1 Title: **Technological and probiotic selection criteria of a bile-adapted *Bifidobacterium***
2 ***animalis* subsp. *lactis* strain**

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ABSTRACT

The technological and probiotic properties of the bile-adapted strain *Bifidobacterium animalis* subsp. *lactis* 4549dOx have been studied in comparison to its parental strain IPLA4549. The survival of the bile-adapted derivative to the simulated gastrointestinal transit was significantly higher than that of the parental strain, except in the presence of 10% skimmed milk. The adherence of strain 4549dOx to the intestinal cell line HT29-MTX almost duplicated that of strain IPLA4549. However, both strains have similar behaviour throughout the shelf-life of fermented milk. In conclusion, the bile-adapted strain 4549dOx presented, in conjunct, additional advantages for its inclusion in dairy probiotic foods.

1 1. INTRODUCTION

2 The term probiotic refers to *live microorganisms which when administered in*
3 *adequate amounts confer a health benefit on the host* (FAO/WHO, 2006), their target being
4 the human gastrointestinal tract (GIT). Probiotic *Bifidobacterium* strains are usually
5 implemented as active cultures in fermented dairy products (Jayamanne & Adams, 2006) and,
6 after being ingested, they must overcome the biological barriers of the GIT in order to

7 We have obtained several bile-adapted bifidobacteria strains by progressive
8 subcultures in gradually increasing bovine bile salt concentrations (Noriega, Gueimonde,
9 Sánchez, Margolles, & de los Reyes-Gavilán, 2004). A significant part of our work has been
10 focused on the couple *Bifidobacterium animalis* subsp. *lactis* IPLA4549 and its derivative
11 strain *B. animalis* subsp. *lactis* 4549dOx. Strain 4549dOx displayed enhanced resistance to
12 the GIT conditions (mainly low pH in the stomach, high bile salt concentration in the intestine
13 and digestive enzymes), mainly linked to an increase in the membrane-bound F₁-F₀-ATPase
14 activity, as well as to changes on the glycolytic flux (Sánchez et al., 2007; Sánchez, de los
15 Reyes-Gavilán, & Margolles, 2006; Sánchez, Ruiz, de los Reyes-Gavilán, & Margolles,
16 2008). *B. animalis* subsp. *lactis* is the most common *Bifidobacterium* species included in
17 fermented dairy products, due to its good tolerance to both acidic and oxidative stress
18 (Gueimonde, Noriega, Margolles, de los Reyes-Gavilán, & Salminen, 2005; Masco,
19 Crockaert, van Hoorde, Swings, & Huys, 2007; Vernazza, Gibson, & Rastall, 2006), being
20 able to maintain higher viability during product storage than other species (Jayamanne, &
21 Adams, 2006)

22 In the present study, we aimed to characterise the technological and some probiotic
23 capabilities of the strains *B. animalis* subsp. *lactis* IPLA4549 and its bile-adapted derivative
24 4549dOx (Ruas-Madiedo, Hernández-Barranco, Margolles, & de los Reyes-Gavilán, 2005).
25 The study was carried out in order to assess whether the acquisition of the bile resistance

phenotype might confer additional advantages for the inclusion of bile-adapted strains in fermented dairy products.

2. MATERIAL AND METHODS

2.1. Bifidobacteria strains

The parental strain *B. animalis subsp. lactis* IPLA4549 and its bile-adapted derivative strain 4549dOx were used in this study. Bifidobacteria were cultured in MRSC [MRS (Biokar Diagnostics, Beauvais, France) with 0.05% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] and incubated for 24 h at 37°C in the anaerobic chamber MG500 (Don Whitley Scientific, West Yorkshire, UK) under 10% (v/v) H₂, 10% CO₂ and 80% N₂. Cultures were collected by centrifugation (10,000 g, 20 min, 5°C), washed twice with PBS solution and resuspended (10-fold concentration) in sterile (11 % w/v) reconstituted skimmed milk (Difco®, Becton Dickinson, Franklin Lakes, NJ, USA). Finally, bacterial suspensions were lyophilised (Freezemobile 12EL, Virtis, Gardiner, NY, USA) for 24 h.

2.2. Manufacture of fermented milk

Two yoghurt starters were used for the manufacture of fermented milk: Fermovac® 1000 V9 (containing *Streptococcus thermophilus*) and Fermovac® 1000 MSK R1 (containing *S. thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*) from Danisco (Copenhagen, Denmark). Both starters were separately propagated (1% w/v) in skimmed milk overnight at 37°C. Cultures were then mixed in equal amounts and used to inoculate (2% v/v) whole milk supplemented with 1% skimmed milk powder previously pasteurised at 85°C for 30 min. When appropriate, lyophilised bifidobacteria strains were added to a final concentration of 10⁸ cfu mL⁻¹ just after the inoculation with the yoghurt starter mixture. Milk fermentations were carried out in a water bath at 42°C until they reached a pH = 5.0 ± 0.1 and, afterwards, each fermented milk type was separated in several aliquots which were stored at 4°C for 24

1 days. Three types of fermented milk were manufactured in triplicate: control (containing the
2 yoghurt starter mixture), IPLA4549 (containing the yoghurt starter mixture and *B. animalis*
3 IPLA4549) and 4549dOx (containing the yoghurt starter mixture and *B. animalis* 4549dOx).
4 Several samples were collected at different points: after milk inoculation (0 h), after 1 h of
5 fermentation, at the final fermentation (FF) time, and after 2, 10, 18 and 24 days of
6 refrigeration.

7 Bacterial counts and pH were determined at each sampling point using different
8 growth media and culturing conditions. After serial dilutions of samples in Ringer solution
9 (Merck, Darmstadt, Germany), *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were
10 enumerated by spread plating on agar-M17 (Oxoid Ltd., Basingstoke, Hampshire, UK) or
11 agar-MRS, respectively. All plates were incubated at 37°C for 48 h. *Bifidobacterium* strains
12 were counted in the same way in agar-MRSC-LP [MRSC supplemented with 0.2 % (w/v)
13 lithium chloride and 0.3% sodium propionate (Sigma)] after growing in an anaerobic chamber
14 at 37°C for 72 h. The residual lactose and the accumulated galactose, as well as the production
15 of acetic and lactic acids were quantified by HPLC and the production of volatile compounds
16 by means of GC-MS as previously reported (Salazar et al., 2009). Results were expressed as
17 mM for sugar and organic acids and in μ M for volatile compounds.

18 **2.3. Resistance to the chemically simulated gastrointestinal transit**

19 The survival of the bifidobacteria strains during the passage through the
20 gastrointestinal tract (GIT) was studied in an *in vitro* model that chemically simulates the
21 physiological conditions, which had been modified from that previously described
22 (Fernández, Boris, & Barbés, 2003). The following components were used: (i) simulated
23 gastric juice (GJ) containing 125 mM NaCl, 7 mM KCl, 45 mM NaHCO₃ and 0.3% pepsin
24 (Sigma) adjusted with HCl to pH 2.0 or to pH 3.0, (ii) simulated duodenal juice (DJ)
25 containing 1% bovine bile (Sigma) adjusted with 10 N NaOH to pH 8.0, and (iii) simulated

intestinal juice (IJ) containing 0.3% bovine bile, 0.1% pancreatin (Pancreas acetone powder porcine, Type I, Sigma, Catalogue number P4251) pH 8.0. To simulate the GIT transit conditions, bifidobacteria from 24 h MRSC-grown cultures of the parental and bile-adapted derivative were harvested by centrifugation (10,000 g, 15 min, 5°C), washed twice with 0.85% NaCl and resuspended in three types of GJ: pH 2.0, pH 3.0 and pH 2.0 containing 10% w/v skimmed milk powder. The three bacterial suspensions of each strain were then incubated for 180 min at 37°C with stirring (200 rpm). Afterwards, samples were centrifuged (10000 g, 15 min), cells were resuspended in DJ and incubated for 10 min at 37°C in an anaerobic chamber. After this step cells were again centrifuged, each resuspended in IJ and incubated for 180 min at 37°C in anaerobic conditions. Viable cell counts were obtained from the initial cultures, after 90 and 180 min of GJ challenge, after 10 min of DJ challenge, and after 90 and 180 min of IJ challenge. Results were expressed as Log cfu mL⁻¹ and the percentage of survival was calculated from the viable counts recovered after the complete chemically simulated GIT transit with respect to the initial counts (% cfu recovered bacteria / cfu initial bacteria). Experiments were carried out in triplicate.

2.4. Adhesion to the HT-29-MTX cell line

The adhesion capability of the strains was assessed with the epithelial intestinal cell line HT29-MTX which was kindly supplied by Dr. T. Lesuffleur (INSERM U843 Paris, France). This cell line is able to constitutively produce mucin (Lesuffleur, Barbat, Dussaulx, & Zweibaum, 1990). The cell line was maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated bovine foetal 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 and incubations took place at 37°C, 5% CO₂ in a SL Waterjacked CO₂ Incubator (Sheldon Mfg. Inc., Cornelius, Oregon, USA). Culture media were changed every two days and the cell lines

1 were trypsinized with 0.25% trypsin-EDTA solution (Sigma) following standard procedures
2 (Sánchez et al., 2009). For experiments, 10^5 cells mL^{-1} were seed in 24-well plates and
3 incubated to confluence (reaching about 10^7 cells mL^{-1}) for 13 ± 1 days.

4 Parental and bile-adapted derivative strains and the reference strain *B. animalis* subsp.
5 *lactis* Bb12 were grown in MRSC for 24 h. Cultures were harvested by centrifugation,
6 washed twice with Dulbecco's PBS buffer (Sigma) and resuspended in DMEM without
7 antibiotics at a concentration of about 10^8 cfu mL^{-1} . HT29-MTX monolayers were washed
8 twice with Dubelco's PBS to remove the antibiotics and then bacterial suspensions were
9 added in a ratio of epithelial cells:bacteria of 1:10. Plates were incubated for 1 h at 37°C , 5%
10 CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany).
11 After the incubation period, supernatants were removed and wells were softly washed three
12 times with Dubelco's PBS buffer to remove the non-attached bacteria. Afterwards, the
13 monolayers were trypsinized and bacterial counts were carried out in agar-MRSC to
14 determine the number of adhered bacteria. Results were expressed as the percentage of
15 bacteria adhered with respect to the amount of bacteria added (% cfu bacteria adhered / cfu
16 bacteria added). Experiments were carried out in duplicate (using two HT29-MTX plates) and
17 in each plate the strains were also tested in duplicate.

18 2.5. Statistical analysis

19 Data were statistically analysed using the SPSS 11.0 software for Windows (SPSS
20 Inc., Chicago IL, USA) by means of one-way ANOVA tests independently performed at each
21 sampling point. When needed, the mean comparison LSD (least-significant difference, $p <$
22 0.05) test was used to assess differences among fermented milk or strains.

3. RESULTS AND DISCUSSION

3.1. Growth and metabolic activity in milk

The pH and bacterial dynamics of milk fermented with a starter mixture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* containing the parental strain *B. animalis* subsp. *lactis* IPLA4549 or its bile-adapted derivative 4549dOx is depicted in Figure 1. No remarkable statistical differences were detected among control and fermented milk containing bifidobacteria during milk fermentation or during cold storage of the fermented products. Once *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* reached their maximum counts (8.45 ± 0.06 and 7.56 ± 0.02 Log cfu mL⁻¹, respectively) (average counts of all fermentations) they remained without noticeable variations until the end of the storage period, although in all cases a trend towards a slight decrease on the viability is observed, regardless the type of fermented milk. Thus, these data indicates that the presence of the bifidobacteria strains did not modify the growth and acidification capability of the starter mixture used except in the first hour of fermentation, in which a quicker acidification in the control milks was observed. In the same way, counts of bifidobacteria did not suffer significant variations during fermentation. It has been suggested that the recommended daily intake for probiotic strains is around 10⁹ viable bacteria per day (Ouweland, Salminen, & Isolauri, 2002), thus both parental and bile-adapted *B. animalis* subsp. *lactis* strains complied with the recommendations in the fermented milk assayed. In fact, this species is widely present in commercial probiotic fermented milk (Gueimonde et al., 2004) probably because it tolerates technologically adverse conditions, such as low pH and the presence of oxygen, better than other bifidobacteria species (Masco, Crockaert, van Hoorde, Swings, & Huys, 2007; Simpson, Stanton, Fitzgerald, & Ross, 2005).

The metabolic activity of the strains in the fermented milk was assessed by studying the evolution of the saccharides and organic acids (Table 1), as well as of some volatile

compounds (Table 2). Similar trends were observed, whether fermented milks contained or not bifidobacteria (Table 1), although the absence of bifidobacteria reduced the post-acidification process at days 10 and 18 of cold storage (Table 1). Noticeably, acetic acid accumulation was slightly higher in the fermented milk samples that contained bifidobacteria, which is in agreement with its fermentative metabolism (Sánchez et al., 2007). This suggests that the co-cultivation of bifidobacteria and starter strains did not produce, in general, any antagonist or synergic effect that could influence the evolution of these metabolites.

Regarding the main volatile compounds produced (Table 2), diacetyl synthesised by the starter strains increased during the fermentation and cold storage and the levels were not affected by the presence of the bifidobacteria strains ($p > 0.05$). However, significant differences ($p \leq 0.05$) in the levels of acetaldehyde and ethanol were detected among the three fermented milk types. In general, lower concentrations of these metabolites were detected in the control batch with respect to the batches containing bifidobacteria. Fermented milk containing the 4549dOx strain, presented higher levels of acetaldehyde at the end of the refrigeration period. This fact could reflect the modification of glucose metabolism that occurs in the bile-adapted derivative strain as a consequence of the adaptation to bile (Ruas-Madiedo, Hernández-Barranco, Margolles, & de los Reyes-Gavilán, 2005; Sánchez, et al., 2007).

3.2. Probiotic selection criteria

As previously stated, one of the criteria for the selection of orally-delivered probiotic strains is their ability to survive the adverse conditions of the gut. Figure 2 shows the dynamics of the parental strain *B. animalis* subsp. *lactis* IPLA4549 and its bile-adapted derivative 4549dOx at different steps during the chemically simulated GIT transit. The greatest difference between both strains was detected when they were challenged to GJ pH 2.0 during 180 min. A drastic reduction of counts was detected in the parental strain (6.9 Log cfu

1 mL⁻¹) whereas in the bile-adapted derivative was lower (3.6 Log cfu mL⁻¹). This different
2 behaviour of the 4549dOx strain could be due to its acid cross-resistance earned as a
3 consequence of the acquisition of bile resistance (Noriega, Gueimonde, Sánchez, Margolles,
4 & de los Reyes-Gavilán, 2004), which is produced by an increase in its F₁F₀-ATPase activity
5 with respect to the parental strain (Sánchez, de los Reyes-Gavilán, & Margolles, 2006).
6 Meanwhile, the acidic challenge at higher pH (pH 3.0) or at pH 2.0 in the presence of 10%
7 skimmed milk (which increased the pH of the GJ to 4.0) did not produce a loss in the viability
8 of both strains after 180 min of GJ stress. Probably, this is due to the high intrinsic resistance
9 to acidic pH of the species *B. animalis* (Matsumoto, Ohishi, & Benno, 2004). After the GJ pH
10 2.0 and DJ (1% bile salt) challenges, the counts of 4549dOx strain showed lower decreases
11 than strain IPLA4549, but decreased abruptly after the subsequent IJ challenge (0.3% bile salt
12 and 0.1% pancreatin) reaching the lowest counts after 180 min of exposure. No viability loss
13 was detected at the end of the process when the 4549dOx strain was initially challenged to GJ
14 pH 3.0 or pH 2.0 + 10% skimmed milk, probably because the strain was initially less stressed
15 in these conditions. Regarding the parental strain IPLA4549, at acidic pH 3.0 and after the DJ
16 and IJ challenges the counts decreased near 1 Log unit due to the bile stress, whereas in the
17 presence of milk the strain kept its viability probably because of the protective effect of milk
18 components. Finally, the calculation of the bacterial survival after the whole GIT transit
19 showed that when the strains were initially challenged to GJ pH 2.0 they hardly survived
20 (counts lower than 5x10² cfu mL⁻¹). However, the same GJ in the presence of 10% of
21 skimmed milk considerably improved the bacterial survival and statistical differences (p<
22 0.05) were detected between the parental and bile-adapted strains (% survival: 19.6±7.6 and
23 60.0±25.3, respectively). Similar behaviour was detected when strains were initially
24 challenged to GJ pH 3.0 (% survival: 9.5±4.5 and 52.6±25.9 for IPLA4549 and 4549dOx,
25 respectively, p< 0.05). All these results showed that the bile-adapted 4549dOx strain survived

1 slightly better *in vitro* the adverse conditions of the GIT, thus probably being able to reach the
2 colon in a higher number. In this way, other authors have shown that higher tolerance to bile
3 also improves the resistance of *Bifidobacterium breve* UCC2003 to the murine GIT transit
4 (Watson, Sleator, Hill, & Gahan, 2008). In addition, several articles reporting the survival of
5 bifidobacteria after the GIT transit have been published in which different study approaches
6 were carried out. Recently, using a bioreactor model of the stomach-intestine, it has been
7 proven that *B. animalis* subsp. *lactis* is the species that shows the highest survival in
8 comparison to other species of the genus *Bifidobacterium* (Ritter, Kohler, & von Ah, 2009).

9 Another criterion for the selection of potential probiotic strains is their capability to
10 transitory colonise the GIT. In our case, we studied the adhesion of both parental and bile-
11 adapted derivative strains to the epithelial intestinal line HT29-MTX, as the percentage of cfu
12 adhered / cfu added bacteria. The results (IPLA4549 = 2.96 ± 1.74 ; 4549dOx = 5.58 ± 1.60)
13 were compared with those of the reference strain *B. animalis* subsp. *lactis* Bb12 (% adhesion
14 = 3.08 ± 1.37), which shows a good adherence capability to epithelial intestinal cell lines and
15 to human mucus (de Palencia, López, Corbí, Peláez, & Requena, 2008; He et al., 2001). No
16 statistical differences ($p > 0.05$) were detected among the three strains, although the bile-
17 adapted 4549dOx strain showed a slightly higher (almost twice as much) percentage of
18 adhesion than the parental and reference strains.

19 20 **4. CONCLUSIONS**

21 We have studied the technological and probiotic potential of the bile-adapted strain *B.*
22 *animalis* subsp. *lactis* 4549dOx with respect to its parental strain IPLA4549. The bile-adapted
23 derivative strain showed better ability to survive in simulated GIT conditions, and slightly
24 higher adhesion capability to the model epithelial intestinal line HT29-MTX. Both parental
25 and bile-resistant derivative strains were able to keep their viability for the shelf-life of the

1 fermented milk. The strain 4549dOx seems to be more suitable for its inclusion on the
2 formulation of functional foods.

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1 **Table 1**

2 Residual lactose, and production of lactic and acetic acids (mM) in milk fermented with a commercial starter of *S. thermophilus* and *L.*
3 *delbrueckii* subsp. *bulgaricus* (control) in the presence of *B. animalis* subsp. *lactis* IPLA4549 (IPLA4549) or its bile-resistant derivative
4 4549dOx (4549dOx) during the fermentation process and during the cold storage of the fermented products.

Period	Time	Sample	Lactose	Lactic acid	Acetic acid
Fermentation	0h	Control	148.65±9.16	2.46±0.23	0.13±0.07
		Control	145.36±10.32	17.80±6.72	0.06±0.09
		IPLA4549	137.88±13.98	10.52±5.47	0.46±0.51
		4549dOx	136.52±36.86	12.92±4.13	0.42±0.35
	Final	Control	125.09±6.08	121.42±10.10	0.08±0.12 ^b
		IPLA4549	111.35±23.59	104.93±15.99	1.79±0.68 ^a
		4549dOx	103.57±30.30	140.37±47.32	0.71±0.51 ^{ab **}
	Cold storage	2d	Control	111.64±11.32	0.07±0.10 ^b
			IPLA4549	105.65±25.09	1.49±1.05 ^a
			4549dOx	90.01±19.37	0.74±0.52 ^{a **}
		10d	Control	116.35±2.46	0.12±0.17 ^b
			IPLA4549	92.28±32.82	1.26±0.94 ^a
			4549dOx	95.98±25.96	0.46±0.34 ^{ab *}
		18d	Control	95.05±16.88	0.07±0.10 ^b
			IPLA4549	95.98±25.96	1.38±0.98 ^a
			4549dOx	78.33±24.70	0.62±0.45 ^{ab **}
		24d	Control	70.05±28.50	0.00±0.00
			IPLA4549	101.57±20.06	1.57±1.11
			4549dOx	77.23±26.94	1.07±0.93

5 One-way ANOVA: * p<0.05. Within each time period the values that do not share a common superscript are statistically different (p<0.05) according to the LSD (least
6 significant difference) mean comparison test.

Table 2

Production of volatile compounds (μM) in milk fermented with a commercial starter of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the presence of *B. animalis* subsp. *lactis* IPLA4549 or its bile-resistant derivative 4549dOx during the fermentation process and during the cold storage of the fermented products.

Period	Time	Sample	Acetaldehyde	Ethanol	Diacetyl	
Fermentation	0 h	Control	1.36±0.22	16.32±5.50	1.17±0.16	
	1 h	Control	6.09±3.52	11.00±2.11	1.92±0.94	
		IPLA4549	14.91±9.84	16.47±4.22	1.12±0.23	
		4549dOx	12.96±9.81	12.33±4.62	0.97±0.14	
	Final	Control	113.73±10.39	16.18±0.21 ^a	2.85±0.72	
		IPLA4549	156.12±35.48	73.46±24.58 ^b	3.88±0.30	
		4549dOx	151.65±38.14	57.42±16.81 ^b **	4.83±1.53	
	Cold storage	2 d	Control	92.66±19.86	35.46±7.76 ^a	6.28±1.43
			IPLA4549	110.75±20.73	136.64±19.59 ^c	6.75±1.89
4549dOx			129.01±34.54	67.36±18.01 ^b ***	6.70±1.04	
10 d		Control	95.63±30.63	49.68±11.99 ^a	10.87±2.72	
		IPLA4549	106.98±18.35	181.04±63.16 ^b	8.00±3.34	
		4549dOx	96.07±28.62	61.91±15.53 ^a **	9.44±2.03	
18 d		Control	72.67±14.48 ^a	45.84±7.07 ^a	9.13±2.61	
		IPLA4549	89.00±19.28 ^a	140.26±17.69 ^c	8.61±2.20	
		4549dOx	131.95±19.85 ^b **	87.66±35.44 ^b **	11.05±1.92	
24 d		Control	66.91±11.32 ^a	35.16±6.57 ^a	12.75±3.38	
		IPLA4549	103.37±35.72 ^{ab}	148.62±17.80 ^c	11.77±3.58	
		4549dOx	121.58±25.65 ^b *	67.27±29.52 ^b ***	9.81±2.09	

One-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Within each time period the values that do not share a common superscript are statistically different ($p < 0.05$) according to the LSD (least significant difference) mean comparison test.

FIGURE CAPTATIONS

Figure 1

Counts (Log cfu mL⁻¹) of *S. thermophilus* (A), *L. delbrueckii* subsp. *bulgaricus* (B), or *B. animalis* subsp. *lactis* (C), in milk fermented with a commercial starter of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in the presence of *B. animalis* subsp. *lactis* IPLA4549 or its bile-resistant derivative 4549dOx. Samples were taken during the fermentation process and during the cold storage of the fermented products, and pH (D) was also monitored. The final fermentation period (FF) was 277 ± 21 min. Fermented milk types: control (white bars), IPLA4549 (grey bars) and 4549dOx (black bars). At each sampling point, columns that do not share a common letter are statistically different ($p < 0.05$).

Figure 2

Counts (Log cfu mL⁻¹) of *B. animalis* subsp. *lactis* IPLA4549 (black circles) and its bile-resistant derivative 4549dOx (white squares) along the chemically simulated gastrointestinal transit. Three simulated gastric juice conditions were tested: pH 2.0 (A), pH 3.0 (B) and pH 2.0 + 10% skimmed milk (C). The modal value of the coefficient of variation (SD/mean) of these results was 4%.

1 **Figure 1**

2

1 **Figure 2**