Publisher's version
DOI: 10.1016/j.ijfoodmicro.2016.01.006

1 Streptococcus thermophilus urease activity boosts Lactobacillus delbrueckii subsp. bulgaricus
2 homolactic fermentation
3

4 Stefania Arioli[a*], Giulia Della Scala[a], Maria Chiara Remagni[b], Milda Stuknyte[a], Stefano
5 Colombo[a], Simone Guglielmetti[a], Ivano De Noni[a], Enzio Ragg[a], Diego Mora[a]
6 [a] Department of Food Environmental and Nutritional Sciences (DeFENS), University of Milan,
7 Italy. [b] Sacco srl, Cadorago (CO), Italy.
8
9
10
11
12 Corresponding author: Stefania Arioli
13 Department of Food Environmental and Nutritional Sciences (DeFENS), University of Milan, via
14 Celoria 2, 20133, Milan, Italy
15 Phone: +39 02 50319133
16 Fax: +39 02 50319238
17 E-mail: stefania.arioli@unimi.it
18
19 Running title: The cooperative behaviour of *S. thermophilus* urease
20

21 ABSTRACT

22

23 The proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp.
24 *bulgaricus* in the yogurt consortium enhances the growth rate and size of each population. In
25 contrast, the independent growth of the two species in milk leads to a slower growth rate and a
26 smaller population size. In this study, we report the first evidence that the urease activity of *S.*
27 *thermophilus* increases the intracellular pH of *L. delbrueckii* in absence of carbon source.
28 However, in milk, in presence of lactose the alkalizing effect of urea-derived ammonia was not
29 detectable. Nevertheless, based on glucose consumption and lactic acid production at different
30 pH_{in}, *L. delbrueckii* showed an optimum of glycolysis and homolactic fermentation at alkaline pH
31 values. In milk, we observed that ammonia provided by urea hydrolysis boosted lactic acid
32 production in *S. thermophilus* and in *L. delbrueckii* when the species were grown alone or in
33 combination. Therefore, we propose that urease activity acts as an altruistic cooperative trait,
34 which is costly for urease-positive individuals but provides a local benefit because other
35 individuals can take advantage of urease-dependent ammonia release.

36

37 1. Introduction

38

39 Most food fermentation processes involve mixed cultures in which different microbial species
40 interact with each other. These interactions may have neutral, positive or negative effects on the
41 fitness of the strains performing the fermentation. Microbial interactions that exert positive effects
42 on the micro-organisms participating in the process are classified as mutualistic interactions
43 (Boucher *et al.*, 1982). The best example of mutualism in a food process is represented by the
44 yogurt consortium, which consists of the lactic acid bacteria (LAB) *Streptococcus thermophilus*
45 and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Sieuwerts *et al.*, 2008). In the yogurt consortium,
46 the interactions that occur between the two LAB species in milk have been described as a proto-
47 cooperative process, which has positive effects on the growth rate and size of each population; in
48 contrast, the independent growth of the two species in milk led to a slower growth rate and a
49 smaller population size (Zourari *et al.*, 1992; Sieuwerts *et al.*, 2010). The seemingly simplistic
50 metabolism of these organisms, the availability of complete genomic information for the two
51 species involved in the yogurt consortium (Bolotin *et al.*, 2004; van de Guchte *et al.*, 2006), and
52 the availability of molecular tools suitable for the design of appropriate recombinant strains have
53 made this microbial consortium an attractive model system for studies of species-species
54 interactions. The molecular interactions that play key roles in the mutualistic behaviour of the
55 yogurt consortium have been investigated using several approaches (Courtin *et al.*, 2002; Liu *et*
56 *al.*, 2009; Herve-Jimenez *et al.,* 2009; Sieuwerts *et al.*, 2010) and have been reviewed by several
57 authors (Sieuwerts *et al.*, 2008; Smid *et al.*, 2013). Validated interactions between *S. thermophilus*
58 and *L. delbrueckii* during the growth of these species in milk have been described in relation to: i)
59 the availability of nitrogen in milk (Courtin *et al.*, 2002), ii) the exchange of formic acid, pyruvic
60 acid and folic acid (Crittenden *et al.*, 2003), iii) the production and utilisation of carbon dioxide
61 (Driessen *et al.*, 1982), iv) and the metabolism of purine, amino acid, long-chain fatty acids

62 (Sieuwerts *et al.*, 2010) and iron (Herve-Jimenez *et al.*, 2009). A schematic summary of these
63 metabolic interactions is presented in Figure 1. As expected, most of the previously described
64 interactions are trophic interactions in which the species feed each other with pyruvate, folate, long
65 chain fatty acids, ornithine, carbon dioxide, peptides, amino acids and putrescine (Sieuwerts *et al.*,
66 2010). Among the LAB involved in dairy fermentation processes, urease activity is present and
67 widely distributed in only *S. thermophilus*, for which urease-negative strains are rare in nature
68 (Driessen *et al.*, 1982;; Mora *et al.*, 2002; Mora *et al.*, 2005; Zourari *et al.*, 1991). Urease is a
69 multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyses the hydrolysis of urea to yield
70 ammonia and carbamate. Urease is encoded by an 11-gene operon that accounts for 0.9 % of the
71 estimated core genome of *S. thermophilus* (Mora *et al.*, 2004; Rasmussen *et al.*, 2008). The urease
72 activity of *S. thermophilus* shares metabolic relationships with the biosynthetic pathways involved
73 in aspartate, glutamine, arginine and carbon dioxide metabolism (Arioli *et al.*, 2007; Arioli *et al.*,
74 2009; Monnet *et al.*, 2005). Notably, urea hydrolysis increases the catabolic efficiency of *S.*
75 *thermophilus* by modulating the intracellular pH and increasing the activity of β-galactosidase,
76 glycolytic enzymes and lactate dehydrogenase (Arioli *et al.*, 2010). Urea hydrolysis results in
77 increases in both intracellular (pH_{in}) and extracellular (pH_{out}) pH due to the rapid diffusion of
78 ammonia outside of the cell. Consequently, in the presence of urea and a urease-positive
79 microorganism, urease-negative microorganisms share the environmental benefit derived from the
80 release of ammonia and the related transient local pH increase (Arioli *et al.*, 2010). In this study,
81 we analysed the effect of urea hydrolysis by *S. thermophilus* on variations in the pH_{in} and lactic
82 acid fermentation of *L. delbrueckii* to obtain new information related to the proto-cooperation that
83 exists between these two species during yogurt production.

84 2. Materials and Methods

85 2.1. Bacterial strains and growth conditions

86 *S. thermophilus* CNRZ385, the urease-negative mutant A16(*ΔureC3*) (Mora *et al.*, 2004), and
87 *Lactobacillus delbrueckii* MIM91 were maintained in lactose M17 broth (Difco, Laboratories,
88 Detroit, MI) containing 20 g/L of lactose and in MRS broth (Difco, Laboratories, Detroit, MI) at
89 42 °C. For pH_{in} measurements, the strains were grown at 42°C in the appropriate medium until the
90 culture reached an OD_{600nm} of 1.0. The cells were then collected by centrifugation at 15,000 x *g*
91 for 1 min and suspended in solution A (9 g/L of NaCl and 100 µg/ml of chloramphenicol-Sigma-
92 Aldrich, Milan, Italy, in distilled water). Chloramphenicol at the concentrations used here was
93 expected to block translation, so that only enzymes already present within the cell are expected to
94 participate in the experiment (Lopez *et al.*, 1998).

95 2.2. Flow cytometric measurement of intracellular pH and D/L lactic acid quantification

96 The internal pH of *S. thermophilus* and *L. delbrueckii* was measured using the pH-sensitive
97 fluorescence probe 5 (and 6-)-carboxyfluorescein succinimidyl ester (cFSE), based on the method
98 originally described by Breeuwer (Breeuwer *et al.*, 1996), and later described by Sawatari and
99 Yokota (Sawatari *et al.*, 2007), with some modifications. The fluorescence intensity of this probe
100 increases at alkaline pH and decreases at acidic pH. Cell suspensions in solution A were obtained
101 as described above, diluted to an OD_{600nm} of approximately 0.5 and supplemented with 4 µM
102 cFDASE (Sigma-Aldrich, Milan, Italy), which is a precursor molecule of cFSE. The suspensions
103 were then incubated for 30 min at 37°C. During this incubation, the membrane-permeating
104 cFDASE was cleaved by intracellular esterases and the resultant cFSE molecules were conjugated
105 to the aliphatic amines of intracellular proteins. After centrifugation at 15,000 x *g* for 1 min and
106 washing with solution A, the cells were suspended in the same volume of solution A.

107 The unconjugated probe was eliminated by the addition of glucose at a final concentration of 16
108 mM and subsequent incubation at 37°C for 1 h. After centrifugation and washing with solution A,

109 the cells were suspended to obtain a final cell concentration that corresponded to 10^9 events per
110 ml. To be sure that the unconjugated and free probes were eliminated by the cells, we monitored
111 the cells fluorescence before and after the treatment with glucose. Moreover, the stability of the
112 cells fluorescence was assessed steadily; the stained cells kept in ice and in solution A after staining
113 maintained a stable fluorescence, indicating that no free cFSE molecules were inside the cells. A
114 part from pH, the intensity of the fluorescence depends on the esterase activity of the cells and
115 could be a species and/or strain dependent feature. In all experiment, after staining we observed a
116 major fluorescence intensity in *L. delbrueckii* than in *S. thermophilus*, reflecting a diverse esterase
117 content in the two species, as previously reported for strains belonging to other bacterial species
118 (Ouzari *et al.*, 2006).
119 Cell counting was performed using an Accuri C6 flow cytometer (BD Biosciences, Milan, Italy).
120 In flow cytometry, particles/cells that pass through the beam will scatter light, which is detected
121 as forward scatter (FSC) and side scatter (SSC). FSC correlates with cell size, cell shape and cell
122 aggregates, whereas SSC depends on the density of the particles/cells (*i.e.*, the number of
123 cytoplasmic granules and membrane size); in this manner, cell populations can often be
124 distinguished based on differences in their size and density (Cronin *et al.*, 2010, Gunasekera *et al.*,
125 2003). The combination of scattered and fluorescent light is measured by a number of detectors as
126 the cells pass by an interrogation point in a fluid stream (Sincock and Robinson, 2001). Cell
127 suspensions that were prepared as described above were analysed using the flow cytometer with
128 the following threshold settings: FSC 5,000, SSC 4,000, and 20,000 total events collected. All of
129 the parameters were collected as logarithmic signals. The 488 nm laser was used to measure the
130 FSC values. The rate of events in the flow was generally lower than 2,000 events/s.
131 The obtained data were analysed using BD Accuri™ C6 software version 1.0 (BD Biosciences,
132 Milan, Italy). The cFSE fluorescence intensity of stained cells was recovered in the FL1 channel
133 (excitation 488 nm, emission filter 530/30, provided by BD Biosciences, Milan, Italy). Density

134 plots of green fluorescence (FL1) and FSC allowed for optimal distinction between the cFSE-
135 stained microbial cells and instrument noise or sample background. An electronic gate on the green
136 fluorescence/FSC density plot was used to select the measured bacterial concentration (events per
137 ml), and the selected data from the bacterial gate were subsequently visualised on a cFSE green
138 fluorescence histogram for further analysis. To measure pH_{in}, 5 ml of a cFSE-labelled cell
139 suspension were incubated at 42 °C in the presence or absence of 2.5 mM urea or 5 mM ammonia
140 for 15 min. Every 5 min, 100 µl of suspension was sampled and the internal pH was determined
141 by measuring the fluorescence intensities in the FL1 channel via flow cytometry. A calibration
142 curve that reported FL1 fluorescence *vs*. pH_{in} was obtained as described below. An aliquot of the
143 cell suspension was washed and suspended in different buffers with pH values ranging from 6.96
144 to 8.25 for *S. thermophilus* and from 6.36 to 7.82 for L. *delbrueckii* and treated with 100 µM
145 gramicidine (Sigma-Aldrich, Milan, Italy), which dissipates the transmembrane proton gradient.
146 The fluorescence intensity was then measured for calibration at appropriate external pH values.
147 (*only for reviewers:* the calibration curves obtained for *S. thermophilus* and *L. delbrueckii* strains
148 are shown in Figure 4S1 and Figure 5S1). Alternatively, pH_{in} was measured in urea-free skimmed
149 milk, obtained by treating reconstituted skimmed milk (Difco, Laboratories, Detroit, MI) with 1.36
150 U ml^{-1} of jack bean urease (Sigma-Aldrich, Milan, Italy) for 3 h at 37°C prior to sterilisation (110°
151 C, 10 min) (Pernoud *et al.*, 2004). In all experiments, external pH (pH_{out}) was measured using a
152 standard pH meter. D/L lactic acid was quantified after 15 min of incubation at 42 °C in urea-free
153 skimmed milk using a D/L lactic acid kit (R-Biopharm, Milan, Italy) according to the
154 manufacturer's instructions. All data are presented as the average of three independent replicates
155 ± SEM.

156 2.3. Flow cytometry total cell count

157 Total cell count of *S. thermophilus* and *L. delbrueckii* culture in reconstituted skimmed milk was
158 measured by flow cytometry after 3 and 6 h of incubation at 42 °C. 500 µl of milk culture was

159 subjected to a clearing procedure by adding an equal volume of Tris-HCl (2 M) EDTA (0.2 M)
160 buffer (pH 8). Following 10 min of incubation at 50 °C the cell suspension was labelled with
161 SYBR green I (Sigma-Aldrich, Milan, Italy). After incubation at 37 °C for 20 min, the labelled
162 cell suspension was diluted to reach approximately 10^6 events per ml and analysed by flow
163 cytometry. The obtained data were analysed using BD Accuri™ C6 software version 1.0 (BD
164 Biosciences, Milan, Italy). The SYBR green I fluorescence intensity of stained cells was recovered
165 in the FL1 channel. Density plots of green fluorescence (FL1) *vs* FSC allowed for optimal
166 distinction between the SYBR green I-stained microbial cells and instrument noise or sample
167 background. An electronic gate on the green fluorescence/FSC density plot was used to select and
168 measure the total bacterial concentration (events per ml) (*only for reviewers:* Fig. 9S1).

169 2.4. In vivo NMR spectroscopy

170 All the NMR spectra were recorded using a Bruker AV-600 spectrometer (Rheinstetten,
171 Germany), operating at a frequency of 600.1 MHz.

172 For ^{13}C- experiments, *L. delbrueckii* cells grown in MRS were collected in the exponential growth
173 phase (*i.e.*, at an $O.D._{600nm}$ of 1.0) and washed and suspended in solution A containing 10 % (v/v)
174 D_2O to obtain a cell density that corresponded to 8-9 mg of protein per ml. The lactose metabolism
175 of non-growing *L. delbrueckii* was studied in energetically discharged cells. Specifically, a cell
176 suspension that was prepared in solution A (see section 2.1) as described above was de-energised
177 via incubation at 42°C for 20 min in the presence of 2 mM NH_3, as previously described for other
178 LAB species (21). After incubation, the cell suspension was washed four times with solution A,
179 the pH was adjusted to 6.5 with lactic acid, and the cells were considered to be energetically
180 discharged cells (EdCs). The NMR experiments were performed using a 5-mm NMR tube
181 containing 600 µl of EdCs. ^{13}C NMR spectra were acquired sequentially after the addition of [1-
182 $^{13}C^{Glc}$]lactose (16 mM) (Omicron Biochemicals Inc., South Bend, IN, USA) with or without 0.5
183 mM ammonia. The ^{13}C-lactic acid generated by homolactic fermentation was monitored non-

184 invasively for 30 min at 42°C. Quantitative kinetic data were taken from resonance intensities of
185 [1-$^{13}C^{Glc}$]-lactose and ^{13}C-lactic acid, directly measured during the *in vivo* experiments. A
186 reference NMR spectrum of a ^{13}C-lactose solution without cells was measured with the same
187 acquisition parameters. Relevant ^{13}C-NMR acquisition parameters were: 30° flip-angle reading
188 pulse; 36 KHz spectral width; 0.9 s acquisition time; 96 scans, 64K time domain; 2 s relaxation
189 delay. Proton broad-band decoupling was achieved by a waltz-type pulse sequence.
190
191 2.5. Assay of β-galactosidase activity
192 β-galactosidase activity of *L. delbrueckii* has been measured on cells prepared as described for
193 NMR analysis. In specific, aliquots of cells suspension in solution A were incubated on ice or at
194 42 °C for 30 min in presence and absence of lactose 16 mM and ammonia 2.5 mM. After
195 incubation the cell suspension was washed in 100 mM Tris-HCl buffer pH 7 and subjected to a
196 mechanical disruption using a Precellys bead beater (Advanced Biotech Italia srl, Seveso, Italy).
197 The amount of total proteins of each lysate was measured using the Bradford method with BSA as
198 the standard. Measurement of the β-galactosidase activity was performed in 100 mM Tris-HCl
199 buffer pH 7, using 10 μl of total cell extract to a final 200 μl volume containing 0.2 mg/ml of 2-
200 nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, Milan, Italy) at 42 °C, by monitoring the
201 optical density at 415 nm with a microplate-reader EON (BioTek Instruments, Inc., CA, USA)
202 programmed for a reading set of 40 repetitions with intervals of 3 min. The β-galactosidase activity
203 was calculated as the max velocity (mO.D.$_{415\,nm}$ per min) per µg of protein using the Gen5 software
204 (BioTek Instruments, Inc., USA) as the mean of four independent determinations.
205 2.6. Measurement of pH_{in}-dependent glycolysis and homolactic fermentation activity
206 To evaluate the pH_{in} dependency of *L. delbrueckii* glycolysis and homolactic fermentation activity,
207 the rate of glucose consumption and lactic acid production were measured in the presence of
208 gramicidine 100 µM at various pHs between 5.0 and 9.0. The cells were collected in exponential

209 grow phase (O.D. $_{600\ nm}$ 1.0) in MRS medium, harvested, and washed with solution A. The cells
210 were concentrated to obtain a cell density corresponding to 7-8 mg of protein per ml in MES buffer
211 100 mM (for pH 5.0 to 7.0), or Tris-HCl 100 mM (for pH 7.5-9.0), in presence of gramicidine 100
212 µM. The reaction was started by the addition of glucose at a final concentration of 16 mM. The
213 mixtures were incubated at 42 °C for 1 h. After being incubated, the mixtures were centrifuged at
214 15000 x g for 5 min at 4°C, and the supernatants were filtered through a 0.2-µm-pore-size
215 membrane (VWR International PBI srl, Milan, Italy). The glucose and lactic acid concentrations
216 of the filtered supernatants were measured by HPLC analysis. The separation of glucose and lactic
217 acid was performed by means of HPLC using a Waters (Milford, MA, USA) equipment consisting
218 of an Alliance 2695 pump system and a 410 refractive index detector. A cation exchange column
219 Aminex HPX-87H (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) maintained at 50
220 °C was used. The isocratic elution was run at a flow rate of 0.6 mL/min using 0.01 N sulphuric
221 acid aqueous solution as a mobile phase. Data were collected and processed using Empower™
222 software (Waters S.p.A., Italy). Glucose and lactic acid concentrations were calculated by the
223 external standard method using analytical grade standard solutions of glucose and lactic acid
224 (Sigma-Aldrich, Milan, Italy) diluted in 0.01 N sulphuric acid. All analyses were run in triplicate.
225 2.7. Effect of urea on the growth of S. thermophilus and L. delbrueckii in mixed culture
226 Urea-free skimmed milk prepared as above described, without and with the addition of 2.5 mM of
227 urea, and pre-heated at 42 °C, were inoculated with overnight cultures of strains CNRZ385 and
228 MIM91, each one at a final concentration of 10^7 CFU/ml. The inoculum concentration was
229 standardized after total cell count performed by flow cytometry and SYBR green I labelling. Total
230 cell count (events per ml) and cultivable count of *S. thermophilus* and *L. delbrueckii* was measured
231 after 3 and 6 h of incubation at 42 °C. Total cell count was measured by flow cytometry as
232 previously described, whereas the viability of the mixed cultures was carried out by standard
233 plating on M17 (lactose 20 g/L) incubated at 37 °C for 24 h and MRS (pH 5.5) incubated at 37 °C

234 for 48 h respectively for *S. thermophilus* and *L. delbrueckii*, in anaerobic condition in presence of

235 Anaerocult® A (Merck, Germany). All data are presented as the average of three independent

236 replicates ± SEM.

237 **3. Results and Discussion**

238

239 3.1. The ammonia released by the urease activity of S. thermophilus modulates the pH_{in} of L.
240 delbrueckii

241 To verify the effect of the urease activity of *S. thermophilus* on the pH_{in} of urease-negative LAB,
242 a preliminary model study was performed using bacterial populations in solution A in the absence
243 of a carbon source. Variations in pH_{in} were monitored based on the pH-dependent fluorescence of
244 cFSE molecules that were used to label urease-negative LAB. An initial experiment was
245 performed using the urease-negative strains *S. thermophilus* A16, a DSM20617 derivative urease-
246 negative (Mora *et al*., 2004) and *L. delbrueckii* MIM91. The addition of 5 mM ammonia to cFSE-
247 labelled cells significantly increased cFSE fluorescence in both species (Fig. 2). The observed
248 increase in fluorescence represented a shift in pH_{in} from 7.04 to >7.80 in *S. thermophilus* and from
249 5.65 to 7.72 in *L. delbrueckii*. Variations of pH_{out} from pH 6.68 to 9.60 and from pH 5.16 to 9.70
250 were observed for *S. thermophilus* and *L. delbrueckii*, respectively. In the absence of added
251 ammonia, the cFSE fluorescence was stable during the time of the experiments (Fig. 2).
252 To verify the effect of the ammonia released by urease activity, a mixed population composed of
253 unlabelled urease-positive *S. thermophilus* CNRZ385 and cFSE-labelled urease-negative *S.*
254 *thermophilus* A16 or *L. delbrueckii* MIM91 was prepared to obtain equivalent numbers of cells
255 per ml. The *S. thermophilus* A16 and MIM91 populations were detected easily and were
256 discriminated from the CNRZ385 population based on the level of cFSE fluorescence (Fig. 3).
257 Fifteen min after urea addition, the release of ammonia caused by the urease activity of the
258 unlabelled CNRZ385 strain mediated an increase in the extracellular pH (pH_{out}) from 6.60 to 8.60
259 and an increase in the cFSE fluorescence of strain A16 that corresponded to an increase of >1.0
260 units of pH_{in} (*i.e.*, from 6.6 to > 7.8) (Fig. 3A). In the absence of urea addition, cFSE fluorescence
261 and the associated pH_{in} were stable during the time of the experiments. These results demonstrate

262 that the ammonia released by the urease-positive population diffuses inside urease-negative cells
263 and causes an increase in pH_{in}. Analogously, the pH_{in} of *L. delbrueckii* became more alkaline as a
264 consequence of the urea hydrolysis performed by *S. thermophilus* cells (Fig. 3B). Remarkably,
265 during the incubation time, we observed an increase in the fluorescence of the unlabelled *S.*
266 *thermophilus* CNRZ 385 population, which likely occurred due to the release of unconjugated
267 cFSE molecules from the labelled populations of *S. thermophilus* A16 and *L. delbrueckii* MIM91.
268 Nevertheless, this phenomena did not interfere with the data analysis. Then, we have further
269 investigated whether ammoniac could affect homolactic fermentation in *L. delbrueckii* MIM91.
270 3.2. Effect of urea and ammonia on pH homeostasis and lactic acid production in milk in S.
271 thermophilus and L. delbrueckii
272 Urease-positive *S. thermophilus* CNRZ385 and urease-negative *L. delbrueckii* MIM91 were tested
273 in urea-free skimmed milk that was preheated to 42 °C. The effect of urea or ammonia alkalisation
274 on pH homeostasis and homolactic fermentation was analysed in each species. In the absence of
275 urea or ammonia, both species exhibited reduced pH_{in} and pH_{out} as a consequence of the production
276 of lactic acid. In *S. thermophilus*, the addition of urea or ammonia resulted in higher values of pH_{in}
277 compared to the previous experimental condition and a significant increase in L-lactic acid
278 production (Fig. 4), which is in accordance with previous observations (Arioli *et al.*, 2010;
279 Pernoud *et al.*, 2004). Similar to *S. thermophilus*, the homolactic fermentation of *L. delbrueckii*
280 appeared to be boosted by ammonia, as revealed by the measured amount of D-lactic acid, which
281 increased from 7.9 ± 0.2 to 9.4 ± 0.1 mM in the absence and presence of ammonia, respectively.
282 In contrast, after the addition of ammonia, the pH homeostasis of *L. delbrueckii* exhibited different
283 behaviour from that of *S. thermophilus*. In *L. delbrueckii,* we observed the formation of a Δ (*i.e.*,
284 $\Delta pH = pH_{in} - pH_{out}$) that was close to zero (Fig. 5), which is a phenomenon that was previously
285 described in other LAB, such as *L. acidophilus* and *L. plantarum*, after exposure to alkaline stress

286 (Sawatari and Yokota, 2007). In specific, it could be hypothesized that in the two species H^+ were
287 not externalized at the same rate as lactate was produced.
288 3.3. Dynamics of lactose consumption and lactic acid production in L. delbrueckii
289 The stimulation of *L. delbrueckii* lactose consumption and lactic acid production by ammonia was
290 confirmed using in vivo NMR analysis of energetically discharged cells (EdCs) (Arioli *et al.*,
291 2010) that were activated with (1-$^{13}C^{Glc}$)-lactose. The dynamics of *in vivo* lactic acid productions
292 (Fig. 6) demonstrate that the presence of ammonia strongly affected homolactic fermentation.
293 Specifically, (1-^{13}C)-lactic acid concentrations remained close to 3.5 mM when the EdCs were
294 supplied with (1-$^{13}C^{Glc}$)-lactose only but reached a final concentration of 4.57 mM when ammonia
295 was supplied to the cell suspension. Conversely, lactose consumption appeared to be faster in the
296 presence of ammonia (Fig. 6), especially during the first minutes of the incubation. Interestingly,
297 the amount of (1-^{13}C)-glucose was always below the detection limit of the instrument, indicating
298 that in *L. delbrueckii*, lactose transport rather than glycolysis is the limiting step of energetic
299 metabolism. The opposite was reported for *S. thermophilus* (Arioli *et al.*, 2010). Like *S.*
300 *thermophilus*, *L. delbrueckii* uses only the glucose moiety of lactose while galactose is supposed
301 to be used in antiporter for the intake of lactose (Chervaux *et al.*, 2000; Leong-Morgenthaler *et*
302 *al.*, 1991). In this context, ammonia could increase the activity of the transport and/or the activity
303 of β-galactosidase. In both the scenario, the lactose transport should increase in efficiency. When
304 NH_4Cl was used instead of ammonia, it was not observed any stimulating effect on lactic acid
305 production or lactose consumption (Fig. 6S1). Moreover, NH_4Cl is a salt molecule and was not
306 able to modify the pH_{in}, as revealed by the absence of cFSE fluorescence increase using cells
307 suspended in solution A (Fig. 6S2); a significant shift at high alkaline pH_{in} values was instead
308 observed using ammonia (Fig. 2B and repeated in Fig. 6S2). The absence of a stimulating effect
309 of NH_4Cl on homolactic fermentation could be due to the inability of *L. delbrueckii* to transport
310 ammonium ions inside the cell. This hypothesis is corroborated by the absence of ammonium

311 specific transport in the publically available genomes of this species. Moreover, a previous study
312 dealing on the physiology of *L. delbrueckii* in a chemical defined medium, revealed that the
313 elimination of ammonium ions from the media formulation did not affect the growth efficiency of
314 the microorganism (Chervaux *et al.*, 2000). We therefore conclude that only ammonia and not its
315 protonated form is actively affecting homolactic fementation in *L. delbrueckii*.
316 NMR spectra (Fig. 6A) showed that only the signals coming from lactose and lactic acid were
317 detectable by ^{13}C-NMR. The absence of a stoichiometric carbon balance between the lactose
318 consumed (4.49 mM in presence of ammonia) and the lactic acid detected (4.57 mM instead of the
319 expected 8.98 mM), can be due to the presence of glycolytic intermediates (i.e. fructose 1,6-
320 bisphosphate, 3-phosphoglycerate, phosphoenolpyruvte) (Neves *et al.*, 1998) with an intracellular
321 concentration unable to produce NMR signals with heights distinguishable from the instrumental
322 noise. In order to evaluate the enzymatic stability, β-galactosidase activity was measured in EdCs
323 before and after 30 min of incubation at 42 °C. The results obtained (data not shown, *only for*
324 *reviewers:* Fig. 6S3) showed that the specific β-galactosidase activity did not significantly change
325 after incubation at 42 °C, thus indicating that this enzyme is stable during the time of the
326 experiment.
327
328 3.4. pH-dependent glucose consumption and lactic acid production in L. delbrueckii
329 Based on glucose consumption and lactic acid production at different pH_{in}, *L. delbrueckii* showed
330 an optimum of glycolysis and homolactic fermentation at the alkaline pH 8 (Fig. 7), a value not
331 dissimilar to what we previously observed for *S. thermophilus* (Arioli *et al.*, 2010). These data
332 highlights that homolactic fermentation enzymes of *L. delbrueckii* are optimized at neutral alkaline
333 pH, such as those of other lactic acid bacteria (Hutkins and Nannen, 1993; Sawatari and Yokota,
334 2007).

335 3.5. Variation of cFSE fluorescence, pH_{out}, and D/L lactic acid production in the yogurt
336 consortium

337 The cFSE fluorescence of the mixed culture yogurt consortium was monitored via flow cytometry
338 in the presence and absence of 2.5 mM urea or 5 mM NH_3. Because the cFSE labelling protocol
339 required several steps of biomass manipulation (see Materials and Methods), which could affect
340 the metabolic activity of the cells but not the cell viability (Breeuwer *et al.*, 1996), both species
341 were labelled in this experiment. As a result, the use of two cFSE-labelled populations did not
342 allow for the calculation of the pH_{in} because the fluorescence values of the *S. thermophilus* and *L.*
343 *delbrueckii* cells were superimposed. Therefore, we monitored the overall variations in the
344 fluorescence of the bacterial consortium (Fig. 8). The flow cytometry data revealed that the cFSE
345 fluorescence values and the corresponding pH_{in} values were affected, with different kinetics, by:
346 i) the release of ammonia due to the urease activity of *S. thermophilus* cells only after 10 min of
347 incubation and ii) by ammonia supplementation in the milk. After 15 min of incubation in milk,
348 milk supplemented with urea, and milk supplemented with ammonia, the pH_{out} values were 6.36
349 ± 0.04, 6.45 ± 0.05 and 6.70 ± 0.04, respectively. Considering that the addition of 5 mM NH_3 to
350 the mixed culture caused an immediate increase in pH_{out} from 6.68 ± 0.03 to 6.79 ± 0.02, the pH_{out}
351 values that were reached after 15 min of incubation revealed that the mixed cultures produced an
352 amount of lactic acid capable of counteracting the alkalising effect of the ammonia molecules
353 added to the milk or generated by urea hydrolysis. D- and L-lactic acid measurements in a mixed
354 culture yogurt consortium confirmed previous observations for single-species cultures (*i.e.,*
355 homolactic fermentation was boosted in the presence of urea and ammonia). The amounts of D-
356 and L-lactic acid were 26 % and 20 % higher than the control value when the milk was
357 supplemented with urea and ammonia, respectively.

358 3.6. Urea hydrolysis by S. thermophilus increases the population size of the yogurt consortium

359 The effect of urea hydrolysis on the population size of a milk mixed culture of strain *S.*
360 *thermophilus* CNRZ385 and *L. delbrueckii* MIM91 was detectable after 6 h of incubation at 42 °C
361 (Fig. 9). In presence of urea, the two populations showed an increment in total count (events per
362 ml) of 38 %. Moreover, plate count analysis on differential media revealed that strain CNRZ385
363 and MIM91 showed a statistically significant increment in CFU/ml of 35 % and 19 %,
364 respectively. These results show that urea metabolism may have an influence on the population
365 size of *S. thermophilus* and *L. delbruekii* in yogurt consortium, most probably due to the effect of
366 urea hydrolysis on homolactic fermentation.
367
368 ## 4. Conclusions
369
370 During milk fermentation, *S. thermophilus, L. delbrueckii*, and most LAB are faced with
371 constantly changing environmental stimuli and stresses, which can affect cellular physiology.
372 These predictable environmental changes include pH variations, the limitation of nutrient
373 availability, and the accumulation of toxic metabolites (*i.e.*, lactic acid) formed by the fermentation
374 process. Exposure to low pH for a long period of time causes an arrest of growth, a dramatic
375 reduction of glycolytic fluxes and a progressive loss of viability (Cotter and Hill, 2003; Hutkins
376 and Nannen, 1993; Siegumfeldt *et al.*, 2000). Urease activity, which is an enzymatic reaction that
377 is known to be a stress response that is activated to counteract acidic environmental pH in a number
378 of bacteria, has been described as a potential metabolic regulatory mechanism for energetic
379 metabolism in the dairy bacterium *S. thermophilus*. In *S. thermophilus*, urease increases the overall
380 change in enthalpy that is generated by microbial metabolism as a consequence of increased
381 glycolytic flux (Arioli *et al.*, 2010). Outside of the 'selfish' utility of urease for cells harbouring
382 this enzymatic activity, the cooperative relevance of urease in an ecological context in which
383 different microbial species share the same environment has been poorly investigated. According

384 to the results presented here and on the basis of previous observations (Arioli *et al.*, 2010; Monnet
385 *et al.*, 2005), urease production should be considered an altruistic cooperative trait, which is costly
386 for urease-positive individuals but provides a local benefit because other individuals can take
387 advantage of the release of ammonia. However, the effect of ammonia molecules on the lactose
388 consumption and lactic acid production of *L. delbrueckii* subsp. *bulgaricus* MIM91 in milk cannot
389 be directly linked to the variation of pH_{in} because intracellular alkalization was detected only in
390 saline solution in absence of carbon source.
391 Unlike other described cooperative behaviours, such as those involving siderophores (Griffin *et*
392 *al.*, 2004), urease first provides a benefit to the individual that harbours the enzymatic activity and
393 then provides a local benefit. In this study, we report the first evidence that the urease activity of
394 *S. thermophilus* affects the physiology of this species during milk fermentation. These findings
395 put a new light in the interactions that occur between the two species in the yogurt consortium, but
396 also between a urease-positive species and the other urease-negative lactic acid bacteria in milk
397 environment. In this context, urease activity should be taken into account as a metabolic booster
398 for the optimal growth of lactic acid bacteria in milk. In every microbial cell, the modulation of
399 cellular catabolism arises from multiple overlapping regulatory mechanisms and from metabolic
400 feedback into regulatory networks. In this context, within the yogurt consortium, urease-dependent
401 ammonia could directly affect kinetic parameters of enzymes involved in homolactic fermentation
402 of both *S. thermophilus* and *L. delbrueckii*. The boost of lactic acid production that is triggered by
403 urease in this microbial consortium occurs when *S. thermophilus* show the maximum urease
404 activity (*i.e.* in the early exponential growth phase, Mora *et al.*, 2005) to support their “selfish”
405 energetic behaviour (*i.e.,* rapid sugar consumption and lactic acid production) (Mora *et al.*, 2013).
406 From an ecological point of view, we should note that microbes do not live as single species in
407 natural environments but co-exist with other microbial species. Therefore, the adaptation process

408 of a defined environmental niche appears to be strongly influenced by the metabolic relationships
409 that exist among different species within the community.
410 The data presented here still do not clarify the role/s of ammonia molecules in the microbial
411 metabolism. The increase of lactic acid production in *L. delbrueckii* could be the consequence of
412 a trophic interaction modulated by ammonia released by *S. thermophilus* urease activity. In
413 microorganisms, carbon and nitrogen metabolisms are largely interconnected, and glycolytic
414 intermediates are also precursors of molecules involved in nitrogen metabolism. Nevertheless, the
415 genome of *L. delbrueckii* highlights the intrinsic evidence for specialization through loss of
416 function provided by the complete absence of a large number of enzymes involved in the
417 biosynthesis of amino acids, as a consequence of the species adaptation to the protein-rich milk
418 environment (van de Guchte *et al.*, 2006). However, the publically available genomes of *L.*
419 *delbrueckii* revealed two enzymes involved in ammonia utilization, glutamine synthase and
420 asparagine synthetase, but a direct link between their activities and the carbon metabolism is far
421 from clear.
422

423 REFERENCES

424

425 Arioli S, Monnet C, Guglielmetti S, Parini C, De Noni I, Hogenboom J, Halami PM, Mora D.
426 2007. Aspartate biosynthesis is essential for the growth of *Streptococcus thermophilus* in milk,
427 and aspartate availability modulates the level of urease activity. Applied and Environmental
428 Microbiology 73: 5789-5796.

429 Arioli S, Ragg EM, Scaglioni L, Fessas D, Signorelli M, Karp M, Daffonchio DG, De Noni I,
430 Mulas L, Oggioni M, Guglielmetti S, Mora D. 2010. Alkalizing reactions streamline cellular
431 metabolism in acidogenic microorganisms. Plos One 5(11): e15520.

432 Arioli S, Roncada P, Salzano AM, Deriu F, Corona S, Guglielmetti S, Bonizzi L, Scaloni A, Mora
433 D. 2009. The relevance of carbon dioxide metabolism in *Streptococcus thermophilus*.
434 Microbiology 155:1953-1965.

435 Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich, Kulakauskas S, Lapidus A, Goltsman E,
436 Mazur M, Pusch GD, Fonstein M, Overbeek R, Kyprides N, Purnelle B, Prozzi D, Ngui K, Masuy
437 D, Hancy F, Burteau S, Boutry M, Delcour J, Goffeau A, Hols P. 2004. Complete sequence and
438 comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nature
439 Biotechnol. 22:1554-1558.

440 Boucher DH, James S, Keeler KH. 1982. The ecology of mutualism. Ann. Rev. Ecol. Syst. 13:
441 315-347.

442 Breeuwer P, Drocourt JL, Rombouts FM, Abee T. 1996. A novel method for continuous
443 determination of the intracellular pH in bacteria with the internally conjugated fluorescent probe
444 5 (and 6-)-carboxyfluorescein succinimidyl ester. Appl. Environ. Microbiol. 62:178-183.

445 Chervaux C, Ehrlich SD, Maguin E. 2000. Physiological study of *Lactobacillus delbrueckii* subsp.
446 *bulgaricus* strains in a novel chemically defined medium. Appl. Environ. Microbiol. 66:5306-
447 5311.

448 Cotter PD, Hill C. 2003. Surviving the acid test: responses of Gram-positive bacteria to low pH.
449 Microbiol. Mol. Biol. Rev. 67:429–453.
450 Courtin P, Monnet V, Rul F. 2002. Cell-wall proteinases PrtS and PrtB have a different role in
451 *Streptococcus thermophilus/Lactobacillus bulgaricus* mixed cultures in milk. Microbiol.
452 148:3413-3421.
453 Crittenden RG, Martinez NR, Playne MJ. 2003. Synthesis and utilisation of folate by yoghurt
454 starter cultures and probiotic bacteria. Int. J. Food Microbiol. 80:217–222.
455 Cronin UP, Wilkinson MG. 2010. The potential of flow cytometry in the study of *Bacillus cereus*.
456 J. Appl. Microbiol. 108:1-16.
457 Driessen FM, Kingma F, Stadhouders J. 1982. Evidence that *Lactobacillus bulgaricus* in yoghurt
458 is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. Neth. Milk Dairy J.
459 36:135–144.
460 Griffin AS, West SA, Buckling A. 2004. Cooperation and competition in pathogenic bacteria.
461 Nature 430:10241027.
462 Gunasekera TS, Veal DA, Attfield PV. 2003. Potential for broad applications of flow cytometry
463 and fluorescence techniques in microbiological and somatic cell analyses of milk. Int. J. Food
464 Microbiol. 85:269-279.
465 Herve-Jimenez L, Guillouard I, Guedon E, Boudebbouze S, Hols P, Monnet V, Maguin E, Rul F.
466 (2009) Postgenomics analysis of *Streptococcus thermophilus* cocultivated in milk with
467 *Lactobacillus delbrueckii* subsp. *bulgaricus*: involvement of nitrogen, purine, and iron
468 metabolism. Appl. Environ. Microbiol. 75:2062-2073.
469 Hutkins RW, Nannen NL. 1993. pH homeostasis in lactic acid bacteria. J. Dairy Sci. 76:2354–
470 2365.

471 Leong-Morgenthaler P, Zwahlen MC, Hottinger H. 1991. Lactose metabolism in *Lactobacillus*
472 *bulgaricus*: analysis of the primary structure and expression of the genes involved. J. Bacteriol.
473 173:1951-1957.
474 Liu M, Siezen RJ, Nauta A. 2009. In silico prediction of horizontal gene transfer events in
475 *Lactobacillus bulgaricus* and *Streptococcus thermophilus* reveals proto-cooperation in yogurt
476 manufacturing. Appl. Environ. Microbiol. 75:4120-4129.
477 Lopez PJ, Marchand I, Yarchuk O, Dreyfus M. 1998. Translation inhibitors stabilize *Escherichia*
478 *coli* mRNAs independently of ribosome protection. Proc. Natl. Acad. Sci. USA 95:6067-6072.
479 Monnet C, Mora D, Corrieu G. 2005. Glutamine synthesis is essential for the growth of
480 *Streptococcus thermophilus* in milk and is linked to the catabolism of urea. Appl. Environ.
481 Microbiol. 71:3376-3378.
482 Mora D, Fortina MG, Parini C, Ricci G, Gatti M, Giraffa G, Manachini PL. 2002. Genetic diversity
483 and technological properties of *Streptococcus thermophilus* strains isolated from dairy products.
484 J. Appl. Microbiol. 93:278–287
485 Mora D, Maguin E, Masiero M, Ricci G, Parini C, Manachini PL, Daffonchio D. 2004.
486 Characterization of urease genes cluster of *Streptococcus thermophilus*. J. Appl. Microbiol.
487 96:209–219.
488 Mora D, C Monnet, C Parini, Guglielmetti S, Mariani A, Pintus P, Molinari F, Daffonchio D,
489 Manachini PL. 2005. Urease biogenesis in *Streptococcus thermophilus.* Res. Microbiol. 156:897-
490 903..
491 Mora D, Arioli S, Compagno C. 2013. Food environments select microorganisms based on selfish
492 energetic behavior. Front. Microbiol. 4:348.
493 Neves AR, Ramos A, Nunes MC, Kleerebezem M, Hugenholtz J, de Vos W, Almeida J, Santos
494 H. 1998. In Vivo Nuclear Magnetic Resonance studies of glycolytic kinetics in *Lactococcus lactis*.
495 Biotechnol bioeng 64(2): 200-12.

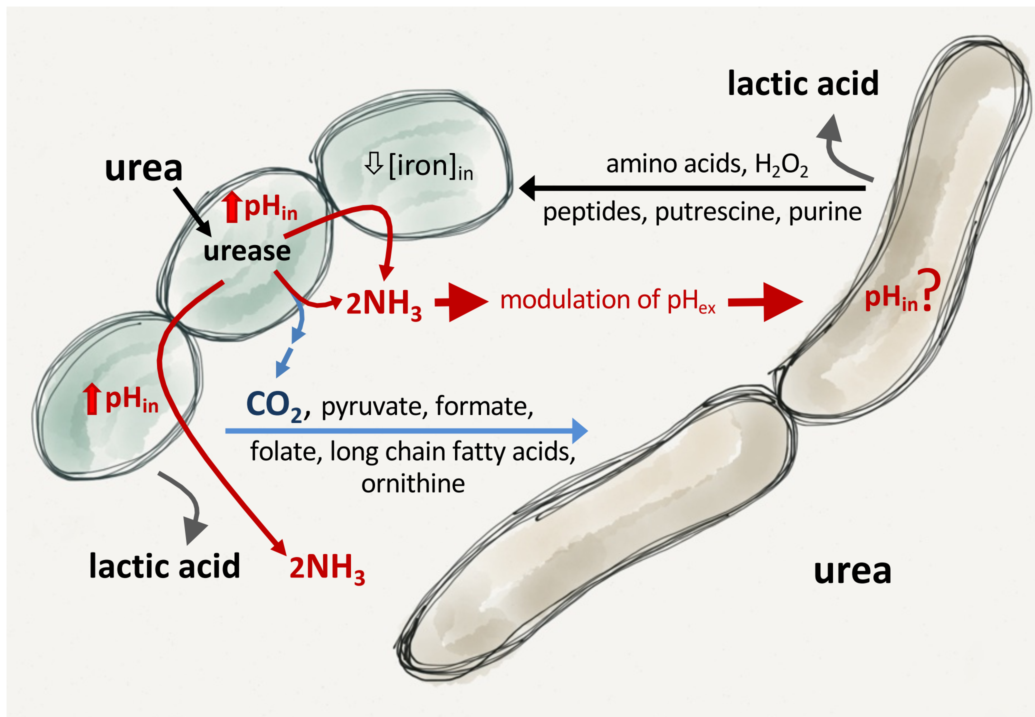
496 Ouzari H, Hassen A, Najjari A, Ettoumi B, Daffonchio D, Zagorec M, Boudabous A, Mora D.
497 2006. A novel phenotype based on esterase electrophoretic polymorphism for the differentiation
498 of *Lactococcus lactis* ssp. *lactis* and *cremoris*. Lett. Appl. Microbiol. 43:351-359.
499 Pernoud S, Fremaux C, Sepulchre A, Corrieu G, Monnet C. 2004. Effect of the metabolism of urea
500 on the acidifying activity of *Streptococcus thermophilus*. J. Dairy Scie. 87:550-555.
501 Rasmussen TB, Danielsen M, Valina O, Garrigues C, Johansen E, Pedersen MB. 2008.
502 *Streptococcus thermophilus* Core Genome: Comparative Genome Hybridization Study of 47
503 Strains. Appl. Environ. Microbiol. 74:4703–4710.
504 Sawatari Y, Yokota A. 2007. Diversity and mechanisms of alkali tolerance in Lactobacilli. Appl.
505 Environ. Microbiol. 73:3909-3915.
506 Siegumfeldt H, Rechinger KB, Jakobsen M. 2000. Dynamic changes of intracellular pH in
507 individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. Appl. Environ.
508 Microbiol. 66:2330–2335.
509 Sieuwerts S, de Bok FAM, Hugenholtz J, van Hylckama Vlieg JET. 2008. Unraveling microbial
510 interactions in food fermentations: from classical to genomics approaches. Appl. Environ.
511 Microbiol. 74:4997-5007.
512 Sieuwerts S, Molenaar D, van Hijum SAFT, Beerthuyzen M, Stevens MJA, Janssen PWM,
513 Ingham CJ, de Bok FAM, de Vos WM, van Hylckama Vlieg JET. 2010. Mixed-culture
514 transcriptome analysis reveals the molecular basis of mixed-culture growth in *Streptococcus*
515 *thermophilus* and *Lactobacillus bulgaricus*. Appl. Environ. Microbiol. 76:7775-7784.
516 Sincock SA, Robinson JP. 2001. Flow cytometric analysis of microorganisms. Meth. Cell Biol.
517 64:511-537.
518 Smid EJ, Lacroix C. 2013. Microbe-microbe interactions in mixed culture food fermetnations.
519 Curr. Opinion Biotech. 24:148-154.

520 van de Guchte M, Pernaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S,
521 Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto J-M, Walunas T, Gibrat
522 J-F, Bessières P., Weissenbach J, Ehrlich SD, Maguin E. 2006. The complete genome sequence
523 of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proc. Nat. Acad.
524 Sci. USA 24:9274-9279.
525 Zourari A, Accolas JP, Desmazeaud MJ. 1992. Metabolism and biochemical characteristics of
526 yogurt bacteria-a review. Lait 72:1-34.
527 Zourari A, Roger S, Chabanet C, Desmazeaud MJ. 1991. Characterization of lactic-acid bacteria
528 isolated from Greek yogurts. 1. *Streptococcus salivarius* subsp. *thermophilus* strains. Lait 71:445–
529 461.
530
531
532

533 ACKNOWLEDGEMENTS

534 We thank Françoise Rul, INRA Jouy-en-Josas France, for providing *S. thermophilus* CNRZ 385.
535 We acknowledge Dr Stefano Amalfitano Istituto di Ricerca sulle Acque (IRSA-CNR)
536 Monterotondo Rome Italy, for his support on flow cytometry applications. This work was partially
537 supported by Sacco srl, Cadorago, Italy.

538



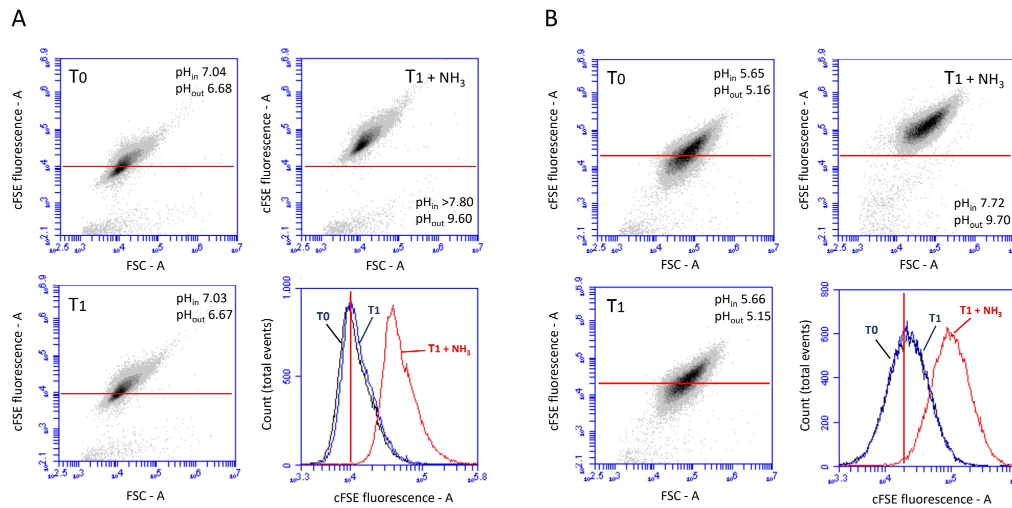
539

540

541

542 **FIG 1** Graphic representation of the molecular interactions that play key roles in the mutualistic
543 behaviour of the yogurt consortium. The effect of *S. thermophilus* urease on urea hydrolysis and
544 the hypothetical role of the NH_3 released by this enzyme on the pH_{in} of *L. delbrueckii* are shown
545 in red.

546

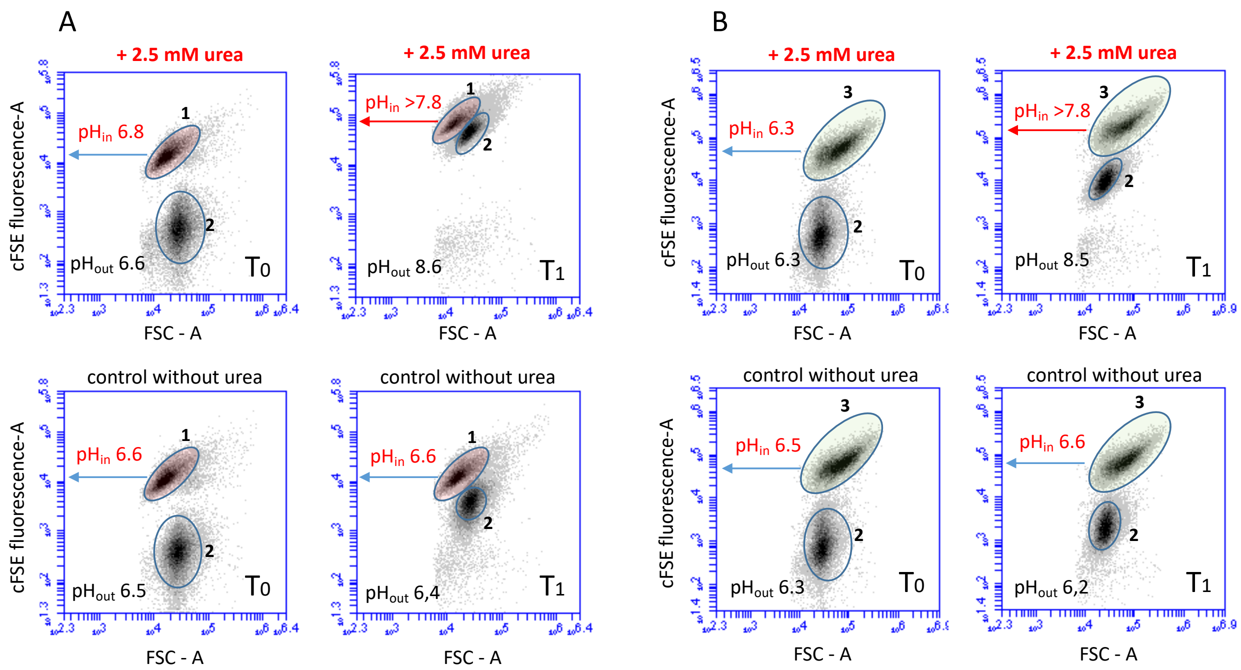


547

548 **FIG 2** The effect of ammonia on variations in the pH_{in} and pH_{ex} of the urease-negative
549 A16(Δ*ureC3*) strain of *S. thermophilus* (A) and *L. delbrueckii* MIM91 (B). The density diagrams
550 (upper and lower left panels) show the FSC of cells in the presence and absence of the addition of
551 5 mM NH_3 *vs.* the green (cFSE) fluorescence levels. T0 and T1 density plots of the *S. thermophilus*
552 population before and after 15 min of incubation at 42°C, respectively. The frequency histograms
553 (lower right panels) show the increasing green fluorescence that occurs due to NH_3-dependent
554 intracellular alkalisation. The horizontal and vertical line refers to the average cFSE fluorescence
555 of the cell population at T0.

556

557



558 **FIG 3** The effect of *S. thermophilus* CNRZ385 urease activity on the pH_{in} of the urease-negative

559 A16(Δ*ureC3*) strain of *S. thermophilus* (A) and *L. delbrueckii* MIM91 (B). The density diagrams

560 show the green (cFSE) fluorescence levels of the cells in the presence and absence of the addition

561 of 2.5 mM urea *vs.* FSC. T0 and T1 density plots of *S. thermophilus*/*L. delbrueckii* mixed

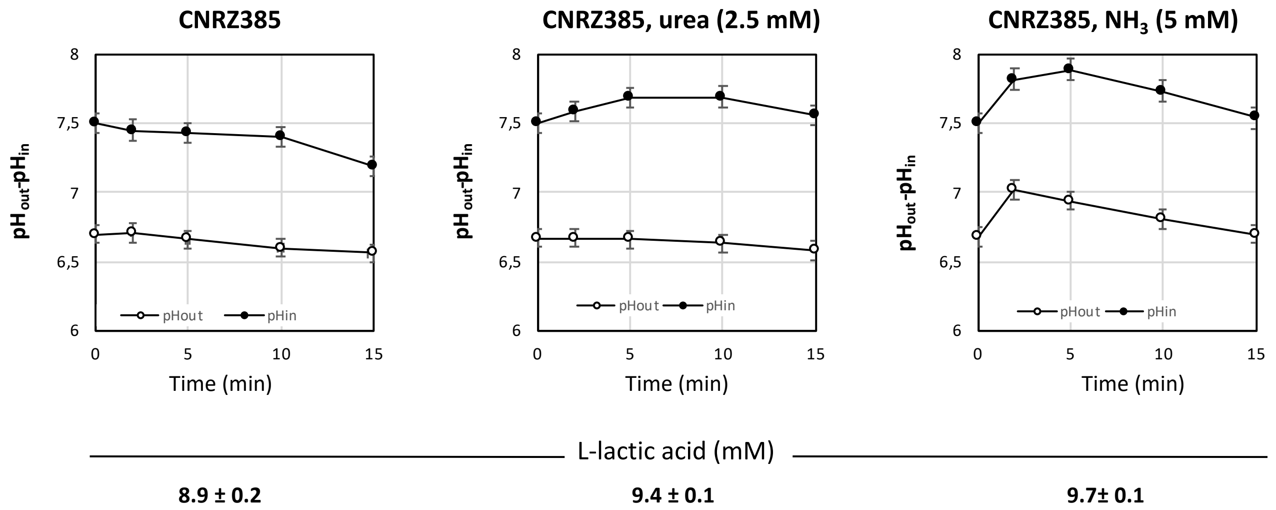
562 populations before and after 15 min of incubation at 42°C, respectively. Gate 1, cFSE-labelled

563 cells of urease-negative *S. thermophilus* A16(Δ*ureC3*). Gate 2, unlabelled cells of urease-positive

564 *S. thermophilus* CNRZ385. Gate 3, cFSE-labelled cells of *L. delbrueckii* MIM91. pH_{in} and pH_{out}

565 are indicated.

566



567

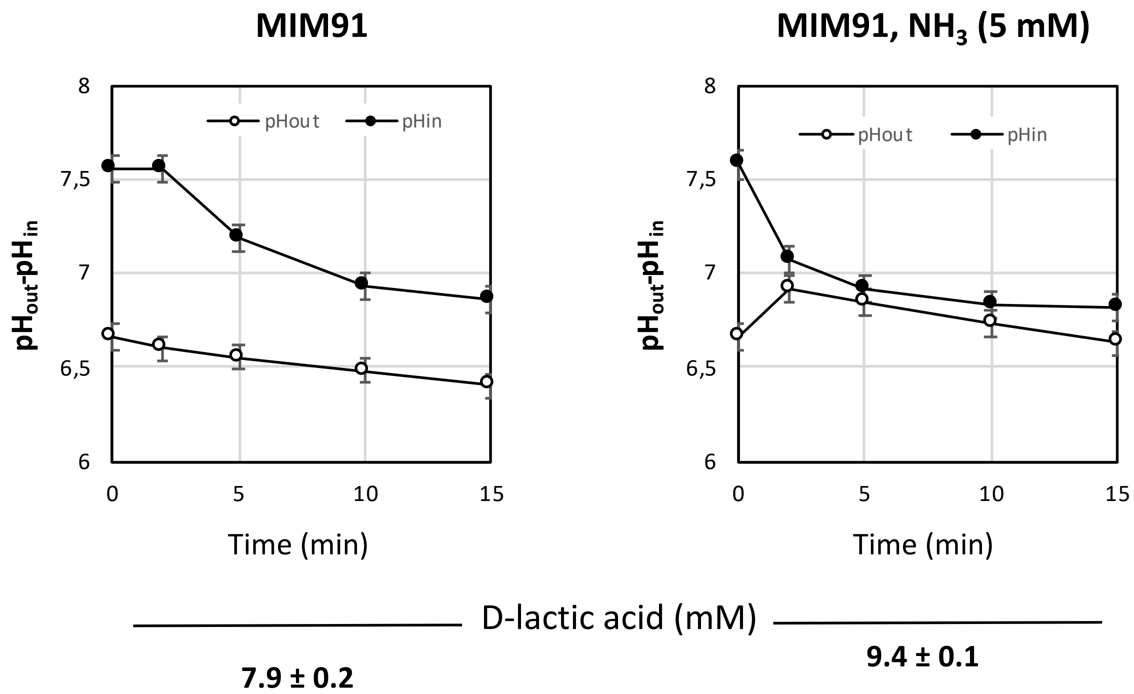
568 **FIG 4** Variations in pH_{in} and pH_{out} and L-lactic acid production in urease-positive *S. thermophilus*

569 CNRZ385 during incubation at 42°C in urea-free reconstituted skimmed milk in the presence and

570 absence of 2.5 mM urea and 5 mM NH_3. The amount of L-lactic acid is reported as the average of

571 three determinations ± SEM.

572



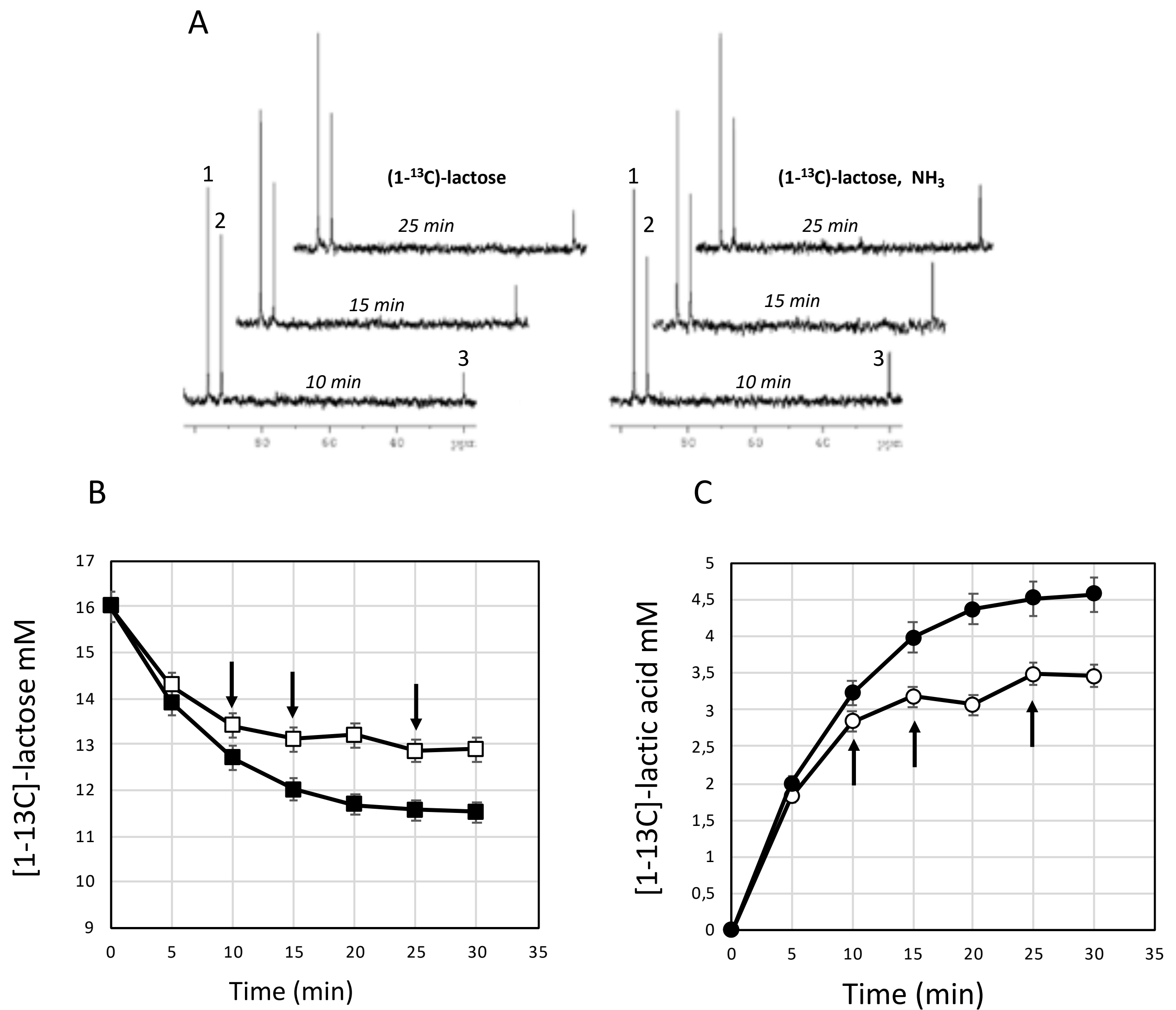
573

574 **FIG 5** Variations in pH_{in} and pH_{out} and D-lactic acid production in *L. delbrueckii* MIM91 during

575 incubation at 42°C in urea-free reconstituted skimmed milk in the presence and absence of 5 mM

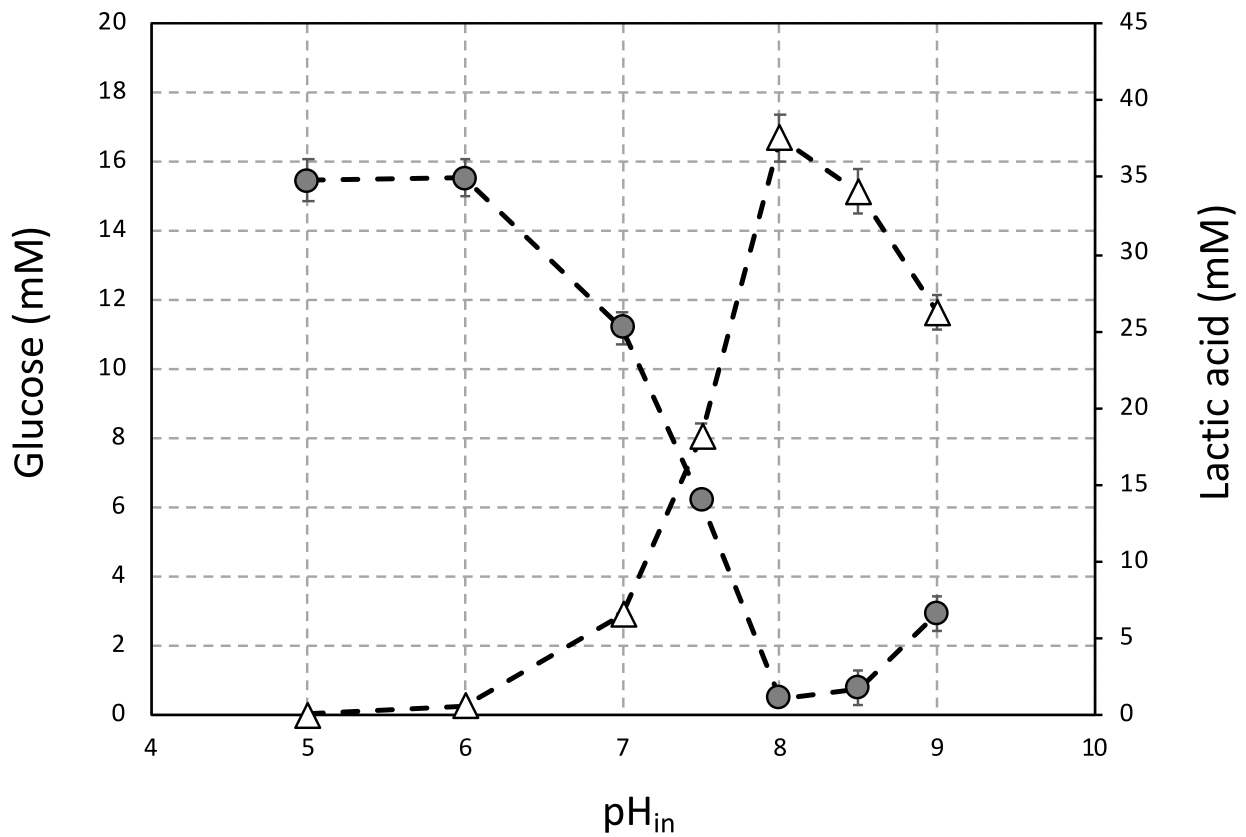
576 NH_3. The amount of D-lactic acid is reported as the average of three determinations ± SEM.

577



578

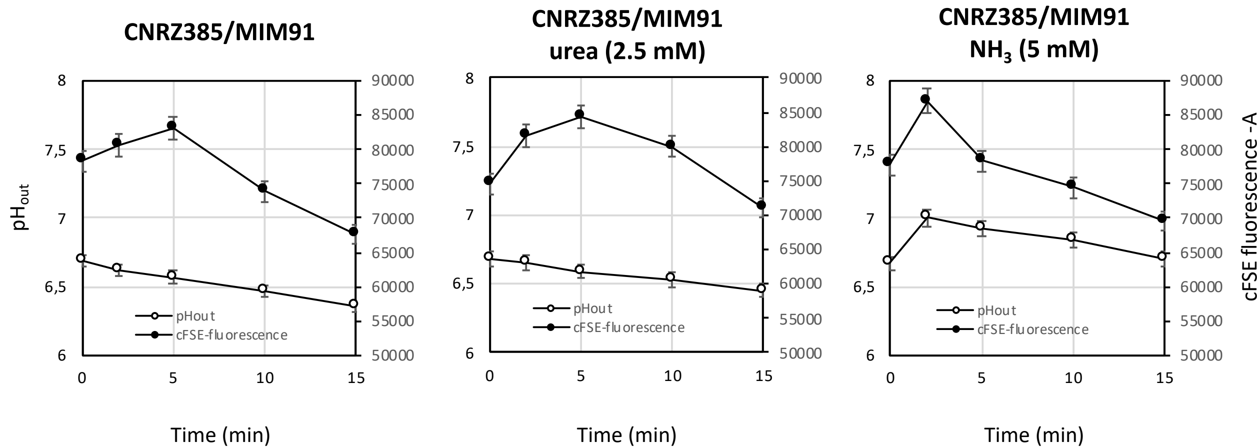
579 **FIG 6** Dynamics of metabolite pools in *L. delbrueckii*, as determined by in vivo NMR during in
580 incubation at 42 °C in solution A. Time course of (1-$^{13}C^{Glc}$)-lactose consumption and (1-^{13}C)-lactic
581 acid formation in *L. delbrueckii* MIM91. A) Each spectrum represents 5 min of accumulation. The
582 spectra after 10, 15 and 25 min from the beginning of the experiment are shown. (1-$^{13}C^{Glc}$)-lactose
583 was added at time zero and each spectrum acquired during the indicated interval. (1), β-(1-$^{13}C^{Glc}$)-
584 lactose, (2) α-(1-$^{13}C^{Glc}$)-lactose; (3) (1-^{13}C)-lactic acid. The metabolite concentrations (B, C) were
585 measured in *in vivo* ^{13}C NMR experiments using EdCs that were activated with 14 mM (1-
586 $^{13}C^{Glc}$)lactose (white symbols) or 14 mM (1-$^{13}C^{Glc}$)lactose and 5 mM NH_3 (black symbols). The
587 black arrows refer to spectra shown in A. The error bars represent the SEM.



588

589 **FIG 7** pH-dependent glucose consumption (grey circles) and lactic acid production (white
590 triangles) in EdCs of *L. delbrueckii* MIM91 that were treated with 100 mM of the uncoupler
591 gramicidine. The error bars represent the SEM.

592



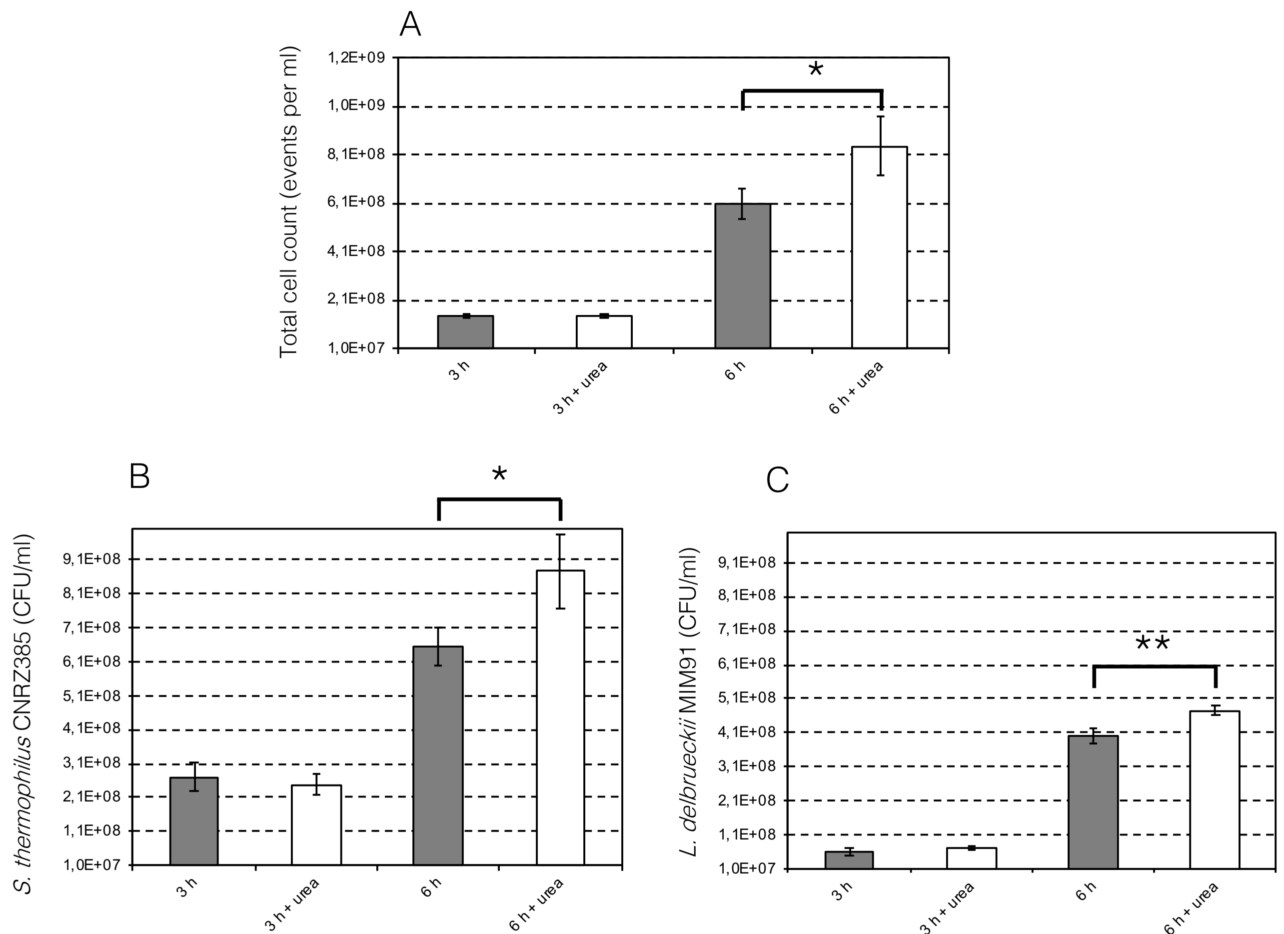
	lactic acid (mM)		
D+L	7.0 ± 0.3	8.8 ± 0.3	8.4± 0.3
L	3.6 ± 0.1	4.0 ± 0.2	4.0± 0.1
D	3.4 ± 0.2	4.8 ± 0.1	4.4± 0.2

593

594 **FIG 8** Variation of cFSE fluorescence, pH_{out}, and D/L-lactic acid production in *S. thermophilus*
595 CNRZ385/*L. delbrueckii* MIM91 mixed cultures during incubation at 42°C in urea-free
596 reconstituted skimmed milk in the presence and absence of 2.5 mM urea or 5 mM NH_3. The
597 amount of D/L-lactic acid is reported as the average of three determinations ± SEM.

598

599
600



601

602 **FIG 9** Population size of *S. thermophilus* CNRZ385/*L. delbrueckii* MIM91 mixed cultures after 3
603 h and 6 h of growth at 42°C in urea-free reconstituted skimmed milk in the presence and absence
604 of 2.5 mM urea. (A) Flow cytometry total cell count. (B) Selective count of viable *S. thermophilus*
605 CNRZ385. (C) Selective count of viable *L. delbrueckii* MIM91. The error bars represent the SEM.
606 Statistically significant differences were determined by an unpaired Student's t test (*, P < 0.05;
607 **, P < 0.01).

608

Fig. 6S1

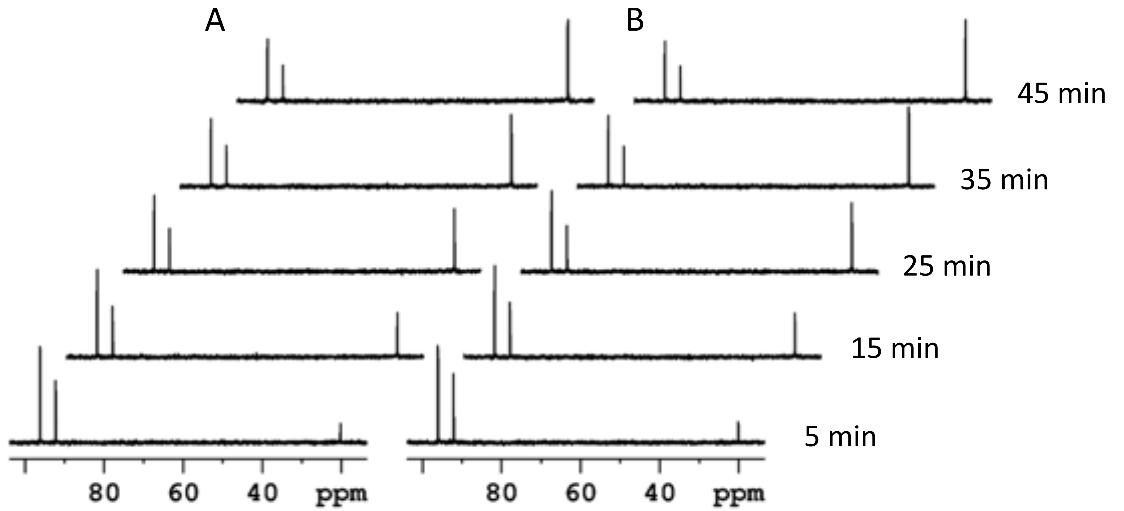


FIG 6S1 Kinetics of lactose metabolism in *L. delbrueckii*, as determined by *in vivo* NMR. Time course of (1-$^{13}C^{Glc}$)-lactose consumption and (1-^{13}C)-lactic acid formation in *L. delbrueckii* MIM91 in absence (A) and in presence of NH_4Cl 0.5 mM (B) during incubation in solution A. Each spectrum represents 5 min of accumulation. The spectra after 5, 15, 25, 35 and 45 min. from the beginning of the experiment are shown. (1-$^{13}C^{Glc}$)-lactose was added at time zero and each spectrum acquired during the indicated interval. (1), β-(1-$^{13}C^{Glc}$)-lactose, (2) α-(1-$^{13}C^{Glc}$)-lactose; (3) (1-^{13}C)-lactic acid.

609

610

611

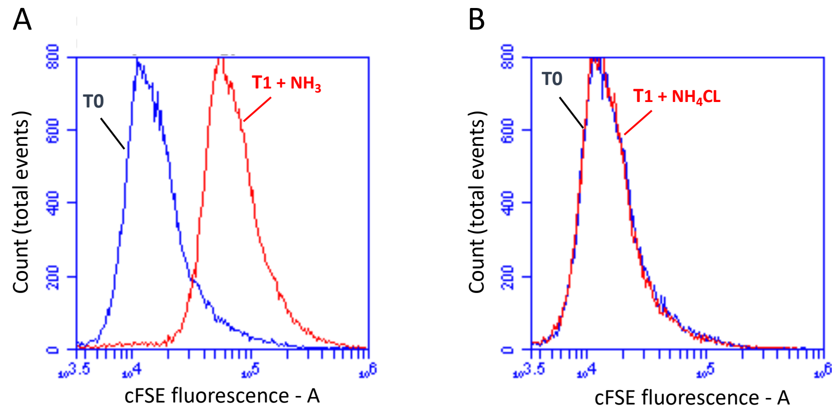


Fig. S2 The effect of ammonia and NH_4Cl on the pH_{in}-dependent variations of cFSE fluorescence of *L. delbrueckii* MIM91. The frequency histograms show the increasing green fluorescence that occurs due to NH_3 (A) or NH_4Cl (B) 5 mM addition. T0 and T1 frequency histograms of *L. delbrueckii* MIM91 population before and after 15 min of incubation at 42°C, respectively.

612