

1 **Fermentation pH influences the dynamic of *Lactobacillus bulgaricus* CFL1**

2 **physiological state during pH-controlled cultures**

3 **Running title: pH affects the dynamic of physiological state**

4

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10 **Keywords:** Lactic acid bacteria, viability, intracellular pH, acidification activity, flow
11 cytometry

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13 **Abbreviation key:** cFDA: carboxyfluorescein diacetate; cFDA-SE: 5(6-)-carboxyfluorescein
14 diacetate succinimidyl ester; D: dead cell concentration; DIBAC₄(3): bis (1,3
15 dibutylbarbituric acid) trimethine oxonol; dpH: pH gradient (pHi-pHext); FSC: forward-angle
16 light scatter; Lac: lactate concentration; N: cultivable cell concentration; NaOH: sodium
17 hydroxide; pHext: extracellular pH; pHi: intracellular pH; PI: propidium iodide; pKa:
18 dissociation constant of lactic acid; SSC: side-angle light scatter; tm: time to reach the
19 maximal rate of NaOH consumption; tpH5.5: time necessary to reach pH 5.5 in milk; V:
20 viable cell concentration; Vm: maximal rate of NaOH consumption

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25 **Abstract**

26 This study aims at better understanding the effects of fermentation pH and harvesting time on
27 *Lactobacillus bulgaricus* CFL1 cellular state, in order to improve knowledge on the dynamic
28 of physiological state and to better manage starter production. The Cinac system and
29 multiparametric flow cytometry were used to characterize and compare the progress of the
30 physiological events that occurred during pH 6 and pH 5 controlled cultures. Acidification
31 activity, membrane damage, enzymatic activity, cellular depolarization, intracellular pH and
32 pH gradient were determined and compared during growing conditions. Strong differences in
33 the time course of viability, membrane integrity and acidification activity were displayed
34 between pH 6 and pH 5 cultures. As a main result, the pH 5 control during fermentation
35 allowed the cells to maintain a more robust physiological state, with high viability and stable
36 acidification activity throughout growth, in opposition to a viability decrease and fluctuation
37 of activity at pH 6. This result was mainly explained by differences in lactate concentration in
38 the culture medium and in pH gradient value. The elevated content of the ionic lactate form at
39 high pH values damaged membrane integrity that led to a viability decrease. On the contrary,
40 the high pH gradient observed throughout pH 5 cultures was associated with an increased
41 energetic level that helped the cells maintain their physiological state. Such results may
42 benefit to industrial starter's producers and fermented products manufacturers, by allowing
43 them to better control the quality of their starters, before freezing or before using them for
44 food fermentation.

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48 **Introduction**

49 Lactic acid bacteria are traditionally employed to produce or to preserve various food
50 products such as fermented milks, meats and vegetables. Their ability to initiate rapid
51 acidification of the raw material is essential to improve flavor, texture and safety of these
52 products (11, 14). In order to prevent poor fermentation yields and to improve the quality and
53 reliability of the products, it is important to maintain proper control starter production. This
54 control may be achieved by studying the effects of process parameters on growth kinetics of
55 the bacteria and on their acidification activity and physiological state in growing conditions.
56 Among all process parameters, pH and harvesting time are key factors that strongly influence
57 the physiological state of lactic acid bacteria after fermentation and stabilization.

58 Lactic acid starters are currently produced using pH-controlled pure cultures (6) during which
59 pH is generally regulated at an optimal value by continuously adding sodium hydroxide or
60 ammonia in the bioreactor (23). Various growth characteristics such as maximal biomass
61 concentration, specific growth rate, fermentation time, sugar consumption or growth and
62 product yields are significantly influenced by the pH control value (1, 4). Optimal pH ranges
63 were therefore determined for several lactic acid bacteria such as *Streptococcus thermophilus*
64 (pH 6.5), *Lactobacillus bulgaricus* (pH 5.8-6) (5, 22) or *Lactococcus lactis* subsp. *cremoris*
65 (pH 6.3-6.9) (8).

66 As compared to acidic fermentations, pH-controlled cultures led to higher growth yields and
67 productivity (9, 23) as a result of the lower level of non-dissociated lactic acid in the culture
68 medium (2, 12, 15). The acidification of the cytoplasm induced by the non-dissociated form
69 of the weak organic acid leads to the collapse of the proton motive force (13). This
70 phenomenon inhibits nutrient transport and enzymatic reactions and leads to DNA alteration
71 and biomass inactivation (12). Maintaining the extracellular pH at a high value helps the cells

72 stabilize their intracellular pH at a sufficiently high value (9), thus decreasing the inhibiting
73 effect of lactic acid.

74 Fermentation pH also acts on energetic parameters, such as internal pH (pHi), pH gradient
75 (dpH), proton motive force, membrane potential, NADH/NAD ratio, ATP level and rate of
76 ATP formation, lactate dehydrogenase and ATPase activity (1, 9, 17). During batch cultures
77 of *Lactococcus lactis* performed with and without pH control, Cachon et al. (9) showed that
78 pH control has a significant influence on the variations of pHi, dpH and NADH/NAD ratio,
79 thus acting on growth parameters. Moreover, in batch cultures, pHi is dependant upon both
80 the external pH and the age of culture. Mercade et al. (17) showed that cultures of *L.*
81 *bulgaricus* at controlled pH 6.4 are inhibited at the level of anabolism but were not energy
82 limited. They are characterized by a high maintenance coefficient in contrast to cultures
83 without pH control which consume intracellular energy for pHi regulation.

84 The effect of pH on cellular physiology is confirmed by other studies which show that it
85 influences acidification activity of lactic acid bacteria (23-25). Whereas Wang et al. (25)
86 indicated that *Lactobacillus acidophilus* cells grown at optimal pH display a higher residual
87 acidification activity than cells grown at lower pH control values, Schepers et al. (24) and
88 Savoie et al. (23) demonstrated that this activity is higher when starters are produced without
89 pH control or at low pH control values. These authors explained that conditions generating
90 high biomass concentrations do not systematically lead to cells with an efficient acidification
91 activity.

92 From this information, the effect of pH control was elucidated on growth and energetic
93 parameters, whereas its effect on the dynamic of cellular physiology, viability and
94 acidification activity during growth is still not determined.

95 A few authors demonstrated that harvesting time has a strong impact on cellular parameters

96 such as viability and acidification activity (3, 20, 24). Béal et al. (6) specified that there is an
97 optimal range of time during which to harvest cells in a good physiological state, i.e. at a high
98 cellular concentration and a high acidification activity. However, as this optimal range is
99 strongly strain and conditions dependant, more information is needed about the influence of
100 harvesting time on physiological parameters.

101 In order to improve knowledge about the effects of fermentation pH and harvesting time on
102 starter's quality, this work aims at applying some rapid and relevant methods to characterize
103 the dynamic of *L. delbrueckii* subsp. *bulgaricus* CFL1 physiological state throughout pH 6
104 and pH 5 fermentations. This may allow industrial starter's producers to better control their
105 fermentations and to achieve high quality starters. Among the available methods, the Cinac
106 system and multiparametric flow cytometry, associated with plate counts, made it possible to
107 determine and compare different physiological parameters such as cultivability, acidification
108 activity (Cinac system), membrane damage, enzymatic activity, cell depolarization,
109 intracellular pH and pH gradient (flow cytometry) (20). Two dynamic schemes of the time
110 course of the physiological state during pH 6 or pH 5 cultures will be proposed and discussed.

111

112 **Materials and methods**

113 **Bacterial strain and preculture conditions**

114 *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 (INRA, Thiverval-Grignon, France) was
115 used throughout this study. Frozen cells were stored at -80°C in MRS broth (AES-Chemunex,
116 Combourg, France) supplemented with 15 % glycerol. Before inoculation in the bioreactor,
117 cells were sub-cultured twice, for 24 h and 8.5 h, at 42°C in 5 ml MRS broth.

118 **Fermentation conditions**

119 The culture medium was composed of 60 g l⁻¹ sweet whey powder (Eurosérum, Port-sur-

120 Saône, France) which was adjusted to pH 5 with 50 % H₂SO₄, heat-treated at 110°C for 20
121 min and centrifuged at 7000 g for 30 min at 4°C. After filtration (0.45 μm), it was
122 supplemented with 20 g l⁻¹ yeast extract (Organotechnie, La Courneuve, France) and
123 introduced into a 2-l bioreactor (Setric Génie Industriel, Toulouse, France). After sterilization
124 at 110°C for 20 min, the initial pH was adjusted at pH 6, and inoculation was done at a low
125 initial level of 20 ± 5.5 CFU ml⁻¹ to allow overnight culture. Fermentations were performed at
126 42°C, 100 rpm and with pH 6 or pH 5-control, by automatic adding of a 5 N NaOH solution
127 in the bioreactor. The NaOH concentration (m, in g l⁻¹) and its consumption rate (dm/dt, in
128 g l⁻¹ min⁻¹) were calculated with WCidus software (INRA, Thiverval-Grignon, France).
129 The fermentation kinetics were therefore established and the reproducibility was assessed
130 according to three descriptors: the maximal rate of NaOH consumption (V_m, in g l⁻¹ min⁻¹),
131 the time necessary to reach V_m (t_m, in min) and the NaOH consumption (dNaOH, in g l⁻¹) at
132 t_m. Samples were taken from the bioreactor at different sampling times, from the beginning of
133 NaOH consumption up to late stationary phase. In order to normalize the data obtained from
134 the different cultures, results were expressed as a function of the time t_m (in min) necessary to
135 reach V_m, which was considered as a reference time.

136 HPLC measurements

137 Lactose, glucose, galactose and lactic acid concentrations were quantified using High
138 Performance Liquid Chromatography (HPLC, Waters Associates, Millipore, Molsheim,
139 France). Before the HPLC analyses, each sample was combined with 120 g l⁻¹ trichloroacetic
140 acid (Prolabo, Paris, France), centrifuged at 4000 g for 30 min at 4°C and filtered (0.22 μm).
141 The HPLC analysis was made on a cation exchange column (Aminex Ion Exclusion HPX-
142 87H 300*7.8 mm, Biorad, Richmond, USA) at 35°C with propionic acid (10 g l⁻¹) as an
143 internal standard. The mobile phase was 0.005 M H₂SO₄ and the flow rate was set at

144 0.6 ml min⁻¹ (LC-6A pump, Shimadzu, Courtaboeuf).

145 The dissociated lactate [A⁻] and non-dissociated lactic acid [AH] concentrations were
146 evaluated as a function of the dissociation constant of lactic acid (pK_a = 3.86), pH and total
147 lactic acid [LA] concentration measured by HPLC. They were calculated by considering the
148 following formula:

$$149 \quad [A^-] / [AH] = 10^{(pH - pK_a)} \quad (\text{eq. 1})$$

$$150 \quad [A^-] + [AH] = [LA] \quad (\text{eq. 2})$$

151 **Measurement of cultivability**

152 Cultivability was evaluated by plate counts. After serial dilutions in peptoned water
153 (bactopeptone, 1 mg l⁻¹, DIFCO Laboratories, Le Pont-de-Claix, France), cells were plated
154 onto solid MRS agar (AES-Chemunex) and incubated at 42°C for 48 h under anaerobic
155 conditions (Genbox 96124, bioMérieux, Marcy l'Etoile, France). Each result (N, in CFU ml⁻¹)
156 was the geometrical mean of at least three counts.

157 **Fluorescent probes and staining protocols**

158 **Viability and mortality assessment**

159 Carboxyfluorescein diacetate (cFDA) was used to assess *Lactobacillus bulgaricus* CFL1
160 viability according to Rault et al. (19). The nucleic acid dye propidium iodide (PI) made it
161 possible to quantify damaged and dead cells. Live/dead assays were done by dual staining of
162 each sample to differentiate viable, dead and damaged cells. Before staining, cell suspensions
163 were diluted in Chemsol B13 buffer (AES-Chemunex) to reach 10⁶ cells ml⁻¹. 1 ml of the
164 diluted suspension was first supplemented with 10⁻¹ PI (1.496 mM in distilled water, Sigma-
165 Aldrich, Lyon, France) and incubated for 20 min at 40°C. 10⁻¹ cFDA (0.217 M in acetone,
166 Invitrogen-Molecular Probes, Eragny-sur-Oise, France) were then added and incubation took
167 place for 10 min before direct analysis by flow cytometry.

168 **Depolarization assessment**

169 Bis (1,3-dibutylbarbituric acid) trimethine oxonol (DIBAC₄(3)) was used to assess the
170 depolarization state of the cells (7). Dual staining was performed in order to differentiate dead
171 cells with propidium iodide and depolarized cells with DIBAC₄(3) in a single analysis. 1 ml
172 of a bacterial suspension was diluted in Chemsol B13 buffer (AES-Chemunex) to reach 10⁶
173 cells ml⁻¹ and incubated with 10⁻¹ PI (1.496 mM in distilled water, Sigma-Aldrich) for 10
174 min at 40°C. 5⁻¹ DIBAC₄(3) (96.78⁻¹ M in dimethylsulfoxide, Invitrogen-Molecular Probes)
175 were then added and incubation took place for 20 min, before direct analysis by flow
176 cytometry.

177 **pHi measurement**

178 The fluorochrome 5-(6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) was used
179 for pHi measurements (21). The method was first calibrated by using pH-equilibrated cells at
180 known pHi (20). Cell suspensions were diluted in a pH 7.3 Mac Ilvaine buffer (citric acid 0.1
181 M, Fisher Chemical, Elancourt, France; disodium dihydrogenophosphate 0.2 M, J.T. Baker,
182 Deventer, NL) to reach 10⁷ cells ml⁻¹. 1 ml of the diluted suspension was incubated with 5⁻¹
183 cFDA-SE (8.96⁻¹ M in acetone, Invitrogen-Molecular Probes) for 10 min at 40°C. After
184 staining, 100⁻¹ of cell suspension were diluted in 1 ml Mac Ilvaine buffers ranging from pH
185 4 to pH 7.5. Intracellular pH was equilibrated with buffer pH by addition of valinomycin and
186 nigericin (Sigma Aldrich) to a final concentration of 1⁻¹ M each. After 1 min incubation at
187 25°C, fluorescence intensity was measured by flow cytometry.

188 A calibration curve was established by plotting the logarithm of the mean fluorescence
189 intensity as a function of pH of equilibrated cells, in the range of pH 4 to pH 7.5, as
190 previously described by Rault et al. (20).

191 Analyses of intracellular pH of samples were carried out according to the same protocol.

192 After staining with cFDA-SE, 100 μ l of cell suspension were diluted in 1 ml of a buffer
193 adjusted to the external pH of the cells. The fluorescence intensity was measured with flow
194 cytometry and the linear calibration curve made it possible to quantify the intracellular pH
195 (20).

196 **Flow cytometry and data analyses**

197 Flow cytometry analyses were performed with a Cyflow cytometer (AES-Chemunex)
198 equipped with a specific volumetric counting system, an air-cooled argon ion laser emitting at
199 488 nm and four band-pass filters: a forward-angle light scatter (FSC) combined with a diode
200 collector, a side-angle light scatter (SSC) and two fluorescence signals collected with
201 photomultiplier tubes. A 530 nm band-pass filter (515 to 545 nm) was used to collect the
202 green fluorescence of carboxyfluorescein or DIBAC₄(3) (FL1 channel) and a 630 nm long-
203 pass filter to collect the red fluorescence of propidium iodide (FL2 channel). The FACSFlo
204 solution (Becton Dickinson, Le Pont-de-Claix, France) was used as sheath fluid. Flow
205 cytometry analyses were performed by using logarithmic gains and specific detector settings
206 adjusted on a sample with unstained cells in order to eliminate cellular autofluorescence. A
207 combination of FSC and SSC was used to discriminate the bacteria from the background.

208 Data was collected and analyzed with Flowmax software (Partec, Sainte-Geneviève-des-Bois,
209 France). Subpopulations were identified by using dot plots of FSC, SSC, green fluorescence
210 and red fluorescence, which separated the different events. Data was analyzed with the aid of
211 statistical tables given by the Flowmax software. It indicated numbers (in cells ml⁻¹) and
212 percentages of stained cells determined by each detector, along with the mean of fluorescence
213 intensity of each fluorescent signal, and the associated coefficients of variation. Each result
214 corresponded to the mean of at least six measurements.

215 **Specific acidification activity measurements**

216 The Cinac system (Ysebaert, Frépillon, France) was used to assess the acidification activity of
217 the cells (10). Acidification was measured in triplicate at 42°C in 100 g l⁻¹ dry skim milk
218 (EPI-Ingredient, Ancenis, France), heat-treated at 110°C for 20 min in 150 ml flasks and
219 stored at 4°C before use. From a previous determination of viable cell concentrations by
220 cFDA staining and flow cytometry, the flasks were inoculated at an initial concentration of
221 10⁵ viable cells ml⁻¹. The pH was continuously measured by the Cinac system and led to the
222 determination of the time necessary to reach pH 5.5 (tpH5.5, in min) as a descriptor to
223 characterize the acidification activity of bacterial suspensions. The higher the tpH5.5, the
224 lower the acidification activity.

225

226 **Results**

227 **Fermentation kinetics**

228 Table 1 summarizes the main kinetic parameters characterizing the fermentations performed
229 at pH 6 and pH 5. As 95 % confidence intervals were low compared to the mean values of the
230 parameters, it was considered that the reproducibility of the cultures was satisfactory, thus
231 making it possible to analyze and compare the physiological characteristics of the cells
232 obtained from the different batch cultures at each controlled pH.

233 As shown on Table 1, the fermentation kinetic was slower at pH 5 than at pH 6, with a
234 difference of about four hours for t_m . This difference was related to the lower maximal rate of
235 NaOH consumption (V_m) observed at pH 5. If the other kinetic parameters and their
236 confidence intervals are considered, pH 5 cultures do not appear to produce significant
237 differences from pH 6 cultures in terms of lactose consumption, galactose and lactic acid
238 production or NaOH consumption.

239 In order to normalize the data obtained from the six cultures, results were expressed as a

240 function of the reference time t_m (in min) necessary to reach the maximal rate of NaOH
241 consumption (Fig. 1a and Fig. 2a). However, as t_m was higher at pH 5 than at pH 6, it must be
242 remembered that the resulting adjusted time was different in these two conditions.

243 **Analysis of *L. bulgaricus* CFL1 physiological characteristics during pH 6 controlled** 244 **fermentations**

245 Viability, mortality, injury, depolarization, pH gradient and intracellular pH of *L. bulgaricus*
246 CFL1 during pH 6 cultures were quantified using multiparametric flow cytometry. The results
247 were associated with plate counts and acidification activity (Fig. 1) and discussed as a
248 function of the time t_m , necessary to reach the maximal NaOH consumption rate (Fig. 1a).

249 As shown in Fig. 1b, whereas a maximal CFU concentration of $3.8 \cdot 10^8 \pm 6 \cdot 10^7$ CFU ml⁻¹ was
250 reached at t_m+100 min, a significant drop in the percentage of viable cells was observed
251 throughout growth, from 93 ± 3 % before t_m-200 min down to 34 ± 2 % at t_m+800 min. The
252 percentage of damaged cells reached a maximum of 19 ± 6 % just before the maximal
253 acidification point, then decreased and stood quite stable at 8 ± 1 %. The viability decrease
254 was inversely correlated to the percentage of dead cells, which rose by 58 ± 2 % in the same
255 period. Depolarized cells depicted a slight rise of 16 ± 1 % during growth, before stabilizing
256 during stationary phase. By comparing these results with dead cells percentages, it was
257 observed that only a small part of dead cells were depolarized.

258 During the stationary phase, CFU concentration stood quite steady at a high level up to 500
259 min after the maximal acidification point before decreasing down to $7 \cdot 10^7$ CFU ml⁻¹ (Fig. 1b).

260 Total viable cell counts (V, in viable+damaged cells ml⁻¹) determined by flow cytometry were
261 well correlated with plate counts (N, in CFU ml⁻¹) with a significant coefficient of
262 determination of 0.977. Nevertheless, they were slightly lower than plate counts with a mean
263 difference of 5 % throughout growth. The corresponding linear relationship was determined

264 as follows:

$$265 \log V = 0.95 (\pm 0.05) \cdot \log N + 0.08 (\pm 0.04) \quad R^2 = 0.977 \quad (\text{eq. 3})$$

266 From Fig. 1c it can be seen that the specific acidification activity of *L. bulgaricus* CFL1 cells,
267 measured by the Cinac system, fluctuated throughout the culture when the pH was controlled
268 at pH 6. During exponential phase, the cellular activity decreased significantly, as tpH5.5
269 increased from 215 ± 10 min to reach a maximum of 461 ± 39 min at the end of the log phase
270 (t_m+100 min). During the stationary phase, the cells recovered better acidification activity, as
271 tpH5.5 decreased down to 316 ± 25 min at t_m+300 min. It remained high and stable at $327 \pm$
272 9 min up to t_m+500 min. Finally, it declined gradually in late stationary phase, from t_m+500
273 min until t_m+800 min.

274 The time course of intracellular pH (pHi) and pH gradient (dpH) was followed during pH 6
275 fermentations and the results are summarized in Fig. 1d. These two parameters were stable at
276 $p_{Hi} = 6.34 \pm 0.01$ and $dpH = 0.34 \pm 0.01$ during the log phase, until the stationary phase was
277 achieved (t_m+100 min). At the beginning of the stationary phase, these two parameters
278 decreased to $p_{Hi} = 6.05 \pm 0.01$ and $dpH = 0.05 \pm 0.01$ at $t_m + 200$ min, then remained stable
279 until the end of fermentation.

280 **Analysis of *L. bulgaricus* CFL1 physiological characteristics during pH 5 controlled** 281 **fermentations**

282 In order to further analyze the effect of the fermentation pH on physiological characteristics
283 of *L. bulgaricus* CFL1, they were quantified throughout pH 5 controlled cultures. Results are
284 shown in Fig.2 and discussed as a function of the time t_m , necessary to reach the maximal
285 NaOH consumption rate (Fig. 2a).

286 Fermentations performed at controlled pH 5 led to a maximal CFU concentration of $5.1 \cdot 10^8 \pm$
287 $4 \cdot 10^7$ CFU ml⁻¹. This maximal concentration was reached at the end of log-phase, at t_m+60

288 min (Fig. 2b). Total viable cell counts (V , in viable+damaged cells ml^{-1}) determined by flow
289 cytometry were 6 % lower than plate counts (N , in CFU ml^{-1}) throughout growth, and these
290 two variables were well correlated. The corresponding linear relationship was determined as
291 follows:

$$292 \log V = 0.94 (\pm 0.1) \cdot \log N + 0.09 (\pm 0.01) \quad R^2 = 0.967 \quad (\text{eq. 4})$$

293 As shown in Fig. 2b, the percentages of viable, dead and injured cells remained constant all
294 along the growth curve, at respectively 67 ± 5 %, 20 ± 3 % and 12 ± 2 %. As cell
295 depolarization was low throughout pH 5 culture, with less than 0.05 % of depolarized cells,
296 results were not significant and therefore not depicted on Fig. 2b.

297 From Fig. 2c it can be seen that specific acidification activity decreased throughout pH 5
298 fermentation, as $t_{\text{pH}5.5}$ increased from 254 ± 9 min until 423 ± 11 min. Fluctuations were
299 less important than at pH 6. After a slight increase during log phase until $t_{\text{m}}-100$ min, the
300 time to reach pH5.5 remained stable at 314 ± 5 min up to $t_{\text{m}}+400$ min. Finally, acidification
301 activity decreased, i.e. $t_{\text{pH}5.5}$ increased up to 423 ± 11 min at $t_{\text{m}}+800$ min.

302 Fig. 2d shows that, in spite of a constant and low external pH 5, the cells maintained a high
303 intracellular pH ($\text{pH}_i > 6$) and a high pH gradient ($\text{dpH} > 1$) until the beginning of the
304 stationary phase. These two characteristics increased from the beginning of the measurements
305 up to a maximum of $\text{pH}_i = 6.5 \pm 0.05$ and $\text{dpH} = 1.5 \pm 0.05$ at the end of the log phase ($t_{\text{m}}+60$
306 min). They then decreased down to $\text{pH}_i = 5.4 \pm 0.08$ and $\text{dpH} = 0.4 \pm 0.08$ at $t_{\text{m}}+800$ min.

307 **Relationship between dead cells and lactate concentration in the culture medium**

308 The final lactate level was fermentation pH-dependent. It was higher ($>15 \text{ g l}^{-1}$) at the end of
309 the cultures conducted at pH 6 than at pH 5 (15 g l^{-1}) and strongly associated to cellular
310 mortality that increased from $1.9 \cdot 10^6$ to $8.2 \cdot 10^7$ cells ml^{-1} with lactate concentration.

311 If we consider that the fraction of dissociated lactate differed according to the pH, the

312 mortality of *L. bulgaricus* CFL1 was related to this data. In Fig. 3, a linear correlation can be
313 observed between the logarithm of dead cell counts (D, in cells ml⁻¹) measured by flow
314 cytometry and the lactate concentration (Lac, in g l⁻¹) in the culture medium. This relationship
315 was corroborated by the following linear relationship:

$$316 \quad \text{Log D} = 0.08 (\pm 0.01) \cdot \text{Lac} + 6.47 (\pm 0.08) \quad R^2 = 0.882 \quad (\text{eq. 5})$$

317

318 **Discussion**

319 **Comparison of *L. bulgaricus* CFL1 physiological state during controlled pH 6 and pH 5** 320 **cultures**

321 As shown in our results, even though *Lactobacillus bulgaricus* CFL1 fermentations at pH 5
322 were longer than at pH 6, as previously observed by Amrane and Prigent (2) with low pH-
323 controlled cultures of *Lactobacillus helveticus*, they did not lead to significant differences in
324 terms of final lactose consumption, galactose and lactic acid production or NaOH
325 consumption. Final CFU concentration was slightly higher at pH 5 than at pH 6, but the
326 difference was not significant if standard deviations are considered. This result is in
327 agreement with that obtained by Mercade et al. (18) who depicted a similar final biomass
328 production when *Lactococcus lactis* subsp. *cremoris* was cultured at pH 6.6 or pH 5.
329 However, the concentrations obtained in the current study were significantly higher than
330 those obtained by Rault et al. (20) when cultures of *L. bulgaricus* CFL1 were performed
331 without pH control (1.3·10⁸ CFU ml⁻¹). This result is consistent with previous studies which
332 compared free and controlled pH cultures of *Lactococcus lactis* (9) and *L. bulgaricus* (17).

333 At both fermentation pH, viable cell counts were well correlated with plate counts, but they
334 remained slightly lower (5 to 6 %, respectively). Such a difference was previously observed
335 with other fluorescent probes by Jepras et al. (16) who indicated that the natural clumping of

336 the cells may affect the flow cytometry counts. As compared to flow cytometry samples that
337 need two dilution steps, plate count samples undergo more dilution steps before being plated
338 onto agar, thus facilitating the break-up of cell clumps. Such a difference was not observed
339 during cultures of *L. bulgaricus* CFL1 performed without pH control (20). This situation may
340 be explained by considering that pH affects *L. bulgaricus* chain length, which decreases with
341 pH (22). This hypothesis was corroborated by microscopic analyses which displayed longer
342 cell chains at pH 6 or pH 5 as compared to cultures without pH control.

343 Comparison of pH 6 and pH 5 cultures displayed significant differences in the dynamics of
344 viability, membrane integrity, acidification activity, intracellular pH and pH gradient. We first
345 demonstrated that cellular acidification activity, viability and membrane integrity were
346 maintained at high levels throughout pH 5 cultures as compared to pH 6 cultures.
347 Consequently, a lower pH control value allowed the cells to maintain a more robust
348 physiological state. This result is consistent with those of Schepers et al. (24) who
349 demonstrated that viability and activity of *L. helveticus* cultures conducted at pH 4.2 were
350 better than pH 5.5 cultures.

351 A significantly higher dpH was observed throughout pH 5 cultures ($\text{dpH} > 1$) than with pH 6
352 cultures ($\text{dpH} < 0.5$). This higher pH gradient at low pH allowed the cells to maintain their
353 pHi at values compatible with growth and cellular enzymatic activities. This result is in
354 agreement with those of Mercade et al. (18) who compared the dpH of *Lactococcus lactis*
355 subsp. *cremoris* grown at pH varying between pH 6.6 and pH 4.4.

356 **The dissociated lactate level influenced cellular viability, membrane integrity and**
357 **depolarization**

358 Differences in viability between the fermentations performed at pH 6 or pH 5 may be
359 explained by the lactate concentration in the culture medium. Lactic acid is a weak acid (pKa

360 = 3.86), with a pH-dependent dissociated lactate level that is prevalent and higher at pH 6
361 than at pH 5. By considering (eq. 1), it diminished with pH, at the expense of the non-
362 dissociated form of lactic acid. The concentration of the lactate form was well correlated with
363 cellular mortality and membrane damage. A high level of dissociated lactate led to a high
364 level of dead cells and then to a poor physiological state of the population. This result
365 indicates that the dissociated form of lactic acid strongly affected the physiological state of *L.*
366 *bulgaricus* CFL1 by damaging membrane integrity. It differed partially from the conclusion
367 of Amrane and Prigent (2) and Even et al. (12) who stated that the non-dissociated form of
368 lactic acid was the main inhibitory compound for growth of lactic acid bacteria. Nevertheless,
369 it was in agreement with the findings of Gonçalves et al. (13) who depicted such a negative
370 influence of the ionic lactate form on *Lactobacillus rhamnosus* at high pH values, whereas the
371 non-dissociated lactic acid was the inhibiting form at low pH. This observation indicates that
372 the lactic acid inhibition mechanism may be described by combining total lactic acid
373 concentration and pH, thus taking into account the membrane integrity damage induced by
374 the ionic form at high pH values. From a mechanistic point of view, this phenomenon can be
375 explained by considering pH homeostasis in the cells. Lactic acid affects pH homeostasis
376 through a mechanism that depends upon both proton concentration, normally referred to the
377 weak acid inhibition mechanism that inhibits cellular reactions, and lactate concentration,
378 which might affect membrane integrity and cellular viability.

379 Whereas high levels of dead cells were observed at pH 6 (> 50 %), most of these cells were
380 not depolarized (< 20 % of depolarized cells). These high levels were linked to the high
381 dissociated lactate concentrations inside ($pH_i > 6$) and outside the cells when $pH_{ext} = 6$. It
382 induced a high level of negative charges inside and outside the cell, thus leading to damaged
383 or dead cells that were not depolarized. Therefore, an enhanced dissociated lactate

384 concentration in the culture medium was associated to an increased cellular mortality but not
385 to cellular depolarization.

386 **The pH gradient influenced the progress of physiological events during *L. bulgaricus***
387 **CFL1 controlled pH cultures**

388 On the basis of viability, acidification activity, intracellular pH and pH gradient
389 measurements, significant differences were observed during the fermentations which made it
390 possible to identify distinct physiological states. Two dynamic schemes representing the
391 progress of the cellular state during fermentations performed at pH 6 and pH 5 are proposed
392 in Fig. 4. Four major periods at pH 6 and three major periods at pH 5 were outlined
393 throughout the fermentations and are discussed below.

394 From cultivability and viability measurements, the first period [1] corresponded, in both
395 cases, to the exponential phase. When the pH was regulated at pH 6, the intracellular pH and
396 the pH gradient remained stable at respectively $pH_i = 6.3$ and $dpH = 0.3$. The same has
397 previously been observed with *L. bulgaricus* that displayed stable $pH_i = 6.5$ and $dpH = 0.1$
398 during log phase of pH 6.4 cultures (17) and with *Lactococcus lactis* which exhibited a
399 constant dpH of 1.1 during growth at pH 6.5 (9). At the same time, the cultivability increased
400 concomitantly to the decrease of viability and acidification activity. When entering the
401 stationary phase, the cells displayed a very low specific acidification activity i.e. a high
402 $tpH_{5.5}$ ($tpH_{5.5} = 461$ min) and a decreasing percentage of viable cells, thus indicating that
403 they were in a bad physiological state. This observation confirms that the metabolic activity
404 of the cells was mainly devoted to their growth during the pH 6 log phase instead of their
405 physiological state.

406 During pH 5 cultures, this behavior differed as acidification activity decreased slightly during
407 the log phase but was maintained at a higher level than at pH 6. The pH_i increased during this

408 first period and was maintained in the range of 6.2 – 6.5, which was associated to compatible
409 pHi values for *L. bulgaricus* growth (17, 20). Even if this pHi range is equivalent to that
410 observed at pH 6, the pH gradient was much more elevated (dpH = 1.2 instead of 0.3). This
411 difference that characterizes a higher energetic level of the whole cell system at pH 5 may be
412 related to the more stable and higher acidification activity, as well as to the stable viability
413 observed at pH 5. This result is in agreement with the findings of Rault et al. (20) obtained
414 during cultures of *L. bulgaricus* CFL1 without pH control.

415 A second period [2] was observed during pH 6 fermentations. It corresponded to the
416 beginning of the stationary phase and to the deceleration of the NaOH consumption rate. It
417 was characterized by the recovery of a high acidification activity associated with a rapid
418 decrease of intracellular pH and of pH gradient, down to pHi = 6.05 and dpH = 0.05,
419 respectively. Viability and membrane integrity continued to decrease while the percentages of
420 injured and depolarized cells remained stable at low levels. A negative relationship was thus
421 demonstrated between the cellular activity and the intracellular pH and pH gradient. These
422 results indicate that the decrease of the pH-gradient makes it possible to deliver energy, thus
423 helping the cells to recover their cellular activity. Such an annulment of dpH at the end of
424 growth has previously been described during *Lactococcus lactis* batch cultures at pH 6.5 (9).

425 During the following period ([2] during pH 5 cultures and [3] during pH 6 cultures), cells
426 were in the stationary phase, as shown by cultivability measurements. Their acidification
427 activity was quite high and stable. Differences between the two pH conditions concerned
428 firstly the viability, which decreased at pH 6 but was high and constant at pH 5. The
429 maintenance of such high cellular activity and viability during the stationary phase has
430 previously been observed during *L. helveticus* cultures at low pH (24). Secondly, a lower
431 lactate level in the culture medium and a higher pH gradient value were observed at pH 5.

432 According to Rault et al. (20), such high dpH values were compatible with high cellular
433 cultivability, viability and acidification activity. By combining these results, it may be
434 proposed that cellular viability and membrane integrity are maintained at high values only if
435 the dpH remains high, as during pH 5 cultures or during cultures without pH control (20). On
436 the contrary, when a low pH gradient was measured, the cells no longer maintained their
437 viability and membrane integrity.

438 The last period ([3] for pH 5 cultures and [4] for pH 6 cultures) was characterized by the
439 decrease of both cultivability and acidification activity. At pH 6, this decrease was related to a
440 reduction of viability that reached less than 50 % of viable cells in the total population,
441 whereas at pH 5, viability and membrane integrity remained high and constant. As previously
442 explained, this difference in cellular viability was associated with the lower lactate level and
443 the significantly higher pH gradient at pH 5 ($\text{dpH} > 0.42$) as compared to pH 6 ($\text{dpH} = 0.05$).
444 Despite these high pH gradient and viability values observed during pH 5 cultures, the pHi
445 decreased to $\text{pHi} = 5.42$. This value was lower than 5.8, which was considered by Rault et al.
446 (20) as the limit value compatible with growth and acidification activity of *L. bulgaricus*
447 CFL1. This situation may explain the decrease in cultivability and acidification activity
448 during this last period.

449

450 **Conclusion**

451 As a final conclusion, this work makes it possible to establish the progress of the
452 physiological events that occur during controlled pH cultures of *L. bulgaricus* CFL1. It points
453 out strong differences in the time course of mortality (membrane damage), viability
454 (enzymatic activity), cellular depolarization, intracellular pH and acidification activity
455 between pH 6 and pH 5 controlled cultures. Viability and acidification activity were higher

456 and more stable during growth when cultures were carried out at pH 5, instead of pH 6. These
457 divergences can mainly be explained by differences in dissociated lactate concentration,
458 intracellular pH and pH gradient during growth. These key factors grant a better
459 understanding of the influence of fermentation pH on *L. bulgaricus* CFL1 cellular state in
460 growing conditions.

461 At an industrial stage, such results may benefit to starter's producers and fermented products
462 manufacturers, as they may allow them to better control the quality of their starters, before
463 freezing or before using them for food fermentation. Within this approach, they may select a
464 well defined physiological state for a given application, and apply well defined conditions
465 (harvesting time and pH) to achieve this physiological state. From an economical point of
466 view, the cost of the flow cytometry method remained quite high, thus representing a
467 drawback to its development. Conversely, the Cinac system is still highly employed, which
468 confer it a great interest.

469 In the future, such discrimination between different physiological states in growing conditions
470 may be useful to standardize the cells in a well defined physiological state during their
471 production. We also aim at explaining the observed differences by analyzing the intracellular
472 proteome and the membrane fatty acid composition of the cells.

473

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477

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559 **Figures:**

560 **Fig. 1:** Time course of *L. bulgaricus* CFL1 physiological characteristics throughout
561 fermentations performed at pH 6. **(a):** NaOH consumption (—, in g l^{-1}) and rate of NaOH
562 consumption (dNaOH/dt , - - , in $\text{g l}^{-1} \text{min}^{-1}$); **(b):** Cultivability (Δ , in CFU ml^{-1}) and relative
563 percentages of viable (\bullet , in %), dead (\circ , in %), damaged (\bullet , in %) and depolarized ($+$, in
564 %) cells; **(c):** Specific acidification activity (tpH5.5 , \blacktriangle , in min); **(d):** Intracellular pH (\blacksquare),
565 extracellular pH (\square) and pH gradient ($\text{dpH} = \text{pHi} - \text{pHext}$) (\blacksquare).

566 Results are the means of at least three independent measurements. They are expressed as a
567 function of the time t_m to reach the maximal rate of NaOH consumption as an adjusted time.

568

569 **Fig. 2:** Time course of *L. bulgaricus* CFL1 physiological characteristics throughout
570 fermentations performed at pH 5. **(a):** NaOH consumption (—, in g l^{-1}) and rate of NaOH
571 consumption (dNaOH/dt , - - , in $\text{g l}^{-1} \text{min}^{-1}$); **(b):** Cultivability (Δ , in CFU ml^{-1}) and relative
572 percentages of viable (\bullet , in %), dead (\circ , in %), damaged (\bullet , in %); **(c):** Specific
573 acidification activity (tpH5.5 , \blacktriangle , in min); **(d):** Intracellular pH (\blacksquare), extracellular pH (\square) and
574 pH gradient ($\text{dpH} = \text{pHi} - \text{pHext}$) (\blacksquare).

575 Results are the means of at least three independent measurements. They are expressed as a
576 function of the time t_m to reach the maximal rate of NaOH consumption as an adjusted time.

577

578 **Fig. 3:** Relationship between the logarithm of dead cell counts measured by flow cytometry
579 (D , in cells ml^{-1}) and dissociated lactate concentration in the culture medium (Lac , in g l^{-1}),
580 during fermentations performed at pH 6 (\blacksquare) and pH 5 (Δ).

581

582 **Fig. 4:** Schematic representation of physiological events during pH 6 or pH 5 *L. bulgaricus*

583 CFL1 cultures. Bold lines correspond to main changes in the cellular state. Distinct cellular
584 phases are numbered.

585 **Tables:**

586 **Table 1:** Kinetic parameters characterizing fermentations of *L. bulgaricus* CFL1 conducted at
587 controlled pH 6 or pH 5.

Parameters	pH 6	pH 5
V _m	-0.32 (± 0.03)	-0.13 (± 0.02)
t _m	806 (± 23)	1045 (± 34)
dNaOH	29.1 (± 1.5)	23.8 (± 4.9)
dlact	35.2 (± 0.8)	32.4 (± 2.1)
dgal	19.2 (± 0.7)	16.9 (± 0.5)
dLA	17.2 (± 0.4)	16.2 (± 0.5)

588 V_m (in g l⁻¹ min⁻¹): maximal rate of NaOH consumption; t_m (in min): time to reach V_m;

589 dNaOH (in g l⁻¹): NaOH consumption at t_m; dlact (in g l⁻¹): total lactose consumption; dgal

590 (in g l⁻¹): total galactose production; dLA (in g l⁻¹): total lactic acid production.

591 Values are means of at least three measurements, with corresponding 95 % confidence

592 intervals in brackets.

Fig. 1

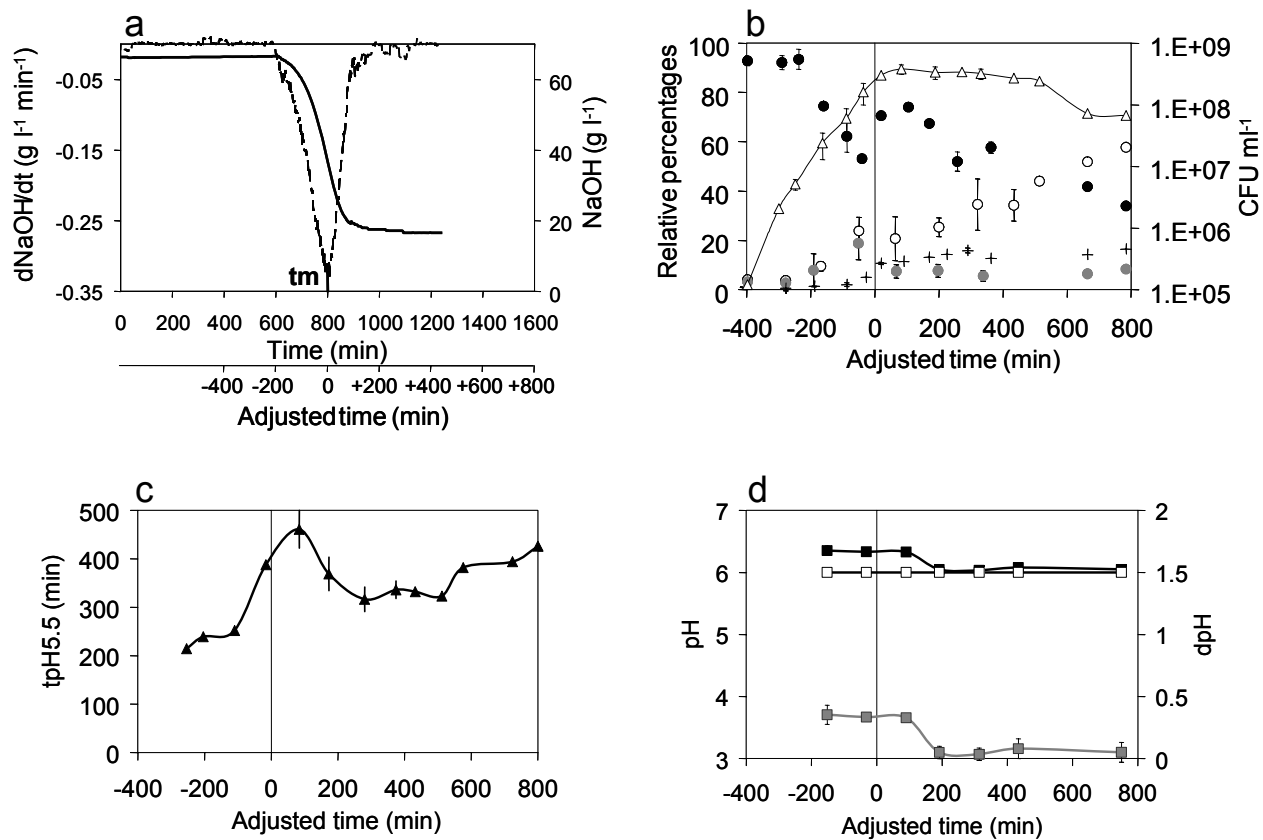
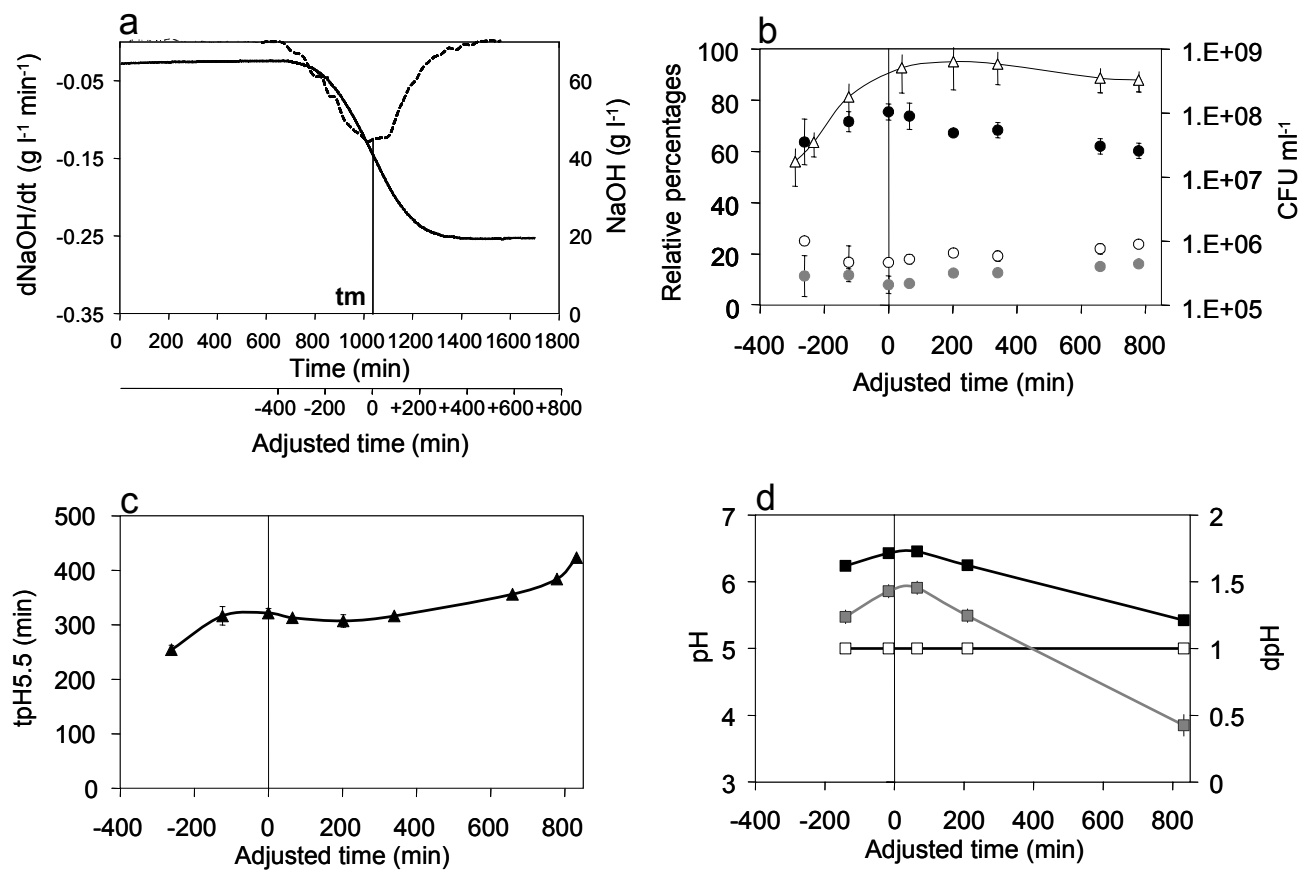


Fig. 2



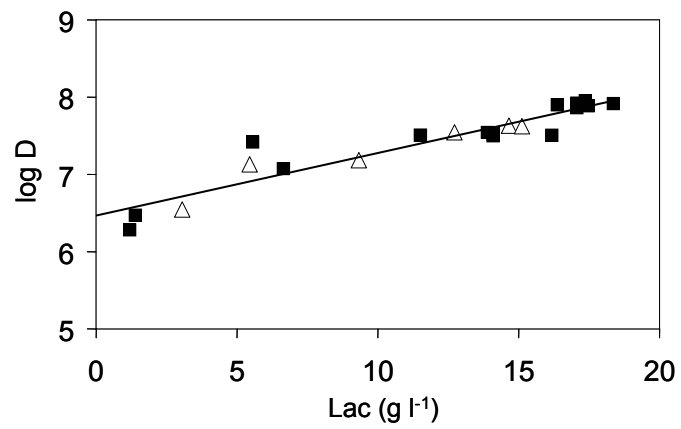


Fig. 3

Fig. 4

