitamin D, and primary BAs (García et al., 2012). Following its GIT passage, most cholesterol is absorbed in the duodenum and proximal jejunum by a passive diffusion process. Reabsorbed cholesterol is incorporated with triglycerides and lipoproteins into transportable complexes called chylomicrons, which return to the liver through the enterohepatic circulation. The cholesterol escaping this re-absorption reaches the colon, where it can be metabolized by the intestinal microbiota and/or excreted with feces (Gérard, 2013).

The metabolism of cholesterol by gut microbes has been described since the 30s (Schoenheimer, 1931) and has been supported by studies on germ-free animal models (Gérard et al., 2007). The microbial activities on cholesterol are based on its enzymatic reduction to produce coprostanone and coprostanol (**Figure 1**), which is poorly absorbable in the intestine. Thus, coprostanol production leads to increased cholesterol excretion into feces, contributing to reduce blood cholesterol level (Lye et al., 2010). Two different pathways have been proposed for this microbial reduction of cholesterol. The first pathway involves the direct reduction of the double bond 5–6 to give coprostanol, by cholesterol reductases (Gérard et al., 2004). The second pathway involves the oxidation of the 3β-hydroxy group and the isomerization of the double bond to produce 4-cholesten-3-one by cholesterol oxidases (ChOx) or 3β-hydroxysteroid dehydrogenases/isomerases (HSD) (García et al., 2012), followed by two reductions to form coprostanone and finally coprostanol (Gérard, 2013). Very limited information is available on the occurrence and distribution of the latter enzymes, although sequences belonging to ChOx are frequently found in the genomes of intestinal bacteria and gut/fecal metagenomes, indicating that cholesterol oxidation is a common activity in the gut microbiota. Remarkably, ChOx-encoding genes are found in the phyla Bacteroidetes, Proteobacteria and Actinobacteria, displaying a lower degree of conservation in Actinobacteria, but are absent in Firmicutes, one of the dominant phyla in the human gut microbiota (**Figure 2** and **Supplementary Figure 1**).

Several factors throughout life, including changes in diet or antibiotics consumption (Korpela and Adlercreutz, 1985; Norin, 1997), have been suggested to affect the gut microbiota's ability to reduce cholesterol to coprostanol, which exhibits higher rates of conversion in elderly individuals (Benno et al., 2009). Indeed, these factors are known to affect the gut microbiota composition in humans, although the real impact of lifestyle and other clinical factors in the microbial reduction of cholesterol, and the particular gut bacteria/activities implicated warrant further investigation.

Several cholesterol-reducing strains have been isolated from the intestine and feces of mammals (Eyssen et al., 1973; Brinkley et al., 1980, 1982). The first described cholesterol-reducing isolate of human origin was the *Bacteroides* sp. strain D8 (Gérard et al., 2007). Otherwise, only a few cholesterol-reducing intestinal bacteria have been identified, most of them belonging to the genus *Eubacterium,* although the genes or enzymes involved in this metabolism have not been well characterized yet.

Some other gut bacterial inhabitants, including lactobacilli and bifidobacteria species usually used as probiotics, have been long studied for their possible cholesterol-lowering activities. Although different mechanisms of action (involving removal, co-precipitation or assimilation) have been proposed (Pereira and Gibson, 2002; Liong and Shah, 2005; Tomaro-Duchesneau et al., 2014; Zanotti et al., 2015), to date, the real contribution of these microbial groups toward cholesterol-lowering and the molecular activities involved remain mostly unknown.

BACTERIAL BILE METABOLISM: IMPLICATIONS ON HEALTH AND DISEASE

The metabolism of BAs by the gut microbiota has been known for decades, although its consequences on human health have only started to be considered (Farina et al., 2009;

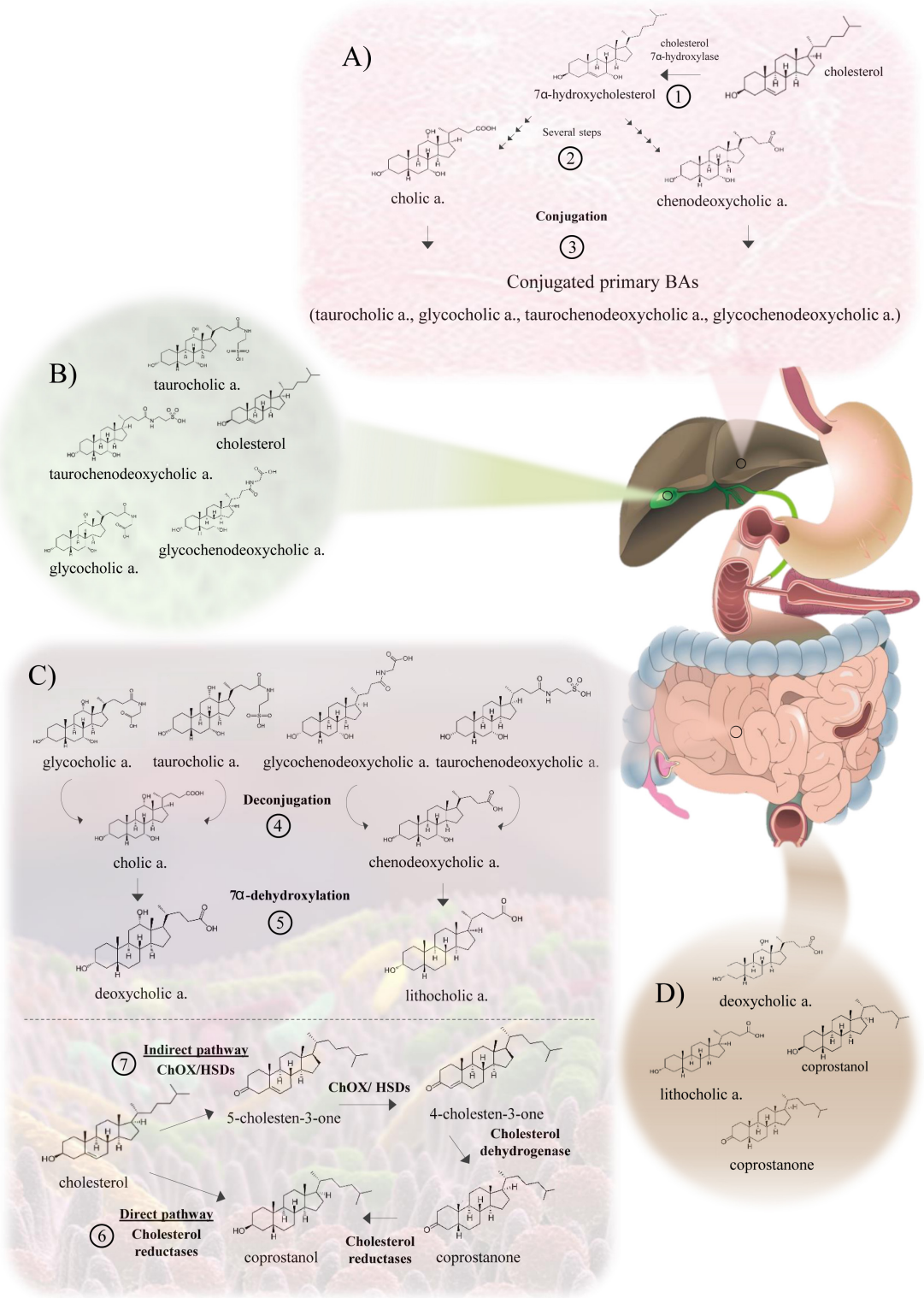

FIGURE 1 | Bacterial cholesterol and bile metabolism in the gut, including microbiota-mediated transformations. **(A)** Metabolism of cholesterol in the hepatocyte. The conversion of cholesterol to primary BAs and their subsequent conjugation is carried out in the hepatocyte. (1) The primary BAs, cholic and chenodeoxycholic acids, are synthesized through the cytochrome P450 pathway. First, 7α-hydroxycholesterol is produced by the action of cholesterol 7α-hydroxylase. (2) Subsequently, several steps mediated by 12α-hydroxylase and 27α-hydroxylase generate the primary BAs. (3) The conjugation with glycine or taurine is mediated by

(Continued)

FIGURE 1 | Continued
the enzymes bile acid CoA synthetase and bile acid-CoA: amino acid N-acyltransferase. These conjugated BAs are excreted into bile by a BA export pump (BSEP) and stored in the gallbladder. **(B)** Bile composition. Conjugated primary BAs (glycocholic, taurocholic, glycochenodeoxycholic and taurochenodeoxycholic acids) are the main components of bile. Cholesterol, fatty acids, bilirubin and phospholipids are present in lower amounts. **(C)** Metabolism of BAs and cholesterol by intestinal bacteria. (4) The first reaction in the metabolism of BAs is the deconjugation or hydrolysis of conjugated BAs, catalyzed by bile salt hydrolases (BSHs). (5) Then, a bile salt 7α-dehydroxylase carries out the conversion of primary BAs to secondary BAs, deoxycholic and lithocholic acids. A part of the cholesterol is absorbed in the duodenum and proximal jejunum, returning to the liver. Remaining cholesterol reaches the large intestine, where it can be further metabolized by the intestinal microbiota or excreted with the feces. (6) Regarding cholesterol metabolism, the main gut microbial activity reaction involves the direct reduction of cholesterol to produce coprostanol, a reaction carried out by cholesterol reductases. (7) The indirect pathway begins with the oxidation of the 3β-hydroxy group by cholesterol oxidases (ChOx) or 3β-hydroxysteroid dehydrogenases/isomerases (HSD) to form 4-cholesten-3-one, and then cholesterol dehydrogenases produce coprostanone. Finally, cholesterol reductases form coprostanol. **(D)** BAs and sterols in feces. The main BAs in feces are secondary BAs, deoxycholic acid and lithocholic acid, with a lower concentration of primary BAs. Feces do also contain products of cholesterol metabolism such as coprostanol and coprostanone, that represent more than 50% of the total fecal sterols.

Islam et al., 2011; Gérard, 2013; Jia et al., 2017; Long et al., 2017), opening a new area of research in the microbiome-host interactions field. Key findings on this microbiota-BA signaling and host health are presented below.

Metabolism of BAs by Intestinal Bacteria

The composition of the BAs pool in humans is determined by the enterohepatic cycle and the microbial metabolism of intestinal BAs. Briefly, the liver synthesizes two primary BAs from cholesterol, cholic acid and chenodeoxycholic acid, which are conjugated to either taurine or glycine before being poured into the bile flow. Conjugated BAs are the primary components of bile, which is stored in the gallbladder before being excreted into the small intestine during digestion. Over 95% of the BAs secreted in bile are reabsorbed in the terminal ileum, returning to the liver through the enterohepatic circulation, and only 5% reach the large intestine, being excreted in feces. In the large intestine, BAs can suffer several microbial-mediated transformations including deconjugation, carried out by bile salt hydrolases (BSHs) that hydrolyze the amide bond, and transformation of primary deconjugated BAs into secondary BAs mainly by a 7α-dehydroxylation (**Figure 1**). Whereas deconjugation reactions are carried out by a broad spectrum of colonic bacteria (**Figure 2** and **Supplementary Figure 1**), 7α-dehydroxylation appears to be restricted to a limited number of intestinal bacteria (Ridlon et al., 2006). Thus, the BAs profile excreted in feces, mainly composed of secondary BAs, largely depends on the gut microbiota metabolism (Perwaiz et al., 2002).

Deconjugation of BAs

Bile salt hydrolases encoding genes have been detected and characterized in diverse gut microbes including species belonging to the genera *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacterium*, among others, being more diverse in members of the phylum Firmicutes (**Figure 2** and **Supplementary Figure 1**) (Jones et al., 2008). BSH activity has been suggested as a BA detoxification mechanism for bacteria, although they may also obtain carbon, nitrogen and even sulfur from BA deconjugation. This latter element has relevance in the production of hydrogen sulfide that may have lasting health consequences as it increases colonocyte turnover and has been associated with inflammation and cancer (Carbonero et al., 2012). Through regulation of key genes involved in cholesterol metabolism and gastrointestinal homeostasis, BSH activity was proposed as a gut microbial activity with capacity to profoundly alter local (gastrointestinal) and systemic (hepatic) host functions as revealed by different studies in mice (Joyce et al., 2014).

7-Dehydroxylation of BAs

The conversion of primary BAs to secondary BAs by 7α-dehydroxylases is probably one of the most physiologically relevant microbial transformations of BAs in humans (Duboc et al., 2013). Through 7α-dehydroxylation, the primary cholic acid is transformed into the secondary deoxycholic acid, and the primary chenodeoxycholic acid is transformed into the secondary lithocholic acid. To date, 7α-dehydroxylation activities have been characterized only in species belonging to the genera *Eubacterium* and *Clostridium,* including the species *Clostridium scindens* and *Clostridium hylemonae* (Ridlon et al., 2010). *C. scindens* is also capable of performing a 7β-dehydroxylation on ursodeoxycholic acid (the 7β epimer of chenodeoxycholic acid), yielding lithocholic acid (Ridlon et al., 2006, 2016).

Other Microbial Enzymatic Activities Acting on BAs

Other BA modifications such as amidation, oxidation-reduction, epimerization, esterification and desulfatation, can be carried out by intestinal microbes. Among them, oxidation-reduction and epimerization have received particular attention as some intestinal microbes synthesize HSD capable of performing reversible oxidation/reduction reactions and hydroxyl groups epimerization (Ridlon et al., 2016). Indeed, BA epimerization reactions have been largely overlooked due to the lack of appropriate analytical methods, although some iso-BAs have been suggested to represent the most abundant BAs in human feces (Hamilton et al., 2007). HSD activities are present in the four major phyla of the intestinal microbiota *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (Wahlström et al., 2016), and the capability to carry out epimerization reactions has been characterized in several intestinal bacteria, including *Clostridium*, *Collinsella*, *Ruminococcus* or *Eubacterium* species (White et al., 1982; Lepercq et al., 2004; Liu et al., 2011; Lee et al., 2013). However, the physiological and functional significance of this metabolic activity remains largely unclear.

Host Health Implications of Microbial Bile Metabolism

The microbial-mediated transformations of BAs at the intestinal level have been shown to be essential for intestinal and

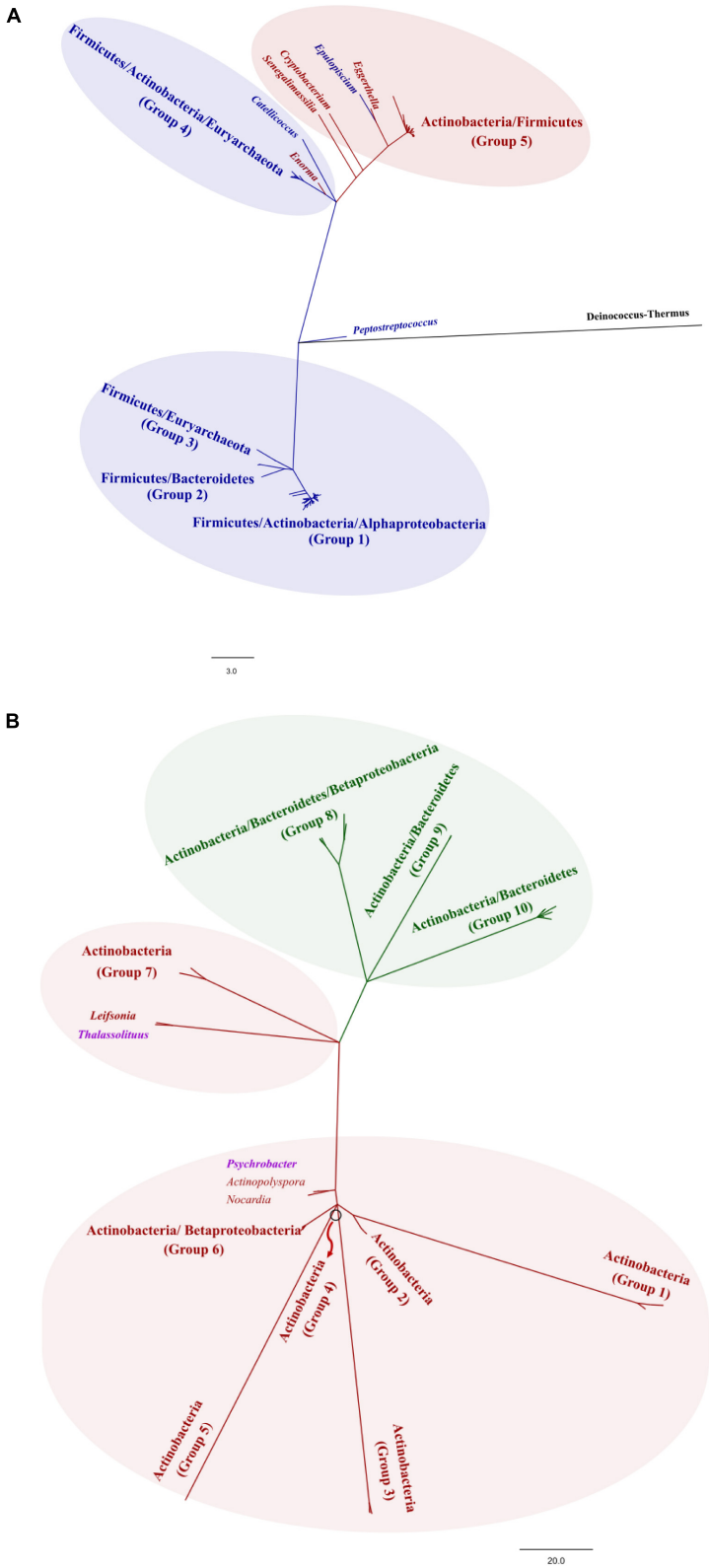

FIGURE 2 | Phylogenetic analysis of bile salt hydrolases (BSH) **(A)** and cholesterol oxidases (ChOx) **(B)**. The construction of the phylogenetic trees and the clustering methods are described in detail in **Supplementary Figure 1**. The edition of the phylogenetic trees was performed with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The trees were divided into groups, depending on the grouping at phylum level.

systemic health maintenance as the intestinal BAs and the gut microbiota mutually influence each other and, accordingly, BA-microbiota crosstalk disruption has been associated with several gastrointestinal, metabolic and inflammatory disorders, including those associated with aging-related decline (Jia et al., 2017), as summarized below.

BAs Metabolism and Inflammation

The gut microbiota-mediated biotransformation of the BA pool regulates BAs signaling by affecting the activation of host BA receptors such as the nuclear receptor farnesoid X receptor (FXR), which governs bile, glucose and lipid metabolism (Gadaleta et al., 2011). Indeed, a disrupted gut microbiota including reduced bile metabolizing bacteria significantly impairs BA metabolism and, consequently, the host metabolic pathways regulated by BA signaling, affecting glucose and cholesterol homeostasis, as well as immune states. Indeed, disorders associated with chronic low-grade inflammation have been linked to gut dysbiosis and altered BA profiles in humans (Chavez-Talavera et al., 2017), although few works have established a connection among specific activities of the microbiota on bile and cholesterol and the physiological alterations observed. As an example, analysis of existing gut metagenomic datasets evidenced that the abundance of the BSH gene *bsh* was significantly reduced in inflammatory bowel disease (IBD) and type-2 diabetes patients (Labbé et al., 2014). Accordingly, IBD patients evidenced increased fecal conjugated and sulphated BAs, and reduced fecal secondary BAs, suggesting the existence of characteristic alterations of bile metabolism associated with gut microbial shifts in IBD (Duboc et al., 2012, 2013). Indeed, some of these changes might be linked to dietary factors such as a diet high in saturated fat and increased sulfur-rich taurine conjugate BAs, which in turn promoted the expansion of the sulphite-reducing pathobiont *Bilophila wadsworthia* in mice. The resulting dysbiosis lead to an associated pro-inflammatory Th1 response and acute colitis in a mouse model, further demonstrating how microbial activity on a particular BA can impact inflammatory states and host health (Devkota et al., 2012).

BAs Metabolism and Colorectal Cancer

The relation between diet, microbial metabolism of BAs and human disorders, including colorectal cancer risk (CRC), is further supported by the fact that dietary fat increases biliary hepatic synthesis and, thus, the quantity of BAs that reach the colon, providing substrate for the synthesis of secondary BAs. These have been described as proinflammatory (Bernstein et al., 2011) and their increase may contribute to the pathogenesis of several gastrointestinal diseases, having been associated with colon polyps (de Kok et al., 1999) and CRC (Bernstein et al., 2005; O'Keefe et al., 2015). Indeed, fecal secondary BAs and microbial genes encoding for 7α-dehydroxylases were more common in African Americans who had a high risk of suffering CRC as compared with rural native Africans (Ou et al., 2013).

BAs Metabolism and Liver Diseases

Several chronic liver-related disorders, including non-alcoholic fatty liver disease (NAFLD), primary sclerosing cholangitis, steatosis and hepatic cancer – frequently associated with obesity – have been related to different intestinal microbial patterns (Adolph et al., 2018). In some of these diseases, an altered liver-microbiota-BAs crosstalk has also been defined. For instance, the ratio between primary and secondary BAs in feces and the levels of conjugated and unconjugated BAs in serum are higher in NAFLD patients (Kakiyama et al., 2013; Mouzaki et al., 2016; Jiao et al., 2018). Interestingly, an increase in taurine metabolizing activities has been evidenced in the gut microbiota of these patients, associated with increased representation of *Bilophila* species, and increased secondary BAs production (Jiao et al., 2018). Additionally, NAFLD is frequently associated with obese patients, for whom specific dysbiosis signatures have been defined (Gao et al., 2018). Consequently, in addition to affecting bile metabolism within the gut, the microbiota might also contribute to NAFLD pathogenesis through other mechanisms including increased energy intake, intestinal permeability and contribution to chronic pro-inflammatory states (Han et al., 2018), which go beyond the scope of this mini review.

Gut Microbiota Shifts in Aging Impact BAs Metabolism and Signaling

Gut microbiota changes throughout life, including loss of diversity, are associated with lifestyle and dietary changes in the elderly population, though they may also modulate elements of aging frailty such as innate immunity or cognitive function. Indeed, recent studies have evidenced that alterations in BAs metabolism accompany these aging-associated microbiota shifts and health decline. For instance, increased fecal excretion of deconjugated BAs has been observed in old mice in association with a shift toward pro-inflammatory states in the gut (Becker et al., 2019). In addition, a reduction in cholic acid and an increase in secondary BAs have been noticed in the serum of patients with Alzheimer disease (AD) (MahmoudianDehkordi et al., 2019), presumably reflecting augmented 7α-dehydroxylase activity in the gut microbiota. In fact, a mice model of AD has evidenced changes in the gut microbiota, including an increase in members of the *Clostridium* group, among which 7α-dehydroxylase activity is frequent (Brandscheid et al., 2017). Nevertheless, comprehensive studies of the gut microbiota and concomitant BAs metabolic changes in AD human cohorts are still lacking.

MICROBIOTA MODULATION OF BILE AND CHOLESTEROL METABOLISM: INFLUENCE ON HOST PHYSIOLOGY AND SIGNALING MECHANISMS INVOLVED

Several studies on germ-free animal models have evidenced the microbiota's involvement in cholesterol and bile metabolism. For instance, the lack of gut microbiota in mice deficient in ApoE (a protein involved in the metabolism of fats) increased the plasma and liver cholesterol levels and reduced hepatic BAs synthesis (Kasahara et al., 2017). Also, the reverse cholesterol

transport from peripheral tissues to the liver is augmented in germ-free mice (Mistry et al., 2017). These observations suggest that specific targeting of the intestinal microbiota could significantly impact cholesterol metabolism and cardiovascular diseases. Furthermore, germ-free animals lack secondary BAs production, and their microbial colonization modifies intestinal and serum BA fingerprinting, increasing total BAs concentrations (Joyce et al., 2014).

Since BAs are ligands of bile-responsive receptors involved in host metabolism, changes in BAs composition orchestrated by the intestinal microbiota activity, may affect their interaction with specific receptors, such as pregnane-activated receptor, vitamin D receptor, sphingosine-1-phosphate receptor, muscarinic receptor (Ridlon et al., 2016). Additionally, FXR, a nuclear transcription factor that regulates a wide range of genes (Teodoro et al., 2011), as well as the plasma membrane-bound G-protein coupled receptor TGR5 (Kawamata et al., 2003), have been remarkably characterized in relation to bile signaling. Both receptors are ubiquitously distributed in several tissues and have different affinity for individual BAs. TGR5 is mainly activated by the secondary BAs litocholic and deoxycholic acids, and recognizes both conjugated and deconjugated forms (Long et al., 2017). The most potent ligand for FXR is chenodeoxycholic acid, with cholic acid, deoxycholic acid and litocholic acid having a lower effect (Wahlström et al., 2016). FXR activation can induce innate immune genes, promote the synthesis of antimicrobial agents acting on the gut microbiota (Inagaki et al., 2006), and regulate BA synthesis (Sinal et al., 2000). On the other hand, TGR5 plays a role in the regulation of BA and energy homeostasis (Wahlström et al., 2016). Therefore, through these receptors, BAs act as signaling factors beyond the GIT. Further, considering that the gut microbiota deeply influences the BAs signature, different microbial communities can differentially impact bile signaling and determine the degree of activation of these receptors, with a concomitant impact on host metabolism. Indeed, BA receptors are currently considered therapeutic targets for several gastrointestinal and hepatic diseases (Firoucci et al., 2007); thus, microbiota-based approaches to modulate their activation may represent novel alternatives for certain disorders and warrant further investigation.

FUTURE PERSPECTIVES: POTENTIAL OF MICROBIOTA-BASED APPROACHES TO MODULATE BILE METABOLISM AND ASSOCIATED CONDITIONS

In light of the recently unearthed gut microbiota-BA-host signaling interactions, microbiota-based approaches, from probiotics to dietary interventions, may become novel strategies to manage specific diseases linked to BAs metabolism dysregulation, as suggested by some *in vivo* studies (Devkota and Chang, 2015; Fukui, 2017). Most studies to date have focused on the potential of probiotics administration to reduce serum cholesterol levels. In this context, administration of probiotic strains to healthy mice increased deconjugation of BAs and fecal excretion (Jeun et al., 2010; Degirolamo et al., 2014) in association with increased BSH activity in the gut and overall modification of the microbiota composition (Degirolamo et al., 2014; Joyce et al., 2014; Tsai et al., 2014; Lye et al., 2017), changes that may have implications for host lipid metabolism. Indeed, a cholesterol-lowering effect was also observed following supplementation of a BSH-positive *Lactobacillus* strain to mice fed high-fat diets (Michael et al., 2017). However, limited studies have been conducted in human subjects in this regard. Remarkably, consumption of a BSH-positive *Lactobacillus* strain significantly reduced cholesterol in hypercholesterolemic subjects (Jones et al., 2012), although the observed effect might be the result of a complex metabolic re-arrangement, rather than solely a consequence of an increase in bile excretion.

Some probiotic interventions have also demonstrated their efficacy to ameliorate liver and inflammatory markers in models of NAFLD and IBD, although results are not yet conclusive (Han et al., 2018; Kobyliak et al., 2018). Besides, the strains tested in most studies were not specifically selected for their activities over bile metabolism, and the impact of the intervention on the fecal or serum BAs profiles, on the fecal microbiota composition or on their metabolic capability over bile and cholesterol, was not always evaluated. This strongly hampers establishing causal relationships between the metabolic activities of the microbiota over these compounds and the physiological effects observed.

Diet is another factor known to affect the gut microbiota and the BAs host signature. For instance, in a dietary intervention study in humans, a diet rich in animal-based fats was associated with increased excretion of secondary BAs, in accordance with an increased overall expression of *bsh* encoding genes in the gut microbiota, and an increase in the representation of potential pathobiont species such as *B. wadsworthia* (David et al., 2014). Thus, dietary strategies aimed at modulating BA metabolism through balancing the microbiota may represent alternative approaches to manage diseases linked to BA dysmetabolism (Ghaffarzadegan et al., 2018). Though these have been scarcely studied in humans, studies in mice models have showed the potential of certain dietary ingredients to modulate gut microbiota and BAs profile. For instance, *Akkermansia muciniphila* enrichment through administration of epigallocatechin-3-gallate prevented diet-induced obesity and regulated bile signaling (Sheng et al., 2018), although the contribution of changes in specific microbial metabolic activities over bile and cholesterol in this model has not been determined.

CONCLUSION

In summary, it has become increasingly clear that BAs exert a much wider range of biological activities than initially recognized and that BAs, gut microbiota and health status are closely linked and hold a yet- underexplored valuable potential to design novel diagnostic and therapeutic approaches based on specific gut microbiota activities. Elucidating the molecular mechanisms underlying the gut microbiota-BA-host health interplay will establish the basis to fully understand the gut microbiota potential to

modulate bile metabolism and host health. Further studies using specifically designed *in vivo* models or human trials, and exploiting microorganisms or activities with demonstrated capacity to specifically act on selected BAs, are necessary for aiding the development of novel microbiome-based approaches for disorders associated with BAs dysregulation.

AUTHOR CONTRIBUTIONS

AM, SD, and BS conceived and organized the manuscript. NM designed the figures. NM, LR, BS, AM, and SD contributed to the writing, critically reviewed the manuscript, and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00185/full#supplementary-material

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Objetivos

Objectives

OBJETIVOS

La microbiota del tracto gastrointestinal humano y su relación con diferentes estados fisiológicos (dieta, edad, trastornos metabólicos, etc.) ha sido objeto de numerosos estudios durante los últimos años, de forma que las poblaciones microbianas del estómago e intestino han sido caracterizadas en detalle. Sin embargo, la microbiota de la bilis y de la vesícula biliar apenas ha sido estudiada hasta el momento, debido fundamentalmente a la dificultad para acceder al material biológico y a la falta de técnicas y protocolos adecuados. Además, las funciones de los microorganismos autóctonos de la bilis y las características que les permiten sobrevivir y colonizar la vesícula biliar no han sido estudiadas.

Teniendo en cuenta estos antecedentes, el **objetivo general** de esta Tesis Doctoral fue caracterizar y estudiar en detalle el microbioma de la bilis, así como determinar su posible relación con la dieta y trastornos biliares, en concreto, la colelitiasis o presencia de cálculos en la vesícula biliar. Para abordar este objetivo general, se establecieron tres objetivos específicos :

Objetivo 1.: Estudio y caracterización de la microbiota biliar humana en distintos estados de salud aplicando técnicas moleculares y -ómicas.

Para esta primera tarea se pusieron a punto técnicas y protocolos para el aislamiento del ADN microbiano presente en muestras de bilis y biopsias de vesícula biliar pertenecientes a dos grupos de estudio: donantes de hígado sin patologías hepatobiliares, que consideramos como grupo control; y pacientes con colelitiasis o

cálculos biliares. Se aplicaron además técnicas metagenómicas y metabonómicas, para describir en profundidad los perfiles de las comunidades microbianas propios de cada grupo, así como las principales actividades y funciones microbianas presentes. Los resultados obtenidos del trabajo correspondiente a este objetivo se presentan en el capítulo 1:

CAPÍTULO 1: *Caracterización de la microbiota biliar humana. Descripción de las modificaciones de esta microbiota asociadas a la presencia de cálculos biliares. Perfil metabólico de la bilis en distintos estados de salud.*

Objetivo 2.: Evaluar la relación entre la microbiota biliar y la dieta.

En el segundo objetivo de esta tesis nos propusimos utilizar la información obtenida en el objetivo 1 para establecer la posible relación existente entre el perfil de la microbiota biliar y la dieta consumida en el grupo de pacientes con colelitiasis. Se analizó la correlación entre los componentes de la dieta y los principales grupos bacterianos presentes en la bilis de estos sujetos. Los resultados de este subobjetivo se muestran en el capítulo 2.

CAPÍTULO 2*: Análisis de la relación entre los componentes de la dieta y el perfil microbiano biliar en sujetos con colelitiasis.*

Objetivo 3.: Aislar y caracterizar microorganismos presentes en la bilis humana.

En el último objetivo de esta tesis nos enfocamos en optimizar las condiciones para aislar y cultivar en el laboratorio microorganismos presentes en muestras de bilis humana. Se utilizaron diversos medios de cultivo que permitieron aislar representantes de los principales filos bacterianos. Dos de ellos, pertenecientes a la clase *Clostridiales*,

fueron caracterizados y estudiados en profundidad desde el punto de vista genómico y metabólico. Los resultados de este último objetivo se presentan en los capítulos 3 y 4 de esta tesis.

CAPÍTULO 3*: Descripción de un nuevo taxón aislado de bilis humana, para la que se propone el nombre de Ruminocoides biliarensis* IPLA60002*.*

CAPÍTULO 4*: Relación entre dos aislados de bilis humana: Ruminococcus gauvreauii IPLA60001 y Ruminocoides biliarensis IPLA60002.*

Trabajo experimental

Experimental work

Capítulo 1

Chapter 1

CAPÍTULO 1

Caracterización de la microbiota biliar humana. Descripción de las modificaciones de esta microbiota asociadas a la presencia de cálculos biliares. Perfil metabólico de la bilis en distintos estados de salud

En el primer capítulo de esta Tesis Doctoral abordamos el estudio de la microbiota biliar humana. Se utilizaron dos grupos de estudio: un primer grupo de 26 sujetos donantes de hígado, sin patologías hepatobiliares descritas, de los que se tomaron muestras de bilis y biopsia de vesícula durante la cirugía de trasplante de hígado y que consideramos como grupo de referencia o control; y un segundo grupo de 14 pacientes diagnosticados de colelitiasis, de los que se tomaron las mismas muestras durante la colecistectomía. Con el fin de homogeneizar en la medida de los posible los grupos, se aplicaron una serie de criterios de inclusión en el grupo de referencia, de modo que finalmente el grupo control quedó formado por 13 sujetos, todos ellos menores de 80 años, con una estancia en la Unidad de Cuidados Intensivos (UCI) menor de 48 horas y que hubieran recibido tratamiento antibiótico previo a la cirugía durante menos de 24 horas.

Debido a que la bilis presenta una alta heterogeneidad en su composición, fue necesario optimizar un protocolo de extracción de ADN total de bilis. Se cuantificó mediante qPCR la concentración bacteriana presente en estas muestras determinándose una carga bacteriana del orden de 10^3 bacterias/ml. Posteriormente, mediante metagenómica filogenética se determinó el perfil microbiano característico de

cada grupo, que mostró diferencias significativas en la abundancia relativa de diversos taxones. Además, se determinó el perfil funcional mediante metagenómica total o *shotgun* de tres muestras de bilis pertenecientes al grupo control, con el fin de analizar las principales actividades presentes en el microbioma. Finalmente se analizó el perfil metabólico biliar representativo de cada grupo de estudio.

Los resultados de este trabajo mostraron la presencia, por primera vez, de una microbiota biliar en sujetos sin patologías hepatobiliares o gastrointestinales previas, además de mostrar diferencias entre los perfiles microbianos y metabólicos de los dos grupos de estudio. Los resultados de este primer capítulo de la tesis se presentan en el artículo publicado en la revista *Microbiome*:

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The human gallbladder microbiome is related to the physiological state and the biliary metabolic profile

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Abstract

Background: The microbial populations of the human intestinal tract and their relationship to specific diseases have been extensively studied during the last decade. However, the characterization of the human bile microbiota as a whole has been hampered by difficulties in accessing biological samples and the lack of adequate methodologies to assess molecular studies. Although a few reports have described the biliary microbiota in some hepatobiliary diseases, the bile microbiota of healthy individuals has not been described. With this in mind, the goal of the present study was to generate fundamental knowledge on the composition and activity of the human bile microbiota, as well as establishing its potential relationship with human bile-related disorders.

Results: Human bile samples from the gallbladder of individuals from a control group, without any record of hepatobiliary disorder, were obtained from liver donors during liver transplantation surgery. A bile DNA extraction method was optimized together with a quantitative PCR (qPCR) assay for determining the bacterial load. This allows the selection of samples to perform functional metagenomic analysis. Bile samples from the gallbladder of individuals suffering from lithiasis were collected during gallbladder resection and the microbial profiles assessed, using a 16S rRNA gene-based sequencing analysis, and compared with those of the control group. Additionally, the metabolic profile of the samples was analyzed by nuclear magnetic resonance (NMR). We detected, for the first time, bacterial communities in gallbladder samples of individuals without any hepatobiliary pathology. In the biliary microecosystem, the main bacterial phyla were represented by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Significant differences in the relative abundance of different taxa of both groups were found. Sequences belonging to the family *Propionibacteriaceae* were more abundant in bile samples from control subjects; meanwhile, in patients with cholelithiasis members of the families *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Veillonellaceae* were more frequently detected. Furthermore, the metabolomics analysis showed that the two study groups have different metabolic profiles.

(Continued on next page)

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Conclusions: Our results indicate that the gallbladder of human individuals, without diagnosed hepatobiliary pathology, harbors a microbial ecosystem that is described for the first time in this study. Its bacterial representatives and metabolites are different from those detected in people suffering from cholelithiasis. In this regard, since liver donors have been subjected to the specific conditions of the hospital's intensive care unit, including an antibiotic treatment, we must be cautious in stating that their bile samples contain a physiologically normal biliary microbiome. In any case, our results open up new possibilities to discover bacterial functions in a microbial ecosystem that has not previously been explored.

Keywords: Bile microbiota, Cholelithiasis, Microbial bile metabolites, Gallstones patients

Background

Bile is a biological fluid, mainly constituted by bile acids (BA), cholesterol, phospholipids, and proteins. Bile is synthesized in the liver and stored in the gallbladder. Its main physiological function is to facilitate fat absorption in the small intestine during digestion [1]. Several bile-related disorders can modify bile functionality, but the most frequent is the generation of gallstones, either in the gallbladder or in the bile duct, the so-called cholelithiasis, with a prevalence among adults normally above 10% [2]. Gallstone formation is attributed to a combination of environmental and genetic causes, typically linked to cholesterol supersaturation. The primary constituent of gallbladder stones is cholesterol, whereas calcium bilirubinate predominates in pigmented bile duct stones. To date, cholecystectomy remains the most effective treatment option for chronic carriers with gallbladder lithiasis [3].

The landscape of microorganisms inhabiting our gastrointestinal tract has been extensively studied during the last few years [4–6]. Furthermore, we currently know that some biological fluids in different locations in our body also have an autochthonous microbiota that suffers alterations depending on the physiological state of the host [7, 8]. However, very little is known about the microbial inhabitants of human bile. Current knowledge is mainly limited to a few species of cultivable bacteria that have been associated with physiological disorders such as cholelithiasis. In this regard, our understanding of the exact contribution of bacteria in gallstone formation is very limited, although a possible association between bacteria and the etiology of gallstones has been suggested and enterobacteria are frequently isolated from bile aspirates or gallbladder bile from cholelithiasis patients [9–12]. Only very recently, a few authors have analyzed, using culture-independent techniques, the microbiota of the biliary tract and the gallbladder and its association with bile-related diseases. In these studies, it was shown that *Enterobacteriaceae* members are abundant microorganisms in the biliary tract of acute cholecystitis and gallstone patients [13, 14]. It was also observed that oral cavity and respiratory tract microorganisms were more prevalent than intestinal microorganisms in the microbiota of the common bile duct of gallstone disease patients [15] or that the genera *Prevotella*, *Streptococcus*, *Veillonella*, *Fusobacterium*, and *Haemophilus* are prevalent in the bile microbial communities of the bile duct of primary sclerosing cholangitis patients [16], among other findings [13, 17, 18]. All these works demonstrated that a microbial community is present in the human gallbladder and the bile duct in some hepatobiliary disorders. Nevertheless, the human bile microbiota in individuals without any bile or liver-related disorder had not been explored until now.

In a previous work, we characterized the gallbladder microbiota of healthy pigs [19]. This fact, together with our experience in bacterial bile resistance mechanisms, led us to believe that the human gallbladder may contain an autochthonous microbiota, and the ability of resident bacteria to live in, survive, and colonize bile must necessarily reflect a specific bacterial physiology, well adapted to face the environmental challenges found in such a specific niche, and thus different from the rest of the microorganisms inhabiting other gastrointestinal habitats. Thus, we performed the first study focused on the characterization of human gallbladder microbiota in a group of individuals free from hepatobiliary diseases (control group) vs a group with diagnosed cholelithiasis (non-lithiasis vs lithiasis), with the aim of generating new knowledge about bile microbial profiles, functions, and activities and to contribute to complete our understanding of the complex landscape of different microbiotas in the human body (Table 1).

Results

Bacterial load in bile samples

In order to perform further molecular analyses, we firstly optimized a method for DNA extraction and quantification from human bile. For this purpose, bile samples from liver donors that did not meet the inclusion criteria for investigating the microbiome (see the "Methods" section for details) were used to extract biliary DNA following a phenol-based protocol. A quantitative PCR (qPCR) assay was developed to determine the bacterial load. General primer pairs, targeting the 16S rRNA gene of prokaryotic microorganisms and the18S rRNA gene

Table 1 Demographic and clinical features of cholelithiasis patients

Subject	Age (years)	Sex	Clinical history	Altered parameters in blood test (with higher levels than those considered normal)[a]
C-01	73	F	Non-cirrhotic portal hypertension. Alteration of liver function due to chemotherapy. Digestive hemorrhage (2 years before collecting the bile sample)	ALP, AST, and GGT
C-02	67	M	Hemicolectomy (3 years before collecting the bile sample)	GGT, TB, DB, and TG
C-03	60	F	Hypercholesterolemia	–
C-04	58	F	–	AST and GGT
C-05	67	M	–	DB and TB
C-06	55	F	Obesity type II (BMI 38.5)	ALT, AST, Glu, GGT, and TC
C-07	27	F	–	–
C-08	50	M	Obesity type I (BMI 32.2) Acute lithiasic pancreatitis (7 months before collecting the bile sample)	GGT, LDL, TC, and TG
C-09	38	F	–	LDL and TC
C-10	33	F	–	–
C-11	36	F	Obesity type II (BMI 36.1)	ALT and GGT
C-12	44	F	–	TG
C-13	50	F	–	ALT and AST
C-14	70	F	–	DB, GGT, and TB

– no relevant information, *BMI* body mass index, *ALP* alkaline phosphatase, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *DB* direct bilirubin, *GGT* gamma-glutamyl transferase, *Glu* glucose, *LDL* cholesterol LDL, *TB* total bilirubin, *TC* total cholesterol, *TG* triglycerides, *F* female, *M* male
[a]At the time of sampling

of eukaryotic cells, were used. DNA quantification and recovery after artificial enrichment of these bile samples with different amounts of bacterial cells was performed. Total DNA was extracted and used as a template for qPCR validation experiments, and the threshold cycle (Ct) values were used to calculate the efficiency and the limit of quantification. Dilution series of eukaryotic and prokaryotic DNA, obtained from cell cultures of *Lactococcus lactis* NZ9000 and HT29 cells, allowed us to perform a linear regression analysis based on the Ct data, yielding a high coefficient of determination ($R^2 > 0.99$). The lowest concentration of the standard was close to the Ct value of the non-template control, so 10^2 bacterial cells/ml was established as the limit of detection of our assay. Afterwards, DNA samples obtained from the bile of a group of 13 liver donors (see the "Methods" section for general characteristic of the selected control group of donors) were analyzed with the qPCR assay and the bacterial load and the ratio of prokaryotic DNA with respect to eukaryotic DNA established. We observed that bacterial cell counts between individuals did not vary substantially (Fig. 1). The proportion of eukaryotic DNA was superior by at least tenfold compared to prokaryotic DNA in all cases.

Overall, the qPCR assay was useful to determine the levels of total bacteria in bile and allowed us to select samples for performing shotgun sequencing.

Functional metagenomic analysis of bile samples

Three bile samples (encoded in this study as H-04, H-05, and H-06) were selected based on the results obtained by qPCR (16S rRNA gene/18S rRNA gene ratio ≤ 0.01) and DNA concentration and quality (≥ 15 ng/μl and a ratio 260/280 ≥ 1.6). The metagenomic analysis performed by shotgun sequencing resulted in a mean of 2,556,103 raw sequences per sample obtained, of which a total of 7,169,256 proved to be of high quality considering the three samples together. After the removal of human DNA, the numbers of final reads were 1,963,038 for H-04, 342,649 for H-05, and 80,384 for H-06. These sets of sequences served as input for further analyses. Taxonomy assignment based on coding reads against non-redundant NCBI database with BLASTx and the metagenome analyzer MEGAN5 revealed that most of the quality-filtered reads resulted from bacteria, although the presence of archaea, virus, and other eukaryotic microorganisms was also noticed (Table 2). Within the bacteria domain, four main phyla, *Firmicutes*,

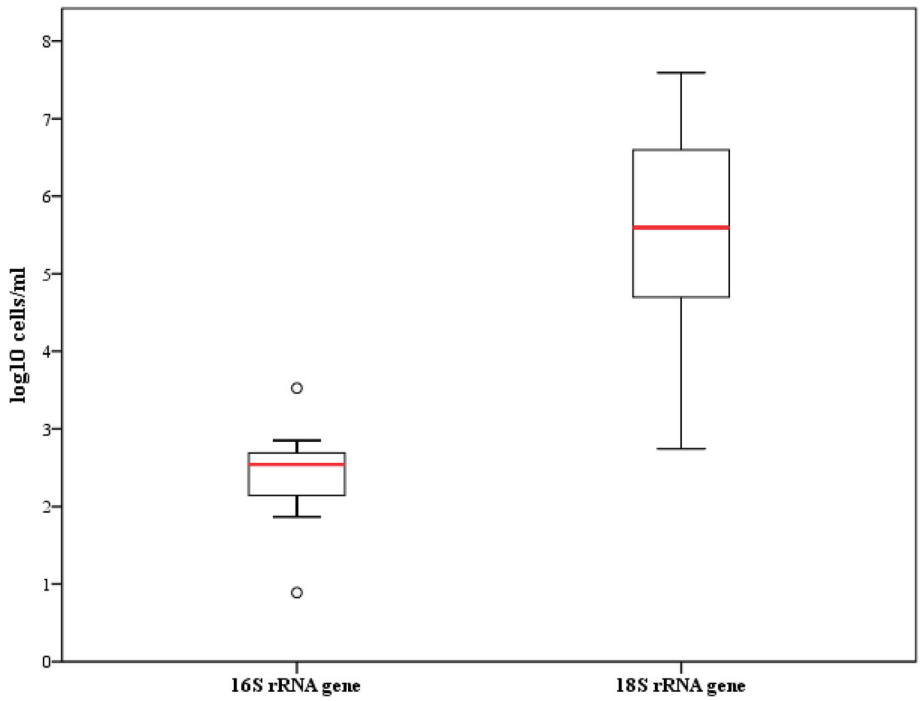

Fig. 1 Boxplots representing 16S rRNA gene and 18S rRNA gene levels in the control group ($n = 13$). The central rectangles represent interquartile ranges (IQR), the lines inside the rectangles show the median, and the whiskers indicate the maximum and minimum values. The dots outside the rectangles are suspected outliers (> 1.5 × IQR). Statistically significant differences (p value < 0.05) between the two variables (16S rRNA gene and 18S rRNA gene) were found (Mann-Whitney U test)

Bacteroidetes, *Actinobacteria*, and *Proteobacteria*, predominate in the bile microbiome. Other phyla, such as *Verrucomicrobia*, *Chlamydiae*, *Acidobacteria*, *Planctomycetes*, *Cyanobacteria*, *Spirochaetes*, and *Fusobacteria*, were noticed in the three samples at low percentages (mean between 0.05 and 0.5%) after normalization against the total number of sequences assigned to bacteria.

When performing the gene annotation and functional classification of the metagenomes obtained for the three samples, assignation results for the main functional categories of COG (Clusters of Orthologous Groups) were closely related. The COG functional classes, as observed in Fig. 2, were also similar to those found in the intestinal microbiome, although between 24 and 31% of the sequences were annotated as "unknown function." The search for activities related to the metabolism of cholesterol and bile salts in the MetaCyc metabolic pathway database showed differences between the three samples. As shown in Table 3, a clear difference was observed for the superpathway of cholesterol biosynthesis and to a minor extent glycocholate metabolism, with 2 to 4 times more genes related with this pathway in samples H-04 and H-05, respectively, as compared to H-06. Additionally, when comparing with similar activities against metagenomes from fecal samples of five healthy individuals (see the "Methods" section for accession numbers and reference), differences were observed for cholesterol oxidase genes, more abundant in bile. On the contrary, bacterial genes for glycocholate metabolism were more abundant in the fecal datasets.

Bile microbiota load and compositional profiles: comparison between lithiasis and non-lithiasis

To compare the human gallbladder microbiota in individuals with no diagnosed hepatobiliary diseases vs a disease state, bile samples were obtained during surgery from patients ($n = 14$) diagnosed with cholelithiasis. The bacterial load was determined by qPCR, as for the control group ($n = 13$), and the results are shown in Fig. 3. No statistical differences were observed between either group for the total number of bacterial cells (Mann-Whitney U test, p value < 0.05), but much more variation (reaching levels of 10^4–10^6 in some cases) was found in the bile of patients with gallstones.

Bile microbial composition was determined by high-throughput sequencing by paired-end Illumina technology of 16S rRNA gene amplicons. In order to evaluate the potential DNA contamination associated to low bacterial biomass samples, three "blank" controls with no template DNA (adding ultrapure molecular biology grade water instead of bile for the DNA extraction

Table 2 Percentages (%) of quality and human filtered reads assigned at the ranks of superkingdom and phylum from three control bile samples subjected to shotgun metagenomics

Taxonomic assignment		Sample code		
Superkingdom	Phylum[a]	H-04	H-05	H-06
Not assigned		32.67	20.96	33.13
Archaea		0.02	0.06	0.04
Eukaryota		2.11	0.21	1.83
Viruses		0.12	5.58	0.20
Bacteria		65.08	73.19	64.79
	Actinobacteria	13.17	2.91	12.23
	Bacteroidetes	34.36	34.22	35.60
	Firmicutes	22.47	56.51	23.10
	Proteobacteria	27.80	5.46	27.06
	Verrucomicrobia	0.16	0.43	0.25
	Chlamydiae	0.73	0.01	0.55
	Acidobacteria	0.18	0.04	0.14
	Planctomycetes	0.20	0.05	0.20
	Spirochaetes	0.14	0.07	0.12
	Fusobacteria	0.09	0.05	0.10
	Tenericutes	0.05	0.08	0.09
	Deinococus-Thermus	0.02	0.00	0.01
	Fibrobacteres	0.00	0.00	0.02
	Synergistetes	0.00	0.02	0.00
	Cyanobacteria	0.11	0.03	0.08
	Chloroflexi	0.03	0.01	0.03
	Chlorobi	0.00	0.00	0.02
	Nitrospirae	0.00	0.00	0.01
	Unclassified bacteria	0.49	0.21	0.38

[a]Abundances of different phyla are related to total assigned bacteria sequences

protocol) were also processed for sequencing. Bacterial profiles and diversity from bile of both groups were compared. On average, a total of 74,885 raw reads per sample were obtained in the control group, and 100,510 in the cholelithiasis group. After quality and chimera filtering, a mean of 63,473 and 41,997 high-quality partial 16S rRNA gene sequences were retrieved, respectively. Sequences were classified, using QIIME and SILVA database. In accordance with the shotgun metagenome results, the main phyla found in bile were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Significant differences in the relative abundance of different taxa present in the bile of both groups were found after the application of the Metastats statistical method with a false discovery rate (FDR correction), adjusted following the Benjamini-Hochberg method to 0.25. At phylum level, *Bacteroidetes* was statistically less abundant in the bile of control subjects (13.49% with respect to 24.00% in cholelithiasis). Sequences belonging to the family *Propionibacteriaceae* were more abundant in bile samples from the control group (mean relative abundance 10.77%) compared with samples from patients with stones in the gallbladder (mean relative abundance 0.59%); meanwhile in patients, members of the families *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Veillonellaceae* were more frequently detected (10.21%, 3.23%, 2.36%, and 1.78%, respectively) (Table 4). Classification was assigned to the genus level when possible; otherwise, the closest taxonomic rank was given, preceded by unknown member "U. m." (Table 5). In cholelithiasis patients, within the *Bacteroidaceae* family, *Bacteroides* was the genus that showed a significantly higher proportion, as compared with the control group (mean 10.21% vs 2.74% of relative abundance, respectively, p value = 0.001). Within the family *Veillonellaceae*, the genus *Dialister* also showed a significantly higher representation in the group of patients with gallstones. Moreover, a significantly higher percentage of assigned reads to the enterobacteria *Escherichia-Shigella* was observed in the bile of these patients. However, sequences assigned to other genera from the Alpha (*Bradyrhizobium*, *Methylobacterium*, and *Sphingomonas*) and Gamma (*Acidibacter* and *Brevundimonas*) divisions of the *Proteobacteria* phylum were more abundant in the control group. In contrast, no amplification was observed from any of the three blank controls included in this study. In this regard, to further support the existence of an autochthonous biliary microbiota, different cultivation techniques were used. We were able to identify several isolates from a selection of bile samples, and in all cases, the microbial profile determined by 16S rRNA gene sequencing contained sequences (at genus or family level) that matched with the corresponding isolates of each sample (data not shown)

The OTU-based microbial diversity was estimated. The intra-subject alpha diversity was estimated on data rarefied to the sequencing depth obtained in the sample with the lowest number of sequences (i.e., 20,000). The Shannon index (H) was determined for each sample. The median of this diversity index was statistically higher in the bile of the control group than that obtained in the patients with gallstones (Mann-Whitney *U* test, p value = 0.038) (Fig. 4). On the other hand, after applying Principal Coordinate Analysis (PCoA) to the weighted UniFrac distance matrix generated from the comparison between the microbiota identified in bile samples of cholelithiasis patients vs non-cholelithiasis (control group), we noticed that the non-cholelithiasis bile samples grouped together showing more similarities to each other, than to those samples from the cholelithiasis patients (Fig. 5). When analysis of molecular variance (AMOVA) was used to assess the statistical significance of the spatial separation observed, significant differences in

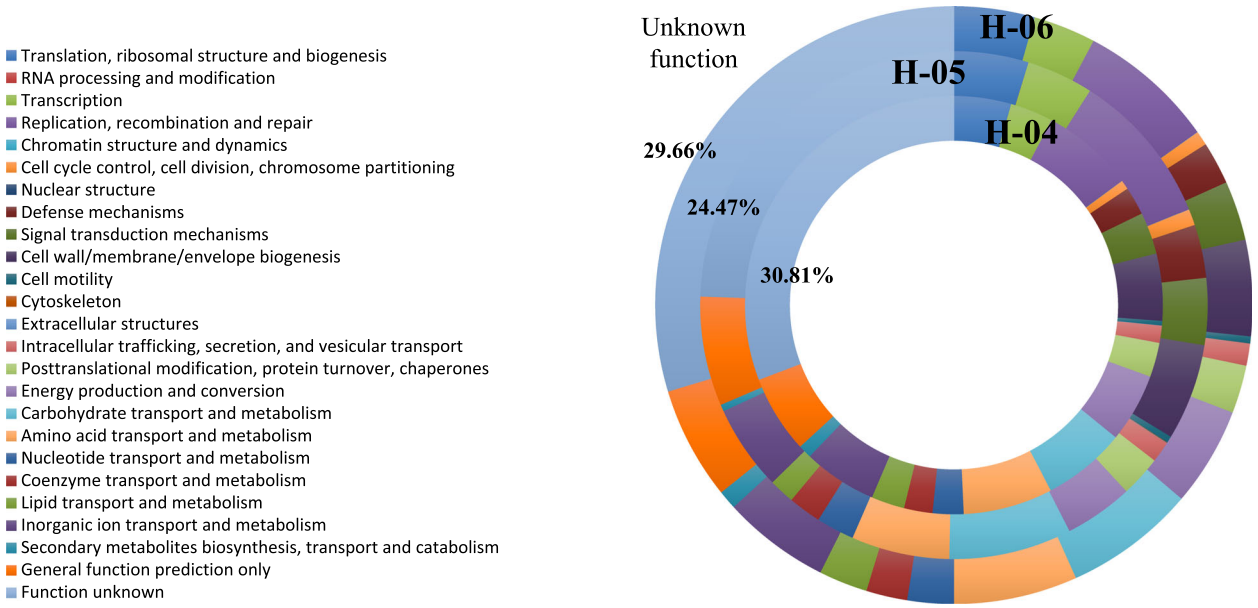

Fig. 2 Distribution of Clusters of Orthologous Groups (COGs) in three bile samples from control group (H-04, H-05, and H-06). The results show the percentage of sequences assigned to different metabolic functions (relative to all sequenced microbes). Secondary metabolite biosynthesis includes antibiotics, pigments, and non-ribosomal peptides. Inorganic ion transport and metabolism includes phosphate, sulfate, and various cation transporters

Table 3 Microbial functional genes related to the metabolism of cholesterol and BA assigned (ratio 1/10,000 reads) through MetaCyc metabolic pathway database in three metagenomes from human bile and five fecal metagenomes from healthy subjects from a previous study [20]

	Bile samples			Fecal samples					Average bile	Average gut
Pathway	H-04	H-05	H-06	2HS	26HS	30HS	31HS	32HS		
Cholesterol biosynthesis I	0.13	0.02	0.29	0.00	0.00	0.00	0.08	0.00	0.15 ± 0.11	0.02 ± 0.03
Cholesterol biosynthesis II (via 24,25-dihydrolanosterol)	0.13	0.02	0.29	0.00	0.00	0.00	0.08	0.00	0.15 ± 0.11	0.02 ± 0.03
Cholesterol biosynthesis III (via desmosterol)	0.13	0.02	0.29	0.00	0.00	0.00	0.08	0.00	0.15 ± 0.11	0.02 ± 0.03
Superpathway of cholesterol biosynthesis	0.37	3.29	15.31	0.12	0.21	0.25	1.06	0.25	6.32 ± 6.47	0.38 ± 0.34
Superpathway of cholesterol degradation I (cholesterol oxidase)	1.06	0.17	0.21	0.41	0.46	0.39	0.48	0.71	0.48 ± 0.41	0.49 ± 0.11
Superpathway of cholesterol degradation II (cholesterol dehydrogenase)	1.52	0.34	0.36	0.54	0.5	0.47	0.62	0.81	0.74 ± 0.55	0.59 ± 0.12
Cholesterol degradation to androstenedione I (cholesterol oxidase)	0.35	0.02	0.07	0.00	0	0.00	0.01	0.02	0.15 ± 0.15	0.01 ± 0.01
Cholesterol degradation to androstenedione II (cholesterol dehydrogenase)	0.74	0.05	0.21	0.00	0.00	0.00	0.06	0.02	0.33 ± 0.29	0.02 ± 0.02
Bile acid biosynthesis, neutral pathway	0.61	0.07	0.29	0.06	0.06	0.02	0.11	0.05	0.32 ± 0.22	0.06 ± 0.03
Glycocholate metabolism (bacteria)	1.58	2.71	0.72	10.58	15.03	12.4	15.53	12.35	1.67 ± 0.81	13.18 ± 1.84
Cholate degradation (bacteria, anaerobic)	0.35	0.46	0.14	0.97	0.77	0.41	0.62	0.38	0.32 ± 0.13	0.63 ± 0.22

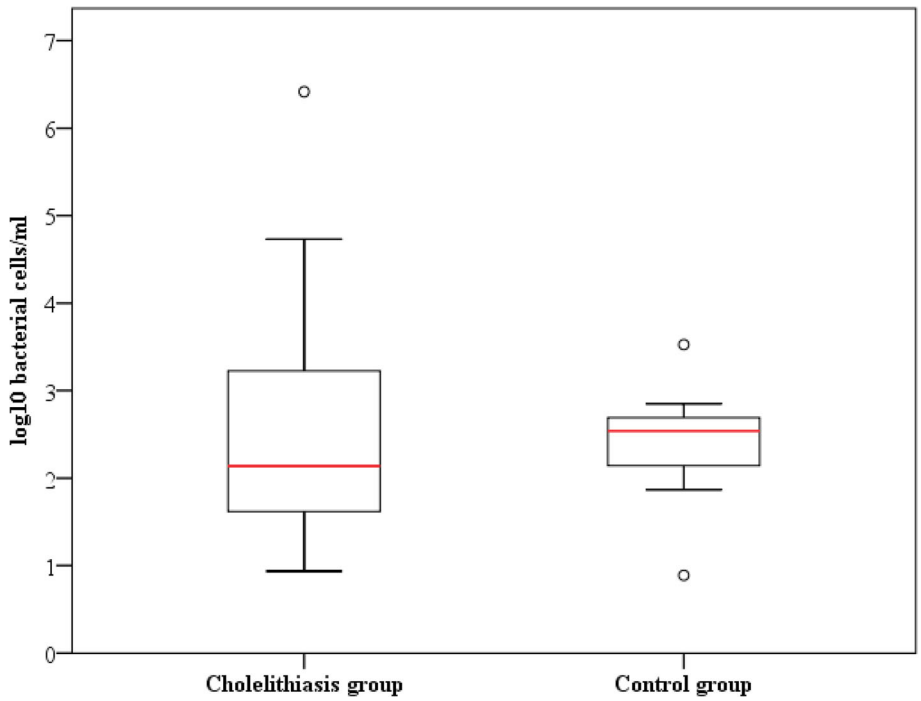

Fig. 3 Boxplots representing 16S rRNA gene levels between cholelithiasis ($n = 14$) and control group ($n = 13$). The central rectangles represent interquartile ranges (IQR), the lines inside the rectangles show the median, and the whiskers indicate the maximum and minimum values. The dots outside the rectangles are suspected outliers (> 1.5 × IQR). Statistically significant differences (p value < 0.05) between the two groups of the study were not found (Mann-Whitney U test)

the microbial clustering between both groups were revealed (p value < 0.001).

Bile metabolomics

Neither the pH values nor the UV/visible spectra showed statistical differences between bile samples of both groups (data not shown). A pH mean value of 7.51 ± 0.88 was obtained for the bile samples. In relation to the absorbance spectra, two samples from the cholelithiasis group (C-09 and C-10) showed a blood-stained aspect, showing a maximum in 415 nm. This was not observed in any of the bile samples from the control group.

The assignment of the signals from ^{1}H-NMR spectrum is depicted in Table 6. Signals from glycine/taurine-conjugated BA, phosphatidylcholine, unspecific methylene groups from glycerides, cholesterol, and primary BA characterized the spectra of the samples of gallbladder bile. Considering the signals of the whole spectra, a statistically significant separation between bile samples of the two groups was detected (p value < 0.05) by principal component analysis (PCA) (Fig. 6). Besides, the split of the spectrum in two sections (0.15–4.20, aliphatic; 5.00–10.00, aromatic) led to a significant discrimination of the samples depending on types of individuals (patients vs controls). Therefore, the differences in the aliphatic section could be related to the content of chenodeoxycholic (CDCA), deoxycholic acid (DCA), and cholic acids (CA), whereas the differences within the aromatic section would be caused by their glycine- and/or taurine-conjugated forms. The highest percentage of explained variance was obtained by considering the whole spectrum, thus explaining a total of 76.23% in a plot of PC1 (63.06 %) vs PC2 (13.17 %). PCA results evidenced that the second principal component was closely related to bile sample type (controls "H" vs cholelithiasis "C"). It was observed that the control samples showed positive values for PC2, whereas those from patients with cholelithiasis presented negative values (Fig. 6).

Discussion

The advent and refinement of novel sequencing methodologies during the 21st century, the so called next-generation sequencing (NGS) methods, together with bioinformatics-based analyses, have revolutionized how we study the human microbiome. Thanks to these techniques, we are able to extract the information encrypted in the genomes of the intestinal microbiota members, as well as to depict the diversity and potential metabolic capabilities of this microbial community. The human fecal microbiota has largely been studied, and numerous links have been established between these microbial communities and different physiological conditions, a

Table 4 Differences in microbial relative abundance (% of sequences) in bile at family level between cholelithiasis patients and the control group. Only families with a mean relative abundance higher than 0.5% are presented. Only families that were detected in more than half of the samples in each group were considered for the analysis

Family	Cholelithiasis group[a]	Control group	*p* value[b]
Acidaminococcaceae	0.63 ± 0.32	0.21 ± 0.26	0.148
Alcaligenaceae	0.56 ± 0.98	0.07 ± 0.15	0.040
Bacteroidaceae	10.21 ± 6.94	2.74 ± 3.77	0.002
Bacteroidales S247 group	2.98 ± 2.14	1.61 ± 2.34	0.135
Beijerinckiaceae	0.03 ± 0.09	0.50 ± 0.64	0.006
Bifidobacteriaceae	3.17 ± 5.37	7.60 ± 17.99	0.607
Bradyrhizobiaceae	0.18 ± 0.44	7.91 ± 9.77	0.001
Caulobacteraceae	0.29 ± 0.28	5.33 ± 6.15	0.002
Chitinophagaceae	0.74 ± 1.05	5.11 ± 5.14	0.004
Christensenellaceae	0.60 ± 0.42	0.32 ± 0.83	0.391
Clostridiales vadin BB60 group	0.69 ± 0.58	0.35 ± 0.51	0.305
Comamonadaceae	0.09 ± 0.20	0.68 ± 0.72	0.009
Enterobacteriaceae	5.35 ± 10.55	1.69 ± 1.14	0.122
Haliangiaceae	0.01 ± 0.04	0.54 ± 0.88	0.006
Helicobacteraceae	10.85 ± 8.47	6.29 ± 9.33	0.208
Lachnospiraceae	15.16 ± 8.06	7.96 ± 11.33	0.070
Methylobacteriaceae	0.04 ± 0.04	1.81 ± 3.77	0.001
Pasteurellaceae	10.43 ± 27.14	0.02 ± 0.04	0.028
Porphyromonadaceae	2.36 ± 1.10	0.96 ± 1.23	0.008
Prevotellaceae	3.23 ± 1.60	0.79 ± 1.16	0.001
Propionibacteriaceae	0.59 ± 0.41	10.77 ± 18.48	0.006
Rikenellaceae	4.17 ± 2.36	2.01 ± 2.59	0.039
Ruminococcaceae	11.23 ± 5.59	5.23 ± 7.92	0.031
Sphingomonadaceae	0.04 ± 0.06	2.89 ± 4.90	0.001
Streptococcaceae	7.19 ± 22.23	14.03 ± 24.29	0.483
Veillonellaceae	1.78 ± 1.53	0.22 ± 0.42	0.002
U. m. of *Rhizobiales* order	0.01 ± 0.03	1.03 ± 1.92	0.001
U. m. of *Sphingomonadales* order	0.34 ± 0.28	1.20 ± 1.68	0.049
U. m. of *Xanthomonadales* order	0.13 ± 0.33	3.15 ± 4.24	0.002

[a]Mean relative abundance ± standard deviation

[b]Statistical significance was considered with a *p* value below 0.05, adjusted for multiple hypothesis testing using a false discovery rate (FDR) correction of 0.25 (grey shadow)

process that has been favored, at least in part, due to the ease of obtaining non-invasive biological samples. The characterization of other human microbial niches has been hampered by difficulties in accessing solid biopsies or liquid fluids and the lack of adequate methodologies to assess molecular studies in microbial ecosystems with a low bacterial load. Within these "other microbiotas," recent evidence suggests that a variety of internal biological fluids possess a native microbiota, among which human milk and blood have received particular attention [7, 8, 21, 22]. However, caution should be taken for the presence of contaminating DNA when applying sequence-based techniques to the study of the microbiome in low biomass environments [23]. In our study, we analyzed the microbial load of human bile by qPCR. Remarkably, our quantitative results might be underestimated as we observed that, after enrichment of bile samples with different amounts of *L. lactis* cells, the bacterial cells quantified by 16S rRNA gene analysis were usually lower than the total number of cells added to the bile sample, suggesting that the primer choice or the cell lysis method could influence quantitative estimations.

To date, the microbiota of the human gallbladder has scarcely been studied, and current results are limited to a few hepatobiliary pathological conditions in which the gallbladder has been resected during surgery. Cultivation and culture-independent techniques have shown that enterobacteria are frequently isolated and detected in cholecystitis and cholelithiasis samples, although the lack of optimal culture conditions suitable for other microorganisms could allow those adapted to the biliary niche to pass unnoticed [11–13]. Remarkably, besides some fecal bacterial indicators of cholelithiasis, such as the genera *Roseburia* and *Oscillospora* [24], one of the few bacteria frequently associated with the presence of

Table 5 Differences in microbial relative abundance (% of sequences) in bile at genus level between cholelithiasis patients and the control group. Only genera with a mean relative abundance higher than 0.5% are presented. Only genera that were detected in more than half of the samples in each group were considered for the analysis

Genera	Cholelithiasis group[a]	Control group	*p* value[b]
Acidibacter	0.13 ± 0.33	3.15 ± 4.24	0.005
Actinobacillus	3.34 ± 12.03	0.00 ± 0.00	0.225
Alistipes	3.85 ± 2.16	1.72 ± 2.31	0.031
Alloprevotella	0.53 ± 0.43	0.23 ± 0.35	0.346
Bacteroides	10.21 ± 6.94	2.74 ± 3.77	0.001
Barnesiella	1.45 ± 0.90	0.73 ± 1.05	0.084
Bifidobacterium	3.01 ± 502	7.60 ± 17.98	0.599
Blautia	1.15 ± 1.22	0.79 ± 1.63	0.560
Bradyrhizobium	0.18 ± 0.44	6.90 ± 8.56	0.004
Brevundimonas	0.15 ± 0.20	2.80 ± 3.31	0.003
Christensenellaceae R.7 group	0.56 ± 0.44	0.08 ± 0.20	0.040
Coprococcus 3	1.19 ± 0.69	0.35 ± 0.67	0.009
Dialister	1.49 ± 1.40	0.12 ± 0.25	0.002
Escherichia-Shigella	4.90 ± 10.52	0.30 ± 0.49	0.001
Eubacterium coprostanoligenes group	2.22 ± 2.53	0.59 ± 0.81	0.010
Faecalibacterium	2.22 ± 1.31	1.53 ± 3.66	0.661
Haemophilus	7.09 ± 25.28	0.02 ± 0.04	0.011
Haliangium	0.01 ± 0.04	0.54 ± 0.88	0.006
Helicobacter	10.84 ± 8.46	6.29 ± 9.32	0.232
Lachnospira	2.75 ± 5.32	0.54 ± 1.32	0.055
Lachnospiraceae NK4A136 group	1.41 ± 0.92	0.63 ± 0.88	0.039
Lactococcus	0.53 ± 1.10	13.14 ± 24.37	0.007
Methylobacterium	0.04 ± 0.04	1.80 ± 3.76	0.003
Parabacteroides	0.68 ± 0.43	0.14 ± 0.28	0.067
Prevotella 9	1.16 ± 1.17	0.17 ± 0.57	0.011
Prevotellaceae NK3B31 group	0.59 ± 0.56	0.06 ± 0.18	0.040
Propionibacterium	0.58 ± 0.40	10.77 ± 18.48	0.010
Pseudobutyrivibrio	0.59 ± 0.36	1.11 ± 3.20	0.977
Ruminococcaceae UCG-002	0.82 ± 0.60	0.17 ± 0.33	0.023
Ruminococcaceae UCG-014	0.56 ± 0.34	0.23 ± 0.35	0.229
Ruminococcus 2	0.51 ± 0.48	0.15 ± 0.42	0.182
Sediminibacterium	0.55 ± 0.94	4.65 ± 4.75	0.008
Sphingomonas	0.03 ± 0.05	2.74 ± 4.68	0.001
Streptococcus	6.67 ± 22.33	0.89 ± 0.81	0.957
Subdoligranulum	1.65 ± 1.24	0.47 ± 0.71	0.013
U. m. of *Bacteroidales* S24-7 group family	1.48 ± 1.05	0.78 ± 1.11	0.111
U. m. of *Caulobacteraceae* family	0.14 ± 0.11	2.52 ± 3.14	0.009
U. m. of *Lachnospiraceae* family	6.05 ± 3.05	3.46 ± 4.33	0.108
U. m. of *Ruminococcaceae* family	1.05 ± 0.58	1.09 ± 2.17	0.986

[a]Mean relative abundance ± standard deviation

[b]Statistical significance was considered with a *p* value below 0.05, adjusted for multiple hypothesis testing using a false discovery rate (FDR) correction of 0.25 (grey shadow)

gallstones is *Salmonella enterica*, which is able to colonize and persist in the human gallbladder [25]. Only recently, using massive sequencing techniques, a few reports analyzed the biliary microbiome of the human gallbladder potentially associated with the generation of gallstones; however, the relatively low number of sequences analyzed and the absence of gallbladder control samples (samples from individuals without hepatobiliary pathology) hampered the yield of physiologically relevant results and made challenging to establish a microbiota profile associated with cholelithiasis. Among these studies, the analysis of a group of 29 Chinese patients with gallbladder gallstones showed that the biliary core microbiome was constituted by 6 phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* being the predominating phyla, and *Bacteroides* the most abundant

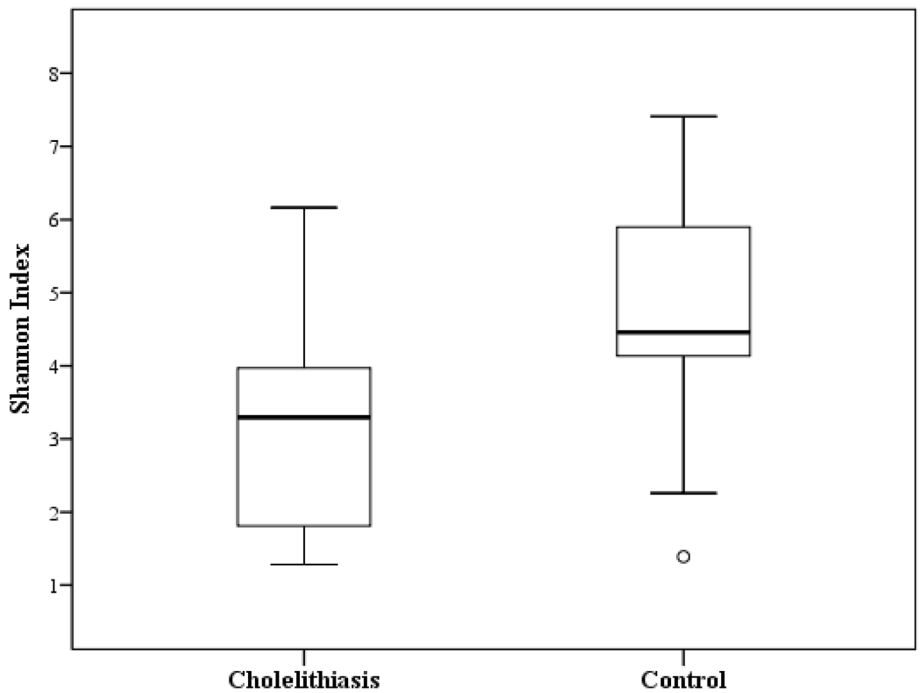

Fig. 4 Comparison of Shannon's diversity indices in cholelithiasis ($n = 14$) and control ($n = 13$) groups. The central rectangles represent interquartile ranges (IQR), the lines inside the rectangles show the median, and the whiskers indicate the maximum and minimum values. The dots outside the rectangles are suspected outliers (> 1.5 × IQR). Statistically significant differences (p value < 0.05) between groups were found (Mann-Whitney U test)

genus [17]. Similar results were found by Saltykova and co-workers, who observed that these four phyla dominated the human gallbladder microbiota of patients with gallbladder gallstones [18]. Our results also point to the predominance of these four phyla in bile obtained from the gallbladders of cholelithiasis patients, all of them with gallbladder gallstones, and the highest number of sequences assigned to a specific genus was for *Bacteroides* (Table 5). On the other hand, a few works have analyzed the human microbiota of samples from the common bile duct, using endoscopic retrograde cholangiopancreatography (ERCP) for sample collection, obtained from patients with gallstones in the common bile duct [14–16]. Overall, these studies showed a high abundance of the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, with a lower representation of *Actinobacteria* and other phyla.

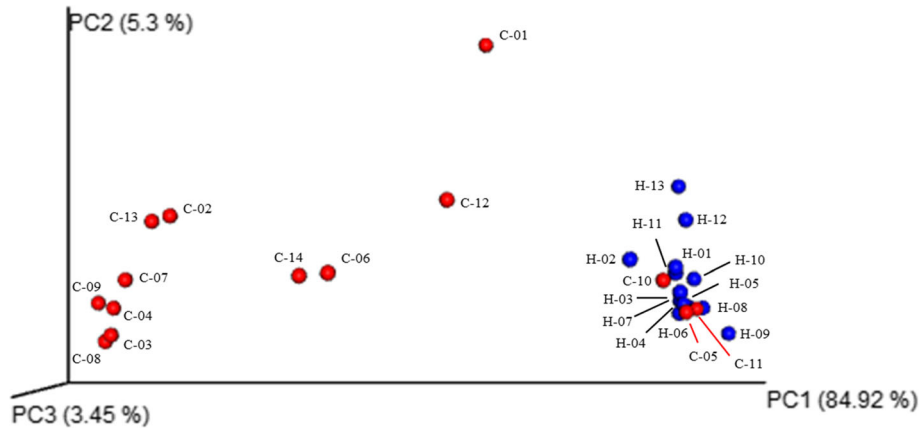

Fig. 5 Principal Coordinate Analysis (PCoA) plot of weighted UniFrac distances, comparing the bacterial communities among samples from cholelithiasis (red circles, $n = 14$) and control group (blue circles, $n = 13$). Percentages shown in the axes represent the proportion of dissimilarities. Analysis of molecular variance (AMOVA) was used to assess the statistical significance of the spatial separation between both groups (p value < 0.001)

Table 6 Signal assignments for ^{1}H-NMR spectra obtained from gallbladder bile

Chemical shift	Compounds
0.00	TSP (internal standard)
0.67	BA (H18)
0.71	Cholesterol (H18)
1.42	Lipids $(CH2)_n$
1.49	BA cholesterol + lipids
2.38	BA cholesterol + lipids
2.74	Lipids
2.78	Lipids
2.81	Lipids
3.07	Conjugated taurine
3.24	Phosphatidylcholine
3.49	CA+CDCA
3.56	DCA + conjugated taurine
3.63	Phosphatidylcholine
3.74	Conjugated glycine
3.84	CA+DCA
3.88	CA, CDA, + PC-glycerol
4.28	PC-glycerol
4.31	Phosphatidylcholine
4.44	PC-glycerol
4.46	PC-glycerol
4.83	Residual water
5.32	Lipids, cholesterol PC-glycerol
7.83	Glycochenodeoxycholic acid
7.86	Glycodeoxycholic acid
7.88	Glycocholic acid
7.99	Taurochenodeoxycholic acid
8.00	Taurodeoxycholic acid
8.01	Taurocholic acid

BA bile acids, *CA* cholic acid (primary), *CDCA* chenodeoxycholic acid (primary), *DCA* deoxycholic acid (secondary), *TSP* 3-(trimethylsilyl) propionicacid-d4, *PC-glycerol* phosphatidylcholine-glycerol

Until now, the microbial populations inhabiting the human gallbladder in individuals without any hepatobiliary disorder had not been studied. Our group has recently shown that healthy pigs have a native microbiota [19], and this led us to think that humans, even in the absence of pathologies, can also hold a native gallbladder microbiota. Thus, in our study, we have tried to take another step in the characterization of the human bile microbiome, and in order to compare the microbial profile of cholelithiasis patient's vs a non-pathological condition, we have established a control or reference group with samples from individuals without any hepatobiliary disease. Given the difficulty of obtaining bile samples from healthy individuals due to obvious ethical reasons, we have tried to overcome the problem to obtain samples from a "healthy group" by collecting samples from liver donors without any record of biliary or hepatic disorders. Since antibiotic prophylaxis is a standard protocol for liver transplantation, with the aim of minimizing the potential effect of antibiotics on the biliary microbiota, we only selected samples from liver donors that had received antibiotics at the intensive care unit (ICU) for less than 24 h before the surgery. Even so, we are aware that this antibiotic treatment (see the "Methods" section for details) may constitute a limitation of our study and could affect the endogenous microbiota of bile. Regrettably, as commented above, this was due to the difficulty in obtaining bile samples from healthy volunteers. In the bile samples of this control group, we found sequences matching some genera of the Alpha division of *Proteobacteria* (*Bradyrhizobium*, *Methylobacterium*, and *Sphingomonas*) that were previously associated with nitrogen fixation and potential contaminants of ultrapure water systems [26]. In this regard, with our template-free "blanks" (in which the bile sample had been substituted by molecular biology grade water in the DNA extraction procedure), no amplification from the 16S rRNA gene was achieved, suggesting an extremely low bacterial load in the water, and sequencing results could not be obtained. Therefore, we were not able to confirm if these genera (representing around 10% of total assigned reads) constitute a potential contamination or background signals, a fact that is plausible in high-throughput sequencing approaches using biological specimens with low bacterial load, as is the case of our control bile samples (Fig. 3). Therefore, we cannot completely rule out the possibility of introducing a minor bias in our sequence-based microbiome study of bile control samples from liver donors, but shotgun metagenomic analyses corroborated the data of 16S rRNA gene profiling, indicating the existence of three main phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*) apart from *Proteobacteria* (Table 2) in human bile samples, which is in accordance with previous literature of this environment [17, 18]. In addition, from the liver donors, we extracted DNA from gallbladder tissues removed during liver transplantation surgery. 16S rRNA gene profiling was carried out in three of these biopsies (from individuals H-04, H-05, and H-06), revealing the presence of a low abundance of bacterial amplicons, mainly belonging to *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* phyla. Observed eukaryotic amplicons ranged between 75 and 85% of the total profile. Based on our previous experience, such results indicate that the vast majority of the retrieved DNA is eukaryotic (data not shown).

Furthermore, weighted PCoA analysis grouped the biliary microbiota of the two study groups (non-lithiasis vs lithiasis patients) differently, showing a higher dispersion

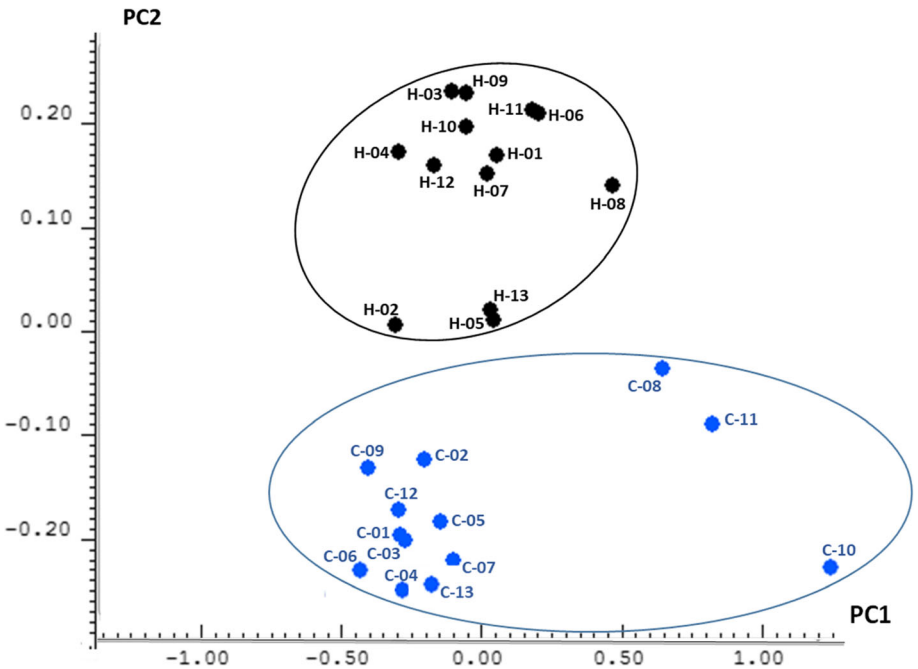

Fig. 6 Principal component analysis (PCA) plot of bile metabolites. PC1 versus PC2 obtained for the whole spectra is represented. Control samples are represented in black. Blue dots belong to bile samples from cholelithiasis patients

among the samples from cholelithiasis patients. Statistical differences in the microbial relative abundances of sequences between both groups, at family and genus level, were also noticeable. Within the different genera detected, the highest statistical significance was observed for *Bacteroides* and *Escherichia-Shigella* (more abundant in the cholelithiasis patients) and *Sphingomonas* (more abundant in the control group). As previously mentioned, enterobacteria are frequent in the bile of cholelithiasis patients [11–13]. Furthermore, it is worth highlighting that half of the cholelithiasis patients have altered levels of total cholesterol or triglycerides at the time of sampling, or a clinical history of hypercholesterolemia and/or obesity (Table 1 and Additional file 1: Table S1). In this regard, high abundance percentages of *Bacteroides* in the fecal microbiota have been linked to the consumption of a diet rich in animal fat [27, 28]. In accordance with our microbiological and metabolic results found in bile from patients with cholelithiasis, it has been previously reported that high-fat intake increases BA secretion, and accordingly, animal-based diets increase the abundance of bile-tolerant microorganisms in the gut, such as *Bacteroides* [28]. Although results on human intestinal microbiota cannot be directly compared with the biliary microbiota described in our work, we hypothesized that diet rich in saturated fat and cholesterol levels, two factors that have been related to the appearance of cholelithiasis [29], might favor microorganisms, such as *Bacteroides*, in the biliary micro-environment.

From the 16S rRNA gene profile analysis, it seems that 3 to 4 phyla constitute the core population of human bile, and relevant differences at family and genera level are highlighted depending on the specific physiological condition of the host.

After a careful selection of samples from the control group, only three of them with a reasonably high 16S/18S ratio were used for shotgun metagenomic analysis. We have seen that the distribution of the main functional categories in the biliary metagenome is similar to that described in the human gut microbiota [30]. However, it is worth highlighting that genes related to the metabolism of cholesterol and BA biosynthesis are present at higher relative abundance in the human bile microbiome with respect to that of the human gut [20]. This could reflect the adaptation of the bile microorganisms to this specific niche. However, the interpretation of these results should be taken with caution, due to the small number of samples analyzed from the control group (3 individuals) and to the particular physiological conditions of the liver donors (a period of stay in the hospital's ICU and the unavoidable standard-of-care antibiotic treatment), considering that the comparison of gallbladder and fecal microbiotas was not carried out within the same group of individuals (liver donors who had suffered a brain accident or stroke vs healthy individuals).

Regarding the biochemical analysis of the bile samples, the pH values of the samples were within the range previously described in bile from gallbladders [31]. Attending to the color analysis, the maximum at 415 nm of two samples coming from patients with cholelithiasis could be associated to hemoglobin [32]. Together, the differences in shapes of the spectra of the rest of samples, although not statistically significant, could be related to the heterogeneity on the concentration of pigments (bilirubin and biliverdin) and bile salts, the aggregation state, and the oscillations of pH values [33, 34].

With respect to the metabolic NMR profiles (Table 6), high levels of the secondary BA deoxycholic acid (DCA) in blood and feces have been associated with increased risk of cholesterol gallstone disease [35]. Statistically significant differences between bile samples from controls and patients were found when considering the compounds of both the aromatic and the aliphatic regions of the spectra (Fig. 6). In agreement with this, several authors have stated that different levels of BA or its glycine and/or taurine conjugates play an important role in cholestasis [36, 37]. Besides, Ijare and colleagues described a major role of the amide proton region of the ^{1}H-NMR spectra of human bile in differentiating cholestatic patterns from normal ones [38]. Further studies are mandatory to characterize in depth the potential relationships between the biliary metabolic and microbial profiles.

Conclusions

This work is the first study pointing to the existence of a human gallbladder microbiota in individuals without any hepatobiliary disease and establishing a link between bile microbiota and pathological conditions. The 16S rRNA gene profiling and the metagenomic analysis allowed us to propose the existence of a microbial bile ecosystem and to access the main taxonomic and functional profiles present in this microbiome. Furthermore, the metabolomics analysis showed that the two study groups have different metabolic profiles. We are aware of the moderate sample size of our work, mainly related to the number of liver transplants carried out in the collaborating hospitals during the one and a half year sampling period and to the strict inclusion criteria for the two study groups. Also, the difficulty of obtaining gallbladder samples from healthy volunteers and the inherent disadvantage of working with biological samples with a low bacterial load must be taken into account. In addition, we must bear in mind that the results of the control group have been obtained with liver donors that have been subjected to the specific protocols of the hospital's ICU, including an unavoidable standard-of-care short antibiotic treatment that could introduce a bias in the physiologically normal biliary microbiota. Even considering these limitations, our results establish the basis for future larger-scale studies on the relationship between bile microbiota, gut microbiota, human metabolism, and health. Future investigations should be oriented to unraveling the role and influence of the biliary microbiota in the pathophysiology of this (lithiasis) and other human diseases related to diet, bile, and cholesterol metabolism. These findings might open promising strategies to search for novel biomarkers associated with the disease or dietary strategies that could help prevention and/or patient care and treatment.

Methods

Bile samples and patients

Human bile samples from the gallbladders of individuals without hepatobiliary disease were obtained during liver transplants from liver donors who had suffered a brain accident or stroke (control group or reference group; H-sample codes). Although a total of 26 donors were initially recruited and sampled, a further selection of bile samples for the microbiological study was applied in order to have a homogenous reference group. The criteria used for selection were as follows: less than 80 years old, no more than a 48-h stay in the hospital's ICU before the transplant, and had not received antibiotics at ICU for more than 24 h. In all selected cases, the antimicrobial treatment consisted on 2 g of amoxicillin-clavulanic acid every 6 h until liver transplantation surgery. Other characteristics of this selected control group ($n = 13$) were as follows: 4 male and 9 female and aged range 37 to 79 years old. The samples of bile from the gallbladders were collected aseptically during the surgery by physicians at the General Surgery Service of HUCA (Central University Hospital of Asturias, Spain). The surgeons also confirmed the absence of gallstones and sludge from the donors.

Bile samples from gallbladders from a similar group in gender and age (3 male and 11 female, age range 27 to 73 years old), with a bile disorder (diagnosed with cholelithiasis), were obtained from 14 patients ($n = 14$) with gallstones, who underwent surgical gallbladder removal at Cabueñes Gijon University Hospital (Asturias, Spain) (Cholelithiasis group; C-sample codes). General characteristics and clinical parameters of the patients are reported in Table 1. None of the patients received previous antibiotic prophylaxis (which was considered as an exclusion criterion), and the intervention was performed in all of them by laparoscopic cholecystectomy. The recruitment of patients was undertaken by physicians of the General and Digestive Surgery Service of Cabueñes Gijon University Hospital, Spain. After bile collection, bile samples were immediately transported refrigerated to the laboratory and stored at − 80 °C until use. Comparison between clinical parameters of both groups of individuals (patients with cholelithiasis and

liver donors as controls), from whom bile samples were obtained in this study, is reported in Additional file 1: Table S1. Additionally, there were no statistical differences with respect to gender (79% female and 21% male in patients with cholelithiasis vs 69% female and 31% male in controls) and age (52 years old in patients vs 59 in controls) between both groups.

Ethical approval for this study was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias n°112/13) in compliance with the Declaration of Helsinki of 1964. All experiments were carried out in accordance with the approved guidelines and regulations.

Microbial analysis of bile samples

DNA extraction and quantification

Total DNA extraction from 1-ml bile samples was performed following an optimized protocol based on a previously described method [19], with slight modifications. Briefly, samples were centrifuged at maximum speed at room temperature for 10 min and pellets re-suspended in 0.5 ml of extraction buffer consisting of 200 mM Tris-HCl pH 7.0, 25 mM EDTA, 250 mM NaCl, and the following enzymes: 20 mg/ml lysozyme (Merck, Darmstadt, Germany), 5 μg/ml of lysostaphin (Sigma-Aldrich, Saint Louis, MO, USA), and 40 U/ml mutanolysin (Sigma-Aldrich). Enzymatic lysis was performed for 1 h at 37 °C; after that, SDS was added to a final concentration of 0.5% (*w*/*v*), and mechanical disruption was performed in a FastPrep FP120 apparatus (Qbiogene, Carlsbad, CA, USA). The lysate solution was treated with proteinase K, 1.5 M NaCl as previously described [39] and extracted with phenol/chloroform. Precipitation of DNA was performed with 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of cold ethanol. The DNA was then pelleted, washed with 70% ethanol, resuspended in 50 μl of molecular-biology grade water (Sigma-Aldrich), and stored at − 20 °C until use. DNA concentration and quality was determined in a BioTek Epoch™ spectrophotometer system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and in parallel in a Qubit fluorometer with dsDNA assay kits (Thermo Fisher Scientific).

Determination of prokaryotic and eukaryotic DNA by qPCR

Differentiation and quantification of eukaryotic and prokaryotic DNA from bile samples were carried out by qPCR using specific primers targeting the 18S rRNA gene of eukaryotic cells [40] and the 16S rRNA gene of prokaryotic microorganisms [41]. Amplification reactions were performed in 96-well optical plates (Applied Biosystems, Foster City, CA, USA) in a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplifications were done in triplicate in a final volume of 25 μl containing 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.2 μM of each primer, and 1 μl of DNA obtained from bile. Primer efficiency was calculated from the slope of the standard curve ($E = 10^{-1/\text{slope}}$). Standard curves were generated by plotting the Ct values against the numbers of cells corresponding to serial tenfold dilutions of cultures of *L. lactis* strain NZ9000 as a reference for prokaryotic DNA and calculated by plate counting [42] and HT-29 cell line as a reference for eukaryotic DNA and titrated under an inverted microscope with a Neubauer Chamber [43]. To simulate different bacterial loads in the bile matrix, human bile was artificially supplemented with serial tenfold dilutions of known concentrations (ranged from 10^2 to 10^7 cfu/ml) of a grown culture of *L. lactis* strain NZ9000.

Shotgun metagenomic sequencing and analysis

Extracted DNA of three bile samples from the control group were used for total shotgun sequencing. DNA was fragmented to 550–650 bp using a BioRuptor machine (Diagenodo, Belgium) and subsequently prepared with the Next era XT Library Preparation kit (Illumina, San Diego, CA, USA). Sequencing was performed at the DNA sequencing facility of GenProbio SRL (Parma, Italy) using an Illumina MiSeq System with MiSeq Reagent Kit v3 chemicals (Illumina).

The fastq files were filtered for reads with quality < 25 and presence of alien DNA, as well as for reads < 150 bp. Bases were also removed from the end of the reads until the average quality in a window of 5 bp was > 25. Only paired data was further analyzed. Assembly of the shotgun dataset and annotation of the reads was performed with the MEGAnnotator platform [44]. Taxonomic classification was obtained using a combination of RapSearch2 software [45] and the non-redundant NCBI database (BLASTx), and MEGAN5, in order to reconstruct taxonomic profiles based on reads covering coding regions. Functional analysis of the reads was achieved using CAZy [46], EggNOG [47], and MetaCyc [48] databases. Specifically, COG functional class profiling was based on the EggNOG database; meanwhile, metabolic pathway prediction was based on the MetaCyc database. Abundance of genes related to the metabolism of cholesterol and BA was compared with those found in fecal metagenomes of five healthy individuals used as controls in a previous study [20]. These dataset sequences are available at the Sequence Read Archive (SRA) database under the accession number SRP125191.

High-throughput sequencing and analysis of 16S rRNA gene amplicons

Partial 16S rRNA gene sequences were amplified from the extracted DNA of 27 bile samples (13 controls and

14 patients) using the primer's pair Probio_Uni and /Probio_Rev, which target the variable region V3 of the bacterial 16S rRNA gene, as previously described [49]. Samples were submitted to 2 × 250 bp paired-end sequencing by means of an Illumina MiSeq System (Illumina). Sequence reads were filtered by the Illumina software to remove low-quality sequences. All Illumina quality-approved, trimmed, and filtered sequences were processed using a custom script based on the QIIME software suite [50]. After joining the paired-end reads, the quality control phase retained sequences with a mean sequence quality score > 20 and a length between 140 and 400 bp. Sequences with homopolymer regions > 7 bp and those with mismatched primers were omitted. In order to calculate downstream diversity measures, 16S rRNA Operational Taxonomic Units (OTUs) were defined at 100% sequence homology using DADA [51]; OTUs not encompassing at least 2 sequences of the same sample were removed. All sequences were classified to the lowest possible taxonomic rank using QIIME and the SILVA database as reference [50, 52]. Similarity of the bacterial communities between samples was calculated by weighted UniFrac method [53]. All raw data was deposited in the SRA of the NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR6872870 to SRR6872896 (for the 16S rRNA gene sequencing data) and SRR6877525 to SRR6877527 (for the shotgun sequencing data).

Metabolic analysis of bile samples

pH and color determination

pH and UV-visible spectra were measured on each of the collected bile samples. A CRISON GLP21 pH-meter was used to the pH measurement. Optical density was measured between 200 and 800 nm in a spectrophotometer Zenyth 200RT (Anthos Labtec Instruments GmbH, Salzburg, Austria).

Sample preparation for NMR analysis of metabolites

The same samples used for the microbiota analysis were used for the NMR analysis, excluding sample C-14 (this sample was not available after the 16S rRNA gene profiling and the optimization of analyses protocols). The intact bile samples were frozen at − 80 °C, freeze-dried, and stored under vacuum at − 80 °C. For NMR analyses, 5 mg of bile and 50 μl of a 1 mM solution in distilled water of the sodium salt of the 3-(trimethylsilyl) propionicacid-d4 (TSP) were mixed with 550 μl of distilled water, and the mix was transferred to 5-mm diameter NMR glass tubes. TSP was added to provide an internal reference standard and a field-frequency lock (0.00 ppm).

1D ^{1}H-NMR experiments

NMR experiments were performed on a Bruker Biospin Avance 700-MHz NMR spectrometer. Spectra were recorded using the following pulse sequences: 1D NOESY (NOESYPRESAT) and CPMG (Carr Purcell Meiboom Gill), both with water suppression. The 1D NMR experiments were acquired with 32k time domain data points, 18 ppm spectral width, 64 scans, and 2 s of relaxation delay. A mixing time of 150 ms was used in the 1D NOESY experiment and an echo time of 50 ms was selected for the CPMG sequence. The free induction decays (FIDs) obtained were processed with Bruker BioSpin TOPSPIN software (version 3.1) as follows: exponential filtering (LB ¼ 1 Hz), Fourier transformation (FT), spectral phasing, and baseline correction.

2D ^{1}H-NMR experiments

Assignments of NMR signals were based on standard two dimensional experiments, namely, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC. Homonuclear water suppressed COSY and TOCSY experiments were performed with 2k data points in t_2 domain and 384 increments in t_1, each with 64 scans. Spectral widths of 13 ppm were employed in both dimensions. Mixing times of 70 ms were used for TOCSY experiments. Heteronuclear HSQC and HMBC experiments of these liquid samples were performed as follows: 2k data points in t_2 domain and 384 increments in t_1, each with 256 scans; spectral widths of 13 ppm for f_2 dimension and 260 ppm for f_1 dimension; coupling constant values of 145 Hz and 10 Hz were employed to set delay durations for short range and long range correlations, respectively. In all cases, two-dimensional data sets were enhanced in the first dimension by forward linear prediction from 384 to 512 real data points followed by zero filling to 1024 data points. In addition, squared sine bell window functions were applied in both dimensions prior to Fourier transformation. Assignment of resonances in the ^{1}H-NMR spectra was based on both spin connectivity information obtained from 2D experiments and the use, as guidelines, of both data reported in the literature [36] and data obtained from HMDB database (Human Metabolite Data Base http://www.hmdb.ca/).

Statistical analyses

The identification of differentially abundant taxa was assessed using the Metastats program [54]. Multiple hypothesis tests were adjusted using the false discovery rate (FDR) correction; an FDR threshold of 0.25 was used to identify significant differences. Multivariable statistical analysis of microbiological sequences was performed by PCoA. Differences in the microbial

distribution were sought by AMOVA. Comparisons among other variables were performed by using the non-parametric Mann-Whitney *U* test. A PCA was developed on NMR signals as discrimination analysis. Statistical analysis was performed using SPSS v. 22.00 (IBM, Armonk, NY, USA) and the free software R (www.r-project.org).

Additional file

Additional file 1: Table S1. Data and clinical characteristics of both groups of individuals (patients with cholelithiasis and liver donors as controls) from whom bile samples were analyzed in this study. (DOCX 19 kb)

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Not applicable

Authors' contributions
IC, CMGB, JIR, SG, JMR, SD, and AM conceived and designed the study. NM, LR, IGD, JS, and ABC conducted the laboratory work. NM, AC, CMGB, and JIR collected and processed the biological samples. ABC developed the DNA extraction protocol. CM, MM, and MV performed the library preparation for NGS and the DNA sequencing. NM, CM, BS, MM, and MV analyzed the 16S data and the metagenomic data. JS, LR, JMR, and IC analyzed the NMR data. CMGB and JIR recruited the subjects. NM, MV, IC, SD, and AM prepared the figures and tables and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All raw data was deposited in the Sequence Read Archive (SRA) of the NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR6872870 to SRR6872896 (for the 16S rRNA gene sequencing data) and SRR6877525 to SRR6877527 (for the shotgun sequencing data).

Ethics approval and consent to participate
Ethical approval for this study was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias n°112/13).

Consent for publication
Not applicable.

Competing interests
The authors declared that they have no competing interests.

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Publisher's Note

Capítulo 2

Chapter 2

CAPÍTULO 2

Análisis de la relación entre los componentes de la dieta y el perfil microbiano biliar de sujetos con colelitiasis

En el segundo capítulo de esta Tesis Doctoral, utilizamos la información obtenida en el capítulo anterior acerca de la microbiota biliar para estudiar la relación existente entre la dieta consumida y el perfil microbiano biliar en los pacientes con colelitiasis.

El trabajo se llevó a cabo con el grupo de 14 pacientes con colelitiasis, y un grupo de 14 sujetos sanos, sin patologías gastrointestinales previas, seleccionados de un trabajo previo (Gutiérrez-Díaz *et al.*, 2016) en base a su edad y sexo para obtener un grupo homogéneo y comparable al grupo con patología biliar. Se llevó a cabo una entrevista personal con los sujetos de estudio y se les realizó un cuestionario en el que se tomaron datos de ingesta. Con esta información, mediante las tablas de composición de nutrientes en los alimentos desarrolladas por el Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID), se determinó la ingesta calórica aproximada, así como el contenido ingerido de distintos macro- y micronutrientes, colesterol, vitaminas, fibra, ácidos grasos de cadena corta y compuestos fenólicos, entre otros. También se obtuvieron datos acerca de su estilo de vida, y se les realizó un análisis de sangre con el fin de obtener información de los niveles de colesterol, triglicéridos y glucosa en suero.

Con todo ello, se analizaron diferencias en los patrones dietéticos y estilo de vida entre ambos grupos. Además se estudió la presencia de asociaciones entre el consumo de distintos alimentos y la abundancia relativa de algunos taxones representativos. Los

resultados mostraron diferencias entre los dos grupos de estudio en los niveles de glucosa, colesterol y triglicéridos en suero; así como en la ingesta de distintos alimentos. Se detectaron asociaciones entre el consumo de ciertos alimentos y la abundancia relativa de algunos miembros de la microbiota biliar.

Este es el primer estudio que establece un vínculo entre la microbiota biliar, la dieta y la formación de cálculos biliares. Los resultados de este segundo capítulo de la tesis se presentan en el artículo publicado en la revista *Nutrients*:

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Article

Diet: Cause or Consequence of the Microbial Profile of Cholelithiasis Disease?

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Abstract: Recent dietary habits and lifestyle could explain the shaping of the gut microbiota composition and, in consequence, the increasing prevalence of certain pathologies. However, little attention has been paid to the influence of diet on microbiotas, other than the gut microbiota. This is important in cholelithiasis, given that changes in the production of bile acids may affect gallbladder microbial communities. Our aim was to assess the association between regular dietary intake and gallbladder microbial composition. Fourteen adults with cholelithiasis and 14 controls, sex-age-matched and without gastrointestinal pathology, were included. Diet was assessed through a food frequency questionnaire and quantification of gallbladder microbiota sequences by Illumina 16S rRNA gene-based analysis. The cholelithiasic patients showed greater intake of potatoes and lower consumption of vegetables, non-alcoholic drinks, and sauces, which resulted in a lower intake of energy, lipids, digestible polysaccharides, folate, calcium, magnesium, vitamin C, and some phenolic compounds. Regarding the altered bile microorganisms in cholelithiasic patients, dairy product intake was negatively associated with the proportions of *Bacteroidaceae* and *Bacteroides*, and several types of fiber, phenolics, and fatty acids were linked to the abundance of *Bacteroidaceae*, *Chitinophagaceae*, *Propionibacteraceae*, *Bacteroides*, and *Escherichia-Shigella*. These results support a link between diet, biliary microbiota, and cholelithiasis.

Keywords: diet; polyphenols; fiber; cholelithiasis; biliary microbiota

1. Introduction

In the last few years, solid scientific evidence has emerged supporting the view that dietary patterns are intimately linked to the composition and activity of the millions of microbes that inhabit along the gastrointestinal tract [1–4]. This close relationship is the result of a co-evolutionary process over almost half a billion years, whereby the diets of our ancestors, rich in polysaccharides and antioxidants, forced evolution towards a microbiota dominated by saccharolytic bacteria capable of extracting additional energy from food, in addition to offering other benefits for the health of the host [2,5–7]. However, during recent times, industrialized countries have undergone a profound change in their dietary habits, resulting in the abandonment of a dietary pattern characterized by the abundance of cereals, tubers, vegetables, and fruits, in favour of a more "Westernized"

pattern, characterized by the high consumption of refined foods, meats, and other products of animal origin [2,8]. This, together with other factors related to current lifestyle, could explain the change in the gut microbiota composition and the increasing prevalence of certain pathologies in the population. However, very little attention has been paid to the influence of diet on microbiotas other than the gut microbiota.

In this regard, in the aetiology of cholelithiasis, one of the most common biliary disorders in adults from developed countries [9], diet has long been recognized as an important risk factor. Some mechanisms, such as the modification of gallbladder motility or the alteration of the composition of bile salts, have been proposed [10] in order to explain this association. However, how dietary components influence this pathology remains unclear. The impact of saturated fat intake on gut microbiota by means of a change in the pool of biliary acids has recently been demonstrated, so it seems possible that diet-driven changes in the production of bile acids affect gut microbes that, in turn, trigger disease [11,12]. Also, the intake of certain types of fiber that may be significant predictors in the pathogenesis of cholelithiasis has been described [13,14]. In this regard, the activity of the gut microbiota could also be linked to the development of cholelithiasis, by altering the concentration of biliary lipids in bile [13] and/or increasing the faecal excretion of bile salts [15,16]. However, and to the best of our knowledge, there are no previous studies examining the association between the regular dietary intake and the microbial composition in this organ. At this moment there is extremely scarce and fragmented information about the microbiota present in the gallbladder. In the last years a few reports analysed the microbiota of the biliary tract and the gallbladder using culture independent techniques. It was shown that *Enterobacteriaceae* dominate the biliary tract of acute cholecystitis and gallstone patients [17,18]. Also, a Chinese study with gallbladder gallstones patients showed that the biliary microbiota was constituted mainly the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria, with *Bacteroides* being the most abundant genus [19]. It was also observed that these four phyla dominated the human gallbladder microbiota of patients with gallbladder gallstones [20]. Apart from this, the description of the microbiota of the human gallbladder has been hampered by difficulties in accessing bile samples and the lack of optimal culture conditions adapted to the biliary ecosystem. However, this information would be of great interest for the identification of the specific dietary components associated with the composition of the gallbladder microbial communities.

2. Subjects and Methods

2.1. Participants

The study sample comprised 14 patients with cholelithiasis (three males, 11 females; mean age 51.50 $\pm$ 14.10 years old) and 14 controls, sex-age-matched without declared gastrointestinal pathology (three males, 11 females; mean age 46.50 $\pm$ 11.47 years old). Subject recruitment was carried out at the General and Digestive Surgery Service of Cabueñes Hospital (Gijon, Spain). Information on clinical manifestations was obtained from clinical records and by personal interviews. Exclusion criteria, for both groups, were previous diagnosis of allergy, diabetes type II, metabolic syndrome, or autoimmune diseases, as well as having undergone medical treatment with antibiotics or glucocorticoids during the previous three months.

Potential volunteers were informed of the objectives of the study. When they agreed to participate, one personal appointment was made to collect dietary information and another to obtain the biological samples. All determinations were performed with fully informed written consent from all participants involved in the study.

Ethical approval for this study (reference code AGL2013-44761-P; grant title "The human bile microbiota: ecology, functionality and relationship to diet and biliary disorders") was obtained from the Bioethics Committee of Consejo Superior de Investigaciones Científicas (CSIC) and from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias, n°112/13) in compliance with the Declaration of Helsinki. The study did not interfere with patients' normal care.

2.2. Nutritional Assessment

Dietary intake was registered using an annual, semi-quantitative Food Frequency Questionnaire (FFQ), detailing 54 items. During a personalized interview with expert dietitians, participants were asked, item by item, whether they usually ate each food and, if so, how much they ate. Methodological issues concerning dietary assessment were detailed [21]. Food intake was analysed for energy, macronutrients, folate, cholesterol, calcium, vitamin C, magnesium, and total dietary fibre content by using the nutrient Food Composition Tables developed by the "Centro de Enseñanza Superior de Nutrición Humana y Dietética" (CESNID) [22]. The intake of fatty acids was converted using the National Nutrient Database for Standard Reference from the United States Department of Agriculture [23]. Also, information about dietary fibre components (soluble and insoluble) was completed using Marlett et al.'s food composition tables [24]. These authors used the enzymatic-chemical method developed by Theander et al. [25], in which pectin content is determined using a calorimetric assay, cellulose and hemicellulose by high-performance liquid chromatography (HPLC), and Klason lignin is estimated as the insoluble material after a Seaman acid hydrolysis [26]. The phenolic compound content in foods was estimated using the Phenol Explorer database, which contains detailed information of over 400 foods regularly consumed in European countries [27].

2.3. Food Groups

Food intake was analysed in 17 food groups according to CESNID classification [22] as follows: cereals: pastry, bread, pasta, and flours and grains; dairy products: milk, yogurt, dairy dessert, fresh, mature, and processed cheeses; fats and oils: olive oil, other oils (sunflower and corn), and solid fats; sugar and sugary products: sweets, chocolate, honey, and jam; vegetables: bulbs, mushrooms, roots, inflorescence, and stem and leaf vegetables; legumes: lentils, chickpeas, beans and peas; meat and derived products: poultry, red meat, processed meat, and others; fish: low omega-3 and high omega-3 fish, and other derivatives; fruits: fresh, dried, and canned fruits; non-alcoholic drinks: coffee, tea, soft drinks, and juice; alcohol drinks: spirits, wine, beer, and cider; seafood: crustaceans and molluscs. Other categories include eggs, potatoes, sauces, snacks, and nuts and seeds.

2.4. Anthropometric Measures

Height was measured using a stadiometer with an accuracy of $\pm$1 mm (Año-Sayol, Barcelona, Spain). The subjects stood barefoot, in an upright position and with the head positioned in the Frankfort horizontal plane. Weight was obtained on a scale with an accuracy of $\pm$100 g (Seca, Hamburg, Germany). Body mass index (BMI) was calculated using the formula: weight (Kg)/height $(m)^2$.

2.5. Biochemical Analysis

Fasting blood samples were drawn by venipuncture after a 12-h fast and collected in separate tubes for serum and plasma. Samples were kept on ice and centrifuged (1000$\times$ *g*, 15 min) within 2–4 h after collection. Plasma and serum aliquots were kept at -20 °C until analyses were performed. Serum glucose, total cholesterol, high-density lipoproteins (HDL), low-density lipoprotein (LDL), and triglycerides were determined by standard methods.

2.6. Bile Sample Collection and Microbiota Analysis Based on Sequencing of 16S rDNA Amplicons

Bile samples were obtained from 14 patients diagnosed with cholelithiasis, who underwent surgical removal at Cabueñes Gijon University Hospital (Asturias, Spain) to eliminate gallstones from the gallbladder. Total DNA extraction from bile samples was performed according to a previously described method [28]. Partial 16S rRNA gene sequences were amplified from the extracted DNA using the primer´s pair Probio Uni and /Probio_Rev, which target the variable region V3 of the bacterial 16S rRNA gene, as previously described [29]. Samples were submitted to 2 $\times$ 250 bp paired-end sequencing by an Illumina MiSeq System (Illumina, San Diego, CA, USA). All quality-approved,

trimmed, and filtered sequences were processed using the QIIME™ v1 open source bioinformatic pipeline (http://qiime.org/) (Flagstaff, AZ, USA) and sequences were classified to the lowest possible taxonomic rank using QIIME and the SILVA database as a reference [30]. Full experimental details will be reported elsewhere and provided in a subsequent full article (Molinero et al., data not shown). Raw sequence data were deposited in the Sequence Read Archive (SRA) of the NCBI (https://www.ncbi.nlm.nih.gov/sra) under the accession numbers SRR6872880 to SRR6872883 and SRR6872887 to SRR6872896.

2.7. Statistical Analyses

Statistical analyses were performed using IBM-SPSS version 24.0 (SPSS-Inc., Chicago, IL, USA). Goodness of fit to normal distribution was analysed with the Kolmogorov-Smirnov test. When the distribution of variables was skewed, the natural logarithm of each value was used in the statistical test. The Mann-Whitney test was used to evaluate the differences in continuous variables between patients with biliary cholelithiasis and control subjects and chi-squared analysis for the categorical ones. For data on biliary microbiota, only those microbial taxa with relative abundancies $\geq$0.5 and detectable in at least one subject were considered. The Spearman correlation method was used to elucidate the relationship between food groups and dietary compounds with major biliary microbiota. Heatmaps were generated under R version 3.4.2 package heatmap.2. The dietary compounds previously correlated to biliary microbiota were selected and placed into a stepwise regression analysis to explore their independent effect. The statistical parameters employed were β (standardized regression coefficient) and R^2 (coefficient of multiple determinations). The conventional probability value for significance (0.05) was used in the interpretation of results.

3. Results

3.1. General Characteristics of the Study Sample

A general description of the studied variables in the cholelithiasic patients and the controls is presented in Table 1.

Table 1. General characteristics of patients with cholelithiasis and control subjects.

Variables	Patients with Cholelithiasis (n = 14)	Control Subjects (n = 14)
Age (year)	51.50 ± 14.10	46.50 ± 11.47
Female (%)	78.6	78.6
BMI (kg/m^2)	26.38 ± 6.04	26.00 ± 3.92
Drug use (%)	50.0	28.6
Probiotics consumption (%)	14.3	35.7
Vitamin and mineral supplements consumption (%)	0.0	35.7 **
Fiber supplementation (%)	0.0	14.3
Ethanol (g/day)	3.98 ± 4.98	5.64 ± 6.70
Current smoker (%)	14.3	14.3
Sedentary lifestyle (%)	50.0	50.0
Serum glucose (mg/dL)	79.14 ± 17.65	91.00 ± 9.83 *
Serum triglycerides (mg/dL)	129.14 ± 84.53	77.00 ± 33.63 *
HDL (mg/dL)	49.93 ± 14.79	61.21 ± 11.18 *
LDL (mg/dL)	117.07 ± 37.78	129.79 ± 35.22
Serum total cholesterol (mg/dL)	192.79 ± 55.33	206.71 ± 39.47

Results derived from Mann-Whitney U test were presented as estimated marginal mean ± standard deviation Differences in categorical variables were examined using chi-squared analysis and presented as percentage (%). BMI: body mass index. HDL: high-density lipoprotein. LDL: low-density lipoprotein. * $p \leq 0.05$, ** $p \leq 0.01$.

While non-significant differences are shown for age, BMI, and sedentary lifestyle among the groups, lower levels of serum glucose and HDL, and higher concentrations of triglycerides were observed in these patients. Regarding dietary habits, even though 57.2% of cholelithiasic patients reported an excellent or good appetite, most of them (64.3%) declared that they excluded some foodstuffs from their regular diet, with legumes, dairy products, red meat, and vegetables being the

most commonly excluded. In consequence, a lower intake of vegetables, sauces, and non-alcoholic drinks, and a higher consumption of potatoes, was found in patients with cholelithiasis (Table 2).

Table 2. Dietary habits and daily intake of the major food groups in patients with cholelithiasis and control subjects.

Dietary Habits	Patients with Cholelithiasis (n = 14)	Control Subjects (n = 14)
Appetite [a] (%)		
Excellent-Good	57.2	-
Normal	28.5	-
Regular	14.3	-
Chewing problems [a] (%)	0.0	-
Special diet [a] (%)	35.7	-
Omit foodstuffs from diet [a] (%)	64.3	-
Legumes	35.7	-
Dairy products	14.3	-
Red meat and meat products	64.3	-
Vegetables	14.3	-
No. of subjects reporting a change in food habits [a] (%)	28.6	-
Food Groups (g/day)		
Cereals	121.14 ± 74.97	142.45 ± 68.39
Dairy products	415.86 ± 206.42	522.15 ± 227.64
Eggs	13.78 ± 13.38	18.60 ± 11.13
Fats and oils	23.30 ± 12.44	31.16 ± 16.99
Sugar and sugary products	16.74 ± 20.78	17.71 ± 17.45
Vegetables	200.17 ± 85.11	318.16 ± 147.14 *
Potatoes	56.46 ± 30.51	33.27 ± 23.51 *
Legumes	27.10 ± 20.82	32.60 ± 26.65
Fruits	244.90 ± 169.37	320.66 ± 151.02
Meat and derived products	129.44 ± 63.15	109.76 ± 34.00
Fish and derived products	60.49 ± 26.76	65.43 ± 31.55
Seafood	12.53 ± 14.30	20.90 ± 24.76
Non-alcoholic drinks	96.94 ± 142.15	369.62 ± 235.93 **
Alcoholic drinks	86.22 ± 110.36	134.96 ± 167.97
Nuts and seed	20.52 ± 55.29	10.72 ± 14.83
Sauces	2.82 ± 4.03	8.29 ± 7.82 *
Snack	2.86 ± 10.69	2.60 ± 4.39

Results derived from Mann-Whitney U test are presented as estimated marginal mean ± SD and percentage (%). "-" (not measured). [a] Variables obtained only for patients with cholelithiasis. * $p \leq 0.05$, ** $p \leq 0.01$.

These differences resulted in a lower intake of energy, some macro-(digestible carbohydrates, total lipids, and polyunsaturated fatty acids (PUFA)) and micronutrients (folate, calcium, magnesium, and vitamin C) in the cholelithiasic group, together with a decreased intake of certain phenolic compounds: flavonoids (anthocyanins, flavanones, flavones, and flavanols), lignans, phenolic acids (hydroxybenzoic, hydroxycinnamic and hydroxyphenylacetic acids), and tyrosols (Table 3).

Table 3. Macro-, micronutrients, and bioactive compounds in patients with cholelithiasis and control subjects.

	Unadjusted		Adjusted	
Dietary Components	**Patients with Cholelithiasis (n = 14)**	**Control Subjects (n = 14)**	**Patients with Cholelithiasis (n = 14)**	**Control Subjects (n = 14)**
Energy (kcal/day)	1660.25 ± 659.28	2079.06 ± 515.84 *	-	-
Carbohydrates (g/day)	170.16 ± 72.05	209.71 ± 51.60	190.12 ± 72.05	189.74 ± 51.60
Digestible carbohydrates	82.04 ± 41.35	114.03 ± 30.63 *	91.77 ± 41.35	104.30 ± 30.63
Digestible polysaccharides	80.46 ± 38.54	84.29 ± 37.14	91.39 ± 38.54	73.36 ± 37.14
Proteins (g/day)	87.20 ± 21.78	100.88 ± 19.97	93.52 ± 21.78	94.56 ± 21.78
Animal	58.22 ± 16.68	66.05 ± 17.71	60.61 ± 16.68	63.63 ± 17.71
Vegetable	25.02 ± 16.46	31.78 ± 18.09	29.44 ± 16.46	27.37 ± 18.09
Lipids (g/day)	66.65 ± 36.77	88.06 ± 34.30 *	78.31 ± 36.77	76.39 ± 34.30
SFA	18.23 ± 9.18	25.78 ± 10.05	21.04 ± 9.18	22.98 ± 10.05
Palmitic fatty acid	8.60 ± 4.05	10.73 ± 4.37	9.82 ± 4.05	9.51 ± 4.37
Stearic fatty acid	3.46 ± 2.57	3.82 ± 1.55	4.11 ± 2.57	3.17 ± 1.55 *

Table 3. *Cont.*

Dietary Components	Unadjusted		Adjusted	
	Patients with Cholelithiasis (n = 14)	Control Subjects (n = 14)	Patients with Cholelithiasis (n = 14)	Control Subjects (n = 14)
MUFA	29.38 ± 13.27	39.10 ± 16.59	33.56 ± 13.27	34.92 ± 16.59
Cis oleic fatty acid	1.28 ± 1.01	1.55 ± 1.07	1.43 ± 1.01	1.41 ± 1.07
Trans oleic fatty acid	0.12 ± 0.14	0.14 ± 0.14	0.13 ± 0.14	0.13 ± 0.14
PUFA	13.52 ± 18.90	15.19 ± 10.25 *	17.54 ± 18.90	11.18 ± 10.25
Linoleic fatty acid	0.19 ± 0.11	0.63 ± 0.95	0.20 ± 0.11	0.63 ± 0.95
Alfa-linolenic fatty acid	0.09 ± 0.08	0.11 ± 0.07	0.10 ± 0.08	0.10 ± 0.07
Docosahexaenoic fatty acid	0.21 ± 0.13	0.23 ± 0.14	0.20 ± 0.13	0.23 ± 0.14
Eicosapentaenoic fatty acid	0.09 ± 0.05	0.11 ± 0.08	0.09 ± 0.05	0.11 ± 0.08
Folate (µg/day)	301.26 ± 148.29	446.34 ± 111.00 **	330.25 ± 148.29	417.36 ± 111.00 *
Cholesterol (mg/day)	243.47 ± 126.33	303.03 ± 86.00	255.53 ± 126.33	290.97 ± 86.00
Calcium (mg/day)	840.95 ± 278.81	1260.04 ± 307.79 **	916.85 ± 278.81	1184.14 ± 307.79 **
Magnesium (mg/day)	348.44 ± 270.31	403.52 ± 110.17 *	403.30 ± 270.31	348.66 ± 110.17
Vitamin C (mg/day)	111.50 ± 61.73	193.63 ± 66.52 **	109.75 ± 61.73	195.39 ± 66.52 **
Phenolic compounds (mg/day)	946.11 ± 473.35	1780.48 ± 899.58 **	1025.80 ± 473.35	1700.78 ± 899.58 *
Flavonoids	211.79 ± 141.50	499.54 ± 393.48 *	248.44 ± 141.50	462.89 ± 393.48
Anthocyanins	7.45 ± 10.69	26.07 ± 35.29 *	5.49 ± 10.69	28.03 ± 35.29 *
Dihydroflavonols	1.04 ± 1.78	1.33 ± 2.84	1.00 ± 1.78	1.38 ± 2.84
Dihydrochalcones	1.78 ± 1.98	2.62 ± 3.97	1.94 ± 1.98	2.46 ± 3.97
Flavanols	167.01 ± 122.61	360.71 ± 397.60	206.56 ± 122.61	321.16 ± 397.60
Flavanones	16.87 ± 33.49	43.54 ± 31.14 **	13.55 ± 33.49	46.86 ± 31.14 *
Flavones	0.54 ± 0.57	5.15 ± 4.15 **	0.20 ± 0.57	5.49 ± 4.15 **
Flavonols	17.06 ± 10.18	37.27 ± 18.16 **	16.71 ± 10.18	37.63 ± 18.16 *
Isoflavonoids	0.03 ± 0.03	22.84 ± 76.64	3.00 ± 0.03	19.88 ± 76.64
Lignans	0.40 ± 0.61	1.14 ± 0.60 **	0.49 ± 0.61	1.05 ± 0.60 *
Phenolic acids	122.64 ± 153.59	336.09 ± 301.89 **	145.93 ± 153.59	312.80 ± 301.89
Hydroxybenzoic acids	7.20 ± 9.24	40.61 ± 28.38 **	7.56 ± 9.24	40.25 ± 28.38 **
Hydroxycinnamic acids	115.37 ± 152.11	294.28 ± 292.73 **	138.21 ± 152.11	271.44 ± 292.73
Hydroxyphenylacetic acids	0.07 ± 0.09	0.98 ± 1.61 **	0.14 ± 0.09	0.91 ± 1.61
Stilbenes	0.68 ± 1.13	0.99 ± 1.85	0.64 ± 1.13	1.04 ± 1.85
Other polyphenols	9.61 ± 12.99	42.22 ± 32.60 **	10.65 ± 12.99	41.18 ± 32.60 **
Alkyphenols	4.75 ± 8.53	18.92 ± 29.75	4.56 ± 8.53	19.11 ± 29.75
Tyrosols	4.05 ± 6.41	19.34 ± 10.83 **	5.29 ± 6.41	18.11 ± 10.83 **
Dietary fiber (g/day)	20.20 ± 8.47	24.21 ± 9.87	22.05 ± 8.47	22.36 ± 9.87
Soluble	2.28 ± 1.18	2.56 ± 0.94	2.42 ± 1.18	2.41 ± 0.94
Pectin	0.74 ± 0.49	0.90 ± 0.43	0.74 ± 0.49	0.90 ± 0.43
Hemicellulose	1.43 ± 0.87	1.51 ± 0.66	1.57 ± 0.87	1.36 ± 0.66
Insoluble	12.77 ± 6.25	15.60 ± 7.40	13.44 ± 6.25	14.92 ± 7.40
Pectin	1.65 ± 0.84	1.84 ± 0.77	1.71 ± 0.84	1.77 ± 0.77
Hemicellulose	4.61 ± 3.04	5.13 ± 3.01	4.99 ± 3.04	4.75 ± 3.01
Klason lignin	1.68 ± 1.13	2.18 ± 0.85	1.84 ± 1.13	2.02 ± 0.85
Cellulose	5.10 ± 2.03	6.26 ± 3.08	5.31 ± 2.03	6.03 ± 3.08

Results derived from Mann-Whitney U test (unadjusted) or multivariate analysis adjusted by energy (adjusted). Variables are presented as estimated marginal mean ± SD. SFA, saturated fatty acids. MUFA, monounsaturated fatty acids. PUFA, polyunsaturated fatty acids. "-" (not measured). * $p \leq 0.05$, ** $p \leq 0.01$.

3.2. Diet and Gallbladder Microbiota in Cholelithiasic Patients

In order to explore the relationship between diet and the major members of the gallbladder microbiota, Spearman correlation analyses were conducted (Figures 1 and 2). The intake of dairy products was negatively correlated to the abundance of the phylum *Bacteroidetes*, the family *Bacteroidaceae* and the genus *Bacteroides*; eggs were inversely associated with the proportions of *Proteobacteria,* and particularly with the family *Xanthomonadaceae*; and seafood and meats showed positive associations with the *Pasteurellaceae* family, and specifically with the genus *Haemophilus.* Moreover, *Pasteurellaceae* correlated directly with sauces, and the intake of legumes was negatively correlated with the relative abundance of this bacterial family (Figure 1). In addition, several dietary compounds were linked to the levels of different biliary microorganisms (Figure 2A,B).

At this point, given the high correlation between all the assessed variables, an additional stepwise regression analysis was conducted to explore the relative importance of dietary compound intake on the gallbladder microbiota (Table 4).

Table 4. Results obtained from a stepwise regression analysis in order to identify the dietary compounds predictors of the relative abundance of the major gallbladder bacterial communities (%).

	Predictors	R^2	β	*p* Value
Phylum				
Actinobacteria	Linoleic fatty acid (g/day)	0.360	−0.600	0.023
Family				
Bacteroidaceae	Hydroxyphenylacetic acids (mg/day)	0.709	0.722	0.001
	Insoluble fiber (g/day)		−0.401	0.031
Chitinophagaceae	Soluble hemicellulose (g/day)	0.524	0.724	0.003
Flavobacteriaceae	Insoluble hemicellulose (g/day)	0.393	0.627	0.016
Lachnospiraceae	Hydroxycinnamic acids (mg/day)	0.383	0.619	0.018
Oxalobacteriaceae	Insoluble pectin (g/day)	0.728	0.853	0.000
Promicromonosporaceae	Magnesium (mg/day)	0.962	1.116	0.000
	Digestible carbohydrates (g/day)		−0.313	0.002
Propionibacteriaceae	Trans oleic fatty acid (g/day)	0.378	−0.615	0.019
Genus				
Actinobacillus	Trans oleic fatty acid (g/day)	0.321	0.566	0.035
Bacteroides	Hydroxyphenylacetic acids (mg/day)	0.709	0.722	0.001
	Insoluble fiber (g/day)		−0.401	0.031
Cellulosimicrobium	Magnesium (mg/day)	0.962	1.161	0.000
	Digestible carbohydrates (g/day)		−0.313	0.002
Cloacibacterium	Insoluble fiber (g/day)	0.549	0.741	0.002
Escherichia-Shigella	Linoleic acid (g/day)	0.286	−0.535	0.049
Lachnosclostridium	Dietary fiber (g/day)	0.316	0.562	0.036
Lachnospira	Hydroxycinnamic acids (mg/day)	0.894	0.946	0.000
Moraxella	Vitamin C (mg/day)	0.652	0.807	0.000
Propionibacterium	Trans oleic fatty acid (g/day)	0.379	−0.616	0.019
Ruminoclostridium 9	Vitamin C (mg/day)	0.430	0.656	0.011
Sediminibacterium	Soluble hemicellulose (g/day)	0.533	0.730	0.003

Results derived from stepwise regression analysis; R^2: coefficient of multiple determinations; β: standardized regression coefficient. Only significant results are presented.

From the compounds previously associated with bile microbiota, linoleic acid was found to be an independent contributor to levels of *Actinobacteria,* and *Escherichia-Shigella*, as well as the intake of *trans* oleic acids to *Propionibacteriaceae* and *Propionibacterium,* and another genus of the *Pasteurellaceae* family: *Actinobacillus*. Whereas *Bacteroidaceae* and *Bacteroides* variations were explained, in part, by the intake of insoluble fiber and hydroxyphenylacetic acids, the relative abundance of *Promicromonosporaceae* and *Cellulosimicrobium* was associated with magnesium and digestible carbohydrate intake. *Lachnoclostridium* levels (from the family *Lachnospiraceae*) were positively associated with dietary fiber intake, a family previously linked to butyrate production [31], while the proportions of *Chitinophagaceae,* and in particular *Sediminibacterium* genus [belonging to the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum (FCB group)], showed a direct relationship with soluble hemicellulose consumption. Also, insoluble fibers were found to be independent contributors to this group of bacteria, specifically the *Flavobacteriaceae* family and the genus *Cloacibacterium,* and insoluble pectin was also found to be an independent contributor to the *Oxalobacteraceae* family (belonging to the β-proteobacteria). Whereas the family *Lachnospiraceae* and the genus *Lachnospira* presented a direct association with hydroxycinnamic acids, the intake of vitamin C was related to the levels of the Gram-negative *Moraxella* and the Gram-positive *Ruminoclostridium* 9 (Table 4).

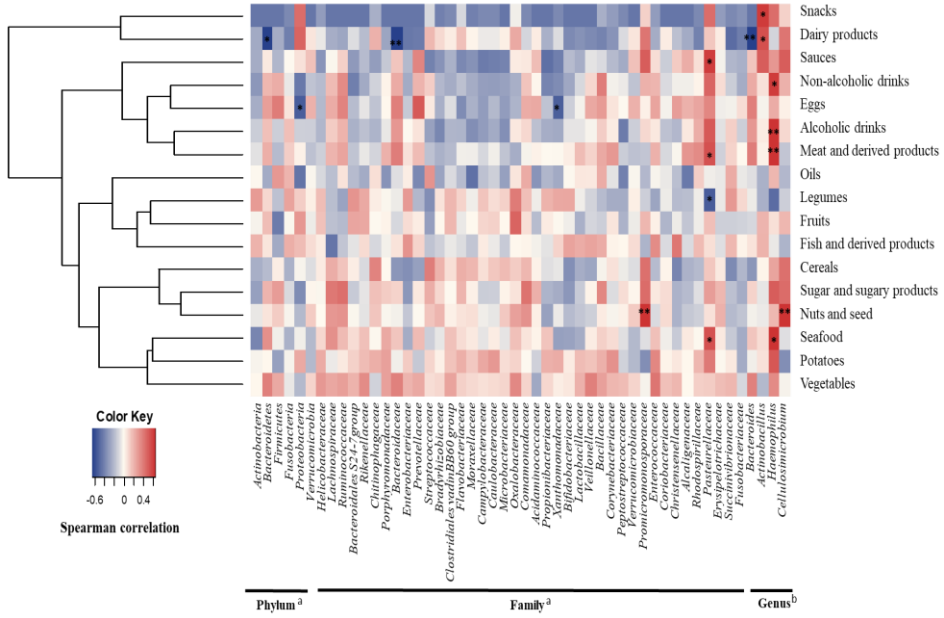

Figure 1. Spearman correlation between main food groups (g/day) from FFQ and gallbladder microbiota (%) in patients with cholelithiasis. [a] Phylum and families showing a relative abundance. [b] Genus belonging to families correlated with some food groups. Columns correspond to major phyla and families of biliary microbiota; rows correspond to the main food groups. Red and blue denote positive and negative association, respectively. The intensity of the colours represents the degree of association between the food groups and biliary microbiota as measured by the Spearman's correlations, and dots indicate significant associations. * $p < 0.05$; ** $p \leq 0.01$.

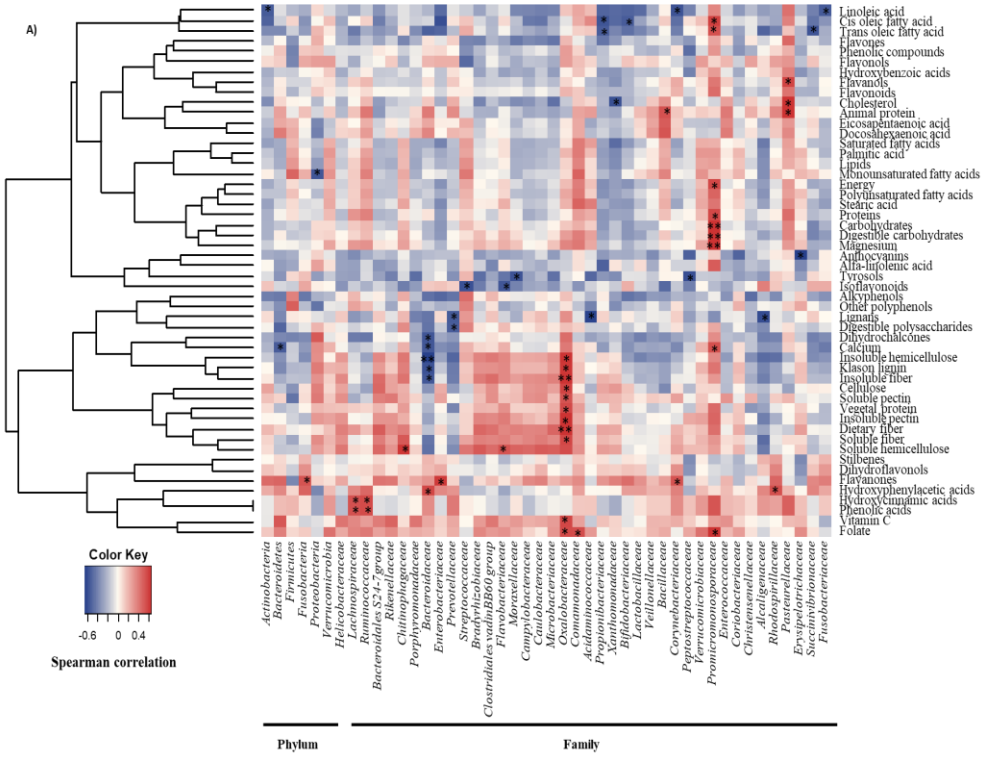

Figure 2. *Cont.*

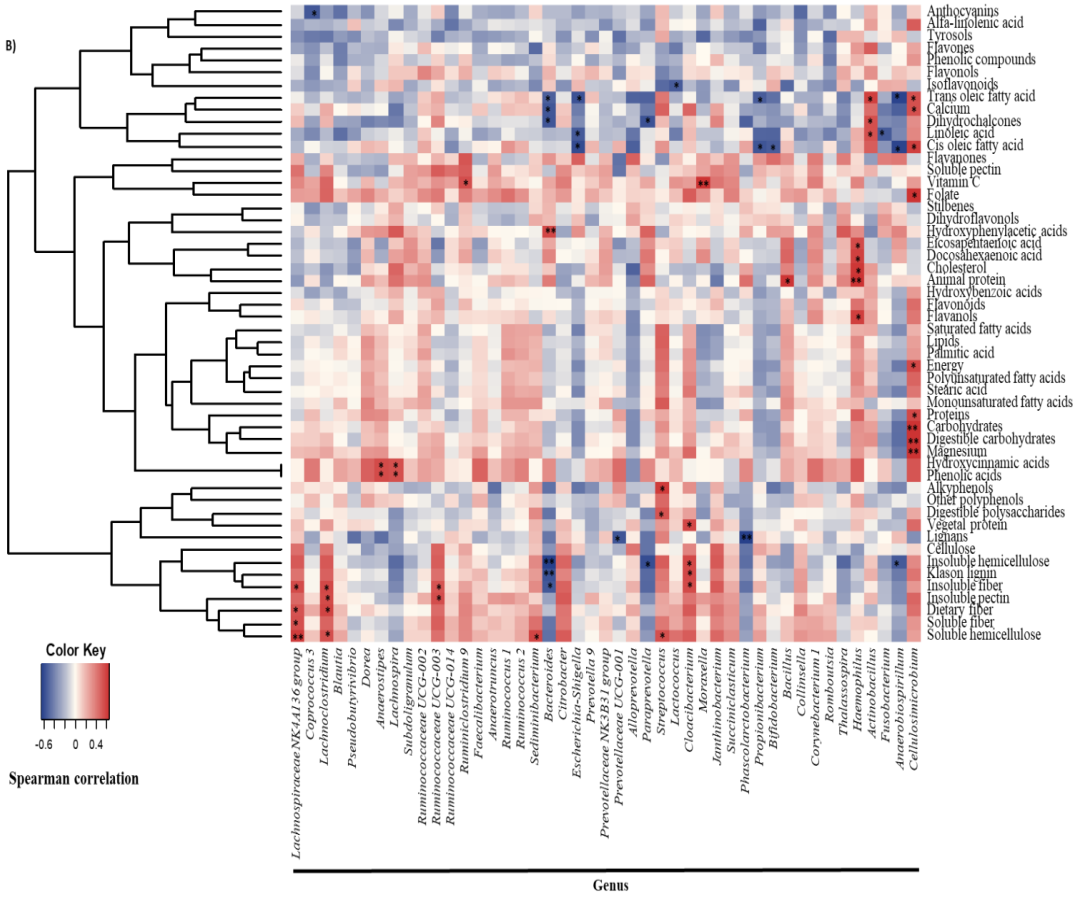

Figure 2. Spearman correlation between macro-, micronutrients, and bioactive compounds from the FFQ and gallbladder bacterial taxa in patients with cholelithiasis. (**A**) Phylum and families with a relative abundance. (**B**) Genera belonging to families correlated with some dietary compounds. Columns correspond to major phyla and families of biliary microbiota; rows correspond to the macro-, micronutrients and bioactive compounds. Red and blue denote positive and negative association, respectively. The intensity of the colours represents the degree of association between the dietary components and biliary microbiota as measured by the Spearman's correlations, and dots indicate significant associations. * $p < 0.05$; ** $p \leq 0.01$.

4. Discussion

Although since the 70's several surveys have been conducted in order to identify the existence of differences in the diets of subjects with cholelithiasis [32–34], the links between dietary factors and the development of the pathology are far from being completely understood. Compelling evidence has shed some light on the existence of microorganisms associated with the occurrence of this pathology. In this regard, it has been demonstrated that strains of *Salmonella* or *Listeria* monocytogenes are able to grow and survive within the gallbladder. In fact, the gallbladder can act as a reservoir for these bacteria, and one of the few bacteria frequently associated with gallstones is *Salmonella enterica* [35–37]. Furthermore, attempts have been made to analyse the biliary tract microbiota potentially associated with gallstones [19,38], using next-generation sequencing methods. Due to the crucial role described for gastrointestinal microbiota in human health, these findings generated novel interest regarding dietary factors able to modulate biliary associated microbial communities.

To our knowledge, there is no consensus on defining a pro-lithiasic diet. While some previous studies have reported an increase in energy intake in cholelithiasic patients [39], others described the inverse situation [40]. As the relationship between energy intake and gallstone formation has been closely linked to the presence of obesity, this could be one of the differential factors between

studies [41]. In our sample, while it has been observed a lower energy intake in the cholelithiasic group, no differences were found in BMI. Given that our patients had been diagnosed at least one year before recruitment, we propose the existence of a reduction in energy intake during this time, because of the exclusion of some foods that could be associated with the symptoms of this pathology [32], such as vegetables, non-alcoholic drinks, and sauces, and the reduction in the appetite reported by 42.8% of the sample, as has been shown in Table 1. Although the mean BMI of patients was indicative of moderate overweight, the findings presented herein do not support the role of obesity as a cholelithiasic risk factor, given the absence of differences with respect to the control group. Also, the lower level of HDL and the higher level of triglycerides observed (Table 1) were consistent with evidence supporting the potential role of cholesterol as a precursor in the formation of micelles with bile salts and phospholipids [41–44].

Analysing the global intake of the major food groups in the sample, a lower intake of vegetables has been found in the group with cholelithiasis [32] (Table 2). For decades, the protective effect of fruits and vegetables in the formation of gallstones was focused on their content of insoluble fibers, mainly lignin, which modifies the turnover of bile salts and cholesterol by means of reducing the lithogenic effect of the diet [45]. Our data do not support the existence of significant differences in the intake of fiber, neither in total nor by subclasses, between groups; nevertheless, a lower intake of most of the evaluated phenolic compounds has been observed in cholelithiasis (Table 3), suggesting a possible inverse association of these compounds in the pathology. Since foods are mixtures of bioactive compounds that could affect microbiota, there is no doubt about the complexity of analysing the associations for these components. For this reason, including the intake of fibers and polyphenols and their combined effect on biliary microbiota is probably the most novel aspect of this study. In addition, both fiber and polyphenol intake has been related in the patient group to the concentration of some of the microorganisms, whose proportion has been found to be altered in the bile of patients with lithiasis or primary sclerosing cholangitis (*Fusobacteria, Bacteroidaceae, Chitinophagaceae, Prevotellaceae*, and *Bacteroides*) [19,31,46], hence it is probable that microbiota could play a role in the protective effect proposed for vegetable sources. In this regard, from all the evaluated compounds hydroxyphenylacetic acid and insoluble fiber were pointed to as independent predictors of *Bacteroidaceae* genus *Bacteroides,* shown to be increased in this pathology [19], whereas cholesterol and *trans* oleic acid were found to be inversely related to the proportions of *Xanthomonadaceae* and *Propionibacteraceae*, respectively (Table 4). As the proportion of these latter microorganisms was found to be reduced in cholelithiasis as compared with healthy liver donors (Molinero et al., unpublished results), our results support the novel hypothesis proposed by Islam et al., suggesting that high-fat diets increase the secretion of bile acids showing strong antimicrobial activity [47]. In order to complete the overview of the role of diet in the gallbladder microbiota, it is worth highlighting the positive association found between the genera *Haemophillus* with the intake of seafood, meat, and drinks (Figure 1). This bacterial genus seems to be directly associated with the intake of flavanols, animal protein, cholesterol, and eicosapentaenoic and docosahexaenoic fatty acids (Figure 2).

The current study is the first to establish a link between biliary microbiota, diet, and gallstone formation. At present, the information about these interactions is limited. Bacterial bile and cholesterol metabolism by the gut communities and its influence on health is already known [48], as well as the effect of diet on bile acid metabolism and gut microbiota composition [11,49]. However, the origin or even the existence of the bile microbiota is more controversial, with only a few studies focusing on that [18,38,46]. The present manuscript represents a proof-of-concept study of this previously unexplored scenario. Some of the findings herein presented, such as the association of certain phenolic compounds with *Bacteroides* and *Lachnospira*, or between vitamin C and *Moraxella* and *Ruminoclostridium,* may pave the way for future research projects focused on this gastrointestinal pathology. One of the limitations is the lower sample size, which could somewhat hamper our ability to detect significant associations by reducing the power of the statistical tests. Although a stepwise regression analysis has been carried out in order to avoid the high degree of correlation

between the analysed variables, it is not possible to establish directionality in the relationship between the diet and the microbiota. The associations between these two variables have been carried out only in the group of cholelithiasic patients, given the impossibility of obtaining gallbladder samples from healthy volunteers. Even considering the above limitation, the knowledge generated in this work allows us to establish a new hypothesis that must be corroborated in the future. If these preliminary data are confirmed, they could be the first step in the rational design of diets adapted to this hepatobiliary disease.

Author Contributions: J.I.R., A.M., S.D. and S.G. designed the experimental work. I.G.-D. and S.G. carried out the nutritional and anthropometric determinations. A.C. and J.I.R. obtained the biological samples. N.M., A.M. and S.D. were involved in sequencing and microbiota analysis, meanwhile I.G.-D. and S.G. performed the statistical analysis. I.G.-D. and S.G. wrote the manuscript. All authors reviewed and approved the submitted version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Capítulo 3

Chapter 3

CAPÍTULO 3

Descripción de un nuevo taxón aislado de bilis humana, para la que se propone el nombre de *Ruminocoides biliarensis* IPLA60002

El tercer objetivo de esta Tesis Doctoral se centró en la caracterización de aislados obtenidos de muestras de bilis humana pertenecientes a sujetos del grupo control, para lo que se utilizaron distintos medios de cultivo en condiciones de aerobiosis y anaerobiosis. Entre otros aislados, se aislaron dos cepas de la clase *Clostridiales*, que consideramos de interés para posteriores estudios. Los dos aislados presentaron una homología de secuencia del ADNr 16S en las bases de datos con *Ruminococcus gauvreauii* (99%) y *Ruminococcus bromii* (94%), respectivamente. Este último con una baja identidad, lo consideramos candidato a una nueva especie.

En este capítulo se presenta la caracterización y la propuesta para describir una nueva especie aislada de bilis humana, perteneciente a la familia *Ruminococcaceae*, que denominamos *Ruminocoides biliarensis* IPLA60002. Se llevó a cabo una caracterización tanto fenotípica como genómica de esta cepa, y se compararon sus características con las de la especie de mayor homología, *Ruminococcus bromii*. A nivel fenotípico, se analizó y comparó con otras cepas de *R. bromii* el crecimiento en distintas fuentes de carbono y en almidón resistente, así como las principales actividades enzimáticas. También se estudiaron los principales productos de fermentación, la susceptibilidad a antibióticos y sales biliares, y la capacidad de esporulación. A nivel genómico se

estudiaron y compararon las principales características, observándose claramente que IPLA60002 se correspondía con un nuevo taxón, distinto de *R. bromii*.

Los resultados de la descripción de *Ruminocoides biliarensis* IPLA60002 se encuentran en fase de revisión en la revista *Systematic and Applied Microbiology*:

Artículo 3. Molinero, N., Conti, E., Sánchez, B., Walker, A., Margolles, A., Duncan, S.H. and Delgado, S. *Ruminocoides* gen. nov., with description of *Ruminocoides biliarensis* sp. nov., an isolate from human bile with autolytic behavior and bile resistance, and reclassification of *Ruminococcus bromii* as *Ruminocoides bromii* comb. nov.

Ruminocoides gen. nov., with description of *Ruminocoides biliarensis* sp. nov., an isolate from human bile with autolytic behavior and bile resistance, and reclassification of *Ruminococcus bromii* as *Ruminocoides bromii* comb. nov.

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Repositories: The whole genome project for *Ruminocoides biliarensis* has been deposited in the Sequence Read Archive (SRA) database of the NCBI under the accession number SRR9209608. The strain has been deposited publicly accessible in the BCCM/LMG culture collection (Belgium, Europe) under accession number LMG 31505, and in the DSMZ German Collection of Microorganisms (Leibniz Institute, Germany) under accession number DSM 110008.

Abbreviations: ANI, Average nucleotide identity; GAMc, Gifu anaerobic medium supplemented with cysteine; CBRF, Clarified bovine rumen fluid; dDDH, Digital DNA-DNA hybridization; GGDC, Genome-to-genome distance calculator, SCFAs, Short-chain fatty acids; GC, Gas chromatography; MICs, minimal inhibitory concentrations; CAZymes, Carbohydrate-Active Enzymes.

Abstract

A strictly anaerobic resistant starch degrading, bile tolerant, autolytic strain belonging to the *Ruminococcaceae* family was isolated from a human bile sample of a liver donor without hepatobiliary disease. Cells were Gram-positive cocci, and 16S rRNA gene and whole genome analysis showed that *Ruminococcus bromii* is the phylogenetically closest related characterized species to the novel strain IPLA60002, with average nucleotide identity (ANI) values below 90%. The DNA G+C content of the strain is 38.9%. Biochemically, the new isolate has metabolic features similar to those described previously in gut *R. bromii* strains, including the ability to degrade a range of different starches. The new isolate, however, produces lactate and shows distinct resistance to the presence of bile salts. Additionally, the novel bile isolate displays an autolytic phenotype after growing in different media. Both IPLA60002 and *R. bromii*, are distinct from other species within the *Ruminococcus* genus. On the basis of phylogenetic, genomic and metabolic data we therefore propose to reclassify *R. bromii* as *Ruminocoides bromii*, and that the novel IPLA60002 strain isolated from human bile be given the name *Ruminocoides biliarensis* sp. nov., within the *Ruminococcaceae* family. Strain ATCC 27255^{T} is proposed as the new type strain for the *Ruminocoides* genus, and *R. bromii* species. IPLA60002^{T} is proposed as the type strain of *Ruminocoides biliarensis*.

Keywords: *Ruminocoides***,** *Ruminococcus*, autolysis, human bile isolate, bile resistance.

Declaration of interest: none.

Introduction

Bacteria belonging to the genus *Ruminococcus* within the *Ruminococcaceae* family are a group of important gut symbionts. Originally isolated from the bovine rumen, ruminococci have been found in numerous mammalian hosts (including ruminants and non-ruminants), but much less is known about the diversity and environmental distribution of this genus [35]. *Ruminococcus* is currently considered a polyphyletic genus, with species members belonging to two separate families: the *Lachnospiraceae* and the *Ruminococcaceae*. It has been suggested that only species of this latter family be considered as "true ruminococci" [34]. Under this definition, there are only six described species of *Ruminococcus* to date, among which is *Ruminococcus bromii*, and the type species of the genus, *Ruminococcus flavefaciens* [39].

The species *R. bromii* was first described in 1972 as a strictly anaerobic Gram-positive coccus predominant in feces of humans [31] with complex nutritional requirements [10]. However, this species displays key phenotypic differences to other true *Ruminococcus* species. For example, it grows particularly well on starches, whereas the other ruminococci appear to be more specialized for growth on non-starch polysaccharides. [43]. Furthermore, 16S rRNA gene-based [34], and more recent whole genome-based [35], analyses have suggested that *R. bromii* is distinct from the other true ruminococci, and may require reclassification into a new genus.

Here, we present the results from a taxonomically novel species from the *Ruminococcaceae* family that was isolated from human bile. This strain is most closely related to *R. bromii* but based on 16S rRNA gene sequencing possesses less than 96% sequence identity with previously described *R. bromii*, and showed low homology with other ruminococci. In the view of its host habitat (human bile) and bile salt resistance, together with its metabolic and genomic relationships to other *R. bromii*, it is proposed that the isolate described here should be placed in a novel genus, be named *Ruminocoides biliarensis* sp. nov., and that *R. bromii* be reclassified as the only other currently known member of the genus.

Material and methods

Isolation and maintenance of a novel Ruminocoides strain from human bile

A human bile sample from the gallbladder of a liver donor without any hepatobiliary disorder was obtained aseptically during liver transplant at the General Surgery Service

of HUCA (Central University Hospital of Asturias) in Spain from a previous study [29] for which ethical approval was obtained from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias n°112/13) in compliance with the Declaration of Helsinki of 1964. The bile sample was immediately transported refrigerated to the laboratory and cultivated fresh in different media, including: Brain Heart Infusion (Oxoid, Waltham, MS, USA), Gifu anaerobic medium (Nissui, Tokyo, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma-Aldrich, San Luis, MO, USA) (named GAMc) and CDC Anaerobic Blood Agar (Anaerobe Systems, CA, USA). The strain reported here, named IPLA60002, was isolated from a 2% agar GAMc plate incubated at 37°C for 72 h in a Don Whitley MG500 anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 gas atmosphere.

The strain IPLA60002 was routinely maintained by growing in GAMc medium. Other media including the semi-defined RUM medium [42] and M2GSC medium supplemented with 30% v/v clarified bovine rumen fluid (CBRF) [28] were also used. Morphology was observed using an optical Leica DMi8 Automated phase-contrast microscope (Leica Microsystems, Germany) and by Scanning Electron Microscopy (SEM) at different times of growth. For the SEM, culture preparations were done following the method of Kubota and co-workers [18] with some modifications. Cultured bacteria were fixed with 2% glutaraldehyde and 1% paraformaldehyde solutions, dehydrated with increasing concentrations of ethanol, and finally dried with CO_2 using a CPD-030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). They were then coated with gold using a SCD 004 Sputtering Coater (Bal-Tec AG) and observed using a JSM-6610LV SEM (JEOL USA Inc, Peabody, MA, USA). Phenotypic characterization of the bile isolate IPLA60002 was carried out in parallel with two strains of *R. bromii,* L2-63 and 5AMG [32,43] isolated from human feces, available at The Rowett Institute, University of Aberdeen, Scotland. All three strains (IPLA60002, L2-63 and 5AMG) were cultured anaerobically in M2GSC broth medium supplemented with 30% CBRF [28] in Hungate tubes at 37°C. Media were prepared anaerobically under 100% CO_2.

Identification by 16S rRNA gene sequencing

Total genomic DNA was extracted and purified from 2 ml of an 18 h grown culture of IPLA60002 on GAMc broth using the GenElute Bacterial Genomic DNA kit (Sigma-

Aldrich), following the manufacturer's recommendations. Purified DNA was used as a template for amplification of the 16S rRNA gene using the universal primer pair 27f (forward primer) and 1492r (reverse primer) of the *Escherichia coli* numbering system [40]. PCR amplification conditions were as previously described [9], and sequencing was carried out using an ABI 373 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at the facilities of Macrogen (Madrid, Spain). The 16S rRNA gene sequence was compared with those deposited in the GenBank database using BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/).

Whole genome sequencing and phylogenetic analysis

Genomic DNA from IPLA60002 was purified using the "DNeasy blood and tissue" kit (Qiagen, Germany) following the manufacturer's protocol. The genome sequencing was carried out using 250-290 paired-end libraries on the Illumina MiSeq Sequencing System (Illumina, USA) at GenProbio SRL (Parma, Italy). Genome assembly was performed with the MIRA assembler v4.0.2 [6]. Open reading frames (ORFs) prediction was performed with Prodigal v2.6 [12]. Automatic annotation of the ORFs was performed with BLAST [1] against the NCBI database, and HMMER software (http://hmmer.org/) against the PFAM database [8] from the European Bioinformatics Institute (EMBL-EBI). Quality optimization of the final contigs was performed with the Burrows-Wheeler Aligner [24], SAMtools suite [23] and VarScan v2.2.3 software [16] packages.

To clarify the identity of the new isolate IPLA60002 we compared its genome with those of five other strains of *R. bromii*. These include strains isolated from human feces from the USA (type strain ATCC27255) [31], the UK (L2-63 and L2-36) [3,43], and Ecuador (5AMG) [32], and also a rumen strain from Australia (YE282) [15]. The genome sequences were retrieved from the NCBI using the genome browser (https://www.ncbi.nlm.nih.gov/genome/genomes). *In silico* DNA-DNA hybridization (DDH) experiments were carried out using the Genome-to-Genome Distance Calculator (GGDC), available online http://ggdc.dsmz.de/ [27]. We additionally used the JSpecies WS online software [38] to perform the Average Nucleotide Identity (ANI) analysis.

Phylogeny reconstruction was carried out by building a phylogenetic tree from human-source isolates of the family *Ruminococcaceae* based on the full-length 16S rRNA gene. The complete sequences of the 16S rRNA gene were aligned by ClustalW and the evolutionary history was inferred by using the Maximum Likelihood method based on

the Kimura 2-parameter model [14]. Trees for the heuristic search were obtained automatically by applying the Neighbor-Joining method (bootstrap=5,000) and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. The tree with the highest log likelihood was selected. Evolutionary analyses were conducted in MEGA7 [19].

Other genome comparisons

To perform additional genomic characterization of the strain IPLA60002, we compared its genomic profile with that of four strains of *R. bromii* isolated from human feces; ATCC 27255^{T}, 5AMG, L2-36 and L2-63. We also carried out dbCAN analysis [41]. dbCAN is a web server and database for carbohydrate-active enzyme activities encoding genes. All data in dbCAN are generated based on the family classification from the CAZy website (http://www.cazy.org/) [26]. Additionally, the PHASTER (PHAge Search Tool Enhanced Release) web server [2] was used to look for presence of prophage sequences within the IPLA60002 genome, in comparison with the *R. bromii* genomes of the L2-63 and 5AMG strains from The Rowett Institute, University of Aberdeen, Scotland.

Carbohydrate utilization and enzymatic profiles

To analyze the growth of IPLA6002 and the *R. bromii* strains L2-63 and 5AMG, in different carbon sources and resistant starches, we performed a series of characterization tests in M2 medium supplemented with 30% CBRF and 0.2% w/v of the different carbon sources [28] in Hungate tubes. Triplicate tubes were inoculated with an overnight culture of the corresponding strain, and the OD_{650} values were measured every two hours up to 48 h in a Novaspec II Spectrophotometer (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey, EEUU). The growth in different resistant starches was recorded by measuring the drop in the pH (pH <6.0 as positive growth) after 48 h incubation, as the opaque nature of starch-containing media made spectrophotometer readings unreliable.These analyses were complemented using the API20A® test system, which contains substrates for carbohydrate utilization and enzymatic reactions (bioMérieux, Marcy l'Etoile, France). A suspension of each strain was prepared by adding the pellet of pure cultures in M2GSC medium supplemented with 30% CBRF into the API Anaerobe suspension medium. The turbidity of the suspension was adjusted to 3.0 of the McFarland standard. The inoculated test strips were incubated in

an anaerobic cabinet at 37°C for 24 h. The strips were read visually based on color changes following the manufacturer's instructions.

Fermentation product analysis

Short-chain fatty acids (SCFAs) concentrations produced by the three strains (*R. biliarensis* IPLA60002, *R bromii* L2-63 and 5AMG) were measured by gas chromatography (GC) as described previously [36] after growing the strains in M2GSC medium supplemented with 30% CBRF. Following derivatization of the samples using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich), the samples were analyzed in triplicate using a Hewlett Packard gas chromatograph system fitted with a silica capillary column linked to a Shimadzu chromatopac integrator (Dyson Instruments Ltd, Tyne and Wear, UK) using helium as the carrier gas.

Antibiotic susceptibility testing

The resistance against different antibiotics of the three strains (*R. biliarensis* IPLA60002, *R bromii* L2-63 and 5AMG) was performed in an anaerobic cabinet by disk diffusion assays in 2% w/v agar plates of modified YCFA medium [43] with a cover of M2GSC with 1% w/v agar and supplemented with 30% CBRF inoculated with the corresponding strain. After 1 h, different antimicrobial susceptibility disks (Oxoid) were put onto the plates using an Antimicrobial Susceptibility Disk Dispenser (Oxoid). The antibiotics, and the concentrations tested, were the following: Fusidic Acid (5 µg), Clindamycin (10 µg), Trimethoprim (2.5 µg), Gentamicin (30 µg), Erythromycin (5 µg), Amoxycillin (25 µg), Rifampicin (30 µg), Ciprofloxacin (10 µg), Amoxycillin/Clavulanic Acid (3 µg), Tetracycline (30 µg), Streptomycin (10 µg), Chloramphenicol (10 µg), Vancomycin (5 µg), Penicillin G (2 µg) and Ampicillin (10 µg).

Bile salt tolerance

The analysis of resistance to different bile salts was performed in triplicate by determining the minimal inhibitory concentrations (MICs) for the three strains in M2GSC 2% agar plates supplemented with 30% CBRF and different concentrations (ranging from 0% to 10% w/v) of Cholic acid, Taurodeoxycholic acid, Glycocholic acid, Taurocholic acid, Glycodeoxycholic acid, Bile extract porcine, Bile Bovine (all

purchased from Sigma-Aldrich) and Mix Bile Salts (Oxoid). The plates were incubated at 37°C in the anaerobic cabinet during 48 h.

Sporulation test

To test for sporulation, *R. biliarensis* IPLA60002 and the *R. bromii* strains L2-63 and 5AMG were grown for 72 h in M2GSC medium and heated at 80°C for 20 min to kill vegetative cells. Subsequently, the cells were treated as described previously [32] and the Schaeffer and Fulton Spore Stain kit was used (Sigma-Aldrich) for the observation of spore germination under an Olympus BX50F microscope (Olympus Corporation, Japan).

Results and discussion

Description of the isolate and growth conditions

The novel strain (IPLA60002) isolated from human bile is a Gram-positive coccus, strictly anaerobic, non-motile, and normally groups in small chains or pairs when examined by phase-contrast microscopy (Suppl. Fig.1). The IPLA60002 strain required 72 h to grow on GAMc agar plates, forming large, white (slightly translucent) rounded colonies with a viscous appearance. Sporulation capability was tested, revealing that IPLA60002 is able to form spores, similar to other related *Ruminococcaceae* such as the species *R. bromii* [32]. In liquid medium (GAMc broth) the IPLA60002 required 16-18 h to reach the maximum OD_{650} of 0.8. We observed that after reaching this point, the strain showed an autolytic phenotype, with a marked reduction in optical density measurements after 24 h (OD_{650} <0.3). IPLA60002 can also grow in RUM medium, exhibiting complete lysis after reaching maximal OD_{650}, as was observed in GAMc medium. Examination of the cultures at different time points under SEM confirmed the autolytic behavior (Fig. 1). In M2GSC broth supplemented with CBRF, the IPLA60002 strain grew faster than in the other two media tested, reaching a maximum OD_{650} of 0.7-0.8 in 12 h and then began autolysis after this point. However, in M2GSC medium it took a longer time to completely lyse. Growth of the IPLA60002 strain in M2GSC medium is represented in Fig. 2.

Molecular and phylogenetic identification

First, we amplified the 16S rRNA gene from the IPLA60002 strain by PCR and sequenced with an ABI sequencer. Sequence comparison using BLASTn against the NCBI GenBank database revealed that the closest fully characterized cultured relative is *R. bromii*, but that the level of homology was less than 96%. Following the criterion applied by Palys and co-workers, sequences with a percentage of identity lower than 97% to those in databases should be allocated to different species [33]. The whole genome of IPLA60002 strain was then sequenced, and after assembling 59 contigs were obtained; these contigs were deposited as a whole-genome shotgun project in the Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under the accession number SRR9209608. The genome analysis of the IPLA60002 strain revealed a genome size of 2.5 Mb, and a DNA G+C content of 38.9%. This analysis also revealed the presence of 2,356 predicted ORFs and 66 tRNAs in the genome of the biliary isolate. In contrast, *R. bromii* has a median total length of 2.21 Mb and a median G+C content of 40.8%, based upon the 16 genomes currently available for this genus from the NCBI genome browser. Genome size estimated for human-derived strains were slightly lower (2.15–2.40 Mb), with all being more similar in size than that reported for the Australian strain YE282 that was isolated from the rumen of cattle (2.53 Mb) [32]. ANI analysis was performed to define the degree of identity at the DNA level. The ANIb values shown in Table 1 correspond with the use of BLAST for the alignment of the sequences, while ANIm represent those obtained based on MUMmer comparisons [21]. The values obtained in both cases were below the boundaries established to limit species definition [17]. The lowest values (73.73% for ANIb and 85.16% for ANIm) were obtained in this case for the comparisons with the rumen strain YE282. In a previous comparative genomic study of *R. bromii* it was observed that the genomic sequences of four *R. bromii* human strains (L2-36, 5AMG, ATCC27255 and L2-63) share 95–100% average nucleotide identity with each other and only 86% identity with the rumen strain [32]. ANI values of approximately 94–96% have been established to represent the boundary for taxonomically circumscribing prokaryotic species. Simultaneously, those values have been shown to mirror the DNA–DNA hybridization range of approximately 70%, classically adopted as the boundary for novel prokaryotic species [17,37]. Additionally, to exclude potential affiliation to the same species, complete dDDH estimates of our IPLA60002 isolate and different *R. bromii* genome sequences were obtained (Table 1). For dDDH, we used the formula #2

(recommended by the GGDC) in which the number of identities is divided by the high-scoring segment pair length. The result of this formula is a distance which is transformed to values analogous to wet DDH through the application of a generalized linear model trained on an empirical reference dataset. These data revealed low sequence similarity between our isolate and the genomes of other *R. bromii*, providing further supporting evidence that the IPLA60002 strain belongs to a new species. The phylogenetic position of the new IPLA60002 isolate was studied by building a phylogenetic tree for the *Ruminococcaceae* family based on the full-length 16S rRNA gene. The analysis involved nucleotide sequences for 30 different members, including the four human *R. bromii* strains (L2-63, L2-36, 5AMG and the type strain ATCC27255). A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter =0.5343)]. The rate variation model allowed for some sites to be evolutionarily invariable [(+I), 53.58% sites]. The maximum-likelihood tree (Fig. 3) showed that IPLA60002 formed a distinct lineage, alongside all four *R. bromii*, that is distinct from the other species in the true *Ruminococcus* genus, including the type species *R. flavefaciens*. However, IPLA60002 also forms a distinct sub-lineage from *R. bromii*, which further supports the other evidence to suggest that it belongs to a distinct species. Our phylogenetic tree is in agreement with previous work, based on 16S rRNA genes [34], and whole genome analysis [35], which both established that the true ruminococci form a monophyletic group within the *Ruminococcaceae* family, with the exception of the *R. bromii* strains, which are more deeply rooted and form a separate clade more closely related to various other species derived from the *Clostridia* Class [35]. We therefore propose that *R. bromii* be re-classified as *Ruminocoides bromii*, and IPLA60002 be classified as a novel species, *Ruminocoides biliarensis*. Given that *R. bromii* is a dominant member of the human gut microbiota, where it is known to be a keystone species for resistant starch degradation [43] and is therefore of considerable research interest, the re-classification with the name "*Ruminocoides*" is suggested as a practical solution, whereby it retains the shorthand name "*R. bromii*". This follows the precedent set in the recent reclassification of *Clostridium difficile* as *Clostridioides difficile* [22].

Phenotypic characteristics

The biochemical traits of the IPLA60002 strain was determined using the commercially available API20A kit. The results for the carbon source utilization are reported in Table

2 where the fermentation capability of IPLA60002 is compared with those of *R. bromii* 5AMG and L2-63. The ability to use different carbon sources was the same for the three strains tested, producing acid from D-fructose and D-maltose, according to the initial nutritional requirements description for this species [10], and unable to metabolize D-glucose, mannitol, lactose, sucrose, D-salicin, L-xylose, D-arabinose, glycerol, cellobiose, D-mannose, melezitose, raffinose, sorbitol, rhamnose, trehalose, inositol, fucose, N-acetylgalactosamine and mucin. As indicated by measuring a decrease in culture pH at the end of the incubations, the IPLA60002 strain was able to hydrolyze resistant starches derived from rice, corn, and potato, as well as different high amylose maize starches such as Hylon VII, Novelose and High Maize. Degradation of resistant starch is a characteristic trait of *R. bromii,* and has previously been described in several studies that report extreme specialization in starch utilization [32,42,43]. Analysis of fermentation products of cultures grown in M2GSC medium supplemented with CBRF showed that acetate and formate were the main products formed by the three strains. However, IPLA60002, but not *R. bromii* 5AMG and L2-63 strains, also produced low levels of lactate (Table 2, Suppl. Fig.2).

We also evaluated resistance to antimicrobials and to bile salts. Regarding antibiotic resistances, the results were obtained by measuring the radius size of the inhibition halo around the antimicrobial disks. The profile of resistance/susceptibility was quite similar for the three tested strains, showing only resistance to trimethoprim and streptomycin, which is rather common among human commensals [5]. Sensitivity to ciprofloxacin was however higher in IPLA60002 than in the other two strains (Table 3).

As regards growth in presence of different bile salts, we observed differences between the IPLA60002 strain and the *R. bromii* strains (Table 4). The MICs were in general much higher for the bile isolate *R. biliarensis* than those found with the *R. bromii* strains 5AMG and L2-63. Of note, was the striking resistance of the IPLA60002 strain, isolated from bile, to the conjugated primary bile salts of the gallbladder, glycocholic and taurodeoxycholic, as well as the resistance to the presence of bovine and mixed bile salts (composed of sodium glycocholate, sodium taurocholate and sodium taurodeoxycholate), with the IPLA60002 strain possessing the ability to grow in 4% (final concentration), which was in marked contrast with the two *R. bromii* strains tested. This phenotype was supported by some genomic traits. The genome of IPLA60002 possesses different genes related to the response to bile salts, which are absent in the genomes of *R. bromii* strains 5AMG and L2-63. This includes a

diaminobutyrate-pyruvate aminotransferase (EC 2.6.1.46) and manganese superoxide dismutase (EC 1.15.1.1), both related with stress response; as well as an G:T/U mismatch-specific uracil/thymine DNA-glycosylase and a methyl-directed repair DNA adenine methylase (EC 2.1.1.72), related to DNA repair proteins. Rather than bile salt hydrolases, whose activity has been suggested as a bile acid detoxification mechanism for bacteria [30], the genome of IPLA60002 possesses many genes encoding efflux transporters likely to export these bile salts, such as ABC-type multidrug transporters and small multidrug resistance (SMR) transporters, which protect bacteria from an array of hydrophobic and cationic antimicrobials [13], and MATE efflux family proteins, which have been related to the extrusion of cholate and deoxycholate in *E. coli* [20] and the presence of sodium-dependent multidrug efflux pumps [7].

Comparative genomics potentially related to the autolytic behavior

dbCAN analysis was carried out to search for sequences related to predicted carbohydrate-degradation enzymatic activities, based on reference sequences in the CAZy database [26]. The results of the dbCAN analysis for the IPLA60002 strain and the four *R. bromii* strains analyzed are represented in the heatmap shown in Fig. 4. The heatmap shows those predicted enzymatic activities that were present more than three times in at least one of the strains. When the IPLA60002 was compared with the other *R. bromii* genomes, the carbohydrate-binding module CBM37 is underrepresented. A previous study already indicated that these four human *R. bromii* genomes, but not the rumen YE282 strain, encode multiple copies of a novel family of accessory ('X') module closest in sequence to CBM37 [32]. An unusually limited Carbohydrate-Active Enzymes (CAZymes) repertoire in *R. bromii* for a carbohydrate utilizing gut bacterium has already been described previously, with the great majority of CAZymes found to be belonging to family GH13 (associated with starch degradation) in this species, and to families (GH23, GH24, GH25) that encode lysozymes [32]. In the genome of IPLA60002 genes of the family GH25 of glycoside hydrolases are overrepresented in comparison with the *R. bromii* genomes (Fig. 4). We found four different genes of this GH25 family of glycosyl-hydrolases that appear to be autolysins (such as glycoside hydrolase family 25-like lysozyme/endolysin), and which may be responsible of the autolytic phenotype observed in IPLA60002. In the genome of IPLA60002 two of these genes were annotated to the enzyme called lysozyme M1 (1-4-beta-N-acetylmuramidase), related to autolysins [25], that were found in the contigs

"Contig_12_1925892_1926980" and "Contig_03_437855_438760". In contrast, in the genomes of the *R. bromii* isolates, we found only one gene annotated to the GH25 family. In addition, a cluster of genes potentially related to exopolysaccharide production in "Contig_12_ 1867726_ 1888884" of IPLA60002 was found (Fig. 5). The genes of this cluster were annotated as different polysaccharide biosynthesis proteins, glycosyltransferases, epimerases, one cDP-glucose4 6-dehydratase and four hypothetical proteins. We hypothesize that this cluster could be associated with exopolysaccharide production [4,11] contributing to the appearance of the extracellular layer covering and connecting the cells of *R. biliarensis* observed in the images obtained by SEM (Fig. 1).

PHASTER was used as a computational method to identify prophages that could be related with the auto-induction of phages and the lysis observed in the IPLA60002 strain after reaching a maximum optical density of 0.8 in liquid media. PHASTER identified the presence of different prophage regions, but only prophages identified as "questionable" were considered (this refers specifically to regions with a total score between 70 and 90). One prophage of 19.6 Kb with a complete transposase, tail and lysin proteins (Suppl. Fig.3) located in contig 11 was identified within the genome of the IPLA60002. The cluster of genes within contig "Contig_11_1630851_ 1650501" were annotated as different phage proteins; toxin secretion/phage lysis holing, phage tail protein, phage protein HK97 gp10 family, phage head-tail adapter protein, phage major capsid protein HK97 family, and a phage portal protein HK97 family terminase. This region showed high homology to HK97 bacteriophage, a dsDNA phage of the family *Siphoviridae* which presence has been observed in *Clostridium* (UniProtKB entry name C1FTH6_CLOBJ). We did not find this cluster in the genomes of *R. bromii* strains 5AMG and L2-63. Although this, we did not observe the presence of free phages in induced cultures with mitomycin (data not shown). More studies are needed for unravelling the molecular mechanisms behind the autolytic behavior of IPLA60002.

Finally, in this work we demonstrate that *Ruminococcus bromii* requires reclassification to a novel genus, *Ruminocoides*, and provide a genotypic and phenotypic description of an additional novel species within this genus, *R. biliarensis*.

Description of *Ruminocoides* gen. nov.

Ruminocoides (Rum.ino.c.o'i.des. N.L. neut. n.Ruminococcus a bacterial genus; L. suff. -oides (from Gr. suff. -eides, from Gr.n.eidos, that which is seen, form, shape, figure), resembling, similar; N.L.neut. n.Ruminocoides, organism similar to Ruminococcus). The type strain of the genus is *R. bromii* ATCC 27255^{T}.

Description of *Ruminocoides bromii* comb. nov.

As originally described in Moore et al. (1972) [31]. The type strain of the species is *R. bromii* ATCC 27255^{T}.

Description of *Ruminocoides biliarensis* sp. nov.

Ruminocoides biliarensis (bil.iar.ensis. N.L. masc. *biliarensis* from bile) from which the type strain was isolated.

Cells are non-motile cocci, with an approximate size of 1 µm, and stain Gram-positive. Cells may sporulate and grow in small chains or pairs. The biochemical characteristics are fermentation on fructose, maltose and a range of resistant starches. It does not grow on arabinose, cellobiose, glucose, lactose, mannose, melezitose, raffinose, rhamnose, sucrose, trehalose and xylose. The major fermentation products are formate, acetate and lactate. The type strain was isolated from human bile and exhibits enhanced tolerance to bile acids relative to *R. bromii*. The DNA G+C content of the type strain is 38.9%. The type strain of the species is IPLA60002^{T}.

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Tables and figures legends

Table 1. ANIb, ANIm and GGDC results for the comparison of the genome of *R. biliarensis* IPLA60002 strain with five different genomes of *R. bromii*.

Table 2. Substrate utilization and major fermentation products of *R. biliarensis* IPLA60002 in comparison with two *Ruminocoides bromii* strains of human origin.

Table 3. Radius of inhibition halo (mm) observed in *R. biliarensis* IPLA60002 and two *R. bromii* strains in presence of different antibiotics.

Table 4. Minimal inhibitory concentrations (MICs) against different bile salts (expressed as %) of *R. biliarensis* IPLA60002 and two human *R. bromii* strains, in M2GSC medium.

Figure 1. Images obtained by scanning electron microscopy (SEM) of the growth of *Ruminocoides biliarensis* IPLA60002 at different time points. A) Culture in GAMcys medium for 12 h. B) Culture in GAMcys medium for 16 h, at which time point some deformed cells can be observed. C) Culture in GAMcys medium after 24 h, where deformed and lysed forms are prevalent.

Figure 2. Growth of *R. biliarensis* IPLA60002 in M2GSC medium supplemented with 30% clarified bovine rumen fluid. The graph shows the average and standard deviation of the OD_{650} values (of triplicate cultures).

Figure 3. Phylogenetic bootstrap consensus tree based on full-length sequences of the 16S rRNA gene from different human isolates within the *Ruminococcaceae* family. The Neighbor-Joining tree is drawn to scale, with branch lengths determined by the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1,241 positions in the final dataset.

Figure 4. Heatmap based on dbCAN analysis, comparing the number of matches to predicted carbohydrate-active enzymes against the reference CAZy database in the genomes of the *R. biliarensis* IPLA60002 and the *R. bromii* strains 5AMG, ATCC27225, L2-36 and L2-63. Color key: the color scale represents a value range of

presence of predicted activities, with darker coloring representing a greater number of matches to the CAZy database, up to a maximum of 22. The up and down arrows represent over and underrepresentation (more than three-fold) of the predicted enzymatic activities.

Figure 5.

Cluster of genes potentially related with exopolysaccharide production detected in Contig_12 (21158bp) in the genome of *Ruminocoides biliarensis* IPLA60002. The genes of this cluster were annotated in the following order: one polysaccharide biosynthesis protein, one tyrosine kinase, one exopolysaccharide biosynthesis protein, one glucose-1-phosphate cytidylyltransferase, two sugar epimerases, three glycosyltransferases, one hypothetical protein, another two glycosyltransferases, one hypothetical protein, one polysaccharide biosynthesis protein and finally two hypothetical proteins.

Supplementary Fig 1. Phase-contrast microscopy image of IPLA60002 isolated from human bile.

Supplementary Fig 2. Short Chain Fatty Acids (SCFAs) production (in mM). In Y axes is represented the normalized concentrations of SCFAs respect to the blank.

Supplementary Fig 3. Prophage cluster detected in *R. biliarensis* IPLA60002. The cluster of 19.6Kb contains in the following order: one integrase, two hypothetical proteins, one chaperone for long tail fiber formation, one lysin followed to one holin, two minor structural proteins, three tail proteins, two hypothetical proteins, one major tail protein, one hypothetical protein, three phage proteins, one major protein of the capsid followed by a protease, one phage portal protein and one terminase.

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Table 1. ANIb, ANIm and GGDC results for the comparison of the genome of *R. biliarensis* IPLA60002 strain with five different genomes of *R. bromii*.

	ANIb		ANIm(%)		GGDC		
Strain for comparison	Identity (%)	Alignment (%)	Identity (%)	Alignment (%)	dDDH	Model C.I. (%)	Distance
R. bromii L2-36	74.97	46.92	85.8	14.9	23.1	20.8-25.5	0.1897
R. bromii ATCC 27255	74.94	48.58	85.76	15.39	22.3	20.0-24.7	0.1966
R. bromii 5AMG	75.69	47.37	86.7	16.1	24.7	22.4-27.2	0.1765
R. bromii YE282	73.73	37.68	85.16	8.03	23.6	21.3-26.1	0.1853
R. bromii L2-63	74.53	46.61	85.73	14.39	22.8	20.5-25.2	0.1923

Abbreviations: GGDC, Genome-to -Genome Distance Calculator; ANIb, Average Nucleotide Identity based on BLAST; ANIm, Average Nucleotide Identity based on MUMmer dDDH, digital DNA-DNA hybridization; C.I., confidence interval.

For dDDH the formula 2 based on a generalized linear model (identities / high-scoring segment pair) was used.

Probability that dDDH > 70% (i.e., same species): 0%.

Table 2. Substrate utilization and major fermentation products of *R. biliarensis* IPLA60002 in comparison with two *Ruminocoides bromii* strains of human origin.

Fermentation of:	***R. biliarensis* IPLA60002**	***R. bromii* 5AMG**	***R. bromii* L2-63**
Arabinose	-	-	-
Cellobiose	-	-	-
Fructose	+	+	+
Glucose	-	-	-
Lactose	-	-	-
Maltose	+	+	+
Mannose	-	-	-
Melezitose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Sucrose	-	-	-
Trehalose	-	-	-
Xylose	-	-	-
Catalase	-	-	-
Esculine	-	-	-
Gelatin	-	-	-
L-tryptophane	-	-	-
Salicin	-	-	-
Urea	-	-	-
Glycerol	-	-	-
Inositol	-	-	-
Mannitol	-	-	-
Sorbitol	-	-	-
Fucose	-	-	-
Mucin	-	-	-
N-acetylgalactosamine	-	-	-
Degradation of starches:			
High Maize	+	+	+
Hylon VII	+	+	+
Novelose	+	+	+
Starch from corn	+	+	+
Starch from potato	+	+	+
Starch from rice	+	+	+
Major fermentation products determined by GC*	A, F, L	A, F	A, F

Abbreviations: GC, Gas Cromatography; A, acetate; F, formate; L, lactate.
+: The strain is able to growth in M2 medium with the corresponding carbon source.
* Production was considered from >2mM.

Table 3. Radius of inhibition halo (mm) observed in *R. biliarensis* IPLA60002 and two *R. bromii* strains in presence of different antibiotics.

Antibiotic	*R. biliarensis* IPLA60002	*R. bromii* L2-63	*R. bromii* 5AMG
Fusidic Acid (5µg)	13.5	9	7
Clindamycin (10µg)	17	18	17
Trimethoprim (2.5µg)	ND	ND	ND
Gentamicin (30µg)	3	1.5	1
Erythromycin (5µg)	4.5	3	5
Amoxycillin (25µg)	15.5	17	15
Rifampicin (30µg)	14	17	13
Ciprofloxacin (10µg)	16.5	6	5
Amoxycillin/Clavulanic Acid (3µg)	14.5	11	10.5
Tetracycline (30µg)	3.5	14.5	3
Streptomycin (10µg)	ND	ND	ND
Chloramphenicol (10µg)	13	12	12
Vancomycin (5µg)	8.5	10	12
Penicillin G (2µg)	15.5	14.5	12
Ampicillin (10µg)	15.5	17	17

ND: no inhibition halo observed.

Table 4. Minimal inhibitory concentrations (MICs) against different bile salts (expressed as %) of *R. biliarensis* IPLA60002 and two human *R. bromii* strains, in M2GSC medium.

Strain	CA	TDC	GC	TC	GDC	Porcine Bile	Bovine Bile	Mix Bile Salts
R. biliarensis IPLA60002	<0.25%	4%	4%	4%	<0.25%	<0.25%	8%	8%
R. bromii 5AMG	0.4%	2%	0.5%	4%	0.1%	0.25%	4%	<0.5%
R. bromii L2-63	0.2%	2%	0.5%	4%	0.1%	0.25%	2%	<0.5%

Abbreviations: CA, Cholic Acid; TDC, Taurodeoxycholic Acid; GC, Glycocholic Acid; TC, Taurocholic Acid; GDC, Glycodeoxycholic Acid.

Figure 1. Images obtained by scanning electron microscopy (SEM) of the growth of *Ruminocoides biliarensis* IPLA60002 at different time points. A) Culture in GAMcys medium for 12 h. B) Culture in GAMcys medium for 16 h, at which time point some deformed cells can be observed. C) Culture in GAMcys medium after 24 h, where deformed and lysed forms are prevalent.

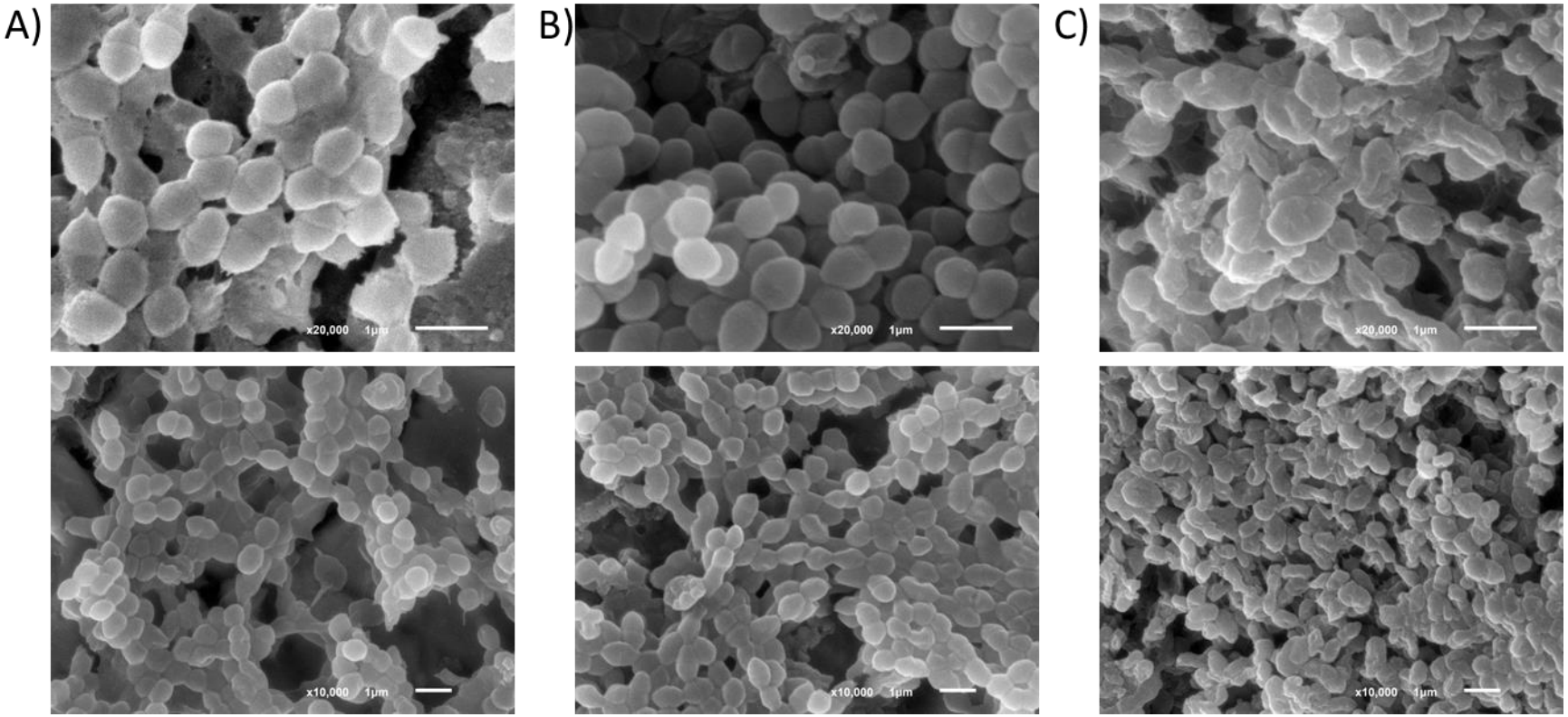


Figure 2. Growth of *R. biliarensis* IPLA60002 in M2GSC medium supplemented with 30% clarified bovine rumen fluid. The graph shows the average and standard deviation of the OD_{650} values (of triplicate cultures).

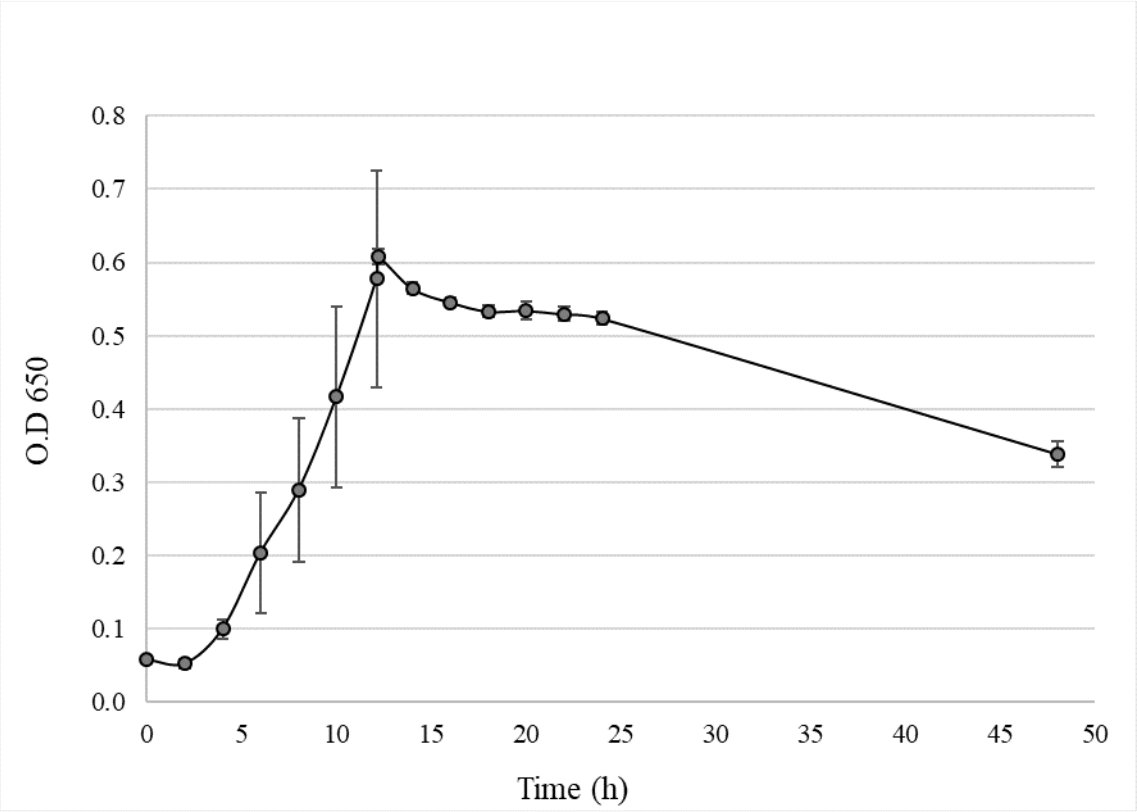


Figure 3. Phylogenetic bootstrap consensus tree based on full-length sequences of the 16S rRNA gene from different human isolates within the *Ruminococcaceae* family. The Neighbor-Joining tree is drawn to scale, with branch lengths determined by the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1,241 positions in the final dataset.

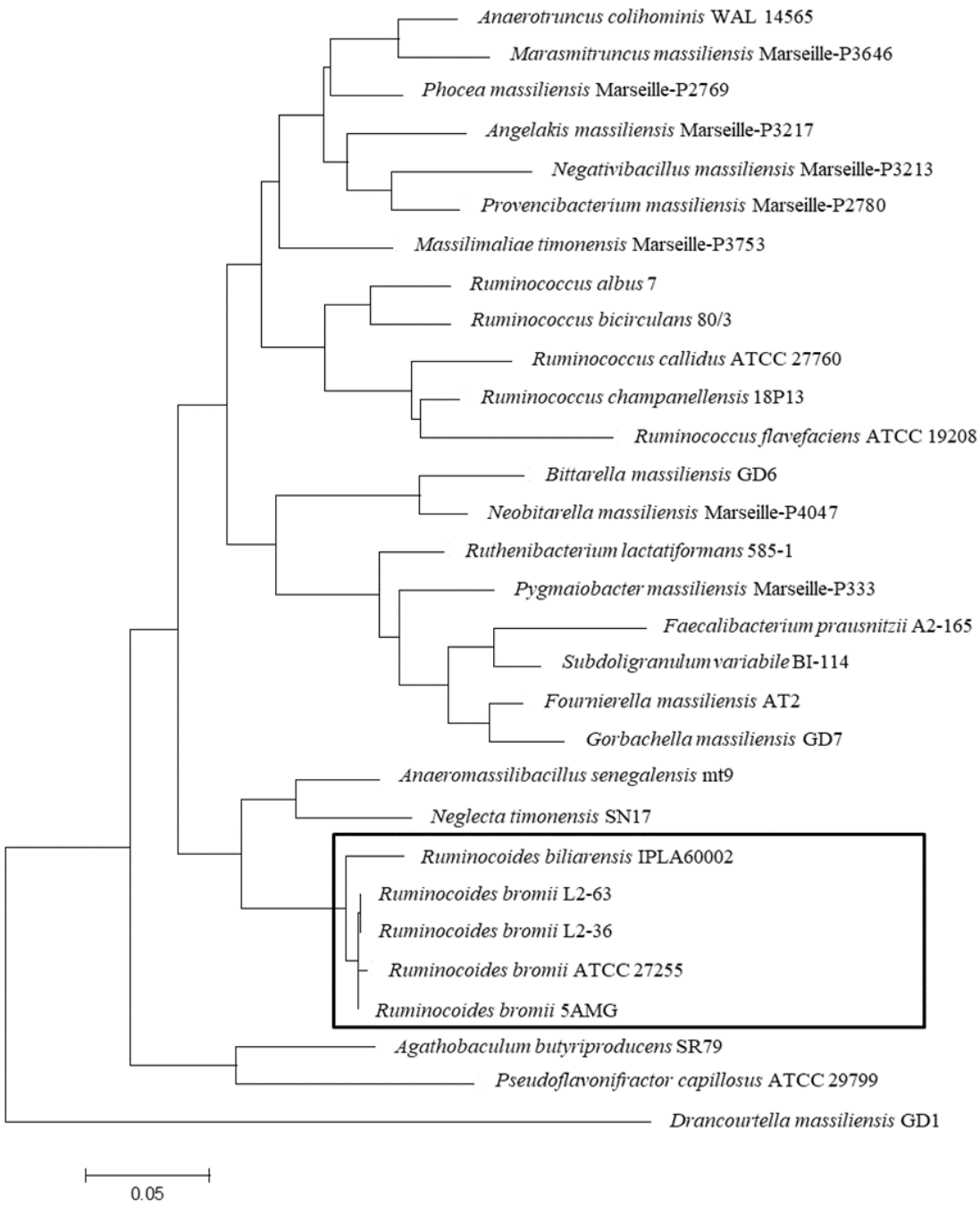


Figure 4. Heatmap based on dbCAN analysis, comparing the number of matches to predicted carbohydrate-active enzymes against the reference CAZy database in the genomes of the *R. biliarensis* IPLA60002 and the *R. bromii* strains 5AMG, ATCC27225, L2-36 and L2-63. Color key: the color scale represents a value range of presence of predicted activities, with darker coloring representing a greater number of matches to the CAZy database, up to a maximum of 22. The up and down arrows represent over and underrepresentation (more than three-fold) of the predicted enzymatic activities.

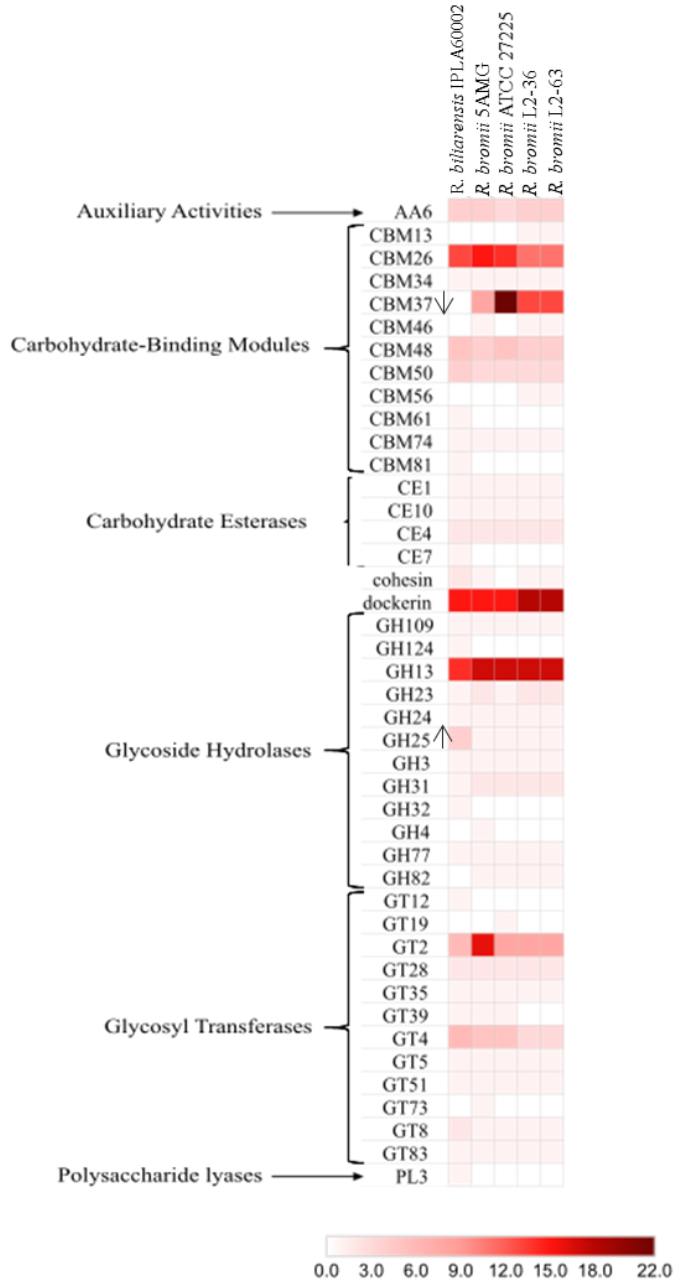
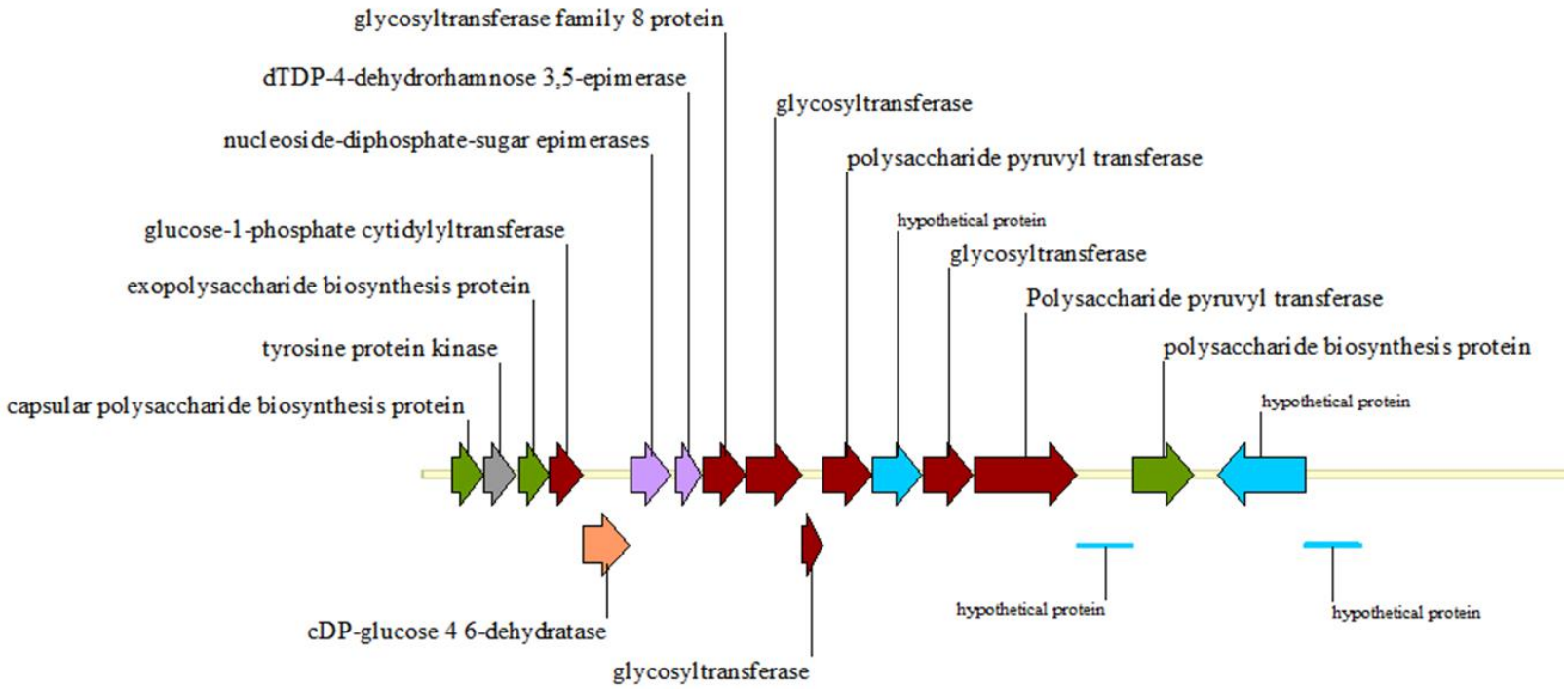
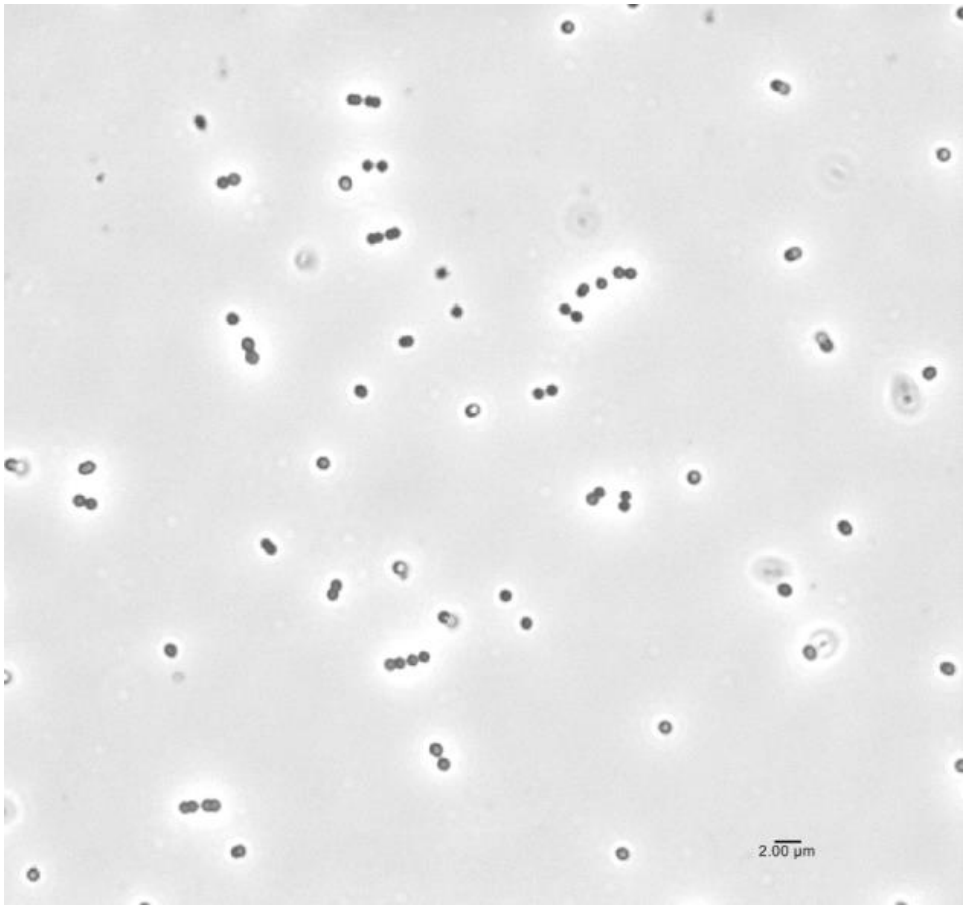


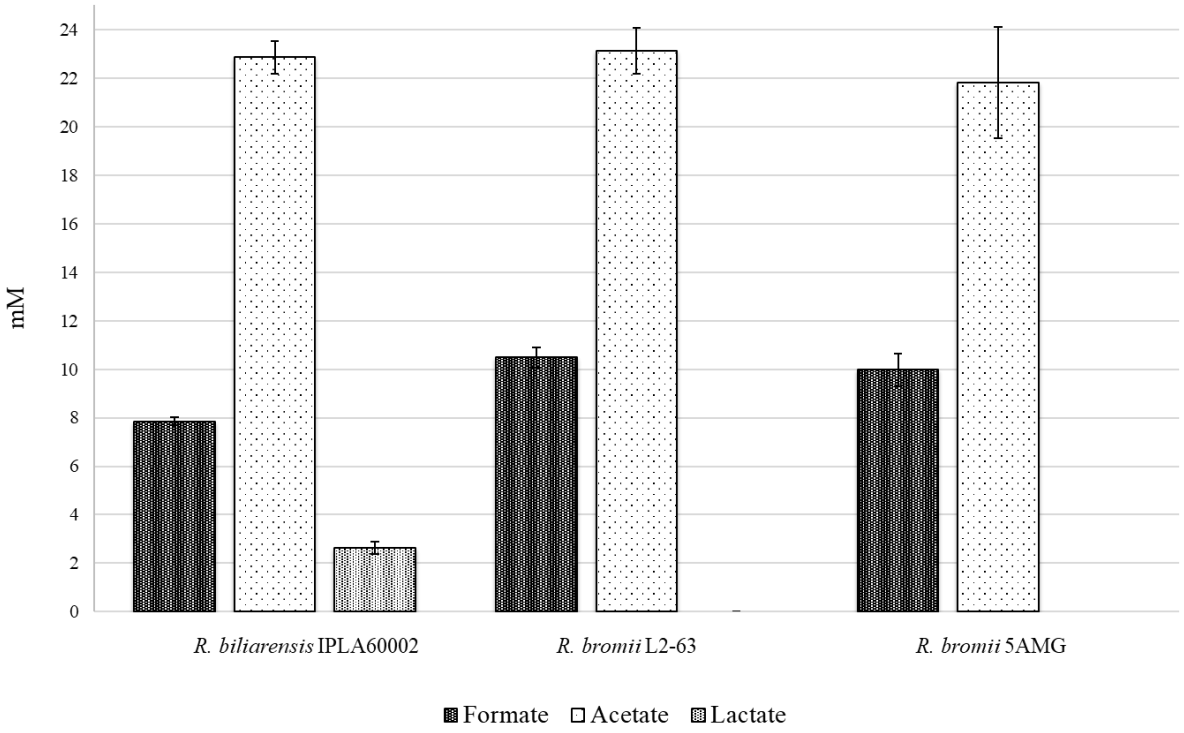
Figure 5. Cluster of genes potentially related with exopolysaccharide production detected in Contig_12 (21158bp) in the genome of *Ruminocoides biliarensis* IPLA60002. The genes of this cluster were annotated in the following order: one polysaccharide biosynthesis protein, one tyrosine kinase, one exopolysaccharide biosynthesis protein, one glucose-1-phosphate cytidylyltransferase, two sugar epimerases, three glycosyltransferases, one hypothetical protein, another two glycosyltransferases, one hypothetical protein, one polysaccharide biosynthesis protein and finally two hypothetical proteins.



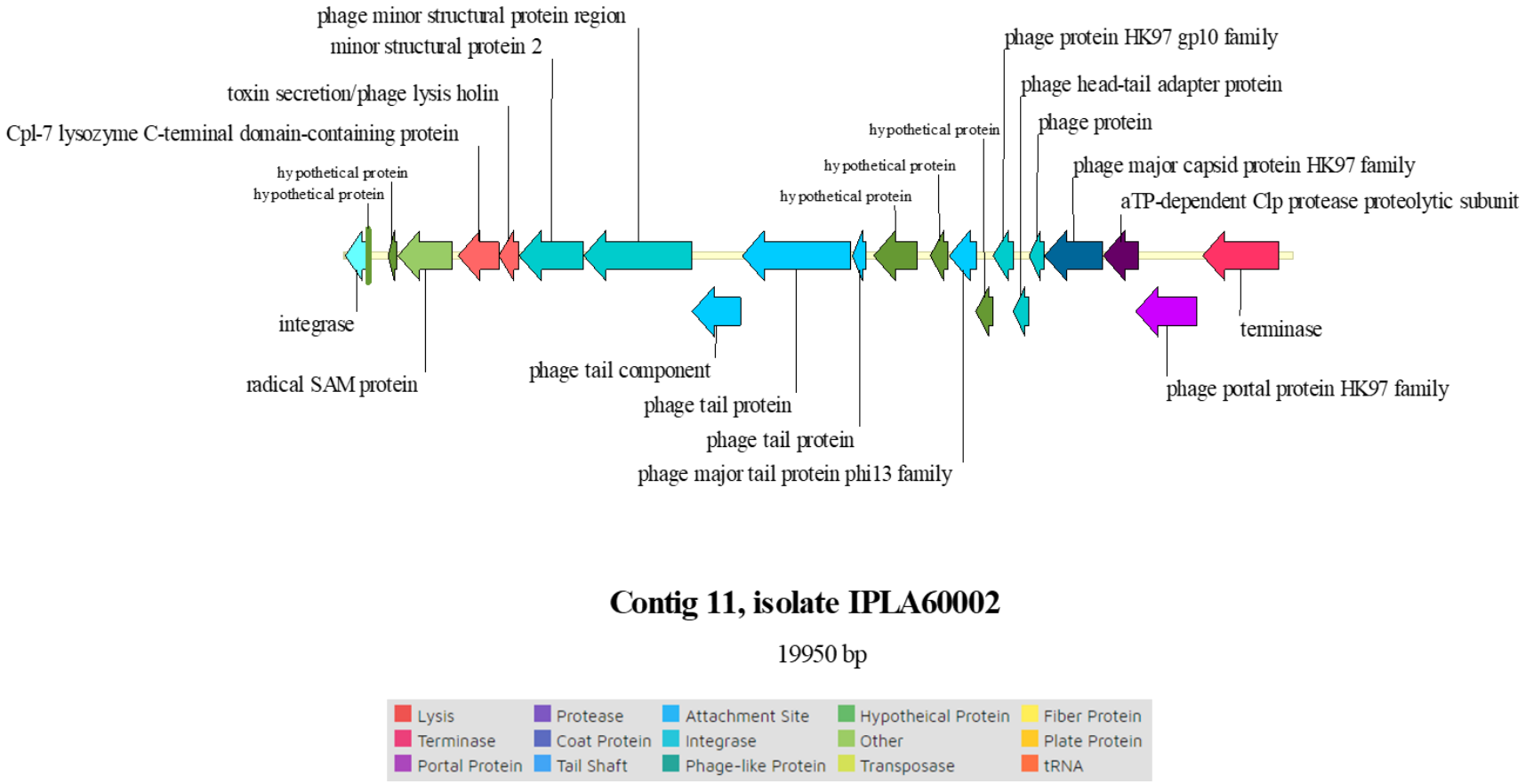
Supplementary Fig 1. Phase-contrast microscopy image of IPLA60002 isolated from human bile.



Supplementary Fig 2. Short Chain Fatty Acids (SCFAs) production (in mM). In Y axes is represented the normalized concentrations of SCFAs respect to the blank.



Supplementary Fig 3. Prophage cluster detected in *R. biliarensis* IPLA60002. The cluster of 19.6Kb contains in the following order: one integrase, two hypothetical proteins, one chaperone for long tail fiber formation, one lysin followed to one holin, two minor structural proteins, three tail proteins, two hypothetical proteins, one major tail protein, one hypothetical protein, three phage proteins, one major protein of the capsid followed by a protease, one phage portal protein and one terminase.



Capítulo 4

Chapter 4

CAPÍTULO 4

Relación entre dos aislados de bilis humana: *Ruminococcus gauvreauii* IPLA60001 y *Ruminocoides biliarensis* IPLA60002.

La segunda parte del tercer objetivo de esta Tesis Doctoral se centró en el estudio de la posible relación entre los dos aislados obtenidos de bilis humana, *Ruminococcus gauvreauii* IPLA60001 y *Ruminocoides biliarensis* IPLA60002. Ambas cepas fueron aisladas de forma conjunta. Por este motivo, decidimos analizar la relación entre ambas utilizando análisis fenotípicos, genómicos y transcriptómicos. Este objetivo fue desarrollado en colaboración con el laboratorio del grupo de Sylvia Duncan y Alan Walker, en el Rowett Institute (Aberdeen, UK).

Para estudiar esta relación, se llevó a cabo un primer análisis de los genomas, que mostró claras diferencias en las rutas de producción de vitaminas y aminoácidos, y en el metabolismo de carbohidratos. Mediante el análisis fenotípico se estudiaron las curvas de crecimiento de ambos aislados y su capacidad para crecer en distintas fuentes de carbono, y se analizaron los principales productos de la fermentación de los mismos. Estos análisis mostraron una relación de alimentación cruzada entre ambos aislados, que se estudió en mayor profundidad mediante RNA-seq, utilizando para ello co-cultivos de ambas cepas en condiciones controladas.

Los resultados obtenidos correspondientes a este capítulo se presentan en forma de manuscrito en el artículo titulado:

Artículo 4. Molinero, N., Conti, E., Sánchez, B., Walker, A., Margolles, A., Duncan, S.H. and Delgado, S. Metabolic relationships between two ruminococci symbionts from human bile. Strategies to survive in a hostile environment.

Metabolic relationships between two ruminococci symbionts from human bile. Strategies to survive in a hostile environment.

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Running title: Interactions of ruminococcal human bile isolates

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Keywords: cross-feeding, co-cultures, ruminococci, bile resistance, SCFAs, stress conditions.

Originality-significance statement

Unique insights into two new bacteria, isolated from the human gallbladder, are presented in this work. One of the isolates was characterised as a new strain of *Ruminococcus gauvreauii* (IPLA60001) and the other, most likely, is a novel species closely related to *R. bromii*, named *Ruminocoides biliarensis* (IPLA60002). This latter strain metabolised a range of resistant starches whilst *R. gauvreauii* failed to do it but grew well in sugar alcohols, including inositol. The two ruminococal isolates revealed marked resistance to bile and possess many efflux transporters, potentially involucred in bile export. The two biliary strains differ markedly in their amino acid catabolism and vitamin synthesis capabilities, a feature that is therefore likely to contribute to their strong synergistic interaction revealed by transcriptomic analysis of co-cultures of both strains. This paper provides new data into the physiology and ecology of two ruminococci from human bile with a particular focus on cross-feeding mechanisms, by which IPLA60002 would produce formate, among other compounds, that will be subsequently used by IPLA60001.

Summary

Little is known about the bacteria that reside in human gallbladder and the mechanisms that allow them to survive within this harsh environmental niche. Certain bacterial species are considered to exhibit antagonistic activities whilst others may form mutualistic interactions through, for example, cross-feeding. We isolated two new strains from a human bile sample, one *Ruminococcus gauvreauii*, belonging to the *Lachnospiraceae* family, and the other constituting a putative new species within the family *Ruminococcaceae*, named *Ruminocoides biliarensis*. The two strains differed markedly in their carbohydrate metabolism as *R. gauvreauii* mainly metabolised sugar alcohols, including inositol, to form acetate as unique fermentation product, meanwhile *Rc. biliarensis* mainly metabolised resistant starches to form formate and acetate as major fermentation end-products. Amino acid and vitamin biosynthesis genomic profiles also differed markedly between the two bile isolates, which is likely to contribute to their synergistic interactions revealed by transcriptomic analysis of co-cultures of both strains. Experimental evidence of formate utilization by the strain *R. gauvreauii* IPLA60001 was obtained in single cultures with limit carbon source. Transcriptome analysis of co-culture combinations of both strains revealed that IPLA60001 is able to grow using the end-products of starch metabolism by *Rc. biliarensis*, and other compounds probably provided by the autolysis of IPLA60002 (such as ethanolamine and inositol). This is the first study providing evidence of a potential syntrophic metabolic cooperation, including ecological aspects (like bile salt resistance, sporulation or autolysis) to tolerate and survive in the bile ecosystem, between two ruminococci isolated from the human biliary microbiota.

Introduction

The human gastrointestinal tract (GIT) is colonised by a variety of different microbial species that plays a vital role for the maintenance and regulation of host homeostasis, through the metabolism of non-digestible diet polysaccharides and producing vitamins and other metabolites with a beneficial effect, like short chain fatty acids (SCFAs), among others (Barko *et al.*, 2018). The bacterial communities that inhabit our GIT have been widely characterized in the last few years, but little is known about the microbial communities present in the gallbladder and bile.

Recently, in a previous study in our group, we characterized the human bile microbiome in subjects without any hepatobiliary pathologies (Molinero *et al.*, 2019). The bile of the gallbladder is colonised by a diverse microbiota, dominated by members of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria phyla. During the course of this previous work (Molinero *et al.*, 2019) we were able to isolate, among other members, two strains from a human bile sample, named IPLA60001 and IPLA6002, belonging to the *Clostridiales* order. The 16S rDNA sequence showed the highest identity with *Ruminococcus gauvreauii* (99%) and *Ruminococcus bromii* (93%), respectively. Strain IPLA60002 showed phenotypical and genomic differences to other *R. bromii* strains, so it was fully characterized and finally postulated as a new species renamed *Ruminocoides biliarensis* DSM 110008 (N. Molinero, unpublished). *Ruminocoides biliarensis* is proposed as a novel specie belonging to *Ruminococcaceae* family of *Clostridium* cluster IV formed by strictly anaerobic Gram-positive cocci. At phenotypic and genomic level *Rc. biliarensis* has some similarities to *R. bromii*, one of the most abundant bacteria constituting the human colonic microbiota and a primary degrader of resistant starches (RS), an important non-digestible dietary polysaccharide (Ze *et al.*, 2012). The fermentation of these substrates provides nutrients for other bacteria and SCFAs, that are beneficial for host health (Flint *et al.*, 2017). *Rc. biliarensis* PLA60002 is able to degrade RS producing formate, acetate and lactate as end fermentation products. This strain forms an extracellular layer connecting the growing cells, that could be related with its autolytic behaviour observed in liquid media (N. Molinero, unpublished).

On the other hand, *R. gauvreauii* are strictly anaerobic Gram-positive cocci, belonging to the *Lachnospiraceae* family and *Clostridium* cluster XIVa, that can use different sugar alcohols as carbon sources such as D-sorbitol, D-mannitol and inositol, and produce acetate as main fermentation product (Domingo *et al.*, 2008).

Microbial interactions in mixed communities may be complex and can be mutualistic or antagonistic. Metabolite cross-feeding may be a key feature of microbial ecosystem, but these likely complex interactions are generally not well characterized. Metabolite cross-feeding involve one strain utilising, for example, macronutrient break down products, vitamins or SCFAs formed by other strain. Of particular importance are cross-feeding interactions that can influence the metabolic pathways involved in fermentative metabolism and hence the energetics of bacterial metabolism. For example, formate, acetate and lactate can be formed by certain species which are then used by other cross-feeded bacteria (Rios-Covian *et al.*, 2015; Moens *et al.*, 2017). A good example is the interactions between *R. bromii* and *Blautia hydrogenotrophica*, as the latter can cross feed on the formate generated by *R. bromii* whilst fermenting RS to generate acetate, employing the Wood-Ljungdahl pathway (Laverde Gomez *et al.*, 2019). In our study, *R. gauvreauii* IPLA60001 and *Rc. biliarensis* IPLA60002 strains were isolated together, and were difficult to cultivate them separately, suggesting a symbiotic relationship between them. In this work, we investigated trophic cooperations between these two human bile isolates using a combination of biochemical, genomic and transcriptomics tools to analyse the production of SCFAs, and go deeply into in the metabolic interactions using dietary starch as the only carbon source in co-culture combinations.

Results

Description of the strains and growth conditions

The strains *R. gauvreauii* IPLA60001 and *Rc. biliarensis* IPLA60002 were isolated from a human bile sample in a previous study (Molinero *et al.*, 2019). IPLA60001 strain grows in 72 hours in GAM plates supplemented with cysteine (GAMc), forming small white colonies, while IPLA60002 strain grows in 72 hours in GAMc plates, forming large white rounded colonies. In GAMc broth, IPLA60001 requires 18-20 h to reach the maximum OD_{650} (0.9-1.1). For *Rc. biliarensis* IPLA60002, it requires 16-18 h to reach the maximum OD_{650} (0.8), and after reaching this point, the strain shows an autolytic phenotype showing a marked reduction in the optical density after 24 h (OD_{650} <0.3) (N. Molinero, unpublished). In M2GSC broth medium supplemented with 30% clarified bovine rumen fluid (CBRF), IPLA60001 strain grows faster than in GAMc broth, reaching the maximum OD_{650} in 12 hours. IPLA60002 also grows faster than in GAMc broth, reaching a maximum OD_{650} in 12 h and beginning autolysis after this point. Growth of the IPLA60001 and IPLA60002 strains in M2GSC medium is represented in Fig. 1.

Molecular and phylogenetic identification

The 16S rRNA genes from the strains IPLA60001 and IPLA60002 were amplified and sequenced. BLASTn results showed that the closest relatives for IPLA60001 and IPLA60002 were *R. gauvreauii* with a homology of 99%, and *R. bromii*, with a homology less than 96%, respectively. We proceeded then to sequence the whole genome of IPLA60001 and IPLA60002 strains. We obtained, after assembling, 60 contigs in the case of *R. gauvreauii* IPLA60001 and 59 contigs in the genome of IPLA60002. The genome analysis of IPLA60001 showed a genome size of 4.14Mb, a G+C percentage of 47.65%, the presence of 3,864 predicted open reading frames (ORFs) and 82 tRNAs, while the genome analysis of IPLA60002 confirmed the low homology sequence of the 16S rRNA genes and revealed a genome size of 2.51Mb, a G+C content of 38.93%, 2,356 predicted ORFS and 66 tRNAs.

The phylogenetic position of IPLA60001 and IPLA6002 strains was analyzed by building phylogenetic trees for the *Lachnospiraceae* and *Ruminococcaceae* family, respectively, based on the full-length 16S rRNA gene. In the case of IPLA60001 strain, the tree was built with 27 different sequences from the main type strains belonging to the *Lachnospiraceae* family of human origin. The analysis with IPLA60002 involved 30 different sequences from strains of the *Ruminococcaceae* family from human origin. The maximum likelihood approach showed that, as we expected, IPLA60001 belongs to the species *R. gauvreauii* (Fig. 2A), meanwhile IPLA60002 strain forms a distinct lineage comparing with other *Ruminococcus bromii* strains (L2-63, L2-36, ATCC 27255 and 5AMG) (Fig. 2B), supporting evidence to rename it as a new species within the *Ruminococcaceae* family. The name *Ruminocoides biliarensis* sp. nov., was given to IPLA60002, which was deposited in the DSMZ collection with number DSM 110008 (N. Molinero, unpublished).

Carbon sources utilisation and major SCFA production

The carbon source utilization of *R. gauvreauii* IPLA60001 and *Rc. biliarensis* IPLA60002 strains was analyzed. The fermentation capability of the strains was compared with related strains, *R. gauvreauii* DSM-19829, and *R. bromii* L2-63 and 5AMG, respectively. The results of the fermentation abilities in the presence of different carbon sources are shown in Table 1. The substrate utilization was the same for the two *R. gauvreauii* strains, being able to use D-glucose, D-mannitol, D-sucrose, D-xylose, D-sorbitol and inositol and producing acetate as major fermentation product. Regarding *Rc. biliarensis* and *R. bromii* strains, the ability to use different carbon sources was the same for the three strains, producing acetate and formate as major fermentation products from D-fructose and D-maltose. Lactate was also produced in the

case of *Rc. biliarensis* IPLA60002. The ability to hydrolyze different RS was assayed as well. IPLA60002 strain and *R. bromii* L2-63 and 5AMG strains were able to hydrolyze RS derived from rice, corn, and potato, as well as different high amylose maize starches such as Hylon VII, Novelose and High Maize (Table 1). The *R. gauvreauii* strains IPLA60001 and DSM-19829 were not able to hydrolyze any of the RS tested.

To test the hypothesis that *R. gauvreauii* IPLA60001 is able to use the formate produced by *Rc. biliarensis*, IPLA60001 strain was grown in M2 medium supplemented with 30% CBRF and different concentrations of formate and limited carbon source (0.1% D-glucose). After 48 h of culture, supernatants were analyzed by gas chromatography showing that, although IPLA60001 was not able to grow with formate as the only carbon source, when the medium was supplemented with glucose the strain metabolized completely the formate added to the medium, either when the concentration was 10mM or 20Mm (Fig. 3A). The SCFA profile at different times of growth in cultures supplemented with formate, butyrate and limited carbon source showed that the formate is metabolized within the first 24 hours, during late exponential phase, when the strain is fermenting the glucose to produce acetate (Fig. 3B). After this time, in the stationary phase and when the glucose is completely metabolized, the strain seemed to metabolize the acetate produced. Butyrate is not consumed by *R. gauvreauii* IPLA60001.

Resistance to bile salts

The resistance to bile salts was tested for all above mentioned strains. The two *R. gauvreauii* strains showed the same resistance phenotype, being able to grow in the highest concentrations tested (8%) of taurodeoxycholate (TDC), glycocholate (GC), taurocholate (TC), bovine bile and a mix of bile salts (Table 2). *R. gauvreauii* strains were more sensitive to cholic acid (CA), glycodeoxycholate (GDC) and porcine bile. Regarding the behavior of IPLA60002 strain in different bile salts, the minimal inhibitory concentrations (MICs) were in general similar to those found in IPLA60001, but much higher in comparison with the *R. bromii* strains 5AMG and L2-63 (excluding CA).

Genome analysis

The genome analyses of *R. gauvreauii* IPLA60001 and *Rc. biliarensis* IPLA60002 strains was performed using different bioinformatic tools. By comparing both genomes we found some differences related to bile salt stress response activities. The genome of *R. gauvreauii* IPLA60001 possesses genes related to activities against osmotic and oxidative stress, as well as some genes related to DNA repair that aren’t present in the genome of IPLA60002. On the

contrary, the genome of the IPLA60002 strain possesses some genes related to stress response that aren't present in the genome of IPLA60001 and some other genes related to DNA repair. Also, IPLA60002 possesses a choloylglycine hydrolase (EC 3.5.1.24), and activity related to bile hydrolysis that is not present in the genome of the IPLA60001 strain. dbCAN analysis provided us information about the ORFs related to predicted carbohydrate-degradation enzymatic activities. The genomes of the IPLA60001 and IPLA60002 were compared with those of closed related representative strains: *R. gauvreauii* DSM-19829 for IPLA60001; and *R. bromii* 5AMG, ATCC 27255, L2-63 and L2-36 for IPLA60002. The results of the dbCAN analysis are represented in a heatmap (Fig. 4). These results revealed that the two *R. gauvreauii* strains had a similar profile, highlighting the presence of 28 genes related to the glycoside hydrolase family GH109. These genes corresponded with myo-inositol 2-dehydrogenases, a fact that would explain the high inositol degradation rate observed for *R. gauvreauii* IPLA60001 (data not shown). Comparing IPLA60002 genome with *R. bromii* genomes, the dbCAN profiles were, except of some differences such as the genes of the family GH25 of glycoside hydrolases, that encode lysozymes, overrepresented in IPLA60002, in comparison with the other *R. bromii* genomes. The vitamin and amino acid biosynthesis and metabolism pathways of IPLA60001 and IPLA60002 were also analysed, showing an apparent cross-relationship (Table 3). About vitamin biosynthesis, comparing the genomes of the two strains, *R. gauvreauii* IPLA60001 have all genes needed to produce mainly coenzyme B12 and cobalamin, not present in IPLA60002 genome. On the contrary, IPLA60002 strain possesses all genes needed to produce coenzyme A, absent in IPLA60001 genome. In relation to the metabolism of amino acids, the results shown that IPLA60001 possesses all genes for putrescine utilization, urea decomposition, methionine and lysine biosynthesis, and creatine and creatinine degradation, not detected in the IPLA60002 genome; and the latter has the genes for glycine and serine utilization, pathways not present in the *R. gauvreauii* genome. The genes detected in at least one of the strains related to these metabolic and biosynthetic pathways are represented in Table Suppl. 2.

Interactions of Rc. biliarensis IPLA60002 and R. gauvreauii IPLA60001 in co-cultivation.

Co-cultures of both strains were performed in order to check a potential cross-feeding relationship. After growth for 6 and 8 h in co-culture in M2 medium with starch from rice as carbon source, a transcriptomic analysis was performed. As shown before, *R. gauvreauii* IPLA60001 is not able to grow in starch from rice as the only carbon source (see Table 1), so if the strain would be able to grow in co-culture with IPLA60002 it means that this *Rc.*

biliarensis strain was producing some products from the metabolism of the RS that IPLA60001 uses to grow. The results obtained with the RNA-seq (see below) confirmed us that IPLA60001 was growing in co-culture with IPLA60002, accordingly with our initial hypothesis. To corroborate the ability of *R. gauvreauii* strain to metabolize formate (see Fig. 3A), in this case produced by *Rc. biliarensis* in co-culture, the SCFAs profiles of the co-cultures and single cultures were tested for the same period of time. The results didn't show the formate consumption previously observed for *R. gauvreauii* in single culture, neither in the 6 h or 8 h samples (Fig. 5), probably because in this case there was not a limitation of carbon source and the time of sampling was earlier in the exponential phase.

Transcriptome analysis and changes in expression profiles

To go deeply into the cross-feeding relationship between *R. gauvreauii* IPLA60001 and *Rc. biliarensis* IPLA60002, RNA-seq was used to analyse the transcriptome profile of each strain alone and in co-cultures. We performed the following comparisons: changes in the transcriptome of *Rc. biliarensis* when growing alone or in co-culture (with IPLA60001) for 8 h; changes in the transcriptome of *Rc. biliarensis* between 6 and 8 h of co-culture (with IPLA60001); and changes in the transcriptome of the *R. gauvreauii* strain between 6 and 8 h of co-culture (with IPLA60002).

About the first comparison, the results of the transcriptomics showed that when IPLA60002 is in co-culture with IPLA60001, some genes of *Rc. biliarensis* are overexpressed. Among others, we found several genes related to sulfate and sulfur metabolism and transport, genes related to thiamine production, and genes related to siroheme synthesis (Fig. Suppl. 1). Also, some activities as a thioredoxin reductase and other stress induced proteins were overexpressed when IPLA60002 was in co-culture.

The most important changes were found when comparing at transcriptional level the profiles of *Rc. biliarensis* among 6 and 8 h of co-culture with IPLA60002 strain. At 6 h, genes related to starch degradation, several ABC transporters, ribosomal proteins and riboflavin uptake were overexpressed, among others (Fig. 6). At 8 h, mainly genes related to stress response were overexpressed, highlighting activities related to sulfate reduction, thiamine production, and some redox enzymes as a thioredoxin reductase and one 4-hydroxybenzoyl-CoA thioesterase. Also, one oxidoreductase was overexpressed, with another stress response and DNA repair proteins as one flavoprotein. In this comparison, also genes for sporulation were overexpressed.

The third comparison performed was to analyse the changes in the transcriptome of *R. gauvreauii* IPLA60001 between 6 and 8 h of growth when it was in co-culture with

IPLA60002. In this case, mainly ribosomal proteins were overexpressed, along to other genes related to replication, transcription and transduction (Fig. Suppl. 2). Highlighted one gene related to ethanolamine utilization (acetaldehyde dehydrogenase (EC 1.2.1.10)). Additionally, the changes in the transcriptome of *R. gauvreauii* when the strain grows in pure culture or in co-culture with IPLA60002 for 8 h were also analysed. In this case, we have to take in account that the single culture of IPLA60001 was performed in glucose (0.2%) since it does not grow in RS. Nonetheless, even considering this limitation, the comparison of the transcriptome showed several genes for ethanolamine and inositol metabolism overexpressed when *R. gauvreauii* IPLA60001 was in co-culture, such as ethanolamine utilization proteins EutA, EutJ and EutQ; one myo-inositol 2-dehydrogenase (EC 1.1.1.18) and one formate dehydrogenase (Table 4). These last findings about formate metabolism corroborate the hypothesis that IPLA60001 is able to metabolize the formate produced by IPLA60002.

Discussion

In this paper two biliary strains were fully characterized through phenotypic, genomic and transcriptomic assays. The strains were initially recovered by culturing together in the same agar plate form the same subject´s sample, and there was difficult to isolate independently them due to the better growth together and the strong relationship between them. Mutualistic interactions including potential cross-feeding were evaluated. Both strains were strict anaerobes belonging to the order *Clostridiales* that were identified based on their 16S rDNA sequence homology. One of the strains, named IPLA60002, presented a percentage of identity less than 97% with other sequences in databases. This percentage was established to represent the boundary for taxonomically circumscribing prokaryotic species (Palys *et al*., 1997) and we proceeded then to sequence the whole genome of IPLA60002 together with the other biliary strain, named IPLA60001, that was assigned to the specie *Ruminococcus gauvreauii*. The phylogenetic analysis of IPLA60001 revealed that this strain forms a distinct lineage compared with *Ruminococcus bromii* (the closest related species) sustaining evidence to propose it as new specie within the family *Ruminococcaceae*. The strain was deposited in the DSMZ collection under number DSM 11008 and the name *Ruminocoides biliarensis* was proposed (N. Molinero, unpublished). On the other hand, phylogenetic reconstruction of IPLA60001 confirmed the taxonomic classification as *R. gauvreauii*, a species within the *Lachnospiraceae* family, isolated from the human gut (Domingo *et al*., 2008). At present, *Ruminococcus* is considered a polyphyletic genus, with species members belonging to the two above mentioned

families: *Lachnospiraceae* and *Ruminococcaceae* (La Reau *et al*, 2016). However, there is an urgent need for reclassifying many of these bacteria into other genera in order to resolve the discrepancy of members belonging to either the family *Ruminococcaceae* or *Lachnospiriaceae* (La Reau and Suen, 2018).

Resistance to different bile salts was showed for both strains isolated from bile, in contraposition with phylogenetically similar strains of fecal origin. The two strains showed in general more resistance to the primary conjugated bile salts, more common in the gallbladder, as GC and TC; as well as to bovine bile and mixed bile salts mainly composed by TC and TDC and GC. The phenotypic results observed for this type of resistance were supported by the genomic analysis, in which some genes potentially related to bile salt resistance and stress response were found. Both biliary strains possesses many genes annotated as different efflux transporters, which could be responsible to export these bile salts, such as ABC-type multidrug transporters and small multidrug resistance (SMR) transporters, which protect bacteria from an array of hydrophobic and cationic antimicrobials (Kermani *et al.*, 2018), as well as MATE efflux family proteins, which have been related to the extrusion of cholate and deoxycholate in *Escherichia coli* (Kuroda and Tsuchiya, 2009). Genomic analysis also revealed the presence of a cholylglycine hydrolase (Dean et al., 2002), in the genome of IPLA60002, an enzyme associated with the hydrolysis of bile salts that could be involucrated in the detoxification of bile salts. The presence of these genes together with those for sporulation sustain their survival in a hostile environment like the biliary tract. In fact, although not presented here we confirmed the sporulation capability of both strains under the microscope with the Schaeffer and Fulton Spore Stain (data not shown). This ability to sporulate was previously reported for some ruminococci of the GIT (Mukhopadhya *et al.*, 2018).

Regarding growth and fermentation capabilities, metabolic interactions between the two biliary strains were shown by biochemical single behavior in monocultures and fermentation abilities, as well as demonstrated by genomic analyses and differential transcriptional responses in co-cultures. We observed that major fermentation products from *Rc. biliarensis* IPLA60002, which was able to grow in RS, were acetate and formate. This last compound is a key metabolite in the energy metabolism of some anaerobic bacteria who cross-fed on it (D´hoe *et al*., 2018). Both formate and hydrogen can be used for interspecies electron transfer (Stams *et al*., 2006), and at least in the specie *R. bromii* the production of acetate and formate is accompanied with ethanol and hydrogen production (Walker *et al*., 2011). Products released from the metabolism of RS by *R. bromii* can be used by other gut bacteria (Ze *et al*., 2012). In this work the strain *R. gauvreauii* IPLA60001 was not able to grow in formate as the only

carbon source. However, it metabolizes formate during its late exponential phase (8 h and ahead), an observation that was in line with the results obtained by SCFAs and RNA-seq analyses at 8 h in co-cultures fermentations on starch with *Rc. biliarensis*. The potential for anaerobic bacteria to grown by converting formate to other compounds such as hydrogen and bicarbonate has been overlooked for years (Morris *et al*., 2013), but reports exits about metabolic interactions and degradation of formate mainly in methanogens (Dolfing *et al*., 2008). These authors observed that after the addition of formate as the sole energy and carbon source, biomass increases only in co-cultures. *R. gauvreauii* IPLA60001 is not able to grow in complex substrates such as starch, so when the strain grows in co-culture with *Rc. biliarensis* IPLA60002 the carbon sources available are the end-products of the metabolism and hydrolysis of the starch. It is possible that upon starch degradation *Rc. biliarensis* IPLA60002 needs at least 24 hours to produce enough formate to be metabolized by IPLA60001, and plausibly during this time *R. gauvreauii* IPLA60001 was metabolizing other secondary products derived from the starch metabolism by IPLA60002, as the utilization of formate was observed at late exponential phase. When comparing *Rc. biliarensis* transcriptome between 6 and 8 h of co-cultivation, gene expression changes from starch degradation and riboflavin uptake, to sporulation and stress response expression at 8 h, which is consistent with the autolytic phenotype observed for this strain after 12 h in pure culture. Co-cultures of both strains might accelerate autolysis, even if both strains do not compete for same substrates, because autolysis phenomenon in bacteria is influenced by a variety of different factors including pH, growth phase or teichoic acids among others (Rice and Bayles, 2008). In line with this, differential expression genes were detected when comparing *Rc. biliarensis* transcriptome at 8 h alone or in co-cultivation, indicating that, also at this time of growth, genes related with stress and sulfur metabolism are activated in the presence of *R. gauvreauii* IPLA60001. The importance of the ability of *R. bromii* (the phylogenetically closest related neighbour of *Rc. biliarensis*) to use sulfate as sulfur source was already indicated long time ago (Herbeck and Bryant, 1974). Additionally, the autolysis behaviour of the *Rc. biliarensis* IPLA60002 strain might provide membrane phospholipids, such as phosphatidylethanolamine and phosphatidylinositol which would be in concordance with the ethanolamine and inositol metabolism overexpression observed in the strain of *R. gauvreauii* in co-cultivation with *Rc. biliarensis* IPLA60002. In the genome analysis of both strains, differences in genes for vitamin, cofactors and aminoacid production pathways were observed, supporting the proposed syntrophic metabolic interaction and benefits between *R. gauvreauii* and *Rc. biliarensis* in the bile ecosystem. The term syntrophy would refer to obligatory mutualistic metabolism, which focuses not only on

microbial metabolic cooperation but also including ecological aspects (Morris *et al.*, 2013). Already described with the initial characterization of the nutritional properties of *R. bromii* (the closest species of *Rc. biliarensis*) was the dependence of growth factors or compounds produced from other bacteria (Herbeck and Bryant, 1974).

Conclusions

Our results points to mechanisms of synergy between these two microorganisms from bile which are of particular ecological interest in this scarcely studied niche. This work represents an important setup forward in the environmental distribution and relatedness of ruminococci symbionts in the human gastrointestinal tract that could be of relevance lastly for the host physiology. Our findings open the door to future efforts to explore microbial syntrophic reactions from other parts of the human body, and to characterize novel bile-related functions that could be unique for bacterial members inhabiting this particular niche.

Experimental procedures

Isolation procedure of bacteria from human bile

Human bile samples from the gallbladder of liver donors without any hepatobiliary disorder was obtained in aseptically conditions during liver transplant at the General Surgery Service of HUCA (Central University Hospital of Asturias) in Spain from a previous study (Molinero *et al.*, 2019). Bile samples were immediately transported refrigerated to the laboratory and cultivated fresh in different media, including Gifu anaerobic medium (Nissui, Tokyo, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma-Aldrich, San Luis, MO, USA) (named GAMc). The strains reported here, named IPLA60001 and IPLA60002, were isolated from a 2% agar GAMc plate incubated at 37°C for 72 h in a Whitley MG500 anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 gas atmosphere. The strains were routinely maintained by growing in GAMc agar plates in anaerobic conditions. Other media, such as the semi-defined RUM medium (Ze *et al.*, 2015), PYG (DSMZ Medium 104) and the M2GSC medium supplemented with 30% v/v CBRF (Miyazaki *et al.*, 1997), were also assayed.

Bacterial strain and growth conditions

Phenotypic analysis of IPLA60001 and IPLA60002 strains were carried out in parallel with two strains of *R. bromii*; L2-63 and 5AMG (Ze *et al.*, 2012; Mukhopadhya *et al.*, 2018)

available at Rowett Institute, and the type strain *R. gauvreauii* DSM-19829 purchased from the DSMZ Collection. Otherwise specified, all strains were routinely maintained by growing anaerobically at 37°C for 16-18 h in M2GSC broth medium supplemented with 30% CBRF in Hungate tubes. Media were prepared anaerobically under a stream of 100% CO2.

Identification of the isolates by 16S rRNA sequencing

Total genomic DNA was extracted from 18h grown cultures on GAMc broth using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Purified DNA was used as a template for amplification of the 16S rRNA gene using the universal primer pair 27 (forward primer) and 1492 (reverse primer) of the *E. coli* numbering system (Weisburg *et al.*, 1991). PCR amplification products were sequenced in an ABI 373 DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at the facilities of Macrogen (Madrid, Spain). The 16S rDNA sequences were compared with those deposited in the GenBank database using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Whole genome sequencing

Genomic DNA from both biliary strains was purified using the "DNeasy blood and tissue" kit (Qiagen, Germany) following manufacturer's protocol. Genome sequencing was carried out using a 250-290 paired-end libraries on an Illumina MiSeq Sequencing System (Illumina, USA) at GenProbio SRL (Parma, Italy). Genome assembly was performed with MIRA assembler v4.0.2 (Chevreux, B., Wetter, T. and Suhai, 1999). ORFs prediction was performed with Prodigal v2.6 (Hyatt *et al.*, 2010). Automatic annotation of the ORFs was performed with BLAST against NCBI database and HMMER software against PFAM database (El-Gebali *et al.*, 2019). Improved quality of the final contigs was performed with Burrows-Wheeler Aligner (Li and Durbin, 2010), SAMtools suite (Li, 2011) and VarScan v2.2.3 (Koboldt *et al.*, 2012) software packages.

Phylogenetic analysis

Phylogeny reconstruction for the IPLA60001 and IPLA60002 strains was carried out by building two phylogenetic trees from human-source isolates of the family *Lachnospiraceae* and *Ruminococcaceae*, respectively, based on the full-length 16S rRNA gene. Accordingly, the complete sequences of the rRNA16S gene were aligned by ClustalW and the evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). Trees for the heuristic search were obtained automatically

by applying the Neighbor-Joining method (bootstrap=5,000) and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. The tree with the highest log likelihood was selected. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

Genome comparisons

We compare the genomes of the two biliary strains IPLA60001 and IPLA60002 with those of related species; *R. gauvreauii* DSM-19829, and *R. bromii* L2-63 and 5AMG, respectively. To perform the comparison between genomes the software RAST (Rapid Annotation using Subsystem Technology) was used, choosing classic RAST tool as gene annotation scheme. We analyzed the different profiles for the vitamin and amino acid synthesis pathways. We also used the dbCAN analysis (Yin *et al.*, 2012), a web server and database for carbohydrate-active enzyme activities encoding genes, to compare the different profiles. All data in dbCAN are generated based on the family classification from CAZy website (http://www.cazy.org/).

Bile salt resistance

The analysis of resistance to different bile salts was performed comparing the two bile isolates IPLA60001 and IPLA60002 with the two *R. bromii* strains from Rowett Institute (L2-63 and 5AMG) and the type strain *R. gauvreauii* DSM-19829. The analysis was carried out in triplicate by determining the MICs in M2GSC 2% agar plates supplemented with 30% CBRF and different concentrations (ranging from 0% to 12% w/v) of CA, TDC GC, TC, GDC, Bile extract porcine, Bile bovine (purchased from Sigma-Aldrich) and Mix Bile Salts (Oxoid, Waltham, MS, USA). The plates were incubated at 37°C in the anaerobic cabinet during 48h.

Carbohydrate utilization and enzymatic profiles

We performed a series of characterization tests in M2 medium supplemented with 30% CBRF and 0.2% w/v of different carbon sources and resistant starches, in Hungate tubes (Miyazaki *et al.*, 1997) to analyze the growth of IPLA60001 and IPLA6002 in comparison with *R. gauvreauii* DSM-19829, *R. bromii* 5AMG and *R. bromii* L2-63 strains. For that, triplicate tubes were inoculated with an overnight culture of the corresponding strain, and the OD_{650} values were recorded every 2 h during 48 h in a Novaspec II Spectrophotometer (Amersham Pharmacia Biotech Inc., NJ, USA). The growth in different resistant starches was recorded by measuring the drop in the pH (pH <6.0 as positive growth) after 48h incubation.

These analyses were completed using the API20A® test system, that contains substrates for carbohydrate utilization and enzymatic reactions, according to manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). The inoculated test strips were incubated anaerobically in the anaerobic cabinet at 37°C for 24 h.

SCFAs determinations

SCFA concentrations and acid production of IPLA60001 and IPLA60002 strains were measured by gas chromatography as described previously (Richardson *et al.*, 1989) after growing the strains in M2GSC medium supplemented with 30% CBRF. Following derivatization of the samples using N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (Sigma-Aldrich), the samples were analyzed, using helium as the carrier gas, in a Hewlett Packard gas chromatograph system fitted with a silica capillary column linked to a Shimadzu chromatopac integrator (Dyson Instruments Ltd, Tyne and Wear, UK). Additionally, we study the ability to metabolize formate and butyrate by *R. gauvreauii* IPLA60001. For that, the strain was grown for 48 h in M2 medium supplemented with 30% CBRF and 0.1% glucose with different concentrations of formate (10 and 20 mM) and butyrate (10 mM).

Finally, the SCFA profiles of co-cultures of IPLA60001 and IPLA60002 strains was checked. For that, supernatants of the co-cultures of the two strains in M2 medium supplemented with 30%CBRF and 0.2% starch from rice at 6 and 8 h of growth were taken in triplicate, and the SCFAs analyzed.

Co-culture conditions

To study the interactions between *Rc. biliarensis* IPLA60002 and *R. gauvreauii* IPLA60001 we made co-cultures combinations in M2 medium with 0.2% starch from rice (Sigma-Aldrich) as only carbon source. The co-culture conditions were as follows: 10ml of initial exponential phase co-cultures of *R. biliarensis* IPLA60002 and *R. gauvreauii* IPLA60001 (6 h, OD_{650} 0.4-0.5), and 10ml co-cultures for middle exponential phase (8 h, OD_{650} 0.6-0.7). The strains were grown in monoculture to have control conditions. Three technical replicates were performed for each condition, and cultures were inoculated at 1%. The tubes were centrifuged 10 min and the pellet was immediately resuspended in 1ml of RNAlater™ (Qiagen) and frozen at -80°C.

RNA extraction and RNA sequencing

Total RNA was extracted from the pellets that were resuspended in 200 µl of lysozyme (20mg/ml) and mutanolysin (40 U/ml) solution and incubated at 37°C for 1 h. Then, 800 µl of

QIAzol (Qiagen) were added and placed in a tube containing 0.8 g of glass beads (106 μm diameter, Sigma). The cells were lysed by shaking the mix on a BioSpec homogenizer (BioSpec Products, Inc., USA) for 2 min followed by 2 min in ice. This step was repeated three times. Samples were further treated with chloroform and then centrifuged at 12,000 rpm for 15 min at 4°C. The upper phase containing the RNA was recovered. Samples were further purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. Quality and integrity of the RNA was checked by an Agilent 2200 Tape Station Nucleic Acid System (Agilent Technologies, Palo Alto, Calif., USA). The amount of RNA was quantified in a Qubit™ 4 fluorometer with Qubit RNA assay kits (Thermo Fisher Scientific, Inc., Waltham, MA, USA). One to 2.5 μg of total RNA was depleted using Ribo-Zero rRNA removal kit (Illumina) to remove rRNA according to the supplier's instructions. The yield of rRNA depletion was checked in the Agilent Tape Station and rRNA-depleted RNA samples were treated with the TruSeq Stranded mRNA sample preparation kit (Illumina). Size evaluation and quantification of RNA samples was performed as above described. Whole transcriptome library was sequenced by means of a NextSeq 550 Sequencing System (Illumina) using a High Output kit v2.5 (75 cycles) flow cell.

Transcriptome analysis

High quality reads for each sample were aligned against the reference genomes of *Rc. biliarensis* IPLA60002 and *R. gauvreauii* IPLA60001. The corresponding associated annotation files in *.gff* format were used to obtain information for downstream analysis. Alignments were generated using bowtie2 v.2.2.6 (Langmead and Salzberg, 2012) and SAMtools v.1.4 (Li, 2011) was used to get the *.bam* files and sort them into a new files suitables for the next step. Read counts were generated using FeatureCounts (Liao *et al.*, 2014). Gene expression for each sample was computed as a measure of the total number of reads uniquely aligning to the reference, binned by genic coordinates. Differential gene expression analysis was performed using the Bioconductor package DESeq2 (Anders and Huber, 2010). Raw read counts thus obtained were normalized to account for differences in sequencing depth and composition using methods implemented within DESeq2. Differential expression of pairwise comparisons (of the different groups) was assessed using the negative binomial test with Benjamini–Hochberg false discovery rate (FDR) adjustment (Benjamini and Hochberg, 1995) applied for multiple testing corrections. For this study, an FDR of 0.05 was applied, and all candidates with a log2 fold change value more or less than 1 and with a *p*-adjusted value of ≤

0.05 was considered to be significantly up- or downregulated. Heatmaps were produced with ClustVis web tool (Metsalu and Vilo, 2015) using the transcript counts as input values.

Data availability

The whole-genome shotgun projects were deposited in the Sequence Read Archive (SRA) database of the NCBI under the accessions number SRR10273262 and SRR9209608. RNA-seq data were deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE140753 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140753).

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Authors contributions

SHD, AWW, AM and SD devised the study. NM and EC performed the experiments. NM, SHD, AWW, AM and SD analysed the data and prepared figures and tables. SHD, NM, AM and SD participated in the elaboration of the manuscript draft. All authors read and approved the final version of the manuscript.

Tables and figures legends

Table 1. Substrate utilization and fermentation products of *Ruminococcus gauvreauii* IPLA60001 and *Ruminocoides biliarensis* IPLA60002 in comparisons with *Ruminococcus gauvreauii* DSM-19829 and *Ruminococcus bromii* L2-63 and 5AMG strains.

Table 2. Minimal inhibitory concentrations (MICs) against different bile salts (expressed as %) of *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001 in M2GSC medium, in comparison with *R. bromii* L2-63 and 5AMG strains, and *R. gauvreauii* DSM-19829.

Table 3. Differences in aminoacid and vitamin pathways between *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001.

Table 4. Transcriptional changes in *Ruminococcus gauvreauii* IPLA60001 when is growing for 8 h alone or in co-culture with *Ruminocoides biliarensis* IPLA60002. The overexpressed genes related to carbohydrate metabolism are represented.

Figure 1. Growth curves for the two biliary isolates IPLA60001 (discontinuous line) and IPLA60002 (continuous line) in M2GSC medium supplemented with 30% clarified bovine fluid. The graph shows the average and standard deviation of the OD_{650} values (of triplicate cultures).

Figure 2. Phylogenetic bootstrap consensus trees based on the complete sequences of the rRNA16S gene from different human isolates. The Neighbour-Joining trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. A) Tree for the *Lachnospiraceae* family. There were a total of 1,284 positions in the final dataset. B) Tree for the *Ruminococcaceae* family. There were a total of 1,241 positions in the final dataset.

Figure 3. A) Growth of *Ruminococcus gauvreauii* IPLA60001 on M2 medium supplemented with 30% clarified bovine fluid, with different concentrations of formate and limited carbon source (0.1% glucose). The results are normalized to time zero. B) Growth of IPLA60001 on M2 medium supplemented with 30% clarified bovine fluid, 10mM formate, 10mM butyrate

and 0.1% glucose. The graph shows the variation in time (h) of short chain fatty acids and glucose (normalized to time zero) as well as the change in optical density (OD_{650}).

Figure 4. Heatmap based on dbCAN analysis comparing the functional family classification from CAZy among the genomes of IPLA60001 strain and *Ruminococcus gauvreauii* DSM-19829; and between the genome of IPLA60002 strain compared to four strains of *Ruminococcus bromi*: 5AMG, ATCC 27225, L2-36 and L2-63. Colour key: each colour represents a value range of presence of activities that was selected for optimal visualization ranging between 0 and 28.

Figure 5. Formate and acetate production of *Ruminococcus gauvreauii* IPLA60001 and *Ruminocoides biliarensis* IPLA60002 in single cultures during 8 h of growth (IPLA60001 in 0.2% D-glucose and IPLA60002 in 0.2% starch from rice, as carbon source) and co-cultures of both strains in starch from rice during 6 and 8 h of growth. The results are normalized to blank and means and standard deviations are represented.

Figure 6. Heatmap of the transcription level of differentially expressed *Ruminocoides biliarensis* IPLA60002 genes between 6 and 8 h of growth in co-culture with *Ruminococcus gauvreauii* IPLA60001. The heatmap only shows the genes differentially expressed with a log2 (fold change) < -1 or > 1 and an adjusted *p*-value of 0.05.

Table Supp.1. Differences in genes for vitamin, cofactors and aminoacid production pathways between *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001. Absence (-) and presence (+) are marked with negative and positive symbols.

Fig. Suppl.1. Heatmap of the transcription level of differentially expressed *Ruminocoides biliarensis* IPLA60002 genes when the strain was growing in pure culture or in co-culture with *Ruminococcus gauvreauii* IPLA60001 for 8 h. The heatmap only shows the genes differentially expressed with a log2 (fold change) < -1 or > 1 and an adjusted *p*-value of 0.05.

Fig. Suppl.2. Heatmap of the transcription level of differentially expressed *Ruminococcus gauvreauii* IPLA60001 genes between 6 and 8 h of growth in co-culture with *Ruminocoides biliarensis* IPLA60002. The heatmap only shows the genes differentially expressed with a log2 (fold change) < -1 or > 1 and an adjusted *p*-value of 0.05.

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Table 1. Substrate utilization and fermentation products of *Ruminococcus gauvreauii* IPLA60001 and *Ruminocoides biliarensis* IPLA60002 in comparisons with *Ruminococcus gauvreauii* DSM-19829 and *Ruminococcus bromii* L2-63 and 5AMG strains.

Fermentation of:	***R. gauvreauii* IPLA60001**	***R. gauvreauii* DMS-19829**	***Rc. biliarensis* IPLA60002**	***R. bromii* 5AMG**	***R. bromii* L2-63**
L-tryptophane	-	-	-	-	-
Urea	-	-	-	-	-
Glucose	+	+	-	-	-
Mannitol	+	+	-	-	-
Lactose	-	-	-	-	-
Fructose	-	-	+	+	+
Sucrose	+	+	-	-	-
Maltose	-	-	+	+	+
Salicin	-	-	-	-	-
Xylose	+	+	-	-	-
Arabinose	-	-	-	-	-
Gelatin	-	-	-	-	-
Esculine	-	-	-	-	-
Glycerol	-	-	-	-	-
Cellobiose	-	-	-	-	-
Mannose	-	-	-	-	-
Melezitose	-	-	-	-	-
Raffinose	-	-	-	-	-
Sorbitol	+	+	-	-	-
Rhamnose	-	-	-	-	-
Trehalose	-	-	-	-	-
Catalase	-	-	-	-	-
Inositol	+	+	-	-	-
Fucose	-	-	-	-	-
N-acetil-galactosamine	-	-	-	-	-
Mucin	-	-	-	-	-
Degradation of RS:					
Starch from rice	-	-	+	+	+
Starch from corn	-	-	+	+	+
Starch from potato	-	-	+	+	+
Hylon VII	-	-	+	+	+
Novelose	-	-	+	+	+
High Maize	-	-	+	+	+
Major fermentation products (GC)[*]	A	A	A, F, L	A, F	A, F

Abbreviations: GC, Gas Chromatography; RS, Resistant Starches; A, acetate; F, formate; L, lactate.

[+]: the strain is able to growth in M2 medium with the corresponding carbon source.

[*]Production was considered from >2mM.

Table 2. Minimal inhibitory concentrations (MICs) against different bile salts (expressed as %) of *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001 in M2GSC medium, in comparison with *R. bromii* L2-63 and 5AMG strains, and *R. gauvreauii* DSM-19829.

Strain	CA	TDC	GC	TC	GDC	Porcine Bile	Bovine Bile	Mix Bile Salts
R. gauvreauii IPLA60001	0.1%	8%	8%	8%	<0.25%	<0.25%	8%	8%
R. gauvreauii DSM-19829	0.1%	8%	8%	8%	<0.25%	<0.25%	8%	8%
Rc. biliarensis IPLA60002	<0.25%	4%	4%	4%	<0.25%	<0.25%	8%	8%
R. bromii 5AMG	0.4%	2%	0.5%	4%	0.1%	0.25%	4%	<0.5%
R. bromii L2-63	0.2%	2%	0.5%	4%	0.1%	0.25%	2%	<0.5%

Abreviations: CA, Cholic Acid; TDC, Taurodeoxycholic Acid; GC, Glycocholic Acid; TC, Taurocholic Acid; GDC, Glycodeoxycholic Acid.

Table 3. Differences in aminoacid and vitamin pathways between *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001.

	***R. gauvreauii* IPLA60001**	***Rc. biliarensis* IPLA60002**
Vitamin		
Coenzyme A biosynthesis cluster	-	+
Coenzyme B12 biosynthesis	+	-
Molybdenum cofactor biosynthesis	+	-
Lipoic acid metabolism	+	-
Cobalamin synthesis	+	-
Aminoacid		
Putrescine utilization pathways	+	-
Urease subunits	+	-
Urea decomposition	+	-
Methionine biosynthesis	+	-
Lysine biosynthesis DAP pathway, GJO scratch	+	-
Creatine and creatinine degradation	+	-
Glycine and serine utilization	-	+

-, genes for this pathway not detected; +, the strain has all genes needed to produce this vitamin or amino acid.

Table 4. Transcriptional changes in *Ruminococcus gauvreauii* IPLA60001 when is growing for 8 h alone or in co-culture with *Ruminocoides biliarensis* IPLA60002. The overexpressed genes related to carbohydrate metabolism are represented.

Gene	log2 fold change	*p*-value	*p* adjusted
Acetaldehyde dehydrogenase (EC 1.2.1.10) ethanolamine utilization cluster	5.369	2.63E-31	8.86E-29
ATP:Cob(I)alamin adenosyltransferase (EC 2.5.1.17) ethanolamine utilization cluster	7.444	1.44E-24	2.42E-22
Ethanolamine ammonia-lyase heavy chain (EC 4.3.1.7)	3.948	5.54E-18	6.22E-16
Ethanolamine ammonia-lyase light chain (EC 4.3.1.7)	2.8383	3.77E-07	1.20E-05
Ethanolamine utilization polyhedral-body-like protein EutL	2.986	1.62E-07	5.68E-06
Ethanolamine utilization protein EutA	3.761	1.97E-08	8.52E-07
Ethanolamine utilization protein EutJ	6.966	4.03E-28	8.48E-26
Ethanolamine utilization protein EutQ	3.123	3.73E-10	2.17E-08
Ethanolamine utilization protein similar to PduT	7.686	2.81E-17	2.95E-15
Ethanolamine utilization protein similar to PduV	3.218	8.42E-06	0.00019
EutN-like protein clustered with choline trimethylamine-lyase	6.89	1.49E-18	1.80E-16
Formate dehydrogenase-O%2C major subunit (EC 1.2.1.2)	2.514	1.14E-10	7.36E-09
Myo-inositol 2-dehydrogenase (EC 1.1.1.18)	0.737	0.00935	0.05546
Sorbitol dehydrogenase	2.053	7.12E-05	0.00122

Figure 1. Growth curves for the two biliary isolates IPLA60001 (discontinuous line) and IPLA60002 (continuous line) in M2GSC medium supplemented with 30% clarified bovine fluid. The graph shows the average and standard deviation of the OD_{650} values (of triplicate cultures).

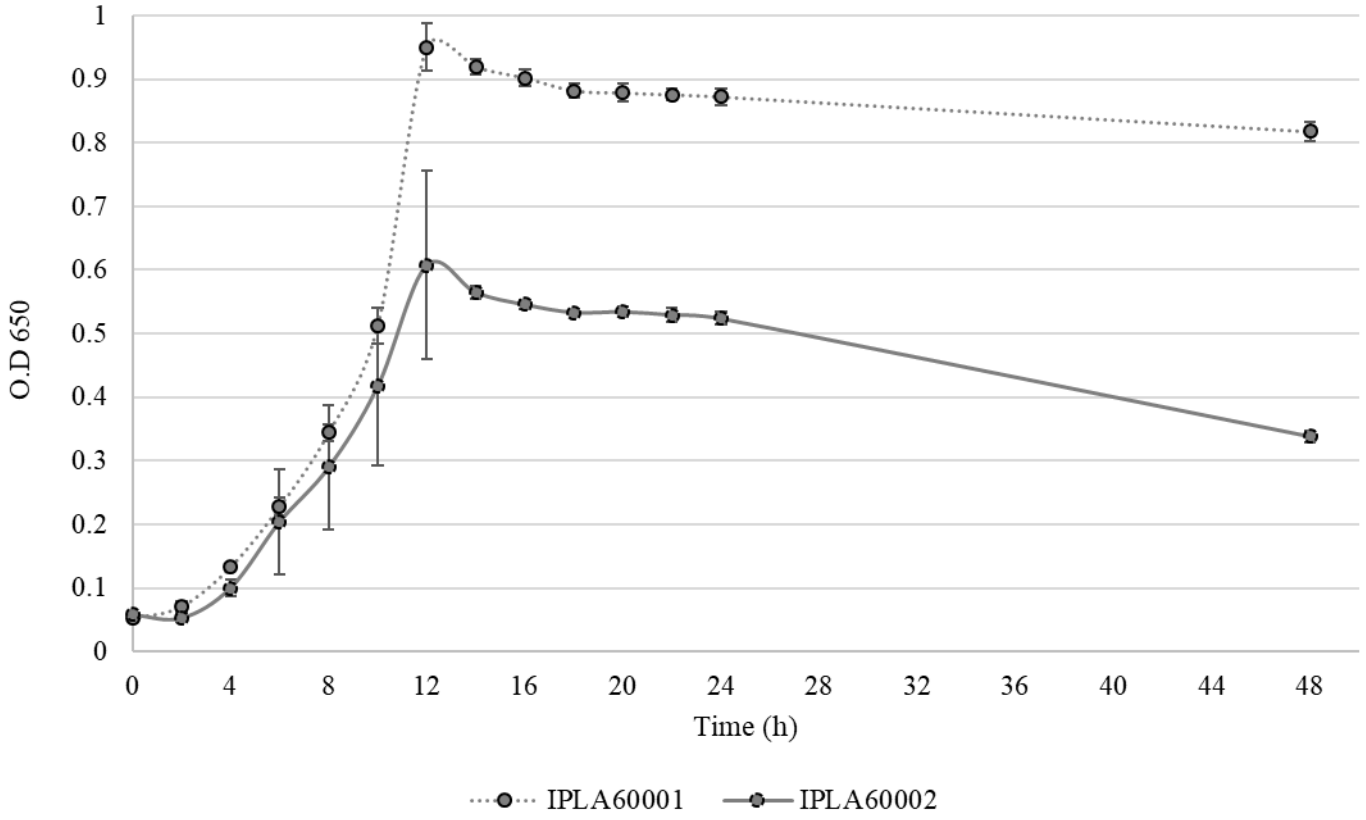


Figure 2. Phylogenetic bootstrap consensus trees based on the complete sequences of the rRNA16S gene from different human isolates. The Neighbour-Joining trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. A) Tree for the *Lachnospiraceae* family. There were a total of 1,284 positions in the final dataset. B) Tree for the *Ruminococcaceae* family. There were a total of 1,241 positions in the final dataset.

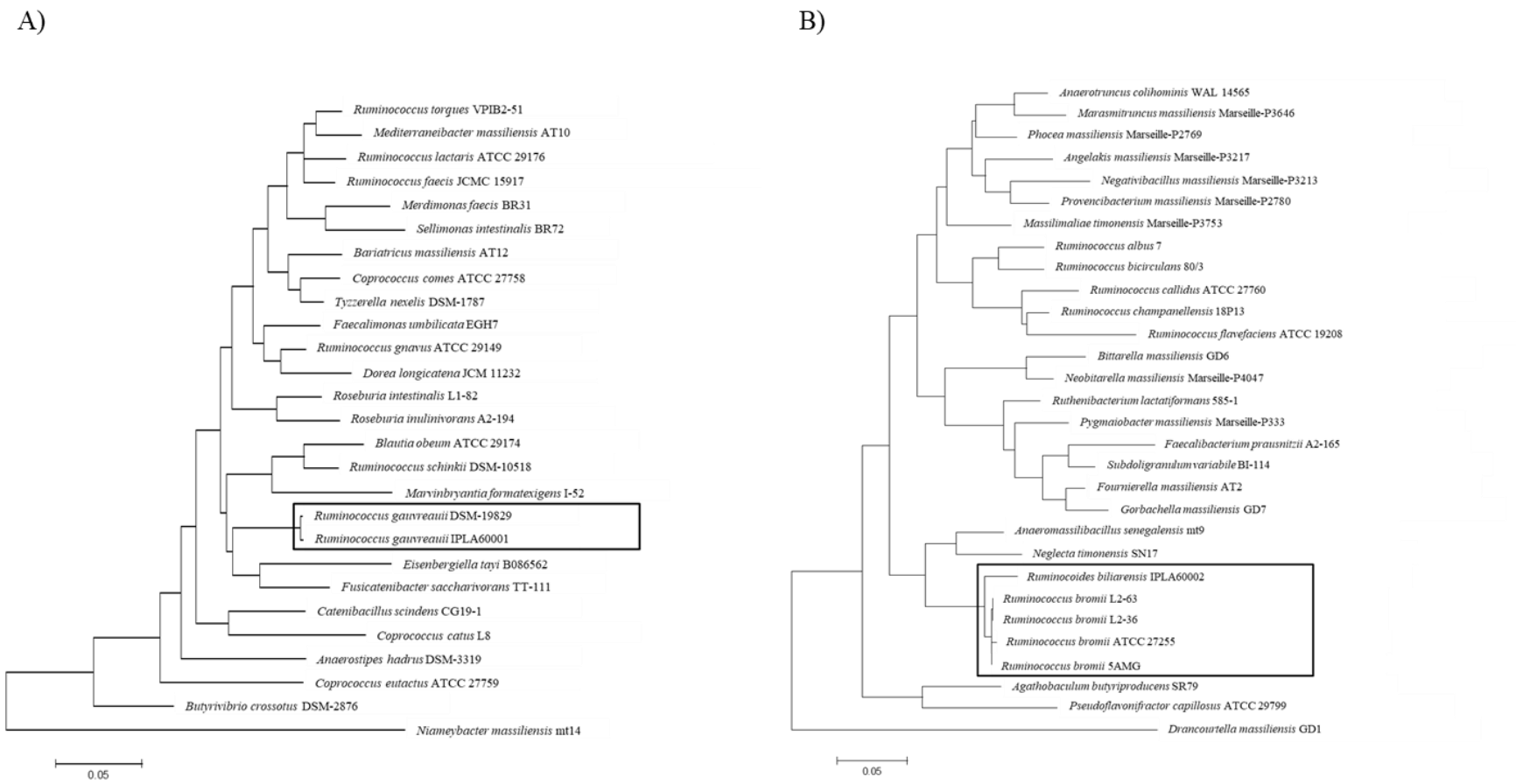
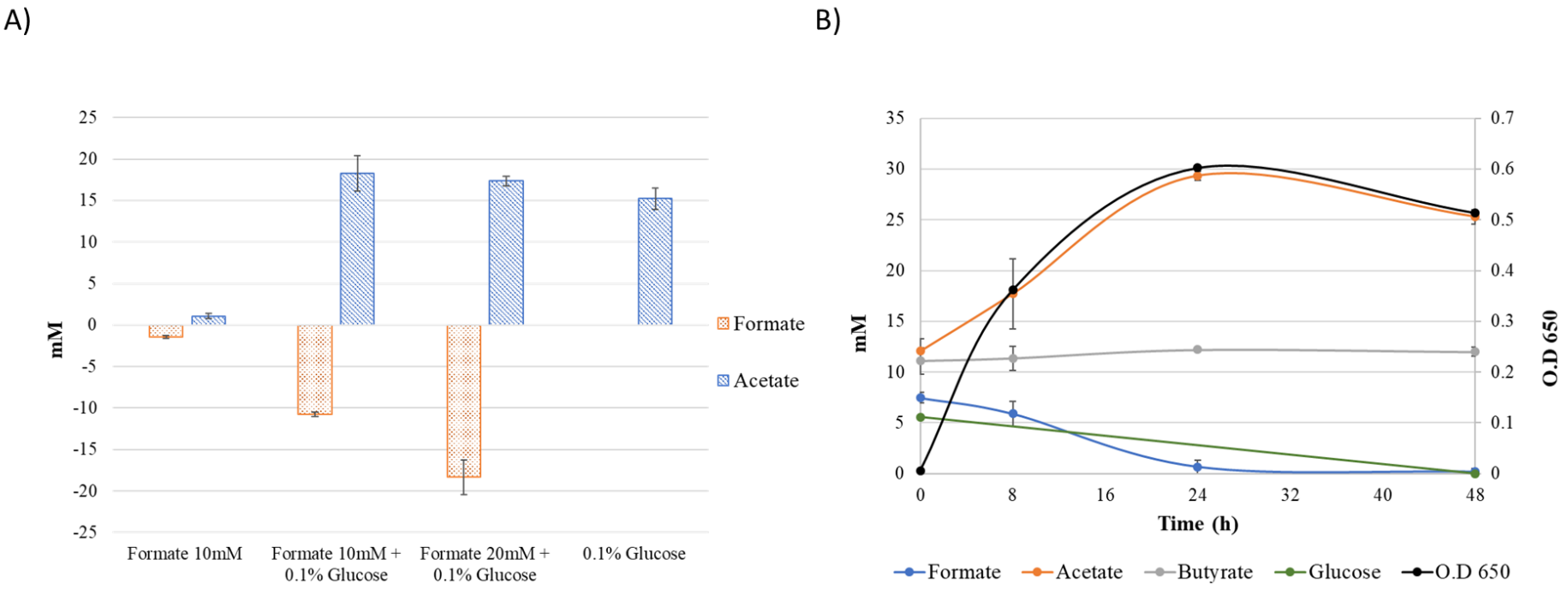


Figure 3. A) Growth of *Ruminococcus gauvreauii* IPLA60001 on M2 medium supplemented with 30% clarified bovine fluid, with different concentrations of formate and limited carbon source (0.1% glucose). The results are normalized to time zero. B) Growth of IPLA60001 on M2 medium supplemented with 30% clarified bovine fluid, 10mM formate, 10mM butyrate and 0.1% glucose. The graph shows the variation in time (h) of short chain fatty acids and glucose (normalized to time zero) as well as the change in optical density (OD_{650}).



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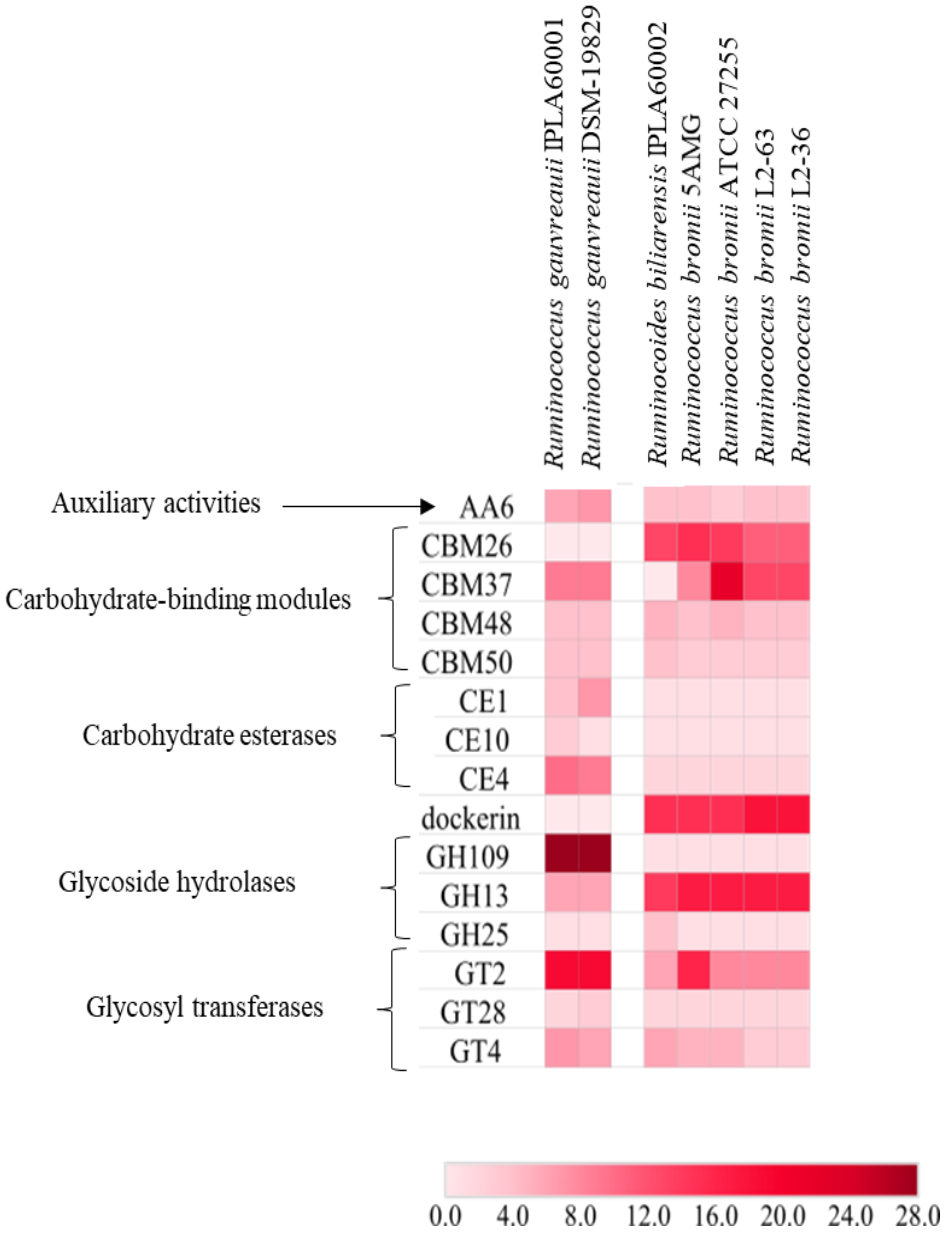
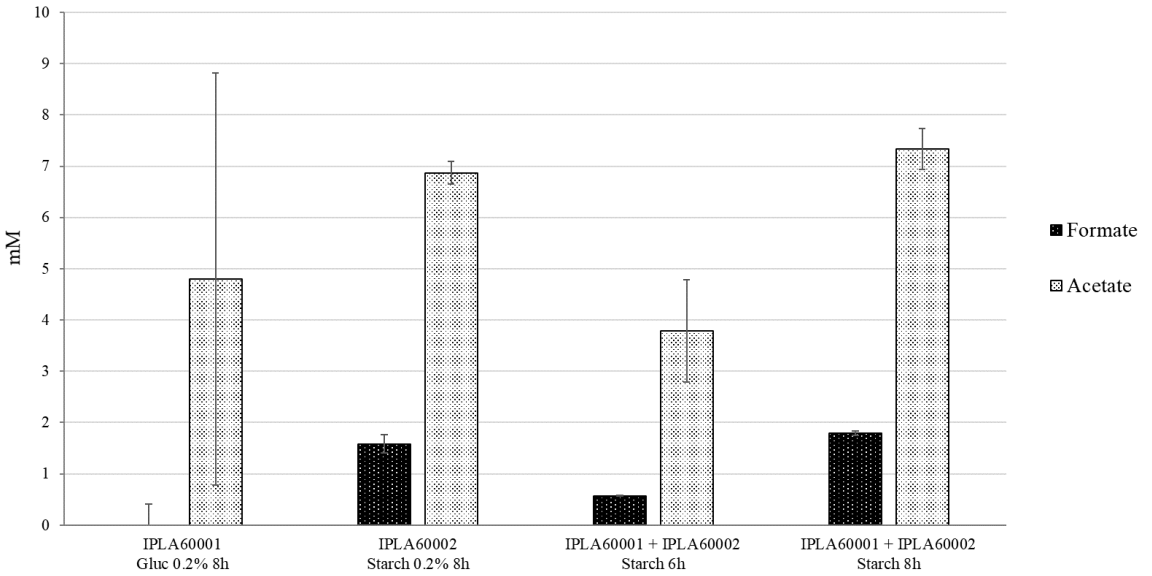


Figure 5. Formate and acetate production of *Ruminococcus gauvreauii* IPLA60001 and *Ruminocoides biliarensis* IPLA60002 in single cultures during 8 h of growth (IPLA60001 in 0.2% D-glucose and IPLA60002 in 0.2% starch from rice, as carbon source) and co-cultures of both strains in starch from rice during 6 and 8 h of growth. The results are normalized to blank and means and standard deviations are represented.



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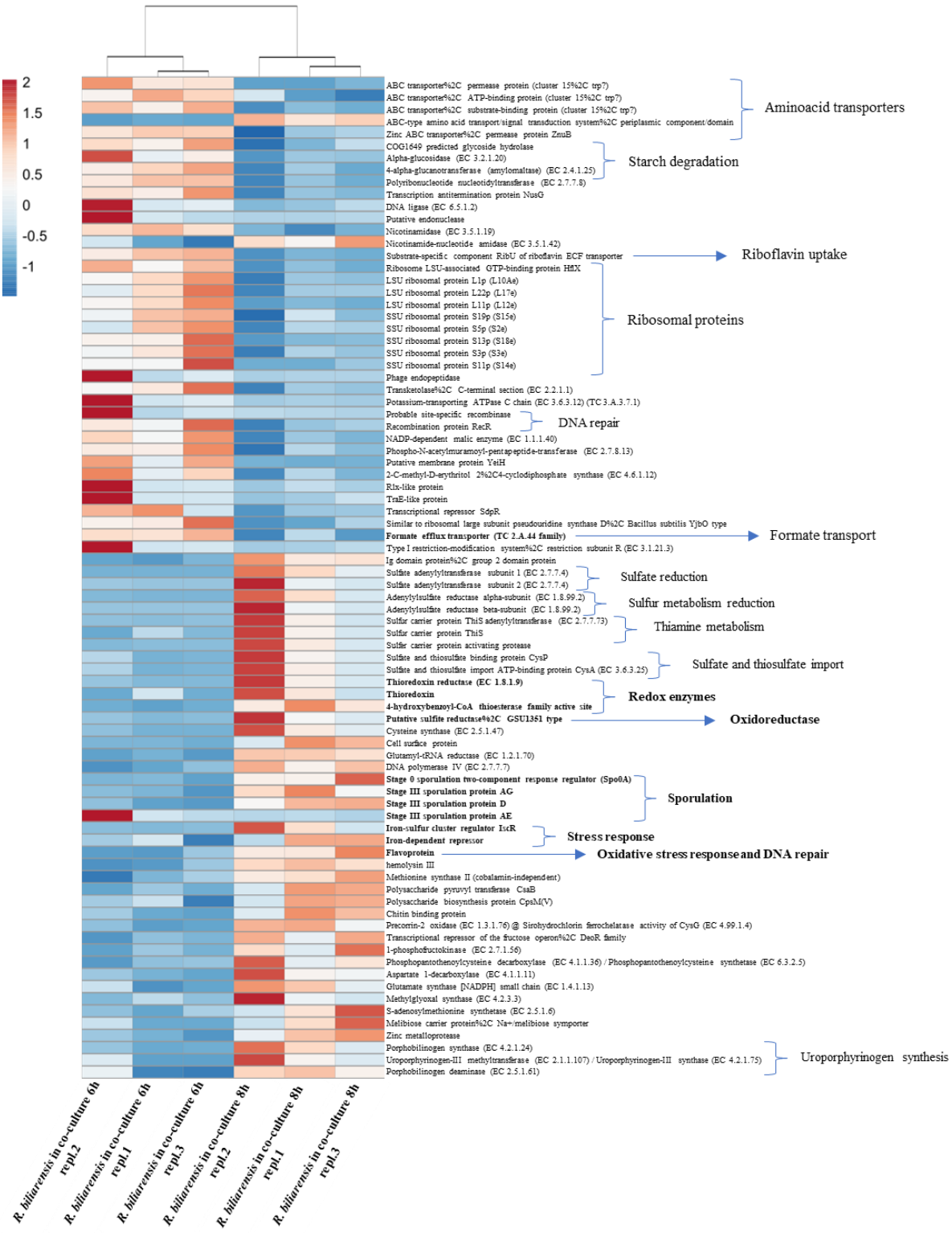


Table Supp.1. Differences in genes for vitamin, cofactors and aminoacid production pathways between *Ruminocoides biliarensis* IPLA60002 and *Ruminococcus gauvreauii* IPLA60001. Absence (-) and presence (+) are marked with negative and positive symbols.

Subsystem	Role	IPLA60001	IPLA60002
Vitamins and cofactors			
Biotin biosynthesis	Substrate-specific component BioY of biotin ECF transporter	+	+
	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	+	-
	3-ketoacyl-CoA thiolase (EC 2.3.1.16)	+	-
	Biotin operon repressor	+	+
	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62)	-	+
	ATPase component BioM of energizing module of biotin ECF transporter	-	+
Biotin biosynthesis experimental	Competence protein F homolog, phosphoribosyltransferase domain	+	+
	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62)	-	+
Thiamine biosynthesis	Sulfur carrier protein adenylyltransferase ThiF	+	+
	tRNA S(4)U 4-thiouridine synthase (former ThiI)	+	-
	Substrate-specific component ThiT of thiamin ECF transporter	+	+
	Thiazole biosynthesis protein ThiG	+	+
	Thiamin pyrophosphokinase (EC 2.7.6.2)	+	+
	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)	+	+
	Sulfur carrier protein ThiS	-	+
	1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)	+	+
	Hydroxyethylthiazole kinase (EC 2.7.1.50)	+	-
	Hydroxymethylpyrimidine ABC transporter, transmembrane component	+	-
	Predicted hydroxymethylpyrimidine transporter CytX	+	-
	Thiamine-monophosphate kinase (EC 2.7.4.16)	+	-
	Hydroxymethylpyrimidine ABC transporter, ATPase component	+	-
Riboflavin, FMN and FAD metabolism	FMN adenylyltransferase (EC 2.7.7.2)	+	+
	Substrate-specific component RibU of riboflavin ECF transporter	+	+
	Riboflavin kinase (EC 2.7.1.26)	-	+
Riboflavin, FMN and FAD metabolism in plants	FIG000859: hypothetical protein YebC	+	+
	Molybdopterin binding motif, CinA N-terminal domain	+	+
	Multi antimicrobial extrusion protein (Na (+)/drug antiporter), MATE family of MDR	+	+
	C-terminal domain of CinA type S	+	+
	6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	+	-
	5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193)	+	-
	tRNA pseudouridine synthase B (EC 4.2.1.70)	+	+
	Riboflavin kinase (EC 2.7.1.26)	+	-
	GTP cyclohydrolase II (EC 3.5.4.25)	+	-
	Riboflavin synthase eubacterial/eukaryotic (EC 2.5.1.9)	+	-
	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26)	+	-
	3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12)	+	-
	FMN adenylyltransferase (EC 2.7.7.2)	-	+
	Substrate-specific component RibU of riboflavin ECF transporter	-	+
	Riboflavin kinase (EC 2.7.1.26)	-	+
Riboflavin to FAD	FMN adenylyltransferase (EC 2.7.7.2)	+	+
	Riboflavin synthase eubacterial/eukaryotic (EC 2.5.1.9)	+	-
	Riboflavin kinase (EC 2.7.1.26)	+	+
	3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12)	+	-
Pyridoxine (Vitamin B6) biosynthesis	Pyridoxine biosynthesis glutamine amidotransferase, synthase subunit (EC 2.4.2.-)	-	+
	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	+	+
	Pyridoxamine 5'-phosphate oxidase (EC 1.4.3.5)	+	+
	Pyridoxal kinase (EC 2.7.1.35)	-	+
	Phosphoserine aminotransferase (EC 2.6.1.52)	+	+
	1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)	+	+
	Pyridoxine biosynthesis glutamine amidotransferase, glutaminase subunit (EC 2.4.2.-)	-	+
	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	+	+
	Predicted transcriptional regulator of pyridoxine metabolism	+	-
Folate biosynthesis cluster	Cell division protein FtsH (EC 3.4.24.-)	+	+
	Pantoate--beta-alanine ligase (EC 6.3.2.1)	-	+
	tRNA(Ile)-lysidine synthetase (EC 6.3.4.19)	+	+
	Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)	+	+
	GTP cyclohydrolase I (EC 3.5.4.16) type 1	+	-
	Dihydropteroate synthase (EC 2.5.1.15)	+	-
	Aspartate 1-decarboxylase (EC 4.1.1.11)	-	+
	Dihydroneopterin aldolase (EC 4.1.2.25)	+	-
	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	+	-
Folate biosynthesis	GTP cyclohydrolase I (EC 3.5.4.16) type 1	+	-
	Dihydrofolate reductase (EC 1.5.1.3)	+	-
	Dihydropteroate synthase (EC 2.5.1.15)	+	-
	Thymidylate synthase (EC 2.1.1.45)	+	-
	Aminodeoxychorismate lyase (EC 4.1.3.38)	+	-
	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	+	-
	Putative DHNTP pyrophosphatase	+	-
	Dihydroneopterin aldolase (EC 4.1.2.25)	+	-
	Dihydrofolate synthase (EC 6.3.2.12)	+	+
	Folylpolyglutamate synthase (EC 6.3.2.17)	+	+
	Substrate-specific component FolT of folate ECF transporter	-	+
	5-formyltetrahydrofolate cyclo-ligase (EC 6.3.3.2)	+	+
	Thymidylate synthase thyX (EC 2.1.1.-)	-	+

	Para-aminobenzoate synthase, amidotransferase component (EC 2.6.1.85)	+	+
	Aspartate 1-decarboxylase (EC 4.1.1.11)	-	+
Coenzyme A biosynthesis	3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11)	-	+
	Dephospho-CoA kinase (EC 2.7.1.24)	+	+
	Pantothenate kinase type III, CoaX-like (EC 2.7.1.33)	+	+
	Aspartate 1-decarboxylase (EC 4.1.1.11)	-	+
	Ketol-acid reductoisomerase (EC 1.1.1.86)	+	+
	Phosphopantetheine adenylyltransferase (EC 2.7.7.3)	+	+
	Phosphopantothenoylcysteine synthetase (EC 6.3.2.5)	+	+
	Pantoate--beta-alanine ligase (EC 6.3.2.1)	-	+
	Phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36)	+	+
	Substrate-specific component PanT of predicted pantothenate ECF transporter	+	-
Amino acids			
Glutamine, Glutamate, Aspartate and Asparagine biosynthesis	Aspartate--ammonia ligase (EC 6.3.1.1)	+	-
	Aspartate aminotransferase (EC 2.6.1.1)	+	+
	NAD-specific glutamate dehydrogenase (EC 1.4.1.2)	+	-
	Glutamate racemase (EC 5.1.1.3)	+	+
	Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	+	+
	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)	+	+
	Glutamine synthetase type I (EC 6.3.1.2)	+	-
	Glutamate synthase [NADPH] small chain (EC 1.4.1.13)	+	+
	Glutamine synthetase type III, GlnN (EC 6.3.1.2)	+	+
	L-asparaginase (EC 3.5.1.1)	-	+
Glutamate dehydrogenases	NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	+	+
	NAD-specific glutamate dehydrogenase (EC 1.4.1.2)	+	-
Glutamine synthetases	Glutamine synthetase type I (EC 6.3.1.2)	+	-
	Glutamine synthetase type III, GlnN (EC 6.3.1.2)	+	+
Polyamine metabolism	ABC transporter, periplasmic spermidine putrescine-binding protein PotD	-	+
	Spermidine Putrescine ABC transporter permease component potC	-	+
	Spermidine Putrescine ABC transporter permease component PotB	-	+
	Ornithine decarboxylase (EC 4.1.1.17)	-	+
	Arginine/ornithine antiporter ArcD	+	-
	Spermidine synthase (EC 2.5.1.16)	+	+
	Carbamate kinase (EC 2.7.2.2)	+	-
	Carboxynorspermidine decarboxylase, putative (EC 4.1.1.-)	+	+
	Putrescine transport ATP-binding protein PotA (TC 3.A.1.11.1)	+	+
	Agmatine deiminase (EC 3.5.3.12)	+	+
	Arginine decarboxylase (EC 4.1.1.19)	+	+
	Carboxynorspermidine dehydrogenase, putative (EC 1.1.1.-)	+	+
	N-carbamoylputrescine amidase (3.5.1.53)	+	+
	Transcriptional regulator, MerR family, near polyamine transporter	+	-
	5'-methylthioadenosine nucleosidase (EC 3.2.2.16)	+	+
Arginine and Ornithine degradation	Arginine/ornithine antiporter ArcD	+	-
	Carbamate kinase (EC 2.7.2.2)	+	-
	Arginine pathway regulatory protein ArgR, repressor of arg regulon	+	+
	Agmatine deiminase (EC 3.5.3.12)	+	+
	Arginine decarboxylase (EC 4.1.1.19)	+	+
	NADP-specific glutamate dehydrogenase (EC 1.4.1.4)	+	+
	Ornithine carbamoyltransferase (EC 2.1.3.3)	+	+
	N-carbamoylputrescine amidase (3.5.1.53)	+	+
	Ornithine decarboxylase (EC 4.1.1.17)	-	+
	Lysine-arginine-ornithine-binding periplasmic protein precursor (TC 3.A.1.3.1)	-	+
Arginine biosynthesis extended	Glutamate N-acetyltransferase (EC 2.3.1.35)	+	-
	Predicted amino-acid acetyltransferase (EC 2.3.1.1)	-	+
	Acetylglutamate kinase (EC 2.7.2.8)	+	+
	Arginine pathway regulatory protein ArgR, repressor of arg regulon	+	+
	Argininosuccinate lyase (EC 4.3.2.1)	+	+
	N-acetylglutamate synthase (EC 2.3.1.1)	+	+
	Argininosuccinate synthase (EC 6.3.4.5)	+	+
	N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	+	+
	Ornithine carbamoyltransferase (EC 2.1.3.3)	+	+
	Acetylornithine aminotransferase (EC 2.6.1.11)	+	+
Arginine biosynthesis	Glutamate N-acetyltransferase (EC 2.3.1.35)	+	+
	Acetylglutamate kinase (EC 2.7.2.8)	+	+
	Arginine pathway regulatory protein ArgR, repressor of arg regulon	+	+
	Argininosuccinate lyase (EC 4.3.2.1)	+	+
	N-acetylglutamate synthase (EC 2.3.1.1)	+	+
	Argininosuccinate synthase (EC 6.3.4.5)	+	+
	N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	+	+
	Ornithine carbamoyltransferase (EC 2.1.3.3)	+	+
	Acetylornithine aminotransferase (EC 2.6.1.11)	+	+
	Predicted amino-acid acetyltransferase (EC 2.3.1.1)	-	+
Arginine Deiminase pathway	Arginine/ornithine antiporter ArcD	+	-
	Arginine pathway regulatory protein ArgR, repressor of arg regulon	+	+
	Carbamate kinase (EC 2.7.2.2)	+	-
	Ornithine carbamoyltransferase (EC 2.1.3.3)	+	+
Methionine degradation	S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	+	+
	Pyruvate-flavodoxin oxidoreductase (EC 1.2.7.-)	+	+
	Methionine ABC transporter ATP-binding protein	+	+
	S-ribosylhomocysteine lyase (EC 4.4.1.21)	+	+
	S-adenosylmethionine synthetase (EC 2.5.1.6)	+	+
	Methionine transporter MetT	+	-
	Methionine gamma-lyase (EC 4.4.1.11)	-	+
Lysine biosynthesis DAP pathway	Diaminopimelate decarboxylase (EC 4.1.1.20)	+	+
	L,L-diaminopimelate aminotransferase (EC 2.6.1.83)	+	+
	Diaminopimelate epimerase (EC 5.1.1.7)	+	-

	Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	+	+
	4-hydroxy-tetrahydrodipicolinate reductase (EC 1.17.1.8)	+	+
	Aspartokinase (EC 2.7.2.4)	+	+
	4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)	+	+
	Meso-diaminopimelate D-dehydrogenase (EC 1.4.1.16)	+	-
Lysine degradation	Lysine decarboxylase (EC 4.1.1.18)	+	+
	Lysine 2,3-aminomutase (EC 5.4.3.2)	+	-
Threonine degradation	Threonine dehydrogenase and related Zn-dependent dehydrogenases	+	-
	Threonine dehydratase (EC 4.3.1.19)	+	+
	L-threonine 3-dehydrogenase (EC 1.1.1.103)	+	-
Cysteine biosynthesis	Cysteine synthase (EC 2.5.1.47)	+	+
	Serine acetyltransferase (EC 2.3.1.30)	+	+
	Sulfate transport system permease protein CysT	-	+
	Sulfate adenylyltransferase subunit 2 (EC 2.7.7.4)	-	+
	Sulfate adenylyltransferase subunit 1 (EC 2.7.7.4)	-	+
	Sulfate and thiosulfate import ATP-binding protein CysA (EC 3.6.3.25)	-	+
	Sulfate and thiosulfate binding protein CysP	-	+
	Sulfate transport system permease protein CysW	-	+
Common Pathway For Synthesis of Aromatic Compounds (DAHP synthase to chorismate)	5-Enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19)	+	+
	2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha (EC 2.5.1.54)	+	+
	3-dehydroquinate synthase (EC 4.2.3.4)	+	+
	Chorismate synthase (EC 4.2.3.5)	+	+
	3-dehydroquinate dehydratase I (EC 4.2.1.10)	+	-
	Shikimate kinase I (EC 2.7.1.71)	+	+
	2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta (EC 2.5.1.54)	+	-
	Shikimate/quinate 5-dehydrogenase I beta (EC 1.1.1.282)	+	-
	Shikimate 5-dehydrogenase I alpha (EC 1.1.1.25)	-	+
	3-dehydroquinate dehydratase II (EC 4.2.1.10)	-	+
Chorismate synthesis	5-Enolpyruvylshikimate-3-phosphate synthase (EC 2.5.1.19)	+	+
	Chorismate mutase I (EC 5.4.99.5)	+	-
	2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha (EC 2.5.1.54)	+	+
	Prephenate dehydratase (EC 4.2.1.51)	+	+
	3-dehydroquinate synthase (EC 4.2.3.4)	+	+
	Chorismate synthase (EC 4.2.3.5)	+	+
	Prephenate dehydrogenase (EC 1.3.1.12)	+	-
	3-dehydroquinate dehydratase I (EC 4.2.1.10)	+	-
	Shikimate kinase I (EC 2.7.1.71)	+	+
	2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta (EC 2.5.1.54)	+	-
	Shikimate/quinate 5-dehydrogenase I beta (EC 1.1.1.282)	+	-
	Shikimate 5-dehydrogenase I alpha (EC 1.1.1.25)	-	+
	Prephenate and/or arogenate dehydrogenase (EC 1.3.1.12)	-	+
	3-dehydroquinate dehydratase II (EC 4.2.1.10)	-	+
Chorismate: Intermediate for synthesis of Tryptophan, PAPA antibiotics, PABA, 3-hydroxyanthranilate and more.	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	+	+
	Anthranilate synthase, amidotransferase component (EC 4.1.3.27)	+	+
	Aminodeoxychorismate lyase (EC 4.1.3.38)	+	-
	Tryptophan synthase alpha chain (EC 4.2.1.20)	+	+
	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	+	+
	Tryptophan synthase beta chain (EC 4.2.1.20)	+	+
	Indole-3-glycerol phosphate synthase (EC 4.1.1.48)	+	+
	Anthranilate synthase, aminase component (EC 4.1.3.27)	+	+
	Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	+	+
	Para-aminobenzoate synthase, aminase component (EC 2.6.1.85)	+	-
	Tryptophan synthase beta chain like (EC 4.2.1.20)	+	+
	Isochorismatase (EC 3.3.2.1)	+	-
	Para-aminobenzoate synthase, amidotransferase component (EC 2.6.1.85)	+	+
Phenylalanine and Tyrosine branches from Chorismate.	Chorismate mutase I (EC 5.4.99.5)	+	-
	Prephenate dehydratase (EC 4.2.1.51)	+	+
	Prephenate dehydrogenase (EC 1.3.1.12)	+	-
	Biosynthetic Aromatic amino acid aminotransferase alpha (EC 2.6.1.57)	+	-
	Prephenate and/or arogenate dehydrogenase (EC 1.3.1.12)	-	+
Tryptophan synthesis	Anthranilate synthase, amidotransferase component (EC 4.1.3.27)	+	+
	Aminodeoxychorismate lyase (EC 4.1.3.38)	+	-
	Tryptophan synthase alpha chain (EC 4.2.1.20)	+	+
	Para-aminobenzoate synthase, aminase component (EC 2.6.1.85)	+	-
	Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	+	+
	Tryptophan synthase beta chain (EC 4.2.1.20)	+	+
	Indole-3-glycerol phosphate synthase (EC 4.1.1.48)		+
	Para-aminobenzoate synthase, amidotransferase component (EC 2.6.1.85)	+	+
	Anthranilate synthase, aminase component (EC 4.1.3.27)	+	+
	Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	+	+
Glycine biosynthesis	Serine hydroxymethyltransferase (EC 2.1.2.1)	+	+
	L-threonine 3-dehydrogenase (EC 1.1.1.103)	+	-
Serine biosynthesis	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	+	+
	Serine hydroxymethyltransferase (EC 2.1.2.1)	+	+
	Phosphoserine aminotransferase (EC 2.6.1.52)	+	+
	Phosphoserine phosphatase (EC 3.1.3.3)	-	+

Fig. Suppl.1. Heatmap of the transcription level of differentially expressed *Ruminocoides biliarensis* IPLA60002 genes when the strain was growing in pure culture or in co-culture with *Ruminococcus gauvreauii* IPLA60001 for 8 h. The heatmap only shows the genes differentially expressed with a log2 (fold change) < -1 or > 1 and an adjusted *p*-value of 0.05.

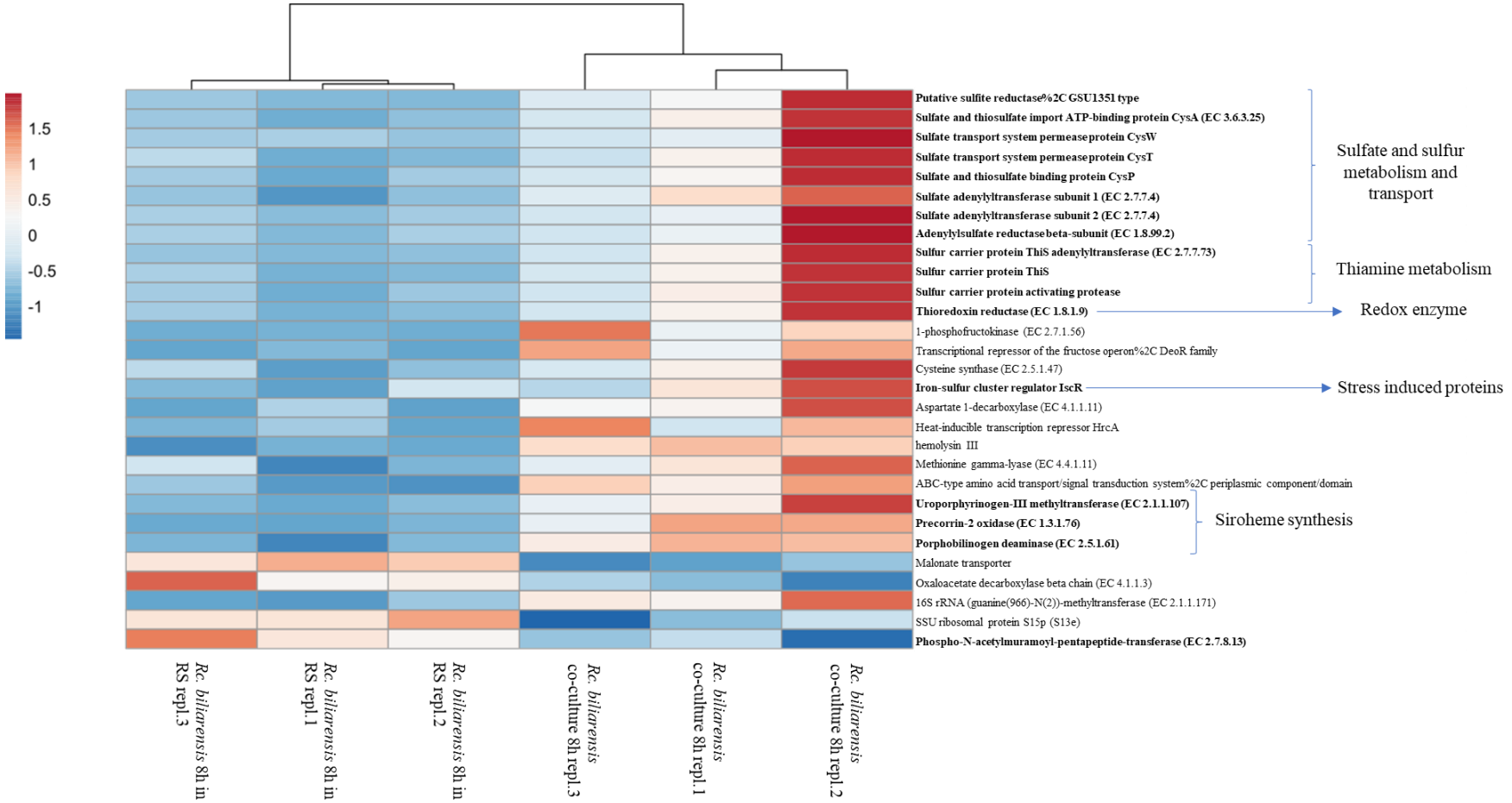
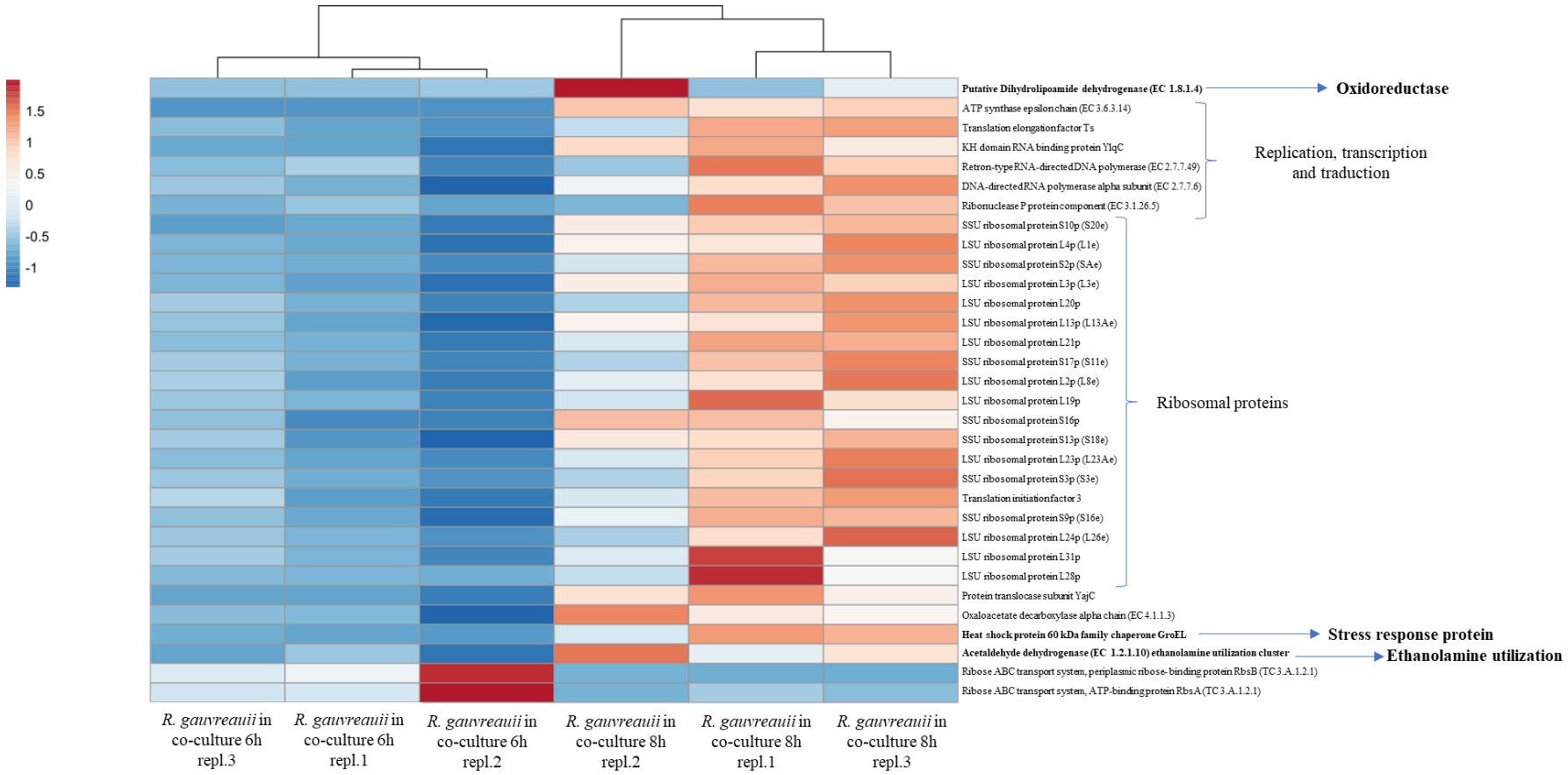


Fig. Suppl.2. Heatmap of the transcription level of differentially expressed *Ruminococcus gauvreauii* IPLA60001 genes between 6 and 8 h of growth in co-culture with *Ruminocoides biliarensis* IPLA60002. The heatmap only shows the genes differentially expressed with a log2 (fold change) < -1 or > 1 and an adjusted *p*-value of 0.05.



Discusión

Discussion

DISCUSIÓN GENERAL

Durante décadas, el estudio de la microbiota humana se centró principalmente en analizar las comunidades microbianas presentes en el TGI y comprender el papel que desempeñan en la salud del hospedador. Con el desarrollo de las NGS y de potentes herramientas bioinformáticas, se produjo una gran revolución, que permitió determinar la gran diversidad de microorganismos presentes en el TGI, así como comprender las relaciones entre los mismos y su función. Además, estas tecnologías abrieron la puerta hacia el estudio de otros ambientes microbianos del cuerpo humano que no habían sido analizados, debido fundamentalmente a la dificultad de obtener muestras y a la falta de técnicas de estudio adecuadas, como la leche materna (Ruiz *et al.*, 2019), la placenta (Aagaard *et al.*, 2014), o en nuestro caso, la bilis humana.

El objetivo principal de esta Tesis Doctoral fue estudiar y caracterizar el microbioma presente en la bilis humana, y analizar su relación con la dieta y la presencia de la patología biliar más frecuente, la colelitiasis. La microbiota biliar humana apenas había sido analizada hasta el momento y la bilis se consideró durante mucho tiempo un ambiente prácticamente estéril en individuos sanos. Para abordar este objetivo general se propusieron una serie de objetivos parciales, siendo el primero de ellos el estudio de la microbiota biliar humana en distintos estados de salud. En esta primera parte del trabajo, debido a la evidente dificultad presente a la hora de tomar muestras de bilis de sujetos sanos, decidimos seleccionar un grupo de sujetos donantes de hígado sin patologías hepatobiliares, que consideramos como grupo de referencia. De estos

individuos se tomaron muestras durante la cirugía de trasplante, lo que nos permitió tomar muestra de bilis directamente de la vesícula biliar, algo novedoso e importante teniendo en cuenta que la mayor parte de los estudios llevados a cabo hasta el momento se tomaban las muestras de bilis del conducto colédoco por ERCP (Shen *et al.*, 2015; Di Carlo *et al.*, 2019). El conducto colédoco tiene una concentración de bilis y sales biliares muy inferior a la presente en la vesícula biliar, además de poseer condiciones fisiológicas diferentes (Behar, 2013). En paralelo, se estudió un grupo de pacientes con colelitiasis de los que también se tomaron muestras de bilis durante la cirugía para eliminar los cálculos. Después de desarrollar un protocolo adecuado para la extracción del ADN de la bilis, analizamos la carga bacteriana presente, obteniendo una media de mil bacterias por mililitro en aquellos sujetos pertenecientes al grupo control. Como se ha comentado, las condiciones fisiológicas normales en la vesícula biliar dificultan el crecimiento y supervivencia bacteriana (Urdaneta and Casadesús, 2017), siendo necesario que la microbiota autóctona desarrolle sistemas de defensa ante un entorno hostil, y por lo tanto, siendo razonable pensar que la bilis presente una carga bacteriana baja en comparación con otros nichos. Sin embargo, los experimentos llevados a cabo sugieren que podríamos estar subestimando la carga bacteriana presente en sujetos sin patologías hepatobiliares, teniendo en cuenta que el protocolo de extracción, la elección de los primers o el límite de detección de la técnica podrían alterar el resultado cuantitativo.

El análisis metagenómico de las muestras de bilis de ambos grupos de estudio nos permitió determinar que la bilis está colonizada fundamentalmente por miembros de los filos Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria. Esto concuerda con los resultados obtenidos por Shen y colaboradores y Saltykova y colaboradores

(Shen *et al.*, 2015; Saltykova *et al.*, 2016), que describen que la bilis de sujetos con colelitiasis está dominada por estos 4 filos principalmente. Por otro lado, comparamos los perfiles microbianos presentes en el grupo control y el grupo con colelitiasis. Los resultados mostraron un agrupamiento entre las muestras de bilis pertenecientes al grupo control, indicando que la composición microbiana presente en la bilis de estos sujetos era similar entre sí y diferente a la del grupo con patología biliar. Los sujetos del grupo control fueron seleccionados de un grupo de referencia más amplio de forma que cumplieran los siguientes criterios de homogeneidad: menores de 80 años, con una estancia en la UCI menor de 48 horas y que no hubieran recibido tratamiento antibiótico previo a la cirugía más allá de 24 horas. El análisis estadístico al comparar ambos grupos de estudio reveló una mayor abundancia relativa en los pacientes con colelitiasis de miembros de los géneros *Bacteroides* y *Escherichia-Shiguella* en comparación con el grupo control. En este sentido, se sabe que el consumo de una dieta rica en grasas animales produce una mayor producción de bilis, favoreciendo el crecimiento de microorganismos tolerantes a la bilis como *Bacteroides* (David *et al.*, 2014). A pesar de que no podemos comparar la microbiota fecal con la microbiota biliar, cabe destacar que la mayoría de pacientes con colelitiasis que formaron parte del estudio presentaban unos niveles altos de colesterol y triglicéridos, o un historial previo de hipercolesterolemia y obesidad, por lo que estos niveles altos de colesterol, probablemente provocados por una ingesta alta en grasas, podrían ser los responsables de la mayor proporción de miembros del género *Bacteroides* en la bilis de estos pacientes. Por otro lado, en trabajos previos se han descrito altos niveles de enterobacterias en la bilis de pacientes con colelitiasis (Capoor *et al.*, 2008; Abeysuriya *et al.*, 2008; Liu *et al.*, 2015a). Estos resultados, junto con los obtenidos del análisis

metabolómico, que mostró perfiles metabólicos distintos entre el grupo control y el grupo con colelitiasis, apoyan la hipótesis de una disbiosis asociada a colelitiasis. A pesar de que existen trabajos que postulan la microbiota biliar como principal responsable de la formación de los cálculos biliares (Swidsinski and Lee, 2001; Wang *et al.*, 2018), el papel causal que juega esta microbiota es aún desconocido, por lo que son necesarios más estudios para poder descifrar en qué medida está involucrada la microbiota biliar en el desarrollo de esta patología.

Los resultados obtenidos del análisis metagenómico a nivel funcional fueron muy similares a las principales funciones presentes en la microbiota intestinal. Sin embargo, las actividades relacionadas con el metabolismo de sales biliares presentaron una mayor abundancia en el microbioma biliar en comparación con el microbioma fecal (Shafquat *et al.*, 2014), hecho que podría relacionarse con una adaptación de los miembros de la microbiota biliar a las condiciones hostiles del ambiente. No obstante, debemos tener en cuenta que la comparación se ha realizado con un pequeño número de muestras, y no se ha realizado con el mismo grupo de individuos sanos, ya que los metagenomas fecales y biliares no provienen del mismo grupo de sujetos, ni estos individuos presentan las mismas condiciones fisiológicas (sanos *vs*. donantes de hígado), por lo que son necesarios más estudios para poder seguir profundizando en este sentido.

Durante la consecución de este trabajo, nos encontramos con algunas limitaciones, como la selección de un grupo de sujetos que pudiésemos considerar como grupo control. El grupo elegido, recibió un tratamiento específico previo al trasplante en la UCI, incluyendo un tratamiento antibiótico corto, hecho que hace que debamos ser cautos y ser conscientes de que podría afectar a los resultados obtenidos. Por otro lado, debemos tener en cuenta que trabajamos con muestras de un ambiente con una baja

carga bacteriana, por lo que no podemos descartar la posibilidad de que parte de los resultados provengan de ADN contaminante, proveniente de los reactivos utilizados para la extracción del ADN o del propio ambiente de laboratorio (Laurence *et al.*, 2014). A pesar de que todos los análisis se llevaron a cabo en paralelo utilizando controles de extracción de ADN (agua de grado molecular), y que éstos controles no dieron lugar a amplificación, algunas de las secuencias obtenidas han sido asociadas en otros trabajos a ADN contaminante (Laurence *et al.*, 2014; Salter *et al.*, 2014), por lo que no podemos excluir completamente la posibilidad de pequeños sesgos en los resultados.

Sin embargo, aun siendo conscientes de estas limitaciones, y de que el número de sujetos por grupo de estudio es reducido (debido fundamentalmente al reducido número de trasplantes de hígado que se produjeron en los hospitales colaboradores durante el tiempo de recogida de muestras), este es el primer estudio enfocado en analizar en profundidad y caracterizar el microbioma presente en la bilis de individuos sin patologías hepatobiliares, además de estudiar la posible relación existente entre la microbiota y los metabolitos biliares. La información derivada del trabajo realizado en el trascurso del primer capítulo de esta tesis supone la base para comprender qué microorganismos son capaces de resistir las condiciones propias de la bilis y proponer la existencia de una microbiota biliar en condiciones fisiológicas. Además, nos permite ahondar en los posibles mecanismos que han desarrollado los miembros de la microbiota biliar para poder sobrevivir, e inferir las funciones que podrían tener sobre nuestra salud. Nuestros resultados establecen una base de conocimiento hasta ahora inexistente para estudios futuros con un mayor tamaño muestral que se centren en caracterizar en mayor profundidad este microbioma, y estudiar su papel en la salud del hospedador.

El segundo objetivo que nos planteamos en esta Tesis Doctoral fue analizar, una vez conocidas las principales comunidades microbianas presentes en la bilis de sujetos sin patologías hepatobiliares y sujetos con colelitiasis, la posible relación entre la microbiota biliar y la dieta. Para ello, se llevaron a cabo cuestionarios nutricionales completos y se tomaron muestras de sangre a todos los sujetos pertenecientes al grupo con colelitiasis. En este caso, debido a la imposibilidad de realizar estos cuestionarios en el grupo de donantes de hígado utilizado en el capítulo anterior, se eligió un grupo de controles sanos reclutados en un trabajo previo (Gutiérrez-Díaz *et al.*, 2016), pareados en número, edad y sexo al grupo de sujetos con colelitiasis, de los que se disponía de datos de cuestionarios nutricionales y de parámetros sanguíneos. Con esta información nutricional y el conocimiento de la microbiota biliar del grupo de pacientes con colelitiasis, se analizaron las posibles asociaciones entre los distintos factores dietéticos y los principales miembros de la microbiota biliar.

Los parámetros en sangre, fundamentalmente los niveles de glucosa, colesterol HDL y LDL, y los triglicéridos mostraron diferencias entre el grupo de sujetos sanos y el grupo de pacientes con colelitiasis. En este sentido, destacan los altos niveles de triglicéridos en el grupo con colelitiasis, hecho que concuerda con trabajos previos en los que se relacionan altos niveles de lípidos en sangre, probablemente debido al consumo de una dieta alta en grasas, con una mayor producción de colesterol, cuya saturación puede producir su precipitación junto con sales biliares y fosfolípidos, y la posterior formación de cálculos biliares (Del Pozo *et al.*, 2017; Di Ciaula *et al.*, 2019). A pesar de que los niveles de colesterol en sangre fueron menores en el grupo con colelitiasis en comparación con el grupo sano, esto puede deberse a que los pacientes con colelitiasis fueron diagnosticados de esta patología con antelación a su ingreso para

colecistectomía antes del comienzo del estudio y de la toma de muestras, por lo que podrían haber cambiado sus hábitos alimenticios y de ingesta calórica, evitando alimentos asociados con los síntomas de esta patología (Sarles *et al.*, 1969). En cuanto a la dieta, se observaron diferencias en la ingesta de distintos macro- y micronutrientes entre el grupo de sujetos sanos y el grupo con colelitiasis. El consumo de una menor cantidad de vegetales en general en el grupo con colelitiasis, es decir, un bajo consumo de fibra dietética entre otros, se ha relacionado con un mayor riesgo de desarrollar cálculos biliares (Di Ciaula *et al.*, 2019). A pesar de que posteriormente el análisis por subclases de los compuestos ingeridos en la dieta no reveló diferencias significativas en la ingesta de fibra entre ambos grupos, sí se detectaron diferencias significativas para la mayor parte de compuestos fenólicos y polifenoles analizados, mostrando valores de ingesta inferiores en el grupo con colelitiasis. Los compuestos fenólicos y polifenólicos se encuentran en casi todos los alimentos de origen vegetal y frutas (Gimeno, 2004), alimentos que se han asociado a un menor riesgo de colelitiasis (Barré *et al.*, 2017). En este sentido, el menor consumo vegetales, y por tanto, de compuestos fenólicos y polifenoles, podría sugerir una posible asociación de los mismos con el desarrollo de la patología.

En cuanto a las posibles asociaciones entre la ingesta de determinados macro- y micronutrientes y la abundancia relativa de algunos miembros en la microbiota biliar, se detectó una posible asociación entre el consumo de fibra y compuestos fenólicos y la abundancia relativa de algunos grupos bacterianos. La ingesta de fibra, dihidrochalcones y lignanos se asoció negativamente con algunos grupos bacterianos que se mostraron alterados en la microbiota del grupo con colelitiasis en comparación con el grupo control, destacando miembros de las familias *Bacteroidaceae* y *Prevotellaceae,* y

concretamente los géneros *Bacteroides* y *Prevotella*, que presentaron mayores abundancias relativas en el grupo con colelitiasis. En este sentido, como se ha mencionado anteriormente, una dieta rica en grasas de origen animal produce una mayor secreción de sales biliares y bilis, favoreciendo el crecimiento de microorganismos tolerantes a la bilis como *Bacteroides* (David *et al.*, 2014). *Bacteroides* también se ha relacionado con el consumo de altos niveles de proteínas de origen animal, así como una variedad de aminoácidos y grasas saturadas (Wu *et al.*, 2011). Por otro lado, un aumento en la abundancia de miembros del género *Prevotella* se ha asociado con dietas altas en consumo de carbohidratos (De Filippo *et al.*, 2010). A pesar de no detectarse diferencias en cuanto al consumo de carbohidratos, proteínas de origen animal y lípidos entre los dos grupos de estudio, sí se detectaron diferencias en cuanto a la ingesta de patata (fuente de carbohidratos) y se observó una cierta tendencia a la ingesta de mayores cantidades de lípidos en la dieta. En este caso, el menor consumo de vegetales y frutas podría estar relacionado con un mayor consumo de carbohidratos, grasas y proteínas de origen animal, siendo este patrón el posible responsable de un aumento en las proporciones de estos grupos microbianos.

Entre otras, cabe destacar la asociación negativa encontrada entre el consumo de fibra insoluble y la abundancia de miembros del género *Bacteroides*, y la asociación positiva de este género con la ingesta del compuesto fenólico ácido hidroxifenilacético. También se encontró una asociación negativa entre el consumo de colesterol y la abundancia de miembros de la familia *Xanthomonadaceae*, y entre el consumo de ácido trans oleico y la proporción de miembros de la familia *Propionibacteriaceae*. Los miembros de estas familias presentaron abundancias relativas menores en el grupo con colelitiasis en comparación con el grupo control, lo que podría deberse a un mayor

consumo de colesterol y grasas. Este consumo podría generar una mayor producción de sales biliares y bilis en el hepatocito, favoreciendo el crecimiento de aquellos microorganismos capaces de tolerar la toxicidad de la bilis, como *Bacteroides* (Pumbwe *et al.*, 2007; Ridlon *et al.*, 2016), y disminuyendo la proporción de los grupos más sensibles (Islam *et al.*, 2011). Finalmente, miembros del género *Haemophilus,* que presentaron una mayor abundancia relativa en el grupo con colelitiasis, mostraron una asociación positiva con el consumo de marisco, carne y bebidas alcohólicas y no alcohólicas, estando su abundancia relativa directamente relacionada con el consumo de proteínas de origen animal, flavanoles, colesterol, y algunos ácidos grasos. Esto parece concordar con trabajos previos en los que se relacionan mayores niveles de *Haemophilus* con dietas altas en proteínas (Jang *et al.*, 2019).

A pesar de las limitaciones que nos encontramos a la hora de abordar este objetivo, como el pequeño tamaño muestral, que podría dificultar la detección de asociaciones con una fuerte significación estadística, o la imposibilidad de obtener bilis de voluntarios sanos para analizar su perfil microbiano biliar (lo que hizo que el análisis de las asociaciones sólo pudiese llevarse a cabo en el grupo con colelitiasis); este trabajo es el primero enfocado en analizar la relación entre la microbiota biliar, la dieta y la formación de cálculos biliares. Aunque los resultados de este trabajo no nos permitan establecer una relación directa entre la microbiota biliar y la dieta, la información obtenida permite establecer nuevas premisas acerca del posible papel de la dieta sobre la modulación de la microbiota biliar, y su implicación en el desarrollo de cálculos biliares. Este estudio aporta información útil para diseñar dietas adaptadas a prevenir o tratar esta patología biliar.

Como tercer objetivo planteado en esta Tesis Doctoral nos propusimos aislar microorganismos de muestras de bilis humana, utilizando para ello distintos medios y condiciones de cultivo, intentando reproducir el entorno biliar. Teniendo en cuenta la baja carga bacteriana presente en la bilis del grupo control, y la dificultad para reproducir las condiciones ambientales y nutricionales de la vesícula biliar, no fue fácil aislar microorganismos de estas muestras. Sin embargo, entre otros aislados, conseguimos aislar y cultivar dos cepas del orden de los *Clostridiales*, denominados IPLA60001 y IPLA60002, cuya secuencia del ADNr 16S presentó una homología con *Ruminococcus gauvreauii* del 99% y con *Ruminococcus bromii* del 94%, respectivamente. Este último, el aislado IPLA60002, mostró una baja identidad con las secuencias del ADNr 16S disponibles en las bases de datos, por lo que se abordó su estudio genómico y filogenético en mayor profundidad proponiéndolo finalmente como una nueva especie. En el capítulo 3 de esta tesis llevamos a cabo los análisis fenotípicos y genómicos de este aislado, para el que se propone el nombre de *Ruminocoides biliarensis* IPLA60002. *Ruminococcus bromii*, la especie más cercana filogenéticamente, se ha descrito como miembro habitual de la microbiota del TGI (Ze *et al.*, 2012), siendo un grupo dominante y fundamental en la degradación de carbohidratos no digeribles, como celulosa, xilano, pectina y almidón resistente (Ze *et al.*, 2015). El análisis fenotípico de IPLA60002, que se llevó a cabo junto con 2 cepas de *R. bromii*, reveló un perfil similar de degradación de carbohidratos y almidón resistente, sin embargo, la cepa de origen biliar mostró una resistencia a sales biliares notablemente superior a la presentada por las cepas de *R. bromii*. Esto sugiere una adaptación a las condiciones presentes en la vesícula biliar, hipótesis que posteriormente se comprobó mediante el análisis genómico, detectándose en el genoma de IPLA60002 diversos genes relacionados con la respuesta

a sales biliares que no estaban presentes en los otros genomas de *R. bromii* analizados. Entre estas actividades destacan proteínas de respuesta a estrés y de reparación de ADN, así como numerosos transportadores de membrana, ampliamente relacionados con la respuesta a la presencia de sales biliares (Hernández *et al.*, 2012; Urdaneta and Casadesús, 2017), como transportadores MDR de tipo ABC y transportadores de tipo SMR ("Small multidrug resistance transporter", Transportadores de resistencia a pequeños fármacos) (Kermani *et al.*, 2018) o transportadores de tipo MATE ("Multi-antimicrobial extrusión transporters", Transportadores de extrusión de antimicrobianos) (Kuroda and Tsuchiya, 2009).

Por otro lado, el aislado IPLA60002 mostraba un fenotipo de autolisis espontánea no descrito previamente en la especie *R. bromii*. Esta lisis espontánea se ha descrito en otros miembros de la microbiota del rumen (Wells and Russell, 1996a), así como en *Helicobacter pylori* y *Propionibacterium freudenreichii* (Lemée *et al.*, 1995; Wang *et al.*, 2015), que se sabe presentan sistemas para sobrevivir en presencia de bilis (Leverrier *et al.*, 2003; Wang *et al.*, 2015). A pesar de que se conoce la existencia de autolisis en varios microorganismos y se conocen incluso los genes implicados, la razón por la que se produce este fenotipo aún no está clara. Se han postulado algunas hipótesis para explicar este fenómeno, entre las que destacan: la esporulación y el intercambio de material genético (Lewis, 2000), la presencia de profagos (Smith *et al.*, 2000; Rice and Bayles, 2008), y la falta de nutrientes en el medio, que provoca la autolisis de aquellas bacterias metabólicamente no activas para liberar proteínas de origen bacteriano y permitir el crecimiento de otros microorganismos (Wells and Russell, 1996b; Liu *et al.*, 2015b). En el genoma de IPLA60002 se encontraron genes relacionados con autolisinas, como glicosil hidrolasas de la familia 25, sobrerrepresentados en comparación con los

otros genomas de *R. bromii* (Mukhopadhya *et al.*, 2018), y dos genes anotados como lisozima M1, una 1-4-β-N-acetilmuraminidasa, relacionada también con autolisis (Lichenstein *et al.*, 1990), que no fueron detectados en los genomas de las cepas de *R. bromii*. Además, se analizó la presencia de profagos en el genoma, detectándose una estructura similar a un profago en IPLA60002, no presente en los genomas de *R. bromii* analizados. Este fenotipo de autolisis podría desencadenarse en respuesta a la falta de nutrientes, o de algún componente en el medio de *quorum sensing* en respuesta a la densidad celular, que provocarían la entrada en fase lítica del profago, o la activación de las autolisinas generando la lisis completa del cultivo (ver microfotografías en el Artículo 3 y el Anexo 6). No se descarta que la capa mucoide observada mediante SEM que rodea los cultivos de *Ruminocoides biliarensis* IPLA60002 pudiera también actuar como señal activadora desencadenante de la autolisis.

Los análisis llevados a cabo, tanto fenotípicos como genómicos, revelaron que IPLA60002 podría pertenecer a una nueva especie no descrita hasta el momento. Aislar una nueva especie de un ambiente prácticamente desconocido como la bilis humana parece razonable teniendo en cuenta que el análisis metagenómico reveló un perfil microbiano diverso, con una amplia proporción de secuencias no asignadas a ningún taxón presente en las bases de datos. Por otro lado, esto concuerda con algunos trabajos previos, que postulan la presencia de especies nuevas en la bilis de sujetos con colelitiasis (Shen *et al.*, 2015). El estudio en profundidad de este tipo de aislados de origen biliar es importante y puede proporcionarnos nuevas cepas de interés no solo por sus características biotecnológicas, sino también por la posibilidad de contar con nuevos microorganismos capaces de tolerar altas concentraciones de bilis, y sobrevivir así mejor al paso por el TGI superior, problema que hoy en día se tiene con muchos de

los probióticos comerciales disponibles. En la actualidad, se utilizan bajas concentraciones de sales biliares para testar los potenciales probióticos y su capacidad de supervivencia en el paso por el TGI (Hu *et al.*, 2018; Nami *et al.*, 2019; Kook *et al.*, 2019), sin embargo, esta concentración puede ser superior en el duodeno (Van Deest *et al.*, 1968), por lo que podría producir una pérdida no esperada de varias unidades logarítmicas de la dosis inicial, llegando una cantidad insuficiente del probiótico al colon (Sahadeva *et al.*, 2011). El estudio de la capacidad probiótica de aislados de bilis podría solucionar en parte este problema, debido a su alta tolerancia a altas concentraciones de sales biliares.

En el último capítulo de esta Tesis Doctoral, correspondiente al tercer objetivo, nos centramos en estudiar la relación existente entre las dos cepas obtenidas de bilis humana, IPLA60001 e IPLA60002. Ambas fueron aisladas de la misma muestra de bilis de forma conjunta, y mostraron mejores tasas de crecimiento antes de su purificación, lo que nos llevó a pensar, junto con el fenómeno de autolisis observado en una posible relación de simbiosis y cooperación. Estudiamos una posible alimentación cruzada entre ambos aislados mediante el análisis comparativo a nivel fenotípico, genómico y transcriptómico tanto de los cultivos puros como de co-cultivos. Esta comparación además se llevó a cabo con la cepa tipo para *R. gauvreauii* (DSM 19829), y dos cepas de *R. bromii* de la colección del "Rowet Institute" (Ze *et al.*, 2012; Mukhopadhya *et al.*, 2018). El análisis fenotípico reveló diferencias en cuanto al consumo de carbohidratos. *R. gauvreauii* IPLA60001, al igual que la cepa tipo DSM 19829 (Domingo *et al.*, 2008), es capaz de metabolizar distintos azúcares alcohol, principalmente inositol. Por otra parte, IPLA60002 es capaz de metabolizar distintos tipos de almidón resistente, capacidad no

detectada en IPLA60001. *Ruminococcus bromii* está altamente especializado en la degradación de almidón, para lo que utiliza generalmente glicosil hidrolasas de la familia 13 (GH13), enzimas con actividad amilasa (Ze *et al.*, 2015; Mukhopadhya *et al.*, 2018). La presencia de 14 genes anotados como GH13 en *Ruminocoides biliarensis* IPLA60002 podría explicar la gran habilidad de este aislado en la degradación de distintos tipos de almidón resistente. En cuanto a los principales productos de fermentación, la cepa IPLA60001 es principalmente productora de acetato, mientras que la cepa IPLA60002 produce acetato, formato y una pequeña cantidad de lactato. Estas diferencias nos llevaron a pensar en una relación de alimentación cruzada a nivel de SCFAs, relación que se corroboró creciendo *R. gauvreauii* IPLA60001 en presencia de formato y detectándose una metabolización completa del mismo al cabo de 48 horas. Se ha descrito que el formato se asimila a través de una de las ramas de la vía Wood-Ljungdahl (Wolin *et al.*, 2003), y en trabajos previos parece que podría utilizarse para producir mayor cantidad de acetato (Laverde Gomez *et al.*, 2019). Sin embargo, la producción de acetato por *R. gauvreauii* IPLA60001 no se vio modificada en presencia de formato, por lo que podría estar utilizando otra vía metabólica, o bien, producir algún tipo de alcohol.

El análisis genómico permitió detectar diferencias en las rutas biosintéticas de producción de vitaminas y aminoácidos, mostrando un patrón de complementariedad entre ambos aislados (IPLA60001 y IPLA60002). Se ha descrito que miembros de *R. bromii* presentan ciertas limitaciones en la producción de algunas vitaminas (Ze *et al.*, 2012; Magnúsdóttir *et al.*, 2015), limitaciones que parecen estar complementadas en este caso por la producción de vitaminas por parte de *R. gauvreauii* IPLA60001. Finalmente, se analizó por RNA-seq el comportamiento de ambos aislados en co-cultivo, para poder comprender la relación de alimentación cruzada que entre ellos se produce.

Los resultados de RNA-seq revelaron que *R. gauvreauii* IPLA60001 es capaz de crecer utilizando los productos de la degradación del almidón resistente por parte de *Ruminocoides biliarensis* IPLA60002. Se ha descrito que uno de los papeles de *R. bromii* es liberar energía del almidón resistente para que otros miembros de la microbiota sean capaces de crecer (Ze *et al.*, 2012), como se ha demostrado en los casos de *Ruminococcus gnavus* (Crost *et al.*, 2018) y *Blautia hydrogenotrophica* (Laverde Gomez *et al.*, 2019). En este caso, la hipótesis es que *Ruminocoides biliarensis* IPLA60002 degrada el almidón resistente liberando así subproductos susceptibles de ser utilizados por *R. gauvreauii* IPLA60001 como fuente de carbono.

Se detectó una mayor expresión de genes implicados en el metabolismo de tiamina cuando *Ruminocoides biliarensis* IPLA60002 está en co-cultivo, un patrón de expresión que se ha descrito previamente cuando *R. bromii* está en co-cultivo con *B. hydrogenotrophica* (Laverde Gomez *et al.*, 2019). La tiamina es una vitamina fundamental en la reacción de la piruvato:ferredoxina oxidorreductasa, una enzima implicada en el metabolismo anaerobio, y, aunque no sabemos por qué estos genes implicados están sobreexpresados en *Ruminocoides biliarensis* IPLA60002 cuando está en co-cultivo, podría deberse a cambios generalizados en el metabolismo anaerobio que podrían estar generando un estrés oxidativo en *Ruminocoides biliarensis*. Se detectaron también cambios en la expresión génica de genes que afectan al metabolismo del sulfato y el siroheme (grupo prostético que es utilizado por algunas enzimas para conseguir la transferencia de electrones necesaria para la reducción del azufre y del nitrógeno), así como una sobreexpresión de genes anotados como oxidoreductasas y proteínas de reparación de DNA, y proteínas de respuesta a estrés celular.

En relación a *R. gauvreauii* IPLA60001, a pesar de que la comparación no es la más correcta teniendo en cuenta que cuando crece en cultivo puro lo hace utilizando D-glucosa como única fuente de carbono, y cuando crece en co-cultivo lo hace utilizando los productos de degradación del almidón resistente; los resultados de RNA-seq revelaron una sobreexpresión de genes relacionados con el metabolismo de la etanolamina, sorbitol e inositol. Estos productos no están presentes en el medio de cultivo, por lo que pueden ser subproductos de la degradación del almidón o bien ser producidos por *Ruminocoides biliarensis*. En el caso del inositol, un fosfolípido indispensable presente en la membrana lipídica, una posible explicación podría ser que el fenómeno de autolisis observado en *Ruminocoides biliarensis* IPLA60002 pudiera liberar este inositol al medio, utilizándolo *R. gauvreauii* IPLA60001 como fuente de carbono. Esta hipótesis se ha comentado anteriormente, pudiendo ser que aquellas bacterias metabólicamente no activas se lisen para liberar proteínas y otros componentes de origen bacteriano y permitir así el crecimiento de otros microorganismos presentes en el medio (Wells and Russell, 1996b; Liu *et al.*, 2015b).

En cuanto al metabolismo del formato, los resultados de RNA-seq revelaron la sobreexpresión de dos genes relacionados con el metabolismo del formato en *R. gauvreauii* IPLA60001, de acuerdo con lo observado a nivel fenotípico. A pesar de que el análisis de los SCFA en los co-cultivos no mostró una degradación del formato producido por *Ruminocoides biliarensis* IPLA60002, esto puede deberse a que las muestras de los co-cultivos fueron tomadas en un periodo de tiempo corto, que quizás no fue suficiente para que ambas cepas pudiesen producir los niveles de SCFAs necesarios para observar este fenómeno de alimentación cruzada que sí se observó en los cultivos de *R. gauvreauii* suplementados con formato.

Esta alimentación cruzada observada entre los dos aislados obtenidos de bilis humana nos hace pensar en una relación de cooperación entre los mismos, que podría llevarse a cabo con el fin de sobrevivir en un entorno hostil como la bilis. Se sabe que existe un equilibrio entre competición y cooperación entre los miembros de la microbiota (Coyte and Rakoff-Nahoum, 2019), sin embargo, esta relación de cooperación parece que va generando cada vez más interés, teniendo en cuenta la variedad de relaciones de alimentación cruzada descritas entre miembros de la microbiota (Li and Durbin, 2010; Rakoff-Nahoum *et al.*, 2016). En ocasiones, estas relaciones de cooperación y alimentación cruzada facilitan el crecimiento en condiciones adversas, pudiendo favorecer incluso la tolerancia a antibióticos (Adamowicz *et al.*, 2018), por lo que en nuestro caso, la relación de cooperación observada entre los aislados IPLA60001 e IPLA60002 podría producirse con el fin de garantizar la supervivencia en la bilis, y tolerar mejor la toxicidad de las sales biliares presentes en la misma. En este sentido, el análisis genómico reveló la presencia de una colilglicina hidrolasa (Dean *et al.*, 2002), en el genoma de IPLA60002, una enzima asociada a la hidrólisis de sales biliares no presente en el genoma de IPLA60001. Esta actividad podría favorecer a *R. gauvreauii* IPLA60001 a través de la detoxificación de las sales biliares, junto con la degradación de almidón para la producción de fuentes de carbono disponibles. En cambio, *R. gauvreauii* produciría aquellas vitaminas y aminoácidos esenciales que *Ruminocoides biliarensis* no es capaz de producir, completando esta relación de simbiosis para que ambos aislados puedan sobrevivir en la bilis humana.

En resumen, a pesar de las limitaciones que hemos ido encontrando a la hora de abordar los distintos objetivos de esta Tesis Doctoral, los resultados nos permiten proponer la existencia de una microbiota biliar humana en un estado fisiológico, que además presenta un perfil microbiano distinto al característico de pacientes con colelitiasis, lo que sugiere una disbiosis asociada a esta patología. Por otro lado, la dieta parece tener un papel crucial en la modulación de la microbiota biliar y en la formación de cálculos biliares, aunque son necesarios más estudios para poder descifrar y comprender esta relación.

Durante esta Tesis Doctoral hemos utilizado técnicas novedosas, como la metagenómica, metatranscriptómica y metabolómica; y hemos sido capaces de crecer en el laboratorio microorganismos provenientes de la bilis humana, con requerimientos nutricionales especiales y necesidad de condiciones de anaerobiosis estricta, además de caracterizar una posible nueva especie bacteriana con características singulares. Creemos que los resultados obtenidos en esta Tesis Doctoral, pioneros en el microambiente biliar, suponen un avance importante en el conocimiento en este campo de estudio y sientan las bases para trabajos futuros orientados a comprender el papel de la microbiota biliar humana en la salud, y su relación con la dieta, con el fin de desarrollar estrategias para prevenir o tratar patologías biliares.

Conclusiones

Conclusions

CONCLUSIONES

1. Mediante técnicas de metagenómica filogenética y funcional hemos accedido a las principales comunidades microbianas presentes en la bilis de individuos sin patologías hepatobiliares y pacientes diagnosticados de colelitiasis, permitiéndonos proponer la existencia de un ecosistema microbiano biliar, así como determinar los principales perfiles taxonómicos y funcionales presentes. Los resultados obtenidos revelan una carga microbiana baja en condiciones fisiológicas, pero diversa, fundamentalmente compuesta por miembros de los filos Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria. Los perfiles microbianos característicos de la bilis de individuos sin patologías hepatobiliares y pacientes con colelitiasis mostraron diferencias significativas en la abundancia relativa de algunos taxones, lo que podría sugerir un estado de disbiosis biliar asociado a la presencia de cálculos en la vesícula biliar.

2. El análisis mediante metagenómica funcional o *shotgun* de la bilis de individuos sin patologías hepatobiliares mostró un perfil funcional similar al descrito en las comunidades microbianas intestinales. Sin embargo, varias actividades relacionadas con el metabolismo de colesterol y sales biliares mostraron una mayor abundancia relativa en el microbioma biliar, sugiriendo una adaptación de las comunidades microbianas presentes en la bilis a las condiciones de este ambiente. Complementando el análisis metagenómico se realizó un análisis metabonómico de las muestras de bilis que mostró

perfiles metabólicos distintos entre los dos grupos de estudio (individuos sin patología hepatobiliar y pacientes con colelitiasis).

3. Se detectaron diferencias significativas en los niveles de algunos parámetros sanguíneos y en la ingesta de distintos micro- y macronutrientes de la dieta entre el grupo de pacientes con colelitiasis y un grupo de sujetos sanos, destacando en el grupo de pacientes un mayor nivel de triglicéridos en sangre y un menor consumo de vegetales. El estudio de la posible relación entre la microbiota biliar y compuestos de la dieta en el grupo de pacientes con colelitiasis reveló asociaciones estadísticamente significativas entre ciertos taxones de la microbiota biliar y compuestos específicos de la dieta, destacando la correlación negativa entre el género *Bacteroides* y la ingesta de fibra insoluble.

4. El cultivo de muestras de bilis de individuos sin patologías hepatobiliares permitió el aislamiento de dos cepas del orden *Clostridiales*. Una de ellas, *Ruminococcus gauvreauii* IPLA60001, pertenece a la familia *Lachnospiraceae.* La otra cepa, denominada IPLA60002, mostró una baja identidad con las secuencias de ADN presentes en las bases de datos, siendo *Ruminococcus bromii*, miembro de la familia *Ruminococcaceae*, la especie con la que mostró un mayor porcentaje de identidad a nivel del gen ARNr 16S. El análisis fenotípico, genómico y filogenético de IPLA60002 reveló que esta cepa se sitúa en una rama filogenética distinta a *R. bromii*, por lo que se propone como una nueva especie denominada *Ruminocoides biliarensis*.

5. *Ruminocoides biliarensis* IPLA60002 mostró características particulares, entre las que destacan una mayor resistencia a sales biliares y bilis, la capacidad de esporular y de metabolizar distintos tipos de almidón resistente, un comportamiento de autolisis en medio líquido y un aspecto mucoide al microscopio electrónico. Aunque durante el desarrollo de este trabajo no hemos sido capaces de dilucidar los mecanismos responsables de su fenotipo, el análisis genómico reveló la presencia de una estructura similar a un profago en el genoma, un grupo de genes relacionados con la producción de exopolisacáridos, así como distintas autolisinas que podrían estar involucradas en el fenómeno de autolisis observado en IPLA60002.

6. El análisis bioquímico, genómico y transcriptómico de co-cultivos llevado a cabo con el fin de analizar las relaciones metabólicas de sintrofia entre las cepas de origen biliar *Ruminococcus gauvreauii* IPLA60001 y *Ruminocoides biliarensis* IPLA60002 mostró una posible relación de alimentación cruzada basada en el formato y otros compuestos, lo que sugiere una relación de simbiosis y cooperación metabólica entre las cepas, con el fin de sobrevivir en la vesícula biliar y facilitar la tolerancia a las condiciones hostiles propias de este nicho.

CONCLUSIONS

1. Through phylogenetic and functional metagenomics, we have uncovered the main microbial communities present in the bile of individuals without hepatobiliary pathologies and patients diagnosed with cholelithiasis. This allows us to propose the existence of a biliary microbial ecosystem, as well as to determine the main taxonomic and functional profiles. The results revealed a low microbial load under physiological conditions, but diverse, mainly composed by members of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria phyla. The microbial profiles of the bile of individuals without hepatobiliary pathologies and patients with cholelithiasis showed significant differences in the relative abundance of some taxa, suggesting a biliary dysbiosis associated with the presence of gallbladder stones.

2. Functional metagenomics or *shotgun* analysis of the bile of individuals without hepatobiliary pathologies showed a functional profile similar to that described in intestinal microbial communities. However, several activities related to the metabolism of cholesterol and bile salts showed a higher relative abundance in the biliary microbiome, suggesting an adaptation of the microbial communities present in the bile to these environmental conditions. The metabonomic analysis of the bile samples showed different metabolic profiles between the two study groups (individuals without hepatobiliary pathology and patients with cholelithiasis).

3. Significant differences were detected in the levels of some blood parameters and in the intake of different micro- and macronutrients between the group of patients with cholelithiasis and a group of healthy subjects. Higher level of blood triglycerides and a lower consumption of vegetables were detected in the group of patients. The study of the possible relationship between the biliary microbiota and dietary compounds in the group of patients with cholelithiasis revealed statistically significant associations between some biliary microbial taxa and specific dietary compounds, standing out the negative correlation between the *Bacteroides* genus and the insoluble fiber intake.

4. The culture of bile samples from individuals without hepatobiliary pathologies allowed the isolation of two strains of the *Clostridiales* order. One of them, *Ruminococcus gauvreauii* IPLA60001, belongs to the family *Lachnospiraceae*. The other strain, called IPLA60002, showed a low identity with the DNA sequences available in the databases, being *Ruminococcus bromii*, a member of the *Ruminococcaceae* family, the species showing a higher percentage of identity at 16S rRNA gene level. The phenotypic, genomic and phylogenetic analysis of IPLA60002 revealed that this strain is located on a different phylogenetic branch than *R. bromii*. Therefore, it is proposed as a new species called *Ruminocoides biliarensis*.

5. *Ruminocoides biliarensis* IPLA60002 showed particular characteristics, among them it is worth noting a higher resistance to bile salts and bile, the ability to sporulate and metabolize different types of resistant starch, an autolysis behavior in liquid medium and a mucoid appearance under electron microscope. Although this work has not been able to elucidate the mechanisms responsible for its phenotype, genomic

analysis revealed the presence in the genome of a structure similar to a prophage, a group of genes related to exopolysaccharide production, as well as different autolysins that could be involved in the autolysis phenomenon observed in IPLA60002 strain.

6. Biochemical, genomic and transcriptomic analysis of co-cultures were carried out to analyze the metabolic relations between *Ruminococcus gauvreauii* IPLA60001 and *Ruminocoides biliarensis* IPLA60002. A possible cross-feeding relationship based on formate and other compounds was detected, which suggests a symbiosis relationship and metabolic cooperation between the strains, in order to survive in the gallbladder and facilitate tolerance to the hostile conditions of this niche.

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Anexos

Annex

ANEXO 1

Informe sobre la calidad de los artículos

La información sobre las revistas en las que se han publicado los artículos se ha recogido de la Web of Science (https://apps.webofknowledge.com/). Se detalla información del factor de impacto (**FI**) de cada revista, correspondiente a los últimos datos publicados por Journal Citation Reports en el año 2018. Se aporta información acerca del **área SCI** a la que está asociada la revista, el Cuartil (**Q**) en el que se encuentra la revista calculado en función a su factor de impacto, y el número de **citas** que tiene el artículo.

➢ **Molinero, N.**, Ruiz, L., Sanchez, B., Margolles, A., & Delgado, S. (2019). Intestinal bacteria interplay with bile and cholesterol metabolism: implications on host physiology. ***Frontiers in physiology***, 10, 185.

Área SCI	Q	FI	Citas
Physiology	2	3.201	3

➢ **Molinero, N.**, Ruiz, L., Milani, C., Gutiérrez-Díaz, I., Sánchez, B., Mangifesta, M., Segura, J., Cambero, I., Campelo, A.B., García-Bernardo, C.M., Cabrera, A., Rodríguez, J.I., González, S., Rodríguez, J.M., Ventura, M., Delgado, S., Margolles, A. (2019). The human gallbladder microbiome is related to the physiological state and the biliary metabolic profile. ***Microbiome***, 7(1), 100.

Área SCI	Q	FI	Citas
Microbiology	1	10.465	0

- Gutiérrez-Díaz, I., **Molinero, N.**, Cabrera, A., Rodríguez, J.I, Margolles, A., Delgado, S., & González, S. (2018). Diet: Cause or Consequence of the Microbial Profile of Cholelithiasis Disease?. ***Nutrients***, 10(9), 1307.

Área SCI	Q	FI	Citas
Nutrition and Dietetics	1	4.171	1

ANEXO 2

Material suplementario artículo 0 (Introducción)

Supplemental Figure 1. A) Phylogenetic tree of bile salt hydrolase sequences retrieved from the NCBI database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model [1]. The tree with the highest log likelihood (-31878.76) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6195)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.32% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 72 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 308 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [3]. The tree was divided into groups, designed as Group 1 to 5, depending on the grouping at phylum level. B) Phylogenetic tree of cholesterol oxidase sequences retrieved from the NCBI database. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman + Freq. model [2]. The tree with the highest log likelihood (-71531.84) is shown. Initial tree(s) for the heuristic search were obtained automatically

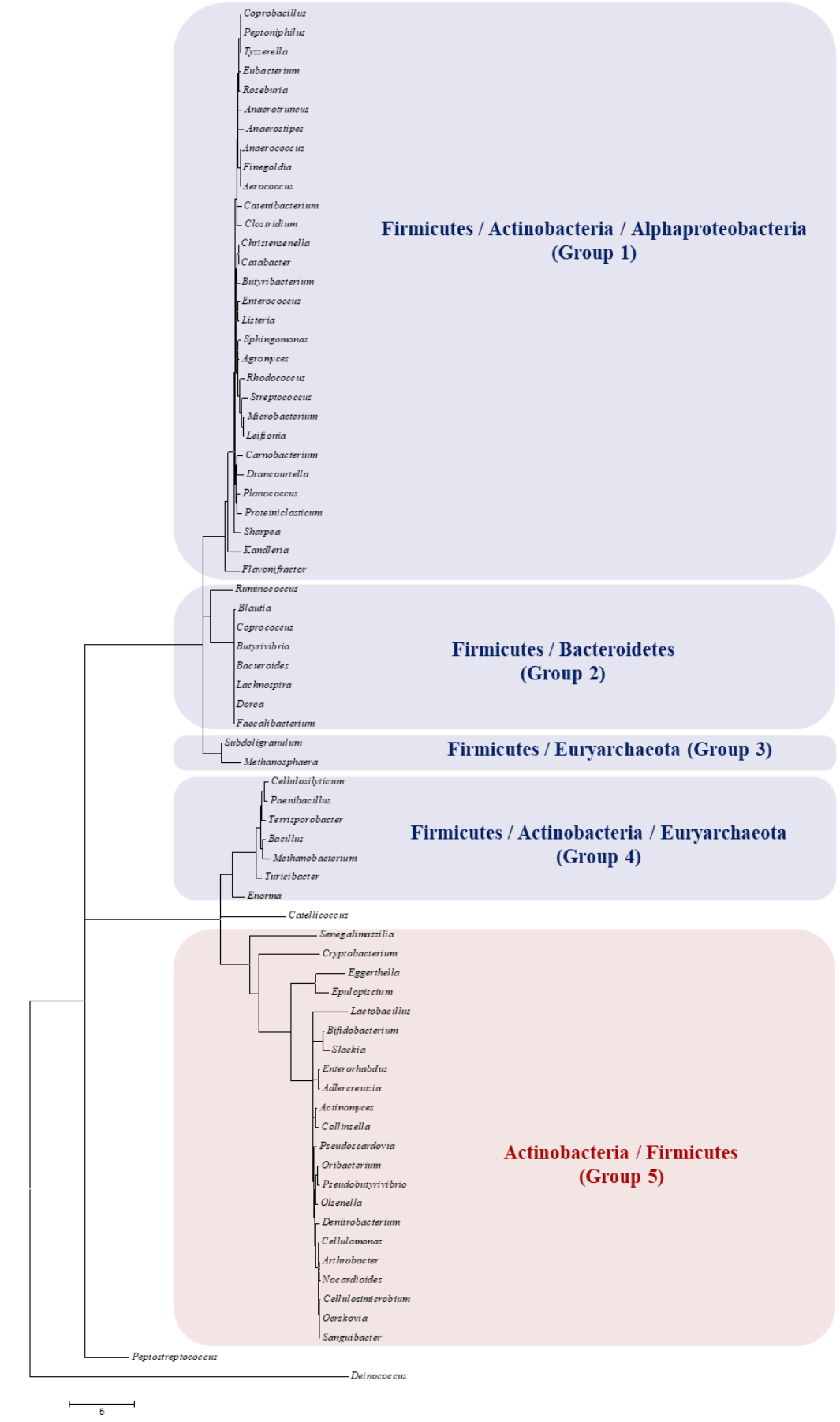
by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 14.7797)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.19% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 59 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [3]. The tree was divided into groups, designed as Group 1 to 10, depending on the grouping at phylum level.

[1]. Le, S.Q., Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol Biol Evol* 25:1307-1320.

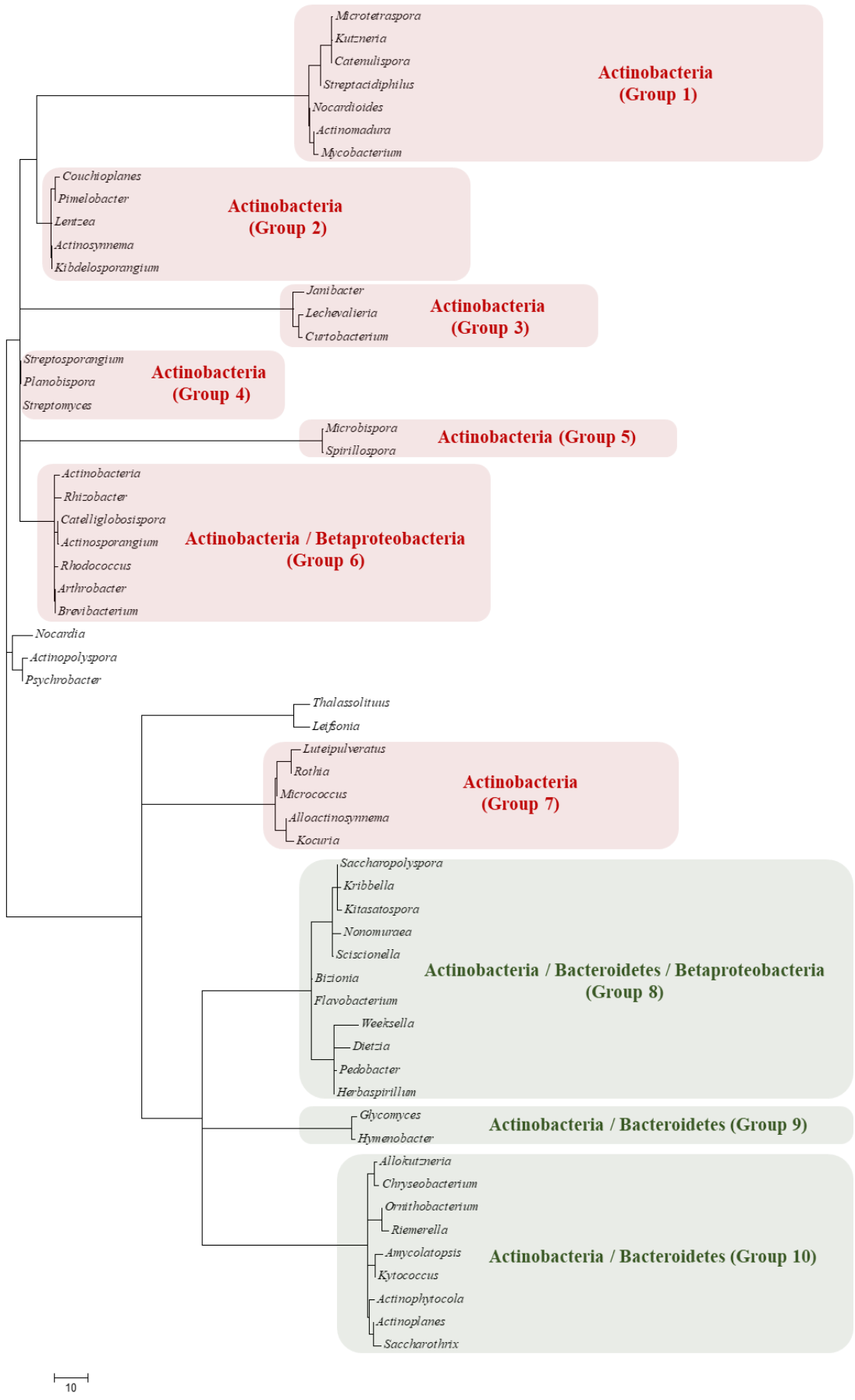
[2]. Whelan, S., Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol Biol Evol* 18:691-699.

[3]. Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mole Biol Evol* 33:1870-1874.

A)


Coprobacillus
Peptoniphilus
Tyzzerella
Eubacterium
Roseburia
Anaerotruncus
Anaerostipes
Anaerococcus
Finegoldia
Aerococcus
Catenibacterium
Clostridium
Christensenella
Catabacter
Butyribacterium
Enterococcus
Listeria
Sphingomonas
Agromyces
Rhodococcus
Streptococcus
Microbacterium
Leifsonia
Carnobacterium
Drancourtella
Planococcus
Proteiniclasticum
Sharpea
Kandleria
Flavonifractor
Firmicutes / Actinobacteria / Alphaproteobacteria
(Group 1)
Ruminococcus
Blautia
Coprococcus
Butyrivibrio
Bacteroides
Lachnospira
Dorea
Faecalibacterium
Firmicutes / Bacteroidetes
(Group 2)
Subdoligranulum
Methanosphaera
Firmicutes / Euryarchaeota (Group 3)
Cellulosilyticum
Paenibacillus
Terrisporobacter
Bacillus
Methanobacterium
Turicibacter
Enorma
Firmicutes / Actinobacteria / Euryarchaeota
(Group 4)
Catellicoccus
Senegalimassilia
Cryptobacterium
Eggerthella
Epulopiscium
Lactobacillus
Bifidobacterium
Slackia
Enterorhabdus
Adlercreutzia
Actinomyces
Collinsella
Pseudoscardovia
Oribacterium
Pseudobutyrivibrio
Olsenella
Denitrobacterium
Cellulomonas
Arthrobacter
Nocardioides
Cellulosimicrobium
Oerskovia
Sanguibacter
Actinobacteria / Firmicutes
(Group 5)
Peptostreptococcus
Deinococcus
5

B)


Microtetraspora
Kutzneria
Catenulispora
Streptacidiphilus
Nocardioides
Actinomadura
Mycobacterium
Actinobacteria (Group 1)
Couchioplanes
Pimelobacter
Lentzea
Actinosynnema
Kibdelosporangium
Actinobacteria (Group 2)
Janibacter
Lechevalieria
Curtobacterium
Actinobacteria (Group 3)
Streptosporangium
Planobispora
Streptomyces
Actinobacteria (Group 4)
Microbispora
Spirillospora
Actinobacteria (Group 5)
Actinobacteria
Rhizobacter
Catelliglobosispora
Actinosporangium
Rhodococcus
Arthrobacter
Brevibacterium
Actinobacteria / Betaproteobacteria (Group 6)
Nocardia
Actinopolyspora
Psychrobacter
Thalassolituus
Leifsonia
Luteipulveratus
Rothia
Micrococcus
Alloactinosynnema
Kocuria
Actinobacteria (Group 7)
Saccharopolyspora
Kribbella
Kitasatospora
Nonomuraea
Sciscionella
Bizionia
Flavobacterium
Weeksella
Dietzia
Pedobacter
Herbaspirillum
Actinobacteria / Bacteroidetes / Betaproteobacteria (Group 8)
Glycomyces
Hymenobacter
Actinobacteria / Bacteroidetes (Group 9)
Allokutzneria
Chryseobacterium
Ornithobacterium
Riemerella
Amycolatopsis
Kytococcus
Actinophytocola
Actinoplanes
Saccharothrix
Actinobacteria / Bacteroidetes (Group 10)
10

ANEXO 3

Material suplementario artículo 1

Table S1. Data and clinical characteristics of both groups of individuals (patients with cholelithiasis and liver donors as controls) from whom bile samples were analyzed in this study.

Parameter	Cholelithiasis[a] (n=14)	Liver donors (n=13)	*p*-value[b]
Age	52±14	59±14	0.274
Triglycerides (50-165mg/dl)[c]	113±82.69	76.73±38.60	**0.044**
Total colesterol (125-240mg/dl)	192.79±53.32	131.64±45.46	**0.011**
HDL (40-80mg/dl)	49.93±14.25	44.27±17.68	0.434
LDL (0-160mg/dl)	117.07±36.40	72.18±35.48	**0.011**
Glucose (65-100mg/dl)	79.07±16.95	123.91±38.16	**0.000**
Urea (10-60mg/dl)	26.80±8.02	36.00±16.53	0.197
Creatinine (0.55-1.2mg/dl)	0.66±0.19	1.01±0.53	0.150
ALT (4-41U/l)	27.64±13.11	53.45±83.24	0.851
AST (4-35U/l)	28.28±10.26	75.5±126.91	0.767
GGT (1-24U/l)	35.64±28.54	44.63±41.57	0.809
Alkaline phosphatase (40-117U/l)	81.85±42.26	68.36±16.45	0.791

[a] Mean ± standard deviation. [b] Comparisons were made with the statistical non-parametric. Mann-Whitney U test and significance (in bold) was considered with a *p*-value below 0.05. [c] Normal ranges in parenthesis

Abbreviations: HDL. Cholesterol HDL; LDL. Cholesterol LDL; GGT. Gamma-glutamyl transferase; ALT. Alanine aminotransferase; AST. Aspartate aminotransferase.

ANEXO 4

Fotografías SEM de cultivo de Ruminocoides biliarensis IPLA60002 a distintos tiempos

Figura 4. Imágenes obtenidas por SEM del crecimiento de *Ruminocoides biliarensis* IPLA60002 en medio GAM suplementado con 0.25% de cisteína, tras 12 horas. Se observan células individuales o en parejas, cubiertas por una capa de matriz extracelular de naturaleza por el momento desconocida.

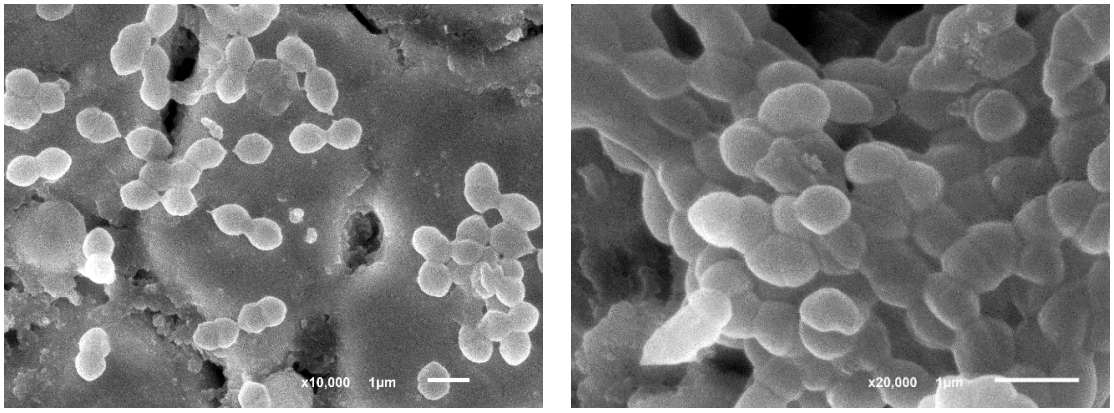


Figura 5. Imágenes obtenidas por SEM del crecimiento de *Ruminocoides biliarensis* IPLA60002 en medio GAM suplementado con 0.25% de cisteína, tras 16 horas. Comienzan a observarse algunas células deformadas.

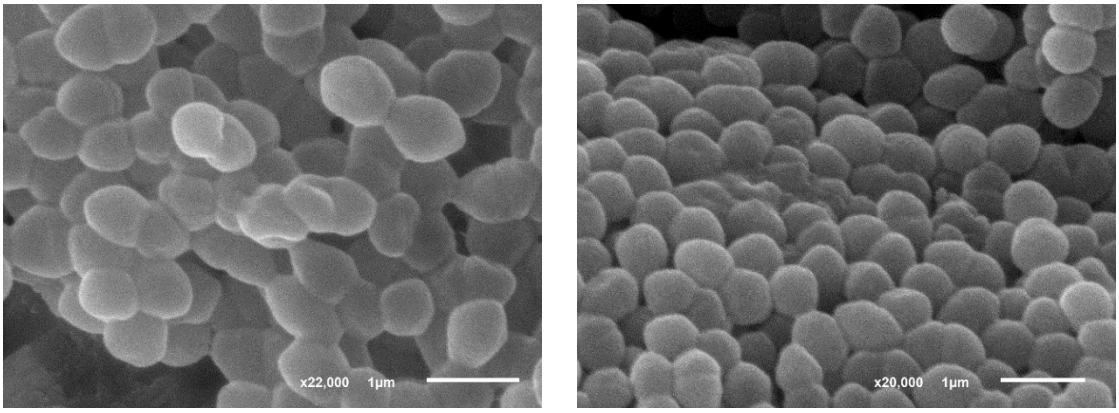


Figura 6. Imagen obtenida por SEM del crecimiento de *Ruminocoides biliarensis* IPLA60002 en medio GAM suplementado con 0.25% de cisteína, tras 24 horas. Prácticamente la totalidad del cultivo se muestra deformado y lisado.