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Fermentation pH influences the dynamic of *Lactobacillus bulgaricus* CFL1 physiological state during pH-controlled cultures

Running title: pH affects the dynamic of physiological state

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Abbreviation key: cFDA: carboxyfluorescein diacetate; cFDA-SE: 5(6-)-carboxyfluorescein diacetate succinimidyl ester; D: dead cell concentration; $DIBAC_4(3)$: bis (1,3 dibutylbarbituric acid) trimethine oxonol; dpH: pH gradient (pHi-pHext); FSC: forward-angle light scatter; Lac: lactate concentration; N: cultivable cell concentration; NaOH: sodium hydroxide; pHext: extracellular pH; pHi: intracellular pH; PI: propidium iodide; pKa: dissociation constant of lactic acid; SSC: side-angle light scatter; tm: time to reach the maximal rate of NaOH consumption; tpH5.5: time necessary to reach pH 5.5 in milk; V: viable cell concentration; Vm: maximal rate of NaOH consumption

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and methods

Bacterial strain and preculture conditions

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

Fermentation conditions

The culture medium was composed of 60 g l^{-1} sweet whey powder (Eurosérum, Port-sur-

Saône, France) which was adjusted to pH 5 with 50 % H_2SO_4, heat-treated at 110°C for 20 min and centrifuged at 7000 *g* for 30 min at 4°C. After filtration (0.45 m), it was supplemented with 20 g l^{-1} yeast extract (Organotechnie, La Courneuve, France) and introduced into a 2-l bioreactor (Setric Génie Industriel, Toulouse, France). After sterilization at 110°C for 20 min, the initial pH was adjusted at pH 6, and inoculation was done at a low initial level of 20 ± 5.5 CFU ml^{-1} to allow overnight culture. Fermentations were performed at 42°C, 100 rpm and with pH 6 or pH 5-control, by automatic adding of a 5 N NaOH solution in the bioreactor. The NaOH concentration (m, in g l^{-1}) and its consumption rate (dm/dt, in g l^{-1} min^{-1}) were calculated with WCidus software (INRA, Thiverval-Grignon, France).

The fermentation kinetics were therefore established and the reproducibility was assessed according to three descriptors: the maximal rate of NaOH consumption (Vm, in g l^{-1} min^{-1}), the time necessary to reach Vm (tm, in min) and the NaOH consumption (dNaOH, in g l^{-1}) at tm. Samples were taken from the bioreactor at different sampling times, from the beginning of NaOH consumption up to late stationary phase. In order to normalize the data obtained from the different cultures, results were expressed as a function of the time tm (in min) necessary to reach Vm, which was considered as a reference time.

HPLC measurements

Lactose, glucose, galactose and lactic acid concentrations were quantified using High Performance Liquid Chromatography (HPLC, Waters Associates, Millipore, Molsheim, France). Before the HPLC analyses, each sample was combined with 120 g l^{-1} trichloroacetic acid (Prolabo, Paris, France), centrifuged at 4000 *g* for 30 min at 4°C and filtered (0.22 m). The HPLC analysis was made on a cation exchange column (Aminex Ion Exclusion HPX-87H 300*7.8 mm, Biorad, Richmond, USA) at 35°C with propionic acid (10 g l^{-1}) as an internal standard. The mobile phase was 0.005 M H_2SO_4 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 (pKa = 3.86), pH and total
147 lactic acid [LA] concentration measured by HPLC. They were calculated by considering the
148 following formula:

149 $[A-] / [AH] = 10^{(pH - pKa)}$ (eq. 1)

150 $[A-] + [AH] = [LA]$ (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 10^6 cells ml^{-1}. 1 ml of the
164 diluted suspension was first supplemented with 10 l PI (1.496 mM in distilled water, Sigma-
165 Aldrich, Lyon, France) and incubated for 20 min at 40°C. 10 l 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 ($DIBAC_4(3)$) 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 $DIBAC_4(3)$ in a single analysis. 1 ml
172 of a bacterial suspension was diluted in Chemsol B13 buffer (AES-Chemunex) to reach 10^6
173 cells ml^{-1} and incubated with 10 l PI (1.496 mM in distilled water, Sigma-Aldrich) for 10
174 min at 40°C. 5 l $DIBAC_4(3)$ (96.78 M in dimethylsulfoxyde, 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 10^7 cells ml^{-1}. 1 ml of the diluted suspension was incubated with 5 l
183 cFDA-SE (8.96 M in acetone, Invitrogen-Molecular Probes) for 10 min at 40°C. After
184 staining, 100 l 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 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 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 $DIBAC_4(3)$ (FL1 channel) and a 630 nm long-
203 pass filter to collect the red fluorescence of propidium iodide (FL2 channel). The FACSFlow
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**

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

Results

Fermentation kinetics

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

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

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

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

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

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

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 reached at tm+100 min, a significant drop in the percentage of viable cells was observed throughout growth, from 93 ± 3 % before tm-200 min down to 34 ± 2 % at tm+800 min. The percentage of damaged cells reached a maximum of 19 ± 6 % just before the maximal acidification point, then decreased and stood quite stable at 8 ± 1 %. The viability decrease was inversely correlated to the percentage of dead cells, which rose by 58 ± 2 % in the same period. Depolarized cells depicted a slight rise of 16 ± 1 % during growth, before stabilizing during stationary phase. By comparing these results with dead cells percentages, it was observed that only a small part of dead cells were depolarized.

During the stationary phase, CFU concentration stood quite steady at a high level up to 500 min after the maximal acidification point before decreasing down to $7 \cdot 10^7$ CFU ml^{-1} (Fig. 1b). Total viable cell counts (V, in viable+damaged cells ml^{-1}) determined by flow cytometry were well correlated with plate counts (N, in CFU ml^{-1}) with a significant coefficient of determination of 0.977. Nevertheless, they were slightly lower than plate counts with a mean difference of 5 % throughout growth. The corresponding linear relationship was determined

as follows:

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

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

The time course of intracellular pH (pHi) and pH gradient (dpH) was followed during pH 6 fermentations and the results are summarized in Fig. 1d. These two parameters were stable at pHi = 6.34 ± 0.01 and dpH = 0.34 ± 0.01 during the log phase, until the stationary phase was achieved (tm+100 min). At the beginning of the stationary phase, these two parameters decreased to pHi = 6.05 ± 0.01 and dpH = 0.05 ± 0.01 at tm + 200 min, then remained stable until the end of fermentation.

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

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

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

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

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

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

From Fig. 2c it can be seen that specific acidification activity decreased throughout pH 5 fermentation, as tpH5.5 increased from 254 ± 9 min until 423 ± 11 min. Fluctuations were less important than at pH 6. After a slight increase during log phase until tm-100 min, the time to reach pH5.5 remained stable at 314 ± 5 min up to tm+400 min. Finally, acidification activity decreased, i.e. tpH5.5 increased up to 423 ± 11 min at tm+800 min.

Fig. 2d shows that, in spite of a constant and low external pH 5, the cells maintained a high intracellular pH (pHi > 6) and a high pH gradient (dpH > 1) until the beginning of the stationary phase. These two characteristics increased from the beginning of the measurements up to a maximum of pHi = 6.5 ± 0.05 and dpH = 1.5 ± 0.05 at the end of the log phase (tm+60 min). They then decreased down to pHi = 5.4 ± 0.08 and dpH = 0.4 ± 0.08 at tm+800 min.

Relationship between dead cells and lactate concentration in the culture medium

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

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

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

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

Discussion

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

As shown in our results, even though *Lactobacillus bulgaricus* CFL1 fermentations at pH 5 were longer than at pH 6, as previously observed by Amrane and Prigent (2) with low pH-controlled cultures of *Lactobacillus helveticus*, they did not lead to significant differences in terms of final lactose consumption, galactose and lactic acid production or NaOH consumption. Final CFU concentration was slightly higher at pH 5 than at pH 6, but the difference was not significant if standard deviations are considered. This result is in agreement with that obtained by Mercade et al. (18) who depicted a similar final biomass production when *Lactococcus lactis* subsp. *cremoris* was cultured at pH 6.6 or pH 5. However, the concentrations obtained in the current study were significantly higher than those obtained by Rault et al. (20) when cultures of *L. bulgaricus* CFL1 were performed without pH control ($1.3 \cdot 10^8$ CFU ml^{-1}). This result is consistent with previous studies which compared free and controlled pH cultures of *Lactococcus lactis* (9) and *L. bulgaricus* (17).

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

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

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

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

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

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

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

Whereas high levels of dead cells were observed at pH 6 (> 50 %), most of these cells were not depolarized (< 20 % of depolarized cells). These high levels were linked to the high dissociated lactate concentrations inside (pHi > 6) and outside the cells when pHext = 6. It induced a high level of negative charges inside and outside the cell, thus leading to damaged or dead cells that were not depolarized. Therefore, an enhanced dissociated lactate

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

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

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

From cultivability and viability measurements, the first period [1] corresponded, in both cases, to the exponential phase. When the pH was regulated at pH 6, the intracellular pH and the pH gradient remained stable at respectively pHi = 6.3 and dpH = 0.3. The same has previously been observed with *L. bulgaricus* that displayed stable pHi = 6.5 and dpH = 0.1 during log phase of pH 6.4 cultures (17) and with *Lactococcus lactis* which exhibited a constant dpH of 1.1 during growth at pH 6.5 (9). At the same time, the cultivability increased concomitantly to the decrease of viability and acidification activity. When entering the stationary phase, the cells displayed a very low specific acidification activity i.e. a high tpH5.5 (tpH5.5 = 461 min) and a decreasing percentage of viable cells, thus indicating that they were in a bad physiological state. This observation confirms that the metabolic activity of the cells was mainly devoted to their growth during the pH 6 log phase instead of their physiological state.

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

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

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

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

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

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

Conclusion

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

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

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

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

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References

1. **Adamberg, K., S. Kask, T. M. Laht, and T. Paalme.** 2003. The effect of

480 temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. Int. J.
481 Food Microbiol. **85:**171-183.
482 2. **Amrane, A., and Y. Prigent.** 1999. Differentiation of pH and free lactic acid effects
483 on the various growth and production phases of *Lactobacillus helveticus*. J. Chem.
484 Technol. Biot. **74:**33-40.
485 3. **Barreto, M. T. O., E. P. Melo, J. S. Almeida, A. M. R. B. Xavier, and M. J. T.**
486 **Carrondo.** 1991. A kinetic method for calculating the viability of lactic starter
487 cultures. Appl. Microbiol. Biotechnol. **34:**648-652.
488 4. **Béal, C., and G. Corrieu.** 1991. Influence of pH, temperature and inoculum
489 composition on mixed cultures of *Streptococcus thermophilus* 404 and *Lactobacillus*
490 *bulgaricus* 398. Biotechnol. Bioeng. **38:**90-98.
491 5. **Béal, C., P. Louvet, and G. Corrieu.** 1989. Influence of controlled pH and
492 temperature on the growth and acidification of pures cultures of *Streptococcus*
493 *thermophilus* 404 and *Lactobacillus bulgaricus* 398. Appl. Microbiol. Biotechnol.
494 **32:**148-154.
495 6. **Béal, C., M. Marin, E. Fontaine, F. Fonseca, and J. P. Obert.** 2008. Production et
496 conservation des ferments lactiques et probiotiques, p. 661-785. *In* G. Corrieu and F.-
497 M. Luquet (ed.), Bactéries lactiques, de la génétique aux ferments. Tec&Doc
498 Lavoisier, Paris.
499 7. **Ben Amor, K., P. Breeuwer, P. Verbaarschot, F. M. Rombouts, A. D. L.**
500 **Akkermans, W. M. De Vos, and T. Abee.** 2002. Multiparametric flow cytometry and
501 cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells
502 during bile salt stress. Appl. Environ. Microbiol. **68:**5209-5216.
503 8. **Bibal, B., G. Goma, Y. Vayssier, and A. Pareilleux.** 1988. Influence of pH, lactose

and lactic acid on the growth of *Streptococcus cremoris*: a kinetic study. Appl. Microbiol. Biotechnol. **28:**340-344.

9. **Cachon, R., P. Antérieux, and C. Diviès.** 1998. The comparative behavior of *Lactococcus lactis* in free and immobilized culture processes. J. Biotechnol. **63:**211-218.

10. **Corrieu, G., H. E. Spinnler, Y. Jomier, and D. Picque.** 1988. Automated system to follow up and control the acidification activity of lactic acid starters. French patent FR 2 629 612.

11. **Daly, C., G. F. Fitzgerald, L. O'Connor, and R. Davis.** 1998. Technological and health benefits of dairy starter cultures. Int. Dairy J. **8:**195-205.

12. **Even, S., N. D. Lindley, P. Loubiere, and M. Cocaign-Bousquet.** 2002. Dynamic response of catabolic pathways to autoacidification in *Lactococcus lactis*: transcript profiling and stability in relation to metabolic and energetic constraints. Mol. Microbiol. **45:**1143-1152.

13. **Gonçalves, L. M. D., A. Ramos, J. S. Almeida, A. M. R. B. Xavier, and M. J. T. Carrondo.** 1997. Elucidation of the mechanism of lactic acid growth inhibition and production in batch cultures of *Lactobacillus rhamnosus*. Appl. Microbiol. Biotechnol. **48:**346-350.

14. **Grattepanche, F., S. Miescher-Schwenninger, L. Meile, and C. Lacroix.** 2008. Recent developments in cheese cultures with protective and probiotic functionalities. Dairy Sci. Technol. **88:**421-444.

15. **Hutkins, R. W., and N. L. Nannen.** 1993. pH homeostasis in lactic acid bacteria. J. Dairy Sci. **76:**2354-2365.

16. **Jepras, R. I., J. Carter, S. C. Pearson, F. E. Paul, and M. J. Wilkinson.** 1995.

Development of a robust flow cytometric assay for determining numbers of viable bacteria. Appl. Environ. Microbiol. **61:**2696-2701.

17. **Mercade, M., F. Duperray, and P. Loubière.** 2004. Energetic analysis of cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus*: identification of the type of control between catabolism and anabolism. Lait **84:**39-47.

18. **Mercade, M., N. D. Lindley, and P. Loubière.** 2000. Metabolism of *Lactococcus lactis* subsp. *cremoris* MG 1363 in acid stress conditions. Int. J. Food Microbiol. **55:**161-165.

19. **Rault, A., C. Béal, S. Ghorbal, J.-C. Ogier, and M. Bouix.** 2007. Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. Cryobiology **55:**35-43.

20. **Rault, A., M. Bouix, and C. Béal.** 2008. Dynamic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 physiological characteristics during fermentation. Appl. Microbiol. Biotechnol. **81:**559-570.

21. **Rechinger, K. B., and H. Siegumfeldt.** 2002. Rapid assessment of cell viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* by measurement of intracellular pH in individual cells using Fluorescence Ratio Imaging Microscopy. Int. J. Food Microbiol. **75:**53-60.

22. **Rhee, S. K., and M. Y. Pack.** 1980. Effect of environmental pH on chain length of *Lactobacillus bulgaricus*. J. Bacteriol. **144:**865-868.

23. **Savoie, S., C. P. Champagne, S. Chiasson, and P. Audet.** 2007. Media and process parameters affecting the growth, strain ratios and specific acidifying activities of a mixed lactic starter containing aroma-producing and probiotic strains. J. Appl. Microbiol. **103:**163-174.

552 24. **Schepers, A. W., J. Thibault, and C. Lacroix.** 2002. *Lactobacillus helveticus* growth
553 and lactic acid production during pH-controlled batch cultures in whey
554 perrmeate/yeast extract medium. Part I. Multiple factor kinetic analysis. Enzyme
555 Microb. Technol. **30:**176-186.
556 25. **Wang, Y., G. Corrieu, and C. Béal.** 2005. Fermentation pH and temperature
557 influence the cryotolerance of *Lactobacillus acidophilus* RD758. J. Dairy Sci. **88:**21-
558 29.

Figures:

Fig. 1: Time course of *L. bulgaricus* CFL1 physiological characteristics throughout fermentations performed at pH 6. **(a):** NaOH consumption (**—,** in g l^{-1}) and rate of NaOH consumption (dNaOH/dt, - -, in g l^{-1} min^{-1}); **(b):** Cultivability (△, in CFU ml^{-1}) and relative percentages of viable (●, in %), dead (○, in %), damaged (●, in %) and depolarized (+, in %) cells; **(c):** Specific acidification activity (tpH5.5, ▲, in min); **(d):** Intracellular pH (■), extracellular pH (□) and pH gradient (dpH = pHi – pHext) (■).

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

Fig. 2: Time course of *L. bulgaricus* CFL1 physiological characteristics throughout fermentations performed at pH 5. **(a):** NaOH consumption (**—,** in g l^{-1}) and rate of NaOH consumption (dNaOH/dt, - -, in g l^{-1} min^{-1}); **(b):** Cultivability (△, in CFU ml^{-1}) and relative percentages of viable (●, in %), dead (○, in %), damaged (●, in %); **(c):** Specific acidification activity (tpH5.5, ▲, in min); **(d):** Intracellular pH (■), extracellular pH (□) and pH gradient (dpH = pHi – pHext) (■).

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

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

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
Vm	-0.32 (± 0.03)	-0.13 (± 0.02)
tm	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 Vm (in g l^{-1} min^{-1}): maximal rate of NaOH consumption; tm (in min): time to reach Vm;

589 dNaOH (in g l^{-1}): NaOH consumption at tm; 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

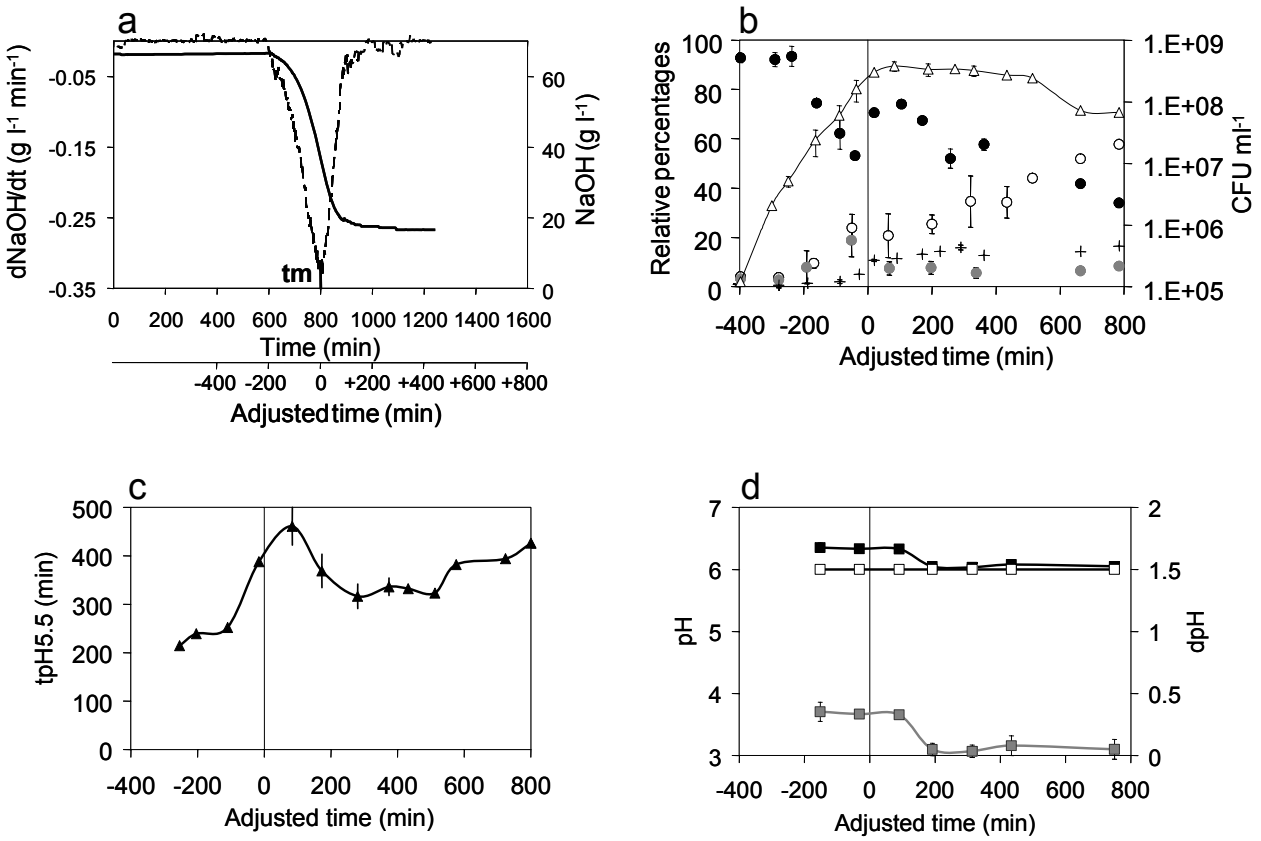

Fig. 2

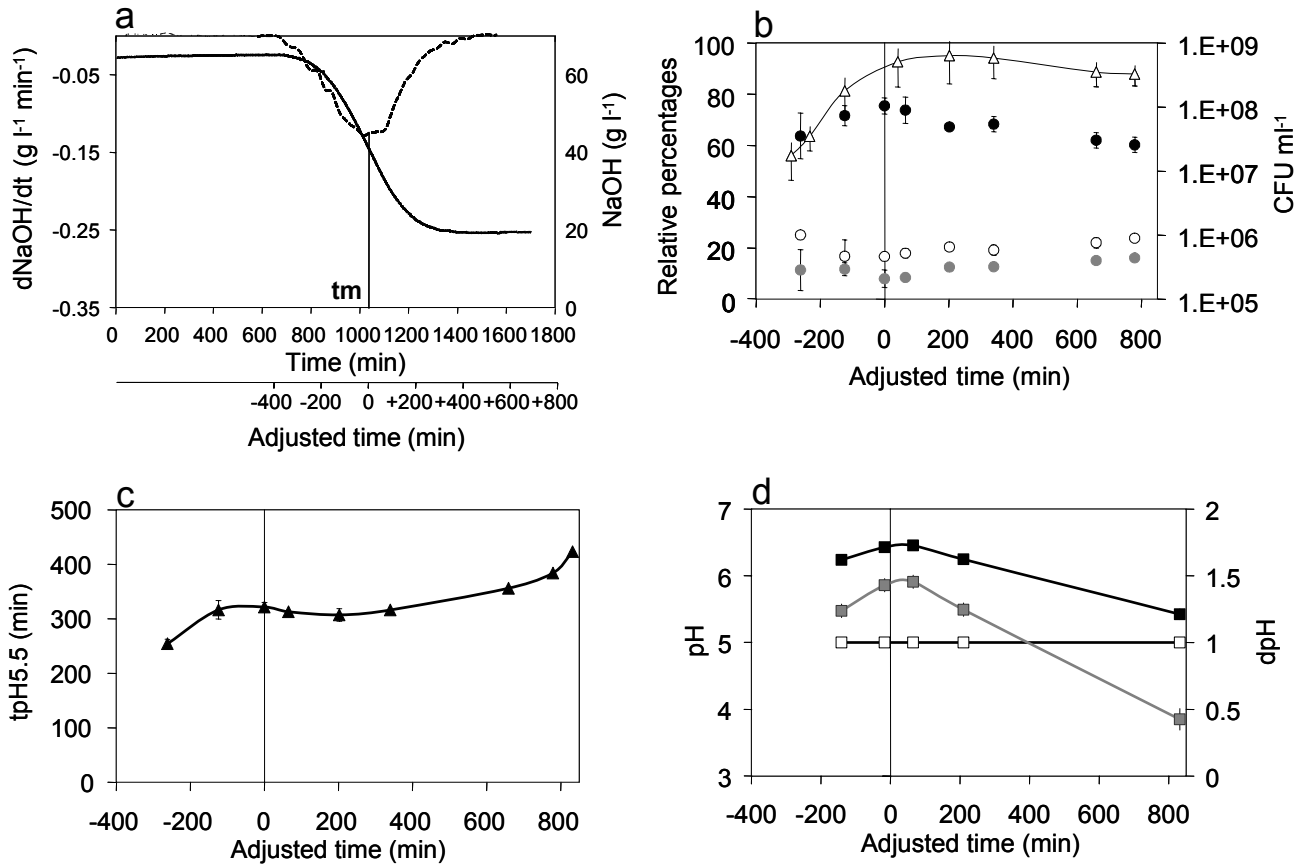

Fig. 3

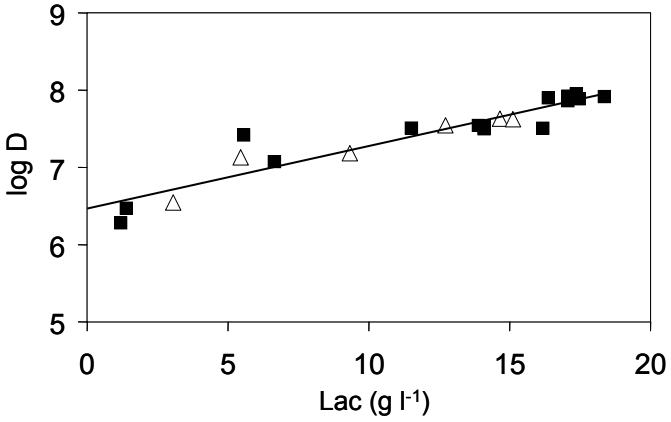

Fig. 4