Characterization and *in vitro* properties of potentially probiotic *Bifidobacterium* strains isolated from breast milk

Running title: Potentially probiotic bifidobacteria from breast-milk

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Abstract

Most of the current commercial probiotic strains have not been selected for specific applications, but rather on the basis of their technological potential for use in diverse applications. Therefore, by selecting them from appropriate sources, depending on the target population, it is likely that better performing strains may be identified. Few strains have been specifically selected for human neonates, where the applications of probiotics may have a great positive impact. Breast-milk constitutes an interesting source of potentially probiotic bifidobacteria for inclusion in infant formulas and foods targeted to both pre-term and full-term infants. In this study six *Bifidobacterium* strains isolated from breast-milk were phenotypically and genotypically characterised according to international guidelines for probiotics. In addition, different *in vitro* tests were used to assess the safety and probiotic potential of the strains. Although clinical data would be needed before drawing any conclusion on the probiotic properties of the strains, our results indicate that some of them may have probiotic potential for their inclusion in products targeting infants.

Keywords: Bifidobacterium, breast-milk, infant mucus, probiotics.

Introduction

Probiotics are defined as *live microorganisms which when administered in adequate amounts confer a benefit on the host* (FAO/WHO, 2006). Different microorganisms are currently used as human probiotics; among them *Lactobacillus* and *Bifidobacterium* constitute the most frequently used genera. Most of the current commercial probiotic strains have not been selected for specific applications but rather selected on the basis of their technological potential. Nevertheless, it is well known that probiotic effects are strain specific, therefore it is very likely that better performing strains may be selected when a rational, use-specific selection process is followed. The selection of strains from appropriate sources depending on the target population, such as neonates, children, pregnant women or the elderly, whose microbiota may differ from that of healthy adults (O'Toole and Claesson, 2010), may constitute a promising approach. In this regard most of the strains currently available have targeted the adult population but few strains have been selected for other groups of age, such as human neonates, where the applications of probiotics may have a great positive impact (Salminen et al., 2009).

Microbial colonization of the neonate gut is a stepwise process that provides important signals for the maturation of the immune system and the development of the intestine (Cebra, 1999; Stappenbeck et al., 2002), thus greatly contributing to the establishment and maintenance of the gut barrier (Penders et al., 2006; Rakoff-Nahoum et al., 2004). Aberrancies in this process can predispose to disease in later life (Kalliomaki et al., 2001, 2008). Thus, initial microbial colonization provides an important starting point for the later

homeostasis and well-being of the individual. One of the factors having a strong influence in this process is the feeding habits of the infant (Harmsen et al., 2000), with breast-feeding constituting the golden standard for infant nutrition. In fact, the protective effect of breast-feeding on allergies, diarrhoea or respiratory infections has been shown in different studies (Björksten et al., 2001; Majamaa et al., 1995). One of the main differences observed between breast and formula-fed infants is the composition of the gut microbiota, with bifidobacteria constituting the most predominant microorganisms in the former group. Increasing bifidobacterial levels is often considered a target in the development of infant formulas (Agget et al., 2003). Traditionally, the higher level of bifidobacteria observed in breast-fed infants has been attributed to the presence of bifidogenic oligosaccharides in breast-milk (Aggett et al., 2003). Also, more recently the presence of bifidobacteria in breast-milk has been reported (Gueimonde et al., 2007a; Martín et al., 2009; Solis et al., 2010), which may also play a role in the dominance of this microorganism observed in breast-fed infants. This fact has directed attention towards the supplementation of infant formulas. However, most of the strains currently used were not isolated from the natural source but from the commercially available strains. The inclusion of breast-milk bifidobacteria in formulas targeted at both pre-term and full-term infants constitutes a very promising area for the development of new products aiming at the establishment of a healthy gut microbiota and a proper intestinal barrier resembling that of breast-fed infants.

In a previous study (Solis et al., 2010) we isolated three *Bifidobacterium longum* and three *Bifidobacterium breve* strains from breast-milk. Now we aim to identify these isolates at strain level by using different phenotypic and

genotypic methods as well as to characterise them by using different *in vitro* tests following the FAO/WHO guideline recommendations (2006).

Material and Methods

Strains and growth conditions

Six *Bifidobacterium* strains, *B. longum* IPLA 20001, 20002 and 20003 and *B. breve* IPLA 20004, 20005 and 20006, previously isolated from breast-milk of five healthy mothers (Solis et al., 2010) were included in this study. *B. longum* strains IPLA 20002 and 20003 were isolated from breast-milk of the same women at different time points (10 days and 1 month postpartum, respectively). The other four strains were isolated from different women at different times; *B. longum* IPLA 20001 at 1 day postpartum, *B. breve* strains IPLA 20004 and 20005 at 1 month and *B. breve* IPLA 20006 at 3 months postpartum. The strains were routinely grown in MRS medium (Difco, Becton-Dickinson and Company, Le Pont de Claix, France) supplemented with a 0.25% L-cysteine (Sigma Chemical Co, St. Louis, MO, USA) (MRSc) and using anaerobic incubation conditions (10% H_2, 10% CO_2 and 80% N_2) in a chamber Mac 500 (Don Whitley Scientific, West Yorkshire, UK) at 37ºC. The pathogens used in the different experiments were *Clostridium difficile* DSMZ 12056, *Salmonella enterica* subsp. *enterica* serovar. Typhimurium LMG 15860, *Cronobacter sakazakii* LMG 5740 (formerly *Enterobacter sakazakii* LMG 5740), *Shigella sonnei* LMG 10473 and *Staphylococcus aureus* CECT 435. These pathogenic microorganisms were selected due to their role as pathogens for humans and their presence in the human gut. For the experiments of *in vitro* growth

inhibition, the strains of *Shigella*, *Salmonella* and *Cronobacter* were grown in Nutrient Agar plates, whilst for *Clostridium* and *Staphylococcus* the agar media used were Brain Heart Infusion (BHI) (Oxoid Ltd., Basingstoke, Hamshire, England) and Trypticase Soy Broth (TSB) (Oxoid), respectively. *Clostridium difficile* was grown at 37ºC under anaerobic conditions whereas the other pathogens were grown in aerobiosis at 32ºC for *Cronobacter* and at 37ºC for the remaining microorganisms.

For adhesion to mucus experiments, all the bacteria were grown in Gifu Anaerobic Medium (GAM) broth (Nissui Pharmaceutical CO., Tokio, Japan) and incubated at 37°C under anaerobic conditions. In adhesion and competitive exclusion assays, bacteria were grown for 18 h, harvested, and then washed twice with phosphate-buffered saline (PBS) buffer. Microorganisms were metabolically labelled by the addition of 10 μL/mL tritiated thymidine (5-3H-thymidine 1.0 mCi/mL, Amersham Biosciences, UK) to the media.

The widely used probiotic *Bifidobacterium animalis* subsp. *lactis* Bb12 was included in the tolerance to acid and bile as well as adhesion assays for comparison purposes.

Species identity of bifidobacterial strains by partial sequence analysis of the 16S rRNA gene.

The strains were identified at species level by partial sequence analysis of the 16S rRNA gene. DNA was extracted from 1 mL of culture by using the GenElute™ Bacterial Genomic DNA Kit (Sigma) following the manufacturer's instructions. 16S rDNA was amplified using *Bifidobacterium* specific primers Bif164 and Bif662 as indicated elsewhere (Ruas-Madiedo et al., 2005).

151 Amplified PCR products were purified using the GenElute™ PCR clean-up Kit
152 (Sigma) and sequencing of the amplicons was done at Secugen SL (Madrid,
153 Spain) in an automated sequencer ABI Prism (Applied Biosystems, Foster City,
154 CA, USA). The sequences obtained were compared with those held at the
155 databases by using BLAST (Altschul et al., 1997).
156
157 *Genetic typing of strains*
158 Randomly amplified polymorphic DNA (RAPD)-PCR. DNA extracts were
159 employed for breast-milk bifidobacteria typing by RAPD-PCR using previously
160 described conditions and the primer 5'-TGCCGAGCTG-3' (Mättö et al., 2004)
161 (Sigma Genosys). PCR reactions were run in a UnoCycler VWR-AD™ (VWR
162 Internacional Eurolab S.L, Spain) thermocycler. Amplification products were
163 subjected to electrophoresis in 1% agarose (Sigma) and gels were stained and
164 visualized by ethidium bromide staining.
165 DNA restriction patterns by pulsed-field gel electrophoresis (PFGE). Intact
166 high molecular weight genomic DNA was isolated and digested in agarose
167 plugs. Cells were grown to an OD_{600} of 1.5, harvested by centrifugation, washed
168 three times in buffer TE pH 8.0 (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and
169 resuspended in 500 μL of the same solution. To form agarose plugs, the cell
170 suspension was heated to 50ºC, mixed with an equal volume of 2 % PFGE
171 agarose (Bio-Rad Laboratories, Richmond, Ca., USA) in 0.5X TBE buffer and
172 added to moulds. Plugs were incubated for 24 h at 37ºC in 1 mL of lysis buffer
173 (per plug) containing 50 mM EDTA pH 8.0, 0.5 mg/mL N-laurylsarcosine, 5
174 mg/mL Brij58, 2 mg/mL deoxicholate, 2 mg/mL lysozyme, 15 U/mL mutanolysin
175 and 2 μg/mL RNase and then deproteinized by incubation at 50ºC for 24 h in a

solution containing 0.5 M EDTA pH 8.0, 40 mM Tris-HCl pH 8.0, 1% w/v SDS and 1.5 mg/mL proteinase K (all reagents were purchased from Sigma). They were finally washed for 1 h in TE and incubated for 24 h at 37ºC in TE pH 8.0 containing 0.29 mg/mL pefabloc SC (Merck, Darmstadt, Germany).

Thin slices of agarose plugs were cut and washed six times for 30 min at room temperature in TE buffer. DNA within the plugs was digested with 20 U of the restriction enzyme *Xba*I. Electrophoresis was carried out at 6 V/cm and 14ºC using a CHEF DRII apparatus (Bio-Rad) in 1 % PFGE certified agarose (Bio-Rad) gels with 0.5 x TBE buffer. Pulse times ranged from 2 to 25 s during the 22 h electrophoresis. A DNA pulse marker (LowRange PFG Marker N0350S, New England Biolabs, Ipswich, MA, USA) was used as the molecular size standard. Gels were stained and visualized by ethidium bromide staining.

Characterization of strains by enzymatic and carbohydrate fermentation profiles

Fermentation profiles of strains were obtained in API 50 CH strips (Bio-Mérieux, Marcy l'Etoile, France) following the manufacturer's instructions. Enzymatic activity profiles were determined using API Zym strips (Bio-Mérieux).

Antibiotic resistance profiles

The minimal inhibitory concentration (MIC) of the strains towards gentamicin, kanamycin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin and chrolamphenicol was determined by the microdilution method using the microtiter VetMIC Lact-1 panel for susceptibility testing of bacteria (Statens Veterinarmedicinska Anstalt, Uppsala, Sweden). The Standard Operating Procedure (SOP) proposed by the EU funded ACE-ART project, and

currently being evaluated for approval as an international standard by the ISO/IDF working group (ISO/DIS 10932|IDF 223), was followed. In brief, strains were grown in MRSc agar, colonies were resuspended in LSM medium (90% Isosensitest medium [Oxoid] + 10% MRS medium [Difco]) supplemented with 0.3 g/L-cysteine to OD_{600nm} 0.2 and the suspension was diluted 1000 times in the same medium. 100 µL were then added to each microtiter plate well and incubated at 37ºC under anaerobic conditions for 48 hours. Growth within each well was determined visually after incubation.

Tolerance to acid and bile

Five mL bacterial cultures were grown overnight at 37ºC under anaerobic conditions. Then, cells were harvested, washed twice with 0.85% NaCl and resuspended in 500 µL of the same solution. 100 µL of bacterial suspensions were added to 900 µL of simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, and 3 g/L pepsin [Sigma], adjusted to pH 2.5 with HCl) or bile juice (45 mM NaCl, 1 g/L pancreatin [Sigma] and 3 g/L Oxgall [Sigma], adjusted to pH 8.0 with NaOH). Suspensions were then incubated in anaerobiosis for 90 or 180 min with acid and bile juices, respectively. Plate counts in MRSc were done at time 0 and after incubation and results represented as % of survival.

In vitro *inhibition of pathogen growth*

The ability of the supernatants from the *Bifidobacterium* strains to inhibit the growth of pathogenic microorganisms was determined using the agar diffusion tests by measuring the diameter of the inhibition zones.

225 Five mL samples were taken from growing cultures of the bifidobacterial
226 strains at two different growth phases; exponential (OD between 1 and 2) and
227 stationary (OD between 4 and 5, depending on the strain). After centrifugation,
228 supernatants were divided in two aliquots, one was adjusted to pH 6.2 and the
229 other one left unadjusted. Supernatants were then stored at -20ºC until use in
230 the agar diffusion tests.
231 Overnight (16 h) pathogen cultures were used to inoculate (1% v/v) agar
232 media, 5 mm wells were cut out of the agar and 25 µL of each supernatant were
233 added to the well. Tetracycline (100 µg/mL) was used as a positive control.
234 Plates were then incubated for 24 h under appropriate conditions for each
235 specific pathogen. After incubation the diameter of the inhibition zone, if any,
236 was measured. Two independent replicates were conducted for each
237 experiment.
238
239 *Adhesion to HT29 cell line*
240 The adhesion capability of the strains was assessed with the epithelial
241 intestinal cell line HT29 (ECACC No. 91072201), derived from human colon
242 adenocarcinoma, purchased from the European Collection of Cell Cultures
243 (Salisbury, UK). The cell line was maintained in McCoy's medium supplemented
244 with 10% (v/v) heat-inactivated bovine foetal serum and a mixture of antibiotics
245 to give a final concentration of 50 µg/mL penicillin, 50 µg/mL streptomycin, 50
246 µg/mL gentamicin, and 1.25 µg/mL amphotericin B. All media and supplements
247 were obtained from Sigma. The incubations took place at 37ºC, 5% CO_2 in an
248 SL Water-jacketed CO_2 Incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA).
249 Culture media were changed every two days and the cell line was trypsinized

250 with 0.25% trypsin-EDTA solution (Sigma) following standard procedures. For
251 experiments, 10^5 cells/mL were seeded in 24-well plates and incubated to
252 confluence (reaching about 10^7 cells/mL) during 13±1 days.
253 Bacterial cultures were harvested by centrifugation, washed twice with
254 Dulbecco's PBS buffer (Sigma) and resuspended in McCoy's medium without
255 antibiotics at a concentration of about 10^8 cfu/mL. HT29 monolayers were
256 washed twice with Dubelco's PBS to remove the antibiotics before adding the
257 bacterial suspensions. Plates were incubated for 1 h at 37°C, 5% CO_2 in a
258 Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold,
259 Germany). After the incubation period, supernatants were discarded and wells
260 were softly washed three times with Dulbecco's PBS buffer to remove the non-
261 attached bacteria. Afterwards, the monolayers were trypsinized and bacterial
262 counts were carried out in agar-MRSc to determine the number of adhered
263 bacteria. Results were expressed as the percentage of bacteria adhered with
264 respect to the amount of bacteria added (% cfu bacteria adhered / cfu bacteria
265 added). Experiments were carried out in replicate (using two HT29 plates) and
266 in each plate the strains were also tested in duplicate.
267
268 *Adhesion to human infant mucus*
269 Human intestinal mucus was obtained from 2 and 6-month-old infant
270 faeces as described earlier (Kirjavainen et al., 1998). Mucus was dissolved (0.5
271 mg protein/mL) in HEPES-Hanks buffer (HH; 10 mM HEPES, pH 7.4).
272 Radiolabeled bacteria optical density (OD_{600} nm) was adjusted to 0.25 ± 0.05 to
273 standardize the bacterial concentration (10^8 bacteria/mL). The adhesion
274 assessment was carried out as previously described (Gueimonde et al., 2005).

Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucus. Adhesion was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

Inhibition of pathogen adhesion to infant mucus

To test the ability of the bifidobacteria to inhibit the adhesion of pathogens, the procedure described by Collado et al. (2005) was used. In brief, unlabeled bifidobacteria (10^8 bacteria/mL) or HH buffer (control) were added to the wells and incubated for 1 h at 37°C and wells were washed twice with HH buffer (to remove unattached bifidobacteria in the corresponding wells). Radiolabeled pathogens (10^8 bacteria/mL) were then added to the wells and incubated at 37°C for 1 h. The wells were then washed and bound bacteria were recovered after lysis. Radioactivity was measured by liquid scintillation. The percentage of adhesion inhibition was calculated as the change in the adhesion of the pathogen in the presence of the different bifidobacterial strains with regard to that in the control (set arbitrarily to value zero). Inhibition was determined in three independent experiments and each assay was performed in duplicate.

Statistical analysis

Data were statistically analysed using the SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA). One-way ANOVA tests were performed to determine differences among strains. When appropriate the post-hoc mean comparison LSD test was additionally used.

Results

Identification at species level

The partial 16S rDNA sequences obtained from the amplification products confirmed the identity of the strains previously determined by using another primer pair (Solis et al., 2010). The sequences obtained are held at Genbank database under deposit numbers HM856586 to HM866591.

Genotypic and phenotypic characterisation of the strains

The six bifidobacterial strains were genotyped by using two different methods. The RAPD profiles obtained allowed us to distinguish the strain *B. breve* IPLA 20006 from the other two *B. breve* strains, which showed identical profiles, and *B. longum* IPLA 20001 from the other two *B. longum* strains, which also showed the same RAPD profile (Figure 1a). PFGE macro-restriction showed identical profiles for the strains *B. breve* IPLA 20004 and IPLA 20005, these being different from that of *B. breve* IPLA 20006 (Figure 1b), and allowed distinguishing among the three *B. longum* strains, including those indistinguishable by RAPD (IPLA 20002 and IPLA 20003). Overall, RAPD produced four different profiles, whilst PFGE showed a higher discriminatory power establishing five different profiles.

With regard to the phenotypic characterisation, none of the six *Bifidobacterium* strains fermented glycerol, erythiol, D-arabinose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, D-cellobiose, D-trehalose, inulin, starch,

xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. All the strains fermented D-ribose, D-galactose, D-glucose, D-maltose, D-lactose, D-mellibiose, D-saccharose and D-raffinose. In addition, only the three *B. longum* strains were able to ferment L-arabinose and D-xylose whilst aesculin was only fermented by the *B. breve* strains. The results obtained for those sugars showing variability within each species are shown in Table 1. Sugar fermentation profiles allowed distinguishing between two *B. breve* strains (IPLA 20004 and IPLA 2005), which showed identical RAPD and PFGE profiles, and corroborates previous reports indicating a higher discriminatory power for the combination of phenotypic and genotypic techniques than for the genotypic methods alone (Gueimonde et al., 2004).

Analysis of enzymatic activity profiles of the six *Bifidobacterium* isolates showed that α-fucosidase, as well as the harmful β-glucuronidase activity, were not present in any strain, whilst all microorganisms presented high levels (≥ 30 nanomoles, results not shown) of leucine arylamidase, β-galactosidase, α-glucosidase and α-galactosidase activities. The presence of this last activity was in accordance with the capacity of these microorganisms to ferment raffinose. The three *B. longum* strains lacked β-glucosidase activity which was present on the *B. breve* strains. Enzymatic activities showing variability are shown in Table 1.

Antibiotic resistance profiles of the strains

B. longum strains showed higher resistance levels than *B. breve*, although strain-specific variability was found (Table 2). The strain *B. longum* IPLA 20001

showed resistance to erythromycin (4 µg/mL) and clindamycin (>16 µg/mL), eight and over 50 times higher, respectively, than that found for any of the other strains tested.

Tolerance to acid and bile

The widely used probiotic strain *B. lactis* Bb12 was included for reference and comparison purposes. Tolerance to low pH was highly variable. *B. lactis* Bb12 showed the highest stability in simulated gastric juice with 74.5% survival after 90 min of exposure (Table 3). Among the tested strains *B. breve* IPLA 20006 displayed the highest survival (23.36%) whilst the recovery of all the other strains was less than 1.5%. Interestingly, simulated bile juice was more toxic than the gastric juice. In fact, survival percentages after exposure for 180 min to bile juice were lower than 1% for all the strains tested. *B. longum* IPLA 20003 showed the highest survival with a 0.84% whereas the reference strain *B. lactis* Bb12 survived only a 0.0004% after bile juice challenge. When the results of tolerance to both gastric and bile juices are taken together it appears that the strain *B. longum* IPLA 20003 has the highest ability to survive during gastrointestinal transit, this being even higher than that of the reference strain *B. lactis* Bb12.

In vitro *inhibition of pathogen growth*

The inhibition ability of supernatants from the different bifidobacterial strains against some pathogens was determined by the agar diffusion test. The positive control used (tetracycline) produced inhibition zones for all pathogens with diameters ranging from 14 to 29 mm. No inhibition was observed for any of

375 the supernatants in which the pH was neutralized (results not shown). None of
376 the *Bifidobacterium* non-neutralized culture supernatants inhibited the growth of
377 *St. aureus* or *C. difficile* (results not shown). For the other three pathogens
378 tested, inhibition was found for some of the non-neutralized stationary phase
379 bifidobacterial cultures supernatants (Table 4). *S. enterica* was inhibited by non-
380 neutralized supernatants of *B. longum* IPLA 20002, *B. longum* IPLA 20003, *B.*
381 *breve* IPLA 20004 and *B. breve* IPLA 20005, but not by those of *B. longum*
382 IPLA 20001 and *B. breve* IPLA 20006. Similarly, all non-neutralized stationary
383 phase culture supernatants, except that of *B. breve* IPLA 2006, inhibited the
384 growth of *Sh. sonnei* and all of them were able to inhibit *Cr. sakazakii* growth.
385 This last microorganism was, in addition, inhibited by the non-neutralized
386 exponential phase supernatants from *B. longum* IPLA 20002 and *B. breve* IPLA
387 20005 (diameter of the inhibition zones: 8 mm), these being the only cases in
388 which inhibition was observed for exponential growth phase culture
389 supernatants (results not shown).
390
391 *Adhesion to intestinal epithelial cells*
392 Adhesion ranged between 0.1 and 5.5% depending on the strain, with
393 some of the breast-milk strains adhering significantly better ($p<0.05$) than the
394 reference strain *B. lactis* Bb12 (Figure 2).
395
396 *Adhesion to human infant mucus*
397 *B. longum* IPLA 20001 was the strain showing a significantly better
398 adhesion to 2-month-old infant mucus (55.3%) (Figure 3). When 6-month-old
399 infant mucus was used, *B. longum* IPLA 20002 and *B. breve* IPLA 20005

showed significantly higher adhesion values than the other strains. The strain *B. breve* IPLA 20004 showed the lowest adhesion values in both models (2 and 6-month-old infant mucus). Interestingly, strains *B. longum* IPLA 20001, *B. breve* IPLA 20006 and the reference strain *B. lactis* Bb12 adhered significantly better to mucus from 2-month-old infant than to that obtained from 6-month-old infants.

Inhibition of adhesion of pathogens to human intestinal mucus

The adhesion of the pathogens tested on 2 and 6-month-old infant mucus was 3.27±0.51 and 3.23±0.79 %, respectively, for *S. enterica*, 1.69±0.48 and 3.83±1.25 % for *Cr. sakazakii*, 13.21±2.18 and 12.50±1.62 % for *Sh. sonnei*, 3.49±1.09 and 5.19±1.10 % for *St. aureus* and 4.26±0.21 and 4.86±0.53 % respectively, for *C. difficile*. *Cr. sakazakii* adhered significantly better ($p<0.05$) to mucus isolated from 6-month-old infants than that from 2-month-old babies.

The ability of the breast-milk bifidobacterial strains to inhibit the adhesion of the pathogens to infant intestinal mucus was determined (Figure 4). *B. lactis* Bb12 was also included for comparative purposes. All the bifidobacterial strains increased significantly the adhesion of *S. enterica* to infant mucus in at least one of the two models (2 and 6-month-old infants), with increases ranging from 4 to 210% when compared to the control (buffer without bifidobacteria). The reference strain *B. lactis* Bb12 caused the highest increase in the adhesion of the pathogen, 210 and 177% in 2 or 6-month-old infant mucus, respectively. None of the strains affected significantly the adhesion of *Sh. sonnei* in these mucus models. *B. breve* IPLA 20004 and *B. lactis* BB12 increased significantly the adhesion of *C. difficile* to 2-month-old infant mucus (22 and 31%,

425 respectively) whilst *B. breve* IPLA 20006 increased the adhesion of the
426 pathogen to 6-month-old infant mucus. Similarly, *B. longum* IPLA 20001 and
427 IPLA 20003 as well as *B. lactis* Bb12 significantly increased the adhesion of *St.*
428 *aureus* to 2-month-old infant mucus, whereas no effect was found with the other
429 strains tested, or in the 6-month-old infant mucus model. The adhesion of *Cr.*
430 *sakazakii* to 2-month-old infant's mucus was significantly increased (70%) by
431 the reference strain *B. lactis* Bb12, whilst *B. breve* IPLA 20005 significantly
432 reduced (43.4%) the adhesion of this pathogen in that model. This was the only
433 case in which the adhesion of a pathogen was significantly inhibited by one of
434 the strains tested.
435
436 **Discussion**
437
438 Human breast-milk constitutes an interesting source to obtain new and
439 specific probiotic strains for neonates aiming at assisting a proper development
440 of the gut microbiota and the immune development in infants who, for different
441 reasons, cannot be breast-fed.
442 Here we confirmed the identity of six *Bifidobacterium* strains, previously
443 isolated from breast-milk (Solis et al., 2010), by partial sequencing of the 16S
444 rRNA gene. Three of the strains were identified as *B. longum* and the other
445 three as *B. breve*. These strains were typified and characterised by using
446 phenotypic and genotypic tests. A step-wise process was used to this end
447 following the guidelines established by the FAO/WHO working group
448 (FAO/WHO, 2006), which recommends to carry out strain identification by
449 phenotypic and genotypic methods as well as *in vitro* functional characterization

and safety assessment before enrolling on clinical trials. In accordance with previous studies, genetic typing techniques have shown a high discriminatory power with PFGE showing a higher resolution than RAPD (Mättö et al., 2004). Our results are in good agreement with previous reports (Gueimonde et al., 2004) indicating that the combination of genotypic and phenotypic methods extended the discriminatory power of the former alone, as shown with the *B. breve* strains IPLA 20004 and IPLA 20005 which displayed identical genetic profiles but different phenotypic traits. In addition, phenotypic tests provide data on the specific properties and the metabolic potential of the strains related to functionality and safety. The presence of potentially deleterious enzymatic activities may, sometimes, be a concern and it was also checked. The enzymatic activity profiles of our strains indicate the absence of potentially deleterious activities such as β-glucuronidase, related to the conversion of pre-carcinogens to carcinogens.

In addition to proper strain identification it is always important to pay attention to safety. *B. longum* and *B. breve* are considered safe and have QPS status according to EFSA (EFSA, 2007). However, when identifying and characterizing new strains it is always advisable to take into account any potential safety concern. Several reports have underlined the importance of establishing antibiotic resistance profiles, which have become mandatory in some countries (Health Canada, 2009). For these reasons we determined the antibiotic resistance profiles of our strains. In general, the antibiotic resistance levels observed are in the range of those previously reported for these species (ISO10932/IDF223; Kushiro et al., 2009; Mättö et al., 2007). Intrinsic resistance to aminoglycosides (streptomycin and gentamicin) is normally present in

475 bifidobacteria due to the anaerobic nature of these microorganisms (Mättö et
476 al., 2007) and the subsequent lack of cytochrome-mediated transport (Bryan
477 and Kwan, 1981). According to the breakpoint values established by EFSA for
478 antibiotic resistance in the genus *Bifidobacterium* (EFSA, 2008) one of the *B.*
479 *longum* strains included in this study (IPLA 20001) presented resistance to
480 erythromycin (4 µg/mL versus breakpoint for *Bifidobacterium* of 0.5 µg/mL) and
481 clindamycin (>16 µg/mL versus breakpoint of 0.25 µg/mL). The presence of
482 resistance to these two antibiotics in the same strain has been previously
483 reported and suggests a common resistance mechanism against both
484 antibiotics. It is known as the macrolide-lincosamide-streptogramin (MLS)
485 phenotype and in a recent study this phenotype was found in 7 out of 17 *B.*
486 *longum* strains tested (Ammor et al., 2008). The MIC for tetracycline obtained
487 for the three *B. longum* strains was also above the breakpoint established by
488 EFSA (EFSA, 2008) for this antibiotic in the genus *Bifidobacterium* (16 µg/mL
489 versus breakpoint value of 8 µg/mL). This suggests the possible presence of
490 tetracycline resistance genes, such as *tet*(W) which has been reported to be
491 commonly present in this species (Ammor et al., 2008). The genetic basis and
492 potential transferability of these resistances, even if they are common in *B.*
493 *longum*, require further clarification. It is worth commenting that strains showing
494 high non-transferable resistance to certain antibiotics provide an interesting tool
495 for co-administration with antibiotics, in order to stabilize the microbiota,
496 potentially avoiding certain side-effects of antibiotics, such as associated
497 diarrhoea. On the contrary, strains harbouring transferable resistances should
498 not be used.

499 It is also important to determine *in vitro* potential probiotic properties of the
500 strains before engaging in long and expensive clinical trials. Tolerance to
501 gastrointestinal transit (acidic pH in the stomach and bile in the small intestine),
502 antimicrobial activity, as well as the ability to adhere to the human intestinal
503 mucosa are among the most frequently used selection tests.
504 Survival in simulated gastric juice and in the presence of simulated bile
505 juice were independently determined as indicators of the survival potential of
506 the strains during gastrointestinal transit. The tolerance to gastric juice was
507 highly variable, *B. lactis* Bb12 being the strain showing higher survival followed
508 by *B. breve* IPLA 20006. Bile juice was more toxic than acidic conditions, *B.*
509 *longum* IPLA 20003 showing the highest survival. Combining the results of both,
510 acid and bile tolerance, *B. longum* IPLA 20003 appears to display the highest
511 ability to survive during gastrointestinal transit. Nevertheless, the relevance of
512 these tests in the selection of probiotics for application in neonates is not clear
513 as newborns have a reduced acid and bile secretion (Bhat et al., 1997;
514 Lebenthal and Lebenthal, 1999) and, therefore, even strains not showing a
515 good *in vitro* tolerance may perform well in the *in vivo* situation.
516 The production of antimicrobial compounds against pathogens by breast-
517 milk isolates was determined using the agar diffusion test. *St. aureus* and *C.*
518 *difficile* were not inhibited by any of the bifidobacterial supernatants. Inhibition of
519 *S. enterica*, *Sh. sonnei* and *Cr. sakazakii* was obtained with non-neutralized
520 supernatants from some of the bifidobacterial strains, whilst no inhibition was
521 observed for any of the supernatants in which the pH had been neutralized.
522 This indicates that most likely the inhibition was due to the production of organic
523 acids. In addition, supernatants taken from stationary growth phase cultures

were more inhibitory than supernatants from exponential growth, supporting the role of acid production in such inhibition.

Adhesion to human intestinal mucus has been shown to vary depending on the age of the host (Ouwehand et al., 1999). Because of this we decided to use in the present work, in addition to the HT29 cell line, mucus from infants as they constitute a very clear target in the human population for breast-milk probiotic strains. The two adhesion models used (HT29 vs. infant mucus) clearly showed different results. In fact, the strain showing higher adhesion to HT29 cells, *B. breve* IPLA 20004, was found to be the less adhesive to infant mucus. Interestingly, all *B. breve* strains and *B. longum* IPLA 20001 adhered significantly better to HT29 cells than the reference strain *B. lactis* Bb12, considered a highly adhesive strain. The same is true for the strain *B. longum* IPLA 20001 when mucus obtained from 2-month-old infants was used, this strain showing the highest adhesion. These results indicate good adhesion ability for some of the breast-milk *Bifidobacterium* strains included in this study. In this regard, the strain *B. longum* IPLA 20001 was isolated from breast-milk from a 1-day old baby's mother and isolates showing identical PFGE profiles were also found in the infant faeces at 1, 10 and 30 days of age (results not shown), which suggests a good colonization ability for this strain.

The mucus adhesion levels observed for the reference strain *B. lactis* Bb12 are comparable to those reported by other authors using infant mucus (Juntunen et al., 2001). In general, the adhesion values obtained are slightly higher than those usually found when using intestinal mucus isolated from adults (Collado et al., 2005; He et al., 2001), which corroborates previous results showing that adhesion to intestinal mucus varies depending on the age

549 or health status of the mucus donor (Ouwehand et al., 1999, 2003). Higher
550 adhesion of some *Bifidobacterium* strains to 2-month-old than to 6-month-old
551 infant mucus, or to mucus isolated from adults or elderly subjects, has
552 previously been reported (Ouwehand et al., 1999), supporting our findings of
553 higher adhesion of some strains in mucus of younger infants.
554 In accordance with previous reports (Collado et al., 2005; Gueimonde et
555 al., 2007b) the inhibition of pathogens' adhesion was found to be rather specific,
556 depending on both the bifidobacterial strain and the pathogen used.
557 Interestingly, some *Bifidobacterium* strains seemed to increase the adhesion to
558 infant intestinal mucus of some of the pathogens tested, suggesting that
559 bifidobacteria may facilitate the adhesion of pathogens through their attachment
560 to the bifidobacterial cells. Increases in the adhesion of pathogens to human
561 mucus in the presence of bifidobacteria and lactobacilli have previously been
562 reported (Collado et al., 2005; Gueimonde et al., 2006, 2007b). However, the
563 biological significance of this phenomenon is unknown, it is possible that the
564 pathogen adhered to the bifidobacteria is no longer available to invade the
565 mucosa. Only one of the strains tested (*B. breve* IPLA 20005) was able to
566 significantly inhibit the adhesion of one of the pathogens tested (*Cr. sakazakii*)
567 in the 2-month-old infant mucus model, whereas none of the strains was
568 inhibitory in the 6-month-old infant mucus model.
569 In short, in this study we characterised phenotypically and genotypically
570 six *Bifidobacterium* strains isolated from breast-milk according to international
571 guidelines for probiotics. In addition, different *in vitro* tests were used to assess
572 the probiotic potential of these strains. Although clinical data would be needed
573 before any conclusion on the probiotic properties of the strains can be drawn,

our results demonstrate that some of the tested strains isolated from breast-milk may have good probiotic potential for their inclusion in products targeting infants.

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744 Table 1. Carbohydrate fermentation and enzymatic activities (expressed as
745 nanomoles according to the API Zym strips manufacturer) demonstrating
746 variability among the strains included in this study.
747

	Strain					
	B. longum			***B. breve***		
	IPLA 20001	**IPLA 20002**	**IPLA 20003**	**IPLA 20004**	**IPLA 20005**	**IPLA 20006**
Carbohydrate						
D-fructose	-	+	+	+	+	+
D-mannose	-	-	-	-	-	+
D-mannitol	-	+	+	+	+	-
D-sorbitol	-	+	+	+	+	-
Salicin	-	-	-	-	-	+
D-melezitose	+	-	-	-	-	-
Glycogen	-	-	-	-	+	-
Gentiobiose	-	-	-	+	-	-
D-turanose	-	+	+	+	+	+
Enzymatic activity						
Alkaline phosphatase	0	0	5	5	5	5
Esterase (c4)	30	20	20	20	20	10
Esterase lipase (c8)	30	10	10	30	20	10
Lipase (c14)	0	5	5	5	5	5
Valine arylamidase	10	10	10	20	10	10
Cystine arylamidase	20	20	20	30	10	5
Trypsin	0	0	0	5	0	5
α-chymotrypsin	5	0	0	0	0	5
Acid phosphatase	30	≥40	≥40	≥40	30	20
Naphthol-AS-BI-phosphohydrolase	10	10	5	10	10	10
β-glucosidase	0	0	0	20	10	≥40
N-Acetyl-β-glucosaminidase	20	30	30	30	30	30
α-mannosidase	30	20	20	5	5	10

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751 Table 2. Antibiotic resistance profiles. MICs (µg/mL) obtained for the different

752 bifidobacterial strains included in this study.

Strain	GE	KA	ST	NE	TE	ER	CL	CH
B. longum IPLA 20001	16	256	16	32	16	4	>16	1
B. longum IPLA 20002	32	512	64	128	16	0.5	0.12	2
B. longum IPLA 20003	64	512	64	>256	16	0.5	0.12	2
B. breve IPLA 20004	2	64	2	16	1	0.12	0.12	1
B. breve IPLA 20005	8	256	4	64	1	0.25	0.25	0.5
B. breve IPLA 20006	32	256	32	64	2	0.12	0.06	1

753 GE, gentamycin; KA, kanamycin; ST, streptomycin; NE, neomycin; TE,

754 tetracycline, ER, erythomycin, CL, clindamycin, CH, chloramphenicol

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Table 3. Percentage of survival of bifidobacterial strains after 90 or 180 min of exposure to simulated gastric or bile juices, respectively.

Strain	**Tolerance to gastric juice** (Mean ± sd)	**Tolerance to bile juice** (Mean ± sd)
B. longum IPLA 20001	0.1625 ± 0.025	0.0006 ± 0.0004
B. longum IPLA 20002	0.2445 ± 0.050	0.0018 ± 0.0013
B. longum IPLA 20003	0.9235 ± 0.132	0.8400 ± 0.1318
B. breve IPLA 20004	0.0160 ± 0.009	0.0002 ± 0.0001
B. breve IPLA 20005	1.1940 ± 0.486	<0.00001
B. breve IPLA 20006	23.3580 ± 6.644	<0.00001
B. lactis Bb12	74.5000 ± 4.950	0.0004 ± 0.0002

Table 4. *In vitro* inhibition of the growth of pathogens by the bifidobacterial strains tested. Results display the diameter of the inhibition zones (mm) obtained for the stationary growth phase non-neutralized supernatants from the *Bifidobacterium* strains and the corresponding pH values.

Strain	Diameter (mm) of inhibition zones					
	IPLA 20001	IPLA 20002	IPLA 20003	IPLA 20004	IPLA 20005	IPLA 20006
pH	*4.5*	*4.3*	*4.4*	*4.3*	*4.3*	*4.5*
Cr. sakazakii	10	11	10	11	11	12
Sh. sonnei	11	13	10	8	10	0
S. enterica	0	10	12	11	10	0

807 **Figure 1. (a)** RAPD-PCR profiles obtained for the *B. breve* (A) and *B. longum*
808 (B) strains included in this study. A: Lane 1, molecular weight marker; lane 2,
809 IPLA 20005; Lane 3, IPLA 20006; Lane 4, IPLA 20004. B: Lane 1, molecular
810 weight marker; lane 2, IPLA 20001; lane 3, IPLA 20002; lane 4, IPLA 20003. **(b)**
811 PFGE macrorestriction profiles obtained with *Xba*I for the *Bifidobacterium*
812 strains included in this study. Lanes: 1, molecular weight marker; 2, *B. longum*
813 IPLA 20001; 3, *B. breve* IPLA 20004; 4, *B. breve* IPLA 20005; 5, *B. longum*
814 IPLA 20002; 6, *B. longum* IPLA 20003; 7, *B. breve* IPLA 20006.
815
816 **Figure 2.** Percentage of adhesion to HT29 cells of the *Bifidobacterium* strains
817 included in this study and the control strain *B. lactis* Bb12. (ANOVA, $p = 0.000$).
818 Bars with different letters differ significantly ($p<0.05$; LSD test).
819
820 **Figure 3.** Percentage of adhesion to intestinal mucus, from 2 month old (black
821 bars) and 6 month old (white bars) infants, of the *Bifidobacterium* strains
822 included in this study and the control strain *B. lactis* Bb12. Bars with different
823 letters within the same mucus group (2 or 6 months) differ significantly (both
824 cases ANOVA, $p = 0.000$) ($p<0.05$; LSD test). Asterisks indicate statistically
825 significant differences between adhesion of the strain to mucus from 2 or 6
826 month-old infants ($p<0.05$).
827
828 **Figure 4.** Inhibition of the adhesion of model pathogens to intestinal mucus
829 isolated from 2 month-old (black bars) or 6 month-old (white bars) infants by the
830 bifidobacteria tested and the control strain *B. lactis* Bb12. An asterisk indicates
831 strains which significantly modified the adhesion with regard to the

corresponding 2 or 6 month mucus control (HH buffer without bifidobacteria, arbitrarily set to value zero). Positive values indicate increases in the adhesion of the pathogens whilst negative values indicate inhibition of adhesion. Results are expressed as mean values, coefficients of variation ranged between 2 and 19%.

Figure 1. Arboleya et al.

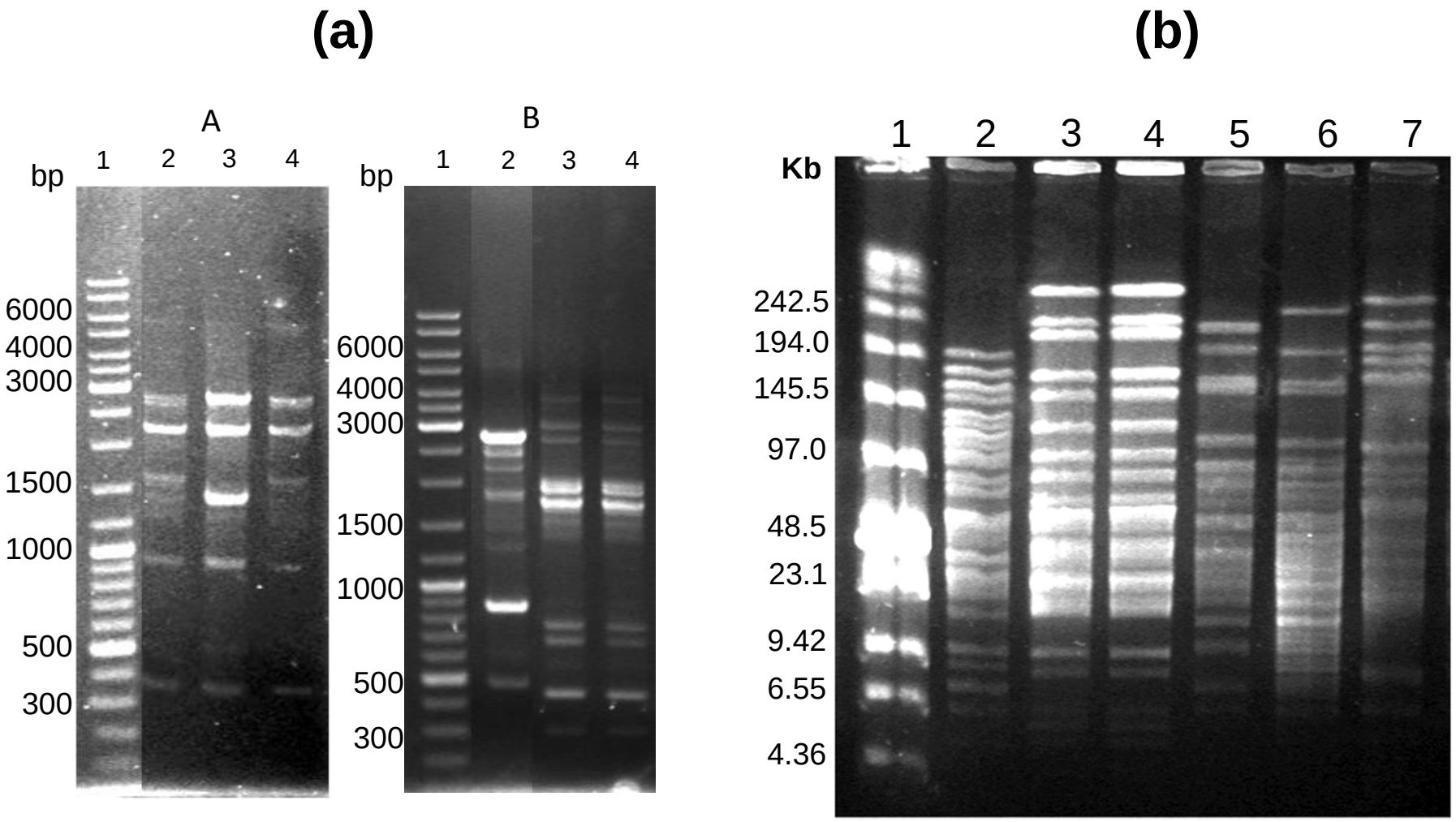

Figure 2. Arboleya et al.

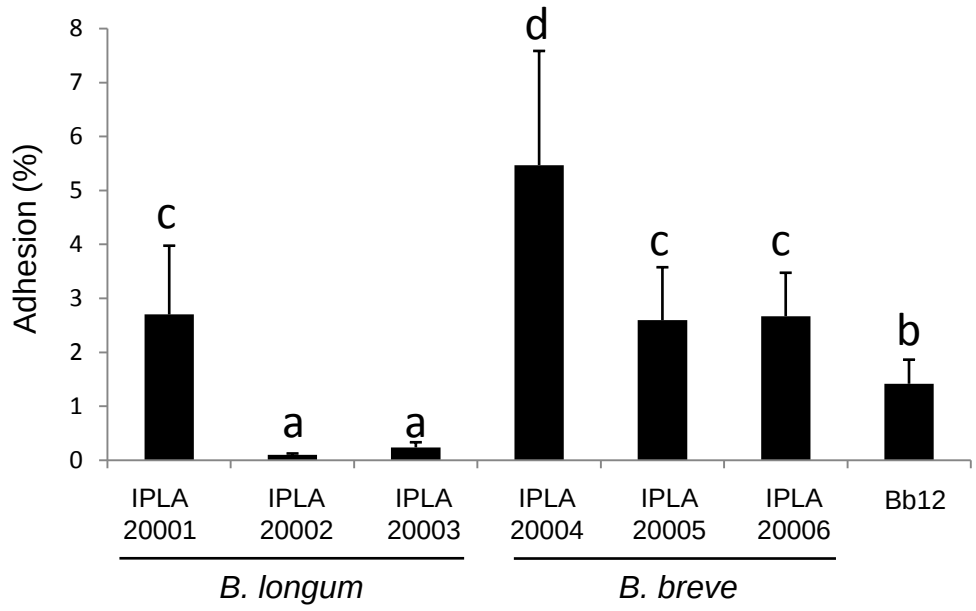

Figure 3. Arboleya et al.

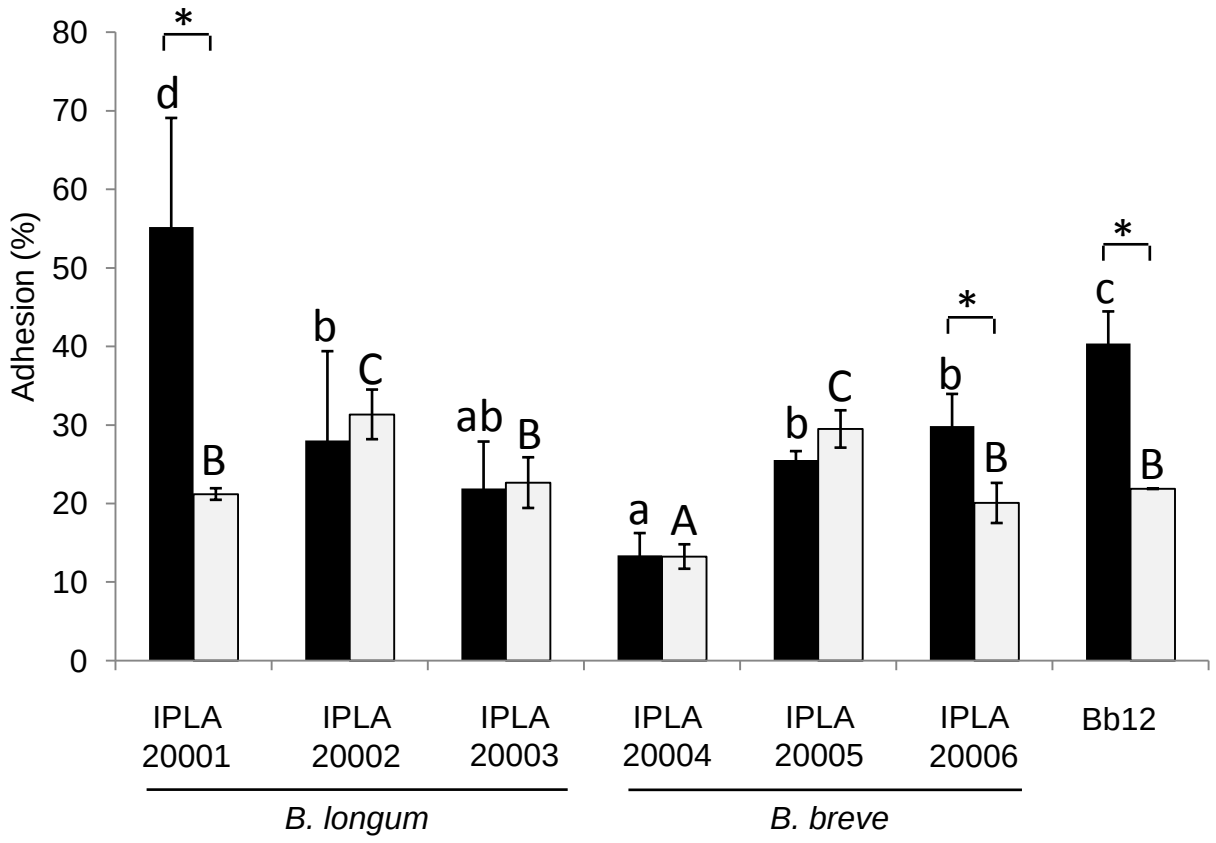

Figure 4. Arboleya et al.