Title: Adhesion of bile-adapted *Bifidobacterium* strains to HT29-MTX cell line is modified after sequential gastrointestinal challenge simulated *in vitro* using human gastric and duodenal juices

Running title: Gut transit of bile-adapted bifidobacteria

Authors: Clara G. de los Reyes-Gavilán[a], Adolfo Suárez[b], María Fernández-García[a], Abelardo Margolles[a], Miguel Gueimonde[a], Patricia Ruas-Madiedo[a]*

Addresses:

[a] Department of Microbiology and Biochemistry of Dairy Products. Instituto de Productos Lácteos de Asturias - Consejo Superior de Investigaciones Científicas (IPLA-CSIC). Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain.

[b] Sección de Aparato Digestivo. Hospital de Cabueñes, C/ Los Prados 395, 33203 Gijón, Asturias, Spain.

greyes_gavilan@ipla.csic.es

adolfo.suarez@hcabuenes.es

mariaf@ipla.csic.es

amargolles@ipla.csic.es

mgueimonde@ipla.csic.es

ruas-madiedo@ipla.csic.es *Correspondence and reprints

Abstract

According to the FAO/WHO, the survival to gastrointestinal tract (GIT) challenges and the ability to colonize the colon are some of the *in vitro* tests proposed for the selection of probiotics for food application. We have used a model that simulates the GIT transit using immersion in, sequentially, gastric and duodenal juices from human origin to evaluate the survival of bile-adapted *Bifidobacterium* strains. *Bifidobacterium animalis* tolerated well the gastric juice, whereas *Bifidobacterium longum* showed poor survival in these conditions. In contrast, *B. animalis* strains were more sensitive to duodenal juice than *B. longum*. The percentage of survival after the GIT transit simulation (GITTS), determined both with plate counts and fluorescent probes, was significantly higher for the bile-adapted strains than for the corresponding parental ones. This suggests that the use of bile-adapted strains is a suitable approach to increase the survival of bifidobacteria to the harsh conditions of the upper GIT. However, the bile-resistance phenotype was not related with any improvement in the adhesion capability, after GITTS, to the intestinal cell line HT29-MTX which constitutively produces mucus. This work shows that the sequential GITTS with human juices modified the *in vitro* adhesion properties of the challenged strains to colonocyte-like cells.

Key-words: bifidobacteria; bile-adapted strain; human gastric juice; human duodenal juice; HT29-MTX cell line; gastrointestinal transit simulation (GITTS).

1. Introduction

Probiotics have been defined as “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2006), with *Bifidobacterium* and *Lactobacillus* being the genera most commonly found in probiotic dairy products for human consumption (Gueimonde et al., 2004; Masco et al., 2005). When ingested, these bacteria must overcome the gastrointestinal tract (GIT) barrier in sufficient numbers to arrive in the colon or in a metabolically active state, in order to transitorily persist in this environment, thus being able to exert their healthy effects. The extremely low pH (ranging from 1.5 to 3) and gastric enzymes in the stomach, followed by the bile salts, pancreatin and other intestinal enzymes that bacteria find in the duodenum, are the main challenges for probiotics (Masco, et al., 2007). One of the strategies to improve the microbial viability in these harsh conditions is the use of acid- and/or bile-resistant bacteria (Chung, et al., 1999; Collado and Sanz, 2006). We have previously obtained a collection of bile-adapted strains by exposure of parental strains to progressively increasing concentrations of bile salts (Margolles et al., 2003; Noriega et al., 2004). Some of these adapted strains have even several improved *in vitro* properties with respect to the parental strains, such as increased adhesion to human mucus (Gueimonde et al., 2005) and inhibition of pathogen adhesion (Gueimonde et al., 2007).

Several publications have reported the survival of different probiotic strains to the simulated conditions of the GIT, using chemically semi-defined gastrointestinal juices (de Palencia et al., 2008; Huang and Adams, 2004; Mainville et al., 2005; Masco et al., 2007). Recently, Mozzi et al. (2009) simulated the transit through the mouth using human saliva and Del Piano et al. (2008) tested the resistance of probiotics to human pancreatic juice. However, to the best of our knowledge, no data are currently available simulating GIT transit with sequential use of human gastric and duodenal juices. On the other hand, several authors have

studied the possible colonization ability of putative probiotic strains by analysing their adhesion capability to epithelial intestinal cell lines, such as Caco-2 and HT29 (Candela et al., 2008; Riedel et al., 2006; Schillinger et al., 2005). A limitation of these studies is that the survival of strains after the GIT transit is assessed by simulation with independent gastric and duodenal juices tests. In addition, as far as we know, there is no information about the influence of GIT transit simulation (GITTS) on the subsequent adhesion ability of probiotic strains.

Taking into account these facts, the aim of the present study was to evaluate whether the acquisition of resistance to bile could influence the survival and adhesion ability of bifidobacteria under the GIT conditions simulated *in vitro*. For this purpose, bifidobacteria were challenged in a model of GITTS using, sequentially, gastric and duodenal juices from human origin and finishing with the study of their adhesion capability to the epithelial intestinal cell line HT29-MTX, which is able to constitutively produce mucin (Lesuffleur et al., 1990).

2. Material and Methods

2.1. Bacterial strains and growth conditions

Three groups of parental and bile-adapted derivative *Bifidobacterium* strains have been used in this study (Gueimonde et al., 2005; Noriega et al., 2004), as indicated in Table 1. Bacteria were cultured in MRSC [MRS (Biokar Diagnostics, Beauvais, France) with 0.05% (w/v), L-cysteine (Sigma Chemical Co., St. Louis, MO)] and incubated for 24 h at 37ºC in an anaerobic chamber MG500 (Down Whitley Scientific, West Yorkshire, UK) under 10% (v/v) H_2, 10% CO_2 and 80% N_2.

2.2. Collection of human gastric and duodenal juices

The collection of the human samples was approved by the Regional Ethics Committee of Clinical Research from the Principado de Asturias (Spain) after the informed consent form was signed by the volunteers. Samples were obtained from 16 donors (7 male and 9 female from 25 to 67 years old) that needed an endoscopic exploration of the upper GIT (oesophagus, stomach and duodenum) due to unspecific digestive disturbances. They had not any previous intestinal pathology or surgery, were not taking acid secretion inhibitors or antibiotic treatment and declared as following a healthy diet. After the endoscopic study, they did not show pathology and thus they were considered as healthy donors. The endoscopy was performed after 6 hours without liquid or food intake using a video-endoscope Olympus GIF-Q 165 (Olympus Europa GmbH, Hamburg, Germany). The gastric juice (GJ) and duodenal juice (DJ) were aspired through the endoscope after its location into the gastric body or the duodenal bulb, respectively. The DJ collected from the duodenal bulb contained gall bladder, pancreatic and duodenal secretions.

For each donor, the 3 ml initially aspired was discarded and the resting juice was collected in a sterile tube which was stored at -20ºC until use. The pH of the GJ samples ranged from 1.4 to 2.5 and that of DJ samples from 7.4 to 8.6. Samples of GJ and DJ from different donors were mixed (giving a final pH of 1.6 and 7.5, respectively) and were filtered through 0.45 μm sterile PTFE-membrane filters (VWR International Eurolab S.L., Barcelona, Spain) before use.

2.3. Survival to the simulated gastrointestinal transit

The simulation of the GIT transit is outlined in Fig. 1. Bacterial cultures were centrifuged (10,000 × *g*, 10 min), washed twice with sterile saline solution (0.85% NaCl), resuspended in 20% of sterile skimmed-milk (Difco, Becton Dickinson, Franklin Lakes, NJ) and added to GJ which increased the pH of the mixture from 1.6 to 1.9. To simulate conditions in the stomach, bacterial suspensions were kept at 37ºC under middle stirring (200

rpm) for 90 min (step 1). Afterwards, and to simulate the duodenal conditions, bacteria were collected by centrifugation, resuspended in DJ and kept under anaerobic conditions for 20 min (step 2). Finally, harvested bacteria were resuspended in diluted DJ (dDJ: DJ diluted 10-fold in saline solution) and kept for 18 h under anaerobiosis (step 3). This last step simulates conditions of the distal part of the small intestine. The experiments were carried out in triplicate for each strain.

Initially, and after each step, samples were taken to determine bacterial counts (CFU ml^{-1}). Serial dilutions were made in Ringer's solution (Merck, Darmstadt, Germany), pour-plated in agar-MRSC and plates incubated at 37ºC under anaerobic conditions for 72 h. Additionally, after step 2 bacterial suspensions were collected and dyed with the Live/Dead® BacLigth bacterial viability kit (Molecular Probes, Invitrogen, Merck) following the manufacturer's instructions. Fluorescence emitted (512 nm for green probe and 620 nm for red probe) by cells after samples excitation at 470 nm was measured in a Cary Eclipse fluorescence spectrophotometer (Varian Ibérica S.A., Madrid, Spain). The ratio between (cultivable and non-cultivable) viable (green) and dead (red) bacteria was used to calculate the percentage of survival after the GIT transit. The correlation coefficient of the calibration survival curves was 0.993±0.003.

2.4. Adhesion to the cell line HT29-MTX

Bacterial suspensions were collected after step 3 of the GITTS to assess their capability to adhere to the epithelial intestinal cell line HT29-MTX. Additionally, bacterial suspensions harvested from MRSC cultures grown for 24 h were used to assess the adhesion ability of the strains not submitted to the GIT challenge. The cell line was maintained in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum and a mixture of antibiotics to give a final concentration of 50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin and 1.25 μg ml^{-1}

amphotericin B. All media and reagents were purchased from Sigma. HT29-MTX cells ($1x10^5$ cells ml^{-1}) were seeded in 24-well plates and incubated to confluence (about $1x10^7$ cells ml^{-1}) for 14±1 days at 37ºC, 5% CO_2 in an SL Waterjacked CO_2 Incubator (Sheldon Mfg. Inc., Cornelius, Oregon). Experiments were carried out using two independent HT29-MTX plates (two consecutive passes) and in each plate bacterial strains were analysed by duplicate. The reproducibility of data, determined by calculating the coefficient of variation [(SD*100)/mean], was on average 19%.

Bacterial suspensions were harvested (10,000 × *g*, 15 min), washed twice with Dulbecco's PBS buffer (Sigma) and resuspended in DMEM without antibiotics at a ratio of about 10:1 (bacteria : eukaryotic cell, respectively). HT29-MTX monolayers were washed twice with Dulbecco's PBS to remove the antibiotics before adding the bacterial suspension and then plates were incubated for 1 h at 37°C, 5% CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany). After the incubation period, supernatants were removed and the wells were softly washed three times with Dulbecco's PBS buffer to remove the non-attached bacteria. Finally, the HT29-MTX monolayers were trypsinised with 0.25% trypsin-EDTA solution (Sigma) following standard procedures. The adhesion percentage was calculated using the quotient "bacteria adhered with respect to the bacteria added". For strains not submitted to the GIT challenge (cultured in MRSC), bacteria were enumerated by plate counting (CFU ml^{-1}) as previously described (Sanchez et al., 2010). According to the results of GITTS survival, the level of non-cultivable but still viable bacteria could be high in the cultures submitted to the GIT challenge. Thereby, to determine the adhesion of strains submitted to the GIT transit, bacterial loads (bacteria ml^{-1}) were determined under optical microscope by using a Neubauer counting chamber (Brand, VWR International Eurolab) which allowed us to enumerate both the cultivable and non-cultivable bacteria.

2.5. Statistical analysis

All experiments have been carried out at least in triplicate. Data were statistically analysed using the SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL). Within each parental / derivative setof strains, independent one-way ANOVA tests were performed to determine differences among strains. For the triad A1, A1dOx and A1dOx-R1, the mean comparison LSD (least significant difference, $p< 0.05$) test was additionally used.

3. Results and Discussion

According to the guidelines of the FAO/WHO (2006) the resistance to the adverse GIT conditions and the ability to adhere to human epithelial intestinal cells are some of the *in vitro* tests recommended for the selection of probiotic bacteria before studying their *in vivo* functionality by means of animal models and/or human interventions trials. In this work we have tested *in vitro* the behaviour of bile-adapted bifidobacteria strains under the GITTS conditions by using human gastric and duodenal juices and the human epithelial intestinal cell line HT29-MTX. Figure 2 depicts the evolution of bifidobacteria counts during the simulated GIT transit. The survival of the bacteria in the human juices was dependent on the strain. Counts of both strains of *Bifidobacterium longum* decreased drastically after the GJ challenge (4.61±2.07 and 2.5±1.50 log CFU ml^{-1} units for the parental and bile-adapted strains, respectively), whereas the count decrease of *Bifidobacterium animalis* strains did not reach 1 log unit. Population levels of strains from both species remained without noticeable variations during the DJ (simulated duodenal conditions) challenge but, after 18 h in diluted DJ (simulating the distal part of the small intestine) the five strains of *B. animalis* showed the highest reduction in their counts, which was also much more pronounced than for *B. longum*. These results suggest that the *B. longum* strains are considerably less resistant to acidic conditions than the *B. animalis* ones, but considerably more tolerant than this last species to

192 the bile and/or enzymes present in the duodenal juice used in our experimental model. Similar
193 findings were previously reported (Masco et al., 2007) indicating that *B. animalis* strains
194 showed the highest resistance to chemical-gastric juice but a low tolerance to pancreatin as
195 compared to other bifidobacteria species. It has also been demonstrated by proteomic
196 approaches that, even when adaptation and response to bile and acid challenges in *B. longum*
197 and *B. animalis* share common features, the expression of some proteins is differentially
198 modified in each species depending on the stress conditions (Sánchez et al., 2008).

199 Regarding the differences between our parental and bile-adapted strains, the final
200 percentage of survival after the combined gastric-duodenal transit (step 2) determined with
201 fluorescent probes (Fig. 3) showed statistically significant differences in favour of all bile-
202 adapted strains ($p<0.05$). In the case of the *B. animalis* A1-triad, only the strain A1dox-R1
203 displayed nearly 100% survival after the simulated gastric-duodenal transit. This fact may be
204 related to the putative protective role of a high molar mass ($1.6x10^6$ Da) exopolysaccharide
205 (EPS) produced by this strain which is not present in the parental A1 and in the bile-adapted
206 A1dOx strains (Ruas-Madiedo et al., 2010). In this way, several authors have suggested that
207 bacterial EPS are involved in the protection against toxic compounds such as bile (Crawford
208 et al, 2008; Hung et al., 2006; Ruas-Madiedo et al., 2009). Finally, as stated above for count
209 evolution, the use of fluorescent probes also showed that *B. longum* had the poorest survival
210 rate after gastric-duodenal transit.

211 Figure 4 represents the percentage of adhesion to the human colon adenocarcinoma
212 HT29-MTX cell line of our bifidobacteria strains not submitted to the GIT challenge (Fig. 4a)
213 and after GITTS (step 3) using human juices (Fig. 4b). The adhesion capability of the strains
214 not previously challenged was higher for the species *B. animalis* than for *B. longum.* Within
215 the three groups of parental / derivative strains, the bile-adapted ones showed higher
216 adherence capability to HT29-MTX line than their corresponding parental strains (significant

differences for strains A1dOx and 667Co, $p<0.05$). Similar behaviour was previously reported by us using a human intestinal mucus adhesion model (Gueimonde et al., 2005). In spite of this, *B. animalis* A1dOx-R1 presented a significant decrease in its adherence to the cell line with respect to both the parental A1 and the bile-adapted A1dOx strains. In this regard, we have demonstrated that the purified EPS A1dOx-R1 interferes in the *in vitro* adhesion of probiotic strains to human intestinal mucus (Ruas-Madiedo et al., 2006). Similarly, the deletion of the EPS-synthesis cluster in *Lactobacillus johnsonii* NCC533 increased the resident time of this strain in the gut of an *in vivo* murine model (Denou et al., 2008). On the other hand, and in contrast to that indicated above, after the GIT challenge the differences in adhesion between strains were much less evident (Fig. 4b) and the higher adherence of the bile-adapted strains in relation to the parental ones was not longer maintained. In fact, the strain 667Co even showed significantly lower adhesion than the corresponding parental strain NB667 ($p<0.05$). Changes in adhesion found in the parental and the bile-adapted strains after the GIT transit could be related to previously observed findings, which indicate that bile exposure modifies the surface characteristics of bifidobacteria (Noriega et al., 2004; Ruiz et al., 2007; Sánchez et al., 2008). Thus, the acquisition of the bile-resistance phenotype is not related to any improvement in the capability to adhere to colonocytes after the transit through the upper part of the gut. Finally, it is worth emphasising that *in vitro* test alone are not enough for supporting the *in vivo* functionality of the strains tested. Nevertheless, the use of *in vitro* intestinal cellular models, despite their limitations, provide a rational starting point for screening new potentially probiotic strains before enrolling in expensive and ethically compromised animal or human studies (Cencic and Langerholc, 2010). In any case, it is clear that to prove the safety and health benefits of a given strain, human intervention studies are required (FAO/WHO, 2006).

To conclude, an overall picture of this study indicates that the bile-adapted bifidobacteria strains were able to *in vitro* survive better in human gastric and duodenal juices than their original counterparts. However the bile-resistance phenotype was not related to any improvement of the *in vitro* adhesion capability after the GIT transit. The next step would be to check whether these *in vitro* findings also apply to the *in vivo* situation where the potential probiotics have to compete for mucosa receptors and nutrients with a plethora of intestinal microorganisms.

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347

348 **Table 1** *Bifidobacterium* strains used in this study

Species	Strains [a]	Origin
B. animalis subsp. *lactis*	IPLA4549 (P)	IPLA collection [b]
	4549dOx (D)	IPLA collection
B. animalis subsp. *lactis*	A1 (P)	Isolated from commercial dairy product
	A1dOx (D)	IPLA collection
	A1dOx-R1 (D)	IPLA collection
B. longum	NB667 (P)	NIZO Culture collection (infant faeces) [c]
	667Co (D)	IPLA collection

349 [a] Parental (P) and bile-adapted derivative (D) strains

350 [b] Isolated as a co-culture of the strain *B. bifidum* CECT4549 (10).

351 [c] NIZO Food Research Collection (Ede, The Netherlands)

352

353 **Legends to figures**

354 **Fig 1.** Schematic representation of the simulated gastrointestinal transit using juices of
355 human origin. GJ: gastric juice (step 1), DJ: duodenal juice (step 2), dDJ-18h: duodenal juice
356 diluted 1/10 with saline solution after 18 h of incubation (step 3).

357

358 **Fig 2.** Counts (log cfu mL^{-1}) along the simulated gastrointestinal transit using juices of human
359 origin of parental (white circles) and bile-resistant derivatives (black symbols)
360 *Bifidobacterium* strains initially suspended in 20% skimmed milk. GJ: gastric juice (step 1),
361 DJ: duodenal juice (step 2), dDJ-18h: duodenal juice diluted 1/10 with saline solution after 18
362 h of incubation (step 3). The coefficient of variation (SD / mean) percentage of this data
363 varied among 0.5 and 10%. At the end of the challenge (dDJ-18 h), symbols that do not share
364 a common letter indicating that counts are statistically different ($p<0.05$).

365

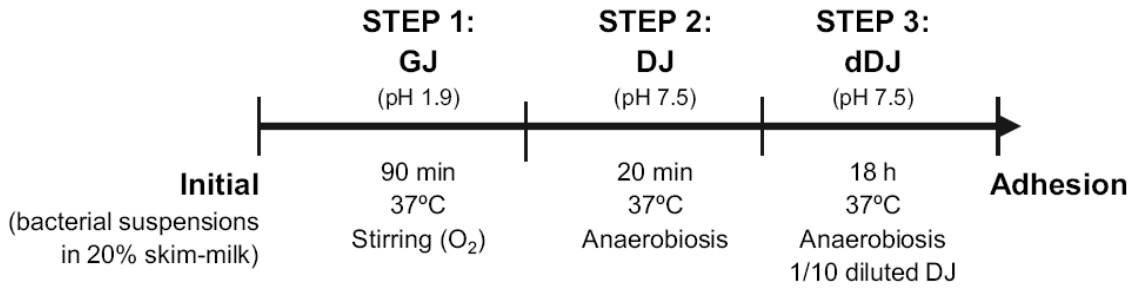
366 **Fig 3**. Percentage of bacterial survival after the gastric and duodenal juice challenges (step 2)
367 determined by the Live/Dead® BacLight kit. For each group of parental /derivative strains,
368 the columns that do not share a common letter are statistically different ($p<0.05$).

369

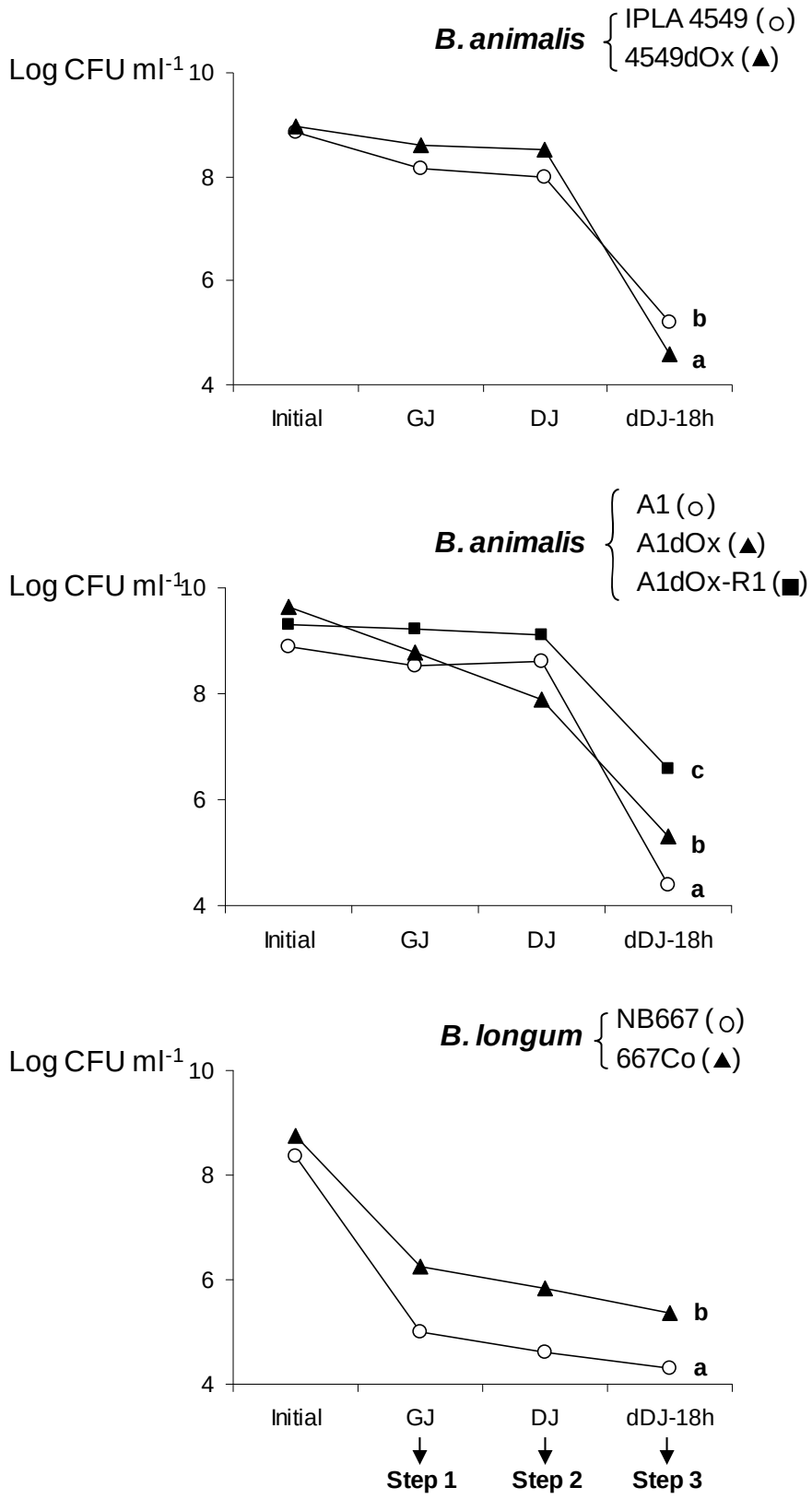
370 **Fig 4**. Percentage of adhesion to the epithelial cell line HT29-MTX of *Bifidobacterium* strains
371 not submitted to the gastrointestinal tract challenge (a) and after the simulated gastrointestinal
372 transit (step 3) using juices of human origin (b). Units: CFU ml^{-1} of adhered with respect to
373 CFU ml^{-1} of added bacteria determined by plating in agar-MRSC (a), and number mL^{-1} of
374 adhered bacteria with respect to number ml^{-1} of added bacteria determined under optical
375 microscope by using a Neubauer counting chamber (b). For each group of parental /derivative
376 strains, the columns that do not share a common letter are statistically different ($p<0.05$).

377

Figure 1

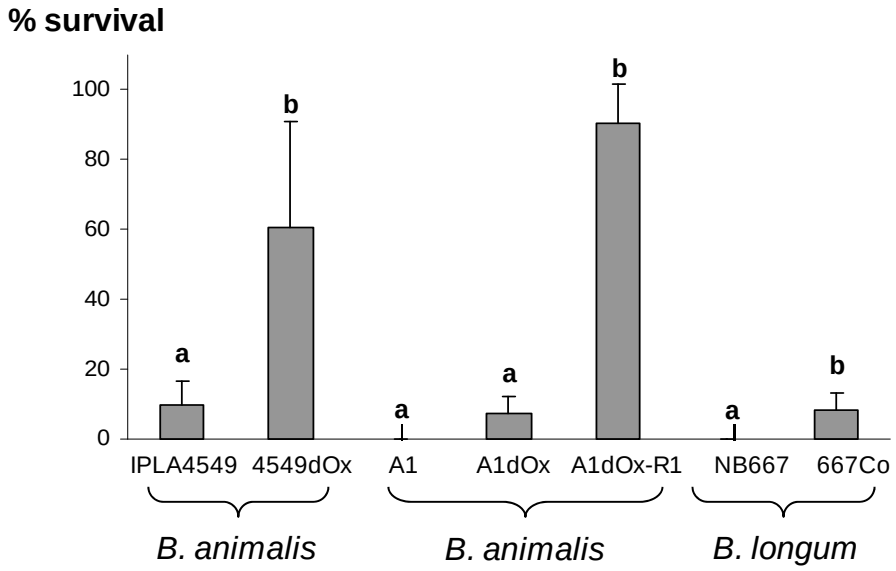

STEP 1:
GJ
(pH 1.9)
STEP 2:
DJ
(pH 7.5)
STEP 3:
dDJ
(pH 7.5)
Initial
(bacterial suspensions
in 20% skim-milk)
90 min
37°C
Stirring (O_2)
20 min
37°C
Anaerobiosis
18 h
37°C
Anaerobiosis
1/10 diluted DJ
Adhesion

380 **Figure 2**



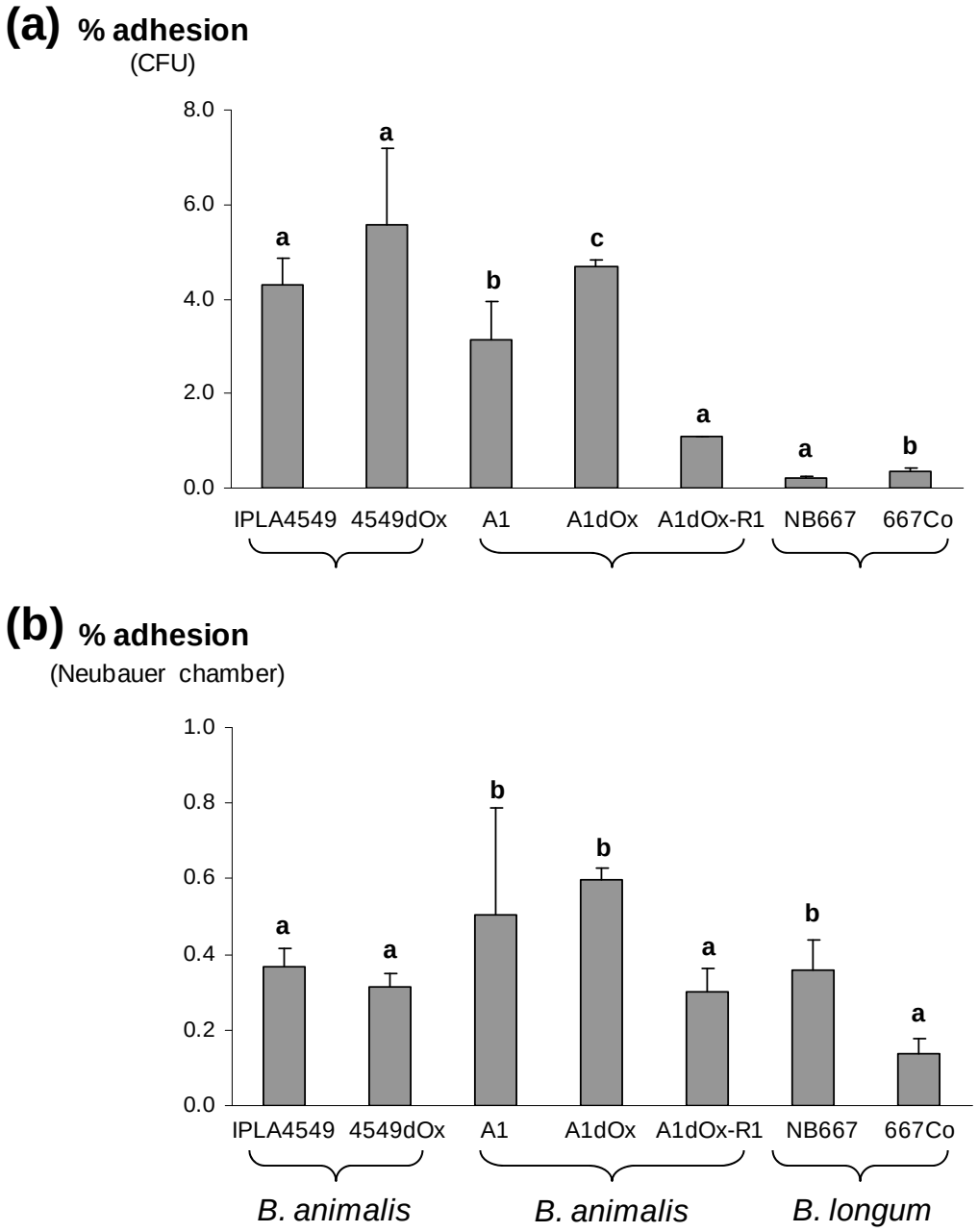
381

382 **Figure 3**



383

384 **Figure 4**



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