UNIVERSIDAD POLITÉCNICA DE VALENCIA

Instituto de Agroquímica y Tecnología de Alimentos

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

CARACTERIZACIÓN MOLECULAR DE LA MICROBIOTA DE INDIVIDUOS CELÍACOS

Memoria presentada para optar al grado de Doctor por:

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Valencia, Junio de 2013

La Dra. Yolanda Sanz Herranz, Investigador científico del Instituto de Agroquímica y Tecnología de Alimentos (IATA) del Consejo Superior de Investigaciones Científicas (CSIC),

Certifica: Que Ester Sánchez Sánchez, licenciada en Biología por la Universidad de Valencia, ha realizado bajo su dirección el trabajo titulado: "Caracterización molecular de la microbiota de individuos celíacos", que presenta para optar al grado de Doctor por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos firma el presente certificado en Valencia, Junio de 2013.

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Resumen

La enfermedad celíaca (EC) es una enteropatía de carácter autoinmune en la que intervienen factores genéticos y ambientales. Es la enfermedad crónica intestinal más frecuente, y aunque puede presentarse a cualquier edad y con una gran variedad de síntomas, los casos típicos suelen manifestarse durante la infancia y cursar con síntomas gastrointestinales. En individuos genéticamente susceptibles a desarrollar la enfermedad la ingesta del gluten de la dieta causa una inflamación crónica en el intestino delgado que conduce a pérdidas en la integridad y la función de la mucosa intestinal. Los cambios histológicos que se generan en la mucosa de los enfermos celíacos pueden provocar la atrofia de las microvellosidades intestinales lo que conlleva malabsorción de nutrientes y manifestaciones intestinales y/o extraintestinales. El único tratamiento que existe para los celíacos es una dieta estricta exenta de gluten durante toda la vida. El mantenimiento de esta recomendación dietética es complejo debido a la presencia de gluten en la mayoría de los alimentos elaborados, de modo que los pacientes siguen expuestos al gluten y algunos continúan teniendo sintomatología, principalmente gastrointestinal, y mayores riesgos de salud. Como consecuencia se han abierto diversas líneas de investigación con el fin de desarrollar terapias complementarias y/o alternativas a la dieta sin gluten.

Además del gluten y las características genéticas de los individuos, en el desarrollo de la EC intervienen otros factores como el tipo de lactancia o la incidencia de infecciones durante la infancia. Estos factores que pueden influir en la microbiota intestinal y en el desarrollo del sistema inmunitario. En los últimos años se ha demostrado que los pacientes celíacos presentan alteraciones en su microbiota intestinal en comparación con sujetos sanos. El **objetivo** de esta tesis ha sido avanzar en la caracterización de las alteraciones de la composición de la microbiota intestinal asociadas de forma primaria o secundaria a la EC, y establecer la posible función de los grupos microbianos relevantes en la patogénesis de la enfermedad. El estudio de las funciones de componentes específicos de la microbiota en la patogénesis y el riesgo de sufrir la enfermedad podrían permitir el posterior desarrollo de estrategias de intervención nutricional basadas en probióticos y/o prebióticos para mejorar el estado de salud de estos pacientes o reducir el riesgo de padecer la enfermedad.

En el primer capítulo (**estudios 1 y 2**) se analizaron las poblaciones bacterianas fecales y duodenales de niños con EC y se compararon con las de controles mediante la utilización de la técnica de electroforesis en gel con gradiente desnaturalizante (DGGE). Mediante el uso de esta técnica se detectaron cambios en la diversidad y composición de las poblaciones bacterianas entre los grupos de individuos en estudiados.

El siguiente capítulo se basó en la caracterización de algunos de los grupos bacterianos relevantes en la EC, bien por las diferencias detectadas a nivel cuantitativo respecto a controles o bien por su posible acción como patógenos oportunistas, mediante el uso de técnicas dependientes de cultivo. Se aislaron representantes de las poblaciones fecales de enterobacterias, estafilococos y bacteroides de individuos celíacos y controles y se identificaron a nivel de especie. Las especies dominantes se caracterizaron con el fin de determinar la presencia/ausencia de factores de virulencia. En estos estudios (**estudios 3, 4 y 5**) se observó que los individuos celíacos mostraban una mayor abundancia de las especies *Escherichia coli, Staphylococcus epidermidis* y *Bacteroides fragilis* que los controles, y en general estas especies presentaban mayor cantidad de genes que codifican factores de virulencia que los clones aislados del grupo control. Además se aislaron e identificaron bacterias cultivables asociadas a la mucosa duodenal de pacientes celíacos y controles con el fin de establecer posibles diferencias. En este estudio (**estudios 6**) se observó que los pacientes con EC presentaban una menor abundancia de miembros de la familia *Streptococcaceae* y una mayor abundancia de miembros de las familias *Enterobacteriaceae* y *Staphylococcaceae*, y en particular de *Klebsiella oxytoca, S. epidermidis* y *Staphylococcus pasteuri*.

El último capítulo (**estudio 7**) se planteó con el fin de caracterizar el proceso de colonización intestinal de especies de *Bacteroides* en recién nacidos con riesgo de desarrollar la EC y determinar su relación con factores ambientales y genéticos. Para ello se monitorizó por DGGE las poblaciones de bacteroides de recién nacidos sanos con riesgo genético a desarrollar la EC. Los resultados determinaron que el genotipo de los recién nacidos sanos puede influir en los patrones de colonización de las poblaciones de *Bacteroides* y por lo tanto en el riesgo a desarrollar la enfermedad.

Resum

La malaltia celíaca (MC) és una enteropatia de caràcter autoimmune en la qual intervenen factors genètics i ambientals. És la malaltia crònica intestinal més freqüent, i encara que pot presentar-se a qualsevol edat i amb una gran varietat de símptomes, els casos típics solen manifestar-se durant la infància i cursar amb símptomes gastrointestinals. En individus genèticament susceptibles a desenvolupar la malaltia, la ingesta del gluten de la dieta causa una inflamació crònica a l'intestí prim que condueix a pèrdues en la integritat i la funció de la mucosa intestinal. Els canvis histològics que es generen en la mucosa dels malalts celíacs poden provocar l'atròfia de les microvellositats intestinals el que comporta mala absorció de nutrients i manifestacions intestinals i/o extraintestinals. L'únic tractament que existeix per als celíacs és una dieta estricta exempta de gluten durant tota la vida. El manteniment d'aquesta recomanació dietètica és complex a causa de la presència de gluten en la majoria dels aliments elaborats, de manera que els pacients segueixen exposats al gluten i alguns continuen tenint simptomatologia, principalment gastrointestinal, i majors riscos de salut. Com a conseqüència s'han obert diverses línies de recerca per tal de desenvolupar teràpies complementàries i/o alternatives a la dieta sense gluten.

A més del gluten i les característiques genètiques dels individus, en el desenvolupament de l'EC intervenen altres factors com el tipus de lactància o la incidència d'infeccions durant la infància, factors que poden influir en la microbiota intestinal i en el desenvolupament del sistema immunitari. En els últims anys s'ha demostrat que els pacients celíacs presenten alteracions en el seu microbiota intestinal en comparació amb subjectes sans. L'objectiu d'aquesta tesi ha estat avançar en la caracterització de les alteracions de la composició de la microbiota intestinal associades de forma primària o secundària a l'EC i establir la possible funció dels grups microbians rellevants en la patogènesi de la malaltia. L'estudi de les funcions de components específics de la microbiota en la patogènesi i el risc de patir la malaltia podrien permetre el posterior desenvolupament d'estratègies d'intervenció nutricional basades en probiòtics i/o prebiòtics per millorar l'estat de salut d'aquests pacients o reduir el risc de patir la malaltia.

En el primer capítol (estudis 1 i 2), es van analitzar les poblacions bacterianes fecals i duodenals de nens amb MC i es van comparar amb les de controls mitjançant la utilització de la tècnica d'electroforesi en gel amb gradient desnaturalitzant (DGGE). Mitjançant l'ús d'aquesta tècnica es van detectar canvis en la diversitat i composició de les poblacions bacterianes entre els grups d'individus en estudiats.

El següent capítol es va basar en la caracterització d'alguns dels grups bacterians rellevants en l'EC, bé per les diferències detectades a nivell quantitatiu respecte a controls o bé per la seva possible acció com patògens oportunistes, mitjançant l'ús de tècniques dependents de cultiu. Es van aïllar representants de les poblacions fecals d'enterobacteris, estafilococs i bacteroides d'individus celíacs i controls i es van identificar a nivell d'espècie. Les espècies dominants es van caracteritzar per tal de determinar la presència/absència de factors de virulència. En aquests estudis (estudis 3, 4 i 5) es va observar que els individus celíacs mostraven una major abundància de les espècies Escherichia coli, *Staphylococcus epidermidis* i *Bacteroides* fragilis que els controls, i en general, aquestes espècies presentaven major quantitat de gens que codifiquen factors de virulència que els clons aïllats del grup control. A més es van aïllar i identificar bacteris cultivables associades a la mucosa duodenal de pacients celíacs i controls per tal d'establir possibles diferències. En aquest estudi (estudis 6) es va observar que els pacients amb MC presentaven una menor abundància de membres de la família *Streptococcaceae* i una major abundància de membres de les famílies *Enterobacteriaceae* i *Staphylococcaceae*, i en particular de *Klebsiella oxytoca*, *S. epidermidis* i *Staphylococcus pasteuri*.

L'últim capítol (estudi juliol) es va plantejar per tal de caracteritzar el procés de colonització intestinal d'espècies de *Bacteroides* en nounats amb risc de desenvolupar l'EC i determinar la seva relació amb factors ambientals i genètics. Per a això es va monitoritzar per DGGE les poblacions de bacteroides de nadons sans amb risc genètic a desenvolupar l'EC. Els resultats van determinar que el genotip dels nadons sans pot influir en els patrons de colonització de les poblacions de *Bacteroides* i per tant en el risc a desenvolupar la malaltia.

Summary

Celiac disease (CD) is an autoimmune enteropathy that involves genetic and environmental factors. It is the most common chronic intestinal disorder and, although it can occur at any age with a variety of symptoms, typical cases with gastrointestinal symptoms usually occur during childhood. In CD patients, the ingestion of gluten from the diet cause chronic inflammation of the small intestine leading to loss the integrity and function of the intestinal mucosa. The intestinal mucosal lesion can cause total villus atrophy, leading to malabsorption syndrome and intestinal and/or extraintestinal manifestations. The only treatment for CD patients is the adherence to a strict lifelong gluten-free diet, but maintaining this dietary recommendation is complex due to the presence of gluten in most processed foods. Therefore, patients are often exposed to gluten and some continue having mainly gastrointestinal symptoms, and increased health risks. As a result, several research lines are opened to develop complementary and/or alternative therapies to the gluten-free diet.

In addition to gluten and the individual genetic background, other factors such as the type of feeding-practices or the incidence of infections during childhood which influence the microbiota and the development of the immune system, could also be implicated in the risk of developing CD. In recent years, it has been shown that CD patients have alterations in their intestinal microbiota in comparison with healthy controls. The **aim** of this thesis has been to progress in the characterization of alterations in the intestinal microbiota composition associated primary or secondary with CD, and to establish the possible role of relevant microbial groups in the pathogenesis of the disease. The study of the functions of specific components of the microbiota in the pathogenesis and risk of the disease may facilitate the further development of nutritional intervention strategies based on probiotics and/or prebiotics to improve the health status of these patients and reduce the risk of developing the disease.

In the first chapter (**studies 1 and 2**) fecal and duodenal bacterial populations of CD children were analyzed and compared with controls by using denaturing gradient gel electrophoresis (DGGE). Using this technique, the studied children´s groups showed differences in diversity and species composition.

Next chapter was focused on characterization of relevant bacterial groups for CD, either by the detection of quantitative differences regarding controls or because of their possible action as opportunistic pathogens, using cultured-dependent techniques. Clones of enterobacteria, staphylococci and bacteroides were isolated from stools of CD patients and control subjects and were identified at species level. Dominant species were characterized to determine the presence/absence of virulence factors. In these studies (**studies 3, 4 and 5**) it was observed that celiac subjects showed a greater abundance of *Escherichia coli*, *Staphylococcus epidermidis* and *Bacteroides fragilis*, and, generally, these isolates harbored a higher number of genes encoding virulence factors. Furthermore, cultivable-associated bacteria were isolated and identified from the duodenal CD mucosa and from control biopsy specimens in order to investigate differences. In this study (**study 6**) it was observed that patients with CD showed lower abundance of species of the family *Streptococcaceae* and a greater abundance of species of the families *Enterobacteriaceae* and *Staphylococcaceae*, in particular *Klebsiella oxytoca*, *S. epidermidis* and *Staphylococcus pasteuri*.

The last chapter (**study 7**) focused on the characterization of the intestinal colonization process of *Bacteroides* spp. in infants with risk of developing CD, and to determine its relation to environmental and genetic factors. For this purpose, *Bacteroides* spp. from healthy infants with high genetic risk of developing CD were monitored. The results indicated that the HLA-DQ genotype of healthy infants can influence in the colonization pattern of *Bacteorides* spp. and, probably, the risk of developing CD.

Agradecimientos

Es difícil entender la importancia de los agradecimientos de una tesis hasta que no se ha terminado. En ese momento te das cuenta de cuánto tienes que agradecer a la gente que ha estado presente durante esta etapa, haciendo posible deje de ser un sueño y pase a ser una realidad.

Este trabajo se ha realizado en el Grupo de Ecofisiología Microbiana y Nutrición del IATA (CSIC) gracias a un contrato asociado al proyecto PETRI (PTR95-0987.OP.02), a la beca concedida por el Instituto Danone, y a la financiación asociada a los proyectos AGL2005-05788-C02-01 (CICYT), 200570F0091 (CSIC), AGL2007-66126-C03-01-03/ALI (CICYT), Consolider Fun-C-Food CSD2007-00063 (MEC), AGL2008-01440/ALI (MICINN).

En primer lugar quiero dar las gracias a la Dra. Yolanda Sanz, directora de esta tesis, por toda su ayuda, confianza, apoyo, disponibilidad e infinita paciencia, fundamentalmente durante la recta final. Le agradezco que me abriera las puertas del grupo dándome la oportunidad de vivir desde dentro el apasionante mundo de la investigación.

Este trabajo tiene un poquito de toda la gente con la que me he ido cruzando por los pasillos, de todos mis compañeros y de aquellos que de forma más o menos temporal pasaron por el grupo. Tengo mucho que agradecer a la gente del laboratorio 103 en especial a Inma por sus consejos, confidencias, risas y lágrimas, y en definitiva por hacerme ver el mundo de otra manera. A Merche que con su alegría completó el trío; al equilibrio formado por el dúo Marcela-Giada (la calma y el terremoto); a Raquel por acompañarme en mis primeros pasos con la DGGE. Gracias a Mª Carmen por su ayuda; a Arlette por sus aclaraciones de última hora. A Moisés por su experiencia, ideas y consejos. Gracias a Marta por su inocencia y dulzura; a Onofrio por ponerle música a la vida; a Paola por su tranquilidad y a Fernando por enseñarme a “parar, relajarme y pensar” (aunque dicho por él suena más bonito). Gracias a Mª Jesús, Ángela, Eva e Iris por renovar el aire del nuevo laboratorio 114. Gracias a Sabina, mi eficaz sustituta, por romper con la rutina del laboratorio. Gracias a Amparo Feria por su experiencia, sus buenas palabras y su sonrisa y a Carmen Laosa por hacer

de cada día una nueva aventura. Y a Jana, Bea y Tamara por sus visitas ¡Gracias a todos por cada uno de los momentos compartidos!

No quiero olvidarme de Juanan, Juan Mario y Óscar (los chicos del fondo del pasillo) ni de Amparo, Rossana y Aleida (las chicas de mitad del pasillo) por todas sus palabras y sonrisas entre paseo y paseo. Ni de toda la gente con la que compartí mesa durante las comidas, en especial a Arancha.

Gracias a la Unidad de Gastroenterología Pediátrica del Hospital Universitario La Fe y al Servicio de Pediatría del Hospital General Universitario por la recogida de muestras y al resto de "voluntarios". Así como a los grupos de Cereales, Ciencia de la carne, Bacterias lácticas y probióticos, Actividad biológica de compuestos alimentarios y Nuevos materiales y nanotecnología por los materiales y equipos prestados.

Además, quiero dar las gracias Al Dr. David R. Arahal y la CECT por ayudarme a dar mis primeros pasos; al Dr. Chema Lagarón y su grupo por darme esa genial oportunidad; y a la Dra. Erika Fernandez-Vizarra y sus mitocondrias por permitirme seguir pensando que vale la pena.

A mis amigos, por haber estado ahí siempre que lo he necesitado.

Y especialmente a mis padres por no haber dejado de creer nunca en mí, por apoyarme, acompañarme y confiar siempre en mí, y por ser el mejor modelo a seguir. A mi hermano por formar parte de mi infancia y por seguir ahí y saber que siempre seguirá haciéndolo. Al resto de familia, la de siempre y la nueva, por vuestros ánimos y palabras de apoyo. A Jony, mi chico, por esperarme estos "tres meses", por preguntar y/o callar, por escucharme, animarme, cuidarme y quererme tanto. Y sobretodo a Diego, mi chiquitín, por acompañarme en todo momento, por darme fuerza y confianza con cada patadita, mirada y sonrisa, por ayudarme a acabar esta etapa para dar lugar a otra mucho mejor y dar un nuevo sentido a mi vida.

ÍNDICE

I. INTRODUCCIÓN

1. ENFERMEDAD CELÍACA

1.1. Epidemiología de la EC

La enfermedad celíaca (EC) se describió por primera vez en la segunda mitad del siglo II después de Cristo por Areteo de Capadocia y se empleaba la palabra griega "koliakos" (de la cual deriva celíaco) para identificar a los pacientes que sufrían del intestino. La presentación clásica de la EC, con malabsorción, fue descrita en 1888 por Samuel Gee (Gee, 1888) y era considerada una especie de indigestión crónica típica de la edad infantil que cursaba con diarrea, cansancio, falta de crecimiento y afectaba sobre todo a la población europea. La etiología de la EC fue desconocida hasta 1940, cuando Willem Karel Dicke (Dicke, 1950) postuló que los cereales (trigo, cebada y centeno) eran agentes etiológicos de la EC y propuso a los enfermos celíacos seguir una dieta estricta sin gluten tras la cual los pacientes mostraban una clara mejoría. Dicke llegó a esta conclusión tras observar que durante la Segunda Guerra Mundial, al escasear los alimentos básicos como el trigo, los pacientes celíacos mejoraban considerablemente y que luego volvían a recaer tras el final de la guerra con la reintroducción en la dieta de dichos alimentos. Hasta 1970 el diagnóstico de la EC se basaba únicamente en la manifestación clínica de la sintomatología típica de la patología (cuadro gastrointestinal típico) y la prevalencia global en la población general era de un 0.03% (Lohi et al., 2007). Debido a la revisión de los criterios necesarios para el diagnóstico de la EC por la Sociedad Europea de Gastroenterología, Hepatología y Nutrición Pediátrica (ESPAGHAN) (Walker-Smith, 1990) y la utilización de marcadores serológicos como método de rastreo, el panorama epidemiológico de la patología varió considerablemente. A partir de estudios de población general realizados en Europa y en Estados Unidos se ha establecido que la prevalencia de la EC en la población general está próxima al 1/100 o es incluso superior (Tommasini et al., 2011; Dube et al., 2005). Además, la enfermedad presenta una prevalencia más alta en los denominados grupos de riesgo. Entre éstos se incluyen los familiares en primer y en segundo grado, y en aquellas personas con otras enfermedades asociadas como diabetes Melitus tipo 1, tiroiditis, artritis, enfermedad hepática autoinmune, síndrome de Sjögren,

miocardiopatía dilatada, nefropatía IgA, síndrome de Down, síndrome de Turner, síndrome de Williams, y déficit IgA (**Tabla 1**) (Green y Jabri, 2006).

Tabla 1. Desordenes asociados con la EC

Enfermedades Autoinmunes
Diabetes Mellitus tipo 1 (3,5 -10%)
Tiroiditis (4 -8%)
Artritis (1,5 -7,5%)
Enfermedad hepática autoinmune (6 -8%)
Síndrome de Sjögren (2- 15%)
Miocardiopatía dilatada (5,7%)
Nefropatía IgA (3,6%)
Enfermedades Genéticas
Síndrome de Down (4- 19%)
Síndrome de Turner (4- 8%)
Síndrome de Williams (4- 8%)
Déficit IgA (7%)

Entre paréntesis, porcentaje de padecer la EC entre esos grupos de población

1.2. Manifestaciones clínicas y sintomatología

Se reconocen tres posibles formas clínicas en las que la EC puede manifestarse (Setty et al., 2008): (i) *clásica*, se caracteriza por una enteropatía grave con atrofia vellositaria inducida por gluten en sujetos con anticuerpos séricos positivos y que pueden presentar síntomas digestivos o extradigestivos; (ii) *silente*, se caracteriza por la presencia de anticuerpos séricos positivos y atrofia vellositaria inducida por gluten que se recupera con una dieta libre, y por la ausencia de síntomas; y (iii) *latente o potencial*, se caracteriza porque los individuos tienen características genéticas e inmunológicas compatibles con la EC, pero presentan una morfología de la mucosa normal y son asintomáticos (**Figura 1**).

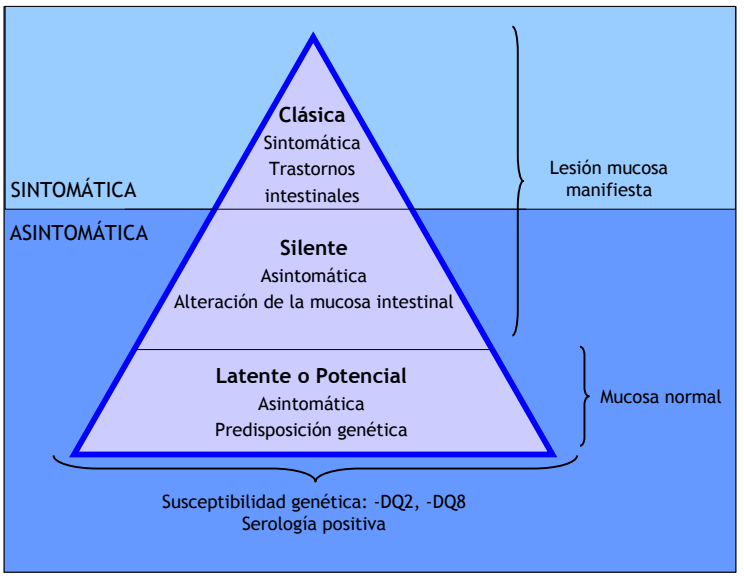

Figura 1. El modelo de iceberg en la EC

Las manifestaciones clínicas de la enfermedad varían considerablemente en función de la edad del paciente (**Tabla 2**) (Rampertab et al., 2006). Los niños generalmente presentan diarrea crónica, distensión abdominal y retraso del crecimiento aunque otros síntomas como vómitos, irritabilidad, falta de apetito y estreñimiento también pueden ser comunes. Los niños mayores y adolescentes a menudo presentan manifestaciones extraintestinales como una baja estatura, síntomas neurológicos o anemia. En la edad adulta la enfermedad suele cursar con manifestaciones digestivas clásicas, y con otros síntomas como deficiencia en hierro, dolores óseos y articulares, diarrea, estreñimiento, intestino irritable, infertilidad, abortos recurrentes, malnutrición, etc. (Fernandez et al., 2010) y suele ser más común en mujeres que en hombres, con un proporción de 3:1 (Fernandez et al., 2010; Lohi et al., 2007).

Tabla 2. Manifestaciones clínicas de la EC según la edad de presentación. (Adaptado de Polanco et al, 2009)

NIÑO	ADOLESCENTE	ADULTO
Síntomas		
Diarrea	Asintomáticos	Dispepsia
Anorexia	Dolor abdominal	Diarrea crónica
Vómitos	Cefalea	Dolor abdominal
Dolor abdominal	Artralgias	Síndrome de intestino irritable
Apatía	Menarquia retrasada	Dolores óseos y articulares
Introversión	Irregularidad menstrual	Infertilidad, abortos recurrentes
Tristeza	Estreñimiento	Parestesias, tetania
	Hábito intestinal irregular	Ansiedad, depresión, ataxia
Signos		
Malnutrición	Aftas orales	Malnutrición
Distensión abdominal	Hipoplasia del esmalte	Edemas periféricos
Hipotrofia muscular	Distensión abdominal	Talla baja
Retraso del crecimiento	Debilidad muscular	Neuropatía periférica
Anemia ferropénica	Talla baja	Miopatía proximal
	Artritis, osteopenia	Anemia ferropénica
	Queratosis folicular	Hipertransaminemia

1.3. Patogénesis de la enfermedad

Se considera que la EC tiene una etiología multifactorial e intervienen factores genéticos y ambientales y un factor desencadenante que es el gluten. La patología está altamente asociada a los genes del sistema de antígenos leucocitarios humanos (HLA, del inglés human leukocyte antigen) que codifican las moléculas DQ2 y DQ8, presentes en la mayoría de pacientes celíacos. No obstante, estos genes también se encuentran presentes en un 30% de la población general sana y, por tanto, su presencia es necesaria pero no suficiente para que se desarrolle la enfermedad (Trynka et al., 2010). El gluten de la dieta es el factor ambiental responsable de la manifestación de la enfermedad; no obstante, cada vez se diagnostican más casos en la edad adulta indicando que la exposición

al gluten no es el único factor que actúa como desencadenante de la enfermedad (Catassi et al., 2010). Estudios epidemiológicos indican que en la EC intervienen otros factores que pueden aumentar o reducir el riesgo de padecer la enfermedad, como por ejemplo el momento y la cantidad de gluten introducido inicialmente en la dieta, el tipo de lactancia y su duración, o la incidencia de infecciones durante la infancia. Esto implica que el desarrollo de la EC en sujetos genéticamente predispuestos podría evitarse mediante la modificación de los factores ambientales que contribuyen al riesgo de padecerla.

1.3.1. Factores genéticos

La contribución de los factores genéticos a sufrir la EC se refleja en la mayor incidencia de la enfermedad en familiares, en los que el riesgo aumenta entre 20 y 30 veces en comparación con la población general (Tosco et al., 2010), y una concordancia del 75% entre gemelos homocigóticos (Nistico et al., 2006; Greco et al., 2002; Bardella et al., 2000).

La predisposición genética puede ser resultado de un efecto colectivo de varios genes, en la región HLA y fuera de ella, siendo los genes de la región HLA de clase II (CELIAC1) los que confieren hasta un 40% del riesgo total. Los genes HLA (DP, DQ y DR) codifican moléculas que se expresan en las células presentadoras de antígeno y están implicadas en el reconocimiento de los péptidos del gluten. Aproximadamente, el 90% de los pacientes celíacos expresan el heterodímero HLA-DQ2 (codificado por los alelos HLA-DQA1*0501/DQB1*0201), que puede ser codificado en *cis* (los dos aleleos en el mismo cromosoma) o en *trans* (cada alelo procede de un cromosoma de los progenitores). El 10% restante, expresan moléculas del tipo HLA-DQ8 (DQA1*0301/DQB1*0201) (**Figura 2**). No obstante, solamente un pequeño porcentaje (2%) de los portadores DQ2 ó DQ8 desarrolla la EC lo que indica que es probable que haya una acumulación de riesgos debido a otros muchos genes.

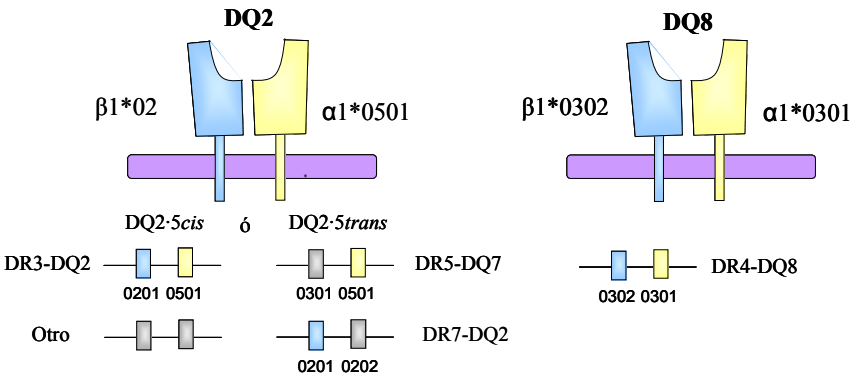

Figura 2. Combinación clásica de los haplotipos que codifican los heterodímeros HLA-DQ2 o HLA-DQ8. Los pacientes celíacos que son DR3 o DR5/DR7 expresan la misma molécula, HLA-DQ2 (α1*0501, ß*02). Los genes DQA1*0501 y DQB1*02 se pueden localizar en *cis* (en el mismo cromosoma) en individuos DR3 o en *trans* (en diferentes cromosomas) en individuos heterozigóticos DR5/DR7. Los pacientes que son DR4 expresan la molécula HLA DQ8 (ß*0302 α1*0301), codificada por los genes DQA1*03 y DQB1*0302.

Los estudios de asociación del genoma completo (GWAS, del inglés Genome-wide association studies) realizados hasta el momento han permitido establecer 26 asociaciones definitivas y otras 13 posibles asociaciones entre loci independientes a la región HLA y la predisposición a desarrollar la EC, y al analizarlos en profundidad se ha visto que muchos de ellos contienen genes implicados en mecanismos de regulación del sistema inmune (Gutierrez-Achury et al., 2011; Trynka et al., 2010). Así por ejemplo, se ha asociado un mayor riesgo a desarrollar la EC con variantes raras del la región cromosómica 4q27 que contiene los genes de la IL-2 y la IL-21, y de hecho pacientes celíacos activos y no activos presentan un aumento en los niveles séricos de IL-21 (Einarsdottir et al., 2011; van Heel et al., 2007).

1.3.2. El gluten como agente desencadenante

En 1950 se describió el trigo como agente causal (antígeno) de la EC (Dicke, 1950) y posteriormente se añadieron la cebada y el centeno como cereales tóxicos para los celíacos. Existen controversias sobre la posible acción tóxica de la avena para los individuos celíacos (Zannini et al., 2012). Debido a la

ubicuidad del trigo en la dieta y a la controversia existente con la avena, estos dos cereales han sido los más estudiados. En la **tabla 3** se muestra diferentes cereales clasificados en función de si contienen o no gluten.

Tabla 3. Granos y semillas que pueden o no contener gluten. (Adaptado de Zanini et al, 2012).

Granos que contienen gluten	**Granos libres de gluten**
El trigo (*Triticum* spp, espelta, farro, kamut, trigo duro)	Amaranto
Centeno (*Secale*)	Trigo sarraceno o alforfón
Triticale (Cruce entre *Triticum* y *Secale*)	Maíz
Cebada (*Hordeum*)	Mijo
Avena	Quinoa
	Arroz
	Sorgo
	Soja
	Legumbres

Actualmente se sabe que los síntomas de la EC se desencadenan por la ingesta de gluten en la dieta. Estrictamente hablando el gluten hace referencia al conjunto de proteínas de almacenamiento presentes en las semillas de ciertos cereales como el trigo, la cebada o el centeno que se obtienen tras eliminar los compuestos solubles en agua (Wieser, 2007).

Las proteínas del gluten pueden separarse, según su solubilidad en alcohol en prolaminas (solubles en etanol al 60%) y gluteninas (insolubles en etanol al 60%). Las prolaminas presenten en el gluten del trigo se denominan gliadinas y en base a su secuencia N-terminal, tamaño y movilidad electroforética se subdividen en α-, ß-, γ- y ω-gliadinas. Las prolaminas análogas de la cebada se denominan hordeínas y las del centeno secalinas. Las gluteninas se encuentran formando agregados de alto peso molecular (HMW-GS, del inglés high molecular weight glutenin subunits) o de bajo peso molecular (LMW-GS, del inglés low molecular weight glutenin subunits) (Silano et al., 2009; Wieser y Koehler, 2008).

Las gliadinas, hordeínas, secalinas y gluteninas se caracterizan por presentar un elevado contenido en los aminoácidos glutamina (Gln, Q) y prolina (Pro, P), que les hace más resistentes a la digestión proteolítica en el tracto gastrointestinal (TGI). Además las enzimas gástricas y pancreáticas carecen de actividad prolil-endopeptidasa, por lo que se acumulan fragmentos peptídicos ricos en prolina y glutamina en la luz intestinal. Numerosos estudios realizados *in vivo* e *in vitro* indican que ciertos epítopos de las gliadinas (Wieser y Koehler, 2008; Londei et al., 2005) y de las gluteninas, fundamentalmente de las HMW-GS (Ellis et al., 2012; Dewar et al., 2006), son tóxicos e inmunógenos para los individuos celíacos.

1.3.3. Otros factores ambientales posiblemente implicados

Se ha descrito que la lactancia materna podría retrasar el momento de aparición de la EC e incluso reducir el riesgo de desarrollarla sobre todo si se mantiene en el momento de la introducción del gluten en la dieta, pues parece contribuir al desarrollo de tolerancia oral a este antígeno (Pinier et al., 2010; Akobeng et al., 2006; Ivarsson et al., 2002; Hernell et al., 2001). Asimismo, otros factores como la dosis y el momento de introducción del gluten en la dieta también pueden influir en el posterior desarrollo de la enfermedad. A partir de estudios epidemiológicos se ha estimado que la introducción de elevadas cantidades de gluten eleva el riesgo de desarrollar la enfermedad en comparación con la introducción del gluten en la dieta en menores cantidades (Silano et al., 2010; Ivarsson et al., 2000); además, se ha establecido una edad de entre 4 y 6 meses como la óptima para comenzar a introducir alimentos complementarios que contienen gluten en la dieta y se considera que la introducción del mismo antes de los 4 meses aumenta el riesgo de padecerla (Pinier et al., 2010; Guandalini, 2007).

También se ha propuesto que otros factores relacionados con el nacimiento pueden influir en el riesgo de padecer la EC ya que los niños nacidos por cesárea o con bajo peso presentan un mayor riesgo (Pinier et al., 2010; Decker et al., 2010). Además, los niños nacidos en verano presentan un riesgo mayor a desarrollar la enfermedad debido, posiblemente, a que en el momento

de la introducción del gluten las infecciones son más comunes (Ivarsson et al., 2005; Ivarsson et al., 2003). En este sentido, la enfermedad se ha vinculado con una mayor incidencia de infecciones de origen bacteriano (*Pneumococcus, Staphylococcus* o *Mycobacterium tuberculosis*) (Walters et al., 2008) y víricas (adenorvirus, rotavirus) (Plot y Amital, 2009; Stene et al., 2006). Sin embargo, otros estudios que proponen una hipótesis opuesta y consideran que las infecciones en edades tempranas tienen un papel protector (Plot et al., 2009). Además, la EC se ha asociado a un desequilibrio en la composición de la microbiota intestinal tanto en fase activa como no activa de la enfermedad (Collado et al., 2009; Nadal et al., 2007; Collado et al., 2007), lo que se describirá con mayor profundidad en apartados posteriores.

Finalmente, otros factores como las recomendaciones nutricionales o el contenido de gluten u otros alérgenos de los alimentos infantiles pueden influir en el riesgo de padecer la enfermedad que se explica por los datos epidemiológicos descritos anteriormente. Entre 1985 y 1995 la incidencia de la EC en la población sueca aumentó considerablemente (del 1% al 3%) debido a un cambio en las recomendaciones sobre el tipo de lactancia y al aumento de las cantidades de gluten incorporado a los productos infantiles utilizados en el momento de introducirlo en la dieta. En los años posteriores se modificaron las recomendaciones nutricionales para los recién nacidos y lactantes y se observó que la incidencia de la EC en Suecia volvió a estabilizarse en un 1%, similar a la encontrada en el resto de la población europea (Ivarsson et al., 2005; Ivarsson, 2000). La gran variación de la incidencia de la enfermedad y el escaso espacio temporal en el que se produjo no pueden explicarse por cambios del componente genético de la población, sino por cambios a nivel socio-cultural. Un resumen de los posibles factores que afectan a la prevención de la EC se recoge en la tabla 4.

Tabla 4. Factores asociados a la reducción de riesgo de desarrollar la EC.

Factores asociados	Referencias
Lactancia materna	
La incidencia de la EC se ve significativamente reducida (~ 50 %) si la lactancia materna se mantiene en el momento de introducción del gluten	(1)
El riesgo de desarrollar la EC disminuye (~ 63 %) en niños amamantados durante más de 2 meses	(2)
El mecanismo de protección de la leche materna no se ha dilucidado, y no se conoce si previene permanentemente o solo retrasa la presentación de la EC	
El momento de la introducción del gluten	
La edad de introducción del gluten parece afectar al desarrollo de la EC	(3)(4)
Combinar la lactancia materna con una lenta introducción del gluten parece ser beneficioso	(1)
Se recomienda evitar la introducción temprana (<4meses) del gluten en la dieta; también se ha sugerido que la introducción tardía (>7meses) puede ser perjudicial pero es una teoría todavía controvertida	(5)
Las infecciones virales	
En individuos predispuestos, una alta frecuencia de infección por rotavirus parece estar correlacionada con un mayor riesgo de desarrollar la EC	(6)
Un péptido reconocido por las IgGs de pacientes celiacos muestra homología con una proteína de rotavirus (VP-7)	(7)
Se ha observado un patrón estacional de presentación de la EC, aumentando el riesgo en niños nacidos en verano, a los que se introduce el gluten en la época de invierno con mayor incidencia de infecciones	(8)
Otros estudios han sugerido la hipótesis contraria que las infecciones pueden proteger frente al desarrollo de la EC	(9)

Referencias: (1) Akobeng et al., 2006; (2) Peters et al. 2001; (3) Norris et al., 2005; (4) Carlsson et al., 2006; (5) Agostoni et al., 2008; (6) Stene et al., 2006; (7) Zanoni et al., 2006; (8) Ivarsson et al., 2003; (9) Pozo-Rubio et al. 2012.

1.3.4. Inmunopatogénesis de la EC

Las proteínas del gluten son parcialmente resistentes a la digestión completa por las enzimas gástricas y pancreáticas debido a su alto contenido en glutamina y prolamina. Esto permite que péptidos derivados de estas proteínas se acumulen en el intestino delgado sin ser hidrolizados, y posteriormente alcancen

la lámina propia. En los celíacos se produce un aumento de la permeabilidad intestinal, relacionado con una mayor expresión de zonulina que es una proteína reguladora de las uniones intercelulares entre los enterocitos, lo que podría permitir el paso de los péptidos del gluten a la lámina propia por vía paracelular (Lammers et al., 2008; Di Sabatino y Corazza, 2009). Otros autores han demostrado que la translocación de péptidos, como el de gliadina 33mer, se puede producir por transcitosis regulada por interferón-γ (IFN-γ) tras su degradación parcial por endocitosis (Schumann et al., 2008). Además, otros péptidos, como el p31-49, pueden transportarse sin degradar a través del receptor CD71 mediante retro-transcitosis con la IgA secretora, lo que promueve el flujo de péptidos intactos y por lo tanto más inmunógenos (Matysiak-Budnik et al., 2008).

Una vez que los péptidos del gluten alcanzan la lámina propria, la transglutaminasa tisular (TGt) aumenta el efecto inmunoestimulatorio de los péptidos tras desaminar específicamente los residuos de glutamina (Gln, Q) a glutamato (Glu, E) de modo que quedan cargados negativamente y aumentan así su afinidad por las moléculas HLA-DQ2/DQ8 de las células presentadoras de antígeno (Qiao et al., 2009). El reconocimiento de los péptidos del gluten presentados en por las moléculas HLA-DQ2/DQ8 en la superficie de las células presentadoras de antígeno activa a los linfocitos T CD4+ que sobre-expresan la producción de citoquinas pro-inflamatorias de tipo Th1, principalmente IFN-γ, (Di Sabatino y Corazza, 2009; Kagnoff, 2007). EL incremento de citoquinas pro-inflamatorias en la mucosa de los celíacos provoca la infiltración de linfocitos intraepiteliales y células Th17 en el epitelio y en la lámina propria (Troncone et al., 1998; Nilsen et al., 1995). Además, los linfocitos T $CD4^+$ activados producen citoquinas anti-inflamatorias que conducen a la expresión clonal de linfocitos B que se diferencian a células plasmáticas. Las células plasmáticas producen anticuerpos anti-gliadina y anti-TGt que inducen cambios en el citoesqueleto de los entericitos. Esto conlleva el desarrollo de las lesiones típicas de la EC, hiperplasia de las criptas y atrofia de las vellosidades (Sollid, 2002).

Recientemente se ha observado que ciertos péptidos como el p31-43/49 tienen una acción tóxica, directa o innata, no relacionada con los linfocitos e

inducen una respuesta inmediata sobre el epitelio. Estos péptidos inducen a los enterocitos, macrófagos y células dendríticas a segregar IL-15. La IL-15 induce la expansión de los linfocitos intraepiteliales y la expresión de la molécula de superficie MICA (del inglés, MHC class I chain-related molecule A) de las células epiteliales. La unión de la molécula MICA de las células epiteliales a los receptores NKG2D de los linfocitos natural-killer promueve la destrucción de los enterocitos (Wieser et al., 2008; Benahmed et al., 2007) (**Figura 3**).

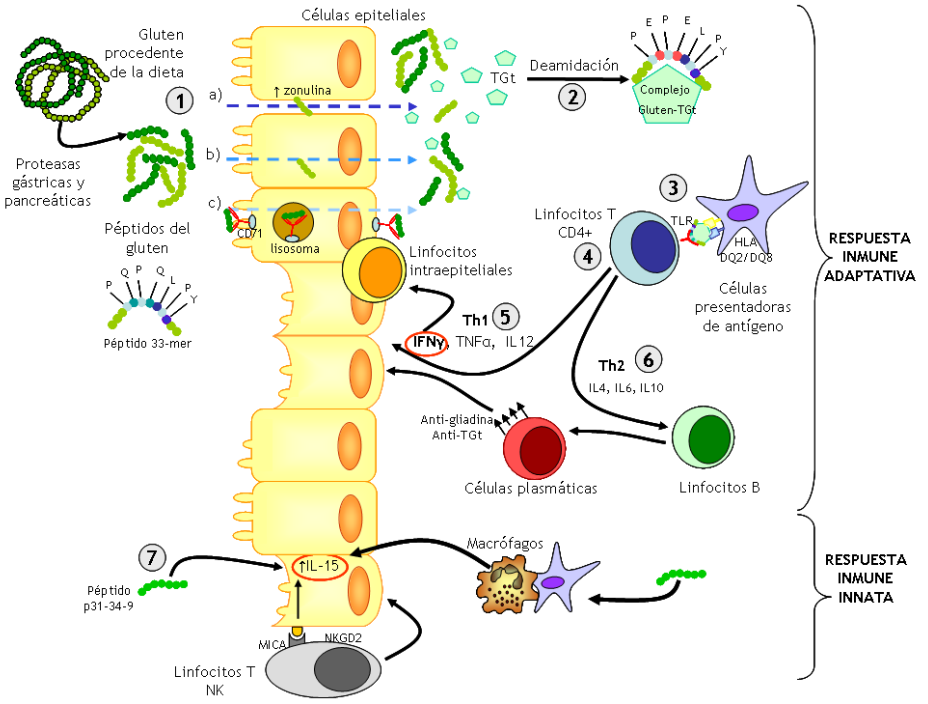

Figura 3. Inmunopatogénesis de la EC. (1) Transporte de los péptidos del gluten a través del epitelio intestinal por vía (a) paracelular, (b) transcelular o (c) retrotranscitosis. (2) Desamidación de los péptidos del gluten por la transglutaminasa tisular (TGt). (3) Aumento de la afinidad por las moléculas HLA-DQ2/DQ8 de las células presentadoras de antígeno. (4) Activación de una respuesta inmune adaptativa mediante la activación de linfocitos T $CD4^+$ que producen (5) citoquinas tipo Th1 y la infiltración de linfocitos intraepiteliales, y (6) citoquinas tipo Th2 que inducen la expansión clonal de linfocitos B que, tras diferenciarse a células plasmáticas, producen anticuerpos anti-gliadina y anti-TGt e inducen cambios en el citoesqueleto de los entericitos. (7) Activación de una respuesta inmune innata mediante la expresión de IL-15 que estimula la interacción entre linfocitos "natural-killer" (NK) y células epiteliales y conduce a la muerte celular.

1.4. Diagnóstico y tratamiento

La variedad tanto de formas (clásica, atípica, silente, latente) como de síntomas clínicos (digestivos, extradigestivas o asintomáticos) que pueden presentar los individuos celíacos dificulta el diagnóstico mediante una valoración exclusiva de los datos clínicos o analíticos. La ESPAGHAN ha desarrollado una serie de criterios necesarios para el diagnóstico de la EC (Husby et al., 2012): detección de marcadores serológicos positivos, identificación de cambios estructurales y/o alteraciones citológicas de la mucosa intestinal y respuesta positiva tras la dieta sin gluten. Los marcadores serológicos utilizados se basan en la detección de anticuerpos anti-endomisio (IgA-EMA) y anti-transglutaminasa tisular (IgA-TGt) (Green y Jabri, 2006), y además son de gran utilidad para la monitorización de los pacientes sometidos a la dieta sin gluten, ya que las transgresiones pueden ser detectadas por una elevación de los marcadores, o para el sondeo en las poblaciones de riesgo. Para el diagnóstico definitivo de la EC es necesario realizar al menos una biopsia intestinal obtenida a nivel duodeno yeyunal, en la que se observe una lesión infiltrativa con aumento de linfocitos intraepiteliales, una elongación de las criptas y una atrofia de las microvellosidades (Husby et al., 2012).

Actualmente, el único tratamiento eficaz para la EC es una dieta estricta exenta de gluten durante toda la vida. Una vez iniciado el tratamiento, se puede observar una mejora de los síntomas a partir de las dos semanas, la normalización serológica entre los 6 y 12 meses y la recuperación de las vellosidades intestinales en torno a los 2 años después de haber iniciado del tratamiento, aunque el patrón de recuperación varía dependiendo de si se trata de niños o adultos. Los pacientes celíacos deben excluir de la dieta el trigo, la cebada, el centeno y la avena y todos sus derivados (**tabla 3**).

1.4.1. Nuevas estrategias terapéuticas

El único tratamiento efectivo para la EC es mantener una dieta estricta exenta de gluten. Sin embargo esto a menudo restringe la actividad social de los pacientes, limita su variedad nutricional, y suele ser costoso y difícil de mantener debido a la presencia de gluten en la mayoría de alimentos elaborados. Por otra

parte, una proporción considerable de pacientes tienen una elevada sensibilidad al gluten y se ven afectados por la presencia de trazas de gluten en algunos alimentos declarados como "libres de gluten" o "con trazas de gluten". El avance del conocimiento sobre la patogénesis de la EC, sobretodo de las bases moleculares y celulares de la respuesta inmune frente al gluten, ha permitido avanzar en la identificación y desarrollo de estrategias terapéuticas alternativas y/o complementarias a la dieta sin gluten, que están en fase de investigación.

Estrategias dirigidas a reducir la toxicidad del gluten en los alimentos

1) Variantes de trigo y modificación genética; se pueden seleccionar cepas de trigo con menor inmunogenicidad y toxicidad como la variedad hexaploide *Triticum aestivum*, que es la más utilizada en la industria alimentaria; o bien por modificación genética de variedades ya existentes, como por ejemplo, la deleción genética de genes gliadina en *T. aestivum* (Schuppan et al., 2009).

2) Estrategias de fermentación de masas madre; se han utilizado ciertas cepas de lactobacilos con actividad prolil-hidrolasa que les permite degradar parte de los péptidos del gluten durante el proceso de fermentación, disminuyendo así su inmunotoxicidad (Rizzello et al., 2007; De Angelis et al., 2006).

Estrategias destinadas a reducir la reacción inmuno-tóxica frente al gluten de los sujetos con EC

1) Prolil-endopeptidasas y glutamin-endopeptidasas. Se ha propuesto su uso por vía oral como estrategia complementaria a la dieta exenta de gluten por su capacidad para romper proteínas que contienen residuos internos de prolina y glutamina, como el gluten. Así se generan péptidos más pequeños que sí pueden servir como sustrato para aminopeptidasas y carboxipeptidasas intestinales (Wieser y Koehler, 2012; Ehren et al., 2008; Gass y Khosla, 2007; Shan et al., 2004). Al hidrolizarse los polipéptidos del gluten hasta los aminoácidos constituyentes se pierde la capacidad lesiva para el intestino consiguiéndose así la detoxificación proteolítica del gluten. No obstante, esta estrategia solo permite reducir el umbral de toxicidad al gluten.

2) Inhibición de la TGt; se pretende evitar el aumento de la afinidad de los péptidos inmunogénos del gluten por las moléculas HLA-DQ2/DQ8, con lo que se impide que se desencadene la respuesta inflamatoria (Siegel y Khosla, 2007). Sin embargo, esta estrategia no impide que otros péptidos del gluten, que no requieren desamidación, puedan mantener o iniciar la respuesta inflamatoria. Además, debido a la ubicuidad y múltiples funciones de la TGt, su inhibición podría tener efectos no deseados, al alterar otras funciones biológicas (reparación tisular) fuera del intestino (Telci y Griffin, 2006).

3) Bloqueo de las moléculas HLA-DQ2/DQ8; mediante péptidos sintéticos no estimuladores (HLA agonistas) que compiten por los lugares de unión de los péptidos del gluten a las moléculas DQ, induciéndose la inactivación funcional de los linfocitos T reactivos (Hoffmann et al., 2009; Xia et al., 2007). El problema de esta estrategia es la gran heterogeneidad de péptidos inmunoestimulantes del gluten así como la síntesis continua de moléculas HLA por parte de las células presentadoras de antígeno.

4) Inmunomodulación; mediante i) citoquinas capaces de regular la respuesta inmune hacia un perfil anti-inflamatorio, como por ejemplo la IL-10 recombinante, que es capaz de inducir la expansión de los linfocitos T reguladores y suprimir la diferenciación de las células T (Salvati et al., 2005); o mediante ii) la administración de inhibidores (antagonistas) de citoquinas proinflamatorias, como fármacos anti-IFN-γ, anti-TNF-α o anti-IL-15 (Yokoyama et al., 2009; De Nito et al., 2009; Di Sabatino et al., 2007; Korzenik y Podolsky, 2006).

5) Inducción de tolerancia; se ha visto que la administración controlada del antígeno (gliadina tratada con TGt) regula la transcripción de citoquinas pro-inflamatorias (IFN-γ), anti-inflamatorias (IL-4 e IL-10) y TGF-β (Senger et al., 2003). Además, la introducción gradual de cantidades controladas de gluten y el momento de introducción en la dieta, puede reducir el riesgo a padecer la EC en individuos genéticamente susceptibles (Ivarsson et al., 2002).

2. MICROBIOTA INTESTINAL

El TGI de recién nacidos se considera prácticamente estéril, pero inmediatamente después del nacimiento los microorganismos de la madre y del ambiente comienzan a colonizarlo y tras unas pocas horas de vida las bacterias empiezan a ser detectadas en las heces. Los primeros colonizadores del TGI son bacterias anaerobias facultativas, como enterobacterias o estreptococos, que reducen el ambiente intestinal permitiendo que pueda ser colonizado por parte de bacterias anaerobias estrictas de los géneros *Bifidobacterium* y *Bacteroides* (Penders et al., 2006; Mackie et al., 1999). Algunos factores, como el tipo de parto o de alimentación, pueden determinar los principales grupos bacterianos que colonizarán el TGI (Biasucci et al., 2010; Biasucci et al., 2008; Mackie et al., 1999). En caso de parto vaginal la composición de la microbiota intestinal del neonato a los 5-10 minutos después de nacer es igual al del cérvix y la vagina de la madre, mientras que en caso de parto por cesárea el ambiente y el personal de enfermería son el vector de transmisión de los microorganismos (Mackie et al., 1999). No obstante, recientemente se ha cuestionado la importancia del tránsito de bacterias a través de la vagina y las heces de la madre en el proceso de colonización inicial del recién nacido, habiéndose demostrado que en ya el meconio de neonatos nacidos sanos por parto natural o por cesárea se encuentran bacterias (Jiménez et al., 2008; Jiménez et al., 2005; Bearfield et al., 2002). El contacto con el ambiente y la madre también influyen posteriormente en la microbiota del recién nacido y, conjuntamente, a esta forma de adquisición bacteriana se le denomina transmisión horizontal. Otro factor determinante para el desarrollo de la microbiota intestinal de los recién nacidos es el tipo de lactancia. En los niños alimentados con leche materna predominan las bifidobacterias mientras que la presencia de clostridios y coliformes es más baja. Sin embargo, los niños alimentados con fórmulas infantiles presentan una microbiota más heterogénea, con presencia de bacteroides, bifidobacterias, clostridios, estreptococos y coliformes (Fanaro et al., 2003; Mackie et al., 1999). La lactancia materna se considera la mejor fuente de factores de defensa para el niño al contener inmunoglobulinas, células inmunocompetentes, compuestos antimicrobianos y factores "bifidogénicos" (galacto-oligosacáridos). Además en estos últimos años se ha puesto de manifiesto que la leche materna es una fuente

de bacterias comensales para el intestino del lactante como, lactobacilos y bifidobacterias (Martin et al., 2009; Gueimonde et al., 2007; Martin et al., 2003).

La composición de la microbiota también varía con la introducción de alimentos sólidos en la dieta, y es a partir de este momento cuando finalmente se establece una microbiota más compleja, estable y similar a la del adulto (Bezirtzoglou, 1997). Globalmente, se considera que un adecuado proceso de colonización es necesario para regular funciones fisiológicas básicas como la permeabilidad intestinal y el desarrollo del sistema inmunitario, que contribuyen al mantenimiento de un buen estado de salud.

Existen aproximadamente 10^{14} células procariotas en el TGI humano adulto y en los últimos años se ha conseguido avanzar enormemente en la determinación de su composición mediante el uso de técnicas dependientes de cultivo y, especialmente, mediante técnicas moleculares. Además, se ha descrito que la composición y diversidad de la microbiota intestinal varía a lo largo de las diferentes partes del tracto. En el estómago y duodeno las poblaciones son menores (10^2-10^3 organismos por gramo) con representantes aerobios fundamentalmente, y van aumentando hasta llegar al ciego y colon (10^{10}-10^{11} organismos por gramo) colonizado principalmente por bacterias anaerobias (Neish, 2009; Sartor, 2008). En el colon la concentración de bacterias anaerobias es varios órdenes de magnitud superior a la aerobias y la mayoría de ellas (60%-90%) pertenecen a los filos *Bacteroidetes* y *Firmicutes* (Eckburg et al., 2005), y existe una representación menor de bacterias pertenecientes a los filos *Proteobacteria* y *Actinobacteria* (Kovatcheva-Datchary et al., 2009; Eckburg et al., 2005). En la **Tabla 5 s**e puede ver una representación de los principales grupos bacterianos que colonizan las diferentes partes del TGI humano.

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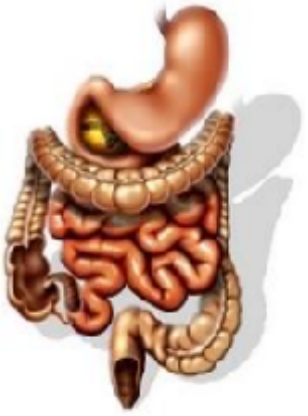
Tabla 5. Grupos microbianos predominantes en el tracto gastrointestinal. (Adaptado de Sartor et al.2008).

	Localización	Concentración	Grupos bacterianos
	Estómago	0-10^4 UFC/g de contenido intestinal	*Candida albicans* *Helicobacter pylori* *Lactobacillus* *Peptostreptococcus* *Streptococcus*
	Duodeno	10^3-10^4 UFC/g de contenido intestinal	*Bacteroides* *Candida albicans* *Lactobacillus* *Streptococcus* *Staphylocuccus*
	Yeyuno	10^5-10^7 UFC/g de contenido intestinal	*Bacteroides* *Candida albicans* *Lactobacillus* *Streptococcus*
	Íleon	10^7-10^8 UFC/g de contenido intestinal	*Bacteroides* *Clostridium* *Enterobacteriaceae* *Enterococcus* *Lactobacillus* *Veillonella*
	Colon	10^{10}-10^{11} UFC/g de contenido intestinal	*Bacteroides* *Bacillus* *Bifidobacterium* *Clostridium* *Enterobacteriaceae* *Enterococcus* *Eubacterium* *Fusobacterium* *Peptostreptococcus* *Ruminococcus* *Streptococcus*

2.1. Microbiota intestinal y enfermedades intestinales

La microbiota intestinal parece desempeñar importantes funciones en el hospedador ya que interviene en numerosos procesos biológicos tales como la estimulación del sistema inmune, la inhibición de microorganismos patógenos o la

metabolización de nutrientes (Hooper et al., 2012; Nicholson et al., 2012; Laparra y Sanz, 2009; Sanz y De Palma, 2009). Por lo tanto, el mantenimiento del equilibrio en la composición de la microbiota intestinal puede influir en el mantenimiento del estado de salud y su alteración se ha asociado con diferentes cuadros clínicos (**Tabla 6**) (Clemente et al., 2012).

Tabla 6. Cambios en la microbiota intestinal asociados a diferentes enfermedades

Microbiota implicada[a]	Cambios de la dinámica de la colonización, la diversidad o la presencia /ausencia de bacterias o grupos específicos	Referencias
Enfermedad celíaca		
Bacteroides spp. ↑ *Bifidobacterium* spp. ↓ *Escherichia coli* ↑ Grupo *Clostridium leptum* ↑ *Staphylocuccus* spp. ↑	Cambios significativos en la microbiota duodenal y fecal se asocian con la EC	(1)(2)(3)
Cáncer gástrico		
H. pylori ↑	Importante para desencadenar el desarrollo de adenocarcinomas	(4)
Obesidad		
Gr. *Bacteroides fragilis* ↑ Gr. *Clostridium coccoides* ↓ *Bifidobacterium* spp. ↓ *Lactobacillus* ↑ *Firmicutes/Bacteroidetes* ↑ *Methanobrevibacter smithii* ↓	Cambios significativos en la microbiota intestinal se asocian con aumento de la obesidad	(5)(6)(7)
EII[b]-Enfermedad de Crohn		
Bacteroides vulgatus ↑ *Bacteroides uniformis* ↓	Menor diversidad en pacientes con enfermedad de Crohn que en sanos	(8)
EII[b] (General)		
Actinobacteria ↑ *Proteobacteria* ↑ *Bifidobacterium spp.*↓ Gr. *Clostridium leptum* ↓ Gr .*Clostridium coccoides* ↓ *Faecalibacterium prasnitzii* ↓ *Firmicutes/Bacteroidetes* ↓	Disbiosis general de la comunidad bacteriana en lugar de una sola especie causal	(9)(10)

[a]Cambios cuantitativos en relación con sujetos sanos. Aumento: ↑. Disminución: ↓

Referencias: (1) Shippa et al., 2010; (2) Collado et al.,2009; (3) Nadal et al., 2007; (4) Lathrop et al., 2011; (5) Santacruz et al., 2009; (6) Turnbaugh et al., 2008; (7) Ley et al., 2005; (8) Dicksved et al., 2008; (9) Spor et al., 2011; (10) Perry et al., 2006

En los últimos años se han realizado numerosos estudios sobre la microbiota intestinal de pacientes con enfermedades inflamatorias crónicas (IBD, del inglés "inflammatory bowel disease"), como la colitis ulcerosa o la enfermedad de Crohn, y se ha descrito una alteración en su composición (disbiosis) (Friswell et al., 2010; Sokol et al., 2006; Conte et al., 2006). En general, se ha detectado una disminución de la diversidad de la microbiota duodenal y fecal en los grupos filogenéticos dominantes, es decir, *Firmicutes* y *Bacteroidetes*. (Sartor, 2008; Frank et al., 2007). Asimismo, se ha identificado la presencia de especies o cepas bacterianas con mayor potencial patogénico en pacientes con estas patologías, como por ejemplo *Mycobacterium avium* subsp. *paratuberculosis* (Schirbel y Fiocchi, 2010; Feller et al., 2007;), cepas de *Escherichia coli* invasivas y con alta capacidad de adhesión al epitelio intestinal (Schirbel y Fiocchi, 2010), o cepas de *Bacteroides fragilis* productoras de enterotoxinas (Rabizadeh et al., 2007; Basset et al., 2004).

2.2. Microbiota intestinal y EC

Durante los últimos años se ha empezado a estudiar la microbiota intestinal de los pacientes celíacos, lo que está ayudando a descubrir nuevos factores que pueden contribuir al desarrollo y patogénesis de la enfermedad. En este contexto, se ha observado que la presentación de esta enfermedad conlleva alteraciones en la composición de la microbiota intestinal y en su actividad metabólica. En los pacientes celíacos, tanto en fase activa como no activa de la enfermedad, se ha descrito una mayor carga bacteriana asociada a la mucosa duodenal (Ou et al., 2009; Nadal et al., 2007; Tursi et al., 2003) y fecal (Nistal et al., 2012; Di Cagno et al., 2011; Schippa et al., 2010; Di Cagno et al., 2009) y además la actividad metabólica asociada a la microbiota intestinal de estos pacientes está caracterizada por niveles más elevados de ácido acético, ácido iso-butírico y ácido iso-valérico que la de los controles (Nistal et al., 2012; Di Cagno et al., 2009; Tjellstrom et al., 2007; Tjellstrom et al., 2005). El análisis de la composición de la microbiota de estos pacientes ha mostrado descensos en la concentración de géneros como *Bifidobacterium* y aumentos de grupos bacterianos con mayor poder patógenico como *Bacteroides* o *E. coli* (Collado et al., 2009; Nadal et al., 2007; Collado et al., 2007). Estas diferencias comienzan a

detectarse a edades tempranas en los recién nacidos sanos con alto riesgo de padecer la EC (De Palma et al., 2012; De Palma et al., 2009) lo que podría indicar que el ambiente genético e inmunológico de los individuos predispuestos influye en el proceso de colonización del recién nacido que podría, a su vez, estar relacionado con el posterior desarrollo de la EC.

Aún se desconoce el papel que los microorganismos potencialmente perjudiciales pueden desempeñar en la patogénesis de la EC y si estas alteraciones son un factor desencadenante o una consecuencia secundaria de la misma. Sin embargo, se sabe que los microorganismos oportunistas o patógenos podían causar un incremento transitorio de la permeabilidad del intestino delgado, que permitiría el acceso de los antígenos del gluten a la lámina propria, aumentando así su capacidad inmunógena, y por lo tanto su capacidad de estimular la producción de INF-γ y TNF-α y activar la actividad de macrófagos (Sanz et al., 2007). Además, estos grupos bacterianos podrían provocan una respuesta inflamatoria capaz de exacerbar la patología de la enfermedad (Cinova et al., 2011; De Palma et al., 2010a).

2.2.1. Modulación de la microbiota intestinal y EC. Uso de probióticos

Como se ha comentado, la lactancia materna parece disminuir el riesgo de padecer la EC por parte de los niños genéticamente susceptibles además de reducir la incidencia de infecciones. La protección ejercida por la leche materna puede deberse tanto al efecto de sus componentes bioactivos (inmunoglobulinas, hormonas, compuestos antimicrobianos, prebióticos, etc.) (Gaskins et al., 2008) como a su influencia sobre el proceso de colonización microbiana del TGI del recién nacido (Solis et al., 2010; Gronlund et al., 2007;).

La microbiota de los niños alimentados con lactancia materna está compuesta mayoritariamente por bifidobacterias, mientras que la de los alimentados con formulas infantiles presenta una composición más heterogénea (Mackie et al., 1999). Estos datos, junto con el hecho de que los pacientes celíacos muestran menores concentraciones de bifidobacterias, convierten a este grupo bacteriano en un buen candidato para su uso como probiótico. Más aún, teniendo en cuenta que las bifidobacterias han demostrado tener mayor

capacidad para inducir respuestas anti-inflamatorias y reguladoras que otras bacterias utilizadas como probióticos como los lactobacilos, pese a que estas propiedades dependen de la cepa (Medina et al., 2008). En este contexto, nuestro grupo de investigación realizó una selección de cepas del género *Bifidobacterium* (*Bifidobacterium longum* ES1 y *Bifidobacterium bifidum* ES2) que han demostrado ser capaces de reducir la síntesis de citoquinas pro-inflamatorias (TNF-α e IFN-γ) inducidas por la microbiota alterada de los pacientes y aumentar la de anti-inflamatorias (IL-10) *in vitro* (Medina et al., 2008). Estudios posteriores en asas intestinales de ratas carentes de gérmenes expuestas a los desencadenantes de la enfermedad (gliadina e IFN- γ) han demostrado que *B. bifidum* ES2 reduce los daños de la mucosa causados por estos factores y enterobacterias (Cinova et al., 2011). Además, un estudio en ratas en las que se indujo enteropatía al gluten tras el nacimiento ha demostrado que la cepa *B. longum* ES1 reduce la respuesta inflamatoria de células T CD4+ a nivel periférico y la síntesis de TNF-α en el intestino delgado y aumenta la de IL-10 (Laparra et al., 2012). Estudios realizados en ratones que expresan las moléculas HLA-DQ8 y con cepas fundamentalmente de lactobacilos han indicado que éstas generan respuestas inductoras más que supresoras, sugiriendo la posibilidad de utilizar alguna de ellas (*Lactobacillus casei* ATCC 9595) como adjuvante en la vacunación para inducir la respuesta de las células T a las gliadinas (D'Arienzo et al., 2009). Un estudio posterior en el mismo modelo pero expuesto oralmente no sólo a gliadinas sino también a indomentacina para inducir daño en la mucosa, indicó que la cepa *L. casei* ATCC 9595 contribuía a reducir los daños histológicos y la producción de TNF-α pero no se observaron efectos positivos sobre la producción de IFN-γ, IL-10 and IL-4 (D'Arienzo et al., 2011; D'Arienzo et al., 2009). No obstante, los efectos de las cepas mencionadas no son comparables debido al uso de diferentes modelos experimentales. Asimismo, será necesario la realización de estudios de intervención en humanos para demostrar si realmente las bacterias evaluadas inicialmente en modelos animales pueden contribuir a mejorar el estado de salud y calidad de vida de la población celíaca.

II. OBJETIVOS

EXPOSICIÓN GENERAL DEL PROBLEMA A INVESTIGAR Y OBJETIVOS

En los últimos años ha aumentado el interés por las investigaciones en torno a la relación entre la microbiota intestinal y el estado de salud con el fin de identificar las características y componentes específicos de la microbiota que pueden agravar o favorecer el desarrollo de diversas patologías y los que, por el contrario, pueden ejercer un efecto protector o reducir su riesgo. Sin embargo, la relación entre la microbiota intestinal y la EC sólo ha sido investigada en los últimos años debido que el factor ambiental desencadenante (las proteínas del gluten) era conocido, limitando las investigaciones sobre la influencia de otros factores en la patogénesis y el riesgo de padecer esta enfermedad. En los últimos años, se ha descrito la existencia de diferencias cuantitativas y cualitativas en la composición de la microbiota fecal y duodenal entre niños con EC e individuos control (Collado et al., 2009; Nadal et al., 2007; Collado et al., 2007) y en niños con riesgo de padecer la enfermedad por sus antecedentes familiares (De Palma et al., 2012; De Palma et al. 2009b), así como diferencias en los metabolitos derivados de la actividad microbiana del intestino de pacientes con EC (Tjellstrom et al., 2007; Tjellstrom et al., 2005).

Teniendo en cuenta estos antecedentes, esta tesis doctoral tuvo como **objetivo global** avanzar en la caracterización de la microbiota intestinal de pacientes diagnosticados con EC en comparación con la de controles no celíacos y de lactantes sanos con riesgo de padecer la enfermedad por sus antecedentes familiares, e investigar la posible patogenicidad de las cepas aisladas de pacientes celíacos.

Este objetivo global se desglosó en los siguientes **objetivos parciales**:

1. Analizar comparativamente la composición de la microbiota fecal y duodenal de individuos sanos y pacientes celíacos en edad pediátrica.

2. Aislar, identificar y caracterizar los factores de virulencia de los géneros y especies bacterianas (enterobacterias, estaflilococos y bacteroides)

posiblemente implicadas en la EC a partir de heces de individuos celíacos y compararlos con los aislados de individuos sanos.

3. Aislar e identificar bacterias asociadas a la mucosa duodenal de individuos celíacos y compararlas con las de controles no celíacos.

4. Analizar el proceso de colonización intestinal de especies del género *Bacteroides* en recién nacidos y lactantes con riesgo de desarrollar la EC y determinar su relación con el tipo de lactancia y el genotipo de riesgo.

III. RESULTADOS Y DISCUSIÓN

Esta tesis es una compilación de una serie de publicaciones que se indican a continuación. El presente apartado de *resultados y discusión* está formado por tales publicaciones introducidas como tal en su orden correspondiente. Además, al final del apartado se incluye una discusión general que engloba y enlaza todos los temas tratados en cada uno de los artículos:

1. Differences in faecal bacterial communities in coeliac and healthy children as described by PCR and denaturing gradient gel electrophoresis. Sanz Y, **Sánchez E**, Marzotto M, Calabuig M, Torriani S, Dellaglio F. FEMS Immunol Med Microbiol. 2007 Dec;51(3):562-8 (**Anexo 1**).

2. Intestinal *Bacteroides* species associated with coeliac disease. **Sánchez E**, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y. J Clin Pathol. 2010 Dec;63(12):1105-11(**Anexo 2**).

3. Reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of coeliac children. **Sánchez E**, Nadal I, Donat E, Ribes-Koninckx C, Calabuig M, Sanz Y. BMC Gastroenterol. 2008 Nov 4;8:50 (**Anexo 3**).

4. Intestinal *Staphylococcus* spp. and virulent features associated with coeliac disease. **Sánchez E**, Ribes-Koninckx C, Calabuig M, Sanz Y. J Clin Pathol. 2012 Sep;65(9):830-4 (**Anexo 4**).

5. Discerning the role of *Bacteroides fragilis* in celiac diease pathogenesis. **Sánchez E**, Laparra JM, Sanz Y. Appl. Environ. Microbiol. 72. 2012 Sep;78(18): 6507 (**Anexo 5**).

6. Duodenal-mucosal bacteria associated with celiac disease in children. **Sánchez, E** C. Ribes-Koninckx, Sanz Y. Appl Environ Microbiol (enviado)

7. Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by *Bacteroides* species. **Sánchez E,** De Palma G, Capilla A, Nova E, Pozo T, Castillejo G, Varea V, Marcos A, Garrote JA, Polanco I, López A, Ribes-Koninckx C, García-Novo MD, Calvo C, Ortigosa L, Palau F, Sanz Y. Appl Environ Microbiol. 2011 Aug;77(15):5316-23 (**Anexo 6**).

Capítulo I:

Analizar comparativamente la composición de la microbiota fecal y duodenal de individuos sanos y pacientes celíacos en edad pediátrica.

- *Differences in faecal bacterial communities in coeliac and healthy children as described by PCR and denaturing gradient gel electrophoresis.*
- *Intestinal* Bacteroides *species associated with coeliac disease.*

Capítulo I

Differences in faecal bacterial communities in coeliac and healthy children as described by PCR and denaturing gradient gel electrophoresis

ABSTRACT

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestinal mucosa. Scientific evidence supports a role of the gut microbiota in chronic inflammatory disorders; yet information is not specifically available for CD. In this study, a comparative denaturing gradient gel electrophoresis analysis of faecal samples from coeliac children and age-matched controls was carried out. The diversity of the faecal microbiota was significantly higher in coeliac children than in healthy controls. The presence of the species *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and *Leuconostoc carnosum* was characteristic of coeliac patients, while that of the *Lactobacillus casei* group was characteristic of healthy controls. The *Bifidobacterium* population showed a significantly higher species diversity in healthy children than in coeliacs. In healthy children, this population was characterized by the presence of *Bifidobacterium adolescentis*. Overall, the results highlighted the need for further characterization of the microbiota in coeliac patients, and suggested a potential role of probiotics and/or prebiotics in restoring their gut microbial balance.

Keywords: coeliac disease; PCR-DGGE; faecal microbiota; lactic acid bacteria; *Bifidobacterium*.

1. INTRODUCTION

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestinal mucosa that involves genetic and environmental factors. The classic form of the disease often manifests in early childhood (9–24 months) with gastrointestinal symptoms and malabsorption (Van Heel and West, 2006). CD is the result of an aberrant Th_1 immune response to gluten peptides within the intestinal mucosa, where IFN-γ is the predominantly secreted cytokine, as well as an innate immune response mediated by IL-15 (Koning et al., 2005). Over the last decades, significant progress has been made in the understanding of the aetiology and pathogenesis of CD. In spite of that, little is known about the roles of environmental factors other than gluten in CD presentation (Barnich and Darfeuille-Michaud, 2007). It has been suggested that a transient infection could increase the permeability of the mucosal epithelial layer to gluten antigens by activating macrophages and dendritic cells with the production of pro-inflammatory cytokines (Kagnoff, 2005).

Moreover, the inflammatory milieu originated by gluten antigens could lead to disturbances in the gut microbial composition that could in turn contribute to perpetuate inflammation (Collado et al., 2007). The development and maintenance of immune homeostasis depends on signals from the gut microbiota. Members of the genera *Lactobacillus* and *Bifidobacterium* are regarded as plausible significant players of gut health, and therefore are intensively investigated for probiotic uses (Isolauri et al., 2001; Thompson-Chagoyan et al., 2005). It has been demonstrated that components of the intestinal microbiota of animal models and inflammatory bowel disease (IBD) patients are involved in the abnormal T cell immune responses leading to loss of tolerance and mucosal inflammation (Barnich and Darfeuille-Michaud, 2007). Furthermore, the administration of probiotics has been found to exert beneficial effects in some disease models and IBD patients by decreasing the production of proinflammatory cytokines (e.g. IFN-γ, and TNF-α) and interfering with harmful bacterial adhesion (Dotan and Rachmilewitz, 2005). At present, little is known about the potential role of the microbiota in CD. Alterations in the composition of faecal short-chain fatty acids as well as the presence of rod-shaped bacteria

associated with the mucosa have been reported in coeliac patients (Forsberg et al., 2004; Tjellstrom et al., 2005). More recently, a quantitative FISH analysis of the faecal microbiota of coeliac children and healthy controls revealed the existence of increased levels of Gram-negative bacteria and *Staphylococcus*, but significant differences in *Lactobacillus* and *Bifidobacterium* numbers were not detected (Collado et al., 2007). Here, denaturing gradient gel electrophoresis (DGGE) analyses using universal and primers specific for *Bifidobacterium* and *Lactobacillus* groups were carried out in order to detect differences in species composition that could be characteristic of an early stage of the disease, and support the use of probiotics and/or prebiotics for restoring the intestinal balance in these patients.

2. MATERIALS AND METHODS

2.1. Reference bacterial strains and growth conditions

The reference strains used as ladders for identification of *Bifidobacterium* spp. by DGGE were: *Bifidobacterium adolescentis* LMG 11037^T, *Bifidobacterium animalis* ssp. *animalis* LMG 10508^T, *B. animalis* ssp. *lactis* LMG 10140 *Bifidobacterium angulatum* LMG 11039^T, *Bifidobacterium bifidum* LMG 11041^T, *Bifidobacterium dentium* LMG 11045^T, *Bifidobacterium longum* biotype *infantis* LMG 11046^T, *B. longum* biotype *longum* LMG 10497^T, *Bifidobacterium pseudocatenulatum* LMG 10505^{T}and *Bifidobacterium ruminantium* LMG 21811^T. The reference strains used as DGGE ladders for identification of lactic acid bacteria were: *Lactobacillus acidophilus* DSM 20079^T, *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842^T, *Lactobacillus ruminis* LMG 10756^T, *Lactobacillus curvatus* LMG 9198^T, *Lactobacillus reuteri* LMG 9213^T, *Lactobacillus brevis* LMG 7944^T, *Lactobacillus gasseri* LMG 9203^T, *Lactobacillus casei* ATCC 393^T, *Lactobacillus sakei* LMG 9468^T, *Lactobacillus plantarum* ATCC 14917^T and *Pediococcus acidilactici* LMG 11384^T. All strains were grown in Man–Rogosa–Sharpe (MRS) medium, supplemented with 0.5 g L^{-1} cysteine to grow bifidobacteria, and incubated in anaerobiosis (Anaerocult, Merck, Darmstadt, Germany) at 37 ºC.

2.2. Subjects and faecal sampling

Altogether, 20 children were included in the study: 10 coeliac patients (age 15–45 months; mean 28 months) and 10 age-matched healthy controls (age 11–40 months; mean 24 months). The group of patients included children who showed clinical symptoms of CD, positive serology markers (antigliadin, antiendomysial and antitransglutaminase antibodies) and signs of severe enteropathy by duodenal biopsy examination. The group of controls included healthy children with no known food intolerance. Samples from coeliac children were collected at the presentation of the disease, when they were still following a normal-gluten-containing diet. The children included in the study were not treated with antibiotics for at least 1 month before the sampling time. The study was approved by the Committee on Ethical Practice of General Universitary

Hospital and CSIC, and children were enrolled in the study after written informed consent was obtained from their parents.

Faecal samples were collected from both groups of children and immediately maintained at 4 ºC, under anaerobiosis (AnaeroGen, Oxoid, Hampshire, UK), and processed in less than 12 h as described previously (Collado et al., 2007). Briefly, faecal samples (2 g of wet weight) were 10-fold diluted in phosphate-buffered saline [PBS, 130 mM sodium chloride, 10 mM sodium phosphate, (pH 7.2)], and homogenized in a Lab Blender 400 stomacher (Seward Medical, London, UK). An aliquot was submitted to low-spin centrifugation (300 *g* for 2 min) and the supernatant was maintained at -80 ºC until analysed.

2.3. Nucleic acid extraction from bacterial cultures and faecal samples

The extractions of genomic DNA from pure cultures of *Lactobacillus* and *Bifidobacterium* used as reference strains as well as total DNA from faecal samples were carried out as previously described (Marzotto et al., 2006).

2.4. PCR amplification and DGGE analysis

PCR fragments of 200 bp representing total faecal bacterial were amplified with the universal primers HDA1-GC and HDA2 (Walter et al., 2000). PCR fragments of 340 bp representing *Lactobacillus*-related species were amplified with the primers Lac1 and Lac2-GC (Walter et al., 2001). PCR fragments of 520 bp representing species of *Bifidobacterium* were amplified with the primers Bif164 and Bif662GC (Satokari et al., 2001). DGGE analysis of PCR amplicons was carried out on the Dcode Universal Mutation Detection System (Bio-Rad, Richmond, CA), essentially as described previously (Marzotto et al., 2006). The linear denaturing gradients of urea and formamide used for separation of amplicons from total microbiota, *Bifidobacterium* and *Lactobacillus*-related species were 30–50%, 45–55% and 30–50%, respectively. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. Selected unknown DGGE bands were excised from the denaturing gels and reamplified with the corresponding primers but without the GC-clamp. The PCR products were purified from agarose gel using the

Qiaex II gel extraction kit (Qiagen, Hilden, Germany) and sequenced at the Bio Molecular Research Center (BMR), University of Padova (Italy). Search analyses to determine the closest relatives of the partial 16S rRNA gene sequences retrieved were conducted in GenBank using the BLAST algorithm.

2.5. Data analysis

DGGE patterns were analysed with the software package $1_{D\text{-}MANAGER}$ and $_{LANEMANAGER}$ V2.0 (TDI, Barcelona, Spain). The similarities between DGGE profiles obtained with universal primers were determined using the Dice coefficient and the unweighted-pair group method with the arithmetic average (UPGMA) clustering algorithm. The number of bands of each individual in every DGGE profile was considered as an indicator of diversity of the faecal microbiota. Differences in diversity between both children groups (coeliacs and controls) were analysed by applying the Mann–Whitney U-test. Differences in species composition between both population groups were analysed using the x^2tests. In every case, analyses were carried out with the STATGRAPHICS software (Manugistics, Rockville, MD), and statistical significance was established at P values < 0.05.

3. RESULTS AND DISCUSION

3.1. DGGE analysis with universal primers

DGGE profiles of PCR amplicons obtained with universal primers were complex and unique for each individual. No amplicon could be unequivocally associated with the presence or the absence of coeliac disease although two major clusters were differentiated: one grouping most coeliac patients and the other most healthy controls (Figs 1 and 2). The individual DGGE profiles showed similarities ranging between 43% and 94%. The diversity of the faecal microbiota, according to the number of bands in DGGE profiles, was significantly higher in coeliac children than in healthy controls (mean 8.40 vs. 7.2, $P < 0.05$; Figs 1 and 2). In contrast, the biodiversity of active faecal bacteria of patients with ulcerative colitis was found to be lower than that of healthy subjects when analysed by temporal temperature gradient gel electrophoresis (Sokol et al., 2006). A recent metagenomic approach also showed a reduction in the diversity of the phylum *Firmicutes* in the faecal microbiota of Crohn's disease patients (Manichanh et al., 2006). Whether these changes are pathogenic, secondary or methodological still remains to be investigated.

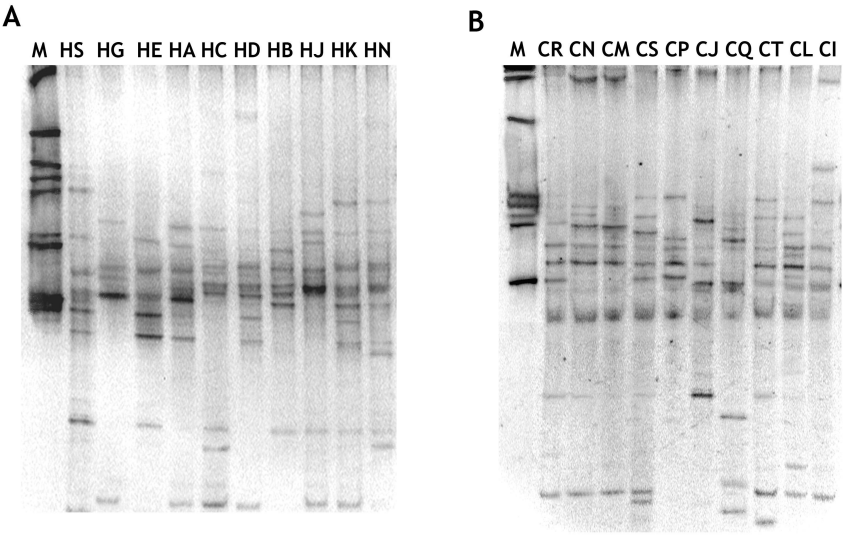


Fig 1. DGGE profiles of total bacteria of faecal DNA from healthy (A) or coeliac (B) children amplified with the universal primers HDA1-GC and HDA2.

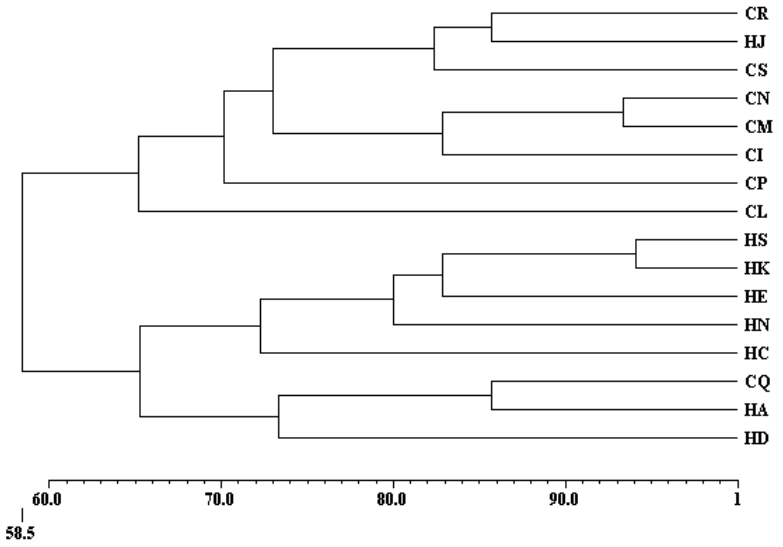


Fig 2. Dendogram derived from DGGE analysis of total faecal bacteria of healthy (H) and coeliac (C) children using universal primers based on Dice's similarity index and the UPGMA clustering algorithm.

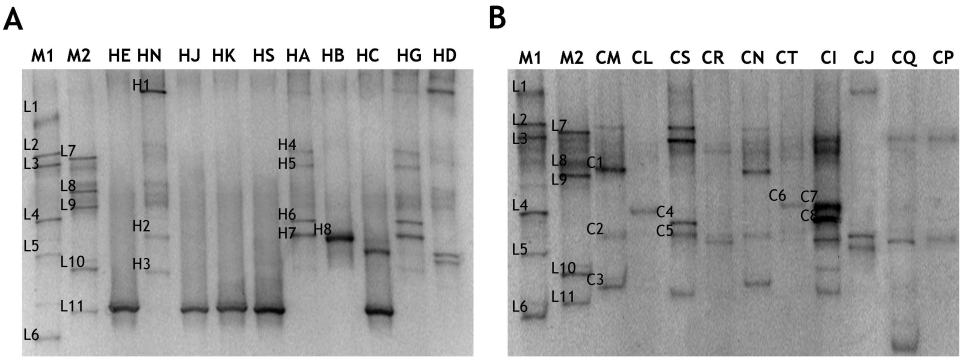


Fig 3. DGGE profiles of 16S rRNA gene fragments of *Lactobacillus* spp. and related lactic acid bacteria of faecal DNA from healthy (A) or coeliac (B) children amplified with the primers Lac1 and Lac2-GC. Lanes M1 and M2, lactic acid bacteria identification ladder (L1, *L. plantarum*; L2, *L. brevis*; L3, *L. acidophilus*; L4, *L. ruminis;* L5, *P. acidilactici*; L6, *L. reuteri*; L7, *L. sakei* ; L8, *L. curvatus*; L9, L. *gasseri*; L10, *L. delbrueckii* subsp. *bulgaricus*; L11, *L. casei*. Numbered arrows refer to sequenced fragments, whose amplicon IDs and closest relatives are shown in Table 1.

3.2. DGGE analysis with Lactobacillus group-specific primers

The DGGE profiles of PCR amplicons obtained with *Lactobacillus* specific primers are shown in Fig. 3. A cluster analysis of the DGGE profiles obtained with *Lactobacillus* group-specific primers was not performed due to their simplicity. The PCR amplicons that were identified by sequencing are shown in Table 1. The profiles of healthy children tended to show a lower diversity than those of coeliac patients but the differences were not significant ($P > 0.05$; Fig. 3. and Table 1). The DGGE profiles of healthy subjects showed between one and four different bands and, in most of them, an amplicon corresponding to the *L. casei* reference strain was the unique or dominant one. In contrast, the DGGE profiles of coeliac children revealed the presence of one to six different *Lactobacillus* group specific bands and species belonging to genera other than *Lactobacillus* were dominant. The prevalence of the *L. casei* group amplicon was significantly higher ($P < 0.05$) in healthy children than in coeliacs (Table 2). According to the specificity of the Lac1 and Lac2-CG primers, the *L. casei* group might correspond to the species *L. casei*, *Lactobacillus paracasei*, *Lactobacillus rhamnosus* or *Lactobacillus zeae* (Walter et al., 2000; Ahrne et al., 2005). In contrast, the prevalence of *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and *Leuconostoc carnosum* was significantly higher ($P > 0.05$) in coeliac patients (Table 2). Most lactic acid bacterial species detected in coeliac patients were likely transient (allochthonous) (Reuter, 2001; Walter et al., 2001), while in healthy samples *L. gasseri* and species of the *L. casei* group could be regarded as both endogenous and food-related bacteria (Walter et al., 2000; Reuter, 2001; Ahrne et al., 2005). Although most of the allochthonous bacterial species found in both groups have been identified previously in faeces of healthy subjects (Walter et al., 2000, 2001; Reuter, 2001), it might be relevant to consider their impact on the consumer as they transit the gut in the digesta (Walter et al., 2001). The proportions of different *Lactobacillus* species might be functionally important because they largely influence the biochemistry, immunology and population dynamics of the host intestinal tract (Walter et al., 2000; Reuter, 2001). DGGE analysis of faeces from Crohn's disease patients and controls indicated that the diversity of lactic acid bacteria varied significantly between

the groups (Scanlan et al., 2006). Alterations in the composition of *Lactobacillus* and other lactic acid bacterial species have also been described in ulcerative colitis patients (Bullock et al., 2004). Although direct evidence of the role of specific Lactobacillus species in human disease has not yet been provided, their composition was found to be different in colitic animal mice (IL-10 – knock out) and controls, and so was the anti-TNFα activity of the isolated species (Pena et al., 2004).

Table 1. *Lactobacillus* and related lactic acid bacterial species identified by sequencing the DGGE bands amplified from faecal DNA of healthy and coeliac children using the *Lactobacillus* group specific primers.

Amplicon ID[a]	Closest relative (accession number)[b]	Identity (%)
Healthy children		
H1	*Lactobacillus algidus* (AB033209)	97
H2	*Leuconostoc mesenteroides* (AY675249)	99
H3	*Leuconostoc inhae* (AY675244)	98
H4	*Lactobacillus sakei* (AY204898)	100
H5	*Lactobacillus curvatus* (AY204894)	100
H6	*Lactobacillus delbrueckii* subsp.*bulgaricus* (AY735407)	98
H7	*Pediococcus* sp. (AM040655)	100
H8	*Leuconostoc mesenteroides* (DQ105649)	99
Coeliac children		
C1	*Weissella viridescens* (M23040)	100
C2	*Leuconostoc mesenteroides* (AY675249)	99
C3	*Leuconostoc carnosum* (AB022925)	99
C4	*Lactobacillus delbrueckii* subsp. *lactis* (AY773950)	96
C5	*Leuconostoc mesenteroides* (AY675249)	100
C6	*Lactobacillus fermentum* (AJ575812)	97
C7	*Lactobacillus delbrueckii* subsp. *lactis* (AY773950)	99
C8	*Lactobacillus paracasei* (DQ199664)	100

[a]Identification labels corresponding to DGGE bands shown in Fig 3 a and b.

[b]Accession numbers of the closets relatives as determined by searching analyses conducted in GenBank using the BLAST algorithm (Koning et al., 2005).

Table 2. Lactic acid bacteria and *Bifidobacterium* species detected by DGGE analysis of faecal DNA from healthy and coeliac children using the *Lactobacillus* group-specific primers and the *Bifidobacterium* species-specific primers, respectively.

Bacterial group	Coeliac children	Healthy children	*P*-value
Lactic acid bacteria			
Lactobacillus algidus	0 (0%)	2 (20%)	0.14
Lactobacillus delbrueckii subsp *bulgaricus*	3 (30%)	2 (20%)	0.60
Lactobacillus brevis	0 (0%)	2 (20%)	0.13
Lactobacillus casei	0 (0%)	5 (50%)	0.01*
Lactobacillus curvatus	4 (40%)	0 (0%)	0.02*
Lactobacillus gasseri	0 (0%)	1 (10%)	0.30
Lactobacillus fermentum	2 (20%)	0 (0%)	0.14
Lactobacillus paracasei	2 (20%)	0 (0%)	0.14
Lactobacillus plantarum	1 (10%)	0 (0%)	0.30
Lactobacillus sakei	2 (20%)	2 (20%)	1.00
Leuconostoc mesenteroides	7 (70%)	2 (20%)	0.02*
Leuconostoc carnosum	4 (40%)	0 (0%)	0.02*
Pediococcus acidolactici	1 (10%)	0 (0%)	0.30
Pediococcus pentosaceus	0 (0%)	2 (20%)	0.14
Weissella viridescens	2 (20%)	0 (0%)	0.14
Leuconostoc inhae	0 (0%)	1 (10%)	0.30
***Bifidobacterium* spp.**			
Bifidobacterium adolescentis	0 (0%)	4 (40%)	0.03*
Bifidobacterium bifidum	4 (40%)	2 (2%)	o.33
Bifidobacterium infantis	3 (30%)	3 (30%)	1.00
Bifidobacterium longum	8 (80%)	10 (10%)	0.14
Bifidobacterium dentium	0 (0%)	2 (2%)	0.14
Bifidobacterium pseudocatenulatum	6 (60%)	7 (7%)	0.64

*Significant difference established at $P < 0.05$ by using the χ^2 test .

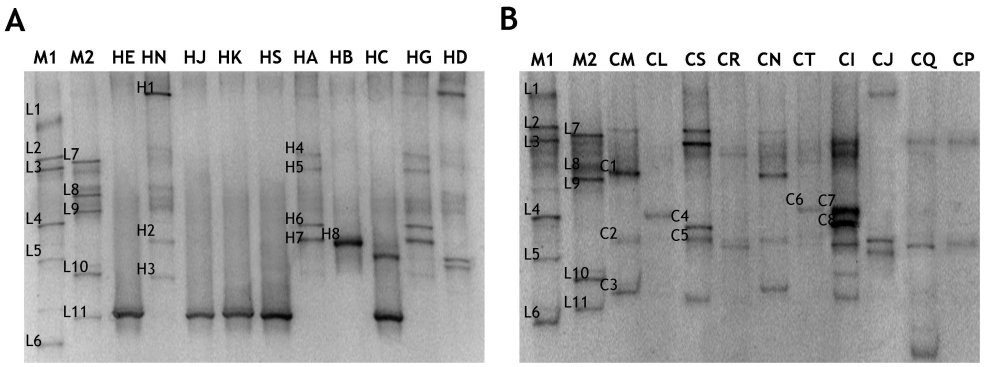


Fig. 4. DGGE profiles of 16S rRNA gene fragments of *Bifidobacterium* spp. of faecal DNA from healthy (A) or coeliac (B) children amplified with the primers Bif164 and Bif662-GC. Lanes M1 and M2, *Bifidobacterium* identification ladder (B1, *B. adolescentis*; B2, *B. infantis*; B3, *B. dentium*; B4, *B. animalis* ssp. *lactis*; B5, *B. bifidum*; B6, *B. longum*; B7, *B. pseudocatenulatum*; B8, *B. animalis* ssp. *animalis*).

3.3. DGGE analysis with Bifidobacterium species-specific primers

The DGGE profiles of PCR amplicons obtained with *Bifidobacterium* specific primers are shown in Fig. 4. The prevalence of different *Bifidobacterium* species is shown in Table 2. All *Bifidobacterium* species could be identified by comparing the migration distances of their respective PCR amplicons with those of reference strains used as ladders. In addition, the identity of six DNA bands was confirmed by sequencing (data not shown). The diversity of *Bifidobacterium* species was significantly higher ($P < 0.05$) in healthy children than in coeliacs. Most DGGE profiles of the healthy subjects (7 out of 10) showed between three and four different *Bifidobacterium* species whereas most DGGE profiles of coeliac children (8 out of 10) only showed one or two species. The prevalence of *B. longum*, *B. pseudocatenulatum* and *B. dentium* tended to be higher in healthy children while that of *B. bifidum* did so in coeliac children, although these differences were not significant. *B. dentium* and *B. adolescentis* were not detected in any coeliac samples and for the last species, the difference was statistically significant ($P < 0.05$, Table 2). Overall, the bifidobacterial population of healthy controls combined both infant- and adult-type features while that of

coeliac patients mainly consisted of infant-type species (*B. bifidum* and *B. infantis*). *Bifidobacterium* species composition is thought to influence host-immune responses as a consequence of the differential immunomodulatory properties shown by diverse *Bifidobacterium* species (Young et al., 2004). Although not in every report (Penders et al., 2006), changes in the composition of *Bifidobacterium* species in the infant gut microbiota have often been related to the development of allergy (Kirjavainen et al., 2001, 2002). In addition, it has been speculated that typical adult-type *Bifidobacterium* species could favour Th_2-biased immune responses characteristic of allergy inflammation (Young et al., 2004). Furthermore, the administration of probiotics and prebiotics to stimulate the proliferation of bifidobacteria has also been linked to beneficial effects in alleviating some IBD and allergic inflammation (Kirjavainen et al., 2002; Dotan and Rachmilewitz, 2005).

In summary, the diversity of total faecal microbiota as well as the species composition of lactic acid bacteria and *Bifidobacterium* have been shown to differ between coeliac children and age-matched controls. The results obtained highlight the need for further characterization of the gut microbiota in coeliac patients and suggest the potential role of probiotic and prebiotics in restoring their microbial gut balance.

4. ACKNOWLEDGEMENTS

This work was supported by grants AGL2005-05788-C02-01 from CICYT (Spain) and 200570F0091 from CSIC (Spain). The grant PTR95-987.OP.01 of E. Sánchez (CICYT, Spain) and the mobility grant CTESPP/2005/055 of Y. Sanz from GVA (Valencia, Spain) are fully acknowledged.

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Capítulo I

Intestinal *Bacteroides* species associated with coeliac disease.

ABSTRACT

Aims: To characterise the predominant species composition of bacterial populations associated with duodenal biopsies of paediatric patients with active and treated coeliac disease.

Methods: 20 biopsy specimens from active coeliac disease and 12 from treated coeliac disease patients and 8 from age-matched controls were evaluated for comparative purpose. *Bacteroides, Bifidobacterium* and lactic acid bacteria (LAB) populations were analyzed by PCR-DGGE using group-specific primers.

Results: *Bacteroides* diversity was higher in biopsy specimens from controls than in active and treated coeliac disease patients. *B. distasonis, B. fragilis*/*B. thetaiotaomicron, B. uniformis* and *B. ovatus* were more abundant in controls than in coeliac disease patients ($P<0.05$). *B. vulgatus* was more frequently detected in controls than in treated coeliac disease patients ($P<0.01$). *B. dorei* was more common in active coeliac CD than in treated CD and control children ($P<0.01$). *Bifidobacterium* diversity was higher in coeliac disease patients than in controls. *B. adolescentis* and *B. animalis* subsp. *lactis* were more commonly detected in active coeliac disease than in treated coeliac disease and control children. A higher LAB diversity was found in treated coeliac disease and controls than in active coeliac disease children. *Weisella* spp. and *L. fermentum* were more frequently detected in treated coeliac disease than in controls and active CD children.

Conclusions: *Bacteroides, Bifidobacterium* and LAB populations of the duodenal microbiota of active and treated coeliac disease patients and controls differ in diversity and species composition, which could contribute to the disease features.

Keywords: *Bacteroides*, coeliac disease**,** intestinal microbiota, PCR-DGGE

1. INTRODUCTION

The human gastrointestinal tract hosts a complex microbial community (microbiota) composed of hundreds of different bacterial species.[1] [2] The microbiota contributes to regulating energy metabolism, and epithelial cell and gut-associated lymphoid tissue functions.[3] [4] The intestinal immune system has to maintain a balance between the need to respond to pathogens and tolerance to the presence of a large community of commensal bacteria, whose disruption might contribute to the pathogenesis of inflammatory conditions.[5] [6] In fact, alterations to the composition of the intestinal microbiota have been implicated in diseases such as allergies,[7] chronic inflammatory bowel conditions[8] and cancer.[9]

Coeliac disease is a chronic inflammatory disorder of the small intestine that presents in genetically predisposed individuals following gluten consumption.[10] Removal of gluten from the diet is currently the only treatment available. In recent years, intestinal dysbiosis has been reported in patients with coeliac disease. The microbiota of these patients is characterised by a breakdown in the balance between potentially protective bacteria (eg, *Lactobacillus* spp. and *Bifidobacterium* spp.) and potentially harmful bacteria (eg, *Bacteroides* spp. and *Enterobacteriaceae*).[11] [12] Some differences in bacterial species composition have also been detected in stool samples of patients with active coeliac disease compared with those of healthy controls;[13] however, patients on a gluten-free diet were not included and duodenal samples were not analysed, limiting the conclusiveness of that preliminary study. A further report provided information on only the faecal and duodenal *Bifidobacterium* species composition in patients with coeliac disease, analysed using real-time PCR.[14]

Denaturing gradient gel electrophoresis (DGGE) has been successfully applied as a rapid culture independent method for the analysis of intestinal microbiota composition.[15] [16] DGGE is based on sequence-specific separation of equal-sized PCR products of the 16S rRNA gene on a polyacrylamide gel. DGGE facilitates the identification of a wider number of bacterial species within a genus than real-time PCR, in which the selection of specific primers restricts the

number of species that can be detected. DGGE also allows the detection of non-cultivable bacterial species, which could represent more than 50% of intestinal bacteria. [17 18]

The objective of this study was to determine the species composition of the genera *Bacteroides* and *Bifidobacterium*, and lactic acid bacteria (LAB), in duodenal biopsy specimens of patients with active and treated coeliac disease, and in those of control children, by PCR-DGGE, in order to obtain more detailed information on the possible contribution of specific species to the disease.

2. MATERIALS AND METHODS

2.1. Subjects

Biopsy specimens from three groups of children were included in this study: 20 patients with active coeliac disease, 12 patients with treated coeliac disease who had been following a gluten-free diet for at least 2 years, and 8 control children without known gluten intolerance. Clinical characteristics of the children are shown in table 1. Untreated patients with coeliac disease were on a normal gluten-containing diet, showed clinical symptoms of the disease, positive coeliac serology markers (anti-gliadin and anti-transglutaminase antibodies) and signs of severe enteropathy, classified as type 3 according to the Marsh classification of coeliac disease based on examination of duodenal biopsies. Patients with treated coeliac disease had been on a gluten-free diet for at least 2 years, showed negative coeliac serology markers, and possessed normal mucosa or infiltrative lesions classified as type 0-1 according to the Marsh classification.[19] Control children were presumptive patients with coeliac disease showing unspecific symptoms (eg, abdominal pain, difficulty to thrive, weight loss, etc), but the diagnosis was negative.

The children included in the study were not treated with antibiotics for at least 1 month before the sampling time. Ethics committee approval was secured for the study from Hospital Universitario de Valencia, Spain; Hospital La Fe Valencia, Spain, and Consejo Superior de Investigaciones Científicas, Madrid, Spain. Written informed consent was obtained from subjects (or their guardians).

Table 1: Clinical characteristics of the children included in this study

	Active coeliac n=20 (%)	Treated coeliac n=12 (%)	Control n=8 (%)
Mean age (months and SD)	51.1±31.8	54.9±25.6	50.1±31.2
Gender (Male/Female)	12(60)/8(40)	7(58)/5(42)	4(50)/4 (50)
Symptoms			
Abdominal pain	5 (25)	0 (0)	1 (12)
Diarrhoea	17 (85)	0 (0)	0 (0)
Weight loss	11 (55)	2 (16)	5 (62)
Anaemia	7 (25)	3 (25)	0 (0)
Anti-gliadin antibodies (AGA+)	20 (100)	0 (0)	0 (0)
Anti-transglutaminase antibodies (tTG+)	20 (100)	0 (0)	0 (0)
Duodenal Biopsy*			
M0-1-2	0 (0)	12 (100)	8 (100)
M3	100 (100)	0 (0)	0 (0)
HLA genetic typing (DQ2/DQ8)	20 (100)	12 (100)	0 (0)
Additional diseases			
Iron deficiency	5 (25)	0 (0)	0 (0)
IgA deficiency	2 (10)	0 (0)	0 (0)

*M0, normal mucosa; M0-1, (infiltrative lesion) seen in patients on a gluten-free diet, and family members of patients with coeliac disease; M2, (hyperplastic type) seen occasionally in dermatitis herpetiforme; M3, 40 intraepithelial lymphocytes per 100 enterocytes, crypts increased and villi atrophy seen in typical cases of coeliac disease. Modified Marsh classification of coeliac disease.[19]

2.2. Sampling preparation and DNA extraction

Duodenal biopsy specimens were obtained by upper intestinal endoscopy or capsule, frozen immediately at -80ºC and kept until further processing. Each specimen (10-15 mg) was diluted 1:10 (w/v) in phosphate-buffered saline (pH 7.2), homogenised by thorough agitation and used for DNA extraction.

DNA from pure cultures of reference bacterial strains (table 2) and from biopsy samples were extracted by using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Genomic DNA concentration was measured using the NanoDrop 2000 spectrophotometer.

Table 2. Bacterial reference strains used as ladder for the identification of *Bacteroides, Bifidobacterium,* LAB species by PCR-DGGE.

Strain	**Source**[a]
Bacteroides distasonis DSM 20701	DSM
Bacteroides fragilis DSM 2451	DSM
Bacteroides ovatus DSM 1896	DSM
Bacteroides thetaiotaomicron DSM 2079	DSM
Bacteroides vulgatus DSM 1447	DSM
Bacteroides coprocola	This study
Bacteroides dorei	This study
Bacteroides masiliensis	This study
Bacteroides uniformis	This study
Bifidobacterium adolescentis LMG 11037T	LMG
Bifidobacterium animalis subsp. *animalis* LMG 10508T	LMG
Bifidobacterium animalis subsp. *lactis* DSM 10140T	DSM
Bifidobacterium angulatum LMG 11039T	LMG
Bifidobacterium. bifidum LMG 11041T	LMG
Bifidobacterium dentium CECT 687	CECT
Bifidobacterium longum subsp *longum* CECT 4503	CECT
Bifidobacterium pseudocatenulatum CECT 5776	CECT
Lactobacillus acidophilus DSM 20079T	DSM
Lactobacillus brevis LMG 7944T	LMG
Lactobacillus sakei LMG 9468T	LMG
Lactobacillus casei ATCC 393T	ATCC
Lactobacillus curvatus LMG 9198T	LMG
Lactobacillus plantarum ATCC 14917T	ATCC
Lactobacillus reuteri LMG 9213T	LMG
Lactobacillus ruminis LMG 10756T	LMG
Pediococcus acidilactici LMG 11384T.	LMG

[a] LMG, Belgian Coordinated Collections of Microorganisms; DSM, German Collection of Microorganisms and Cell Cultures; CECT, Spanish Type Culture Collection; ATCC, American Type culture Collection.

2.3. PCR amplification and DGGE analyses

The bacterial reference strains used as reference ladder for the identification of *Bacteroides*, *Bifidobacterium* and LAB species by PCR-DGGE are shown in table 2. The 16S rRNA gene of *Bacteroides*, *Bifidobacterium* and the LAB group present in biopsy specimens was partially amplified with the primer pair listed in table 3. For amplification of the 16S rRNA gene of *Bacteroides*, *Bifidobacterium* and the LAB group present in biopsy specimens, three separate PCR reactions were performed. Each PCR mixture (30 µl) contained 3 µl 10 X buffer stock (containing 1.5 mM $MgCl_2$), 1.5 µl bovine serum albumin (10 mg/ml), 0.5 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 2.5 U Taq polymerase (Ecotaq; Ecogen, Barcelona, Spain) and 30 ng genomic DNA. One PCR core program was used for all amplifications: an initial denaturation step at 95ºC for 3 min; 35 cycles of denaturation at 94ºC for 30 s, annealing at a primer-specific temperature (table 3) for 1 min, and extension at 72ºC for 1 min; and a final extension step at 72ºC for 7 min. PCR amplification products of 293, 520 and 340 bp, representing *Bacteroides*, *Bifidobacterium* and LAB, respectively, were checked by electrophoresis in ethidium-bromide-stained 1.5% agarose gels and stored at 20ºC. Visualisation of the LAB population required the inclusion of a nested PCR, using 27δ and 1238-r primers as an initial amplification round.[23]

DGGE analysis of PCR amplicons was carried out on the Dcode Universal Mutation Detection System (Bio-Rad, Richmond, California, USA), essentially as described previously.[24] The linear denaturing gradients of urea and formamide used for PCR product separation were 25-45%, 40-50% and 25%-45% for *Bacteroides*, *Bifidobacterium* and LAB species, respectively. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. Selected unknown DGGE bands (S1-S11) were excised from the denaturing gels and checked using a new DGGE. Then, the PCR products were purified using the GFXtm PCR DNA and Gel Band DNA Purification Kit (GE Healthcare Chalfont St Giles, UK). The identification of the unknown DGGE bands was carried out by DNA sequencing using an ABI PRISM-3130XL Genetic Analyser (Applied Biosystems, Foster City, California, USA). Search analyses to determine the closest relatives of the partial 16S rRNA gene

sequences retrieved were conducted in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm. Sequences with more than 97% similarity were considered to be of the same specie.

Table 3. Primers used in the study.

Target group	Primer	Primer sequence (5´-3´)	Amplicon size (bp)	Annealing temp (°C)	DGGE gradient range (%)[a]	References
Bacteroides	Bfra531-f	ATACGGAGGATCCGAGCGTTA	293	60	25-45	18
	Bfra766-r-GC	GC clamp[b]-CTGTTTGATACCCACACT				
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	520	62	40-50	19
	Bif662-GC-r	GC clamp[c]-CACCGTTACACCGGGAA				
LAB group[d]	Lac1	AGCAGTAGGGAATCTTCCA	340	61	25-45	20
	Lac2-GC	GC clamp[c]-ATTYCACCGCTACACATG				
Nested PCR	27-δ	AGAGTTTGATCCTGGCTCAG	1200	54		21
	1220-r	TTGTAGCACGTGTGTAGCCC				

[a] A 100% denaturant acrylamide was defined at 7 M urea and 40% (v/v) formamide.

[b] GC clamp sequence: GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

[c] GC clamp sequence: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC

[d] LAB group which comprises the genera *Lactobacillus* and other such as *Leuconostoc*, *Pediococcus* and *Weisella*.

2.4. Statistical and clustering analyses

Similarities between the banding patterns generated by PCR-DGGE analyses were analysed using the Dice coefficient and the unweighted-pair group method with the arithmetic average (UPGMA) clustering algorithm and were shown graphically as a dendrogram. The Shannon-Wiener index of diversity (H')[25] was used to determine the diversity of taxa present in biopsy specimens of patients with active and treated coeliac disease, and in those of control children. The diversity index was calculated using the following equation:

$$\text{Shannon-Wiener index } (H') = - \sum_{i=1}^{s} pi \; ln \; p_i$$

where *s* is the number of species in the sample and *pi* is the proportion of species *i* in the sample. Generally, the diversity data were non-uniformly distributed; therefore, a nonparametric analysis using a Mann-Whitney U test was performed. Analyses were carried out with the PAST software (PAlaeontological Statistics), and statistical significance was established at P values < 0.05.

Differences in species composition in every DGGE profile between active and treated coeliac disease groups and control group were analyzed using the x^2 tests. Analyses were carried out with the STATGRAPHICS software (Manugistics, Rockville, MD), and statistical significance was established at P values <0.05.

RESULTS

2.5. DGGE analysis of Bacteroides *species*

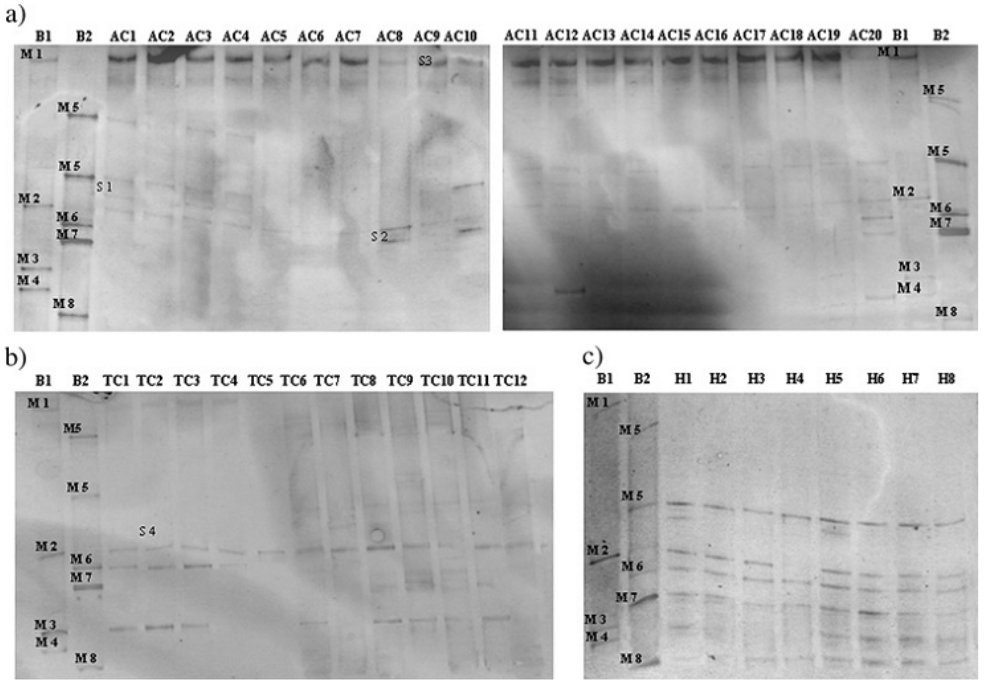

Figure 1. DGGE profiles of 16S rRNA partial gene from *Bacteroides* species detected in DNA from biopsy specimens of patients with active (A) and treated (B) coeliac disease and control children (C), with the primers Bfra531-f/Bfra766-r-GC. Lanes B1 and B2 include *Bacteroides* identification reference ladders: M1, *B. dorei*; M2, *B. massiliensis*; M3, *B. coprocola*; M4, *B. uniformis*; M5, *B. vulgatus*; M6, *B. ovatus*; M7, *B. thetaiotaomicron*/*B. fragilis*; M8, *B. distasonis*. Numbered bands (S) refer to sequenced fragments whose amplicon identifications and closest relatives are shown in table 4.

The DGGE profiles of PCR amplicons obtained with *Bacteroides*-specific primers from patients with active and treated coeliac disease and control children are shown figure 1. It was not possible to differentiate between the species pair *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* because the two DNA bands had the same migration distance. Clustering analysis (Dice/UPGMA) of the DGGE *Bacteroides* profiles showed two differentiated

clusters: cluster I grouped DGGE *Bacteroides* profiles from the control group samples at 72% similarity; and cluster II was divided into two major subgroups, one containing DGGE *Bacteroides* profiles from patients with treated coeliac disease clustered at 64% similarity, and another containing profiles from patients with active and treated coeliac disease clustered at 58% similarity figure 2).

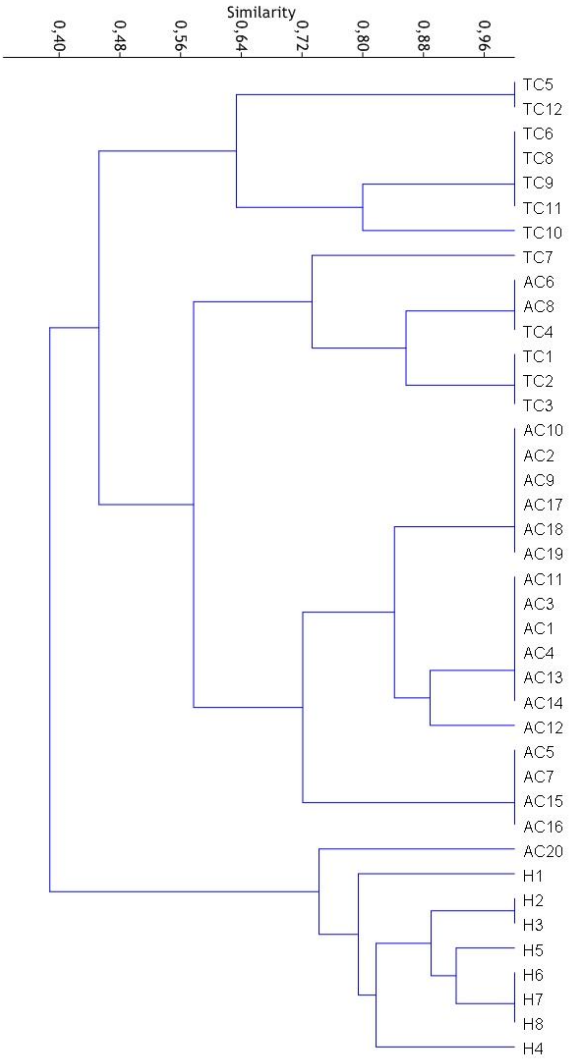

Figure 2 Dendrogram derived from DGGE analysis of 16S rRNA gene fragments of *Bacteroides* species from biopsy specimens of control children (H), and patients with active coeliac (AC) and treated coeliac (TC) disease, using Bfra531f and Bfra766r-GC primers based on Dice's similarity index and the UPGMA clustering algoritm.

The DGGE *Bacteroides* profiles revealed that control children showed the greatest biological diversity, with five to seven bands (*H´*=1.73), followed by patients with active coeliac disease with two to four bands (*H´*=1.19, p=0.005) and patients with treated coeliac disease with one to three amplicons (*H´*=0.82, p=0.002).

The PCR amplicons that were identified by sequencing are shown in table 4. The prevalence of different *Bacteroides* species detected by PCR-DGGE in biopsy specimens from the children included in this study is shown in table 5. Amplicons most closely related to *Bacteroides distasonis*, *B. fragilis*/*B. thetaiotaomicron* and *Bacteroides uniformis* were significantly more abundant in DGGE profiles from samples of the control group than from those of patients with coeliac disease, regardless of the status of the disease ($p<0.05$). The prevalence of *Bacteroides ovatus* was also higher in biopsy samples from the control group than in those from treated and active coeliac disease groups ($p<0.05$). Significant differences were also observed for *B. ovatus* between biopsy specimens of patients with treated and active coeliac disease ($p<0.05$). *Bacteroides vulgatus* was more frequently detected in control children than in samples from patients with treated coeliac disease ($p<0.01$). Amplicons most closely related to *Bacteroides dorei* were more frequently detected in samples from patients with active coeliac disease than in those from patients with treated coeliac disease and control children ($p<0.01$), and *Bacteroides plebeius* was more frequently detected in biopsy samples from patients with active disease than in those from patients with treated coeliac disease ($p=0.03$). In contrast, *Bacteroides coprocola* was more frequently detected in samples from patients with treated coeliac than in samples from patients with active coeliac disease and control samples ($p<0.01$ and $p=0.02$, respectively).

Table 4. *Bacteroides* and lactic acid bacteria (LAB) species identified by sequencing the PCR-DGGE bands obtained with different group-specific primers from biopsy specimens of the active and treated coeliac disease patients and control groups.

Amplicon ID*	Closest relative (Accession number)†	Identity (%)
Bacteroides spp.		
S 1	*Bacteroides plebeius* (AB200222)	97
S 2	*Bacteroides ovatus* (DQ 100446)	100
S 3	*Bacteroides dorei* (EU722737)	98
S 4	*Bacteroides massilienssis* (EU136696)	97
LAB		
S 5	*Lactobacillus fermentum* (FJ172345)	99
S 6	*Weisella soli* (AY028260)	98
	Weisella kimchii (AY281294)	98
	Weissella cibaria (FJ390109)	98
S 7	*Leuconostoc citreum* (FJ378896)	99
S 8	*Weisella cibaria* (AJ295989)	98
	Weisella confusa (AB023241)	98
S 9	*Pediococcus pentosaceus* (AB481102)	100

*Identification labels corresponding to DGGE bands shown in Fig. 1 and 3.

†Accession number of the closets relatives as determined by searching analysis conducted in GenBank using the BLAST algorithm.

Table 5. *Bacteroides*, *Bifidobacterium* and LAB species detected by PCR-DGGE analysis from biopsy specimens of active and treated coeliac disease patients and controls children using group-specific primers.

Bacterial group	Active coeliac n=20 (%)	Treated coeliac n=12 (%)	Controls n=8 (%)
Bacteroides spp.			
Bacteroides coprocola	0 (0%) [a]	8 (67%) [b]	1 (13%) [a]
Bacteroides distasonis	0 (0%) [a]	0 (0%) [a]	8 (100%) [b]
Bacteroides dorei	20 (100%) [a]	4 (33%) [b]	0 (0%) [b]
Bacteroides masiliensis	20 (100%)	12 (100%)	8 (100%)
Bacteroides ovatus	3 (15%) [a]	6 (50%) [b]	8 (100%) [c]
Bacteroides plebeius†	7 (35%) [a]	0 (0%) [b]	2 (25%) [a,b]
Bacteroides thetaiotaomicron/fragilis	1 (5%) [a]	0 (0%) [a]	8 (100%) [b]
Bacteroides uniformis	2 (10%) [a]	0 (0%) [a]	4 (50%) [b]
Bacteroides vulgatus	14 (70%) [a]	0 (0%) [b]	8 (100%) [a]
Bifidobacterium spp.			
Bifidobacterium adolescentis	10 (50%) [a]	0 (0%) [b]	0 (0%) [b]
Bifidobacterium animalis ssp. *lactis*	11 (55%) [a]	0 (0%) [b]	0 (0%) [b]
Bifidobacterium catenulatum†	2 (10%)	4 (33%)	3 (38%)
Bifidobacterium dentium	4 (20%)	0 (0%)	0 (0%)
Bifidobacterium longum bv. *infantis*†	1 (5%)	4 (33%)	0 (0%)
Bifidobacterium longum bv. *longum*	3 (15%)	2 (17%)	0 (0%)
Bifidobacterium pseudocatenulatum	4 (20%)	4 (33%)	0 (0%)
LAB			
Lactobacillus casei	7 (35%)	6 (50%)	6 (75%)
Lactobacillus fermentum†	4 (20%) [a]	12 (100%) [b]	2 (25%) [a]
Lactobacillus plantarum	17 (85%)	7 (58%)	8 (100%)
Lactobacillus sakei	8 (40%)	4 (33%)	6 (75%)
Lactobacillus reuteri	0 (0%) [a]	4 (33%) [b]	0 (0%) [ab]
Lactobacillus ruminis	0 (0%)	0 (0%)	1 (13%)
Leuconostoc citreum†	2 (10%)	0 (0%)	2 (25%)
Pediococcus acidolactici	0 (0%)	0 (0%)	1 (13%)
Pediococcus pentosaceus†	0 (0%) [a]	5 (45%) [b]	0 (0%) [ab]
Weisella spp.†	2 (10%) [a]	6 (50%) [b]	0 (0%) [a]

Different letters (a to c) in the same row and within the same bacterial group indicate significant differences between the groups of the studied children. Significant differences were established at $P< 0.05$ by using the χ^2 test.

†Species identification by sequencing the PCR-DGGE bands.

2.6. DGGE analysis of bifidobacterial species

DGGE profiles for *Bifidobacterium* species contained zero to four bands (data not shown) and revealed that the *Bifidobacterium* community associated with biopsy samples in control children was significantly less diverse (0-1 amplicons, H'=0.00) than that from patients with treated (0-3 amplicons, H'=0.3, $p<0.04$) and active coeliac disease (0-4 amplicons, H'=0.48, $p<0.03$). Due to the simplicity of *Bifidobacterium* -specific DGGE profiles, no cluster analysis was performed.

The bifidobacterial PCR products identified by sequencing are shown in table 4. The prevalence of the different *Bifidobacterium* species detected by PCR-DGGE in biopsy samples of patients with active and treated coeliac disease and the control group is shown in table 5. Amplicons most closely related to *Bifidobacterium adolescentis* and *Bifidobacterium animalis* subsp *lactis* were more commonly detected in biopsy samples from patients with active coeliac disease than in samples from patients with treated coeliac disease and controls ($p<0.03$ and $p<0.001$, respectively). *Bifidobacterium catenulatum* was only detected in biopsy samples of the control group.

2.7. DGGE analysis of LAB

The DGGE profiles of PCR amplicons obtained with LAB primers from patients with active and treated coeliac disease and control children are shown in figure 3. Clustering analysis did not allow differentiation of samples according to disease status (data not shown).

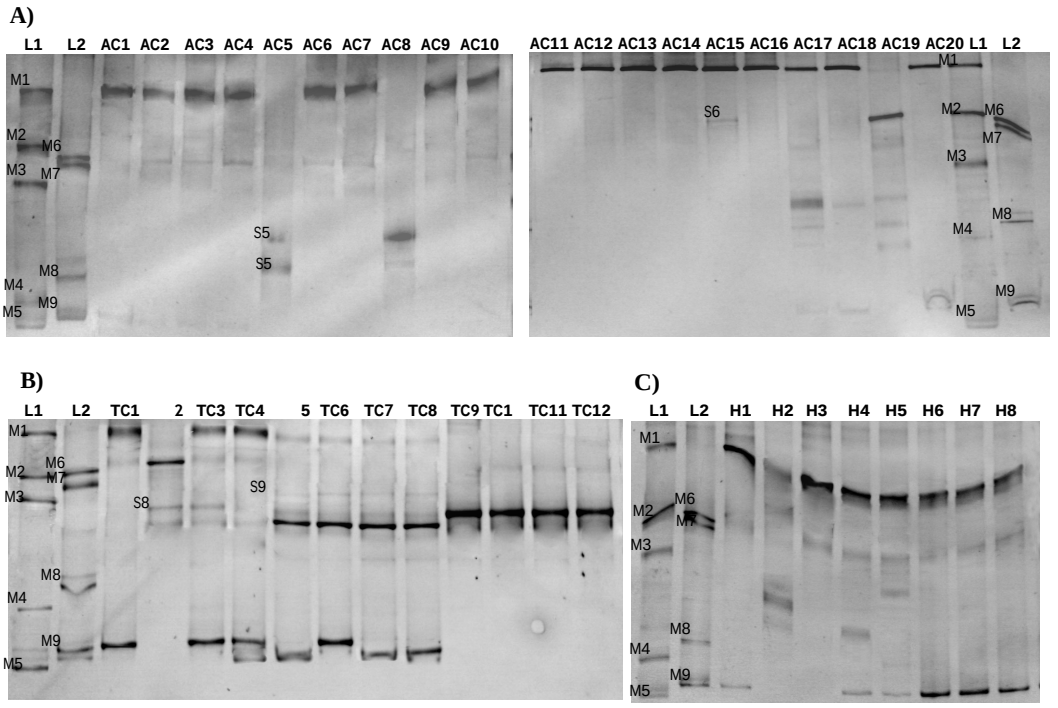

Figure 3 DGGE profiles of 16S rRNA gene fragments from lactic acid bacteria detected in DNA from biopsy specimens of patients with active (A) and treated (B) coeliac disease, and control children (C), with the primers Lac1 and Lac2-GC. Lanes L1 and L2 include the lactic acid bacteria identification reference ladders: M1, *Lactobacillus plantarum*; M2, *Lactobacillus sakei*; M3, *Lactobacillus acidophilus*; M4, *Pediococcus acidilactici*; M5, *Lactobacillus reuteri*; M6, *Lactobacillus brevis*; M7, *Lactobacillus curvatus*; M8, *Lactobacillus ruminis*; M9, *Lactobacillus casei*. Numbered bands (S) refer to sequence fragments whose amplicon identifications and closest relatives are shown in table 4.

DGGE profiles showed that the LAB diversity was higher in biopsy specimens from patients with treated coeliac disease (*H´*=3.58) and control children (*H´*=3.25) than in those from patients with active coeliac disease (*H´*=2.05, =0.003 and p=0.01, respectively).

The PCR amplicons that were identified by sequencing are shown in table 4. The prevalence of the different LAB species detected by PCR-DGGE in biopsy samples of patients with active and treated coeliac disease and the control group

is shown in table 5. The prevalence of amplicons related to the genus *Weissella* and to *Lactobacillus fermentum* was significantly higher in patients with treated coeliac disease than in controls and patients with active coeliac disease ($p<0.05$). The same trend was found for *Lactobacillus reuteri* and *Pediococcus pentosaceus*, but differences were only significant between patients with treated and active coeliac disease ($p=0.01$). The control group showed higher prevalence of *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus ruminis*, *Leuconostoc citreum* and *Pediococcus acidilactici* amplicons than children with active and treated coeliac disease, but these differences were not statistically significant.

3. DISCUSION

The present study confirms the hypothesis that typical cases of coeliac disease are associated with intestinal dysbiosis, which involves specific bacterial species. In this study, a decrease in the *Bacteroides* diversity index was shown for duodenal samples from patients with coeliac disease compared with those from control individuals. This may mean that the increased *Bacteroides* counts detected in biopsy specimens from children with coeliac disease[11 12] could involve only a limited number of *Bacteroides* species that could magnify their individual effects in these patients. Furthermore, it was shown that the duodenal *Bacteroides* species profiles of patients with treated coeliac disease were more similar to those of patients with active coeliac disease than to those of controls. This finding indicates that a gluten-free diet does not lead to a complete restoration of the balance of *Bacteroides* species in the duodenal mucosa of patients with coeliac disease. In patients with active coeliac disease, histological lesions can lead to nutrient malabsorption, which could result in modifications in the composition of the intestinal microbiota. However, alterations in the duodenal microbiota were detected in untreated and treated patients without mucosal lesions; therefore, nutrient malabsorption cannot be considered the only reason for such microbial changes. With a few exceptions,[26 27] in patients with inflammatory bowel disease (IBD) the concentrations of *Bacteroides* associated with the mucosa were also shown to be higher and increased with the severity of the disease.[28-31]

Due to the different potential pathogenicity of *Bacteroides* species, related to a variety of virulence factors, [32 33] the identification of specific *Bacteroides* species associated with a disease is epidemiologically important. In this context, some studies have suggested a relationship between enterotoxigenic *B. fragilis* and clinically-active IBD in humans [34-36] and in animal models.[36 37] Patients with Crohn disease have also been shown to have a lower relative abundance of *B. uniformis* and higher prevalence of *B. ovatus* and *B. vulgatus*.[38] It has been proposed that *B. vulgatus* and *B. ovatus* could be implicated in the disruption of the integrity of the intestinal epithelial barrier, thereby contributing to the initiation of the inflammatory response of IBD.[39-41] In

contrast, the present study reports an increased prevalence of *B. dorei* and a decreased prevalence of *B. ovatus* and *B. uniformis* in patients with coeliac disease.

The *Bifidobacterium* population associated with the duodenum of the children included in this study was relatively simple. This simplicity in the mucosa-associated bifidobacterial populations has also been reported in biopsy samples from the ascending, transverse and descending parts of the colon of healthy individuals[42]. In our study, the prevalence of *B. adolescentis* and *B. animalis* subsp *lactis* was higher in patients with active coeliac disease than in patients with treated coeliac disease and controls. In contrast, a previous study using real-time PCR showed that the prevalence of *B. animalis* subsp *lactis* was higher in groups with treated coeliac disease groups than in those with active coeliac disease and control groups.[14] Also, in that study, the prevalence of *B. catenulatum* was found to be higher in biopsy samples from controls than in those from patients with active and non-active coeliac disease.[14] *B. catenulatum* was the only species found in control children in the present study. It is thought that the composition of *Bifidobacterium* species could influence host immune responses in inflammatory conditions.[43] [44] The prevalence of different *Bifidobacterium* species in the microbiota of infants has been suggested to be related to the incidence of allergic diseases.[45] *Bifidobacterium* species have been demonstrated to have a species-specific and strain-specific influence on immunity, and could exert different effects on the T-helper 1 pro-inflammatory response characteristic of the coeliac disease.[46] However, the association between the prevalence of different *Bifidobacterium* species and coeliac disease is still unclear.

Although the LAB present in the gut depend greatly on dietary intake, these species were analysed because they are also part of the indigenous microbiota, and are of interest for their possible roles as probiotics in inflammatory conditions. DGGE analysis showed that LAB profiles were complex and specific to each host. The diversity index was higher in patients with treated coeliac disease than in patients with active coeliac disease and controls. In healthy subjects, lack of stability in this bacterial group has been described, and

has been attributed to the fact that a significant proportion of LAB populations are related to food-associated species.[22,47] *Lactobacillus* species showing differences in prevalence between patients with treated coeliac disease and those with active coeliac disease, have been reported to be regularly present in fermented foods.[48] Therefore, some of the differences detected in our study could be due to the disease and also to dietary differences.

In summary, this study has demonstrated changes in diversity and species composition of the genera *Bacteroides*, *Bifidobacterium* and LAB in the duodenal microbiota of Spanish children with typical coeliac disease compared with those of control children. The changes detected in the microbiota of patients with active and treated coeliac disease do not seem to be completely dependent on the inflammatory status of the mucosa, particularly in the case of *Bacteroides*, whose potential role in the pathogenesis of the disease deserves further investigation.

4. ACKNOWLEDGEMENTS

This work was supported by grants AGL2007-66126-C03-01/ALI and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Science and Innovation. The scholarship to E. Sánchez from Institute Danone is fully acknowledged.

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Capítulo II:

Aislar, identificar y caracterizar los factores de virulencia de los géneros y especies bacterianas (enterobacterias, bacteroides y estaflilococos) posiblemente implicadas en la enfermedad celíaca a partir de heces de individuos celíacos y compararlos con los aislados de individuos sanos.

- *Reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of coeliac children*
- *Intestinal* Staphylococcus *spp. and virulent features associated with coeliac disease*
- *Discerning the role of* Bacteroides fragilis *in celiac disease pathogenesis*

Capítulo II

Reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of coeliac children

ABSTRACT

Background: Coeliac disease is an immune-mediated enteropathology triggered by the ingestion of cereal gluten proteins. This disorder is associated with imbalances in the composition of the gut microbiota that could be involved in its pathogenesis. The aim of the present study was to determine whether intestinal *Enterobacteriaceae* populations of active and non-active coeliac patients and healthy children differ in diversity and virulence-gene carriage, so as to establish a possible link between the pathogenic potential of enterobacteria and the disease.

Methods: *Enterobacteriaceae* clones were isolated on VRBD agar from faecal samples of 31 subjects (10 active coeliac patients, 10 symptom-free coeliac patients and 11 controls) and identified at species level by the API 20E system. *Escherichia coli* clones were classified into four phylogenetic groups A, B1, B2 and D and the prevalence of eight virulence-associated genes (type1 fimbriae (*fimA*), P fimbriae (*papC*), S fimbriae (*sfaD/E*), Dr haemagglutinin (*draA*), haemolysin (*hlyA*), capsule K1 (*neuB*), capsule K5 (*KfiC*) and aerobactin (*iutA*) was determined by multiplex PCR.

Results: A total of 155 *Enterobacteriaceae* clones were isolated. Non-*E. coli* clones were more commonly isolated in control children than in coeliac patients. The four phylogenetic *E. coli* groups were equally distributed in control children, while in both coeliac patients most commensal isolates belonged to group A. Within the virulent groups, B2 was the most prevalent in active coeliac disease children, while D was the most prevalent in non-active coeliac patients. *E coli* clones of the virulent phylogenetic groups (B2+D) from active and non-active coeliac patients carried a higher number of virulence genes than those from control individuals. Prevalence of P fimbriae (*papC*), capsule K5 (*sfaD/E*) and

haemolysin (*hlyA*) genes was higher in *E. coli* isolated from active and non-active coeliac children than in those from control subjects.

Conclusion: This study has demonstrated that virulence features of the enteric microbiota are linked to coeliac disease.

1. INTRODUCTION

The human gastrointestinal tract is a complex ecosystem integrated by up to 10^{14} bacteria. These microorganisms may belong to more than 500 different bacterial species, although 99% of the total community consists of only 30–40 species. The intestinal microbiota plays an important role in human health as they contribute to inhibiting pathogen colonization, boosting the immune system, and metabolising nutrients.[1-3] Alterations in the intestinal microbiota have also been linked to inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, as well as to other immune-related disorders. IBD patients have been shown to carry higher concentrations of mucosa-associated bacteria than control groups. In addition, the gut microbiota of these patients was characterized by a decrease in protective bacteria, such as *Bifidobacterium* and *Lactobacillus*, and an increase in harmful bacterial groups like *Bacteroides* and *Escherichia coli*. This phenomenon has been named "dysbiosis".[4,5]

Escherichia coli was the first bacterial species to be identified in human gastrointestinal samples. This widespread commensal Gram-negative bacterium found in the intestinal tract of healthy individuals can occasionally cause intestinal and extraintestinal diseases. The pathogenic potential of *E. coli* stains could be influenced by host endogenous factors, as well as by the genetic structure and ecological distribution of the strains in a particular host. Human infections by *E. coli* strains commonly occur in immunocompromised individuals or when the normal gastrointestinal barrier is broken,[6] enabling opportunistic pathogens to evade host surveillance mechanisms. Phylogenetic studies based on the analysis of 38 metabolic enzymes, have shown that *E. coli* strains fall into four main phylogenetic groups, denominated "A", "B1", "B2" and "D".[7] Strains causing extra-intestinal infections mainly belong to group B2, and to a lesser extent to group D, whereas commensal *E. coli* strains mostly belong to A and B1 groups.[8] *E. coli* strains belonging to B2 and D groups carry more virulence-associated genes than strains from A and B1 groups.[9,10] Therefore, the presence or absence of certain *E. coli* strains or an altered distribution of their relative abundance can enhance the pathogenic potential of the enteric population in predisposed individuals.

Coeliac disease is an immune-mediated enteropathology triggered by the ingestion of wheat-gluten and similar proteins of rye, barley and, probably, oats. Coeliac disease can be present at any age with a variety of clinical features; however, typical cases manifest in early childhood with gastrointestinal symptoms.[11] In coeliac patients, the inflammatory milieu caused by gluten antigens could lead to imbalances in gut microbial composition, characterised by higher numbers of Gram-negative bacteria and decreased numbers of beneficial Gram-positive bacteria, as compared to healthy individuals. In previous studies, *E. coli* proportions were significantly higher in duodenal biopsies of active coeliac patients than in controls;[12] moreover, a similar trend was detected in faeces although the differences were not so remarkable.[13] However, little is known about the possible association between enterobacterial population and coeliac disease pathogenesis.

The aim of the present study was to determine whether intestinal *Enterobacteriaceae* populations of active and non-active coeliac patients and healthy children differ in diversity and virulence-gene carriage, so as to establish a possible link between the pathogenic potential of enterobacteria and the disease.

2. MATERIALS AND METHODS

2.1. Subjects and sampling

Thirty-one children were included in this study, 10 active coeliac patients, on a normal gluten-containing diet, showing clinical symptoms of the disease, positive coeliac serology markers and signs of severe enteropathy by duodenal biopsy examination (mean age 3.86 years, range 1.0–8.86 years); 10 symptom-free coeliac patients (non-active coeliac), who had been on a gluten-free diet for 1–2 years (mean age 6.2 years, range 1.0–12.0 years); and 11 control children without known food intolerance (mean age 3.51 years, range 0.1–7.75 years). None of the children included in the study had been treated with antibiotics for at least 1 month before the sampling time. Faecal samples were collected and immediately stored at 4°C, under anaerobic conditions (AnaeroGen, Oxoid, Hampshire, UK), and analysed in less than 12 h. The study protocol was approved by the local Ethics Committee, and children were enrolled in the study after written informed consent was obtained from their parents.

2.2. Isolation and identification of Enterobacteriaceae *clones from faeces*

Faecal samples (2 g wet weight) were 10-fold diluted in phospahate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, [pH 7.2]), and homogenized in a Lab Blender 400 Stomacher (Seward Medical, London, UK). Serial dilutions were made in PBS and aliquots were plated on Violet Red Bile Dextrose Agar, VRBD (Scharlau, Barcelona, Spain) and incubated under aerobic conditions at 37°C for 48 h. Then, 5 individual *Enterobacteriaceae*-like colonies differing in size, shape, colour or mucoid appearance on VRBD agar were isolated and subcultured in Plate Count Agar (Scharlau) under aerobic conditions at 37°C for 24 h. The identity of the presumptive *Enterobacteriaceae* isolates was confirmed by conventional microbiological methods, including colony and cellular morphology and Gram staining. The selected isolates were further identified at species level by using the API20E system (BioMerieux, Lyon, France), in order to select *E. coli* and non-*E. coli* clones.

2.3. Phylogenetic classification and virulence-associated gene carriage of E. coli *isolates*

Escherichia coli isolates were assigned to one of the four phylogenetic groups A, B1, B2 or D by a triplex PCR technique as previously described.[14] The 25 µl PCR mixture consisted of 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 1 µM of each primer pair for the genes *ChuA, YjaA* and *TspE4C2*, 200 µM, dNTPs and 2.5 U of Taq polymerase (Ecotaq, ECOGEN, Spain). A piece of a bacterial colony was collected directly from a plate and added to the reaction mixture as a source of DNA. The PCR programme was as follows: initial denaturalization at 94°C for 4 min; and 30 cycles of 5 s at 94°C, 10 s at 59°C; and a final extension step of 5 min at 72°C. The PCR products were separated in a 2% agarose gel electrophoresis and visualised by ethidium-bromide staining. The isolates were assigned to phylogenetic groups as follows: $ChuA^+YjaA^+$, group B2; $ChuA^+YjaA^-$, group D; $ChuA^-TspE4C2^+$, group B1; $ChuA^-TspE4C2^-$, group A.

The same isolates were also characterized by two sets of multiplex PCR as described by Nowrouzian et al. (2001).[15] The first PCR detected the presence of the following virulence-associated genes: *fimA* (type-1 fimbriae), *papC* (P fimbriae), *sfaD/E* (S fimbriae) and *draA* (Dr haemagglutinin). The second PCR detected the presence of the following genes: *hlyA* (haemolysin), *neuB* (capsule K1), *KfiC* (capsule K5) and *iutA* (aerobactin). The 25 µl PCR reaction mixture consisted of 10 mM Tris HCl (pH 8.3), 1.5 mM of $MgCl_2$ for the first multiplex PCR and 2.0 mM $MgCl_2$ for the second multiplex PCR, 0.45 µM of each primer, 100 µM dNTPs and 2.5 U of Taq polymerase (Ecotaq, ECOGEN, Spain). A piece of a bacterial colony was used as a source of DNA as indicated above.

2.4. Statistical analyses

Differences in prevalence of different species, phylogenetic groups or virulence genes were established by applying the chi-square test, and when appropriate by the two tailed Fisher's exact test and Kolmogorov-Smirnof test. In each case, analyses were carried out with the Statgraphics software (Manugistics, Rockville, MD), and statistical differences were established at *P* value below 0.05.

3. RESULTS

3.1. Diversity of Enterobacteriaceae and E. coli *clones from coeliac and healthy children*

Of the 31 individuals analyzed in this study, in only 14 (45%) *Enterobacteriaceae* species other than *E. coli* were isolated. The latter species was always recovered in healthy individuals and in eight of them (73%) at least one other non-*E. coli* species was detected. Non-*E. coli* species were detected in only four (40%) active and two non-active coeliac patients (20%). Non-*E. coli* clones were recovered more abundantly in control children than in non-active coeliac patients ($P = 0.03$).

Citrobacter freundii, Enterobacter agglomerans, Klebsiella oxytoca and *Escherichia vulneris* were identified in control children harbouring non-*E. coli* species. *Klyuvera* spp, *Enterobacter cloacae, Citrobacter freundii, Shigella* spp, *Chryseomonas luteola* and *Klebsiella oxytoca* were also isolated in active coeliac disease children and *Klebsiella oxytoca, Aeromonas hydrophila/caviae* and *Klebsiella pneumoniae* were identified in non-active coeliac patients.

3.2. Phylogenetic classification of E. coli *clones from coeliac and healthy children*

The phylogenetic classification of *E. coli* clones isolated from faeces of the three groups of children is shown in Figure 1. The total number of isolates belonging to both commensal groups (A+B1) and the total number of isolates belonging to both virulent groups (B2+D) did not differ significantly between the three populations of children under study. However, differences were observed in the abundance of each of the four phylogenetic groups. In control children, A and B1 *E. coli* groups were equally distributed, while in active and non-active coeliac patients almost all the commensal isolates belonged to group A ($P<0.001$), revealing lower *E. coli* diversity in clones from coeliac patients. Significant differences were found between the prevalence of virulent clones isolated from active or non-active coeliac patients and healthy children. In controls, virulent isolates were equally represented by both groups B2 and D. In

contrast, group B2 was the main virulent group isolated from active coeliac patients (*P*=0.0002), and group D was that most common in non-active coeliac patients (*P*=0.0001).

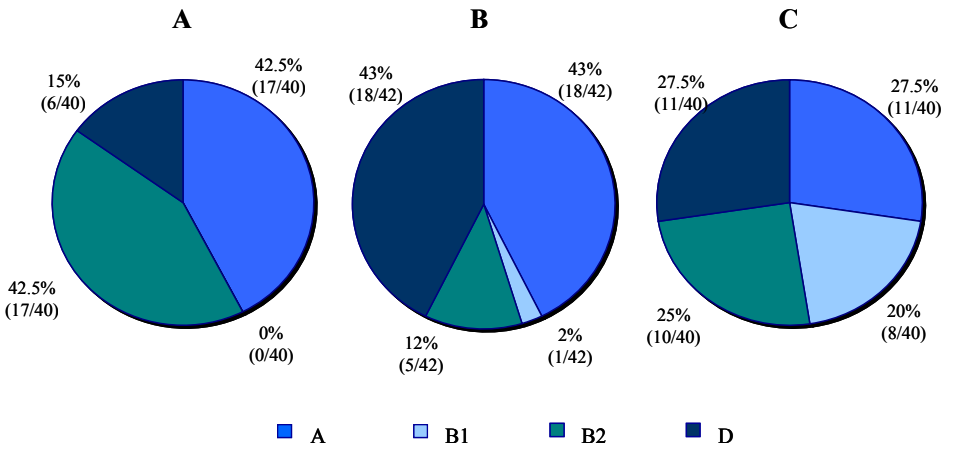

Figure 1. Distribution of the phylogenetic *E. coli* groups in active coeliac patients (A), non-active coeliacs (B) and control children (C). A and B1 commensal groups and B2 and D virulent *E. coli*. Data are expressed as percentage (number of positive clones/number of total analysed clones).

3.3. Virulence-associated gene carriage in E. coli *clones from coeliac and control children*

The prevalence of virulence-associated genes in commensal groups (A+B1) and virulent groups (B2+D) of *E. coli* clones isolated from active and non-active coeliac and control children are shown in Table 1. Overall, virulent *E. coli* clones (B2+D) from healthy individuals carried fewer virulence-associated genes than those from active and non active coeliac patients. Both active and non-active coeliac patients had a significantly higher prevalence of P fimbriae gene (*papC*), (*P*=0.002–0.050) than healthy controls. In addition, type-1 fimbriae gene (*fimA*) prevalence was higher in both active and non-active coeliac patients than in healthy controls but the differences were not significant (*P*=0.06). Capsule K1 gene (*neuB*) was also more prevalent in both active and non-active coeliac patients than in healthy controls; however, differences were not significant. Capsule K5 gene (*KfiC*) was significantly more common in virulent *E. coli* isolates from active coeliac children than in those from non-active coeliac children

(*P*=0.02), suggesting its association with the active phase of the disease. Haemolysin gene (*hlyA*) prevalence was higher in both coeliac disease subjects than in healthy controls, showing significant differences between non-active coeliac disease patients and controls (*P*=0.01). In commensal groups (A+B1), S fimbriae (*sfaD/E*) gene prevalence was significantly higher in non-active coeliac patients than in healthy and active coeliac patients (*P* =0.04 and *P*=0.02), whereas capsule K1 gene (*neuB*) was significantly less common in commensal *E. coli* isolates from active coeliac children than in the other groups (*P*<0.001). Moreover, aerobactin gene (*iutA*) was more common in commensal *E. coli* clones isolated from healthy controls than those from active coeliac individuals (*P*<0.001).

Table 1. Prevalence of virulence-associated genes in A+B1 (commensal) and B2+D (virulent) *E. coli* clones isolated from active and non-active coeliac patients and control children.

	Virulent B2 + D clones (%)			Commensal A + B1 clones (%)		
Virulence factor (gene)	Active coeliac (n=23)	Non-active coeliac (n=23)	Control (n=21)	Active coeliac (n=17)	Non-active coeliac (n=19)	Control (n=19)
Type 1 fimbriae (*fim A*)	18 (78)	18 (78)	10 (48)	11 (65)	14 (82)	16 (84)
S fimbriae (*sfaD/E*)	8 (35)	9 (39)	8 (38)	0 [a]	6 (35) [b]	1 (5) [a]
P fimbriae (*papC*)	5 (22) [a]	9 (39) [a]	0 [b]	0	0	4 (21)
Dr haemagglutinin (*draA*)	0	0	0	0	0	0
Haemolysin (*hlyA*)	5 (22) [ab]	9 (39) [b]	1 (5) [a]	0	1 (6)	4 (21)
K1 (*neuB*)	8 (35)	5 (22)	5 (24)	0 [a]	10 (59) [b]	8 (42) [b]
K5 (*KfiC*)	6 (26) [a]	0 [b]	2 (10) [ab]	0	0	1 (5)
Aerobactin (*iutA*)	4 (17)	4 (17)	7 (33)	1 (6) [a]	6 (35) [ab]	12 (63) [b]

[*]Different letters (a to b) in the same row and within the same phylogenetic group (virulent B2+D or commensal A+B1) shown significant differences in the gene-carriage.

Figure 2 shows the prevalence of virulence-associated genes in commensal groups (A+B1) and virulent groups (B2+D) of *E. coli* clones isolated in this study. S fimbriae (*sfaD/E*), P fimbriae (*papC*), haemolysin (*hlyA*) and capsule K5 (*KfiC*) genes were significantly more common in virulent *E. coli* clones than in commensal clones (P < 0.05). Accordingly, P fimbriae (*papC*), haemolysin (*hlyA*) and capsule K5 (*KfiC*) genes prevalence was associated with one or both coeliac patient groups, which would suggest that *E. coli* clones from patients have a higher pathogenic potential than those from controls. No differences were detected in the carriage of type 1 fimbriae (*fimA*), Dr haemagglutinin (*draA*), capsule K1 (*neuB*) and aerobactin (*iutA*) genes between the commensal and virulent *E. coli* groups. Furthermore, the distribution of these genes was either less remarkable or bore no relationship with coeliac disease.

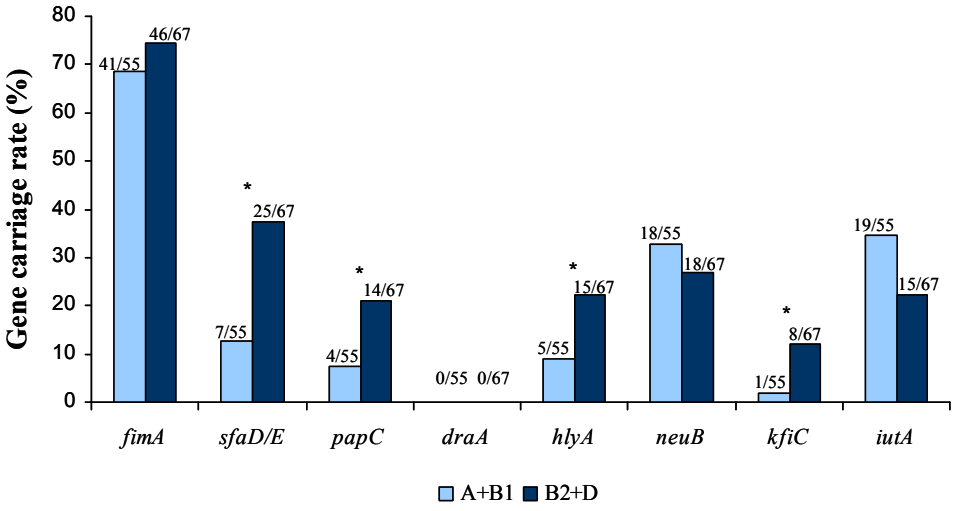

Figure 2. Percentage and prevalence values of virulence-associated genes in A+B1 commensal and B2+D virulent *E. coli* groups. Data in bars are expressed as percentage and data in labels as prevalence. *Significant difference at *P* < 0.05 by applying the Fisher's exact test.

4. DISCUSION

Significant differences were revealed in the diversity and virulence-gene carriage of *Enterobacteriaceae* and *E. coli* clones isolated from faeces of active and non-active coeliac patients and healthy controls, suggesting that this bacterial group could be primarily or secondarily involved in coeliac disease.

E. coli was the most common member of the *Enterobacteriaceae* family isolated from human faecal samples of healthy and coeliac disease children. This result is consistent with a previous study of 49 coeliac and healthy children, in which the main *Enterobacteriaceae* species detected was *E. coli*. In the aforementioned study, the ratio of *E. coli* to *Enterobacteriaceae* was greater in active coeliac children than in healthy controls.[13] Furthermore, biopsy specimens taken from paediatric patients with inflammatory bowel disease also revealed that *E. coli* was the most common *Enterobacteriaceae* species detected in MacConkey agar.[16] Thus, a reduction in the relative abundance of non-*E. coli* species is associated with coeliac disease and could favour the predominance of harmful clones in the gut ecosystem.

Although several *Enterobacteriaceae* colonies were often selected from a sample, because they differed morphology and were suspected to represent different strains, they often turned out to be identical regarding their phylogenetic classification and virulence-associated gene patterns, usually some of them represented the same clone. This can be explained by the fact that only one clone usually dominates human gastrointestinal gut so that the other clones are not detectable.[17]

The phylogenetic groups A, B1, B2 and D were found in different proportions in healthy, active coeliac and non-active coeliac patients. This fact also suggests links between lower diversity of intestinal *E. coli* population and coeliac disease. In healthy children, isolates of each of the four groups were found in equal proportions. By contrast, isolates belonging to groups A and B2, followed by those of group D dominated the composition of *E. coli* microbiota in active coeliac children, while integrants of group B1 were not isolated; group D was the most common in non-active coeliac disease, followed by groups A, B2, and B1. These results reflect imbalances in the composition of the four *E. coli*

phylogenetic groups in coeliac disease children, revealing the most remarkable differences between non-active coeliac patients and healthy controls. In patients with IBD, such as Crohn's disease and ulcerative colitis, B2 and D phylogenetic groups were more prevalent than in the control group. Moreover, a relationship was established between the presence of serine protease autotransporter proteins (SPATE) or adhesins in B2+D positive *E. coli* isolates.[18] In active coeliac patients, the dominance of group B2 virulent clones could provide pathogenic inflammatory factors either causative or consequence of the inflammatory status associated with gluten intake in the active phase of this disorder creating a vicious circle. Nowrouzian et al.[19] concluded that group D strains preferentially colonize infants with less complex intestinal microbiota, since this group is more abundant than others in one-year-old infants. Both genetic make-up and gluten-free diet could contribute to changes in the gut milieu of non-active coeliac patients, leading to a predominant colonization of the virulent D group in detriment to the others. In fact, lower total bacterial populations have been detected in duodenal samples taken from this population group when compared with active coeliac patients and controls.[12]

Overall, *E coli* clones of the virulent phylogenetic groups (B2+D) isolated from active and non-active coeliac patients, carried a higher number virulent genes than those from healthy controls. A higher prevalence of P fimbriae gene (*papC*) was found in coeliac patients as compared to healthy children, regardless of the phase of this disorder; however, the association of type-1 fimbriae (*fimA*) and S fimbriae (*sfaD/E*) gene carriage with coeliac disease was not so evident. Significant differences in the carriage of type-1 fimbriae (*fimA*) gene were not detected in the group of children under study but *E. coli* clones from active and non-active coeliac disease isolates tended to carry fewer type-1 fimbriae (*fimA*) genes than those from healthy children. Previous studies have indicated that the proportion of type-1 fimbriated *E. coli* is lower in IgA-deficient subjects than in control individuals[20,21] and this may also be the case for coeliac disease patients since this disease is associated with IgA deficiency. *E. coli* adhesins, including P fimbriae, confer mannose-resistant (MR) adherence to intestinal epithelial cells. MR adhesions are well-know virulence factors in urinary-tract infection, septicaemia and meningitis. Strains that persist in the human intestinal

microbiota (resident strains) are more often P fimbriated, whereas S fimbriated *E. coli* are not associated with long-term persistence in the gut of healthy individuals.[20,22] Thus, the significantly higher prevalence of P fimbriae *E. coli* clones in treated and untreated coeliac patients constitutes a novel link between gut microbiota and coeliac disease, and revealed that gut health may be compromised in these subjects, even when subjected to a gluten-free diet.

A higher prevalence of capsular K5 gene (*kfiC*) of *E. coli* clones belonging to the virulent groups was associated with active coeliac disease, while a similar but less remarkable trend was found in non-active coeliac patients, as compared to controls. Capsular polysaccharides are known to render bacterial surfaces hydrophilic and negatively charged, making the bacterium resistant to entrapment in mucus. In addition, capsules contribute to virulence by protecting bacteria from phagocytosis and possibly from serum killing, in part by blocking activation of the alternative complement pathway.[23,15]

Moreover, a higher prevalence of the haemolysin (*hlyA*) gene in *E coli* clones of the virulent groups was associated with non-active coeliac patients in particular. α-haemolysin is the most common cytolytic protein secreted by haemolytic *E. coli* strains. Haemolysin activity might contribute to persistence by attacking enterocytes and releasing nutrients for the bacteria. In fact, *E. coli* appears to use membrane lipids as its main nutrient source in the large intestine.[23,24]

It has also been confirmed that *E. coli* clones of the virulent groups (B2+D) carried more virulent-associated genes than those of commensal groups (A+B1), with the exception of the aerobactin gene (*iutA*). Remarkably, genes for S (*sfaD/E*) and P fimbriae (*papC*), K5 capsule (*kfiC*) and haemolysin (*hlyA*) were significantly more common in virulent *E. coli* clones than in commensal strains of our population groups. In agreement, genes coding for virulence-associated genes have been found more frequently in pathogenic strains than in commensal strains.[8,23] In addition, virulent *E. coli* isolates (B2+D) have previously been shown to carry virulence-associated genes more commonly than commensal clones.[10,25,26] Virulence-associated genes are usually encoded on pathogenicity islands (PAIs) providing a mechanism for coordinated horizontal transfer of

virulence genes, thus favouring dissemination of pathogenic determinants, as could be the case in the gut ecosystem of coeliac patients.[27]

5. CONCLUSION

This study has demonstrated changes in *Enterobacteriaceae* diversity and increases in virulence-gene carriage in E. coli clones isolated from coeliac patients when compared to those from healthy controls. Thus, the results support the hypothesis that dysbiosis may constitute a virulence factor contributing to pathogenesis and full expression of coeliac disease. Further studies should be carried out to correlate the virulence gene carriage with specific pathogenic roles that *E. coli* clones could play in this disorder.

6. ACKNOWLEDGEMENTS

This work was supported by grants AGL2005-05788-C02-01 and Consolider Fun-*C*-Food CSD2007-00063 from the Spanish Ministry of Science and Education (MEC). The scholarship to E. Sánchez from Danone S.A. is fully acknowledged.

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Capítulo II

Intestinal Staphylococcus spp. and virulent features associated with coeliac disease

ABSTRACT

Aim To determine whether intestinal *Staphylococcus* spp. and their pathogenic features differed between coeliac disease (CD) patients and healthy controls.

Methods 60 children, including active CD (n=20) and non-active CD (n=20) patients and healthy controls (n=20), were studied. Staphylococci were isolated from faeces and identified by PCR and DNA sequencing. The carriage of virulent genes, including adhesion (*atlE* and *fbe*), cell aggregation (*icaD*), global regulatory (*agr* and *sar*) and methicillin-resistant (*mecA*), was analysed by PCR.

Results *Staphylococcus epidermidis* was more abundant in active and non-active CD patients than in controls, *Staphylococcus haemolyticus* was more abundant in active CD patients than in controls, whereas *Staphylococcus aureus* was less abundant in active CD patients than in the other child groups. *Staphylococcus* spp. diversity was higher in active CD patients than in non-active CD patients and controls. The presence of the *mecA* gene and the simultaneous presence of both the *mecA* and *atlE* genes were higher in *S. epidermidis* clones isolated from CD patients, with active and non-active disease, than in those from control subjects. The individual presence of the other virulent genes was lower in *S. epidermidis* from active CD patients than in those from the other child groups.

Conclusions Increased abundance of *S. epidermidis* carrying the mecA gene, in active and non-active CD patients, most likely reflects increased exposure of these subjects to opportunistic pathogens and antimicrobials.

1. INTRODUCTION

Coeliac disease (CD) is an autoimmune enteropathy caused by a permanent intolerance to cereal gluten proteins (gliadins).[1] Currently, the only available therapy for CD is the adherence to a strict, lifelong, gluten-free diet; however, the compliance with this dietary recommendation is complex and other alternative strategies are needed.[2]

Scientific evidence suggests that environmental factors, other than gluten, may play a role in CD pathogenesis. It has been suggested that early infections may increase the risk of suffering CD in susceptible individuals.[3 4] In addition, imbalances in the gut microbiota of CD patients[5 6] and infants at genetic risk of CD[7] have been reported. Greater numbers of *Staphylococcus* spp. have been found in faecal and biopsy samples of CD patients, suggesting it may be related with the active phase of CD.[8]

Staphylococcus spp. are widespread in various environments, including skin and mucosal membranes of humans and many animals, and some species are clinically important because they cause chronic infections.[9] For instance, *Staphylococcus epidermidis* is the most frequent causative agent of nosocomial infections.[10] The pathogenesis of staphylococci and, particularly, of *S. epidermidis*, depends on the presence of genes coding for virulent factors mainly related to biofilm production and antibiotic resistance. The bacterial biofilm is produced in two steps comprising the initial bacterial attachment to the surface followed by a second stage consisting of bacterial proliferation, intercellular adhesion (cell aggregation) and production of an extracellular slimy substance. The AtlE, a cell surface protein exhibiting vitronectin-binding activity is one of the most important factors involved in the primary attachment of *S. epidermidis*.[11 12] *S. epidermidis* can also bind to other extracellular matrix-related proteins, like fibrinogen, via fibrinogen-binding proteins encoded by the *fbe* gene.[13] Cell aggregation is mediated by the products of the chromosomal *ica* locus, which comprises four intercellular adhesion genes (*icaA*, *icaB*, *icaC* and *icaD*). The *ica* operon encodes enzymes for the biosynthesis of PIA (polysaccharide intercellular adhesin) leading to multilayer biofilm

development.[12 14] PIA is also important for haemagglutination of erythrocytes by *S. epidermidis*.[15]

Quorum-sensing and global regulatory systems also play an important role in the regulation of virulent factors. The genes of two global regulatory loci, *agr* and *sar* seem to be involved in the regulation of the virulent factors of *S. epidermidis*.[16 17] *S. epidermidis* is considered an emergent pathogen causing increasing numbers of human infections, probably because a methicillin-resistant gene complex is widespread among isolates of this species.[9 18] Methicillin resistance is due to the expression of a modified penicillin-binding protein PBP2a (PBP2´) encoded by the *mecA* gene and located on the mobile element staphylococcal cassette chromosome mec (SCCmec), a genomic island.[9]

The purpose of this study was to analyse the diversity and species composition of clones of the genus *Staphylococcus*, isolated from faecal samples of active and non-active CD patients and age-matched controls. Furthermore, the presence of well-known virulent genes was examined in order to establish a possible link between the pathogenic potential of *Staphylococcus* spp. and CD.

2. MATERIALS AND METHODS

2.1. Subjects and sampling

A total of 60 children were included in this study: (1) active CD patients (n=20), who were on a normal gluten-containing diet, showed clinical symptoms of the disease, positive CD serology markers and severe enteropathy, classified as type 3 according to the Marsh classification of CD by duodenal biopsy examination; (2) patients with non-active CD (n=20), who had been on a gluten-free diet for at least 2 years, showed negative CD serology markers and normal mucosa, or infiltrative lesions classified as type 0-1 and absence of disease symptoms; and (3) healthy control subjects (n=20) without known food intolerance. None of the children in the trial had been treated with antibiotics for at least 1 month prior to sampling. Clinical and paraclinical characteristics of the children are shown in table 1.

Faecal samples from the trial subjects were kept under anaerobic conditions (AnaeroGen, Oxoid, Hampshire, UK) and analysed within 12 h. Aliquots (2 g wet weight) were 10 fold diluted in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, (pH 7.2)), homogenised, plated on Baird Parker agar (Scharlau, Barcelona, Spain) and incubated under aerobic conditions at 37 ºC for 48 h.

Table 1: Clinical and paraclinical characteristics of subjects included in this study.

Clinical and paraclinical data*	Active CD n=20	Non-active CD n=20	Control n=20
Mean age (months)	57.4 (37.6)	67.3 (38.4)	54.0 (34.1)
Sex (M/F)	12(60)/8(40)	11(55)/9(45)	12(60)/8(40)
Symptoms			
Abdominal pain	5 (25)	0 (0)	0 (0)
Diarrhoea	8 (40)	0 (0)	0 (0)
Weight loss	11 (55)	2 (16)	0 (0)
Anaemia	6 (30)	1 (5)	0 (0)
Iron deficiency	4 (25)	2 (10)	0 (0)
Anti-gliadin antibodies	20 (100)	0 (0)	N/A†
Anti-transglutaminase antibodies	20 (100)	0 (0)	N/A†
Duodenal biopsy**			
M0-1	0 (0)	20 (100)	N/A†
M3	20 (100)	0 (0)	N/A†
HLA typing (DQ2/DQ8)	20 (100)	20 (100)	N/A†

*Data are expressed as absolute numbers and percentages shown in brackets, except for age, which is expressed as mean with its standard deviation in brackets.

**Modified Marsh classification of CD.[19] M0, normal mucosa; M0-1, (infiltrative lesion) seen in patients on a gluten-free diet, patients with dermatitis herpetiformis, and family members of patients with coeliac disease; M2, (hyperplastic type) seen occasionally in dermatitis herpetiformis; M3, > 40 intraepithelial lymphocytes per 100 enterocytes, crypts increased and villi with atrophy seen in cases of typical coeliac disease.

N/A†, not applicable

2.2. Identification of bacterial isolates

Five individual colonies from the highest dilution plate of each subject were randomly selected and grown in Brain Heart Infusion broth (BHI, Scharlau). Colony and cellular morphology under Gram staining, and DNAse and coagulase activities were evaluated. Species-level identification was carried out by PCR with the primers listed in table 2. Isolates were identified as *Staphylococcus aureus* or *S. epidermidis* by a multiplex PCR based on the *dnaJ* gene (i).[20] Other

isolated species were identified by sequencing of an 883 bp fragment, amplified with *dnaJ* degenerate primers (ii),[21] or by 16SrDNA partial sequencing (iii).[22]

Amplicons were purified using GFXtm PCR DNA Kit (GE Healthcare, Buckinghamshire, UK) and sequenced with an ABI PRISM-3130XL Gene Analyzer (Applied Biosystems, California, USA). Search analyses to determine the closest relatives of the retrieved gene sequences were conducted in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm. Sequences with more than 97% similarity were considered to be of the same species.

2.3. Pathogenicity genes of S. epidermidis *strains*

The presence of *fbe*, *atlE*, *icaD*, *agrA*, *sarA* and *mecA* genes was determined in *S. epidermidis* isolates (table 2). Multiplex PCR was performed for combined amplification of (iv) *atlE*, *fbe* and *icaD* genes;[20] and (v) for that of *agrA* and *sarA* genes;[23] and a simplex PCR (vi) was performed for *mecA* gene amplification.[23]

The haemolytic activity of the isolates was determined on Columbia agar supplemented with 5% horse blood (COH, BioMerieux). A positive-biofilm phenotype of *S. epidermidis* strains was determined using Congo Red agar assay.[24]

Table 2. Oligonucleotide primers used for the genetic study of *Staphylococcus* isolates from children included in this study

Target*	Primer	Primer sequence (5´-3´)	Product size (bp)
Staphylococcus spp.(i)	J-StGen	TGGCCAAAAGAGACTATTATGA	
S. aureus (i)	J-StAur	GGATCTCTTTGTCTGCCG	337
S. epidermidis (i)	J-StEpi	CCACCAAAGCCTTGACTT	249
Staphylococcus spp. (ii)	SA-f	GCCAAAAGAGACTATTATGA	883
	SA-r	ATTGYTTACCYGTTTGTGTACC	
16SrDNA (iii)	U968-f	AACGCGAAGAACCTTAC	430
	L1401-r	CGGTGTGTACAAGACCC	
Fbe gene (iv)	Fbe-f	TAAACACCGACGATAATAACCAAA	472
	Fbe-r	GGTCTAGCCTTATTTTCATATTCA	
atlE gene (iv)	atlE-f	CAACTGCTCAACCGAGAACA	682
	atlE-r	TTTGTAGATGTTGTGCCCCA	
icaD gene (iv)	icaD-f	CAGACAGAGGCAATATCCAAC	225
	icaD-r	ACAAACAAACTCATCCATCCG	
agrA gene (v)	agrA-f	CAACAACGAAACATGGTGCT	923
	agrA-r	TGTCATCGAAAATGGTTACTTTG	
sarA gene (v)	sarA-f	TGGTCACTTATGCTGACAGATT	313
	sarA-r	TTTGCTTCTGTGATACGGTTG	
mecA gene (vi)	mecA-f	GTAGAAATGACTGAACGTCCGATAA	310
	mecA-r	CCAATTCCACATTGTTTCGGTCTAA	

*Primers used in simplex and multiplex PCRs for the identification of *Staphylococcus* ssp. (i, ii and iii); and for the determination of *S. epidermidis* virulence genes (iv, v, and vi).

2.4. Statistical analyses

Shannon-Wiener diversity index (*H'*) was used to determine the diversity of taxa present in faecal samples of active and non-active CD patients and control children, using PAlaeontological STatistics software.

Differences in *Staphylococcus* spp. abundance and in the presence of virulent genes were established by applying x^2 test and, when appropriate, by applying the two-tailed Fisher's exact test. Analyses were carried out with Statgraphics software (Manugistics, Rockville, Maryland, USA).

3. RESULTS

3.1. Diversity of Staphylococcus *spp. in CD patients and control children*

A total of 300 isolates were randomly isolated from faecal samples of the children under study. The abundance of *Staphylococcus*-like species recovered from active and non-active CD patients and control subjects is shown in table 3.

S. epidermidis isolates were more frequently recovered from active and non-active CD patients than from control subjects ($p<0.01$ and $p=0.03$, respectively). Furthermore, *Staphylococcus haemolyticus* was more common in active CD patients than in controls ($p<0.04$). In contrast, *S. aureus* isolates were less frequently recovered from active CD patients than from non-active CD patients and controls ($p<0.01$). The percentage of DNAse and coagulase-positive *S. aureus* isolates was similar in all three groups of children. *Staphylococcus warneri* was less frequently isolated in non-active CD patients than in active CD patients and controls ($p<0.01$). The diversity of *Staphylococcus*-like spp. was calculated applying the Shannon-Wiener diversity index (*H'*), which considers both the number and evenness of species. Active CD patients showed higher species diversity (*H'*=1.71) than non-active CD patients (*H'*=1.45) and controls (*H'*=1.44).

Interestingly, 72 isolates were recovered from Braid Parker agar and identified as *Enterococcus faecalis* or *Enterococcus faecium* (table 2). The abundance of isolates belonging to the species *E. faecium* was higher in control children than in CD patients with either active disease or non-active disease. *Enterococcus* spp. were less frequently isolated in active CD patients (14%) than in non-active CD (26%, $p=0.05$) and in controls (32%, $p<0.01$).

Table 3. Abundance of isolates belonging to *Staphylococcus*-like spp. in active and non-active CD patients and control children*

	Presumptive *Staphylococcus* isolates per group		
Isolate identification	**Active CD (100)**	**Non-active CD (100)**	**Control (100)**
Staphylococcus spp.	86 (86)	74 (74)	70 (70)
S. aureus	20 (20)[a]	37 (37)[b]	44 (44)[b]
S. epidermidis	36 (36)[a]	26 (26)[a]	13 (13)[b]
S. haemolyticus	7 (7)[a]	5 (5)[a,b]	0[b]
S. hominis	0[a]	2 (2)[a]	0[a]
S. pasteuri	4 (4)[a]	2 (2)[a]	0[a]
S. simulans	5 (5)[a]	2 (2)[a]	0[a]
S. warneri	14 (14)[a]	0[b]	13 (13)[a]
Non-*Staphylococcus* spp.	14 (14) [a]	26 (26) [b]	30 (30) [b]
Enterococcus faecalis	14 (14)[a]	26 (26)[a]	21 (21)[a]
Enterococcus faecium	0[a]	0[a]	9 (9)[b]

*Data expressed as number of isolates belonging to each species (% respect to the total number of isolates) in each child group under study. Different letters (a, b) in the same row indicate significant differences among active and non-active CD patients and control children by applying x^2 test ($P<0.05$).

3.2. Characterisation of S. epidermidis *isolates*

A total of 75 *S. epidermidis* isolates were identified in the study, and the presence of virulence-associated genes is shown in table 4.

The adhesion-encoding genes (*atlE* and *fbe*) were less frequently detected in *S. epidermidis* isolates recovered from active CD patients than in those recovered from non-active CD patients ($p<0.01$) and controls ($p<0.01$). The cell-aggregation gene *icaD*, was also less frequently detected in isolates recovered from active CD patients than in those from non-active CD patients ($p<0.01$) and controls ($p=0.02$). There was a good correlation between the presence of this gene and the results obtained with the Congo Red agar assay (data not shown), which determines biofilm production potential. No differences

were detected between the haemolytic activities in *S. epidermidis* strains isolated from the analysed groups of children (data not shown).

The virulent genes corresponding to the global regulatory loci *agr* and *sar* were less common in *S. epidermidis* isolates from active CD patients than in those from controls (p=0.01 and p=0.03, respectively) and non-active CD patients (p<0.01 and p<0.01, respectively). In contrast, the methicillin-resistant gene (*mecA*) was most frequently detected in *S. epidermidis* isolates from active (p<0.01) and non-active CD patients (p<0.01) than in those from controls. The isolates carrying both the *atlE* and the *mecA* genes simultaneously were also more abundant in active and non-active CD patients (p<0.01) than in controls.

Table 4. Analysis of virulence determinants among the *S. epidermidis* isolates from active and non-active CD patients and control children

	***S. epidermidis* isolates per group**		
	Active CD	**Non-active CD**	**Control**
Genes	**n=36**	**n=26**	**n=13**
agrA	16 (44)[a]	26 (100)[b]	12 (92)[b]
atlE	21 (58)[a]	26 (100)[b]	13 (100)[b]
fbe	3 (8)[a]	16 (62)[b]	8 (62)[b]
icaD	0[a]	11 (42)[b]	3 (23)[b]
mecA	27 (75)[a]	23 (88)[a]	0[b]
sarA	21 (58)[a]	26 (100)[b]	12 (92)[b]

*Data are expressed as absolute numbers of positive *S. epidermidis* isolates for virulence genes in each child group under study and their percentages in brackets. Different letters (a, b) in the same row indicate significant differences in the presence of virulence genes among active CD, non-active CD and control children by applying x^2 test (P<0.05).

4. DISCUSSION

Staphylococcus spp. are important inhabitants of the human skin and mucosa[25] and also of the intestinal tract. Various studies have reported increased numbers of *Staphylococcus* spp. in the gut microbiota of active CD patients,[5 8] which is similar in infants with inflammatory bowel disease (IBD)[27 28] or allergy.[29 30] This would suggest there is a relationship between this bacterial group and immune-mediated pathologies. Herein, we show that CD is associated with alterations in species diversity and composition of the faecal *Staphylococcus* population. *S. epidermidis* was more abundant in CD patients, regardless of the disease stage, than in controls. Meanwhile, *S. haemolyticus* was more abundant in active CD patients than in controls. It is known that both species are involved in human infections.[31]

S. epidermidis is currently considered as an important opportunistic pathogen, especially in healthcare-associated infections.[10 32] *S. epidermidis* strains are well equipped with determinants promoting persistence in specific ecosystems, related with the evasion of host defences and biofilm formation.[12] Cell surface proteins could contribute to the attachment of *S. epidermidis* to host cells and tissues,[33 34] which can increase their pathogenicity. In this study, the individual presence of genes related to biofilm formation (*atlE*, *fbe* and *icaD*) was more common in *S. epidermidis* isolates from non-active CD patients and controls, which could lead to a more stable colonisation. In fact, biofilm formation is a common colonisation mechanism of both commensal and pathogenic bacteria, facilitating their persistence in different environments, including the gastrointestinal tract;[35 36] this phenomenon has also been related to microbial alterations in IBDs.[37]

Our study also reports there is an increased abundance of methicillin-resistant *S. epidermidis* isolates associated with active and non-active CD patients. Antibiotic resistance, and particularly methicillin resistance, is a widespread characteristic of *S. epidermidis* strains. Methicillin is an antibiotic of first choice against staphylococcal infections, and other antibiotic-resistant genes are more often found in methicillin-resistant than in methicillin-susceptible strains.[38]

Furthermore, most *S. epidermidis* infections are hospital-acquired or healthcare related, and methicillin-resistant strains are commonly found in these patients. Hospital visits are frequent before CD diagnosis[39] and severe infections are commonly associated with CD,[40] [41] which could explain the increased abundance of methicillin-resistant *S. epidermidis* in our cohort of CD patients. Hospitalized IBD patients are also at an increased risk of methicillin-resistant *S. aureus* infections compared with non-IBD patients.[28] The simultaneous presence of both adhesin-encoding genes, which could confer increased biofilm formation capacity, and antibiotic-resistant genes in *S. epidermidis* isolates from CD patients under study, may explain the persistence of this species in these patients even after the administration of a long-term gluten-free diet.[42] [43]

Unlike *S. epidermidis*, the abundance of *S. aureus* was higher in healthy children than in active CD patients. Consistent with this, it has previously been reported that *S. aureus* is often present in stools of healthy infants;[44] it was suggested that parental skin *S. aureus* strains can easily establish in the infant gut probably due to poor competition from other gut bacteria. Similarly, it could be speculated that the increased abundance of *S. epidermidis* and the increased diversity in *Staphylococcus* spp. is associated with the reduced abundance of *S. aureus* in active CD patients due to higher competition among species of the same genus for the same ecological niche.

This study demonstrates that CD is associated with shifts in *Staphylococcus* species diversity and abundance in the intestinal microbiota. *S. epidermidis* isolates carrying the *mecA* gene, and both the *mecA* and *atlE* genes, were more abundant in CD patients than in controls. This suggests that children with CD have greater exposure to opportunistic staphylococcal pathogens and antimicrobials, which in turn affects the composition/ features of their intestinal microbiota.

5. ACKNOWLEDGEMENTS

The contract (PTR95-0987.OP.01 from MICINN and scholarship (Institute Danone) to E. Sánchez are fully acknowledged.

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Capítulo II

Discerning the role of *Bacteroides fragilis* in celiac disease pathogenesis

ABSTRACT

Celiac disease (CD) is associated with intestinal dysbiosis, which can lead to dysfunctions in host-microbe interactions and contribute to the disease. In this study, possible differences in *Bacteroides* spp. and their pathogenic features between CD patients and controls were investigated. *Bacteroides* clones (n=274) were isolated, identified and screened for the presence of genes coding for metalloproteases (*bft* and *mpII*). The proteolytic activity of selected *Bacteroides fragilis* strains was evaluated by zymography and, after gastrointestinal digestion of gliadin, by HPLC and MS/MS spectrometry. The effects of *B. fragilis* strains on Caco-2 cell culture permeability and the inflammatory response to digested gliadin were determined. *B. fragilis* was more frequently identified in CD patients than in controls, in contrast to *B. ovatus*. *B. fragilis* clones carrying genes coding for metalloproteases were more abundant in CD patients than in controls. Representative *B. fragilis* strains carrying metalloprotease genes, showed gelatinase activity and exerted the strongest adverse effects on the integrity of the Caco-2 cell monolayer. All *B. fragilis* strains also showed gliadin-hydrolyzing activity, and some of them generated immunogenic peptides that preserved or increased inflammatory cytokine production (TNFα) and showed increased ability to permeate through Caco-2 cell cultures. These findings suggest that increased abundance of *B. fragilis* strains with metalloprotease activities could play a role in CD pathogenesis, although further *in vivo* studies are required to support this hypothesis.

1. INTRODUCTION

Celiac disease (CD) is the most common chronic intestinal inflammatory disorder triggered by ingestion of dietary gluten. This disease is considered as both a food hypersensitivity and an autoimmune disorder that involves genetic and environmental factors.[40] The human leukocyte antigen (HLA) class II genes encoding for DQ2 and DQ8 heterodimers are the main hereditary factors predisposing to CD and are present in most CD patients (95%). Nevertheless, while 30 to 35% of the general population are carriers of these genes, only 2 to 5% actually develop CD, indicating that other factors contribute to precipitating the disease.[33] The intake of gluten proteins is the critical environmental element responsible for the signs and symptoms of the disease and, in fact, typical cases manifest in early childhood after introduction of gluten into the diet. However, the disease is also being increasingly diagnosed in adulthood,[5] suggesting that early exposure to gluten is not the only environmental trigger.

Gluten proteins and their toxic components (gliadins) are partially resistant to proteolytic degradation and can accumulate and interact with the small intestinal mucosa.[14] Enzyme deficiency in the small intestinal mucosa of CD patients does not seem to be causally related to the disease.[3] However, in CD patients, some peptides, such as the 33-mer of -gliadin and others containing its main structural epitopes (PFPQPQLPY and PQPQLPYPQ), preferentially drive an adaptive immune response by binding to HLADQ2/DQ8 molecules of antigen-presenting cells and activating T-helper 1 (Th1) and Th17 inflammatory responses within the mucosa, with the resulting production of inflammatory cytokines (e.g., gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], and interleukin-21 [IL-21]) leading to severe inflammation.[24] Other gliadin peptides activate an innate immune response characterized by increased production of IL-15 by epithelial and antigen-presenting cells, which activate the effector function and cytotoxic activity of intraepithelial lymphocytes.[15] Gliadin peptides also induce upregulation of the zonulin innate immunity pathway, which leads to increased intestinal permeability and enables paracellular translocation of gliadin and its subsequent interaction with antigen-presenting cells within the intestinal submucosa.[11]

In recent years, alterations in the composition of the intestinal microbiota have been associated with CD. Increased bacterial numbers of the *Bacteroides-Prevotella* group or the *Bacteroides fragilis* group have been demonstrated compared to healthy controls in CD patients.[8,26] *Bacteroides* spp. are generally considered commensals or symbionts inhabiting the human gastrointestinal tract, representing ca. 25% of the total bacterial cells. Nonetheless, members of the normal microbiota can also potentially cause disease in cases of failure of the host defenses and major dysbiosis and can then be considered "pathobionts".[35] In spite of the abundance of *Bacteroides* spp. in the gut microbiota, their ecological distribution, composition, and impact on health remain unclear.[46] Species such as *B. fragilis* and *Bacteroides vulgatus* seem to be implicated in the disruption of the integrity of the intestinal epithelial barrier, thereby contributing to the development of inflammation in experimental animal models[38,42] and, possibly, in patients with inflammatory bowel disease (IBD).[10] The potential pathogenicity of *Bacteroides* spp. is related to the expression of a variety of virulence factors, including proteolytic and other hydrolytic enzymes.[4] Enterotoxigenic *B. fragilis* (ETBF) strains produce an enterotoxin, termed *B. fragilis* toxin (BFT), which is a 20-kDa zinc-dependent metalloprotease that has been associated with diarrhea in humans and young animals.[37] The *B. fragilis* toxin gene (*bft*) is located in a pathogenicity island, present exclusively in ETBF strains and is associated with another gene (*mpII*) that encodes a second metalloprotease.[12]

Here, we hypothesized that changes in the composition of *Bacteroides* spp. and associated virulence features can turn these commensal bacteria into pathogenic inhabitants of the human intestinal tract that, acting in consortium with gluten peptides, can contribute to CD. To address this question, we determined differences in the diversity of *Bacteroides* spp. isolated from the feces of patients with active and non-active CD in comparison with healthy controls and evaluated their virulence features and potential participation in the generation of gliadin peptides with immunotoxic effects on intestinal epithelial cells.

2. MATERIALS AND METHODS

2.1. Subjects and sampling

Three groups of children were included in the present study: (i) patients with active CD (*n*=20; mean age, 3.9 years; range, 1.0 to 8.8 years), who were on a normal gluten-containing diet, showing clinical symptoms of the disease, positive CD serology markers (anti-gliadin antibodies and anti-transglutaminase antibodies), and signs of severe enteropathy, classified as type 3 according to the Marsh classification of CD by duodenal biopsy examination; (ii) patients with non-active CD(*n*=18; mean age, 6.2 years; range, 3.3 to 12.2 years), who were on a gluten-free diet for at least 2 years, showed negative celiac serology markers and normal mucosa or infiltrative lesions classified as type 0-1 according to Marsh classification, and absence of disease symptoms; and (iii) healthy control children (*n*=20; mean age, 5.7 years; range, 2.5 to 10.8 years). None of the children included in the study were treated with antibiotics for at least 1 month before the sampling time. The study was conducted in accordance with the ethical standards of the responsible institutional committees on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in 1983. Children were enrolled in the study after written informed consent was obtained from their parents.

2.2. Isolation of **Bacteroides** *from child feces*

Fecal samples were collected in sterile containers, kept under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, United Kingdom), stored at 4°C, and analyzed in less than 12 h to avoid alterations in viability of *Bacteroides* spp. Samples (2 g [wet weight]) were diluted (1:10 [wt/vol]) in phosphate-buffered saline (PBS; 130 mM sodium chloride, 10 mM sodium phosphate [pH 7.2]) and homogenized in a Lab Blender 400 Stomacher (Seward Medical, London, United Kingdom). Serial dilutions were prepared in PBS, and aliquots were plated on Schaedler agar (Scharlau, Barcelona, Spain) supplemented with kanamycin (100 mg/liter), vancomycin (7.5 mg/liter), and vitamin K (0.5 mg/liter) and then incubated under anaerobic conditions at 37°C for 48 h. In order to analyze the

dominant clones in each subject,[21] five presumably different individual colonies were isolated from the highest dilution plate from each subject, and their cellular morphology and Gram-staining characteristics were examined.

2.3. Strain typing and species identification

The isolated clones were identified at the species level by partial 16S rRNA gene sequencing using the primer pair Bfra531-f and Bfra766-r.[45] The PCR products obtained were purified using GFX PCR DNA and a gel band DNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom) for DNA sequencing. DNA sequencing was carried out by an ABI Prism 3130XL genetic analyzer (Applied Biosystems, California). The closest relatives of the partial 16S rRNA gene sequences were sought in GenBank using the basic local alignment search tool (BLAST) algorithm, and sequences with 97% similarity were considered to belong to the same species. RAPD (random amplified polymorphic DNA)-PCR was performed to differentiate the isolated clones at the strain level by colony PCR and using the M13 primer as previously described [7] (Table 1).

Table 1. Primers for PCR and sequencing used in this study

Primer	Fragment name	Fragment length	Primer sequence (5´→3´)
M13	M13		TTATGAAACGACGGCCAGT
Bfra 531F	Bfra	289 bp	ATACGGAGGATCCGAGCGTTA
Bfra 766R			CTGTTTGATACCCACACT
GBF-201			GAACCTAAAACGGTATATGT
GBF-312	*Bft-1*	190 bp	CCTCTTTGGCGTCGC
GBF-322	*Bft-2*	175 bp	CGCTCGGGCAACTAT
GBF-334	*Bft-3*	287 bp	TGTCCCAAGTTCCCCAG
LO1	*mplI*	350 bp	CCACCGTGCCAATGTCAGATA
RO1			CTGAAGAACGAGGCGGTATC

2.4. Pathogenicity markers and proteolytic activity of B. fragilis *strains*

The presence of *bft* and *mpII* genes was screened in all of the isolated *B. fragilis* clones by PCR (Table 1). One forward primer (GBF-201) and three reverse primers (GBF-312 for *bft-1*, GBF-322 for *bft-2*, and GBF-334 for *bft-3*) were used in the same amplification reaction for the detection of the three isoforms of the *bft* gene by multiplex PCR.[17] *B. fragilis* clones were also screened for the presence of the *mpII* gene by PCR.[23] PCR products were separated in a 2% agarose gel by electrophoresis and visualized by ethidium bromide staining.

B. fragilis clones were analyzed by RAPD-PCR, and nine different strains were identified (A to I). The proteolytic activity of one representative of each *B. fragilis* strains was determined in gelatin and gliadin zymograms. For this, strains were grown in brain heart infusion broth (Scharlau, Barcelona, Spain) supplemented with 0.05% (wt/vol) cysteine (Sigma, St. Louis, MO) under anaerobic conditions for 24 h. Bacterial cells were collected by centrifugation (6,000 *g* for 15 min), washed, and resuspended in PBS at a final concentration of 10^8 CFU/ml. Cell suspensions were separated in a discontinuous SDS-PAGE system that consisted of (i) a running gel containing 15% acrylamide (pH 8.8) and either 0.5% gelatin or gliadin and (ii) a stacking gel containing 4% acrylamide (pH 6.8). Gels were run at a constant voltage (120 V) in a MiniProtean 3 cell system (Bio-Rad, Richmond, CA). Gels were washed in 2.5% Triton X-100 at room temperature for 1 h and then incubated in reaction buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ [pH 7.4]) at 37°C over night. Hydrolysis bands were visualized as a clear zone after Coomassie brilliant blue R-250 staining (Bio-Rad, Richmond, CA).

2.5. In vitro digestion of gliadins.

Gliadins were subjected to a simulated human gastrointestinal digestion as previously described.[19] Aliquots (150 mg) of a commercially available extract of gliadin (Sigma) were dissolved in 3 ml of a saline solution (140 mM NaCl, 5 mM KCl [pH 3]) at 60°C for 30 min, with gentle agitation. Gastric digestion with pepsin (800 to 2,500 U/mg in 0.1 M HCl [pH 3] for 1 h), and intestinal digestion with pancreatin (4 USP specification) and bile (Sigma) in 0.1 $NaHCO_3$ at pH 7 for 2 h were conducted at 37°C with agitation. After the gastric digestion, the

intestinal digestion was carried out in the upper part of a two-chamber system in six-well plates separated by a 15,000-molecular-weight cutoff dialysis membrane (Spectrum Medical, Gardena, CA). Aliquots of the gastric digested samples were loaded into the upper chambers in the presence or absence of *B. fragilis* cell suspensions (10^8 CFU/ml) and incubated for 4 h. Then, saline solution from the basal chamber was recovered for further analysis. The total protein concentrations in both dialysates and retentates were quantified using a Lowry-based commercial kit (Sigma). The stability of bacteria during digestion was confirmed by plate counting under optimal conditions, which remained at 10^8 CFU/ml.

2.6. Reversed-phase HPLC and MS/MS analysis

Gliadin-derived peptides were analyzed after simulated gastrointestinal digestion, as described elsewhere.[20] The separation was conducted in a BioBasicC_{18} column (5 µm; 4.6 by 250 mm; Thermo, Waltham, MA) using an Agilent highpressure liquid chromatography (HPLC) system connected in-line to an Esquire-LC electrospray system equipped with a quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The elution phases consisted of 15% (vol/vol) acetonitrile (ACN)–0.1% (vol/vol) trifluoroacetic acid (TFA) (solvent A) and 80% (vol/vol) ACN–0.1% (vol/vol) TFA (solvent B). Aliquots (100 µl) of the dialysates were injected in each analysis. The gradient program started with 95% solvent A and 5% solvent B, and changed linearly to reach 10% solvent A and 90% solvent B within 30 min. UV absorbance was recorded at 214 nm. BioTools version 2.1 (Bruker Daltonics) software was used to process the tandem mass spectrometry (MS/MS) data and to identify peptide sequences by comparison to available gliadin sequences (accession numbers: α/ß, AAZ94420; γ, AAQ63856; and ω, AAT74547). Three independent dialysates were analyzed in each case.

2.7. Caco-2 cell culture conditions

The human colon carcinoma Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD) at passage 14 and used in experiments at passages 19 to 23. Caco-2 cells were grown in Dulbecco modified

Eagle medium (DMEM; AQ Media; Sigma), containing 4.5 g of glucose (Sigma/liter), 25 mM HEPES buffer (Sigma), 0.1% (vol/vol) antibiotic mixture (penicillin, streptomycin, and gentamicin; Sigma), and 10% (vol/vol) fetal bovine serum (Sigma). Cells were grown and maintained at 37°C in 5% CO_2 and 95% air, and the culture medium was changed every 2 days.[19] Cells at 70% confluence were detached from the flasks by using a trypsin solution (2.5 g/liter; Sigma) and resuspended in DMEM.

2.8. Evaluation of intestinal Caco-2 cell monolayer integrity

Caco-2 cells were seeded at a density of 50,000 cells/cm^2 onto polyethylene terephthalate membrane inserts (0.4-µm pore size; Millipore, Billerica, MA) and placed in six-well plates (Costar). In this bicameral system, 1.5 ml of treatment medium was loaded into the apical compartment, and 2 ml of saline solution was loaded into the basal compartment. Cell cultures were used at 7 days after seeding.

To determine the influence of selected *Bacteroides* strains on the integrity of the intestinal cell monolayer, bacterial cell suspensions (10^8 CFU/ ml) of *B. fragilis* strains A, B, C, and I grown for 20 h were prepared in DMEM (without antibiotics) and loaded into the upper chamber alone and together with the dialysates of gliadin digested in the presence of these strains. After incubation (4 h), the basal medium was recovered and mixed with 100 µl of 1 M NaOH, and the diffusion of phenol red was determined by measuring the absorbance at 558 nm.

To determine the translocation of gliadin-derived peptides, Caco-2 cells were exposed basolaterally to TNF-α (10 ng/ml) for 24 h to simulate inflammatory conditions.[31] *In vitro* digestions of gliadins in the presence of cell suspensions of *B. fragilis* A, B, C, or I strains were loaded into the upper chamber of the *in vitro* system. After 4 h of incubation, the basal medium was recovered to determine the concentration of permeated gluten peptides by enzyme-linked immunosorbent assay (ELISA), as described below.

2.9. Gluten quantification

A commercially available quantitative immune-based ELISA kit, designed to detect the toxic fraction of gluten from food samples, was used according to the manufacturer's instructions (GlutenTox; Biomedal, Seville, Spain).[25] The analyses were performed in the fraction that reached the basal compartment after crossing the monolayer of Caco-2 cells subjected to inflammatory conditions and the gliadin samples digested in gastrointestinal conditions and in the presence or absence of selected *B. fragilis* strains.

2.10. Analysis of inflammatory markers.

In the supernatants of Caco-2 cell cultures exposed to the dialysates from digests of gliadins inoculated with the different *B. fragilis* strains, TNF- (eBioscience, San Diego, CA), and IL-1 (eBioscience) were determined by ELISA according to the manufacturer's instructions. The sensitivity for these methods is 4 pg/ml.

2.11. Statistical analyses

The Renyi diversity index was used to explore differences in *Bacteroides* species among the different child groups. This index provides three further diversity index values: species richness (*S*), the Shannon index (*H´*), and the Simpson index (1-D).[44] The chi-square test was used to establish differences in the abundance of *Bacteroides* spp. and virulence genes. A *P* value of <0.05 was considered statistically significant. The Bonferroni adjustment test was applied to correct the significance for multiple comparisons among the three child groups studied (active and non-active CD patients and controls), which has the advantage of reducing type I errors and the disadvantage of increasing type II errors.

For experimental studies with Caco-2 cell cultures, one-way analysis of variance (ANOVA) and the Fisher least significant difference (LSD) *post hoc* test were applied. Statistical significance was established at $P<0.05$, using the SPSS software (v.15; SPSS, Inc., Chicago, IL).

3. RESULTS

3.1. Diversity of Bacteroides *spp. in the fecal microbiota of CD patients.*

The species richness (*S*), Shannon (*H´*), and Simpson (1-D) indexes between active CD patients (*S*=12, *H´*=1.95, and 1-D=0.82), non-active CD patients (*S*=14, *H´*=2.15, and 1-D=0.84) and controls (*S*=13, *H´*=2.09, and 1-D=0.84), indicating a similar diversity distribution between the studied child groups. Renyi diversity curves showed that active CD patients had a lower diversity than controls, whereas the diversity curves from non-active CD patients intersected with the diversity curves from both active CD patients and controls and therefore could not be compared (Fig. 1).

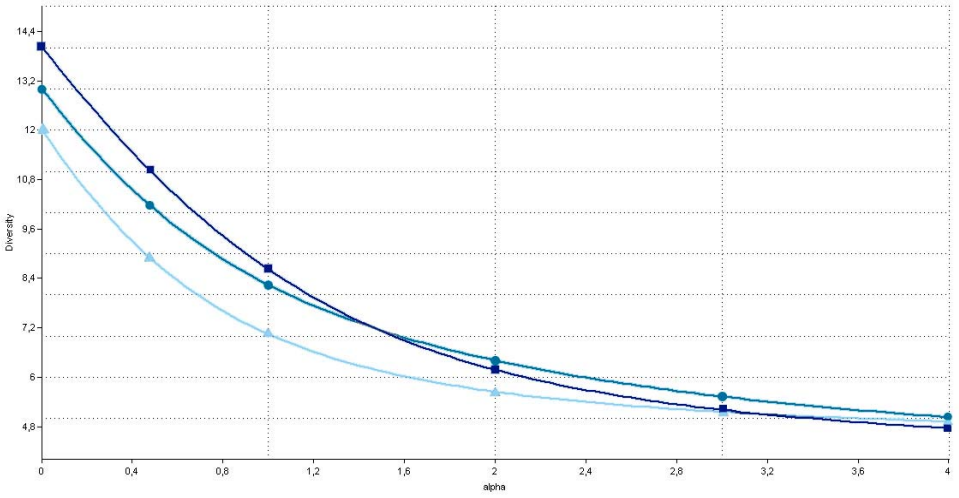

Figure 1. Renyi index curves of the *Bacteroides* spp. in active CD patients (▲), non-active CD (■) patients and control (●) children. The Renyi index estimates total richness for α=0, Shannon index for α=1 and Simpson index for α=2.

The total number of clones (n= 274) recovered from feces of healthy subjects and CD patients that were identified as *Bacteroides* spp. or *Parabacteroides* spp. is shown in Table 2. *B. fragilis* was more frequently isolated from CD patients with either active (*P*=0.007) or non-active (*P*=0.009) disease

than from healthy controls. This species represented up to 12% of the total clones from both groups of CD patients, which differs markedly from the 1%isolated from the controls. *Parabacteroides distasonis* was more frequently isolated from active CD patients than from non-active CD patients and controls (*P*=0.001). In contrast, *Bacteroides ovatus* was more frequently detected in controls than in CD patients (*P*=0.014), irrespective of the phase of the disease (active or non-active), and *Bacteroides finegoldii* was more frequently isolated from controls than from active CD patients (*P*= 0.014).

Table 2. *Bacteroides* and *Parabacteroides* spp. isolated from fecal samples of active and non-active CD patients and control children.

	Species abundance (%)*		
	Active CD	**Non-active CD**	**Control**
Species	**n=97**	**n=82**	**n=95**
B. acidofaciens	0	2 (2.4)	2 (2.1)
B. caccae	1 (1.0)	4 (4.9)	6 (6.3)
B. dorei	4 (4.1)	4 (4.9)	11 (11.6)
B. finegoldii	0 [a]	1 (1.2) [ab]	7 (7.4) [b]
B. fragilis	12 (12.4) [a]	10 (12.2) [a]	1 (1.1) [b]
B. intestinalis	1 (1.0)	3 (3.7)	0
B. massiliensis	2 (2.1)	0	0
B. ovatus	5 (5.2) [a]	4 (4.9) [a]	16 (16.8) [b]
B. stercoris	1 (1.0)	1 (1.2)	2 (2.1)
B. thetaiotaomicron	1 (1.0)	2 (2.4)	1 (1.1)
B. uniformis	22 (22.7)	23 (28.0)	27 (29.5)
B. vulgatus	20 (20.6)	19 (23.2)	13 (13.7)
B. xylanisolvens	0	4 (4.9)	2 (2.1)
P. distasonis	24 (24.7) [a]	4 (4.9) [b]	6 (6.3) [b]
P. merdae	4 (4.1)	1 (1.2)	1 (1.1)

*Percentage of clones belonging to one specific species related to total number of clones isolated from each children group (active CD patients, non-active CD patients or controls).

[(a,b)] Statistical differences were calculated by using Chi-square test 2×2, corrected for a multiple comparison test (3 children groups) by using Bonferroni adjustment. Significantly difference between groups was considered at P<0.017.

3.2. Pathogenicity markers and proteolytic activity of B. fragilis *strains.*

A total of 23 *B. fragilis* isolates were identified from the three groups of children and, according to RAPD-PCR fingerprint analyses, these included nine different strains (A to I). The carriage of *bft* and *mpII* virulence genes was analyzed in all isolates, and the same pattern was detected for isolates belonging to the same strain as defined by RAPD-PCR. The virulence gene carriage in the different *B. fragilis* strains is summarized in Table 3. The *bft* gene was detected in 13 *B. fragilis* clones from CD patients, 6 of which (identified as strain E or G) were associated with active disease and seven (strain H or I) with non-active disease. Considering the *bft*-positive clones, the *bft-2* isoform was significantly more prevalent (85%) than the isoforms *bft-1* (15%, *P*= 0.007) and *bft-3* (0%, *P*=0.001). The *bft-2* isoform was detected in all of the *bft*-positive *B. fragilis* clones (strains E, G, and H) isolated from active and non-active CD patients, whereas the *bft-1* isoform was only detected in two *bft*-positive clones (strain I) isolated from non-active CD patients that also carried the *bft-2* isoform. The *mpII* gene was detected in three of the *B. fragilis* clones (strains B and D) isolated from active CD patients. Only one *B. fragilis* clone (strain A) was identified in the healthy controls, and it was *bft* and *mpII* negative.

Table 3. Characterization of virulence-associated genes (*bft* and *mpII*) in *B. fragilis* strains isolated from active CD patients, non-active CD patients and control children.

***B. fragilis* strain (nº clones)**	**Virulence genes***	
	Bft	***mpII***
Control children		
Strain A (1)	-	-
Active CD patients		
Strain B (2)	-	+
Strain C (2)	-	-
Strain D (1)	-	+
Strain E (2)	+	-
Strain F (1)	-	-
Strain G (4)	+	-
Non-active CD patients		
Strain F (3)	-	-
Strain H (2)	+	-
Strain I (5)	+	-

*Detection of virulence-associated genes (*bft* and *mpII*). Presence +, Absence -

In zymograms of gelatin, a common clear band of 20 kDa was detected for both *bft*-or*mpII*-positive *B. fragilis* strains (B, D, E, G, H, and I) representing all isolated clones; the clearest bands corresponded to *B. fragilis* strains B and H. In contrast, hydrolysis bands were not detected in gelatin gels corresponding to the *B. fragilis* strains A, C, F, and J, which were *bft* and *mpII* negative (data not shown). In zymograms of gliadins, a proteolytic band of 120 kDa was detected in all of the *B. fragilis* strains tested (Fig. 2). These activities were partially or totally inhibited in the presence of EDTA (data not shown), indicating that metalloproteases were responsible for the gliadin hydrolysis in the gels. According to these results, *B. fragilis* strains A, B, C, and I, which represent strains of different origins and gene carriages, were selected for further studies

on their possible differential pathogenic effects on intestinal epithelial cells and ability to hydrolyze gliadin peptides.

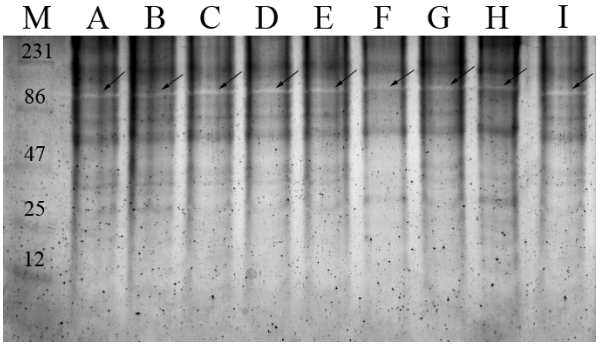

Fig 2. Gliadin zymograms of the *B. fragilis* strains (A to I) without EDTA. The identified gliadin-degrading protease pattern is characterized by one clear band (arrow), present in all the samples with an approximate molecular mass of 120 kDa. (M) Molecular mass of the protein markers in kDa.

3.3. Effects of B. fragilis *strains on monolayer integrity of Caco-2 cells*

The possible direct adverse effects of the selected *B. fragilis* strains on cell monolayer integrity were evaluated by a phenol red diffusion assay, whereby diffusion takes place only across the tight junctions (Fig. 3). There was a significant increase ($P<0.05$) in the basal content of phenol red in Caco-2 cell cultures incubated with *B. fragilis* strains B, C, and I compared to control cultures, indicating alteration of the integrity of the cell monolayer. *B. fragilis* strain B (positive for the *mpII* gene and found in active CD patients) and, secondarily, *B. fragilis* strain I (positive for *bft*) caused the most marked impairment of cell monolayer integrity.

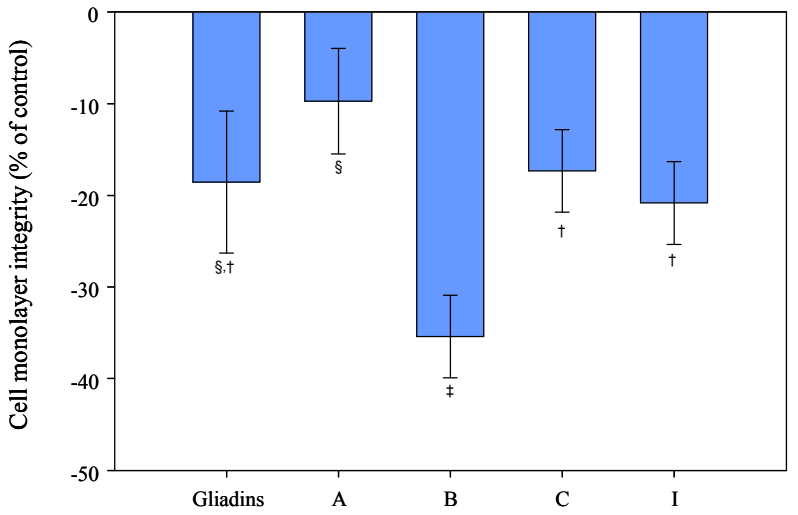

Fig 3. Permeability to phenol red after 4h of incubation with dialyzed fraction of gliadins digested in the presence of *B. fragilis* strains (A, B, C, I) on sensitized (TNFα) Caco-2 cells monolayer. Values are expressed as means ± standard deviations (n=4). Different symbols (†, ‡, and §) indicate statistically significant differences (P<0.05) by applying ANOVA and the LSD *post hoc* test

3.4. Effects of B. fragilis *strains in the generation of gliadin-derived peptides during digestion.*

The amino acid sequences of gliadin-derived peptides generated during simulated gastrointestinal digestion, in the presence or absence of the selected *B. fragilis* strains, and that cross the dialysis membrane, are shown in Table 4. The total protein content of the dialysates from *in vitro* gliadin digestions, not inoculated with *B. fragilis* strains, was 1.05±0.16 mg, representing up to 7% of the total protein loaded in the upper chamber of the *in vitro* system. However, inoculation of the selected *B. fragilis* strains during digestions increased the total dialyzable protein fraction to 2.27±0.38 mg, which constitutes up to 13.3 to 18.7% of the total protein content in the upper chamber, indicating an increase in the degree of gliadin hydrolysis. Peptides generated during *in vitro* digestions showed high variability in their molecular masses, which ranged from 465.7 to 4,869.8 Da. In samples of gliadins digested in the absence of *B. fragilis* strains, peptides with amino acid sequences such as α/ß-gliadin (amino acids 80 to 89) (α/ß-Gld[80-89]) and α/ß-Gld[80-100], which are inflammatory, and α/ß-

Gld[124-133], which has an amino acid sequence similar as those that interact with the chemokine receptor CXCR3, were identified.[18, 20] In samples inoculated with the different *B. fragilis* strains different peptides with amino acid sequences of the main epitopes found in the immunodominant 33-mer peptide of α/ß-Gld[56-88] were identified. For example, the peptide sequences for α/ß-Gld[62-81], α/ß-Gld[82-90], and α/ß-Gld[77-85] were identified in gliadin samples digested in the presence of *B. fragilis* strain A; the sequences for α/ß-Gld[84-96] and α/ß-Gld[72-87] were identified in gliadin samples digested in the presence of *B. fragilis* strain I; the sequences for α/ß-Gld[56-68], α/ß -Gld[77-94], and α/ß-Gld[50-86] were identified in gliadin samples digested in the presence of *B. fragilis* strain C; and the sequences for α/ß-Gld[54-94] and α/ß-Gld[82-90] were identified in gliadin samples digested in the presence of *B. fragilis* strain B. The molecular masses of the peptides identified could not be associated with *bft* or *mpII* gene carriage, but the shorter peptides were identified in samples inoculated with the strains *B. fragilis* strains A and I.

Table 4. Gliadin-derived peptides from dialyzed fraction of gliadin digestions alone or inoculated with *B. fragilis* strains.

	Peptide	Amino acid sequence	Observed *m/z*	Calculated *m/z*	Ion *(m/z)* selected for MS(n) (charge)
Gliadins only					
	α/β-Gld[90-93]	QPQP	465.7	468.3	464.7 (1)
	α/β-Gld[82-89]	PQPQLPYP	933.4	928.5	932.4 (1)
	α/β-Gld[217-224]	SQVSFQQPQ	1033.4	1033.5	1032.4 (1)
	α/β-Gld[124-133]	QQQQQQILQQ	1270.3	1269.7	1270.0 (1)
	α/β-Gld[75-87]	YLQLQPFPQPQLPYPQ	1472.7	1471.8	1471.7 (1)
	α/β-Gld[75-92]	YLQLQPFPQPQLPYPQPQ	2172.8	2172.1	1085.9 (2)
	α/β-Gld[80-100]	PFPQPQLPYPQPQPFRPQQPY	2541.7	2541.8	1270.4 (2)
Gliadins plus strain:					
A	γ-Gld [24-46]	QPFSQQPQQIFPQPQQTPHQPQQ	2371.5	2370.2	2371.5 (1)
	α/β-Gld [62-81]	YPQPQPFPSQQPYLQLQPF	2402.0	2400.2	2402.0 (1)
	α/β-Gld [82-90]	PQPQLPYPQ	1067.5	1068.2	1067.5 (1)
	α/β-Gld [239-248]	QNPQAQGSFQ	1106.5	1105.1	1106.5 (1)
	α/β-Gld [77-85]	QLQPFPQPQ	1085.0	1083.2	1085.0 (1)
	ϖ-Gld [258-267]	QQPQQPYPQQ	1240.9	1241.6	1240.9 (1)
	α/β-Gld [97-105]	QQPYPQPQP	1082.9	1082.5	1082.9 (1)

Table 4. Continuation

Peptide		Amino acid sequence	Observed *m/z*	Calculated *m/z*	Ion *(m/z)* selected for MS(n) (charge)
Gliadin plus strain:					
B	α/β-Gld[248-279]	FQPQQLPQFEAIRNLALQFLPAMCNVYIPPYC	3708.6	3706.8	1854.3 (2)
	α/β-Gld[54-94]	QQPFPPQQPYPQPQPFPSQQPYLQLQPFPQPQLPYPQPQPF	4869.8	4868.4	2434.9 (2)
	α/β-Gld[254-272]	PQFEAIRNLALQTLPAMCN	2131.0	2130.1	1065.5 (2)
	α/β-Gld[82-90]	PQPQLPYPQ	1067.9	1068.2	1067.9 (1)
C	γ-Gld [82-95]	FPQTQQPQQPFPQS	1658.7	1658.8	1659.7 (1)
	ϖ-Gld [376-391]	YPQQQPYGSSLTSIGG	1683.1	1683.8	1684.1 (1)
	α/β-Gld [56-68]	PFPPQQPYPQPQP	1520.8	1521.7	1521.8 (1)
	α/β-Gld [77-94]	QLQPFPQPQLPYPQPQPF	2149.3	2150.1	2150.3 (1)
	α/β-Gld [50-86]	FPGQQQPFPPQQPYPQPQPFPSQQPYLQLQPFPQPQL	4343.3	4343.2	1448.1 (3)
I	α/β-Gld[207-231]	QQQQQQQQPLSQVSFQQPQQQYPSG	2944.4	2943.4	981.8 (3)
	α/β-Gld [97-113]	QQPYPQPQPQYSQPQQP	2041.3	2040.2	2042.3 (1)
	α/β-Gld[148-167]	QQHNIAQGRSQVLQQSTYQL	2329.2	2328.5	2330.2 (1)
	ϖ-Gld [355-371]	QTISQQPQQPFPQQPHQ	2018.2	2018.2	2019.2 (1)
	α/β-Gld [84-96]	PQLPYPQPQPFRP	1565.3	1565.8	1566.3 (1)
	α/β-Gld[72-87]	QQPYLQLQPFPQPQLP	1924.8	1923.2	1925.8 (1)

Inflammatory cytokine production by intestinal Caco-2 cells exposed to gliadin digestion

TNF-α and IL-1ß production by Caco-2 cell cultures exposed to gliadin digestions, inoculated or not with *B. fragilis* strains, is shown in Fig. 4. After gastrointestinal digestion, gliadin-derived peptides induced TNF-α and IL-1ß production in with or without the *B. fragilis* strains tested in comparison to controls (P<0.05). *B. fragilis* strains B and C induced the highest TNF-α production, whose values were higher than those induced by gliadins digested alone (P<0.05), indicating that these *B. fragilis* strains could increase the gliadin-mediated proinflammatory potential. *B. fragilis* strains C and I induced the highest production of IL-1ß, but the increase was only significant in comparison to control, not comparing with gliadin alone.

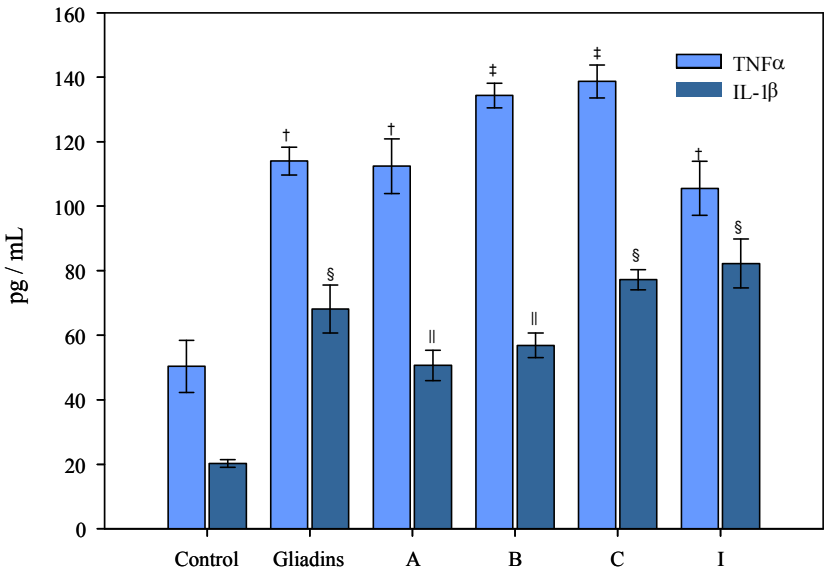

Fig 4. TNF-α and IL-1β production by Caco-2 cell cultures exposed to the dialyzed fraction of gliadin digested with *B. fragilis* strains. Values are expressed as mean ± standard deviation (n=5). Different symbols indicate significant differences for TNF-α (†,‡) or for IL-1β ($^{\S,\|}$) by applying ANOVA and LSD *post hoc* test (P<0.05).

3.5. Effect of B. fragilis *strains on the permeability of the Caco-2 cell monolayer to gliadins.*

To evaluate whether *B. fragilis* strains could increase the permeability of Caco-2 cells to gliadin peptides by their direct deleterious effects on intestinal Caco-2 cell monolayer integrity and by increasing the amount of dialyzable and soluble fraction of gliadin through proteolytic degradation, Caco-2 cells were exposed simultaneously to the bacterial cell suspensions and the dialyzable fraction of gliadins digested in the presence of each *B. fragilis* strain. The toxic fraction of gluten initially loaded in the apical part of the two-chamber system was quantified by ELISA and also in the basal compartment after being incubated with the bacterial strains located in the apical compartment of the two-chamber system. The initial toxic fraction of gluten was significantly (P<0.05) lower in the samples of gliadins digested alone (104.9± 4.4 g/g) than in samples of gliadins digested in the presence of *B. fragilis* strains A (124.1±1.1 g/g), B (126.4 ± 1.9 g/g), C (124.8±3.3 g/g), and I (118.4 ±1.3 g/g). The toxic fractions of gluten detected in the basolateral compartment of Caco-2 cultures exposed to these gliadin digestions, together with the cell suspensions of *B. fragilis* strains, were higher (P< 0.05) than in cell cultures exposed to gliadins digested without bacteria (Fig. 5). *B. fragilis* strain B caused the highest increase in gluten permeability in Caco-2 cells, probably due to its having the most deleterious effect on monolayer integrity related to the presence of the *mpII* gene and its ability to generate peptides that activated a stronger inflammatory response. This strain also generated the most immunogenic 33-mer (α/ß-Gld[54-94], Table 4) identified in α-gliadin.

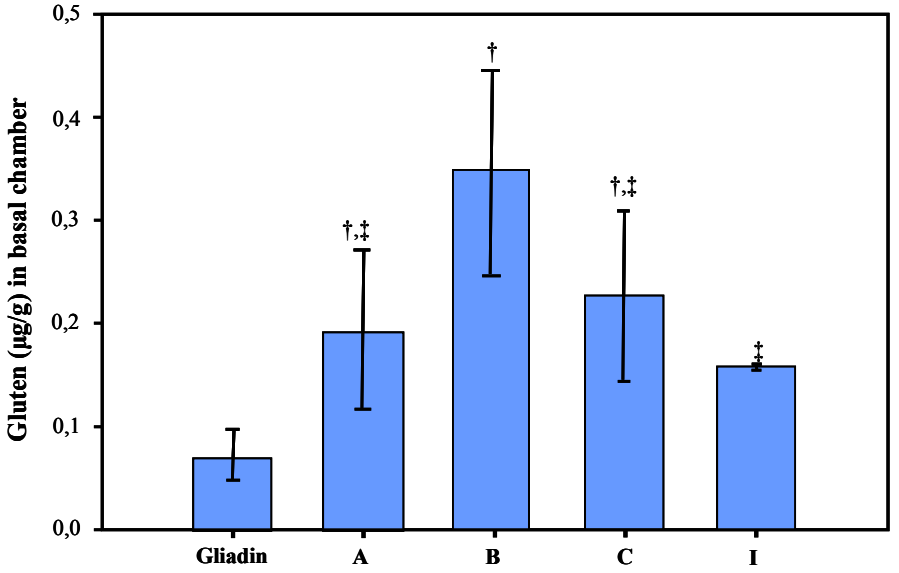

FIG 5 Gluten content quantified in the basal chamber of Caco-2 cell cultures exposed to gliadins digested in the presence of cell suspensions of the different *B. fragilis* strains. Values are expressed as means the standard deviations (n=4). Different symbols († and ‡) indicate statistically significant differences (P<0.05) by applying ANOVA and the LSD *post hoc* test.

4. DISCUSSION

This study reports on the composition and potential virulence features of intestinal *Bacteroides* spp. in CD patients in comparison to healthy controls. *Bacteroides* spp. are commensal inhabitants of the human gastrointestinal tract, but have also been associated with chronic inflammatory bowel disorders (IBDs), such as ulcerative colitis and Crohn's disease,[2, 41, 43] and with CD.[8, 9, 26] In IBD patients, bacteroides and enterobacteria have been considered responsible for 60% of the biofilm mass in the mucosa.[41] Our study shows that active CD patients had a higher abundance of *B. fragilis* and a lower abundance of *B. ovatus* than controls, and these differences were not restored after long-term adherence to a glutenfree diet, suggesting this could play a primary role in the disease. Nevertheless, differences in the abundance of *P. distasonis* and *B. finegoldii* between active CD patients and controls were restored after adherence to the gluten-free diet. Although differences between fecal and duodenal mucosal bacteria may exist, our previous studies reported that *B. fragilis* numbers of feces and biopsy specimens from CD patients correlated, and their differences in comparison with healthy controls were similar,[8] justifying the present study conducted with bacteroides from feces. In accordance with our results, *B. fragilis* is one of the least common species inhabiting the intestinal tract of healthy subjects, and yet it is most frequently isolated from clinical specimens and is the most virulent species.[46] In contrast, *B. vulgatus* has been generally considered one of the most common intestinal *Bacteroides* species of healthy subjects,[46] although in our study no such association was found. Nevertheless, other human studies have also reported associations between increased abundance of *B. vulgatus* and/or *B. ovatus* and chronic IBDs,[10, 22] which partly contrast with our findings in CD patients.

It is known that the pathogenic potential of *Bacteroides* spp. depends on the presence of different virulence factors (e.g., agglutinins, polysaccharide capsules, or lipopolysaccharides) and a variety of proteolytic and hydrolytic enzymes.[29, 46] Moreover, the comparative analyses of the whole genomes of several *B. fragilis* strains are revealing even larger genomic differences among strains (e.g., polysaccharide biosynthesis), which could determine their different

virulence as opportunistic pathogens and ability to evade the immune defense mechanisms.[27, 28] This evidence stresses the need to characterize the isolates at strain level and determine their specific virulence features to understand their potential pathogenicity in a specific ecosystem. In our study, the presence of genes encoding for metalloproteases was evaluated. *B. fragilis* clones with genes encoding for metalloproteases were frequently isolated from CD patients, which suggests a role of at least this species and probably these genes in disease pathogenesis according to our preliminary *in vitro* studies. Enterotoxigenic *B. fragilis* (ETBF) strains have been related to the inflammatory process in humans[2, 30] or animal models of IBD.[30, 32] The enterotoxin produced by ETBF strains is encoded by the *bft* gene, which can be in three different isoforms; *bft-1*, *bft-2*, and *bft-3*.[37] In the present study, *bft-2* was the most common isoform detected in the *B. fragilis* clones isolated from CD patients, which also seemed to be the most frequently present in clones that colonize the guts of children in comparison to those that colonize adult guts.[36] The *B. fragilis* strains tested exhibited a genetic pattern where only either the *bft* gene or the *mpII* gene was present. In accordance with the size of the protease bands detected in our zymograms (Fig. 1), the *bft* gene encodes a zinc-dependent *B. fragilis* toxin (BFT), which is translated as a prepro-protein (44.4 kDa) further processed to a biologically active toxin of 168 amino acid residues, with a molecular mass of 20.7 kDa.[13] In addition, the *mpII* gene also encodes a metalloprotease (20 kDa) predicted to be a zinc-dependent protein with 56% similarity to BFT protein.[12]

The BFT is a soluble virulence factor secreted to the extracellular medium[13] that increases permeability of the intestinal epithelium by cleavage of the trans-membrane adhesion protein E-cadherin.[47] A reduced expression of E-cadherin has been reported in CD patients,[1] although its relation to the microbiota has not yet been directly established. Our study suggests that the carriage of metalloprotease virulence genes by *B. fragilis* strains is associated with the ability of these strains to increase the permeability of the intestinal epithelium *in vitro* and that tight junctions are one of the early sites injured in Caco-2 cell cultures. This property was associated with both *bft* and *mpII* gene carriage, but the most remarkable effects were detected in the strain carrying the *mpII* gene. It has been found that both *bft-1*/*bft-2* isoforms and *mpII* genes

are clustered in pathogenicity islands,[12] and suggested that MPII is synthesized as a precursor protein similar to BFT.[13] The highly conserved sequence of MPII found in different *B. fragilis* strains has led to hypotheses of important roles for this protein in ETBF strains and/or in ETBF-induced disease, although, to the best of our knowledge, no biological activity has yet been identified for this protein. *B. fragilis* strains not endowed with these metalloprotease genes and gelatinase activity also reduced the integrity of the Caco-2 cell monolayer to some extent, suggesting that other factors may also be responsible for this effect.

All of the *B. fragilis* strains studied exhibited gliadin-hydrolyzing activity. In a previous study, gliadin-hydrolyzing activity from microbial origin was found in biopsy specimens of CD patients, in contrast to controls, suggesting a pathogenic role for this activity, although this has yet to be confirmed.[3] For this reason, we also evaluated whether *B. fragilis* could modify the peptide generated from gliadins and their potential immunotoxicity. Our study shows that the *B. fragilis* strains studied hydrolyzed gliadins, producing several peptides with the immunogenetic amino acid sequences of the main epitopes of the immunodominant 33-mer of -Gld[56-88], while the partial digestion of gliadins by the gastrointestinal proteolytic enzymes used in the *in vitro* system did not produce these particular toxic sequences.[19] In order to understand the possible pathological consequences of the hydrolytic activities of *B. fragilis* strains in this disorder, the inflammatory effects of the peptides generated were evaluated, and we demonstrated that they preserve or even increase their ability to induce inflammatory cytokine production (TNF-α). These increases in TNF-α production by epithelial cells could have adverse consequences on the pathogenesis of CD because this cytokine, in conjunction with IL-1ß, increases paracellular permeability, facilitating the translocation of immunogenic peptides derived from gliadin to the lamina propria,[39] and also mediates the infiltration of lymphocytes in the intestinal epithelium, thereby promoting tissue inflammation.[16]

Although all *B. fragilis* strains were shown to have gliadin-hydrolyzing activity, the effects of different *B. fragilis* strains and of the peptides generated were slightly different, probably due to different levels of expression of the

possible enzymes responsible or slightly different specificities.[6] In addition, the gliadin-hydrolyzing activity of all *B. fragilis* strains increased the degree of proteolytic degradation and lowered the molecular masses of the peptides generated, increasing the protein content in the bio-accessible fraction that could facilitate interactions with the apical chemokine CXCR3 receptor of enterocytes, triggering inflammatory events.[18, 20] In addition, the permeation of immunogenic peptides generated by *B. fragilis* from α-gliadins across intestinal epithelia was favored, which could promote the interaction of the peptides with the tissue transglutaminase and with antigen-presenting cells that ultimately activate the T cells responsible for the full expression of the disease.[34]

Conclusions: We have demonstrated here that the species *B. fragilis* is more abundant in the intestinal microbiota of CD patients, whereas *B. ovatus* is less abundant in comparison to healthy controls. These differences were also detected in CD patients after adherence to a gluten-free diet, suggesting that these alterations are not secondary to the underlying disease. We also demonstrated that *B. fragilis* clones carrying the *bft* and *mpII* metalloprotease genes and activity are abundant in CD patients and cause alterations in epithelial permeability. *B. fragilis* clones are also endowed with additional metalloproteases with gliadin specificity that generate immunotoxic peptides during *in vitro* intestinal digestion of gliadin. The generated peptides preserve or even increase their inflammatory properties on intestinal cells and more easily permeate the intestinal epithelial layer, which could favor their interaction with professional immunocompetent cells in the submucosa, although the magnitude of the effects are strain dependent. All in all, our findings indicate that the increased abundance of *B. fragilis* strains with metalloprotease activities in CD patients could play a pathogenic role, although evidence from *in vivo* studies is needed to confirm such a hypothesis.

5. ACKNOWLEDGEMENTS

This study was supported by grants AGL2011-25169 and Consolider Fun*C*-Food CSD2007-00063 from the Spanish Ministry of Science and Education. Scholarships to E. Sánchez from the Institute Danone and a postdoctoral contract to J. M. Laparra within the program Juan de la Cierva (MICINN, Spain) are acknowledged.

We thank L. Izquierdo for statistical advice.

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Capítulo III:

Aislar e identificar bacterias asociadas a la mucosa duodenal de individuos celíacos y compararlas con las de controles no celíacos

- *Duodenal-mucosal bacteria associated with celiac disease in children*

Capítulo III

Duodenal-mucosal bacteria associated with celiac disease in children

ABSTRACT

Celiac disease (CD) is an immune-mediated enteropathy triggered by the ingestion of cereal gluten proteins. This disorder is associated with imbalances in gut microbiota composition that could be involved in the pathogenesis of CD. The aim of this study was to characterize the composition and diversity of the culturable duodenal mucosa-associated bacteria of CD patients and control children. Duodenal biopsy specimens from patients with active disease under a gluten-containing diet (n=32) and patients with non-active disease after adherence to a gluten-free diet (n=17) and controls (n=8) were homogenized and plated on Plate Count, Wilkins-Chalgren, Brain Heart and/or Yeast, Casitone, Fatty Acid media. The isolates were identified by partial 16S rRNA sequencing. Renyi diversity profiles showed the highest diversity values for active CD patients followed by non-active CD and control individuals. Members of the phylum *Proteobacteria* were more abundant in patients with active CD than in the other child groups. *Enterobacteriaceae* and *Staphylococcaceae* were more abundant in patients with active disease than in controls and, particularly, the species *Klebsiella oxytoca*, *Staphylococcus epidermidis* and *Staphylococcus pasteuri*, and these alterations were not completely restored after a gluten-free diet adherence. In contrast, *Streptococcaceae* were less abundant in patients with active CD than in those with non-active disease and controls. Furthermore, *Streptococcus anginosus* and *Streptococcus mutans* groups were more abundant in controls than in patients with both active and not active disease. The findings indicated that the disease is associated with overgrowth of possible pathobionts that exclude symbionts or commensals that are characteristic of the healthy small intestinal microbiota.

Key words: Celiac disease; duodenal microbiota, culture-dependent methods; diversity

1. INTRODUCTION

Celiac disease (CD) is a chronic intestinal disorder caused by a deregulated immune response to gluten proteins from wheat, rye and barley, and their cross-related varieties in genetically susceptible individuals. CD presents a set of diverse clinical features, which typically includes fatigue, weight loss, diarrhea and anemia. Damage of CD intestinal mucosa is characterized by intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy.[1] In CD patients, the pathologic response to gluten proteins involves both adaptive and innate immunity. It is known that gliadin-specific CD4+ T cells develop an inflammatory reaction by production of Th1 cytokines (e.g. IFN-γ) at the mucosal level, which also induces CD8+ cells to kill epithelial cells contributing to tissue damage.[2] In addition, a new subset of T cells, termed Th17 cells, was shown to contribute to CD pathogenesis by producing pro-inflammatory cytokines (such as IL-17, IFNγ, IL-21), although it can also produce mucosa-protective and regulatory factors (IL-22 and TGFß).[3,4] Some gluten peptides that are not recognized by T cells can induce tissue damage by activating components of innate immunity; thus, peptide p31-43/49 activates the production of IL-15 and Natural Killer-receptor-mediated cytotoxicity by intraepithelial lymphocytes, contributing to tissue injury.[5-7] Improvement of the pathological lesions occurring in the intestinal mucosa of sensitive individuals is usually observed after gluten withdrawal from diet; however compliance with this dietary recommendation is complex and other alternative strategies are being investigated.[8]

HLA class II molecules, DQ2 and DQ8, are the major risk factors predisposing individuals to CD and account for 35% of genetic risk.[9] Although the role of these molecules has been well established in CD pathogenesis, their frequency in the general population is approximately 30%, whereas only 1-3% actually develop the disease.[10] This data would suggest that the presence of HLA molecules is a necessary factor but not sufficient alone for disease development. Although gluten is the main environmental trigger of CD, its intake does not fully explain disease development and, thus, other environmental factors are thought to be involved. In recent years, early microbial infections [11,12] and imbalances in the gastrointestinal microbiota composition [13-20] have also been associated with CD. Molecular techniques have shown that, compared to healthy individuals, the

fecal and duodenal microbiota of CD patients is characterized by the presence of higher numbers of Gram-negative bacteria (bacteroides and enterobacteria) and lower numbers of Gram-positive bacteria, like bifidobacteria using molecular techniques.[19,20] *In vitro* assays have shown that this altered microbiota and some enterobacteria isolated from CD patients could activate pro-inflammatory pathways, while some bifidobacteria could inhibit the inflammatory or toxic effects induced by the same isolated enterobacteria and gluten peptides.[21-24] Alterations in the intestinal microbiota are also involved in the pathogenesis of chronic inflammatory bowel disease (IBD) [25,26] and other immune-related disorders.[27-29] For instance, IBD patients have altered duodenal bacterial populations in comparison to healthy controls.[30-32] Nevertheless, neither the specific bacteria involved in pathologies affecting the small intestine nor their possible pathogenic modes of action are fully understood.

This study was designed to establish whether live culture-dependent bacteria associated with the duodenal mucosa of patients with active and non-active CD and controls differ in composition and biodiversity, as reported in previous molecular studies, with a view to exploring their potentially pathogenic features in the future.

1. MATERIALS AND METHODS

1.1. Subjects

Three groups of children were included in this study: 32 biopsy samples from patients with active CD (mean age 5.1 years, range 2–14 years) on a normal gluten-containing diet; 17 from patients with non-active CD (mean age 5.9 years, range 3–8 years) after following a gluten-free diet for at least 2 years; and 8 biopsy samples from control children (mean age 6.9 years, range 3–13 years) without known gluten intolerance. Biopsy specimens of the control group were obtained from children who were investigated for weight loss, growth retardation or functional intestinal disorders of non-CD origin confirmed by showing a normal villous structure after diagnosis by biopsy examination. CD was diagnosed according to criteria given by the European Society for Pediatric Gastroenterology, Hepathology and Nutrition.(33) Children included in the study were not treated with antibiotics for at least 1 month before sampling time.

The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), following EEC Good Clinical Practice guidelines (Document 111/3976/88, July 1990) and current Spanish law, which regulates clinical research in humans (Royal Decree 561/1993). The study protocol was approved by the Committee on Ethical Practice from CSIC and the Hospital Universitario La Fe (Valencia, Spain). Written informed consent was obtained from the parents of children included in the study. Clinical characteristics of the children are shown in **Table 1**.

Table 1. Clinical characteristics of the study subjects.

Clinical data*	Active CD n=32	Non-active CD n=17	Control n=8
Mean age (years)	5.1 (3.2)	5.9 (1.2)	6.9 (4.2)
Sex (M/F)	14(44)/18(56)	8(47)/9(53)	4(50)/4(50)
Symptoms			
Abdominal pain	5 (15.6)	0 (0)	2 (25)
Diarrhea	3 (9.4)	0 (0)	5 (62.5)
Weight loss	5 (15.6)	3 (17.6)	1 (12.5)
Anemia	9 (28.1)	2 (11.8)	0 (0)
Iron deficiency	17 (53.1)	0 (0)	0 (0)
Anti-gliadin antibodies	32 (100)	0 (0)	0 (0)
Anti-transglutaminase antibodies	32 (100)	0 (0)	0 (0)
Duodenal biopsy**			
M0-1	0 (0)	17 (100)	8 (100)
M3	32 (100)	0 (0)	0 (0)
HLA typing (DQ2/DQ8)	32 (100)	17 (100)	N/A†

*Data are expressed as absolute numbers and percentages related to the total numbers in brackets, except for age that is expressed as mean and its standard deviation in brackets.

**Modified Marsh classification of CD.(1) M0, normal mucosa; M0-1, (infiltrative lesion) seen in patients on a gluten-free diet (suggesting minimal amounts of gliadin are being ingested), patients with dermatitis herpetiformis, and family members of patients with CD; M2, (hyperplastic type) seen occasionally in dermatitis herpetiformis; M3, > 40 intraepithelial lymphocytes per 100 enterocytes, crypts increased and villi with atrophy (partial or complete villous atrophy) seen in cases of typical CD.

N/A†, not applicable.

1.2. Sample preparation and bacterial isolation

Duodenal biopsy specimens were obtained by capsule endoscopy, kept under anaerobic condition (AnaeroGen; Oxoid, Hampshire, United Kingdom) and analyzed in less than 2 h to avoid alterations in bacterial viability. Biopsy specimens were homogenized in 200 µl phosphate buffered saline solution (PBS, 130 mM-sodium chloride, 10 mM-sodium phosphate, 0.05% cysteine, pH 7.2), by thorough agitation in a vortex (10 sec). Each homogenized sample was plated on two different culture media (100 µl).

The following media were used: Plate Count agar (PCA, Scharlau, Barcelona, Spain),[34] Wilkins-Chalgren agar (Scharlau, Barcelona, Spain)[35], Brain Heart agar (BH, Scharlau, Barcelona, Spain) [36], and Yeast, Casitone, Fatty Acid agar (YCFA).[37] PCA plates were incubated under aerobic conditions at 37 ºC for 48 hours, whereas Wilkins-Chalgren, BH and YFCA plates were incubated under anaerobic conditions at 37 ºC for 48 hours.

All the viable and culturable bacteria recovered from duodenal biopsy samples (mucus and mucosa-associated bacteria) were isolated and re-streaked onto the same agar media. All isolates were stored at -80ºC in presence of glycerol (20%, v/v) until use for further characterization.

1.3. DNA extraction

For DNA extraction, bacterial isolates were grown in the same isolation broth media and harvested at the late-log growth phase. The bacterial suspensions were centrifuged for 5 min at 6000 g, the pellets were resuspended in 100 µL of suspension buffer (10 mM Tris–HCl, 1 mM sodium EDTA, pH 8.0) with lysozyme (50 mg/mL) (Sigma, St. Louis, MO) and the homogenates were incubated at 37ºC for 1 h. Bacterial DNA extraction procedure was adapted from a standard cetyltrimethylammonium bromide (CTAB) DNA purification method .[38] DNA samples were stored at -20℃ until used as templates for PCR.

1.4. Identification of bacterial isolates

Bacterial DNA of each isolate was amplified with 16S rRNA-gene target primers: 968f, 5´-AACGCGAAGAACCTTA-3´; and 1401-r 5´-CGGTGTGTACAAGACCC-3´.[39] Amplification reactions were carried out in a 50 µl volume containing 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 1 µM of each primer, 200 µM of dNTPs and 2.5 U of Taq polymerase (Ecotaq, ECOGEN, Spain). The amplification programme was 1 cycle at 94ºC for 5 min; 30 cycles at 94ºC for 1 min, 50ºC for 1 min, and 72ºC for 2 min; and finally 1 cycle at 72ºC for 7 min. The amplification products were subjected to gel electrophoresis in 1% agarose gels, purified using GFXtm PCR DNA and Gel Band DNA Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced in an ABI PRISM-3130XL Genetic Analyzer (Applied Biosystems, California, USA). Search analyses to determine the closest relatives of the retrieved partial 16S rRNA gene sequences were conducted in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm, sequences with more than 97% similarity were considered to be of the same species.

1.5. Statistical analyses

Renyi diversity index was used to explore differences in the mucosa-associated bacteria among active and non-active CD patients and control children. This index provides three further diversity index values: species richness (S), Shannon diversity index (*H´*) and Simpson dominance index (1-D) that were determined using PAlaeontological STatistics (PAST) software.[40]

Differences in the relative abundance of the duodenal mucosa-associated bacteria (estimated as isolates belonging to a specific taxa related to total isolates recovered from samples of each child group) were established applying the Chi-square tests, and when appropriate the two-tailed Fisher's exact test. Analyses were carried out with the Statgraphics software (Manugistics, Rockville, MD), and statistical differences were established at a P value less than 0.05.

2. RESULTS

2.1. Subjects

Clinical characteristics of the groups of children included in the study are shown in **Table 1**. No statistically significant differences were detected for gender ratio representation in the study. Patients with active CD, on a normal gluten-containing diet, showed clinical symptoms of the disease, positive CD serology markers (anti-gliadin antibodies and anti-transglutaminase antibodies) and signs of severe enteropathy by duodenal biopsy examination, classified as type 3 according to the Marsh classification of CD.(1) Patients with non-active , following a gluten-free diet for at least 2 years, showed negative CD serology markers and normal mucosa or infiltrative lesion classified as type 0–1 according to the Marsh classification of CD. A total of 32 biopsy specimens from children (mean age 5.1 years) were included in the group of patients with active CD; a. total of 17 biopsy specimens from children (mean age 5.9 years) were included in the group of patients with non-active; and finally, a total of eight biopsy specimens from children (mean age 6.9 years), without known gluten intolerance, were included in the control group for comparative purposes.

2.2. Influence of the culture media on bacterial species recovered

Four different culture media, including PCA, Wilkins-Chalgren, BH and YFCA, were used for isolating bacteria from biopsy specimens of CD patients and controls. The same number of biopsy specimens from active CD (n=16), non-active CD (n=8 [PCA and Wilkins-Chalgren]; or n=9 [BH and YFCA]) and control (n=4) children were cultured in each medium and, therefore, the suitability of each medium to recover duodenal bacteria could be analyzed independently of subject health status.

The abundance of culturable bacterial species associated with the mucosa of the subjects included in this study is shown in **Table 2**. Some differences were detected in the bacterial phyla, genera and species isolated from different culture media.

When the isolates were classified into different phyla, differences were found for *Firmicutes*, whose members were more frequently isolated in BH than in YFCA medium (P=0.02), and for *Proteobacteria* whose members were more frequently recovered in PCA, followed by YFCA, Wilkins-Chalgren and BH media; significant differences were detected between PCA and BH media (P<0.01) and between YCFA and BH media (P=0.02). Differences among culture media were not detected for isolates belonging to the phylum *Actinobacteria*.

In relation to families and species, members of the family *Staphylococcaceae* were more frequently isolated in PCA and Wilkins-Chalgren than in BH (P<0.01 and P=0.01, respectively) and YCFA media (P=0.01 and P=0.05, respectively). Of the staphylococcal species, *Staphylococcus epidermidis* was more frequently isolated in Wilkins-Chalgren than in YCFA medium (P=0.03); and *Staphylococcus pasteuri* was isolated significantly more frequently in PCA than in Wilkins-Chalgren, BH and YFCA media (P=0.03, P<0.01 and P<0.01, respectively).

Members of the family *Streptococcaceae* were more frequently isolated in BH than in PCA and Wilkins-Chalgren media (P<0.01 and P=0.01 respectively). Within this family, the *Streptococcus anginosus* group was significantly more abundant in biopsy samples cultured in BH than in Wilkins-Chalgren media (P=0.03).

Finally, members of the *Clostridiaceae* family were more frequently isolated in Wilkins-Chalgren agar than in BH (P=0.02); and those of the *Enterobacteriaceae* family were more frequently isolated in PCA and YFCA media than in BH (P=0.02).

Table 2. Cultivable bacterial taxa isolated in Plate Count Agar (PCA), Wilkins-Chalgren, Brain Heart (BH) and Yeast, Casitone, Fatty Acid (YCFA) media from active and non-active CD patients and control subjects.

	PCA N=29 (%)	WilkinsChalgren N=50 (%)	BH N=142 (%)	YCFA N=81 (%)
Non-identificable bacteria	0	1 (2.0)	7 (4.9)	10 (12.3)
Phylum *Actinobacteria*	2 (6.9)	1 (2.0)	12 (8.5)	5 (6.2)
Actinomycetaceae	0	0	7 (4.9)	3 (3.7)
Actinomyces odontolyticus (GQ131411)	0	0	7 (4.9)	3 (3.7)
Corynebacteriaceae	0	0	1 (0.7)	0
Corynebacterium accolens (KC113137)	0	0	1 (0.7)	0
Micrococcaceae	2 (6.9)	1 (2.0)	3 (2.1)	1 (1.2)
Kocuria kristinae (JF718430)	2 (6.9)	1 (2.0)	1 (0.7)	1 (1.2)
Rothia mucilaginosa (NR074690)	0	0	2 (1.4)	0
Propionibacteriaceae	0	0	1 (0.7)	1 (1.2)
Propioniacterium acnes (JN700236)	0	0	1 (0.7)	1 (1.2)
Phylum *Firmicutes*	20 (69.0) [a, b]	42 (84.0) [a, b]	118 (83.1) [a]	56 (69.1) [b]
Carnobacteriaceae	0	1 (2.0)	5 (3.5)	1 (1.2)
Granulicatella adiacens (FR822389)	0	1 (2.0)	5 (3.5)	1 (1.2)
Clostridiaceae	0 [a, b]	5 (10.2) [a]	2 (1.4) [b]	2 (2.5) [a, b]
Clostridium bifermentans (JX267097)	0	3 (6.0)	1 (0.7)	0
Clostridium butyricum (HQ830243)	0	2 (4.0)	0	0
Clostridium perfringens (NR074482)	0	0	1 (0.7)	2 (2.5)
Enterococcaceae	1 (3.5)	0	1 (0.7)	0
Enterococcus faecalis (KC484866)	1 (3.5)	0	1 (0.7)	0
Unclassified *Bacillales*	0	1 (2.0)	3 (2.1)	0
Gemella haemolysans (JF803540)	0	1 (2.0)	2 (1.4)	0
Gemella sanguinis (NR026419)	0	0	1 (0.7)	0
Lactobacillaceae	0	0	0	2 (2.5)
Lactobacillus fermentum (KC207719)	0	0	0	2 (2.5)
Staphylococcaceae	10 (34.5) [a]	13 (26.0) [a]	14 (9.9) [b]	9 (11.1) [b]
Staphylococcus aureus (JX866757)	0	0	0	3 (3.7)
Staphylococcus epidermidis (KC213963)	5 (16.7) [a, b]	8 (16.3) [a]	13 (9.2) [a, b]	3 (3.7) [b]
Staphylococcus hominis (KC417307)	0	2 (4.0)	0	1 (1.2)
Staphylococcus pasteuri (JQ993883)	6 (20.0) [a]	2 (4.1) [b]	1 (0.7) [b]	2 (2.5) [b]

Table 2. Continuation

Closest relative (Accession number) †	PCA N=29 (%)***	WilkinsChalgren N=50 (%)	BH N=142 (%)	YCFA N=81 (%)
Streptococcaceae	9 (31.0) [a]	22 (44.0) [a]	90 (63.4) [b]	40 (49.4) [a, b]
Streptococcus anginosus gr. (GU907516)	0 [a, b]	0 [a]	16 (11.3) [b]	4 (4.9) [a, b]
Streptococcus australis (NR036936)	0	0	4 (2.8)	0
Streptococcus bovis gr. (DQ148957)	0	0	3 (2.1)	1 (1.2)
Streptococcus gallolyticus (NR074987)	0	0	1 (0.7)	0
Streptococcus gordonii (NR074516)	0	1 (2.0)	0	0
Streptococcus mitis gr (JF496732)	6 (20.7)	8 (16.0)	20 (14.1)	7 (8.6)
Streptococcus mutans gr. (NR074983)	0	0	1 (0.7)	4 (4.9)
Streptococcus pneumoniae (HE983624)	0	0	11 (7.8)	3 (3.7)
Streptococcus salivarius gr. (AB680534)	1 (3.5)	11 (22.0)	19 (13.4)	15 (18.5)
Streptococcus sanguinis gr. (JF496736)	2 (6.9)	2 (4.0)	13 (9.3)	6 (7.4)
Streptococcus suis (FJ434466)	0	0	2 (1.4)	0
Veillonellaceae	0	0	3 (2.1)	2 (2.5)
Veillonella atypica (HQ012015)	0	0	1 (0.7)	1 (1.2)
Veillonella dispar (NR041879)	0	0	1 (0.7)	0
Veillonella parvula (GU574206)	0	0	1 (0.7)	2 (2.4)
Phylum *Proteobacteria*	7 (24.14) [a]	6 (12.0) [a, b]	5 (3.5) [b]	10 (12.6) [a]
Burkholderiaceae	1 (3.5)	0	0	0
Burkholderia cepacia (JX979120)	1 (3.5)	0	0	0
Enterobacteriaceae	5 (17.2) [a]	5 (10.0) [a, b]	5 (3.5) [b]	10 (12.3) [a]
Enterobacter cloacae (JX979128)	2 (6.9)	2 (4.0)	0	2 (2.5)
Escherichia coli (KC429776)	0	0	2 (1.4)	2 (2.5)
Klebsiella oxytoca (AB749211)	3 (10.3)	3 (6.0)	3 (2.1)	6 (7.4)
Neisseriaceae	1 (3.5)	0	0	0
Neisseria flavescens (KC178511)	1 (3.5)	0	0	0
Pseudomonadaceae	0	1 (2.0)	0	0
Pseudomonas stutzeri (KC253270)	0	1 (2.0)	0	0

†Accession numbers of the closets relative as determined by searching analyses conducted in GenBank using the BLAST algorithm, sequences with more than 97% similarity were considered to be of the same species.

*Data are expressed as absolute numbers of the clones isolated belonging to one specific taxonomic group (phylum, family, or species) and its percentage related to the total number of isolates from each culture media (PCA, Wilkins-Chalgren, BH and YCFA).

[(a,b)] Different letters within a row denote statistical significant differences estimated by using Chi-square test 2×2 and when appropriate the Fisher´s exact test, at $P<0.05$.

The species richness (S), Shannon species diversity (*H´*) and Simpson species dominance (1-D) indexes were calculated for PCA (S=12, *H´*=2.32 and 1-D=0.88), Wilkins-Chalgren (S=17, *H´*=2.49 and 1-D=0.89), BH (S=29, *H´*=2.80 and 1-D=0.92) and YFCA media (S=23, *H´*=2.79 and 1-D=0.92), in order to apply the Renyi index. Renyi diversity profiles showed that the use of PCA and Wilkins-Chalgren media led to the recovery of bacteria with lower species diversity than the use of either BH or YFCA media. Renyi diversity profiles also showed that PCA and Wilkins-Chalgren curves intersect with each other, and the same was observed for BH and YFCA curves; therefore, the diversity of these pairs could not be compared (**Figure 1**).

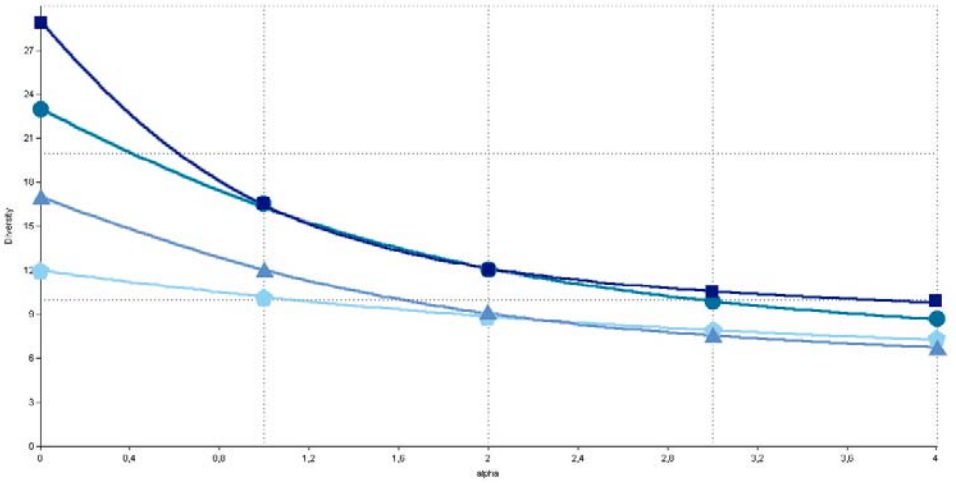

Figure 1. Renyi index profiles of the cultivable bacterial groups associated with the duodenal mucosa of the child study groups in PCA (⬟), Wilkins-Chalgren (▲), BH (■) and YCFA (●) media. The Renyi index estimates total richness for α=0, Shannon index for α=1 and Simpson index for α=2.

Duodenal mucosa-associated bacteria in CD patients and controls

The proportion of biopsy specimens inoculated in each culture medium was similar (~25%) for each group of individuals (patients with active CD, patients with non-active CD and controls) and, therefore, the total number of bacteria recovered in the different media was considered to represent the differences among the study groups, regardless of the different culture media used.

The relative abundance of culturable bacterial associated with the duodenal mucosa of the different child groups that could be isolated and their differences are shown in **Table 3**. In relation to phyla, members of the phylum *Proteobacteria* were more abundant in biopsy samples of patients with active CD (15.1%) than in those of controls (1.4%, P<0.01), while the relative abundance of members of *Actinobacteria* and *Firmicutes* did not differ among the child groups. In relation to families, members of the families *Enterobacteriaceae* were more abundant in patients with active CD than in control individuals (P<0.01). In particular, *Klebsiella oxytoca* isolates were more abundant in patients with active CD than in control children (P=0.02). In addition, members of the family *Staphylococcaceae* were more abundant in patients with active CD than in patients with non-active CD and control individuals (P=0.02 and P<0.01, respectively). In particular, *S. epidermidis* and *S. pasteuri* isolates were more abundant in patients with active CD than in control children (P=0.01 and 0.02, respectively). The same trends were detected between non active CD patients and controls, although differences did not reach statistical significance. Furthermore, members of the family *Streptococcaceae* were less abundant in patients with active CD than in patients with non-active CD and in control children (P<0.01). Statistically significant differences were also detected in the abundance of some particular *Streptococcus* groups; thus, *S. anginosus* and *Streptococcus mutans* groups were more abundant in control individuals than in patients with active (P<0.01 and P=0.02 respectively) and non-active CD (P<0.01 and P=0.02 respectively), whereas *Streptococcus mitis* group was more abundant in patients with non-active CD patients than with active CD (P=0.01).

Table 3. Relative abundance of cultivable bacterial taxa isolated in PCA, Wilkins-Chalgren, BH or YCFA media from biopsy specimens of active and non-active CD patients and control subjects.

Closest relative (Accession number) †	Active CD N=146 (%)*	Non-active CD N=85 (%)	Control N=71 (%)
Phylum *Actinobacteria*	14 (9.6)	2 (2.6)	4 (5.6)
Actinomycetaceae	8 (5.4)	0	2 (2.8)
Actinomyces odontolyticus (GQ131411)	8 (5.4)	0	2 (2.8)
Corynebacteriaceae	1 (0.7)	0	0
Corynebacterium accolens (KC113137)	1 (0.7)	0	0
Micrococcaceae	5 (3.5)	0	2 (2.8)
Kocuria kristinae (JF718430)	3 (2.0)	0	2 (2.8)
Rothia mucilaginosa (NR074690)	2 (1.4)	0	0
Propionibacteriaceae	0	2 (2.4)	0
Propioniacterium acnes (JN700236)	0	2 (2.4)	0
Phylum *Firmicutes*	107 (73.3)	72 (84.7)	57 (80.3)
Carnobacteriaceae	2 (1.4)	4 (4.7)	1 (1.4)
Granulicatella adiacens (FR822389)	2 (1.4)	4 (4.7)	1 (1.4)
Clostridiaceae	6 (4.1)	2 (2.4)	3 (4.2)
Clostridium bifermentans (JX267097)	1 (0.7)	0	3 (4.2)
Clostridium butyricum (HQ830243)	2 (1.4)	2 (2.4)	0
Clostridium perfringens (NR074482)	3 (2.0)	0	0
Enterococcaceae	2 (1.4)	0	0
Enterocuccus faecalis (KC484866)	2 (1.4)	0	0
Lactobacillaceae	0	0	2 (2.8)
Lactobacillus fermentum (KC207719)	0	0	2 (2.8)
Unclassified *Bacillales*	2 (1.4)	2 (2.4)	0
Gemella haemolysans (JF803540)	1 (0.7)	2 (2.4)	0
Gemella sanguinis (NR026419)	1 (0.7)	0	0
Staphylococcaceae	35 (23.7) [a]	9 (10.6) [b]	2 (2.8) [b]
Staphylococcus aureus (JX866757)	3 (2.1)	0	0
Staphylococcus epidermidis (KC213963)	21 (14.4) [a]	6 (7.1) [a, b]	2 (2.8) [b]
Staphylococcus hominis (KC417307)	1 (0.7)	2 (2.4)	0
Staphylococcus pasteuri (JQ993883)	9 (6.9) [a]	1 (1.2) [a, b]	0 [b]
Veillonellaceae	3 (2.0)	2 (2.4)	0
Veillonella atypica (HQ012015)	1 (0.7)	1 (0.7)	0
Veillonella dispar (NR041879)	1 (0.7)	0	0
Veillonella parvula (GU574206)	1 (0.7)	1 (1.2)	0

Table 3. Continuation

Closest relative (Accession number) †	Active CD N=146 (%)*	Non-active CD N=85 (%)	Control N=71 (%)
Phylum *Firmicutes* (continuation)			
Streptococcaceae	59 (39.9) [a]	53 (62.4) [b]	49 (69.0) [b]
Streptococcus anginosus group (GU907516)	0 [a]	0 [a]	20 (28.2) [b]
Streptococcus australis (NR036936)	4 (2.7)	0	0
Streptococcus bovis group (DQ148957)	0 [a]	4 (4.7) [b]	0 [a,b]
Streptococcus gallolyticus (NR074987)	0	1 (1.2)	0
Streptococcus gordonii (NR074516)	1 (0.7)	0	0
Streptococcus mitis group (JF496732)	14 (9.5) [a]	19 (22.4) [b]	8 (11.3) [a,b]
Streptococcus mutans group (NR074983)	0 [a]	0 [a]	5 (7.1) [b]
Streptococcus pneumoniae (HE983624)	7 (4.7)	6 (7.1)	1 (1.4)
Streptococcus salivarius group (AB680534)	25 (16.9)	15 (17.7)	6 (8.5)
Streptococcus sanguinis group (JF496736)	8 (5.4)	6 (7.1)	9 (12.7)
Streptococcus suis (FJ434466)	0	2 (2.4)	0
Phylum *Proteobacteria*	22 (15.1) [a]	5 (5.9) [a, b]	1 (1.4) [b]
Burkholderiaceae	0	0	1 (1.4)
Burkholderia cepacia (JX979120)	0	0	1 (1.4)
Enterobacteriaceae	21 (14.2) [a]	4 (4.7) [b]	0 [b]
Enterobacter cloacae (JX979128)	6 (4.1)	0	0
Escherichia coli (KC429776)	4 (2.7)	0	0
Klebsiella oxytoca (AB749211)	11 (7.4) [a]	4 (4.7) [a, b]	0 [b]
Neisseriaceae	0	1 (1.2)	0
Neisseria flavescens (KC178511)	0	1 (1.2)	0
Pseudomonadaceae	1 (0.7)	0	0
Pseudomonas stutzeri (KC253270)	1 (0.7)	0	0
Non-identificable bacteria	3 (2.0) [a]	6 (7.0) [a,b]	9 (12.7) [b]

†Accession numbers of the closets relative as determined by searching analyses conducted in GenBank using the BLAST algorithm, sequences with more than 97% similarity were considered to be of the same species.

*Data are expressed as absolute numbers of the clones isolated belonging to one specific taxonomic group (phylum, family, or species) and its percentage related to the total number of isolates from each group of children (patients with active CD, patients with non-active CD and controls).

(a,b) Different letters within a row denote statistical significant differences estimated by using Chi-square test 2×2 and when was appropriate the Fisher´s exact test, at $P<0.05$.

The species richness (S), Shannon species diversity (*H´*) and Simpson species dominance (1-D) indexes were different between active CD patients (S=29, *H´*=3.16 and 1-D=0.90), non-active CD patients (S=19, *H´*=2.62 and 1-D=0.87) and controls (S=14, *H´*=2.33 and 1-D=0.84), indicating different species diversity between the studied child groups. Renyi diversity profiles showed that active CD patients had the highest biodiversity of duodenal culturable bacteria, followed by that of non-active CD patients and controls (**Figure 2**).

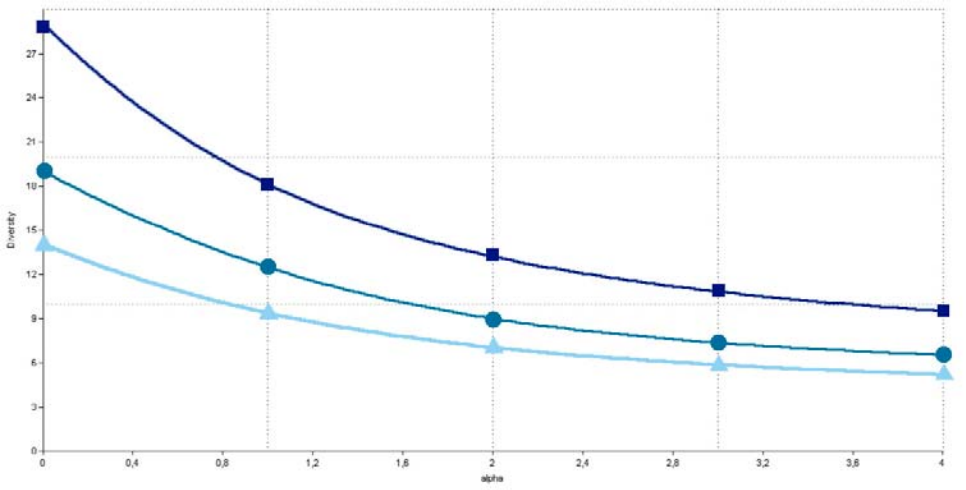

Figure 2. Renyi index curves of the cultivable bacterial groups associated with the duodenal mucosa of active CD patients (■), non-active CD (●) patients and control children (▲). The Renyi index estimates total richness for α=0, Shannon index for α=1 and Simpson index for α=2.

3. DISCUSSION

The study reported herein demonstrates that the microbiota associated with the duodenal mucosa of CD patients has a characteristic deviation from normal microbiota structure, which may characterize the disease. The alterations reported in the present study are partly consistent with those previously detected by molecular techniques using specific primers or probes.[19,20] Thus, our results support the hypothesis that normal components of the microbiota are excluded and replaced by others that could act as pathobionts in this specific disease environment. Although such associations do not demonstrated causality between the altered microbial groups and the disease, they provide a rationale for further studies on the possible pathogenic modes of action of such alterations and specific bacteria in CD.

To obtain bacterial isolates that are representative of those inhabiting the duodenal mucosa in both numbers and diversity, four different culture media were used previously described in literature.[34-37] In general, the greatest species diversity and quantitative recovery of mucosal associated bacteria were obtained using BH and YFCA culture media.

These differences could be linked to the high nutritional requirements of intestinal bacteria, which are better met by the composition of these media; also incubation conditions may have been more appropriate as they were more anaerobic. Even though, to completely reproduce the *in vivo* environmental conditions is rather complicated since the diverse morphology of the small intestine favor a precise spatial relationship of strains within particular intestinal nutritional and micro-aero environments.[41] Also the possibility of having lost some anaerobic bacteria due to oxygen exposure during sample manipulation cannot be disregarded even if the duodenum environment is not strictly anaerobic.

We also analyzed whether some of the media used proved better to isolate specific bacteria. In this regard, PCA and Wilkins-Chalgren seemed to favor the growth and isolation of members of the family *Staphylococcaceae*, but hindered the growth of members of the family *Streptococcaceae*. BH medium favored the growth of the family *Streptococcaceae* but hampered that of *Enterobacteriaceae*. Wilkins-Chalgren also favored the recovery of members of the family

Clostridiaceae. We confirm that none of the media or incubation conditions tested were suitable to recover all viable bacteria detected in the samples analyzed and, therefore, various media must be used to improve the representativeness of live bacteria inhabiting the gut.

We observed increased diversity of the culturable mucosa-associated bacteria of CD patients compared to controls, and these differences were restored after adherence to a gluten-free diet. In concordance, denaturing or temperature gradient gel electrophoresis (DGGE and TGGE, respectively) analysis of duodenal samples showed higher bacterial diversity associated with the small intestinal microbiota of CD patients.[13,18] However, several recent molecular studies [42-45] have reported that reduced mucosal bacterial diversity is associated with inflammatory bowel disease (IBD), although the conditions and techniques used are not always comparable and the section of the intestinal tract studied was not the same.

Considering isolates from all subject groups under study, our results show that the most abundant were those belonging to the phylum *Firmicutes*, followed by those of the phyla *Proteobacteria* and *Actinobacteria*. This is in concordance with a previous culture-independent study, where the same three phyla dominated the proximal small intestine of CD patients, followed by other phyla such as *Bacteroidetes* or *Fusobacteria*.[46] Although our previous culture-independent studies also detected increased numbers of duodenal and fecal *Bacteroides* spp. compared with controls,[19,20,47] this bacterial group was not isolated with the culture conditions applied, probably due to exposure to oxygen during the homogenization process of biopsies and the use of non-selective media for bacteroides, which could have limited the growth of more competitive bacteria, less anaerobic and less nutritionally demanding. Culture-independent studies indicate that normal human gut microbiota belong mainly to two phyla: *Firmicutes* and *Bacteroidetes*, with a smaller number of bacteria belonging to *Proteobacteria* and *Actinobacteria*.[44,48] In addition, previous data suggest that only 12% of the total species richness were detected by applying both molecular and cultivation-based approaches.[50] Remarkably, with both approaches, *Firmicutes* represented the most abundant group; *Proteobacteria* were relatively poorly detected by molecular approaches; and *Bacteroidetes* were less abundant

when was assessed with cultivation-based approaches than with molecular techniques.[48-50] In relation to CD, differences were identified in phylum representation and, in particular, isolates belonging to *Proteobacteria* were more abundant in active CD patients than in non-active CD patients and controls. In this context, microbial diversity studies have also associated an increase in *Proteobacteria* with IBD and, in particular, an increase in adherent-invasive *E. coli*, *Campylobacter concisus* and enterohepatic *Helicobacter*.[51]

In addition, CD seemed to be associated with a decreased abundance of *Streptococcaceae*, specifically of *S. anginosus* and *S. mutans* groups; and with increased proportions of *Enterobacteriaceae* and *Staphylococcaceae*, particularly *Klebsiella oxytoca*, *S. epidermidis* and *S. pasteuri*. In concordance with these observations, recent culture-independent studies indicate that duodenal and fecal microbiota of CD patients are characterized by higher numbers or proportions of *Escherichia coli* and *Staphylococcus*.[19, 20] Furthermore, previous studies using cultured-dependent techniques have shown increased levels of *S. epidermidis* [16] in feces from both active and non-active CD patients in comparison with healthy controls, and a lower prevalence of salivary *S. mutans* associated with CD.[52] It seems that dominant genera in the normal microbiota of healthy individuals, which may act as symbionts, like *Streptococcus* spp., are replaced by potential pathobionts in CD microbiota, like *Staphylococcus* spp. (*S. epidermidis*) and enterobacteria, which could contribute to breaking down the normal dynamics and balance of the ecosystem.

To our knowledge, this is the first time that culturable mucosal-associated bacteria of patients with active and non-active CD has been studied, because previous studies were focused on the characterization of CD microbiota using molecular tools, such as DGGE and TGGE,[13,15,53] fluorescence in situ hybridization (FISH)[20] or real-time PCR.[19] Culture-dependent studies are intrinsically biased by the culture media used, the impact of potential oxygen exposure and the inability to detect viable but non-culturable bacteria present in biological samples; notwithstanding, the results obtained in the present study are coherent with previous studies based on molecular techniques that overcome these limitations. Therefore the use of culture-dependent techniques has allowed the characterization of the active fraction of the mucosal microbiota of CD patients

and will facilitate future investigation into the possible pathogenic role that isolated bacteria play in the development of CD.

Conclusions

This study has demonstrated that the duodenal-mucosal microbiota of CD patients presents alterations in the diversity and abundance of different culturable bacterial taxa (phylum, family, genera and species), which could be a consequence of the pathogenesis of CD, involving massive destruction of small bowel mucosa and consequent release of intracellular contents and serum into the gut. These alterations are attenuated after long-term adherence to a gluten-free diet but not completely restored, which also suggests their potential use as hallmarks of the disease, regardless of inflammatory status. In the active phase of the disease, the mucosa-associated bacteria were characterized by a higher abundance of members of the phylum *Proteobacteria* and the families *Enterobacteriaceae* and *Staphylococcaceae*, apparently excluding members of the family *Streptococcaceae*, which are normal inhabitants of the healthy small intestine.

4. ACKNOWLEDGEMENTS

This work was supported by grants AGL2011-25169 and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Economy and Competitiveness. The scholarship to E. Sánchez from Institute Danone is fully acknowledged.

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Capítulo IV:

Analizar el proceso de colonización intestinal de especies del género* Bacteroides *en recién nacidos y lactantes con riesgo de desarrollar la enfermedad celíaca y determinar su relación con el tipo de lactancia y el genotipo de riesgo

- *Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by* Bacteroides *species*

Capítulo IV

Influence of environmental and genetic factors linked to celiac disease risk on infant gut colonization by *Bacteroides* species

ABSTRACT

Celiac disease (CD) is an immune-mediated enteropathy involving genetic and environmental factors whose interaction might influence disease risk. The aim of this study was to determine the effects of milk-feeding practices and the HLA-DQ genotype on intestinal colonization of *Bacteroides* species in infants at risk of CD development. This study included 75 full-term newborns with at least one first-degree relative suffering from CD. Infants were classified according to milk-feeding practice (breast-feeding or formula feeding) and HLA-DQ genotype (high or low genetic risk). Stools were analyzed at 7 days, 1 month, and 4 months by PCR and denaturing gradient gel electrophoresis (DGGE). The *Bacteroides* species diversity index was higher in formula-fed infants than in breast-fed infants. Breast-fed infants showed a higher prevalence of *Bacteroides uniformis* at 1 and 4 months of age, while formula-fed infants had a higher prevalence of *B. intestinalis* at all sampling times, of *B. caccae* at 7 days and 4 months, and of *B. plebeius* at 4 months. Infants with high genetic risk showed a higher prevalence of *B. vulgatus*, while those with low genetic risk showed a higher prevalence of *B. ovatus*, *B. plebeius*, and *B. uniformis*. Among breast-fed infants, the prevalence of *B. uniformis* was higher in those with low genetic risk than in those with high genetic risk. Among formula-fed infants, the prevalence of *B. ovatus* and *B. plebeius* was increased in those with low genetic risk, while the prevalence of *B. vulgatus* was higher in those with high genetic risk. The results indicate that both the type of milk feeding and the HLA-DQ genotype influence the colonization process of *Bacteroides* species, and possibly the disease risk.

1. INTRODUCTION

The newborn intestine is colonized immediately after birth by microorganisms from the mother and the environment.[12,21] At birth, the intestinal milieu of neonates shows a positive redox potential, and early bacterial colonization begins with facultative anaerobes (*Enterobacteriaceae*, *Lactobacillus*, etc.) that gradually consume the oxygen, permitting the growth of strict anaerobes *Bifidobacterium*, *Bacteroides*, *Clostridium*, etc.).[18,22] Subsequently, milk-feeding practices play an important role in the microbiota composition of the infant gut.[2,18, 22] In breast-fed infants, the microbiota is less diverse and is dominated by *Bifidobacterium* species, while a more diverse microbiota develops only after complementary feeding commences. In contrast, the bacterial composition of formula-fed infants is dominated by members of diverse genera (*Enterobacteriaceae*, *Streptococcus*, *Bacteroides*, *Clostridium*, and *Bifidobacterium*).[1,9] Intestinal colonization influences diverse physiological functions, which may have an impact on the host's health and disease risk.[16, 18, 25] Nevertheless, there is still limited information on the initial establishment of *Bacteroides* species and its possible influence on health .[11, 19, 30]

Celiac disease (CD) is a multifactorial disorder involving both genetic and environmental factors. This disease is associated with human leukocyte antigen (HLA) genes of the major histocompatibility complex (MHC), and approximately 95% of patients are positive for HLA-DQ2 or -DQ8.[28] Studies of twins also showed that in 25% of cases, one twin of the pair did not develop CD, supporting the role of environmental factors in disease development.[10,20] Breast-feeding seems to exert a protective effect against CD development,[4,15] but its possible connection with modulation of the intestinal microbiota is unknown. A preliminary study suggested an association between increased *Bacteroides-Prevotella* Group proportions and the HLA-DQ genotype, but the sample size was limited.[8]

The aim of this study was to determine the influence of milk-feeding practices (breast-feeding versus formula feeding) and HLA-DQ genotype on the intestinal colonization process of *Bacteroides* species in a representative group of infants with a familial risk of developing CD. To do so, PCR and denaturing gradient gel electrophoresis (DGGE) analyses were performed. The final purpose

of the study was to shed light on the interactions between the intestinal colonization process, diet, and genotype and on their overall influence on CD risk.

2. MATERIALS AND METHODS

2.1. Subjects.

This study included 75 full-term newborns with at least one first-degree relative suffering from CD, selected from an ongoing prospective observational 3-year study. Seventy-four percent of the infants had a sibling suffering from CD, 17% of the infants had a parent with CD, and 9% of the infants had both one sibling and one parent with CD. The distributions of the type and number of relatives suffering from CD were similar for the subgroups of infants considered for statistical comparisons (Table 1). Fifty-one percent of the samples were from infants born in Madrid (center of Spain), and the rest (49%) were from infants born in Eastern Spain. Exclusion criteria included prematurity, maternal infections or clinical illness during pregnancy, maternal antibiotic or probiotic administration during the last 2 weeks of gestation and intrapartum, and babies given antibiotic prophylaxis or therapy. Infants were divided according to feeding practice, into formula-fed infants (n=35), eligible if they were fed exclusively with formula from birth, and breast-fed infants (n=40), eligible if they were breast-fed exclusively during the 4-month period under study. Infants were also classified into two main CD genetic risk groups, a high-risk group (n=39) and a low-risk group (n=36), on the basis of their HLA-DQ genotype. The classification was based on the criteria of Bourgey et al.[3] and considering the HLA distribution of the Eastern Spanish population.[5] The demographic data and dietary history of every subject were recorded (Table 1). The study was conducted in accordance with the ethical rules of the Helsinki Declaration (Hong Kong revision, September 1989), following EEC Good Clinical Practice guidelines (document 111/3976/88, July 1990) and current Spanish law, which regulates clinical research in humans (Royal Decree 561/1993 regarding clinical trials). The study was approved by the local ethics committees of the CSIC and the hospitals involved (Hospital Universitario Sant Joan de Reus, Hospital Universitario Sant Joan de Deu, Hospital Clínico Universitario of Valladolid, Hospital Universitario La Paz, Hospital Infantil Universitario La Fe, Hospital Universitario Infantil Niño Jesús,

and Hospital Universitario Nuestra Señora Candelaria). Written informed consent was obtained from the parents of children included in the study.

Table 1. Demographic characteristics of the study cohort of infants.

	Low risk (<1% probability)		High risk (7-28% probability)		
	Breast-fed (n=20)	Formula-fed (n=16)	Breast-fed (n=20)	Formula-fed (n=19)	P value
Delivery					0.900
Vaginal	17	13	18	16	
Caesarean	3	3	2	3	
Size (cm)	49.2 ± 1,4	50 ± 1.9	49.6 ± 2.2	50.5 ± 1.7	0.309
Weight (g)	3253 ±307.7	3269±519.3	3364±514.7	3459 ±795.3	0.640
Weeks of gestation	38.3 ± 3.7	38.3 ± 1.3	39.3 ± 1.1	39.5 ± 1.4	0.332

*Infants were classified in two main CD risk groups, according to their HLA-DQ haplotype[19] and considering the HLA distribution of the Eastern Spanish population.[20]

2.2. Bacterial strains

The reference strains used as ladders for *Bacteroides* species identification by DGGE were *Bacteroides distasonis* DSM 20701, *B. fragilis* DSM 2451, *B. ovatus* DSM 1896, *B. thetaiotaomicron* DSM 2079, and *B. vulgatus* DSM 1447. Another six strains used as ladders were isolated from human stools and identified by 16S rRNA gene sequencing with primers 27d and 1401r as described elsewhere,[13] using an ABI Prism 3130XL genetic analyzer (Applied Biosystems, CA). Search analyses were conducted in GenBank, using the Basic Local Alignment Search Tool (BLAST) algorithm. Sequences of our isolates showed >97% similarity to sequences of the species *B. dorei*, *B. massiliensis*, *B. caccae*, *B. coprocola*, *B. intestinalis*, and *B. uniformis* (GenBank accession numbers EU722737.1, AB510703.1, AB510697.1, AB200223.1, AB437413.1, and AB247141.1, respectively) and were assigned to these species. *Bacteroides* strains were grown in Schaedler agar medium (Scharlau, Barcelona, Spain) supplemented with kanamycin (100 mg/liter), vancomycin (7.5 mg/liter), and vitamin K (0.5

mg/liter) and incubated under anaerobic conditions (AnaeroGen; Oxoid, Hampshire, United Kingdom) at 37°C.

2.3. DNA extraction, PCR amplification, and DGGE analysis

Stool samples were collected from every infant at 7 days, 1 month, and 4 months of age and kept at -20°C until analysis. Samples were diluted and homogenized. DNAs from diluted stool samples and from bacterial strains used as ladders were extracted by using a QIAamp DNA stool minikit (Qiagen, Hilden, Germany) following the manufacturer's instructions. *Bacteroides* genus-specific PCR was performed with 16S rRNA gene primers Bfra531-f (5´-ATACGGAGGATCCGAGCGTTA-3´) and Bfra766GC-r (5´-CTGTTTGATACCCACACT-GCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3´). Each reaction mixture of 30 µl was composed of 30 µl 10X buffer stock (containing 1.5 mM $MgCl_2$), 1.5 µl bovine serum albumin (10 mg/ml), a 0.5 µM concentration of each deoxynucleoside triphosphate, 1 µM (each) primers, 2.5 U *Taq* polymerase (Ecotaq; Ecogen, Barcelona, Spain), and 30 ng DNA. DGGE analysis was carried out on a Dcode universal mutation detection system (Bio-Rad, Richmond, CA) (23). Unknown DGGE bands were excised from the gels, reamplified, and purity checked by DGGE. The PCR products were purified using a GFX PCR DNA and gel band DNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom) and were identified by DNA sequencing using an ABI Prism 3130XL genetic analyzer (Applied Biosystems, CA). Search analyses were conducted as described above, and sequences with >97% similarity were considered to be of the same species.

2.4. Statistical and cluster analyses.

Gels were aligned using ladders, and bands were estimated visually and coded as present or absent. The relatedness of microbial communities was expressed as similarity clusters, using the Dice coefficient and the unweighted-pair group method using average linkages (UPGMA), and the diversity of taxa present in fecal samples was calculated with the Shannon-Wiener index (*H´*), using PAST (Palaeontological Statistics) software. The Levene test was used to establish variance homogeneity and diversity data distributions, and the effects

of feeding practices and genetic risk on *Bacteroides* diversity were determined by using a linear mixed-model analysis for repeated measures in which sampling time was the repeated variable, using SPSS 17.0 software (SPSS Inc., Chicago, IL). *P* values of < 0.05 were considered statistically significant. The interaction between feeding practices and genetic risk of CD in the studied cohort of infants was not statistically significant by applying the linear mixed-model analysis. Differences in *Bacteroides* species prevalence were established by applying x^2 tests and, where appropriate, by two-tailed Fisher's exact test. Analyses were carried out with Statgraphics software (Manugistics, Rockville, MD), and statistical differences were established in cases with *P* values of < 0.05.

3. RESULTS

3.1. Infant gut colonization by **Bacteroides** *species during the first months of life*

Considering the whole cohort of infants, significant differences in *Bacteroides* species diversity indexes (*H´*) were not detected based on the infants' ages, with values of 1.45 at 7 days, 1.42 at 1 month, and 1.36 at 4 months.

Some profiles clustered according to infant age, but others were subject specific, so cluster analysis did not classify *Bacteroides* profiles according to infant age (data not shown), nor did the prevalence of *Bacteroides* species differ significantly in the cohort of infants at different ages (data not shown).

3.2. Influence of milk-feeding practices on **Bacteroides** *spp. colonization*

The *Bacteroides* diversity index (*H´*) was significantly higher for formula-fed infants than for breast-fed infants during the study period, as determined by applying a linear mixed model analysis for repeated measures (*P*= 0.01).

Clustering analysis of DGGE profiles obtained for infant stools at 7 days, 1 month, and 4 months of age revealed some associations between the types of milk-feeding practices. At 7 days of age, some breast-fed and formula-fed infant profiles were grouped together. Cluster A contained 7 formula-fed infant profiles (70% similarity). Moreover, another three clusters containing breast-fed infant profiles were found: cluster B contained 5 profiles, cluster D contained 9 profiles, and cluster E contained 4 profiles, with 63%, 70%, and 82% similarity, respectively (Fig. 1). At 1 month of age, cluster I included most of the breast-fed infant profiles (33), with 55% similarity, whereas cluster II contained 7 formula-fed infant profiles (65% similarity) (Fig. 2). At 4 months of age, 24 breast-fed infant profiles were grouped in cluster D (63% similarity). However, this cluster also contained another 2 subclusters (E and F) including 4 formula-fed infant profiles each, with similarities of 80%. This analysis also revealed another cluster (B), grouping 8 formula-fed infant profiles at 54% similarity (Fig. 3).

The prevalence of *Bacteroides* species in the cohort of infants, given according to feeding practices, are shown in Table 2. At 7 days of age, the prevalence of *B. vulgatus* was higher in breast-fed infants than in formula-fed infants (P=0.03). In contrast, *B. caccae* and *B. intestinalis* were detected more frequently in formula-fed infants than in breast-fed infants (P<0.01). At 1 month of age, the prevalence rates of *B. vulgatus* and *B. caccae* were equal in both the breast-fed and formulafed groups; nevertheless, the prevalence of *B. intestinalis* continued to be increased in formula-fed infants compared with breast-fed infants (P<0.01). In contrast, *B. uniformis* was more prevalent in breast-fed infants than in formula-fed infants (P=0.04). Interestingly, at 4 months of age, the prevalence of *B. uniformis* was also increased in breast-fed compared with formula-fed infants (P=0.04), while that of *B. intestinalis* was higher in formula-fed infants than in breast-fed infants (P<0.01). Furthermore, the prevalences of *B. caccae* and *B. plebeius* were higher in formula-fed infants than in breast-fed infants (P=0.01 and P=0.03, respectively).

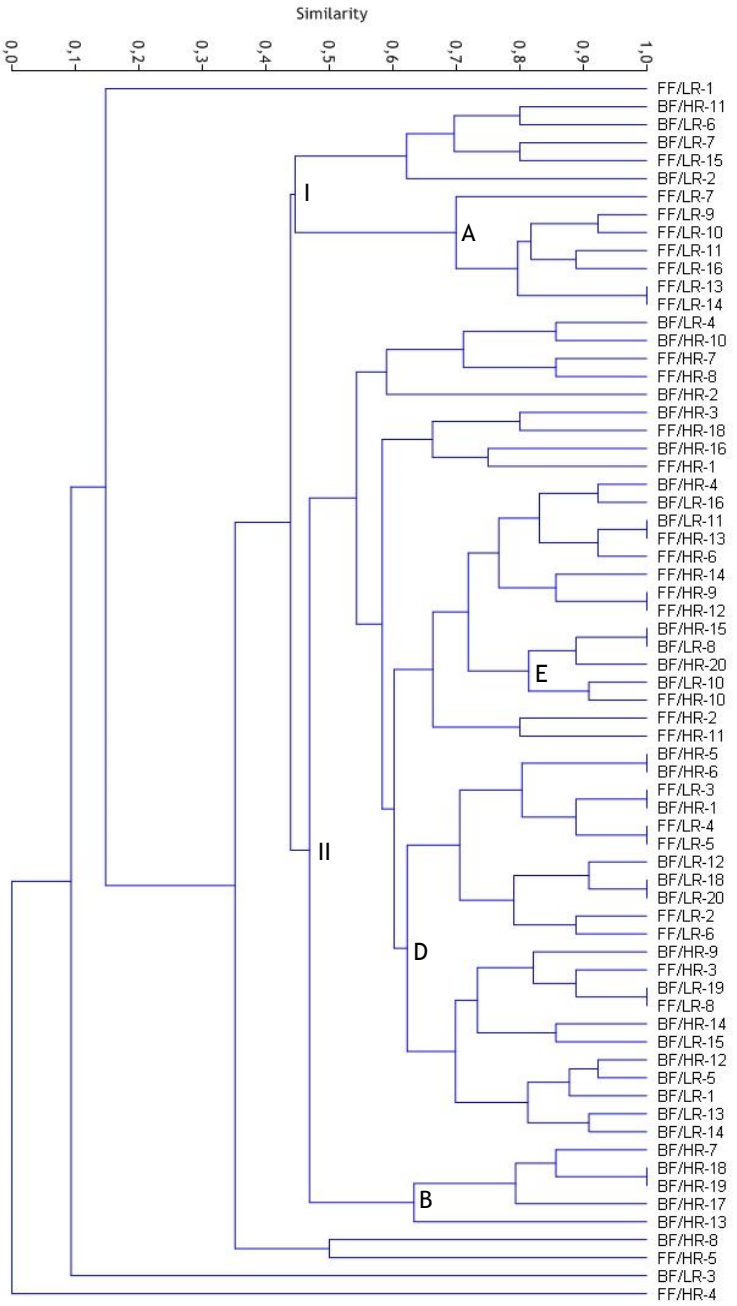

FIG. 1. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 7 days of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

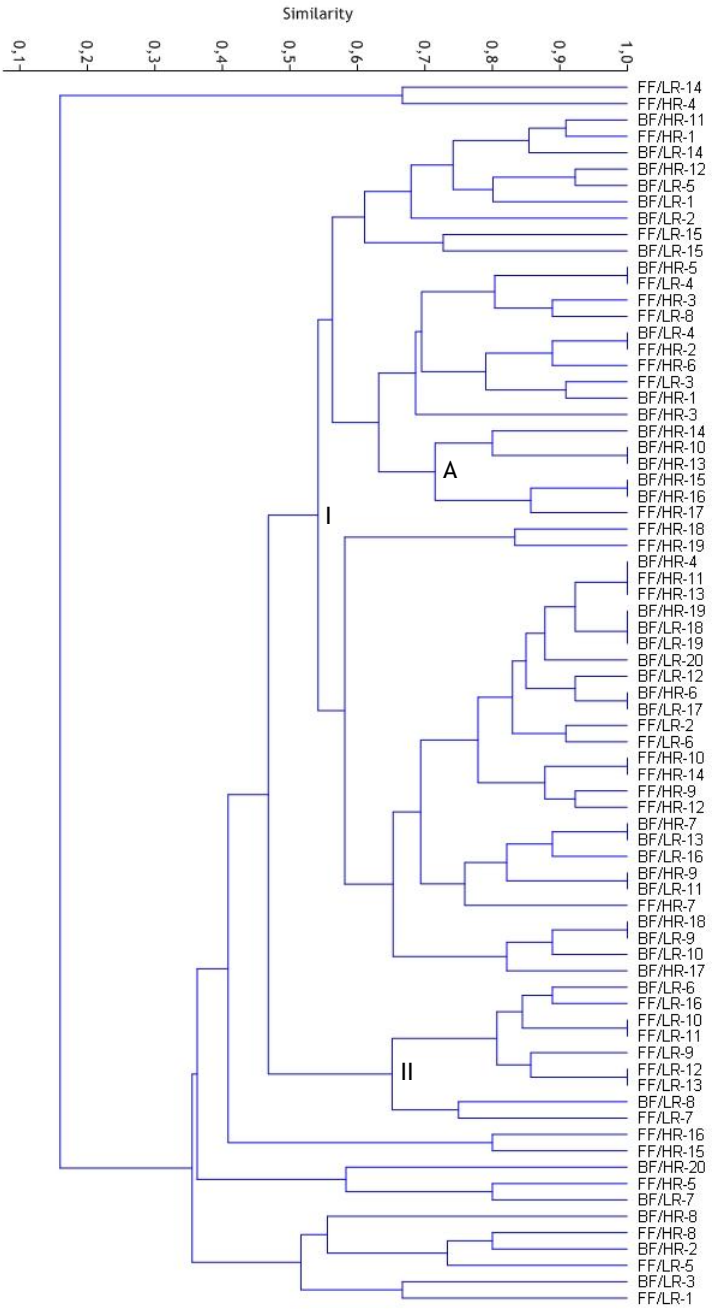

FIG. 2. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 1 month of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

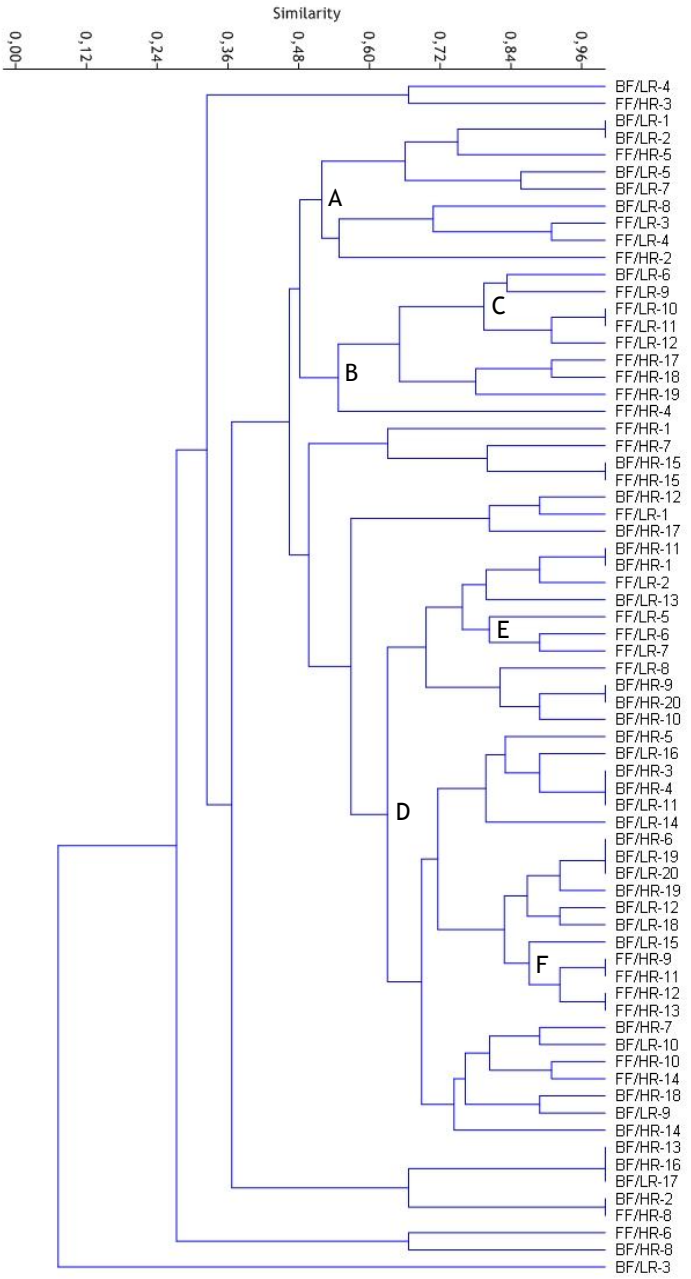

FIG. 3. Dendrogram derived from DGGE analysis of *Bacteroides* species in fecal samples of the study cohort of infants at 4 months of age, based on Dice's similarity index and the UPGMA clustering algorithm. Infants were divided according to feeding practice (breast-fed [BF] and formula-fed [FF]) and genetic risk of CD development (low risk [LR] and high risk [HR]). Letters correspond to clusters of samples.

Table 2. Prevalence of *Bacteroides* species detected by PCR-DGGE analysis of faecal DNA from the study cohort of breast- and formula-feeding infants at 7 days, 1 month and 4 months of age using Bfra531-f and Bfra766-GC-r primers

	7 days		1 month		4 months	
	Breastfed n=39 (%)	Formula-fed n=31 (%)	Breastfed n=40 (%)	Formula-fed n=35 (%)	Breastfed n=40 (%)	Formula-fed n=30
B. caccae	4 (10)	12 (39)*	9 (23)	12 (34)	5 (13)	12 (40)*
B. coprocola	16 (41)	14 (45)	19 (48)	17 (49)	23 (58)	16 (53)
B. distasonis	18 (46)	15 (48)	15 (38)	20 (57)	16 (40)	14 (47)
B. dorei	12 (31)	6 (19)	9 (23)	8 (23)	7 (18)	5 (17)
B. fragilis/B. thetaiotaomicron	28 (72)	27 (87)	31 (78)	30 (86)	31 (78)	27 (90)
B. intestinalis	0	9 (29)*	0	4 (11)*	0	6 (20)*
B. massiliensis	14 (36)	5 (16)	11 (28)	7 (20)	10 (25)	5 (17)
B. ovatus	13 (33)	17 (55)	18 (45)	20 (57)	17 (43)	17 (57)
B. plebeius†	4 (10)	8 (26)	4 (10)	9 (26)	2 (5)	7 (23)*
B. uniformis	24 (62)	17 (55)	27 (68)	15 (43)*	29 (73)	14 (47)*
B. vulgatus	33 (85)	19 (61)*	32 (80)	23 (66)	25 (63)	17 (57)

*Significant differences in *Bacteroides* species detected at 7 days, 1 month and 4 months of age between breastfeed and formula-feed infants. Statistically significant at $p < 0.05$, applying the Fisher´s exact test.

†Species identification by sequencing the PCR-DGGE bands.

Influence of HLA-DQ genotype on Bacteroides *spp colonization*

The diversity index of *Bacteroides* species colonizing the infant gut, calculated by applying a linear mixed-model analysis for repeated measures, proved to be significantly higher in infants with low genetic risk than in those with high genetic risk of CD development (P=0.04).

Clustering analysis of DGGE profiles obtained for fecal samples at 7 days and 1 and 4 months of age revealed some associations between the *Bacteroides* spp. profiles and the genetic risk. At 7 days of age, profiles were divided into two main groups: cluster I contained 11 profiles of infants with low genetic risk (45% similarity), and cluster II contained almost all profiles (31) for infants with high genetic risk (48% similarity) but also included one subgroup (C) containing 15 profiles of infants with low genetic risk (62% similarity) (Fig. 1). At 1 month of age, only two small clusters were detected: cluster II included 9 profiles for infants with low genetic risk (65% similarity), and cluster A included 6 profiles for infants with high genetic risk (70% similarity) (Fig. 2). At 4 months of age, 12 profiles for infants with low genetic risk were grouped into two different clusters (A and C), with 52% and 80% similarity, respectively, while others could not be differentiated clearly from those for infants with high genetic risk (Fig. 3).

The prevalence of *Bacteroides* species in the cohort of infants, stratified according to their genetic risk of CD development, are shown in Table 3. At 7 days of age, the prevalence of *B. vulgatus* was higher in high-risk than in low-risk infants (P<0.01); in contrast, *B. ovatus*, *B. plebeius*, and *B. uniformis* were more prevalent in low-risk than in high-risk infants (P<0.01). The same differences were detected for these bacterial species at 1 month of age (P=0.03, P=0.04, P<0.01, and P<0.01, respectively) and 4 months of age (P=0.02, P=0.04, P=0.14, and P=0.02, respectively).

Table 3. Prevalence of *Bacteroides* species detected by PCR-DGGE analysis of faecal DNA from the study infants with family risk of CD development at 7 days, 1 month and 4 months of age using Bfra531-f and Bfra766-GC-r primers.

	7 days		1 month		4 months	
	Low Risk	High Risk n=36 (%)	Low Risk n=36 (%)	High Risk n=39 (%)	Low Risk n=32 (%)	High Risk n=38 (%)
B. caccae	8 (24)	8 (22)	10 (28)	11 (28)	8 (25)	9 (24)
B. coprocola	13 (38)	17 (47)	18 (50)	18 (46)	17 (53)	22 (58)
B. distasonis	16 (47)	17 (47)	19 (53)	16 (41)	14 (44)	16 (42)
B. dorei	7 (21)	11 (31)	7 (19)	10 (26)	4 (13)	8 (21)
B. fragilis/B. thetaiotaomicron	26 (76)	29 (81)	27 (75)	34 (87)	26 (81)	32 (84)
B. intestinalis	2 (5.9)	7 (19)	1 (2.8)	3 (7.7)	1 (3.1)	5 (13)
B. massiliensis	12 (35)	7 (19)	10 (28)	8 (21)	10 (31)	5 (13)
B. ovatus	22 (65)	8 (22)*	26 (72)	12 (31)*	21 (66)	13 (34)*
B. plebeius†	10 (29)	0*	13 (36)	0*	9 (28)	0*
B. uniformis	24 (71)	17 (47)*	25 (69)	17 (44)*	23 (72)	20 (53)
B. vulgatus	20 (59)	32 (89)*	22 (61)	33 (85)*	14 (44)	28 (74)*

* Significant differences in *Bacteroides* species detected at 7 days, 1 month and 4 months of age between infants with low (<1% probability) or high genetic risk of development CD (8-28% probability). Statistically significant at $p < 0.05$, applying the Fisher´s exact test.

†Species identification by sequencing the PCR-DGGE bands.

Influence of genotype on* Bacteroides *spp. colonizing the guts of breast-fed and formula-fed infants

The prevalence of *Bacteroides* species are shown in Table 4, presented according to the genotypes of breast-fed and formula-fed infants. Among breast-fed infants, the prevalence of *B. uniformis* was significantly higher in infants with low genetic risk than in those with high genetic risk during the study period, but significant differences were detected only at 7 days and 1 month of age ($P<0.01$ and $P=0.02$, respectively). Among formula-fed infants, the prevalence rates of *B. ovatus* and *B. plebeius* were higher ($P=0.001$ to 0.002) in infants with low genetic risk than in those with high genetic risk of CD development, while the prevalence of *B. vulgatus* was higher in infants with high genetic risk ($P=0.003$ to 0.030).

Table 4. Prevalence of *Bacteroides* species detected by DGGE analysis of faecal DNA 7 days, 1 month and 4 months of age in either breast-fed or formula-fed infants as a function of the genetic risk of CD development.

		Breastfed			Formula-fed		
		Low risk	High risk	P value	Low risk	High risk	P value
B. caccae	7 days	3/19 (15.8)	1/20 (5)	0.342	5/15 (33.3)	7/16 (43.8)	0.716
	1 month	5/20 (25)	4 /20 (20)	1	5/16 (31.3)	7/19 (36.8)	1
	4 months	4/20 (20)	1/20 (5)	0.342	4/12 (33.3)	8/18 (44.4)	0.709
B. coprocola	7 days	6 /19 (31.6)	10/20 (50)	0.333	7/15 (46.7)	7/16 (43.8)	1
	1 month	10/20 (50)	9/20 (45)	1	8/16 (50)	9/19 (47.4)	1
	4 months	10/20 (50)	13/20 (65)	0.523	7/12 (58.3)	9/18 (50)	0.722
B. distasonis	7 days	8/19 (42.1)	10/20 (50)	0.751	8/15 (53.3)	7/16 (43.8)	0.724
	1 month	9/20 (45)	6/20 (30)	0.353	10/16 (62.5)	10/19 (52.6)	0.734
	4 months	9/20 (45)	7/20 (35)	0.748	5/12 (41.7)	9/18 (50)	0.722
B. dorei	7 days	6/19 (31.6)	6/20 (30)	1	1/15 (6.7)	5/16 (31.3)	0.172
	1 month	6/20 (30)	3/20 (15)	0.289	1/16 (6.3)	7/19 (36.8)	0.047*
	4 months	4/20 (20)	3/20 (15)	1	0/12 (0)	5/18 (27.8)	0.066
B. fragilis /B. thetaiotaomicron	7 days	13/19 (68.4)	15/20 (75)	0.731	13/15 (86.7)	14/16 (87.5)	1
	1 month	14/20 (70)	17/20 (85)	0.289	13/16 (81.3)	17/19 (89.5)	0.642
	4 months	15/20 (75)	16/20 (80)	1	11/12 (91.7)	16/18 (88.9)	1

Table 4. Continuation

		Breastfed			Formula-fed		
		Low risk	High risk	P value	Low risk	High risk	P value
B. intestinalis	7 days	0/19 (0)	0/20 (0)	-	2/15 (13.3)	7/16 (43.8)	0.113
	1 month	0/20 (0)	0/20 (0)	-	1/16 (6.3)	3/19 (15.8)	0.608
	4 months	0/20 (0)	0/20 (0)	-	1/12 (8.3)	5/18 (27.8)	0.358
B. massiliensis	7 days	10/19 (52.6)	4/20 (20)	0.048*	2/15 (13.3)	3/16 (18.8)	1
	1 month	8/20 (40)	3/20 (15)	0.155	2/16 (12.5)	5/19 (26.3)	0.415
	4 months	8/20 (40)	2/20 (10)	0.065	2/12 (16.7)	3/18 (16.7)	1
B. ovatus	7 days	8/19 (42.1)	5/20 (25)	0.320	14/15 (93.3)	3/116 (18.8)	<0.001*
	1 month	12/20 (60)	6/20 (30)	0.111	14/16 (87.5)	6/19 (31.6)	0.002*
	4 months	9/20 (45)	8/20 (40)	0.762	12/12 (100)	5/18 (27.8)	<0.001*
B. plebeius†	7 days	2/19 (10.5)	2/20 (10)	1	8/15 (53.3)	0/16 (0)	0.001*
	1 month	4/20 (20)	0/20 (0)	0.106	9/16 (56.3)	0/19 (0)	<0.001*
	4 months	2/20 (10)	0/20 (0)	0.244	7 /12(58.3)	0/18 (0)	0.002*
B. uniformis	7 days	16/19 (84.2)	8/20 (40)	0.008*	8/15 (53.3)	9/16 (56.3)	1
	1 month	18/20 (90)	9/20 (45)	0.019*	7/16 (43.8)	8/19 (42.1)	1
	4 months	17/20 (85)	12/20 (60)	0.155	6/12 (50)	8/18 (44.4)	1
B. vulgatus	7 days	15/19 (78.9)	18/20 (90)	0.695	5/15 (33.3)	14/16 (87.5)	0.003*
	1 month	15/20 (75)	17/20 (85)	0.695	7/16 (43.8)	16/19 (84.2)	0.030*
	4 months	11/20 (55)	14/20 (70)	0.353	3/12 (25)	14/18 (77.8)	0.008*

*Statistical differences between genetic risk group were calculated for each feeding practice by using the x^2 test (P<0.05).

4. DISCUSION

The present study provides the first evidence of the effects exerted by both milk-feeding practices and CD genetic risk factors on the intestinal colonization process of *Bacteroides* species in infants at early life. Our study indicates that milk-feeding practices influence both *Bacteroides* diversity and species prevalence. This study reports an increased prevalence of *B. vulgatus* in breast-fed infants at 7 days of age, which is not in agreement with the results of the only published related study, which reported that *B. vulgatus* and *B. thetaiotaomicron* were predominant in fecal samples from 5- to 6-days-old formula supplemented newborns[23].

Genetic predisposition to CD has been linked to the MHC region on chromosome 6p21, with over 95% of CD patients expressing HLA-DQ2 or -DQ8.[26] In our study, infants were classified into two groups according to HLA-DQ genotype, with one including those with a high probability of developing CD (7 to 28%) and the other including those with a low probability (1%) of developing the disease.[5] Infants with high genetic risk had an increased prevalence of *B. vulgatus* and a reduced prevalence of *B. ovatus*, *B. plebeius*, and *B. uniformis* compared with infants with low genetic risk. Enterocytes that are in close proximity with the intestinal contents and bacteria can express HLA-II molecules of the MHC to a certain extent and are able to act as antigen-presenting cells.[16] Moreover, HLA-II molecules are expressed primarily by dendritic cells present in the lamina propria, which can extend dendrites across this epithelium, capture bacteria and other antigens at the mucosal surface, and present the antigens to B or T lymphocytes.[6] This process is a critical step in the initiation of a mucosal immune response, and its possible role in restricting bacterial colonization cannot be disregarded.[14] In fact, the polysaccharide of *B. fragilis* has been proven to associate with MHC-II molecules with a high affinity, through a mechanism mirroring peptide presentation, which could mediate MHC-II antigen presentation and, ultimately, T cell recognition during bacterial infection.[7]

In a preliminary study of exclusively breast-fed infants, larger proportions of the *Bacteroides-Prevotella* group were detected in infants with high genetic risk than in those with low genetic risk of CD development;[8] however, the effect

of milk-feeding type was not considered. The present study provides a wider characterization of the *Bacteroides* population at the species level and also takes into account the variable of milk-feeding type. In this study, we demonstrated that the prevalence of *B. vulgatus* was increased in infants with high genetic risk, considering both the total cohort and formula-fed infants only. Furthermore, in the whole cohort of infants, prevalence rates for *B. uniformis*, *B. ovatus*, and *B. plebeius* were also increased in the group with low genetic risk, and this trend was confirmed by considering the subgroup of either breast-fed or formula-fed infants. These results indicate that the HLA-DQ genotype has an independent effect on the colonization of these species, in addition to that exerted by the type of milk feeding, which could also influence the disease risk. The colonization process of the intestine shortly after birth seems to be important for adequate immune response maturation and to the risk of suffering immune-mediated diseases. A few studies suggest that *B. fragilis* could be involved in this process, influencing Toll-like receptor 4 (TLR4) mRNA in peripheral blood monocytes[27] or the number of circulating IgA and IgM antibody-producing cells.[11,19] *In vitro* studies also indicate that exposure to polysaccharide A of *B. fragilis* activates CD4 T cells, resulting in a Th1 response, which could modify the risk of suffering CD.[29] Nevertheless, neither *in vitro* nor *in vivo* information is available regarding the potential immunological role of other *Bacteroides* species.

In summary, the results indicate that both, milk-feeding type and HLA-DQ genotype, influence *Bacteroides* species colonization process early in life. An increased *B. vulgatus* prevalence was associated with infants at high genetic risk of CD development, while increased *B. uniformis* prevalence was associated with infants at low genetic risk of CD development and with breastfeeding, which could partly explain the protective effect of breastfeeding. Further research is under way to disclose a possible relationship between these findings and CD development in a follow-up study of this cohort of infants.

5. ACKNOWLEDGEMENTS

This work was supported by public grants AGL2007-66126-C03-01-03/ALI and Consolider Fun-C-Food CSD2007-00063 from the Spanish Ministry of Science and Education (MEC). Scholarships to E. Sánchez from the Institute Danone and to G. De Palma and T. Pozo from CSIC are fully acknowledged.

The assistance of Laura Barrios in statistical analysis is also fully acknowledged.

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IV. DISCUSIÓN GENERAL

La estructura de la microbiota fecal de individuos celíacos difiere de la de sanos

Uno de los estudios incluidos en esta tesis se centró en el análisis del perfil de especies bacterianas característico de la microbiota fecal de niños con EC y se comparó con la de sanos mediante la técnica de DGGE y utilizando cebadores universales (HDA1-GC y HDA2) que amplifican la región V2-V3 del gen 16S rDNA (Walter et al., 2000).

Las muestras fecales analizadas mostraron un perfil de bandas complejo y único para cada uno de los individuos estudiados y ninguna banda pudo relacionarse claramente con la enfermad. No obstante el análisis de agrupamiento (UPGMA, aplicando el coeficiente de Dice) de los perfiles de DGGE obtenidos permitió diferenciar claramente dos grupos, uno incluyendo la mayoría de los perfiles de los niños celíacos y otro incluyendo los perfiles del grupo control. Estudios posteriores han demostrado resultados similares al comparar muestras fecales de niños y adultos celíacos con sanos mediante DGGE y TGGE (Nistal et al., 2012; Di Cagno et al., 2011; Schippa et al., 2010; Di et al., 2009). Además, en nuestro estudio, la diversidad en especies bacterianas estimada como el número de bandas presentes en cada individuo, fue mayor en el grupo de niños celíacos que en el grupo control, que también fue observado con posterioridad por Schippa et al. (2010) al analizar muestras similares por TGGE

La composición en especies del género* Bifidobacterium *de la microbiota fecal y duodenal de individuos celíacos está alterada en comparación con la de sanos

Las especies del género *Bifidobacterium* son habitantes naturales del TGI humano y son cuantitativamente importantes, especialmente, en los primeros años de vida (Picard et al., 2005). Se considera que la proporción de las especies de este género podría ser de gran importancia para el adecuado desarrollo del sistema inmunitario y para la regulación de respuestas patológicas del sistema inmune a antígenos ambientales como ocurre en la EC (Laparra et al., 2012; De Palma et al., 2010; Medina et al., 2008). En esta tesis doctoral se realizó el

estudio de la composición en especies del género *Bifidobacterium* de sujetos celíacos y sanos mediante DGGE utilizando cebadores específicos (Bif164-f y Bif662-GC-r) que amplifican parcialmente el gen 16S rDNA (Satokari et al., 2001). El análisis comparativo de las muestras fecales indicó que los celíacos mostraban una menor diversidad en especies de bifidobacterias que el grupo control, resultados que también han sido observados, posteriormente, al comparar adultos celíacos y sanos (Nistal et al., 2012). En cuanto a la composición en especies, los celíacos presentaron una prevalencia menor de *B. adolescentis, B. longum, B. pseudocatenulatum* y *B. dentium* que el grupo control, aunque las diferencias en estas tres últimas especies no fueron estadísticamente significativas. Otros análisis realizados por PCR cuantitativa en nuestro grupo también indicaron que en heces de niños celíacos había una menor prevalencia de *B. adolescentis* y *B. dentium* (Collado et al., 2008).

El análisis de la composición en especies del género *Bifidobacterium* en biopsias duodenales en celíacos, en fase activa y no activa, y de controles no celíacos demostró que su complejidad era menor que en heces y se detectaron diferencias en los índices de diversidad y en la prevalencia de especies de este género. Los pacientes celíacos en fase activa mostraron mayor diversidad en especies seguidos por los celíacos no activos y finalmente por los controles, al contrario que lo observado en heces. La prevalencia de *B. adolescentis* y *B. animalis* subsp. *lactis* fue mayor en biopsias de pacientes en fase activa que en pacientes tratados con dieta exenta de gluten y controles; estos resultados fueron similares a los obtenidos por nuestro grupo por PCR cuantitativa (Collado et al., 2008). En estudios de PCR cuantitativa realizados por nuestro grupo se han descrito reducciones en las poblaciones de *B. longum* como consecuencia de la dieta sin gluten, tanto al comparar individuos celiacos en fase activa y no activa de la EC (Collado et al., 2008) como al comparar individuos sanos antes y después de someterse a una dieta exenta de gluten (De Palma et al., 2009b).

Los resultados obtenidos, a partir de biopsias y de heces, parecen indicar que las especies del género *Bifidobacterium* colonizan de manera diferencial distintas partes del TGI, tanto a nivel de género como a nivel de especie, y confirman una alteración en su diversidad y composición asociada a la EC.

La composición en especies del grupo de bacterias ácido lácticas (BAL) de la microbiota fecal y duodenal de individuos celíacos está alterada en comparación con la de sanos

En el TGI humano el grupo de BAL no es tan abundante, cuantitativamente, como otros y su presencia es bastante dependiente de la dieta; sin embargo, se analizó la relación entre su composición en especies y la EC debido a que se ha propuesto el posible uso de algunas cepas de BAL para el tratamiento de IBD. Al igual que en el caso de las bifidobacterias, se caracterizó la microbiota fecal y duodenal de niños celíacos y se comparó con la de individuos control mediante la amplificación parcial del gen 16S rDNA con cebadores específicos, Lac-1 y Lac2-GC, y DGGE (Walter et al., 2001). En heces se observó que la diversidad en especies de BAL fue mayor en los individuos celíacos que en controles y que los individuos celíacos presentaron mayor prevalencia de la especie *L. casei* y menor de *L. curvatus*, *L. mesenteroides* y *L. carnosus*. Sin embargo, al igual que ocurrió en el caso de las bifidobacterias, estas características no se detectaron en muestras duodenales. En la mucosa duodenal la diversidad en especies de BAL fue menor en individuos celíacos con enfermedad en fase activa que en los celíacos no activos y en los controles. En cuanto a la prevalencia en especies, los pacientes celíacos no activos se caracterizaron por presentar una prevalencia mayor de *Leuconostoc fermentum* y *W.* spp. y menor de *L. reuteri* y *P. pentosaceus* que los enfermos en fase activa y el grupo control. Así pues, estas diferencias podrían estar más relacionadas con el efecto de la dieta sin gluten que con la EC. En un estudio realizado por nuestro grupo en sujetos sanos sometidos a una dieta sin gluten por PCR cuantitativa se observó también que el número de BAL se reducía como consecuencia de la dieta (De Palma et al., 2009b), y en estudios de DGGE realizados por otros autores indican que la diversidad de las poblaciones de BAL de adultos celíacos en tratamiento es menor que la de celíacos activos (Nistal et al., 2012). Estos cambios pueden deberse a que durante la dieta sin gluten se reduce la ingesta de polisacáridos (Kinsey et al., 2008), y estos compuestos suelen llegar a la parte distal del colon parcialmente indigeridos y constituyen una de las principales fuentes de energía para la microbiota intestinal.

La composición en especies del género* Bacteroides *de la microbiota duodenal de individuos celíacos está alterada en comparación con la de sanos

El filo *Bacteroidetes* es uno de los dominantes del TGI junto con el de *Firmicutes (*Kovatcheva-Datchary et al., 2009; Rajilic-Stojanovic et al., 2007; Eckburg et al., 2005) y, en particular, el género *Bacteroides* es uno de los grupos más abundantes (Wexler, 2007). En esta tesis doctoral se caracterizó la composición en especies de este género en muestras duodenales de niños celíacos (con enfermedad activa y no activa) y se comparó con la de individuos control mediante la amplificación parcial del gen 16S rDNA con cebadores específicos (Bfra531-f y Bfra766-GG-r) y DGGE (Vanhoutte et al., 2006). En la microbiota duodenal de pacientes celíacos con enfermedad en fase activa observamos una menor diversidad de especies del género *Bacteroides* que en pacientes celíacos no activos y controles. Igualmente a lo detectado al analizar las poblaciones de bifidobacterias y BAL, en muestras fecales de pacientes adultos otros autores han detectado una diversidad mayor de especies de *Bacteroides* en enfermos celíacos (activos y tratados) que en controles, aunque estas diferencias no fueron significativas (Nistal et al., 2012). En cuanto a la prevalencia en especies, en biopsias de niños celíacos activos detectamos una prevalencia mayor de *B. dorei* y menor de *B. distasonis, B. thetaiotaomicron* y/o *B. fragilis, B. ovatus* y *B. uniformis* en comparación con el grupo control. A excepción de *B. dorei,* estas diferencias también se observaron entre el grupo de celíacos tratados y el grupo control, lo que indicaría que las poblaciones de *Bacteroides* no se reestablecen completamente con la dieta sin gluten. Debido a que algunas especies del género *Bacteroides* se consideran potencialmente patógenicas en función de la presencia de diversos factores de virulencia (Botta et al., 1994), en una fase posterior de la tesis se consideró interesante abordar el aislamiento y la caracterización de las especies de este género y de sus factores de virulencia en individuos celíacos y su comparación con las de sanos.

***Los aislados de la especie* E. coli *de pacientes celíacos presenta un mayor número de genes que codifican factores de virulencia que los de sanos*.**

La familia *Enterobacteriaceae* incluye numerosos géneros que son capaces de colonizar el TGI humano tanto comensales como patógenos. *Escherichia coli* es una de las especies más representativas de este género y puede incluir cepas comensales y virulentas (Picard et al., 1999).

Para esta tesis doctoral nos planteamos aislar y caracterizar enterobacterias presentes en heces de niños celíacos, en fase activa y no activa, y controles. Según nuestros datos, *E. coli* fue la especie más abundante entre las enterobacterias aisladas, sobre todo en los grupos de individuos celíacos tanto activos como no activos. Además, mediante estudios moleculares de FISH o PCR cuantitativa también se ha observado un incremento en las poblaciones fecales de *E. coli* en niños celíacos en fase activa comparadas con las de controles. Al diferenciar los aislados de *E. coli* entre comensales y virulentos, según la clasificación establecida por (Picard et al., 1999), observamos que mientras que en el grupo control los aislados comensales se clasificaban en los dos grupos filogenéticos, A y B1, en los niños celíacos activos y no activos eran dominantes los aislados del grupo A; en cuanto a los aislados virulentos, en el grupo control se detectaron proporciones similares de los dos grupos filogenéticos B2 y D, mientras que en los celíacos activos la mayoría de los aislados eran del grupo B2, y en los de celíacos no activos del D. Se ha descrito que pacientes con IBD presentan niveles mayores de aislados de *E. coli* del grupo filogenético B2, que es el que presentar una mayor cantidad de factores de virulencia (Nowrouzian et al., 2006; Sabate et al., 2006) y se ha relacionado con infecciones (Sepehri et al., 2011; Petersen et al., 2009). Además, nosotros detectamos una mayor prevalencia de los genes que codifican para la fimbria tipo P (*papC*), la hemolisina (*hlyA*) y la cápsula K5 (*KfiC*) en los aislados virulentos de los pacientes celíacos que en aquellos aislados a partir de sanos, lo que sugiere que tienen un potencial patogénico mayor.

La colección de enterobacterias aisladas en este estudio permitió la selección de dos cepas (*E. coli* CBL 2 y *Shigella* CBD8) para el análisis de su

posible papel en la estimulación de la respuesta inmune y la función barrera de la mucosa intestinal en modelos *in vitro* e *in situ* utilizando asas intestinales. Así se vio que las dos cepas de enterobacterias eran capaces de estimular la producción de citoquinas pro-inflamatorias en células mononucleares de sangre periférica (De Palma et al., 2010) e inducían cambios en el intestino delgado de ratas gnotobióticas como reducción del número de células productoras de mucus, secreción masiva de mucina y alteración de las proteínas que median las uniones intercelulares, comprometiendo la función barrera intestinal y favoreciendo la translocación de gliadinas a la lamina propia (Cinova et al., 2011). Estos resultados apoyan la hipótesis de que la población de enterobacterias podría agravar la patogénesis de la EC.

Los aislados de la especie* Staphylococcus epidermidis *que codifican resistencia a meticilina son más abundantes en heces de pacientes celíacos que en las de sanos

Otro de los grupos estudiados en los pacientes con EC ha sido el género *Staphylococcus* debido a que se detectaron niveles mayores de este grupo en muestras de heces y biopsias de niños con EC activa que en controles (Collado et al., 2009). En recién nacidos con riesgo a desarrollar la EC también se ha detectado una asociación entre el aumento de los niveles de *Staphylococcus* spp. y un mayor riesgo genético de sufrir EC, tanto en niños alimentados con leche materna como con fórmulas infantiles (Palma et al., 2012).

A fin de conocer la posible distribución en especies de estafilococos y su virulencia, se aislaron en medio selectivo a partir de muestras fecales de pacientes celíacos con enfermedad activa o no y de controles. Los análisis demostraron que la diversidad en especies de este género fue mayor en los niños con EC activa que en niños con EC no activa y controles. Además, la abundancia de las especies *S. epidermidis* y *S. haemolyticus* fue mayor y la de *S. aureus* menor en los pacientes con EC activa que en controles. Se podría especular que, en pacientes con EC activa, el aumento de la diversidad de las poblaciones de *Staphylococcus* spp. y el aumento en la abundancia de *S. epidermidis* se asocia con la reducción en la abundancia de *S. aureus*, así esta especie podría verse

desplazada por *S. epidermidis* y el resto de especies de estafilococos que compiten por el mismo nicho ecológico (Otto et al., 2001).

Además caracterizamos las cepas de *S. epidermidis* aisladas en función de la presencia/ausencia de genes que codifican factores de virulencia. La presencia de genes relacionados con la formación de biofilm (*atlE*, *fbe* e *icaD*) fue más frecuente en aislados procedentes de pacientes celíacos no activos y controles, lo que podría conducir a una colonización más estable. Sin embargo, los aislados de la especie *S. epidermidis* resistentes a meticilina fueron más abundantes en pacientes celíacos, tanto activos como no activos, que en controles sanos. *S. epidermidis* se ha convertido en uno de los principales agentes causales de infecciones nosocomiales en las que es frecuente la adquisición de cepas resistentes a antibióticos (Otto, 2009). Los resultados obtenidos sugieren que los pacientes celíacos presentan mayor susceptibilidad a infecciones nosocomiales o mayor exposición a antimicrobianos que los sujetos sanos. De hecho, estudios observacionales indican que los pacientes con EC tienen mayor riesgo de infecciones graves y visitan más frecuentemente los hospitales (Plot y Amital, 2009; Ludvigsson et al., 2008), también posiblemente por las dificultades encontradas durante el diagnóstico de la EC (Dickey y McConnell, 1996).

Los aislados de especie* Bacteroides fragilis *que codifican metaloproteasas son más abundantes en heces de pacientes celíacos que en las de sanos

En estudios previos de nuestro grupo se detectó un número mayor de *Bacteroides* spp. en pacientes celíacos en fase activa y no activa que en controles tanto en heces como en biopsias (Collado et al., 2009), por lo que se abordó también la caracterización de los aislados de este género a partir de heces para poder profundizar en su posible implicación en la patogénesis de enfermedad. Al igual que en las muestras de biopsias analizadas previamente por PCR-DGGE (Sánchez et al., 2010), los niños celíacos en fase activa presentaron una menor diversidad en especies del género *Bacteroides* que los del grupo control. Además, los pacientes celíacos presentaban una abundancia mayor de

las especies *B. fragilis* y *B. distasonis* y menor de *B. ovatus* y *B. finegodii*, diferencias que solo para *B. distasonis* y *B. finegodii* se reestablecieron tras es seguimiento de una dieta sin gluten. Aunque estos resultados no son del todo comparables con los obtenidos a partir de las muestras de biopsias duodenales, se observó que en ambos estudios *B. ovatus* se detectó en menor número en heces, y con menor prevalencia en biopsias, en pacientes celíacos activos y no activos comparada con controles; mientras que *B. distasonis* se detectó en mayor número en heces, y con menor prevalencia en biopsias, en pacientes con EC activa y no activa en comparación con controles.

Debido a que la especie *B. fragilis* fue más abundante en pacientes celíacos con enfermedad activa y no activa que en controles y a la asociación descrita entre cepas de *B. fragilis* enterotoxigénicas y las IBD (Rabizadeh et al., 2007; Basset et al., 2004) nos planteamos caracterizar las cepas de *B. fragilis* aisladas. En nuestra población de estudio los genes *bft* y *mpII,* que codifican dos metaloproteasas diferentes pero con funciones similares, sólo se detectaron en cepas aisladas de niños celíacos en fase activa y no activa. Tras exponer a células Caco-2 a cepas de *B. fragilis* observamos que la integridad de la monocapa de las células se veía comprometida y el efecto se agravaba en el caso de las cepas portadoras de metaloproteasas. Se ha descrito que las metaloproteasas y particularmente la BFT constituye un factor de virulencia que al interaccionar con las células epiteliales inicia una cascada de transmisión de señales que conducen a la degradación de la E-cadherina, alterando así la adhesión intercelular y aumentado la permeabilidad del epitelio intestinal y a la activación de rutas pro-inflamatorias (MAPK y FN-κB) y producción de citoquinas como la IL-8 que pueden agravar los procesos inflamatorios (Sears, 2009; Franco et al., 1997). Además, las cepas de *B. fragilis* eran capaces de digerir las gliadinas en fragmentos que no perdían su capacidad para inducir la síntesis de citoquinas, e incluso la aumentaban, y eran más permeables a la moncapa de células Caco-2, lo que podría favorecer la interacción de estos péptidos con células inmunocompetentes de la lámina propia. El análisis de la secuencia de péptidos por espectrometría de masas también permitió detectar péptidos inmunógenicos con secuencias de aminoácidos de los principales epítopos del péptido 33-mer de

las α-gliadinas, considerado como uno de los más potentes inmunoestimuladores en la EC.

Así pues, los pacientes con EC presentan una mayor abundancia de cepas de *B. fragilis*, productoras de metaloproteasas, que podría desempeñar junto al gluten un papel patogénico que agravara esta enfermedad.

La microbiota duodenal cultivable de pacientes celíacos difiere de la de controles

En un estudio adicional abordamos la caracterización de aislados asociados a la mucosa duodenal de niños celíacos, en fase activa y no activa, y controles a fin de poder realizar posteriores estudios sobre su potencial patogénico. Según nuestros resultados, los niños celíacos en fase activa mostraron una diversidad de especies de bacterias cultivables asociadas a la mucosa duodenal mayor que los pacientes en fase no activa y controles. En estudios realizados por otros autores también se detectó una mayor proporción de bacterias con forma bacilar en la mucosa duodenal por microscopía electrónica (Ou et al., 2009; Forsberg et al., 2004), así como una mayor diversidad bacteriana en la mucosa duodenal de pacientes celíacos por DGGE y TGGE (Di Cagno et al., 2011; Schippa et al., 2010). En los tres grupos de niños aquí estudiados, las poblaciones bacterianas aisladas pertenecían al filo *Firmicutes* seguido por el de *Proteobacteria* y *Actinobacteria*; sin embargo, en el grupo de celíacos activos el número de aislados pertenecientes al filo *Proteobacteria* fue superior al de controles. Coincidiendo con esto, en un estudio basado en técnicas independientes de cultivo se observó que los miembros de estos tres filos eran también los más detectados en el intestino delgado proximal de pacientes celíacos seguidos por *Bacteroidetes* y *Fusobacteria* (Ou et al., 2009).

Según nuestros datos, la microbiota duodenal de enfermos celíacos presentaba un menor número de miembros de la familia *Streptococcaceae*, y en particular de los grupos *S. anginosus* y *S. mutans*; y un mayor número de miembros de las familias *Enterobacteriaceae* y *Staphylococcaceae*, en particular de *S. epidermidis*; y estas diferencias se restablecieron con la dieta sin gluten. Los resultados sugieren que las familias dominantes de la microbiota duodenal normal de los individuos sanos, como *Streptococcaceae*, son sustituidos por otros, como *Enterobacteriaceae* y *Staphylococcaceae,* que podrían contribuir a la ruptura de la dinámica normal del ecosistema intestinal de los pacientes celíacos.

Las alteraciones observadas en la microbiota cultivable asociada a la mucosa intestinal de los pacientes celíacos en comparación con la de sanos coincide parcialmente con las diferencias detectadas en heces. Globalmente, los resultados ponen de manifiesto la existencia de una asociación entre la EC y alteraciones en la abundancia de las familias *Streptococcaceae*, *Enterobacteriaceae* y *Staphylococcaceae,* y factores de virulencia de *E. coli* y *S. epidermidis;* estas características son especialmente marcadas en la fase activa de la enfermedad pero no se restablecen totalmente tras el seguimiento de la dieta sin gluten, lo que apoya la hipótesis de que una mejora en el ecosistema intestinal de estos pacientes podría mejorar su estado de salud.

La colonización intestinal de las especies del género Bacteroides *en niños con riesgo de EC está influenciada por la lactancia y el genotipo HLA-DQ*

Tras el nacimiento, el intestino del recién nacido se considera prácticamente estéril pero tras el parto es colonizado rápidamente por bacterias anaerobias facultativas que reducen el ambiente dando paso a la colonización de otras más anaerobias como bacteroides y bifidobacterias. La sucesión bacteriana que se produce puede estar influenciada por factores, como el tipo de lactancia (Penders et al., 2006; Mackie et al., 1999). El proceso de colonización del intestino se considera la principal forma de exposición a los antígenos ambientales para el recién nacido y parece ser esencial para el adecuado desarrollo del sistema inmune innato y adaptativo (Gaboriau-Routhiau et al., 2011). Es importante que se establezca una buena colonización bacteriana debido a que esta juega importantes funciones fisiológicas que pueden tener un impacto en la salud del hospedador (Tlaskalova-Hogenova et al., 2011). En nuestra población de estudio, los niños alimentados con lactancia materna presentaron una menor diversidad de las poblaciones de *Bacteroides* que los alimentados con fórmulas infantiles, y en particular una prevalencia menor de las especies *B. intestinales, B. caccae* y *B. plebeius* y mayor de *B. uniformis* en comparación con los alimentados con fórmulas infantiles. Además el riesgo genético de los recién nacidos a desarrollar la EC, basado en el genotipo HLA-DQ, también pareció influir en el patrón de colonización de las especies de *Bacteroides*. Un elevado riesgo genético se asoció con una prevalencia mayor de

B. vulgatus y menor de *B. ovatus*, *B. plebeius* y *B. uniformis* durante todo el período de estudio. De Palma et al. (2012) también observaron mediante PCR cuantitativa que en este grupo de población existía una asociación positiva entre el riesgo genético a sufrir la EC y el número de *Bacteroides*. De Palma et al. (2012) también observaron mediante PCR cuantitativa que en este grupo de población existía una asociación positive entre el riesgo genético a sufrir la EC y el número de *Bacteroides*. El conjunto de resultados indica que el genotipo HLA-DQ junto con la lactancia influyen en el proceso de colonización del recién nacido y podrían modificar el riesgo de sufrir la enfermedad en etapas posteriores de la vida.

V. CONCLUSIONES

1. La microbiota fecal de niños con EC presentó una diversidad mayor de bacterias totales y de bacterias ácido lácticas y una diversidad menor de bifidobacterias que la de sujetos sanos analizada por DGGE.

2. La microbiota duodenal de niños con EC presentó una diversidad mayor de bacterias totales y bifidobacterias y menor de bacterias ácido lácticas y bacteroides que el grupo control analizada mediante técnicas moleculares (PCR-DGGE) o dependientes de cultivo.

3. La microbiota fecal de niños con EC presentó una prevalencia mayor de las especies *Lactobacillus curvatus*, *Leuconostoc mesenteroides* y *Leuconostoc carnosum* y menor de *Bifidobacterium adolescentis* y *Lactobacillus casei* que la de niños sanos analizada por DGGE.

4. La microbiota duodenal de pacientes con EC activa presentó una prevalencia mayor de las especies *B. adolescentes*, *Bifidobacterium animalis* subsp. *lactis* y *Bacteroides dorei*, y menor de *Bacteroides distasonis*, *Bacteroides fragilis* y/o *Bacteroides thetaiotaomicron*, *Bacteroides uniformis* y *Bacteroides ovatus* que los del grupo control, al analizarla mediante DGGE. Las diferencias en la prevalencia de especies de bifidobacterias se restablecieron con la dieta sin gluten, mientras que la de las especies de bacteroides se mantuvieron con la excepción de *B. dorei*.

5. Las bacterias cultivables asociadas a la mucosa duodenal de pacientes con EC activa presentaron una abundancia mayor de aislados del filo *Proteobacteria* y de las familias *Enterobacteriaceae* y *Staphylococcaceae* y menor de la familia *Streptococcaceae* que el grupo control. Las diferencias se restablecieron con la dieta sin gluten.

6. Los clones virulentos (B2+D) de *Escherichia coli* aislados en heces de niños con EC activa y no activa presentaron una abundancia mayor de genes que codifican la fimbria tipo P (*papC*), la hemolisina (*hlyA*) y la cápsula K5 (*KfiC*) que los del grupo control.

7. La abundancia de los aislados de la especie *Staphylocuccus epidermidis* fue mayor y la de *Staphylocuccus aureus* menor en heces de niños con EC activa y no activa que en controles. Los clones de *S. epidermidis* aislados de pacientes con EC activa presentaron una prevalencia mayor del gen que codifica resistencia a meticilina (*mecA*) que los de controles.

8. Los aislados del género *Bacteroides* pertenecientes a la especie *B. fragilis* portadores de los genes que codifican metaloproteasas (*bft* y/o *mpII*) fueron más abundantes en heces de niños con EC activa y no activa que en controles, y presentaron mayor capacidad para alterar la integridad de la monocapa de células Caco-2. Las cepas de *B. fragilis* analizadas fueron capaces de hidrolizar gliadinas generando péptidos de menor masa molecular que mantenían o aumentaban sus propiedades inflamatorias y eran más permeables a través del epitelio intestinal.

9. Los niños alimentados con lactancia materna presentaron una prevalencia mayor de las especies *B. uniformis* y menor de *Bacteroides intestinalis*, *Bacteroides caccae* y *Bacteroides plebeius* que los alimentados con fórmulas infantiles. Los niños con alto riesgo genético de desarrollar la EC presentaron una prevalencia mayor de *B. vulgatus* y menor de *B. ovatus*, *B. plebeius* y *B. uniformis* que los de bajo riesgo.

VI. BIBLIOGRAFÍA

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ABREVIATURAS

Abreviaturas

AGA: Anticuerpos anti-gliadina
ANOVA: *"ANalysis Of VAriance"*
ATCC: *"American Type Culture Collection"*

BAL: Bacterias Ácido Lácticas
BFT: "Bacteroides fragilis Toxin"
BH: *"Brain Heart agar"*
BLAST: "B*asic Local Alignment Search Tool"*

ºC: Grado centígrado
CD: *"Celiac Disese"*
CFU: *"Colony Forming Unit"*
CTAB: Bromuro de cetiltrimetilamonio

DGGE: *"Denaturing Gradient Gel Electrophoresis"*
DNA: *"DeoxyriboNucleic Acid"*
dNTPs: *"Desoxinucleótidos trifosfato"*
DSMZ: *"Deutsche Sammlung von Mikroorganismen"*

EC: Enfermedad Celíaca
EDTA: Ácido etilendiamino tetracético
ELISA: *"Enzyme-Linked Immuno orbent Assay"*
EMA: Anticuerpos anti-endomisio
ESPAGHAN: *"European Society for Pediatric Gastroenterology, Hepatology, and Nutrition"*
ETBF: *"Enterotoxigenic B. fragilis"*

FISH: *"Fluorescence* In Situ *Hybridization"*

g: gramo
Gld: Gliadina
Gln: Glutamina (Q)
Glu: Glutamato (E)
GWAS: *"Genome-Wide Association Studies"*

h: hora
H´: Índice de diversidad de Shannon Wiener
HLA: *"Human Leukocyte Antigen"*
HMC: *"Major Histocompatibility Complex"*
HMW-GS: *"High Molecular Weight Glutenin Subunits"*
HPLC: *"H*igh-Pressure Liquid Chromatography*"*

IBD: *"Inflammatory Bowel Disease"*
IFN: Interferón
Ig: Inmunoglobulina
IL-: Interleuquina

KDa: KiloDalton

L: Litro
LAB: *"Lactic Acid Bacteria"*
LMW-GS: *"Low Molecular Weight Glutenin Subunits"*

M: Concentración molar
MICA: *"MHC class I chain-related molecule A"*
min: Minuto
mg: Miligramo
mL: Mililitro
mM: Milimolar
MRS: *"Man Rogosa and Sharpe Agar"*
M/F: *"Male/Female"*

n: Número
NK: *"Natural Killer"*

pb: Pares de bases
PBS: *"Phospahate Buffered Saline"*
PCA: *"Plate Count Agar"*
PCR: *"Polymerase Chain Reaction"*
Pro: Prolina (P)

RAPD: *"Random Amplified Polymorphic DNA"*
RNA: *"RiboNucleic Acid"*
RNAr: *"RiboNucleic Acid"*

s: segundos
SD: *"Standard Deviation"*
SDS-PAGE: *"Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis"*

TGF: *"Transforming Growth Factor"*
TGGE: *"Temperature* Gradient Gel Electrophoresis*"*
TGI: Tracto gastrointestinal
TGt: Transglutaminasa tisular
Th: *"T helper*
TNF: *"Tumor Necrosis Tactor"*
Tris: Tris 2-amino-2 (hidroximetil)-1,3propanodiol.

U: *"Unit"*

UPGMA: *"Unweighted-Pair Group Method with the Arithmetic means"*

VRBD: *"Violet Red Bile Dextrose Agar"*
v/v: Relación volumen/volumen

wt/vol: *"weight/volume"*

YFCA: *"Yeast, Casitone, Fatty Acid agar"*

χ^2**:** Test de Chi-cuadrado

µL: microlitro
µM: micromolar

VII. ANEXOS

RESEARCH ARTICLE

Differences in faecal bacterial communities in coeliac and healthy children as detected by PCR and denaturing gradient gel electrophoresis

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Received 18 May 2007; revised 28 July 2007; accepted 21 August 2007.
First published online November 2007.

DOI:10.1111/j.1574-695X.2007.00337.x

Editor: Willem van Eden

Keywords
coeliac disease; PCR-DGGE; faecal microbiota; lactic acid bacteria; *Bifidobacterium*.

Abstract

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestinal mucosa. Scientific evidence supports a role of the gut microbiota in chronic inflammatory disorders; yet information is not specifically available for CD. In this study, a comparative denaturing gradient gel electrophoresis analysis of faecal samples from coeliac children and age-matched controls was carried out. The diversity of the faecal microbiota was significantly higher in coeliac children than in healthy controls. The presence of the species *Lactobacillus curvatus*, *Leuconostoc mesenteroides* and *Leuconostoc carnosum* was characteristic of coeliac patients, while that of the *Lactobacillus casei* group was characteristic of healthy controls. The *Bifidobacterium* population showed a significantly higher species diversity in healthy children than in coeliacs. In healthy children, this population was characterized by the presence of *Bifidobacterium adolescentis*. Overall, the results highlighted the need for further characterization of the microbiota in coeliac patients, and suggested a potential role of probiotics and/or prebiotics in restoring their gut microbial balance.

Introduction

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestinal mucosa that involves genetic and environmental factors. The classic form of the disease often manifests in early childhood (9–24 months) with gastrointestinal symptoms and malabsorption (Van Heel & West, 2006). CD is the result of an aberrant Th_1 immune response to gluten peptides within the intestinal mucosa, where IFN-γ is the predominantly secreted cytokine, as well as an innate immune response mediated by IL-15 (Koning *et al.*, 2005). Over the last decades, significant progress has been made in the understanding of the aetiology and pathogenesis of CD. In spite of that, little is known about the roles of environmental factors other than gluten in CD presentation (Barnich & Darfeuille-Michaud, 2007). It has been suggested that a transient infection could increase the permeability of the mucosal epithelial layer to gluten antigens by activating macrophages and dendritic cells with the production of pro-inflammatory cytokines (Kagnoff, 2005). Moreover, the inflammatory milieu originated by gluten antigens could lead to disturbances in the gut microbial composition that could in turn contribute to perpetuate inflammation (Collado *et al.*, 2007). The development and maintenance of immune homeostasis depends on signals from the gut microbiota. Members of the genera *Lactobacillus* and *Bifidobacterium* are regarded as plausible significant players of gut health, and therefore are intensively investigated for probiotic uses (Isolauri *et al.*, 2001; Thompson-Chagoyan *et al.*, 2005). It has been demonstrated that components of the intestinal microbiota of animal models and inflammatory bowel disease (IBD) patients are involved in the abnormal T cell immune responses leading to loss of tolerance and mucosal inflammation (Barnich & Darfeuille-Michaud, 2007). Furthermore, the administration of probiotics has been found to exert beneficial effects in some disease models and IBD patients by decreasing the production of proinflammatory cytokines (e.g. IFN-γ, and TNF-α) and interfering with harmful bacterial adhesion (Dotan & Rachmilewitz, 2005). At present, little is known about the

Original article

Intestinal *Bacteroides* species associated with coeliac disease

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Accepted 31 August 2010
Published Online First 23 October 2010

ABSTRACT

Aims To characterise the predominant species of bacterial populations associated with duodenal biopsies of paediatric patients with active and treated coeliac disease.

Methods 20 biopsy specimens from patients with active coeliac disease, 12 from patients with treated coeliac disease, and eight from age-matched controls were evaluated for comparative purposes. *Bacteroides*, *Bifidobacterium* and lactic acid bacteria (LAB) populations were analysed by PCR-denaturing gradient gel electrophoresis using group-specific primers.

Results *Bacteroides* diversity was higher in biopsy specimens from controls than in those from patients with active and treated coeliac disease. *Bacteroides distasonis*, *Bacteroides fragilis/Bacteroides thetaiotaomicron*, *Bacteroides uniformis* and *Bacteroides ovatus* were more abundant in controls than in patients with coeliac disease ($p<0.05$). *Bacteroides vulgatus* was more frequently detected in controls than in patients with treated coeliac disease ($p<0.01$). *Bacteroides dorei* was more common in patients with active coeliac disease than in those with treated coeliac disease and control children ($p<0.01$). *Bifidobacterium* diversity was higher in patients with coeliac disease than in controls. *Bifidobacterium adolescentis* and *Bifidobacterium animalis* subsp *lactis* were more prevalent in patients with active coeliac disease than in patients with treated coeliac disease and control children. A higher LAB diversity was found in patients with treated coeliac disease and controls than in patients with active coeliac disease. *Weissella* spp and *Lactobacillus fermentum* were more frequently detected in patients with treated coeliac disease than in controls and patients with active coeliac disease.

Conclusions *Bacteroides*, *Bifidobacterium* and LAB populations in the duodenum of Spanish children with typical coeliac disease (active and treated) and controls differ in diversity and species composition; this could contribute to features of the disease.

INTRODUCTION

The human gastrointestinal tract hosts a complex microbial community (microbiota) composed of hundreds of different bacterial species.[1 2] The microbiota contributes to regulating energy metabolism, and epithelial cell and gut-associated lymphoid tissue functions.[3 4] The intestinal immune system has to maintain a balance between the need to respond to pathogens and tolerance to the presence of a large community of commensal bacteria, whose disruption might contribute to the pathogenesis of inflammatory conditions.[5 6] In fact, alterations to the composition of the intestinal microbiota have been implicated in diseases such as allergies,[7] chronic inflammatory bowel conditions[8] and cancer.[9]

Coeliac disease is a chronic inflammatory disorder of the small intestine that presents in genetically predisposed individuals following gluten consumption.[10] Removal of gluten from the diet is currently the only treatment available. In recent years, intestinal dysbiosis has been reported in patients with coeliac disease. The microbiota of these patients is characterised by a breakdown in the balance between potentially protective bacteria (eg, *Lactobacillus* spp and *Bifidobacterium* spp) and potentially harmful bacteria (eg, *Bacteroides* spp and *Enterobacteriaceae*).[11 12] Some differences in bacterial species composition have also been detected in stool samples of patients with active coeliac disease compared with those of healthy controls[13]; however, patients on a gluten-free diet were not included and duodenal samples were not analysed, limiting the conclusiveness of that preliminary study. A further report provided information on only the faecal and duodenal *Bifidobacterium* species composition in patients with coeliac disease, analysed using real-time PCR.[14]

Denaturing gradient gel electrophoresis (DGGE) has been successfully applied as a rapid culture-independent method for the analysis of intestinal microbiota composition.[15 16] DGGE is based on sequence-specific separation of equal-sized PCR products of the 16S rRNA gene on a polyacrylamide gel. DGGE facilitates the identification of a wider number of bacterial species within a genus than real-time PCR, in which the selection of specific primers restricts the number of species that can be detected. DGGE also allows the detection of non-cultivable bacterial species, which could represent more than 50% of intestinal bacteria.[17 18]

The objective of this study was to determine the species composition of the genera *Bacteroides* and *Bifidobacterium*, and lactic acid bacteria (LAB), in duodenal biopsy specimens of patients with active and treated coeliac disease, and in those of control children, by PCR-DGGE, in order to obtain more detailed information on the possible contribution of specific species to the disease.

MATERIALS AND METHODS

Subjects

Biopsy specimens from three groups of children were included in this study: 20 patients with active coeliac disease, 12 patients with treated coeliac disease who had been following a gluten-free diet for at least 2 years, and 8 control children

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Reduced diversity and increased virulence-gene carriage in intestinal enterobacteria of coeliac children

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Published: 4 November 2008

BMC Gastroenterology 2008, **8**:50 doi:10.1186/1471-230X-8-50

Received: 14 May 2008

Accepted: 4 November 2008

This article is available from: http://www.biomedcentral.com/1471-230X/8/50

Abstract

Background: Coeliac disease is an immune-mediated enteropathology triggered by the ingestion of cereal gluten proteins. This disorder is associated with imbalances in the composition of the gut microbiota that could be involved in its pathogenesis. The aim of the present study was to determine whether intestinal *Enterobacteriaceae* populations of active and non-active coeliac patients and healthy children differ in diversity and virulence-gene carriage, so as to establish a possible link between the pathogenic potential of enterobacteria and the disease.

Methods: *Enterobacteriaceae* clones were isolated on VRBD agar from faecal samples of 31 subjects (10 active coeliac patients, 10 symptom-free coeliac patients and 11 healthy controls) and identified at species level by the API 20E system. *Escherichia coli* clones were classified into four phylogenetic groups A, B1, B2 and D and the prevalence of eight virulence-associated genes (type-1 fimbriae [*fimA*], P fimbriae [*papC*], S fimbriae [*sfaD/E*], Dr haemagglutinin [*draA*], haemolysin [*hlyA*], capsule K1 [*neuB*], capsule K5 [*KfiC*] and aerobactin [*iutA*]) was determined by multiplex PCR.

Results: A total of 155 *Enterobacteriaceae* clones were isolated. Non-*E. coli* clones were more commonly isolated in healthy children than in coeliac patients. The four phylogenetic *E. coli* groups were equally distributed in healthy children, while in both coeliac patients most commensal isolates belonged to group A. Within the virulent groups, B2 was the most prevalent in active coeliac disease children, while D was the most prevalent in non-active coeliac patients. *E coli* clones of the virulent phylogenetic groups (B2+D) from active and non-active coeliac patients carried a higher number of virulence genes than those from healthy individuals. Prevalence of P fimbriae (*papC*), capsule K5 (*sfaD/E*) and haemolysin (*hlyA*) genes was higher in *E. coli* isolated from active and non-active coeliac children than in those from control subjects.

Conclusion: This study has demonstrated that virulence features of the enteric microbiota are linked to coeliac disease.

JCP Online First, published on June 20, 2012 as 10.1136/jclinpath-2012-200759

Intestinal *Staphylococcus spp.* and virulent features associated with coeliac disease

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Accepted 9 May 2012

ABSTRACT

Aim To determine whether intestinal *Staphylococcus spp.* and their pathogenic features differed between coeliac disease (CD) patients and healthy controls.

Methods 60 children, including active CD (n=20) and non-active CD (n=20) patients and healthy controls (n=20), were studied. Staphylococci were isolated from faeces and identified by PCR and DNA sequencing. The carriage of virulent genes, including adhesion (*atlE* and *fbe*), cell aggregation (*icaD*), global regulatory (*agr* and *sar*) and methicillin-resistant (*mecA*) genes, was analysed by PCR.

Results *Staphylococcus epidermidis* was more abundant in the microbiota of active and non-active CD patients than in controls. *Staphylococcus haemolyticus* was more abundant in active CD patients than in control subjects. *Staphylococcus aureus* was less abundant in active CD patients than in the other child groups. *Staphylococcus spp.* diversity was higher in active CD patients than in non-active CD patients and controls. The presence of the *mecA* gene and the simultaneous presence of both the *mecA* and *atlE* genes were higher in *S. epidermidis* clones isolated from CD patients, with active and non-active disease, than in those from control subjects. The individual presence of the other virulent genes was lower in *S. epidermidis* from active CD patients than in those from the other -child- groups.

Conclusions Increased abundance of *S. epidermidis* carrying the *mecA* gene, in active and non-active CD patients, most likely reflects increased exposure of these subjects to opportunistic pathogens and antimicrobials.

INTRODUCTION

Coeliac disease (CD) is an autoimmune enteropathy caused by a permanent intolerance to cereal gluten proteins (gliadins).[1] Currently, the only available therapy for CD is the adherence to a strict, lifelong, gluten-free diet; however, the compliance with this dietary recommendation is complex and other alternative strategies are needed.[2]

Scientific evidence suggests that environmental factors, other than gluten, may play a role in CD pathogenesis. It has been suggested that early infections may increase the risk of suffering CD in susceptible individuals.[3 4] In addition, imbalances in the gut microbiota of CD patients[5 6] and infants at genetic risk of CD[7] have been reported. Greater numbers of *Staphylococcus spp.* have been found in faecal and biopsy samples of CD patients, suggesting it may be related with the active phase of CD.[8]

Staphylococcus spp. are widespread in various environments, including skin and mucosal membranes of humans and many animals, and some species are clinically important because they cause chronic infections.[9] For instance, *Staphylococcus epidermidis* is the most frequent causative agent of nosocomial infections.[10] The pathogenesis of staphylococci and, particularly, of *S. epidermidis*, depends on the presence of genes coding for virulent factors mainly related to biofilm production and antibiotic resistance. The bacterial biofilm is produced in two steps comprising the initial bacterial attachment to the surface followed by a second stage consisting of bacterial proliferation, intercellular adhesion (cell aggregation) and production of an extracellular slimy substance. The AtlE, a cell surface protein exhibiting vitronectin-binding activity is one of the most important factors involved in the primary attachment of *S. epidermidis*.[11 12] *S. epidermidis* can also bind to other extracellular matrix-related proteins, like fibrinogen, via fibrinogen-binding proteins codified by the *fbe* gene.[13] Cell aggregation is mediated by the products of the chromosomal *ica* locus, which comprises four intercellular adhesion genes (*icaA*, *icaB*, *icaC* and *icaD*). The *ica* operon encodes enzymes for the biosynthesis of PIA (polysaccharide intercellular adhesin) leading to multilayer biofilm development.[12 14] PIA is also important for haemagglutination of erythrocytes by *S. epidermidis*.[15]

Quorum-sensing and global regulatory systems also play an important role in the regulation of virulent factors. The genes of two global regulatory loci, *agr* and *sar* seem to be involved in the regulation of the virulent factors of *S. epidermidis*.[16 17] *S. epidermidis* is considered an emergent pathogen causing increasing numbers of human infections, probably because a methicillin-resistant gene complex is widespread among isolates of this species.[9 18] Methicillin resistance is due to the expression of a modified penicillin-binding protein PBP2a (PBP2′) encoded by the *mecA* gene and located on the mobile element staphylococcal cassette chromosome mec (SCCmec), a genomic island.[9]

The purpose of this study was to analyse the diversity and species composition of clones of the genus *Staphylococcus*, isolated from faecal samples of active and non-active CD patients and age-matched controls. Furthermore, the presence of well-known virulent genes was examined in order to establish a possible link between the pathogenic potential of *Staphylococcus spp.* and CD.

METHODS

Subjects and sampling

A total of 60 children were included in this study: (1) active CD patients (n=20), who were on a normal gluten-containing diet, showed clinical

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Discerning the Role of *Bacteroides fragilis* in Celiac Disease Pathogenesis

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Celiac disease (CD) is associated with intestinal dysbiosis, which can theoretically lead to dysfunctions in host-microbe interactions and contribute to the disease. In the present study, possible differences in *Bacteroides* spp. and their pathogenic features between CD patients and controls were investigated. *Bacteroides* clones ($n = 274$) were isolated, identified, and screened for the presence of the virulence genes (*bft* and *mpII*) coding for metalloproteases. The proteolytic activity of selected *Bacteroides fragilis* strains was evaluated by zymography and, after gastrointestinal digestion of gliadin, by high-pressure liquid chromatography/electrospray ionization/tandem mass spectrometry. The effects of *B. fragilis* strains on Caco-2 cell culture permeability and inflammatory response to digested gliadin were determined. *B. fragilis* was more frequently identified in CD patients than in healthy controls, in contrast to *Bacteroides ovatus*. *B. fragilis* clones carrying virulence genes coding for metalloproteases were more abundant in CD patients than in controls. *B. fragilis* strains, representing the isolated clones and carrying metalloprotease genes, showed gelatinase activity and exerted the strongest adverse effects on the integrity of the Caco-2 cell monolayer. All *B. fragilis* strains also showed gliadin-hydrolyzing activity, and some of them generated immunogenic peptides that preserved or increased inflammatory cytokine production (tumor necrosis factor alpha) and showed increased ability to permeate through Caco-2 cell cultures. These findings suggest that increased abundance of *B. fragilis* strains with metalloprotease activities could play a role in CD pathogenesis, although further *in vivo* studies are required to support this hypothesis.

Celiac disease (CD) is the most common chronic intestinal inflammatory disorder triggered by ingestion of dietary gluten. This disease is considered as both a food hypersensitivity and an autoimmune disorder that involves genetic and environmental factors (40). The human leukocyte antigen (HLA) class II genes encoding for DQ2 and DQ8 heterodimers are the main hereditary factors predisposing to CD and are present in most CD patients (95%). Nevertheless, while 30 to 35% of the general population are carriers of these genes, only 2 to 5% actually develop CD, indicating that other factors contribute to precipitating the disease (33). The intake of gluten proteins is the critical environmental element responsible for the signs and symptoms of the disease and, in fact, typical cases manifest in early childhood after introduction of gluten into the diet. However, the disease is also being increasingly diagnosed in adulthood (5), suggesting that early exposure to gluten is not the only environmental trigger.

Gluten proteins and their toxic components (gliadins) are partially resistant to proteolytic degradation and can accumulate and interact with the small intestinal mucosa (14). Enzyme deficiency in the small intestinal mucosa of CD patients does not seem to be causally related to the disease (3). However, in CD patients, some peptides, such as the 33-mer of α-gliadin and others containing its main structural epitopes (PFPQPQLPY and PQPQLPYPQ), preferentially drive an adaptive immune response by binding to HLA-DQ2/DQ8 molecules of antigen-presenting cells and activating T-helper 1 (Th1) and Th17 inflammatory responses within the mucosa, with the resulting production of inflammatory cytokines (e.g., gamma interferon [IFN-γ] and interleukin-21 [IL-21]) leading to severe inflammation (24). Other gliadin peptides activate an innate immune response characterized by increased production of IL-15 by epithelial and antigen-presenting cells, which activate the effector function and cytotoxic activity of intraepithelial lymphocytes (15). Gliadin peptides also induce upregulation of the zonulin innate immunity pathway, which leads to increased intestinal permeability and enables paracellular translocation of gliadin and its subsequent interaction with antigen-presenting cells within the intestinal submucosa (11).

In recent years, alterations in the composition of the intestinal microbiota have been associated with CD. The bacterial numbers of the *Bacteroides-Prevotella* group or the *Bacteroides fragilis* group in CD patients have been demonstrated to be increased compared to those in healthy controls (8, 26). *Bacteroides* spp. are generally considered commensals or symbionts inhabiting the human gastrointestinal tract, representing ca. 25% of the total bacterial cells. Nonetheless, members of the normal microbiota can also potentially cause disease in cases of failure of the host defenses and major dysbiosis and can then be considered "pathobionts" (35). In spite of the abundance of *Bacteroides* spp. in the gut microbiota, their ecological distribution, composition, and impact on health remain unclear (46). Species such as *B. fragilis* and *Bacteroides vulgatus* seem to be implicated in the disruption of the integrity of the intestinal epithelial barrier, thereby contributing to the development of inflammation in experimental animal models (38, 42) and, possibly, in patients with inflammatory bowel disease (IBD) (10). The potential pathogenicity of *Bacteroides* spp. is related to the expression of a variety of virulence factors, including proteolytic and other hydrolytic enzymes (4). Enterotoxigenic *B. fragilis* (ETBF) strains produce an enterotoxin, termed *B. fragilis* toxin (BFT), which is a 20-kDa zinc-dependent metalloprotease that has been associated with diarrhea in humans and young animals (37).

Received 22 February 2012 Accepted 28 June 2012
Published ahead of print 6 July 2012
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doi:10.1128/AEM.00563-12

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2011, p. 5316–5323 Vol. 77, No. 15
0099-2240/11/$12.00 doi:10.1128/AEM.00365-11

Influence of Environmental and Genetic Factors Linked to Celiac Disease Risk on Infant Gut Colonization by *Bacteroides* Species▽

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Received 17 February 2011/Accepted 26 May 2011

Celiac disease (CD) is an immune-mediated enteropathy involving genetic and environmental factors whose interaction might influence disease risk. The aim of this study was to determine the effects of milk-feeding practices and the HLA-DQ genotype on intestinal colonization of *Bacteroides* species in infants at risk of CD development. This study included 75 full-term newborns with at least one first-degree relative suffering from CD. Infants were classified according to milk-feeding practice (breast-feeding or formula feeding) and HLA-DQ genotype (high or low genetic risk). Stools were analyzed at 7 days, 1 month, and 4 months by PCR and denaturing gradient gel electrophoresis (DGGE). The *Bacteroides* species diversity index was higher in formula-fed infants than in breast-fed infants. Breast-fed infants showed a higher prevalence of *Bacteroides uniformis* at 1 and 4 months of age, while formula-fed infants had a higher prevalence of *B. intestinalis* at all sampling times, of *B. caccae* at 7 days and 4 months, and of *B. plebeius* at 4 months. Infants with high genetic risk showed a higher prevalence of *B. vulgatus*, while those with low genetic risk showed a higher prevalence of *B. ovatus*, *B. plebeius*, and *B. uniformis*. Among breast-fed infants, the prevalence of *B. uniformis* was higher in those with low genetic risk than in those with high genetic risk. Among formula-fed infants, the prevalence of *B. ovatus* and *B. plebeius* was increased in those with low genetic risk, while the prevalence of *B. vulgatus* was higher in those with high genetic risk. The results indicate that both the type of milk feeding and the HLA-DQ genotype influence the colonization process of *Bacteroides* species, and possibly the disease risk.

The newborn intestine is colonized immediately after birth by microorganisms from the mother and the environment (12, 21). At birth, the intestinal milieu of neonates shows a positive redox potential, and early bacterial colonization begins with facultative anaerobes (*Enterobacteriaceae*, *Lactobacillus*, etc.) that gradually consume the oxygen, permitting the growth of strict anaerobes (*Bifidobacterium*, *Bacteroides*, *Clostridium*, etc.) (18, 22). Subsequently, milk-feeding practices play an important role in the microbiota composition of the infant gut (2, 18, 22). In breast-fed infants, the microbiota is less diverse and is dominated by *Bifidobacterium* species, while a more diverse microbiota develops only after complementary feeding commences. In contrast, the bacterial composition of formula-fed infants is dominated by members of diverse genera (*Enterobacteriaceae*, *Streptococcus*, *Bacteroides*, *Clostridium*, and *Bifidobacterium*) (1, 9). Intestinal colonization influences diverse physiological functions, which may have an impact on the host's health and disease risk (16, 18, 25). Nevertheless, there is still limited information on the initial establishment of *Bacteroides* species and its possible influence on health (11, 19, 30).

Celiac disease (CD) is a multifactorial disorder involving both genetic and environmental factors. This disease is associated with human leukocyte antigen (HLA) genes of the major histocompatibility complex (MHC), and approximately 95% of patients are positive for HLA-DQ2 or -DQ8 (28). Studies of twins also showed that in 25% of cases, one twin of the pair did not develop CD, supporting the role of environmental factors in disease development (10, 20). Breast-feeding seems to exert a protective effect against CD development (4, 15), but its possible connection with modulation of the intestinal microbiota is unknown. A preliminary study suggested an association between increased *Bacteroides-Prevotella* group

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▽ Published ahead of print on 3 June 2011.