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**A proteomic approach towards understanding the cross-talk
between *Bacteroides fragilis* and *Bifidobacterium longum* in
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Manuscripts

1 **A proteomic approach towards understanding the cross-talk between**
2 ***Bacteroides fragilis* and *Bifidobacterium longum* in co-culture**

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Abstract

A better understanding of the interactions among intestinal microbes is needed in order to decipher the complex cross-talk that takes place within the human gut. *Bacteroides* and *Bifidobacterium* genera are among the most relevant intestinal bacteria and it has been previously reported that co-culture of these two microorganisms affect their survival. Therefore, co-cultures of *Bifidobacterium longum* NB667 and *Bacteriodes fragilis* DSMZ2151 were performed with the aim of unravelling the mechanisms involved in their interaction. To this end we applied proteomic (2D-DIGE) analyses and quantified by chromatographic techniques the bacterial metabolites produced during co-incubation. Co-culture stimulated the growth of *B. longum* retarding that of *B. fragilis*, with concomitant changes in the production of some proteins and metabolites of both bacteria. The combined culture promoted up-regulation of the bifidobacterial pyruvate kinase and down-regulation of the *Bacterioides* phosphoenolpyruvate carboxykinase, two enzymes involved in the catabolism of carbohydrates. Moreover, *B. fragilis* FKBP-type peptidyl-prolyl cis/trans isomerase, a protein with chaperone-like activity, was found to be over-produced in co-culture, suggesting the induction of a stress response in this microorganism. This study provides mechanistic data to deepen our understanding on the interaction between *Bacterioides* and *Bifidobacterium* intestinal populations.

Keywords: *Bifidobacterium*, *Bacterioides*, cross-talk, interaction, co-culture

48 The intestinal tract constitutes one of the habitats displaying higher bacterial
49 diversity in the human body (Human Microbiome Project Consortium 2012) This
50 complex microbial ecosystem plays an essential role in the maintenance of human
51 health and the disruption of its homeostasis has been linked to several health disorders
52 (Clemente et al. 2012). In spite of its large bacterial diversity, recent studies have
53 indicated that at high phylogenetic level, this ecosystem is dominated by a limited
54 number of bacterial phyla. Among them, Bacteroidetes and Firmicutes are clearly the
55 most abundant in adults, with other phyla such as Actinobacteria, Proteobacteria or
56 Verrucomicrobia, being also present at lower levels (Eckburg et al. 2005).

57 Among Bacteroidetes, the genus *Bacteroides* represents the most abundant
58 population, constituting about 20-50% of the colon microbiota (Eckburg et al. 2005).
59 These microorganisms have a predominantly saccharolytic metabolism and are able to
60 ferment host and dietary-derived glucans to succinic, acetic, lactic and propionic acids
61 as the main metabolic end-products (Rios-Covian et al. 2013). The ability of these
62 microorganisms to break-down complex carbohydrates, such as indigestible plant
63 polysaccharides, makes *Bacteroides* a key player within the intestinal ecosystem
64 (Sonnenburg et al. 2005). Different species of this genus can be present in the intestine,
65 *Bacteriodes thetaiotaomicron* being the most frequently found in humans (Eckburg et
66 al. 2005). The genus *Bacteroides* also harbors species that, although often present in the
67 intestinal ecosystem, are important opportunistic pathogens, such as *Bacteroides fragilis*
68 (Chaudhry and Sharma 2011).

69 The genus *Bifidobacterium* is the main representative of the Actinobacteria
70 phylum within the human colon, accounting for up to 3% of the adult microbiota
71 (Matsuki et al. 2002). Bifidobacteria metabolize sugars by the fructose-6-phosphate
72 phosphoketolase pathway, which leads to the production of acetic and lactic acids as

73 main metabolic end-products (Margolles et al. 2011). This genus is among the dominant
74 intestinal bacteria found in the intestine of breast-fed infants, their levels drop later on
75 with weaning and remains stable during the adulthood (Tojo et al. 2014). Diverse
76 species of the genus populate the human gut and *Bifidobacterium longum* is one of the
77 most abundant. Alterations on the levels and/or species composition of the intestinal
78 bifidobacterial populations have been related with different disease states (Tojo et al.
79 2014)

80 Whereas *Bacteroides* and *Bifidobacterium* are among the most relevant intestinal
81 microorganisms, the potential interactions between these two bacteria, and of them with
82 complex carbohydrates that may be present in the gut, have attracted the attention of
83 researchers (Adamberg et al. 2014; Falony et al. 2009; Rios-Covian et al. 2013; Rios-
84 Covian et al. 2015). In a previous work we observed that the co-culture of *B. fragilis*
85 DSMZ2151 with *B. longum* NB667 enhanced the survival of the later microorganism
86 (Rios-Covian et al. 2013). In the present study we performed co-culture experiments
87 and proteomic analyses in order to advance a step further into the understanding of such
88 interactions and the potential mechanisms involved.

89 With this aim, *B. longum* NB667 (NIZO food research culture collection, EDE,
90 The Netherlands) and *B. fragilis* DSMZ2151 (DSMZ bacterial collection,
91 Braunschweig, Germany) were incubated in mono-culture and co-culture. Bacterial
92 strains were recovered in MRSc and GAMs broths, respectively, and mono-culture and
93 co-culture experiments were carried out in mCFBM medium supplemented with 0.3 %
94 (w/v) glucose as described previously (Rios-Covian et al. 2013). Mono-cultures were
95 inoculated at 1% (v/v) with overnight cultures of *B. fragilis* or *B. longum* in mCFBM
96 whereas in co-cultures each of the two strains was inoculated at this percentage. Two
97 samples were collected at T1 (14 ± 1 hours of incubation, mid-late exponential growth

98 phase, corresponding with OD_{600} 0.75 ± 0.05 for mono-cultures and 1.05 ± 0.05 for the
99 co-culture), and T2 (18.5 ± 1 h of incubation, early-stationary growth phase,
100 corresponding with OD_{600} 1.25 ± 0.1 for mono-cultures and co-culture). Bacterial
101 growth (cell counts mL^{-1}) was estimated by qPCR using previously described primers
102 and conditions (Arbolea et al. 2012).

103 Cell-free supernatants of mono-cultures and co-cultures were acidified with HCl
104 to pH 2.5 ± 0.5 and the SCFA produced during incubation were quantified in the
105 supernatants by Gas Chromatography using a system composed of a 6890 gas
106 chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA) connected with a FID
107 detector (Agilent) as described previously (Salazar et al. 2008). Organic acids and
108 glucose were analyzed by HPLC consisting on an Alliance 2695 separation module and
109 a photo diode array (PDA) detector (Waters 996) at 210 nm for organic acids, a
110 refractive index detector for glucose (Waters 2414), and Empower software (Waters,
111 Mildford, MA, USA). Chromatographic conditions were those published elsewhere
112 (Salazar et al. 2009). Concentrations are expressed in millimolar (mM).

113 Differences in the proteome of each strain in mono-culture with respect to co-
114 culture at both T1 and T2, were assessed separately by two-dimensional difference gel
115 electrophoresis (2D-DIGE) as described previously (Rios-Covian et al. 2015). Mono-
116 cultures were labelled with Cy3 dye (green color) whereas the co-culture was labelled
117 with Cy5 dye (red color) (Supplementary Fig. 1A). Another gel was performed with a
118 synthetic mixture of the proteins from both mono-cultures labeled with Cy3 and the co-
119 culture labeled with Cy5 (Supplementary Fig. 1C). This gel was needed in order to
120 detect any new protein spot that may have appeared as a consequence of the co-
121 cultivation but that was not synthesized by mono-cultures (Supplementary Fig. 1D). No
122 new spots were detected in such conditions. Therefore, we assumed that when

123 comparing mono-culture vs. co-culture (Supplementary Fig. 1A), any spot appearing in
124 the co-culture that was not also present in the corresponding mono-culture (either
125 *Bacteroides* or *Bifidobacterium*) belonged to the other microbial partner of the co-
126 culture and, hence, the spot was not considered in the analyses (Supplementary Fig.
127 1B). Gels were analyzed with the 2D Imagemaster (GE Healthcare) software. During
128 the analysis, overlapping spots were excluded. As proteins marked in the co-culture
129 belong to two different microorganisms, an average abundance ratio between proteins in
130 the co-culture relative to the mono-culture was calculated for each strain, representing
131 the average of the abundance ratio obtained for all the spots available in the co-culture
132 for this microorganism. All ratios were normalized by this value and *t* tests were run
133 between mono-culture and co-culture. Spots displaying a significant change on their
134 production (fold-change higher than 2 or lower than -2) were excised from gels and
135 identified as previously described (Rios-Covian et al. 2015).

136 The relative expression of genes coding for proteins with significant changes in
137 their production on the basis of the 2D-DIGE experiments were further analyzed. RNA
138 was extracted from 10 mL of cultures taken at the end of the incubation (T2) using the
139 RNeasy mini kit (Qiagen) as reported by Ulve et al. (2008). Gene expression was
140 determined by reverse transcription qPCR (RT-qPCR) using the $\Delta\Delta$ Ct method as
141 described previously (Gueimonde et al. 2007). Primers used in the present study were: F
142 5'-ATCACTGAACCGACTCCGACA-3' and R 5'-
143 GCAATGATAAGAATGCAGCACC-3' for *B. fragilis* phosphoenolpyruvate
144 carboxykinase; F 5'-AAAATCTCCTGGTTCAGTTCGC-3' and R 5'-
145 AGGCAACTTCAGCTTCCAGG-3' for *B. fragilis* FKBP-type peptidyl-prolyl cis-
146 transisomerase; and F 5'-GGATCTCTTCGAGGTTCTCC-3' and R 5'-
147 CACGAGATCATGGACGAGGAA-3' for *B. longum* pyruvate kinase. The 16S rRNA

gene was employed as endogenous control by using previously described primers (Arbolea et al. 2012) F 5'-GATTCTGGCTCAGGATGAACGC-3' and R 5'-CTGATAGGACGCGACCCCAT-3' for *Bifidobacterium* and F 5'-GAGAGGAAGGTCCCCCAC-3' and R 5'-CGCKACTTGGCTGGTTCAG-3' for *Bacteroides*.

One way ANOVA statistical tests were run for the analyses of values obtained for the different parameters considered between mono-cultures and co-cultures using the IBM SPSS software, version 22.00 (IBM, Armonk, New York, USA).

The results obtained corroborated previous data (Rios-Covian et al. 2013) indicating that co-incubation of *B. longum* NB667 with *B. fragilis* DSMZ2151 stimulated the growth of the former microorganism, resulting in significantly higher levels ($p < 0.05$) of *B. longum* in co-culture than in the corresponding mono-culture (9.07 vs. 8.11 log cells mL⁻¹) at the end of the incubation (Table 1). In contrast, the growth of *B. fragilis* was delayed by the presence of the bifidobacteria, as denoted by the significantly lower ($p < 0.05$) bacterial levels obtained in the co-culture at the first sampling point (T1; representing mild-late exponential phase) (8.66 vs. 8.93 log cells mL). This difference, however, disappeared later (T2; early-stationary phase), with *B. fragilis* population reaching similar levels in co-culture than in the corresponding mono-culture (8.93 vs. 8.89 log cells mL⁻¹) (Table 1).

As expected from the bacterial growth patterns, the consumption of glucose was higher in the co-culture than in mono-cultures, these differences achieving statistical significance ($p < 0.05$) at the later sampling point analyzed (Table 1). The enhanced consumption of glucose in co-culture led to higher concentrations of acetate and lactate ($p < 0.05$), which suggests a predominant production by the bifidobacteria. These data are in good agreement with the increased bifidobacterial growth observed for *B. longum*

173 when it was grown with *B. fragilis*. Moreover, in accordance with the delayed growth of
174 *B. fragilis* in co-culture, the levels of propionate, succinate and formate (mainly
175 produced by *Bacteroides* with no or very limited production by bifidobacteria) were
176 lower in such conditions than in the corresponding mono-culture (Table 1). These
177 results are in agreement with previous reports (Rios-Covian et al. 2013).

178 To further elucidate the mechanisms involved in this microbe-microbe
179 interaction, the protein pools of *B. longum* NB667, *B. fragilis* DSMZ2151 and its co-
180 culture were analyzed and compared by means of 2D-DIGE. At this point, it is
181 important to underline that the proteomic 2D-DIGE analysis of bacterial co-cultures
182 possess a serious methodological limitation because several spots cannot be analyzed as
183 a consequence of the same gel-migration properties of some proteins of both
184 microorganism, therefore overlapping in the gel (supplementary Fig. 1). In spite of this
185 limitation the proteomic analyses allowed us to identify some spots differentially
186 produced in co-culture with regard to the corresponding mono-cultures. By using as a
187 threshold the occurrence of statistically significant ($p < 0.05$) changes in production for
188 values greater or lower than two-fold, three spots were found to be affected (Table 2).
189 One of these spots corresponded to the enzyme pyruvate kinase of *B. longum* which was
190 found to be over-produced at both sampling times (T1 and T2). Moreover, RT-qPCR
191 analyses of the expression of the corresponding gene (*pyk*) at the end of the incubation
192 period (T2) confirmed this observation with more than six-fold induction of the gene
193 expression (Table 2). This enzyme, that catalyzes the conversion of
194 phosphoenolpyruvate to pyruvate, constitutes one of the key steps of sugar metabolism
195 in bifidobacteria (de los Reyes-Gavilán et al. 2005) (Fig. 1). This suggests a stimulation
196 of the catabolism of carbohydrates by *B. longum* when co-cultured with *B. fragilis*, thus
197 providing an explanation for its improved growth and the shift on its carbohydrate

metabolism in co-culture. Interestingly, the pyruvate kinase has been previously found to be over-produced by *B. longum* (Sanchez et al. 2005; Sanchez et al. 2007) and other microorganisms (Len et al. 2004) under stress conditions. As regarding proteins of *B. fragilis*, the proteomic results evidenced three spots with significant changes in their production in co-cultures with respect to mono-cultures, at least in one of the time points analyzed (Table 2). Two of these spots, likely isoforms, were identified by MALDI TOF/TOF tandem mass spectrometry as a FKBP-type peptidyl-prolyl cis/trans isomerase. This enzyme catalyzes the cis/trans isomerization of peptide bonds adjacent to proline residues (Janowski et al. 1997). In bacteria, this enzyme plays an important role in native protein folding and re-naturalization of denatured proteins and, therefore, it has chaperone-like activity (Jo et al. 2015). Different proteins involved in protein folding and turnover have been found to be up- or down-regulated in *B. fragilis* when grown in complex carbohydrates, such as bifidobacterial exopolysaccharides (Rios-Covian et al. 2015). Moreover, chaperones production in bacteria is often affected by stress factors (Ruiz et al. 2011). In this context, our results suggest that the presence of *B. longum* in the co-culture promotes a response in *B. fragilis* leading to a modification on the production of this chaperone. However, the direction of this modification remains unclear as according to the proteomic results there is an up-regulation of the protein production at both sampling times (T1 and T2) but, on the contrary, the expression analyses at the end of incubation (T2) indicate a repression of the gene expression (Table 2). To this regard, discrepancies between protein and gene expression analyses are not uncommon (Aakko et al. 2014; Wu et al. 2011). It must be pointed out that we employed two different techniques, each with its own performance and limitations. RT-qPCR quantifies the mRNA, whose life-span is relatively short, whilst proteomics quantify the protein accumulated in the cell at a given time, including the

effect of post-translational regulation. These differences may account for the apparently contradictory results obtained.

The second *B. fragilis* protein found to be affected by the co-incubation with *B. longum* was the phosphoenolpyruvate carboxykinase, a key enzyme in the catabolism of *Bacteroides*. This enzyme, catalyzing the conversion of phosphoenolpyruvate to oxalacetate (Figure 1), was found to be under-produced at T1 by proteomics and was found down-regulated at the end of incubation (T2) by the RT-qPCR experiments (Table 2). This down-regulation suggests that co-cultivation with *B. longum* promotes a shift on the central sugar catabolism of *B. fragilis*, which may be focused to the use of complex carbohydrates and proteins in order to outcompete *B. longum*. Supporting this hypothesis are our previous results showing down-regulation of the phosphoenolpyruvate carboxykinase and an activation of the catabolism of amino acids by *B. fragilis* when grown in media containing bacterial exopolysaccharides (complex carbohydrates that promote slow growth) as carbon source with respect to glucose (Rios-Covian et al. 2015). *Bacteroides* is well adapted to the use of complex polysaccharides and an expansion on its ability to degrade these substrates has been observed when gnotobiotic animals mono-colonized by *Bacteroides* were compared with those co-colonized by *Bacteroides* and *B. longum* (Sonnenburg et al. 2006). Changes observed by us on the *Bacteroides* metabolism in co-culture with *B. longum* could account for the delayed bacterial growth and reduced metabolites production observed in such conditions.

To sum up, the results of this study provide new insights into the physiological and molecular mechanisms that determine the interaction of *B. fragilis* and *B. longum*, two important intestinal microorganisms, when growing together. *B. fragilis* in the co-culture seems to stimulate the growth and metabolic activity of *B. longum* when glucose

is available as a carbon source. The contrary seems to be true for *B. fragilis*, whose growth was inhibited and the metabolites production reduced in the presence of *B. longum*. The proteomic data allowed identifying the central sugar catabolism pathways as key regulators of this interaction, underlining the role of metabolic interactions among microorganisms as drivers of the cross-talk between these two intestinal bacteria. Increasing our understanding on the microbe-microbe interactions among intestinal bacteria will allow the development of high efficacious and specific microbiota-modulation strategies for improving human health.

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Table 2. Identification of proteins affected by co-cultivation of *B. fragilis* DSMZ2151 and *B. longum* NB667 and expression of the corresponding genes

COG orthology	Spot no. ^a	Putative function ^b	GI number ^c	Mass ^d	pI ^d	MASCOT score	No. of peptides matched ^e	Coverage	Protein change fold ^f		Gene expression change fold ^g
									T1	T2	T2
Energy production and conversion	Bac25	phosphoenolpyruvate carboxykinase	gi 53715725	59333	5.73	333	15	25	-2.15	ND	-3.06±1.47
Posttranslational modification, protein turnover, chaperones	Bac42	FKBP-type peptidyl-prolyl cis-transisomerase	gi 547946109	51380	4.69	153	15	28	3.76	2.02	-2.84±0.80
	Bac44	FKBP-type peptidyl-prolyl cis-transisomerase	gi 547946109	51380	4.69	155	11	19	2.99	ND	
Carbohydrate transport and metabolism	Bif8	Pyruvate kinase	gi 312133003	52310	5.24	525	29	49	ND	2.95	6.55±1.25
	Bif9	Pyruvate kinase	gi 312133003	52310	5.24	587	34	54	ND	4.54	
	Bif10	Pyruvate kinase	gi 312133003	52310	5.24	527	33	49	2.22	2.17	

^aSpot numbers refer to the proteins labelled in 2D-DIGE gels. *B. fragilis*, Bac; *B. longum*, Bif.

^bPutative functions were assigned from the NCBI gene database

^cGI number in the NCBI database for *B. fragilis* DSMZ2141 and *B. longum* NB667

^dAs given by the NCBI database for *B. fragilis* DSMZ2151 and *B. longum* NB667. Molecular masses are expressed in kilodaltons

^eNumber of tryptic peptides observed that contributed to the percentage of amino acid coverage.

^fNormalized change fold for each protein production derived from cells grown in mono-culture with respect to the protein derived from cells grown in co-culture. ND, ratio below 2.

^gGene expression change fold for each gene derived from cDNA obtained from cells grown in mono-culture with respect to the cDNA obtained from cells grown in co-culture. 16S RNA gene was used as housekeeping gene.

377

378 **FIGURE LEGENDS**

379 **Figure 1.**Schematic representation of *B. fragilis* and *B. longum* metabolism. Final
380 metabolites are rounded. Dark arrows indicate one reaction step, dashed arrows indicate
381 more than one reaction. Phospoenolpyruvate, PEP; Oxalacetate, OAA.1, pyruvate
382 kinase; 2, phospoenolpyruvate carboxykinase.

383

Draft

Figure 1. Schematic representation of *B. fragilis* and *B. longum* metabolism. Final metabolites are rounded.

Dark arrows indicate one reaction step, dashed arrows indicate more than one reaction.

Phosphoenolpyruvate, PEP; Oxalacetate, OAA. 1, pyruvate kinase; 2, phosphoenolpyruvate carboxykinase.

317x166mm (200 x 200 DPI)