

# Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease

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Received 9 September 2013; revised 10 December 2013; accepted 24 January 2014.  
Final version published online 3 March 2014.

DOI: 10.1111/1574-6941.12295

Editor: Julian Marchesi

## Keywords

gluten metabolism; 33-mer hydrolysis; gluten proteolysis; gut microbiota; proteolytic bacteria.

## Abstract

Gluten, a common component in the human diet, is capable of triggering coeliac disease pathogenesis in genetically predisposed individuals. Although the function of human digestive proteases in gluten proteins is quite well known, the role of intestinal microbiota in the metabolism of proteins is frequently underestimated. The aim of this study was the isolation and characterisation of the human gut bacteria involved in the metabolism of gluten proteins. Twenty-two human faecal samples were cultured with gluten as the principal nitrogen source, and 144 strains belonging to 35 bacterial species that may be involved in gluten metabolism in the human gut were isolated. Interestingly, 94 strains were able to metabolise gluten, 61 strains showed an extracellular proteolytic activity against gluten proteins, and several strains showed a peptidasic activity towards the 33-mer peptide, an immunogenic peptide in patients with coeliac disease. Most of the strains were classified within the phyla *Firmicutes* and *Actinobacteria*, mainly from the genera *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Clostridium* and *Bifidobacterium*. In conclusion, the human intestine exhibits a large variety of bacteria capable of utilising gluten proteins and peptides as nutrients. These bacteria could have an important role in gluten metabolism and could offer promising new treatment modalities for coeliac disease.

## Introduction

Coeliac disease (CD) is a chronic small intestinal immune-mediated enteropathy precipitated by the exposure to gluten proteins in genetically predisposed individuals (Ludvigsson *et al.*, 2012). In patients with CD, gluten proteins generally induce intestinal symptoms and severe mucosal damage due to an abnormal immune response to incompletely digested gliadin peptides by human digestive enzymes. Currently, a strict life-long gluten-free diet is the only efficient treatment available for CD (Kagnoff, 2007; Fasano, 2009).

*In vitro* studies have shown that gluten proteins are recalcitrant to complete digestion by human digestive proteases, releasing metastable Pro/Gln-rich peptides of

up to 30–40 amino acids in length into the gut lumen. Some of these peptides are capable of triggering the inflammatory process associated with CD (Shan *et al.*, 2002; Bethune & Khosla, 2012). Incompletely digested dietary proteins enter the human large intestine as a complex mixture of protein and peptides (Davila *et al.*, 2013), and a portion of the gluten proteins ingested in the diet and not absorbed in the small intestine is eliminated through faeces (Caminero *et al.*, 2012; Comino *et al.*, 2012). Thus, gluten proteins and peptides arrive in the large intestine where microbiota derive energy from dietary compounds that escape digestion in the stomach and the small intestine. Because of the large amount of bacterial diversity in the large intestine, it is worth considering that these bacteria may participate in the metabolism of

gluten proteins. There is some evidence suggesting the role of the human microbiota in the metabolism of gluten proteins. Indeed, the oral cavity is colonised with microorganisms that produce proteases capable of hydrolysing peptides rich in proline and glutamine residues (Helmerhorst *et al.*, 2010; Zamakhchari *et al.*, 2011; Fernandez-Feo *et al.*, 2013), and our group has recently described a faecal glutenase activity related to diet-ingested gluten, an activity that is most likely derived from bacterial metabolism (Caminero *et al.*, 2012). Additionally, some studies have shown that certain bacterial strains isolated from faeces belonging to *Bifidobacterium* and *Bacteroides fragilis* are capable of digesting gliadin-derived peptides (Laparra & Sanz, 2010; Sánchez *et al.*, 2012).

Despite the ubiquity of wheat in the diet and the importance of gluten proteins for patients with CD, the bacteria that are involved in gluten metabolism and the role of the human gut microbiota in this metabolic process remain unknown. Accordingly, the aim of this study was the isolation and characterisation of the human gut microbiota involved in the metabolism of gluten proteins.

## Materials and methods

### Subjects

A total of 22 volunteers on a normal gluten diet (mean age, 32.05 years; range, 22–47 years; nine men and 13 women) were enrolled in this study. None of the subjects had been treated with antibiotics for at least 2 months prior to the sampling time. The study was conducted according to the guidelines outlined in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local ethics committee of our hospital. Written informed consent was obtained from all of the subjects.

### Faecal sampling

Fresh stools were kept under anoxic conditions immediately after collection. The faecal samples were homogenised and used for culturing as soon as they were received in the laboratory.

### Culture media

The following three culture media with gluten as principal nitrogen source (MCG) were designed: MCG-1–20 g L<sup>-1</sup> glucose, 30 g L<sup>-1</sup> gluten, 0.05 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.07 g L<sup>-1</sup> ZnSO<sub>4</sub>, 0.05 g L<sup>-1</sup> L-cysteine, 0.1% Tween 80, 60 mM phosphate buffer (pH 6.5), 16 g L<sup>-1</sup> agar; MCG-2–20 g L<sup>-1</sup> glucose, 25 g L<sup>-1</sup> gluten, 5 g L<sup>-1</sup> gluten

peptone, 0.05 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.07 g L<sup>-1</sup> ZnSO<sub>4</sub>, 5 g L<sup>-1</sup> NaCl, 0.15 g L<sup>-1</sup> Ca(OH)<sub>2</sub>, 0.05 g L<sup>-1</sup> L-cysteine, 2 g L<sup>-1</sup> meat extract, 5 g L<sup>-1</sup> meat peptone, 60 mM phosphate buffer (pH 6.5), agar 16 g L<sup>-1</sup>; MCG-3–10 g L<sup>-1</sup> glucose, 25 g L<sup>-1</sup> gluten, 5 g L<sup>-1</sup> gluten peptone, 0.05 g L<sup>-1</sup> CaCl<sub>2</sub>, 0.07 g L<sup>-1</sup> ZnSO<sub>4</sub>, 5 g L<sup>-1</sup> NaCl, 0.15 g L<sup>-1</sup> Ca(OH)<sub>2</sub>, 1 g L<sup>-1</sup> sodium pyruvate, 0.5 g L<sup>-1</sup> sodium succinate, 0.05 g L<sup>-1</sup> L-cysteine, 1 g L<sup>-1</sup> L-arginine, 0.01 g L<sup>-1</sup> haemin, 0.25 g L<sup>-1</sup> soluble pyrophosphate, 0.001 g L<sup>-1</sup> vitamin K1, 0.001 g L<sup>-1</sup> thiamine, 0.001 g L<sup>-1</sup> riboflavin, 0.001 g L<sup>-1</sup> biotin, 1 g L<sup>-1</sup> meat extract, 1 g L<sup>-1</sup> meat peptone, 0.4 g L<sup>-1</sup> NaHCO<sub>3</sub> and 16 g L<sup>-1</sup> agar. Gluten peptone was obtained by incubating 0.5 g gluten with 1.7 g of pepsin in 500 mL MilliQ H<sub>2</sub>O (pH 2) for 2 h at 200 r.p.m. Liquid MCG contained the same components without the addition of agar.

### Screening and isolation of bacteria

A 1-g sample of each faecal specimen was homogenised in 5 mL of 0.9% NaCl + 0.05 g L<sup>-1</sup> L-cysteine using a Stomacher 80 Biomaster blender (Seward Medical), and 0.3 mL of the homogenate was inoculated in 30 mL of liquid MCG and incubated for 48 h at 37 °C under anoxic conditions. Serial 10-fold dilutions (of each faecal sample and liquid cultures in MCG) were plated on the corresponding solid MCG medium and incubated for 48 h at 37 °C under anoxic conditions.

### Bacterial identification

The bacteria were identified by 16S rDNA sequencing. DNA was extracted using SpeedTools Tissue DNA Extraction (Biotools), and 16S rDNA amplification was performed using the universal primers 27F and E939R. Each sequence obtained was compared with those in the GenBank database using the BLASTN algorithm (Thompson *et al.*, 1994). To complete the molecular identification of these sequences, a phylogenetic analysis was performed using MEGA v. 4.0 software (Tamura *et al.*, 2007). Sequences with 98% identity to the sequences of known bacteria were considered to be the same species.

### Glutenase activity and gluten utilisation for growth

The extracellular proteolytic activity towards gluten proteins and gelatine was determined by the presence of a hydrolytic halo surrounding the colony. Glutenase activity was tested using the MCG medium, and gelatinase activity was tested on MCG medium, exchanging the gluten with gelatine (MCGel). Trichloroacetic acid (40%) was used to precipitate the proteins in the gelatine media.

The solid media were incubated at 37 °C for 48 h under anoxic conditions. All the isolated strains were tested for the ability to grow on the isolation medium without gluten (MSG). Each MSG medium has the same composition as the corresponding MCG medium but without gluten.

### Gluten quantification

The amount of gluten in the culture media was measured with the Competitive Elisa GlutenTox Kit (Biomedal) according to the manufacturer's protocol.

### Determination of 33-mer hydrolysis

A synthetic 33-mer peptide (LQLQPFQPQLPYPQPQLPYPQPQLPYPQPQPF) was synthesised at a purity of 95% (Proteogenix). The reaction mixtures (60 µL) contained 3.4 µL of bacterial culture supernatant and 60 µM of 33-mer peptide in PBS (pH 7.3) and were incubated at 37 °C for 24 h. Bacterial supernatant was obtained after incubation of each bacterium in 10 mL of liquid MCG for 48 h at 37 °C under anoxic conditions. The reactions were stopped by incubation at 100 °C for 10 min, and 40 µL of each reaction was subjected to reverse-phase HPLC using a C-18 column (Lichrospher 100 RP18 column 5 µm, 4 × 250 mm Teknokroma SL). The elution phases consisted of (A) MilliQ H<sub>2</sub>O containing 0.1% trifluoroacetic acid (TFA) (v/v) and (B) acetonitrile and 0.1% TFA (v/v). Aliquots (10 µL) of the reaction were injected by an automatic injector. The gradient programme started with 100% of solution A for 2 min and changed linearly to reach 100% of solution B at a flow rate of 1.0 mL min<sup>-1</sup> over 20 min. The column was cleaned with 100% of solvent B for 5 min and equilibrated with the initial conditions for 5 min. The eluate was monitored by UV absorbance at 215 nm.

### PCR-DGGE (denaturing gradient gel electrophoresis)

Genomic DNA was obtained using SpeedTools Tissue DNA Extraction kit (Biotools), and PCR fragments of 200 bp were amplified using the universal primers HDA1-GC and HDA2 (Nistal *et al.*, 2012). A DGGE analysis of the PCR amplicons was performed using the DCode Universal Mutation Detection System (Bio-Rad). The linear denaturing gradient of urea and formamide used for the separation of the amplicons was 35–55%. Selected DGGE bands were reamplified with the corresponding primers without the GC-clamp, and the resulting PCR products were cloned with the StrataClone PCR cloning kit (Stratagene) and sequenced. The sequences

were compared with those in the GenBank database using the BLASTN algorithm. A phylogenetic analysis was performed to complete the molecular identification. Sequences with ≥ 98% identity to the sequences of known bacteria were considered to be the same species.

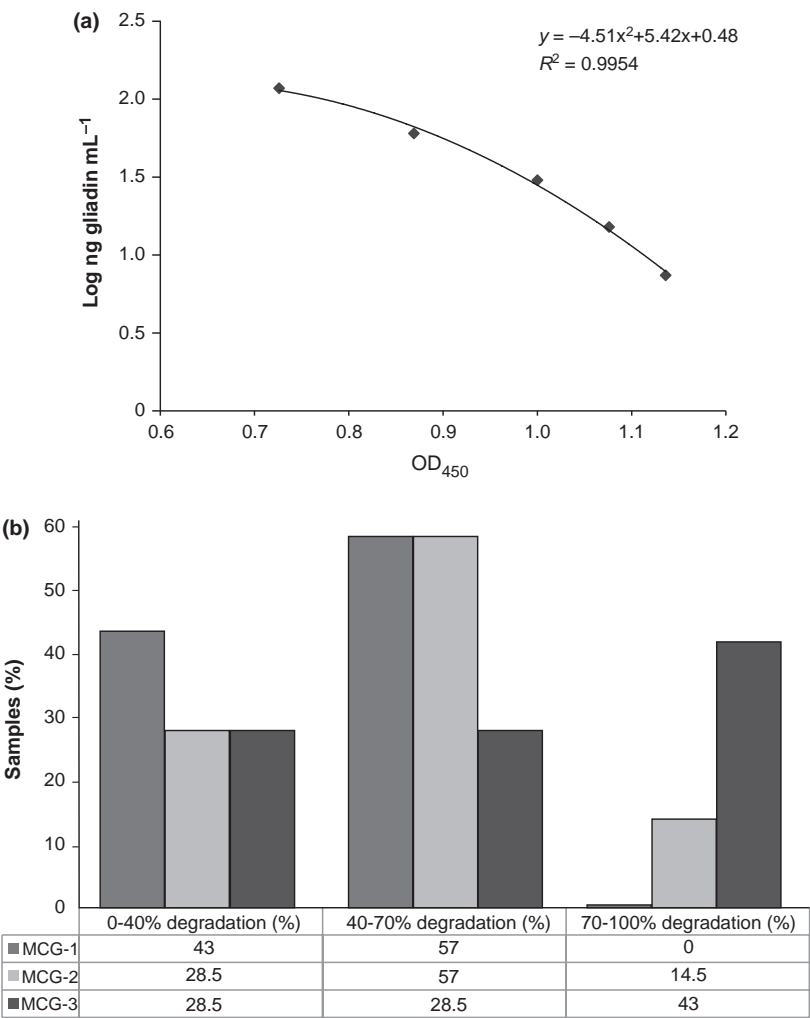
## Results

### MCG1, MCG2 and MCG3 media promote gluten hydrolysis

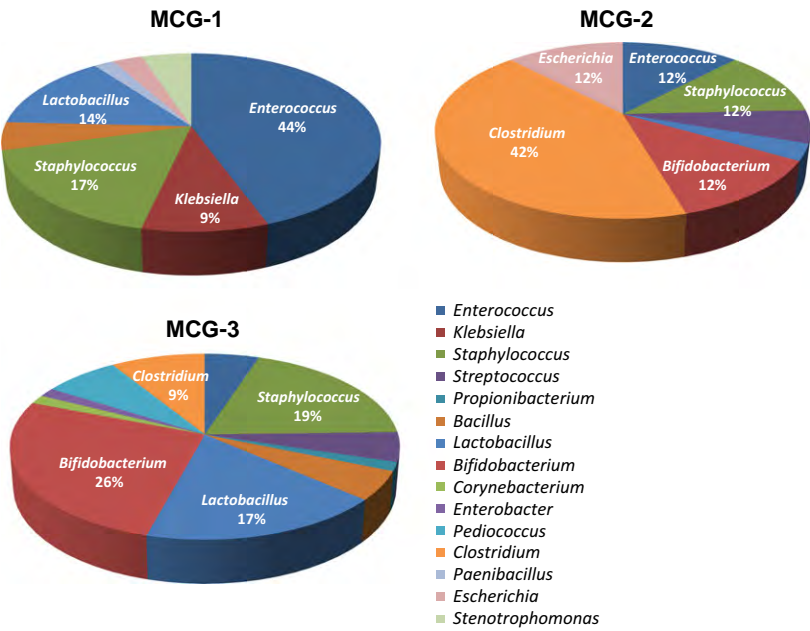
We developed three culture media to grow and isolate microorganisms from the human gut able to participate in the metabolism of gluten proteins. To test whether these media promoted gluten hydrolysis, faecal samples from seven human volunteers were inoculated in MCG1, MCG2 and MCG3. After incubation for 48 h at 37 °C under anoxic conditions, the gluten remaining in the culture medium was quantified using the Competitive Elisa GlutenTox Kit. The results showed that the three media promote gluten consumption (Fig. 1), with the highest gluten degradation achieved in MCG2 and MCG3. Almost half of the samples inoculated in MCG3 were able to consume between 70% and 100% of the gluten proteins present in the culture medium.

### MCG3 promotes the highest microbial diversity

Faecal samples were cultured in two ways to isolate microorganisms involved in gluten metabolism in the gut: (1) faecal samples were diluted in saline and plated directly on MCG1, MCG2 and MCG3; and (2) faecal samples were inoculated in liquid MCG media, and after incubation, an aliquot of serial dilutions from each liquid culture was plated on the corresponding solid medium. Bacteria were isolated based on colony morphology; we isolated those colonies that looked different in size, colour, etc. All the colonies were tested for (1) glutenase activity and (2) the ability to metabolise gluten. Only the bacteria positive for any of these tests were selected and identified by partial sequencing of the 16S rDNA gene, and the results revealed difference in the bacterial diversity grown on the three tested media (Fig. 2). Forty-four bacterial strains were selected and identified from MCG-1; 33 bacterial strains were selected and identified from MCG-2, and 67 bacterial strains were selected and identified from MCG-3. MCG1 is a nutrient-poor medium with gluten as the sole nitrogen source; the diversity on this medium was the lowest, and most of the colonies isolated (44%) belonged to the genus *Enterococcus*. MCG2 is a nutrient-rich medium and showed higher bacteria diversity than MCG1; however, many of the bacteria we isolated using this medium were not related to gluten



**Fig. 1.** (a) Standard curve prepared following the manufacturer's instructions for gluten quantification. (b) Gluten consumption in three media (MCG-1, MCG-2 and MCG-3) inoculated with seven different faecal samples.



**Fig. 2.** Bacterial diversity isolated using the three media tested (MCG-1, MCG-2 and MCG-3) inoculated with 22 different faecal samples.

metabolism. The best medium was MCG3 because it showed the highest bacterial diversity, with almost all of the colonies being related to gluten metabolism; most of the bacteria isolated on MCG1 and MCG2 were isolated using MCG3 (Table 1). MCG-3 is also a rich nutrient medium containing gluten as the main nitrogen source but it also has other protein sources in lower concentrations such as meat extract.

### **Bacteria involved in gluten metabolism are mainly *Firmicutes* and *Actinobacteria***

We isolated 144 strains belonging to 35 bacterial species that could be involved in gluten metabolism in the human gut (Table 1). Most of the strains were classified within the phylum *Firmicutes* (c. 73%), mainly from the genera *Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Clostridium*. Some 15% of the isolates were classified within the phylum *Actinobacteria*, mostly from the genus *Bifidobacterium*. Only 12% of the isolates were Gram-negative bacteria from the phylum *Proteobacteria*.

Of the 144 selected strains, 97 were able to metabolise gluten; moreover, 61 of these 144 strains showed an extracellular proteolytic activity against gluten proteins and gelatine. This glutenase activity was present in strains from *Bacillus licheniformis*, *B. subtilis*, *B. pumilus*, *Bifidobacterium longum*, *Clostridium sordellii*, *C. perfringens*, *C. botulinum/ sporogenes*, *C. butyricum/beijerinckii*, *Enterococcus faecalis*, *E. faecium*, *Propionibacterium acnes*, *Pediococcus acidilactici*, *Paenibacillus jamilae*, *Staphylococcus epidermidis*, *S. hominis* and *Stenotrophomonas maltophilia*.

### **Several bacterial species show peptidasic activity against the 33-mer peptide**

Gluten proteins are recalcitrant to human digestive enzymes; as a consequence, high molecular weight oligopeptides are present in the intestinal lumen and serve as substrates for bacterial peptidases. The 144 strains isolated were tested for their ability to hydrolyse the 33-mer peptide, and we found that several bacterial strains belonging to 11 bacterial species exhibited activity (Table 1). The peptidasic activity towards the 33-mer peptide could be due to an extracellular peptidase or due to an intracellular peptidase released by a low level of cell lysis during growth or during the preparation of the supernatants. Although bacterial strains belonging to *Enterococcus faecalis* or *Bacillus licheniformis* exhibited activity, the peptide was not completely hydrolysed. In contrast, other bacteria, such as *Lactobacillus mucosae* (strains B1c and D5a1), *L. rhamnosus* (strains LA2a, LE3 and D1a) and *Clostridium botulinum/ sporogenes* (all isolated strains) appeared to have a higher activity with regard to the

33-mer peptide than other bacteria tested as *Enterococcus faecalis* or *Bacillus licheniformis* (Fig. 3).

### **PCR-DGGE reveals that part of the bacterial community grown on MCG3 was not isolated on solid medium**

Bacterial strains involved in gluten metabolism were isolated by plating in solid media either directly from faeces or from liquid enrichment cultures. However, the milieu present in the liquid cultures could allow the growth of bacteria involved in gluten metabolism but that are unable to grow in the solid media. To study the microbiota grown in liquid MCG3 a molecular study was performed by PCR-DGGE. The DGGE profiles of the PCR amplicons and the bands identified are indicated in Fig. 4. We identified the presence of 17 bacterial species. Among them, *Bifidobacterium longum* and *Bacteroides dorei* were present in more than 75% of the cultures analysed. The presence of *Faecalibacterium prausnitzii* and *Lactobacillus helveticus/gallinarum/acidophilus*, which were detected in 41% of the samples, was also important. A total of 10 of the 17 bacteria identified by PCR-DGGE were not isolated by plating on solid MCG3. These bacteria could be involved in the metabolism of gluten proteins but their role in it is difficult to know because they were not isolated.

## **Discussion**

Gluten is an important source of dietary protein for most of the humans; however, the gluten protein is the environmental trigger of CD (Fasano, 2009). *In vitro* studies have shown that gluten proteins are unusually resistant to digestion by human digestive proteases (Shan *et al.*, 2002). As a result, a mixture of undigested proteins and peptides is available for bacterial metabolism in the gastrointestinal tract. However, the role of intestinal microbiota in this metabolic process has been scarcely studied (Davila *et al.*, 2013). Importantly, recent studies have shown the presence of bacteria-derived proteolytic activities with the ability to hydrolyse gluten peptides in saliva, the duodenum and faeces (Bernardo *et al.*, 2009; Helmerhorst *et al.*, 2010; Caminero *et al.*, 2012).

Commensal bacteria that comprise the intestinal microbiota primarily belong to five microbial phyla, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria*, which are distributed throughout the gut in different numbers, likely as a result of varying microbial ecosystems (Turroni *et al.*, 2014). *Firmicutes* is the dominant phylum of the human adult microbiota. We isolated from faeces 144 bacterial strains that could be involved in the metabolism of gluten proteins in the human gut: 73% of

**Table 1.** Isolated bacteria from human faeces

Phylum	Species	Isolation medium	Strain	Growth in MCG/MSG	Gluten hydrolysis	Gelatine hydrolysis	33-mer hydrolysis (strain)
Firmicutes	<i>Enterococcus faecalis</i>	MCG1,2,3	A-1, A-2, A-3, A-4, A-5, A-6, A-47, A-45, A-62, A-63, A-138, HF1, HF2	+/+	++	++	A-1, A-2, A-3, A-5, A-6, A-47, A-45, A-62, A-63, A-138
	<i>Enterococcus faecium</i>	MCG1,2,3	A-7, A-8, A-9, A-32, A-33	+/–	–	–	–
			A-97	+/+	++	++	–
	<i>Lactobacillus mucosae</i>	MCG1,2,3	A-67, A-68, A-71, A1a, LB1a	+/–	–	+	–
			LE1a, B1c, D5a1, B2b, B3a, D2a, D2d, LD1a	+/–	–	–	B1c, D5a1
	<i>Lactobacillus amylovorus</i>	MCG1,3	A2c, D3b, LA1b, D1d, D4a, D5a2, E1a	+/–	–	–	E1a
	<i>Lactobacillus gasseri</i>	MCG3	A-108	+/–	–	+/–	–
	<i>Lactobacillus ruminis</i>	MCG3	A-39, A-44	+/–	–	–	–
	<i>Lactobacillus fermentum</i>	MCG3	A-17	+/–	–	–	–
	<i>Lactobacillus rhamnosus</i>	MCG1,3	LA2a, LE3, D1a, LA2d, LD2a, LE2a	+/–	–	–	LA2a, LE3, D1a
	<i>Pedococcus acidilactici</i>	MCG3	A-111, A-113, A-116, A-118	+/–	++	++	–
	<i>Streptococcus sanguinis</i>	MCG2,3	A-105, A-101	+/–	–	–	–
	<i>Streptococcus gallolyticus</i>	MCG2	A-65	+/–	–	–	–
	<i>Streptococcus intermedius</i>	MCG3	A-38	+/–	–	–	–
	<i>constellatus/anginosus</i>						
	<i>Bacillus licheniformis</i>	MCG3	A-104, A-109, A-110	+/+	++	++	A-104, A-109, A-110
	<i>Bacillus subtilis</i>	MCG1	HA4, HA5	+/+	++	++	HA4, HA5
	<i>Bacillus pumilus</i>	MCG1	HC2, HC6, HC9	+/+	++	++	HC2, HC6, HC9
	<i>Paenibacillus jamilae</i>	MCG1	B4a, B4a2	+/+	++	++	B4a, B4a2
	<i>Staphylococcus epidermidis</i>	MCG1,2,3	A-15, A-16, A-25, A-28, A-64, A-139, A-140	+/–	–	–	–
			A-27, A-106	+/+	+	+	–
	<i>Staphylococcus hominis</i>	MCG1,2,3	A-36, A-42	+/–	–	–	–
			A-78	+/+	+	+	–
	<i>Staphylococcus haemolyticus</i>	MCG2,3	A-18, A-100, E3c	+/–	–	–	E3c
	<i>Staphylococcus saprophyticus</i>	MCG3	A-43	+/–	–	–	–
	<i>Staphylococcus pasteurii</i>	MCG3	A-46	+/–	–	–	–
	<i>warneri</i>						
	<i>Staphylococcus aureus</i>	MCG3	A-47, A-58	+/–	–	–	–
	<i>Clostridium botulinum</i>	MCG2,3	A-30, A-49, A-102, A-103, A-117, A-119, A-141	+/+	++	++	A-30, A-49, A-102, A-103, A-117, A-119, A-141
	<i>sporogenes</i>						
	<i>Clostridium perfringens</i>	MCG2,3	A-40, A-41, A-74, A-75, A-76, A-77	+/+	++	++	–
	<i>Clostridium sordellii</i>	MCG2	A-19, A-20, A-21, A-22	+/+	++	++	–
	<i>Clostridium butyricum</i>	MCG2	A-114, A-115	+/+	++	++	–
	<i>beijerinckii</i>						

(continued)



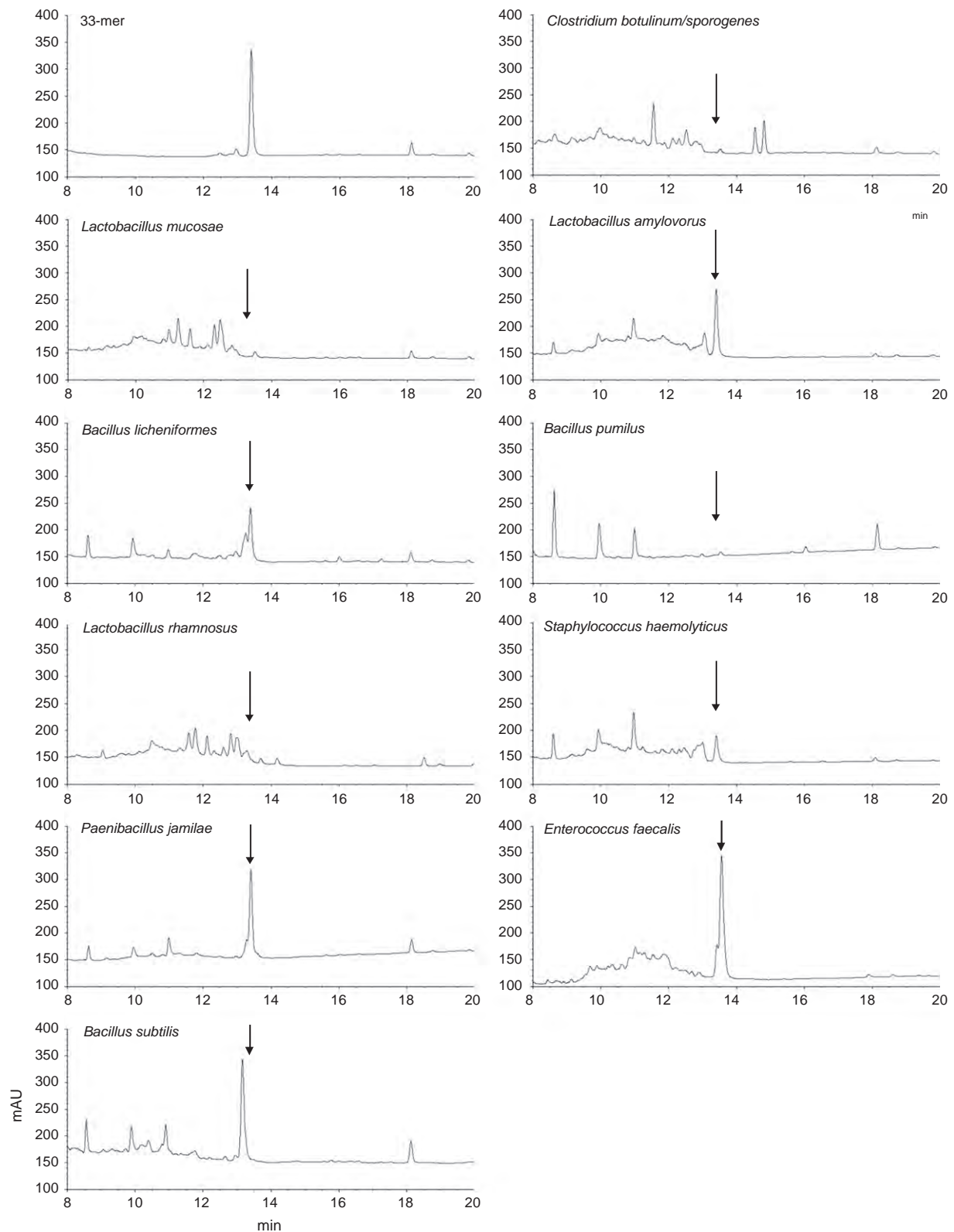
Table 1. Continued

Phylum	Species	Isolation medium	Strain	Growth in MCG/MSG	Gluten hydrolysis	Gelatine hydrolysis	33-mer hydrolysis (strain)
Actinobacteria	<i>Corynebacterium accolens</i> / <i>pseudogenitalium</i>	MCG3	A-31	+/-	-	-	-
	<i>Propionibacterium acnes</i>	MCG3	A-10, A-137	+/-	++	++	-
	<i>Bifidobacterium longum</i>	MCG2,3	A-11, A-12, A-26, A-35, A-48, A-66, A-73, A-95, A-122, A-125, A-98, A-99	+/-	-	-	-
	<i>Bifidobacterium adolescentis</i>	MCG1,2,3	A-10, A-34, A-72	+/-	+	+	A-98, A-99
	<i>Bifidobacterium catenulatum</i> / <i>pseudocatenulatum</i>	MCG3	A-123, A-124	+/-	-	-	-
	<i>Bifidobacterium animalis</i>	MCG3	A-120, A-121	+/-	-	-	-
	<i>Enterobacter aerogenes</i>	MCG3	A-81	+/-	-	-	-
	<i>Klebsiella pneumoniae</i>	MCG1	A-80, A-96, HD1, HD2	+/-	-	-	-
	<i>Escherichia coli</i> / <i>Shigella flexneri</i>	MCG1,2	A-13, A-14, A-23, A-24, A-60, A-61	+/-	-	-	-
	<i>Stenotrophomonas maltophilia</i>	MCG1	HE1, HE2, HB1, HB3, HB9, HB18	+/-	+	+	-
Proteobacteria							

these strains belong to *Firmicutes*, 15% to *Actinobacteria* and 12% to *Proteobacteria*.

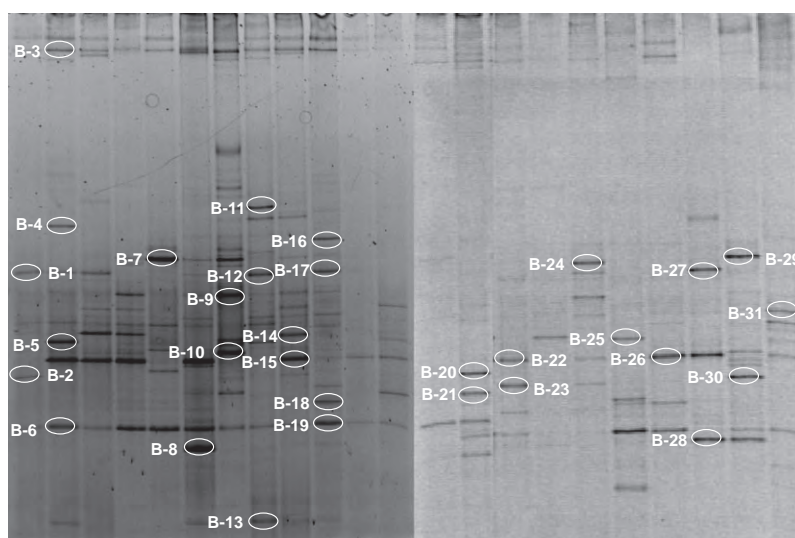
Lactic acid bacteria (LAB) account for 39% of the strains isolated from human faeces. Four groups of LAB were primarily isolated: lactobacilli, enterococci, streptococci and pediococci. There are many facts that support the involvement of LAB in the metabolism of gluten proteins. LAB have evolved complex proteolytic and peptidolytic systems because these bacteria utilise amino acids as both a nitrogen source and also for energy metabolism. These systems include extracellular or membrane-bound proteases, membrane transport proteins catalysing the intracellular uptake of oligo-, di- and tripeptides and a multitude of intracellular peptidases (Kunji *et al.*, 1996; Pessione, 2012). Proteases from LAB have been described as playing an important role in the digestion of not-fully hydrolysed proteins in the human gut and may shorten long- and medium-sized peptides, particularly those peptides derived from dairy proteins (Pessione, 2012). It is very interesting that most of the LAB isolated in our study were able to metabolise gluten; these data are very important because other nitrogen sources were available in the MCG3 medium. Therefore, these bacteria are suspected to have an important role in gluten metabolism in the gut. *Lactobacillus* was the predominant bacteria isolated (20% of the isolated bacteria), and one important feature that supports the involvement of this genus is that the gluten-free diet consumed by healthy volunteers and patients with coeliac significantly affects its populations (De Palma *et al.*, 2009; Nistal *et al.*, 2012). Moreover, proteolysis against gliadin proteins and peptides by certain *Lactobacillus* strains has been described mainly for strains isolated from sourdough (Di Cagno *et al.*, 2002; Gerez *et al.*, 2012). *Lactobacillus* is considered GRAS (generally regarded as safe), and some strains are considered to be health-promoting microorganisms (Snydman, 2008). In this study, we isolated several *Lactobacillus* strains that are good candidates as probiotics for the treatment of CD; these strains were isolated from human volunteers and have the ability to completely hydrolyse the 33-mer peptide. However, the degradation products generated after the 33-mer hydrolysis are unknown and could also be highly immunogenic. We are actually studying these degradation products to determine whether the immunogenic epitopes are destroyed.

Currently, the majority of probiotic bacteria that are commercially exploited belong to two genera, *Bifidobacterium* and *Lactobacillus*, and both appear to be involved in gluten metabolism in the human gut. We isolated 19 strains from four different *Bifidobacterium* species, all of which were able to metabolise gluten, suggesting the involvement of these bacteria in gluten metabolism. In



**Fig. 3.** Hydrolysis of the 33-mer peptide, as analysed by HPLC. One strain from each species is shown as an example.





Closest relative	Amplicon ID	V % (n = 22)
<i>Bacteroides dorei</i>	B3	77.3 (17/22)
<b><i>Bifidobacterium adolescentis/ruminantium</i></b>	B12,B23	18.1 (4/22)
<i>Bifidobacterium bifidum</i>	B13	22.7 (5/22)
<b><i>Bifidobacterium longum</i></b>	B2,B6,B15,B19,B20,B22,B26,B28	86.4 (19/22)
<b><i>Clostridium perfringens</i></b>	B21	18.1 (4/22)
<i>Clostridium symbiosum</i>	B31	36.4 (8/22)
<b><i>Enterococcus faecalis</i></b>	B7,B9	22.7 (5/22)
<i>Eubacterium bifforme</i>	B16	22.7 (5/22)
<i>Eubacterium hadrum/Anaerostipes sp</i>	B14	22.7 (5/22)
<i>Faecalibacterium prausnitzii</i>	B1,B24	40.9 (9/22)
<i>Lactobacillus coleohominis/panis/pontis</i>	B10	13.6 (3/22)
<b><i>Lactobacillus fermentum</i></b>	B4	13.6 (3/22)
<i>Lactobacillus helveticus/gallinarum/acidophilus</i>	B17,B29	40.9 (9/22)
<b><i>Lactobacillus mucosae</i></b>	B30	9.1 (2/22)
<b><i>Lactobacillus ruminis</i></b>	B5,B25	22.7 (5/22)
<i>Mitsuokella jalaludinii</i>	B18	18.1 (4/22)
<i>Streptococcus agalactiae</i>	B17	9.1 (2/22)

**Fig. 4.** The frequency of bacterial species identified by sequencing the DGGE bands amplified using universal primers from DNA obtained from MCG-3 cultures inoculated with the faecal samples of the 22 volunteers (V). Bacteria isolated by plating in solid MCG are marked in bold.

addition, the gluten-free diet appears to reduce the diversity and amount of *Bifidobacterium* species, including *B. longum* (De Palma *et al.*, 2009; Nistal *et al.*, 2012). It is also important to note that PCR-DGGE revealed that *B. longum* was present in 86% of the samples analysed (19 of 22). Some *Bifidobacterium* species, such as *B. longum*, *B. animalis* and *B. bifidum*, of human origin have been previously described as digesting gliadin peptides (Laparra & Sanz, 2010). Indeed, *B. longum* IATA-ES1 was able to hydrolyse the immunogenic peptide 33-mer and modulate an immune response (Laparra *et al.*, 2012). This bacterium is commercially available in food as a probiotic bacterium for patients with CD.

However, not all the bacteria involved in gluten metabolism are health promoting. Bacterial proteases of certain

groups isolated in this work, including *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli*, *Clostridium perfringens* and *C. sordellii*, may be related to inflammatory bowel disease (Pruteanu *et al.*, 2011; Steck *et al.*, 2012). Although an intestinal dysbiosis has been reported in CD, there are few studies focussing on the relationship between CD and bacterial proteases. Bernardo *et al.* (2009) have described a specific gliadinase pattern in duodenal samples from patients with CD, and these authors have suggested that specific bacteria are responsible for these proteolytic activities.

It is very interesting that the bacterial groups related to gluten metabolism are some of those that are altered in patients with CD, including *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Staphylococcus*, *Clostridium* and *Escherichia*

*coli* (Collado et al., 2009; De Palma et al., 2010; Sanz et al., 2011). We are currently studying whether these bacteria have a protecting and/or a pathogenic role in CD.

## Acknowledgements

This research was supported by grants from: the Instituto de Salud Carlos III, Fondo de Investigación Sanitaria cofunded by FEDER (FIS PI10/02447); Junta de Castilla y León, Consejería de Sanidad (GRS 520/A/10) and Junta de Castilla y León, Consejería de Educación (LE156A11-2), Alexandra R. Herrán received a grant from the Junta de Castilla y León, cofunded by Fondo Social Europeo. Jenifer Pérez-Andrés received a grant from the Ministerio de Educación, Cultura y Deporte del Gobierno de España. The authors have no conflicts of interest to declare.

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