1 **Supplementary material:** Hidalgo-Cantabrana, *et al*.

2 **MATERIAL AND METHODS**

3 **Bacterial strains and growth conditions**

4 Three closely related *B. animalis* subsp. *lactis* strains were used in this study: A1 (parental,
5 isolated from a dairy fermented product), A1dOx (adapted to OxGall [Sigma] by exposure of strain A1 to
6 increasing concentrations of these bile salts) and A1doxR (derivative of A1dOx which, after consecutive
7 generations in the absence of bile, spontaneously acquired a ropy phenotype). The three strains are able to
8 synthesise EPS, but only the strain A1dOxR has a ropy phenotype. These strains were cultivated in
9 MRSC [MRS containing 0.25% L-cysteine-HCl (Sigma-Chemical Co., St. Louis, MO)] at 37ºC under
10 anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) in a MG500 chamber (Don Whitley Scientific, West
11 Yorkshire, UK). For routine manipulation and counting, MRS from Biokar (Biokar Diagnostics,
12 Beauvais, France) was used; whereas, the growth curves and proteomic and transcriptimic analyses were
13 performed in MRS from Difco (Difco, BD Biosciences, San Diego, CA).

14 Strains from stocks (stored in MRSC +10% glycerol at -80ºC) were plated on the surface of agar-
15 MRSC and a single colony per strain was picked up to inoculate 10 ml MRSC broth. After overnight
16 incubation this culture was used to inoculate (1%) different volumes of MRSC depending on the
17 experimental setup. For growth curves, 300 ml of fresh MRSC were inoculated, homogenized by stirring
18 and distributed in different volumes (from 15 ml up to 50 ml) and incubated at variable lengths of time
19 during 24 h. At each sampling point, one tube was removed from the anaerobic chamber to perform the
20 corresponding analyses. For proteomic analysis, 50 ml of MRSC were (1%) inoculated and incubated
21 until they reached an $OD_{600\ nm}$ of about 1 (middle exponential phase, around 6±1 h). For gene expression
22 analyses, 10 ml of MRSC were inoculated and incubated, as previously described, for variable times
23 depending on the gene to be tested.

24 **Proteomic analysis**

25 The proteome of the strains A1dOx and A1dOxR was determined by means of two dimensional
26 (2D) electrophoresis analyzing three biological replicates, each one done at least in duplicated gels.

27 Middle-exponential phase cultures were collected by centrifugation and washed twice with PBS before
28 proceeding with the lysis protocol. The cells were broken with ultrasonic treatment (Sonics & materials,
29 Vibra-cell, model VC600, 20 kHz) by sonication twice for one minute and keeping them on ice for one
30 minute between the sonications. Next, unbroken cells and cell debris were removed by centrifugation at
31 (15 000 × g at 4 °C, 5 minutes). The proteins were precipitated by methanol-chloroform (3:1, vol/vol)
32 according to the method described by Wessel and Flugge (6), and the protein concentration of the extract
33 was determined using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL,
34 USA) according to the manufacturer's instructions. The protein extracts were frozen at -20 °C until
35 further use.

36 The proteome of these strains was analysed using different 2D-electrophoresis techniques: i) 2D-
37 gel electrophoresis with traditional blue Coomassie staining (2D-PAGE), to obtain reference maps to
38 spot pick for mass spectrometry analysis, and ii) 2D-difference in gel electrophoresis (2D-DIGE). For
39 2D-PAGE, 500 µg of protein were solubilized in 450 µl Destreak Rehydration Solution (GE HealthCare,
40 Biosciences, Uppsala, Sweeden) supplemented with 0.5 % IPG Buffer (pH 4-7, GE Healthcare). These
41 solutions were used to rehydrate Immobiline™ Drystrips (pH 4-7, 24 cm, GE Healthcare) using an Ettan
42 IPGphor device (GE Healthcare) and focused for 60,000 V. Focused strips were then equilibrated for 15
43 min in equilibration buffer (1 M Tris-HCl, pH 6.8, containing 6 M urea, 30% [vol/vol] glycerol) and 1%
44 (wt/vol) sodium dodecyl sulfate) supplemented with 0.83% (wt/vol) dithiothreitol in the first equilibration
45 step and with 7.5% (wt/vol) iodoacetamide in the second one. The second-dimension separation was
46 performed in 12.5% polyacrylamide gels in an Ettan DALTsix electrophoresis unit (GE Healthcare) at a
47 constant current of 16 mA per gel, at 4 °C. After the electrophoresis, the gels were stained with
48 Gelcode™ Blue Safe Protein Stain (Thermo Fisher Scientific) according to the manufacturer's
49 instructions and finally, the gels were scanned with Imagescanner using Labscan software (Amersham
50 Biosciences). The same protein extracts were also analyzed by 2D-DIGE using fluorescent dyes
51 (Amersham CyDye DIGE Fluors, GE Healthcare). After protein precipitation, samples were solubilized
52 in 20 µl lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea and 4 % (w/v) CHAPS at pH 8.5. Each sample

53 was labeled with different Amersham CyDye DIGE Fluors (minimal dyes: Cy2, Cy3 and Cy5) for Ettan

54 DIGE (GE Healthcare). Briefly, 333 pmol of dye was used for labeling 50 µg of protein and the samples

55 were incubated on ice for 30 min in darkness. After the incubation, 1 µl of 10 mM lysine was added to

56 stop the reaction and the samples were incubated in the same conditions for 10 min. Then, 20 µl of 2X

57 Sample buffer [8 M Urea, 130 mM DTT, 4 % (w/v) CHAPS, 2 % (v/v) IPG Buffer, pH 4-7, GE

58 Healthcare] was added to the samples and they were incubated on ice in darkness for 10 min. Samples for

59 the same gels were pooled together and 330 µl of rehydration buffer [8 M Urea, 4 % (w/v) CHAPS, 1 %

60 (v/v) IPG Buffer, pH 4-7 and 13 mM DTT] was added to the pooled samples. Immobiline™ Drystrips

61 (pH 4-7, 24 cm, GE Healthcare) were rehydrated with the protein solutions and both first-dimensional

62 isoelectric focusing and second dimension separation were performed as described before. In this case, 50

63 µg of protein labeled with fluorescent dyes were loaded in the gels. Finally, gels were scanned in a

64 Typhoon 9400 scanner (GE Healthcare) at a resolution of 100 µm. Spots showing clear differences

65 among strains were chosen for further analysis. Selected spots were excised from the 2D-PAGE gels

66 visualized with Gelcode™ stain and sent to "Unidad de Proteómica- Fundación Centro Nacional de

67 Investigaciones Cardiovasculares Carlos III" (Madrid, Spain) for in-gel trypsin digestion and

68 identification by matrix-assisted laser desorption/ionization coupled to time of flight mass spectrometry

69 (MALDI-TOF) analysis.

70 **Physiological and gene expression analyses**

71 Growth curves of the three strains under study were followed for 24 h and samples were taken

72 every 2 h to monitor the pH decrease, the increase in OD, the bacterial counts, the EPS and organic acids

73 production, as well as the expression of some specific genes. Growth curves were performed, at least, in

74 two biological replicates and measurements in each replicate were done in duplicate. The pH decrease

75 was monitored by using the Crison pH-Meter basic 20 (Crison Instruments SA, Barcelona, Spain). The

76 $OD_{600\ nm}$ was determined using the BioPhotometer plus apparatus (Eppendorf, Hamburg, Germany); from

77 these data, the specific growth rate (µ) and the generation time were calculated from the linear regression

78 curves of ln $OD_{600\ nm}$ vs. time corresponding with the growth phase. For bifidobacterial counting, serial

79 dilutions of samples were made in Ringer, pour plated in agar-MRSC and incubated under anaerobic
80 conditions for 48 h. The organic acids production (expressed in mM) was directly measured in the
81 bacteria-free supernatants (50 µl injection volume) by means of ion-exchange HPLC. The
82 chromatographic system from Waters (Waters, Milford, MA) and the column ICSep-ION-300
83 (Transgenomic, San José, CA) previously described (1) were used.

84 To follow the EPS production during growth, a method based on the use of fluorescent-conjugated
85 lectins was optimised. Samples from each incubation point were centrifuged (10,000*xg*, 15 min at 4°C),
86 washed twice with PBS and resuspended in 1 ml of PBS. When necessary (from 0 to 6 h), samples were
87 concentrated in PBS to have around $1x10^9$ cfu/ml. To favour the attachment of the lectin to the EPS
88 matrix surrounding the bacterial surface, the cellular suspensions were treated in a Vibra-Cell™ sonicator
89 (Sonics & Materials Inc., Danbury, Connecticut) using 2 cycles of continuous ultrasound of 1 min with an
90 intermediate period of cold-storage of 1 min. Afterwards, cells were collected by centrifugation,
91 resuspended in the same volume of PBS and 475 µl of these bacterial suspensions were mixed with 25 µl
92 of concanavalin A-Alexa Fluor488-conjugate (Molecular Probes Invitrogen, Merck, Darmstadt,
93 Germnay) to give a final lectin-concentration of 50 µl/ml. The staining procedure and fluorescence
94 determination was performed in a Cary Eclipse fluorescence-spectrometer (Varian Ibérica SA, Madrid,
95 Spain) as previously described (5). Values of fluorescence emitted were corrected by the number of
96 bifidobacteria obtained at each sampling point and, finally, the relative fluorescence was calculated with
97 respect to the initial point (0 h, relative fluorescence = 1). The linearity range between fluorescence
98 emitted and number of bifidobacteria cells was found to be between $5x10^8$ and 10^{10} cfu/ml, with
99 correlation coefficients of $R^2 = 0.989 \pm 0.002$. Additionally, to discard the quantification of components
100 from MRSC broth potential adhered to bifidobacterial surface, the biomass from each strain was
101 harvested from the surface of agar-MRSC plates (3). These cellular suspensions were processed as
102 previously described, obtaining similar linearity range ($R^2 = 0.976 \pm 0.005$). Finally, the ropy phenotype
103 was visually determined by the occurrence of a filament when a sterile tip was introduced in each
104 sampling-point culture (4).

Quantitative reverse transcription-PCR (qPCR) was used to achieve the expression of the two genes coding for the priming-GTF and the three genes involved in the rhamnose biosynthesis described within the *eps* cluster from *B. animalis* subsp. *lactis* A1dOxR, as well as one gene coding for a DNA-binding protein which was located outside the *eps* cluster. The *16S rRNA* and *recA* genes were used as endogenous reporter controls. The genome location of these genes, accordingly to the annotation of the type strain DSM10140, and the specific primers used in this study are indicated in Table 1. After cell collection, bifidobacteria were mixed with RNA protective bacterial reagent (Qiagen GmbH, Hilden, Germany) and stored at -20ºC until use. The RNA was extracted with the RNeasy minikit (Qiagen) following the manufacturer´s instructions with modifications previously published (5); 3 µg was reverse transcribed into cDNA to perform the qPCR using the ABI Prism 7500 equipment (Applied Biosystems, Foster City, CA) and the SYBR green PCR master mix (Applied Biosystems). Thermal cycling was: initial step 95ºC for 10 min and 35 cycles of 95ºC for 15 s and 60ºC for 1 min. Data obtained for each strain and culture condition, were referred to their reporter genes.

Statistical analysis

Physiological data were analysed by means of independent one-way ANOVA tests in order to assess differences among the three strains and, when necessary, the mean comparison LSD (least significant difference) test was applied (significance level, $p<0.05$). The statistical package SPSS/PC 15.0 (SPSS Inc., Chicago, IL) was used for this analysis.

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142 **Table S1** Primers used in this study for gene expression analyses by means of quantitative-reverse transcriptase-PCR
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Name	Target gene[1]	Predicted function	Sequence 5'→3'	Reference
gtf01207	Balat_1371	Undecaprenyl-phosphate sugar phosphotransferase (RfbP)	**F**: CGTGCTGAGTCGAAAGAATCG **R**: TTGTAGAACGTGATCGGCTCA	(5)
gtf00468	Balat_1392	Galactosyltransferase (CpsD)	**F**:TGACGACTCGTTTGCAACTGA **R**:GCGCAGGCAGCGGAATAC	(5)
Rh1	Balat_1376	dTDP-glucose pyrophosphorylase	**F**: GGAGCCATCGCCAAGCA **R**: GATTATCTCGACGCCGAAGGA	This work
Rh2	Balat_1377	dTDP-4-dehydrorhamnose 3,5-epimerase	**F**: TGCACCTTGGCGGTACCA **R**: CAATCTGACCCTCGTGCATGT	This work
Rh3	Balat_1378	dTDP-D-glucose 4,6-dehydratase	**F**: CCGGCACCAGTTCGTCAA **R**: CGAGTTCGTACATGGCAACATC	This work
Rh1-Rh2	Balat_1376-1377	Intergenic region and part of genes rh1 and rh2	**F**: CGGCAAGAATAATGCCTTTCAT **R**: CCTATGTCGAAGTCGAACTGAAGAA	This work
Rh2-Rh3	Balat_1377-1378	Intergenic region and part of genes rh2 and rh3	**F**:AAATTCCATAGTTCACGCTTTCCA **R**: GGGCCAGTGAATGCTTTGAAT	This work
Hb1	Balat_0642	DNA-binding protein HB1	**F**: AGCAGACCGGTGAGCTTGAG **R**: AATGCTTTCCGCGATGTCTTC	This work
RecA	Balat_1114	Recombinase A	**F**: GGTGGGAATCACCTCGATGT **R**: GCTCTGCCATGCGTTTGG	This work
Bifido	Balat_1279	16S rDNA	**F**: GATTCTGGCTCAGGATGAACGC **R**: CTGATAGGACGCGACCCCA	(2)

144 [1]: gene location according to the genome annotation of the strain *Bifidobacterium animalis* subsp. *lactis* DSM10140 (accession number CP001606