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

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Running title: The cooperative behaviour of S. thermophilus urease
ABSTRACT

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

Most food fermentation processes involve mixed cultures in which different microbial species interact with each other. These interactions may have neutral, positive or negative effects on the fitness of the strains performing the fermentation. Microbial interactions that exert positive effects on the micro-organisms participating in the process are classified as mutualistic interactions (Boucher et al., 1982). The best example of mutualism in a food process is represented by the yogurt consortium, which consists of the lactic acid bacteria (LAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Sieuwerts et al., 2008). In the yogurt consortium, the interactions that occur between the two LAB species in milk have been described as a proto-cooperative process, which has positive effects on the growth rate and size of each population; in contrast, the independent growth of the two species in milk led to a slower growth rate and a smaller population size (Zourari et al., 1992; Sieuwerts et al., 2010). The seemingly simplistic metabolism of these organisms, the availability of complete genomic information for the two species involved in the yogurt consortium (Bolotin et al., 2004; van de Guchte et al., 2006), and the availability of molecular tools suitable for the design of appropriate recombinant strains have made this microbial consortium an attractive model system for studies of species-species interactions. The molecular interactions that play key roles in the mutualistic behaviour of the yogurt consortium have been investigated using several approaches (Courtin et al., 2002; Liu et al., 2009; Herve-Jimenez et al., 2009; Sieuwerts et al., 2010) and have been reviewed by several authors (Sieuwerts et al., 2008; Smid et al., 2013). Validated interactions between *S. thermophilus* and *L. delbrueckii* during the growth of these species in milk have been described in relation to: i) the availability of nitrogen in milk (Courtin et al., 2002), ii) the exchange of formic acid, pyruvic acid and folic acid (Crittenden et al., 2003), iii) the production and utilisation of carbon dioxide (Driessen et al., 1982), iv) and the metabolism of purine, amino acid, long-chain fatty acids.
(Sieuwerts et al., 2010) and iron (Herve-Jimenez et al., 2009). A schematic summary of these metabolic interactions is presented in Figure 1. As expected, most of the previously described interactions are trophic interactions in which the species feed each other with pyruvate, folate, long chain fatty acids, ornithine, carbon dioxide, peptides, amino acids and putrescine (Sieuwerts et al., 2010). Among the LAB involved in dairy fermentation processes, urease activity is present and widely distributed in only S. thermophilus, for which urease-negative strains are rare in nature (Driessen et al., 1982; Mora et al., 2002; Mora et al., 2005; Zourari et al., 1991). Urease is a multi-subunit urea amidohydrolase (EC 3.5.1.5) that catalyses the hydrolysis of urea to yield ammonia and carbamate. Urease is encoded by an 11-gene operon that accounts for 0.9 % of the estimated core genome of S. thermophilus (Mora et al., 2004; Rasmussen et al., 2008). The urease activity of S. thermophilus shares metabolic relationships with the biosynthetic pathways involved in aspartate, glutamine, arginine and carbon dioxide metabolism (Arioli et al., 2007; Arioli et al., 2009; Monnet et al., 2005). Notably, urea hydrolysis increases the catabolic efficiency of S. thermophilus by modulating the intracellular pH and increasing the activity of β-galactosidase, glycolytic enzymes and lactate dehydrogenase (Arioli et al., 2010). Urea hydrolysis results in increases in both intracellular (pH$_{in}$) and extracellular (pH$_{out}$) pH due to the rapid diffusion of ammonia outside of the cell. Consequently, in the presence of urea and a urease-positive microorganism, urease-negative microorganisms share the environmental benefit derived from the release of ammonia and the related transient local pH increase (Arioli et al., 2010). In this study, we analysed the effect of urea hydrolysis by S. thermophilus on variations in the pH$_{in}$ and lactic acid fermentation of L. delbrueckii to obtain new information related to the proto-cooperation that exists between these two species during yogurt production.
2. Materials and Methods

2.1. Bacterial strains and growth conditions

*S. thermophilus* CNRZ385, the urease-negative mutant A16(AureC3) (Mora *et al.*, 2004), and *Lactobacillus delbrueckii* MIM91 were maintained in lactose M17 broth (Difco, Laboratories, Detroit, MI) containing 20 g/L of lactose and in MRS broth (Difco, Laboratories, Detroit, MI) at 42°C. For pH*in* measurements, the strains were grown at 42°C in the appropriate medium until the culture reached an OD*600nm* of 1.0. The cells were then collected by centrifugation at 15,000 x *g* for 1 min and suspended in solution A (9 g/L of NaCl and 100 µg/ml of chloramphenicol-Sigma-Aldrich, Milan, Italy, in distilled water). Chloramphenicol at the concentrations used here was expected to block translation, so that only enzymes already present within the cell are expected to participate in the experiment (Lopez *et al.*, 1998).

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

The internal pH of *S. thermophilus* and *L. delbrueckii* was measured using the pH-sensitive fluorescence probe 5 (and 6-)carboxyfluorescein succinimidyl ester (cFSE), based on the method originally described by Breeuwer (Breeuwer *et al.*, 1996), and later described by Sawatari and Yokota (Sawatari *et al.*, 2007), with some modifications. The fluorescence intensity of this probe increases at alkaline pH and decreases at acidic pH. Cell suspensions in solution A were obtained as described above, diluted to an OD*600nm* of approximately 0.5 and supplemented with 4 µM cFDASE (Sigma-Aldrich, Milan, Italy), which is a precursor molecule of cFSE. The suspensions were then incubated for 30 min at 37°C. During this incubation, the membrane-permeating cFDASE was cleaved by intracellular esterases and the resultant cFSE molecules were conjugated to the aliphatic amines of intracellular proteins. After centrifugation at 15,000 x *g* for 1 min and washing with solution A, the cells were suspended in the same volume of solution A. The unconjugated probe was eliminated by the addition of glucose at a final concentration of 16 mM and subsequent incubation at 37°C for 1 h. After centrifugation and washing with solution A,
the cells were suspended to obtain a final cell concentration that corresponded to $10^9$ events per ml. To be sure that the unconjugated and free probes were eliminated by the cells, we monitored the cells fluorescence before and after the treatment with glucose. Moreover, the stability of the cells fluorescence was assessed steadily; the stained cells kept in ice and in solution A after staining maintained a stable fluorescence, indicating that no free cFSE molecules were inside the cells. A part from pH, the intensity of the fluorescence depends on the esterase activity of the cells and could be a species and/or strain dependent feature. In all experiment, after staining we observed a major fluorescence intensity in *L. delbrueckii* than in *S. thermophilus*, reflecting a diverse esterase content in the two species, as previously reported for strains belonging to other bacterial species (Ouzari *et al.*, 2006).

Cell counting was performed using an Accuri C6 flow cytometer (BD Biosciences, Milan, Italy). In flow cytometry, particles/cells that pass through the beam will scatter light, which is detected as forward scatter (FSC) and side scatter (SSC). FSC correlates with cell size, cell shape and cell aggregates, whereas SSC depends on the density of the particles/cells (*i.e.*, the number of cytoplasmic granules and membrane size); in this manner, cell populations can often be distinguished based on differences in their size and density (Cronin *et al.*, 2010, Gunasekera *et al.*, 2003). The combination of scattered and fluorescent light is measured by a number of detectors as the cells pass by an interrogation point in a fluid stream (Sincock and Robinson, 2001). Cell suspensions that were prepared as described above were analysed using the flow cytometer with the following threshold settings: FSC 5,000, SSC 4,000, and 20,000 total events collected. All of the parameters were collected as logarithmic signals. The 488 nm laser was used to measure the FSC values. The rate of events in the flow was generally lower than 2,000 events/s.

The obtained data were analysed using BD Accuri™ C6 software version 1.0 (BD Biosciences, Milan, Italy). The cFSE fluorescence intensity of stained cells was recovered in the FL1 channel (excitation 488 nm, emission filter 530/30, provided by BD Biosciences, Milan, Italy). Density 6
plots of green fluorescence (FL1) and FSC allowed for optimal distinction between the cFSE-
stained microbial cells and instrument noise or sample background. An electronic gate on the green
fluorescence/FSC density plot was used to select the measured bacterial concentration (events per
ml), and the selected data from the bacterial gate were subsequently visualised on a cFSE green
fluorescence histogram for further analysis. To measure pH_in, 5 ml of a cFSE-labelled cell
suspension were incubated at 42 °C in the presence or absence of 2.5 mM urea or 5 mM ammonia
for 15 min. Every 5 min, 100 µl of suspension was sampled and the internal pH was determined
by measuring the fluorescence intensities in the FL1 channel via flow cytometry. A calibration
curve that reported FL1 fluorescence vs. pH_in was obtained as described below. An aliquot of the
cell suspension was washed and suspended in different buffers with pH values ranging from 6.96
to 8.25 for S. thermophilus and from 6.36 to 7.82 for L. delbrueckii and treated with 100 µM
gramicidin (Sigma-Aldrich, Milan, Italy), which dissipates the transmembrane proton gradient.
The fluorescence intensity was then measured for calibration at appropriate external pH values.
(only for reviewers: the calibration curves obtained for S. thermophilus and L. delbrueckii strains
are shown in Figure 4S1 and Figure 5S1). Alternatively, pH_in was measured in urea-free skimmed
milk, obtained by treating reconstituted skimmed milk (Difco, Laboratories, Detroit, MI) with 1.36
U ml⁻¹ of jack bean urease (Sigma-Aldrich, Milan, Italy) for 3 h at 37°C prior to sterilisation (110°C,
10 min) (Pernoud et al., 2004). In all experiments, external pH (pH_out) was measured using a
standard pH meter. D/L lactic acid was quantified after 15 min of incubation at 42 °C in urea-free
skimmed milk using a D/L lactic acid kit (R-Biopharm, Milan, Italy) according to the
manufacturer’s instructions. All data are presented as the average of three independent replicates
± SEM.

2.3. Flow cytometry total cell count

Total cell count of S. thermophilus and L. delbrueckii culture in reconstituted skimmed milk was
measured by flow cytometry after 3 and 6 h of incubation at 42 °C. 500 µl of milk culture was
subjected to a clearing procedure by adding an equal volume of Tris-HCl (2 M) EDTA (0.2 M) buffer (pH 8). Following 10 min of incubation at 50 °C the cell suspension was labelled with SYBR green I (Sigma-Aldrich, Milan, Italy). After incubation at 37 °C for 20 min, the labelled cell suspension was diluted to reach approximately $10^6$ events per ml and analysed by flow cytometry. The obtained data were analysed using BD Accuri™ C6 software version 1.0 (BD Biosciences, Milan, Italy). The SYBR green I fluorescence intensity of stained cells was recovered in the FL1 channel. Density plots of green fluorescence (FL1) vs FSC allowed for optimal distinction between the SYBR green I-stained microbial cells and instrument noise or sample background. An electronic gate on the green fluorescence/FSC density plot was used to select and measure the total bacterial concentration (events per ml) (only for reviewers: Fig. 9S1).

2.4. In vivo NMR spectroscopy

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

For $^{13}$C- experiments, L. delbrueckii cells grown in MRS were collected in the exponential growth phase (i.e., at an O.D.$_{600\text{nm}}$ of 1.0) and washed and suspended in solution A containing 10 % (v/v) D$_2$O to obtain a cell density that corresponded to 8-9 mg of protein per ml. The lactose metabolism of non-growing L. delbrueckii was studied in energetically discharged cells. Specifically, a cell suspension that was prepared in solution A (see section 2.1) as described above was de-energised via incubation at 42°C for 20 min in the presence of 2 mM NH$_3$, as previously described for other LAB species (21). After incubation, the cell suspension was washed four times with solution A, the pH was adjusted to 6.5 with lactic acid, and the cells were considered to be energetically discharged cells (EdCs). The NMR experiments were performed using a 5-mm NMR tube containing 600 µl of EdCs. $^{13}$C NMR spectra were acquired sequentially after the addition of [1-$^{13}$C]$^{\text{Glc}}$lactose (16 mM) (Omicron Biochemicals Inc., South Bend, IN, USA) with or without 0.5 mM ammonia. The $^{13}$C-lactic acid generated by homolactic fermentation was monitored non-
invasively for 30 min at 42°C. Quantitative kinetic data were taken from resonance intensities of $[1^{13}\text{C}_{\text{Glc}}]$-lactose and $^{13}\text{C}$-lactic acid, directly measured during the *in vivo* experiments. A reference NMR spectrum of a $^{13}\text{C}$-lactose solution without cells was measured with the same acquisition parameters. Relevant $^{13}\text{C}$-NMR acquisition parameters were: $30^\circ$ flip-angle reading pulse; 36 KHz spectral width; 0.9 s acquisition time; 96 scans, 64K time domain; 2 s relaxation delay. Proton broad-band decoupling was achieved by a waltz-type pulse sequence.

2.5. Assay of β-galactosidase activity

β-galactosidase activity of *L. delbrueckii* has been measured on cells prepared as described for NMR analysis. In specific, aliquots of cells suspension in solution A were incubated on ice or at 42 °C for 30 min in presence and absence of lactose 16 mM and ammonia 2.5 mM. After incubation the cell suspension was washed in 100 mM Tris-HCl buffer pH 7 and subjected to a mechanical disruption using a Precellys bead beater (Advanced Biotech Italia srl, Seveso, Italy). The amount of total proteins of each lysate was measured using the Bradford method with BSA as the standard. Measurement of the β-galactosidase activity was performed in 100 mM Tris-HCl buffer pH 7, using 10 µl of total cell extract to a final 200 µl volume containing 0.2 mg/ml of 2-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, Milan, Italy) at 42 °C, by monitoring the optical density at 415 nm with a microplate-reader EON (BioTek Instruments, Inc., CA, USA) programmed for a reading set of 40 repetitions with intervals of 3 min. The β-galactosidase activity was calculated as the max velocity ($\text{mO.D.}_{415\text{nm}}$ per min) per µg of protein using the Gen5 software (BioTek Instruments, Inc., USA) as the mean of four independent determinations.

2.6. Measurement of pH<sub>in</sub>-dependent glycolysis and homolactic fermentation activity

To evaluate the pH<sub>in</sub> dependency of *L. delbrueckii* glycolysis and homolactic fermentation activity, the rate of glucose consumption and lactic acid production were measured in the presence of gramicidine 100 µM at various pHs between 5.0 and 9.0. The cells were collected in exponential
grow phase (O.D. 600 nm 1.0) in MRS medium, harvested, and washed with solution A. The cells were concentrated to obtain a cell density corresponding to 7-8 mg of protein per ml in MES buffer 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 µM. The reaction was started by the addition of glucose at a final concentration of 16 mM. The mixtures were incubated at 42 °C for 1 h. After being incubated, the mixtures were centrifuged at 15000 x g for 5 min at 4°C, and the supernatants were filtered through a 0.2-µm-pore-size membrane (VWR International PBI srl, Milan, Italy). The glucose and lactic acid concentrations of the filtered supernatants were measured by HPLC analysis. The separation of glucose and lactic acid was performed by means of HPLC using a Waters (Milford, MA, USA) equipment consisting of an Alliance 2695 pump system and a 410 refractive index detector. A cation exchange column Aminex HPX-87H (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) maintained at 50 °C was used. The isocratic elution was run at a flow rate of 0.6 mL/min using 0.01 N sulphuric acid aqueous solution as a mobile phase. Data were collected and processed using Empower™ software (Waters S.p.A., Italy). Glucose and lactic acid concentrations were calculated by the external standard method using analytical grade standard solutions of glucose and lactic acid (Sigma-Aldrich, Milan, Italy) diluted in 0.01 N sulphuric acid. All analyses were run in triplicate.

2.7. Effect of urea on the growth of S. thermophilus and L. delbrueckii in mixed culture

Urea-free skimmed milk prepared as above described, without and with the addition of 2.5 mM of urea, and pre-heated at 42 °C, were inoculated with overnight cultures of strains CNRZ385 and MIM91, each one at a final concentration of 10⁷ CFU/ml. The inoculum concentration was standardized after total cell count performed by flow cytometry and SYBR green I labelling. Total cell count (events per ml) and cultivable count of S. thermophilus and L. delbrueckii was measured after 3 and 6 h of incubation at 42 °C. Total cell count was measured by flow cytometry as previously described, whereas the viability of the mixed cultures was carried out by standard plating on M17 (lactose 20 g/L) incubated at 37 °C for 24 h and MRS (pH 5.5) incubated at 37 °C
for 48 h respectively for *S. thermophilus* and *L. delbrueckii*, in anaerobic condition in presence of Anaerocult® A (Merck, Germany). All data are presented as the average of three independent replicates ± SEM.
3. Results and Discussion

3.1. The ammonia released by the urease activity of *S. thermophilus* modulates the pH$_{in}$ of *L. delbrueckii*

To verify the effect of the urease activity of *S. thermophilus* on the pH$_{in}$ of urease-negative LAB, a preliminary model study was performed using bacterial populations in solution A in the absence of a carbon source. Variations in pH$_{in}$ were monitored based on the pH-dependent fluorescence of cFSE molecules that were used to label urease-negative LAB. An initial experiment was performed using the urease-negative strains *S. thermophilus* A16, a DSM20617 derivative urease-negative (Mora et al., 2004) and *L. delbrueckii* MIM91. The addition of 5 mM ammonia to cFSE-labelled cells significantly increased cFSE fluorescence in both species (Fig. 2). The observed increase in fluorescence represented a shift in pH$_{in}$ from 7.04 to >7.80 in *S. thermophilus* and from 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 were observed for *S. thermophilus* and *L. delbrueckii*, respectively. In the absence of added ammonia, the cFSE fluorescence was stable during the time of the experiments (Fig. 2).

To verify the effect of the ammonia released by urease activity, a mixed population composed of unlabelled urease-positive *S. thermophilus* CNRZ385 and cFSE-labelled urease-negative *S. thermophilus* A16 or *L. delbrueckii* MIM91 was prepared to obtain equivalent numbers of cells per ml. The *S. thermophilus* A16 and MIM91 populations were detected easily and were discriminated from the CNRZ385 population based on the level of cFSE fluorescence (Fig. 3). Fifteen min after urea addition, the release of ammonia caused by the urease activity of the unlabelled CNRZ385 strain mediated an increase in the extracellular pH (pH$_{out}$) from 6.60 to 8.60 and an increase in the cFSE fluorescence of strain A16 that corresponded to an increase of >1.0 units of pH$_{in}$ (i.e., from 6.6 to > 7.8) (Fig. 3A). In the absence of urea addition, cFSE fluorescence and the associated pH$_{in}$ were stable during the time of the experiments. These results demonstrate...
that the ammonia released by the urease-positive population diffuses inside urease-negative cells and causes an increase in pH\textsubscript{in}. Analogously, the pH\textsubscript{in} of \textit{L. delbrueckii} became more alkaline as a consequence of the urea hydrolysis performed by \textit{S. thermophilus} cells (Fig. 3B). Remarkably, during the incubation time, we observed an increase in the fluorescence of the unlabelled \textit{S. thermophilus} CNRZ 385 population, which likely occurred due to the release of unconjugated cFSE molecules from the labelled populations of \textit{S. thermophilus} A16 and \textit{L. delbrueckii} MIM91. Nevertheless, this phenomena did not interfere with the data analysis. Then, we have further investigated whether ammoniac could affect homolactic fermentation in \textit{L. delbrueckii} MIM91.

3.2. Effect of urea and ammonia on pH homeostasis and lactic acid production in milk in \textit{S. thermophilus} and \textit{L. delbrueckii}

Urease-positive \textit{S. thermophilus} CNRZ385 and urease-negative \textit{L. delbrueckii} MIM91 were tested in urea-free skimmed milk that was preheated to 42 °C. The effect of urea or ammonia alkalisation on pH homeostasis and homolactic fermentation was analysed in each species. In the absence of urea or ammonia, both species exhibited reduced pH\textsubscript{in} and pH\textsubscript{out} as a consequence of the production of lactic acid. In \textit{S. thermophilus}, the addition of urea or ammonia resulted in higher values of pH\textsubscript{in} compared to the previous experimental condition and a significant increase in L-lactic acid production (Fig. 4), which is in accordance with previous observations (Arioli \textit{et al.}, 2010; Pernoud \textit{et al.}, 2004). Similar to \textit{S. thermophilus}, the homolactic fermentation of \textit{L. delbrueckii} appeared to be boosted by ammonia, as revealed by the measured amount of D-lactic acid, which increased from 7.9 ± 0.2 to 9.4 ± 0.1 mM in the absence and presence of ammonia, respectively. In contrast, after the addition of ammonia, the pH homeostasis of \textit{L. delbrueckii} exhibited different behaviour from that of \textit{S. thermophilus}. In \textit{L. delbrueckii}, we observed the formation of a $\Delta$ (i.e., $\Delta$pH = pH\textsubscript{in} – pH\textsubscript{out}) that was close to zero (Fig. 5), which is a phenomenon that was previously described in other LAB, such as \textit{L. acidophilus} and \textit{L. plantarum}, after exposure to alkaline stress.
(Sawatari and Yokota, 2007). In specific, it could be hypothesized that in the two species H+ were not externalized at the same rate as lactate was produced.

3.3. Dynamics of lactose consumption and lactic acid production in L. delbrueckii

The stimulation of L. delbrueckii lactose consumption and lactic acid production by ammonia was confirmed using in vivo NMR analysis of energetically discharged cells (EdCs) (Arioli et al., 2010) that were activated with (1-13C)Glc-lactose. The dynamics of in vivo lactic acid productions (Fig. 6) demonstrate that the presence of ammonia strongly affected homolactic fermentation. Specifically, (1-13C) lactic acid concentrations remained close to 3.5 mM when the EdCs were supplied with (1-13C)Glc-lactose only but reached a final concentration of 4.57 mM when ammonia was supplied to the cell suspension. Conversely, lactose consumption appeared to be faster in the presence of ammonia (Fig. 6), especially during the first minutes of the incubation. Interestingly, the amount of (1-13C)-glucose was always below the detection limit of the instrument, indicating that in L. delbrueckii, lactose transport rather than glycolysis is the limiting step of energetic metabolism. The opposite was reported for S. thermophilus (Arioli et al., 2010). Like S. thermophilus, L. delbrueckii uses only the glucose moiety of lactose while galactose is supposed to be used in antiporter for the intake of lactose (Chervaux et al., 2000; Leong-Morgenthaler et al., 1991). In this context, ammonia could increase the activity of the transport and/or the activity of β-galactosidase. In both the scenario, the lactose transport should increase in efficiency. When NH4Cl was used instead of ammonia, it was not observed any stimulating effect on lactic acid production or lactose consumption (Fig. 6S1). Moreover, NH4Cl is a salt molecule and was not able to modify the pHin, as revealed by the absence of cFSE fluorescence increase using cells suspended in solution A (Fig. 6S2); a significant shift at high alkaline pHin values was instead observed using ammonia (Fig. 2B and repeated in Fig. 6S2). The absence of a stimulating effect of NH4Cl on homolactic fermentation could be due to the inability of L. delbrueckii to transport ammonium ions inside the cell. This hypothesis is corroborated by the absence of ammonium
specific transport in the publically available genomes of this species. Moreover, a previous study dealing on the physiology of <i>L. delbrueckii</i> in a chemical defined medium, revealed that the elimination of ammonium ions from the media formulation did not affect the growth efficiency of the microorganism (Chervaux <i>et al.</i>, 2000). We therefore conclude that only ammonia and not its protonated form is actively affecting homolactic fermentation in <i>L. delbrueckii</i>.

NMR spectra (Fig. 6A) showed that only the signals coming from lactose and lactic acid were detectable by $^{13}$C-NMR. The absence of a stoichiometric carbon balance between the lactose consumed (4.49 mM in presence of ammonia) and the lactic acid detected (4.57 mM instead of the expected 8.98 mM), can be due to the presence of glycolytic intermediates (i.e. fructose 1,6-bisphosphate, 3-phosphoglycerate, phosphoenolpyruvte) (Neves <i>et al.</i>, 1998) with an intracellular concentration unable to produce NMR signals with heights distinguishable from the instrumental noise. In order to evaluate the enzymatic stability, β-galactosidase activity was measured in EdCs before and after 30 min of incubation at 42 °C. The results obtained (data not shown, <i>only for reviewers</i>: Fig. 6S3) showed that the specific β-galactosidase activity did not significantly change after incubation at 42 °C, thus indicating that this enzyme is stable during the time of the experiment.

3.4. pH-dependent glucose consumption and lactic acid production in <i>L. delbrueckii</i>

Based on glucose consumption and lactic acid production at different pH<sub>in</sub>, <i>L. delbrueckii</i> showed an optimum of glycolysis and homolactic fermentation at the alkaline pH 8 (Fig. 7), a value not dissimilar to what we previously observed for <i>S. thermophilus</i> (Arioli <i>et al.</i>, 2010). These data highlights that homolactic fermentation enzymes of <i>L. delbrueckii</i> are optimized at neutral alkaline pH, such as those of other lactic acid bacteria (Hutkins and Nannen, 1993; Sawatari and Yokota, 2007).
3.5. Variation of cFSE fluorescence, pH$_{\text{out}}$, and D/L lactic acid production in the yogurt consortium

The cFSE fluorescence of the mixed culture yogurt consortium was monitored via flow cytometry in the presence and absence of 2.5 mM urea or 5 mM NH$_3$. Because the cFSE labelling protocol required several steps of biomass manipulation (see Materials and Methods), which could affect the metabolic activity of the cells but not the cell viability (Breeuwer et al., 1996), both species were labelled in this experiment. As a result, the use of two cFSE-labelled populations did not allow for the calculation of the pH$_{\text{in}}$ because the fluorescence values of the *S. thermophilus* and *L. delbrueckii* cells were superimposed. Therefore, we monitored the overall variations in the fluorescence of the bacterial consortium (Fig. 8). The flow cytometry data revealed that the cFSE fluorescence values and the corresponding pH$_{\text{in}}$ values were affected, with different kinetics, by:

1. the release of ammonia due to the urease activity of *S. thermophilus* cells only after 10 min of incubation and
2. by ammonia supplementation in the milk. After 15 min of incubation in milk, milk supplemented with urea, and milk supplemented with ammonia, the pH$_{\text{out}}$ values were 6.36 ± 0.04, 6.45 ± 0.05 and 6.70 ± 0.04, respectively. Considering that the addition of 5 mM NH$_3$ to the mixed culture caused an immediate increase in pH$_{\text{out}}$ from 6.68 ± 0.03 to 6.79 ± 0.02, the pH$_{\text{out}}$ values that were reached after 15 min of incubation revealed that the mixed cultures produced an amount of lactic acid capable of counteracting the alkalising effect of the ammonia molecules added to the milk or generated by urea hydrolysis. D- and L-lactic acid measurements in a mixed culture yogurt consortium confirmed previous observations for single-species cultures (*i.e.*, homolactic fermentation was boosted in the presence of urea and ammonia). The amounts of D- and L-lactic acid were 26 % and 20 % higher than the control value when the milk was supplemented with urea and ammonia, respectively.

3.6. Urea hydrolysis by *S. thermophilus* increases the population size of the yogurt consortium
The effect of urea hydrolysis on the population size of a milk mixed culture of strain *S. thermophilus* CNRZ385 and *L. delbrueckii* MIM91 was detectable after 6 h of incubation at 42 °C (Fig. 9). In presence of urea, the two populations showed an increment in total count (events per ml) of 38 %. Moreover, plate count analysis on differential media revealed that strain CNRZ385 and MIM91 showed a statistically significant increment in CFU/ml of 35 % and 19 %, respectively. These results show that urea metabolism may have an influence on the population size of *S. thermophilus* and *L. delbruekii* in yogurt consortium, most probably due to the effect of urea hydrolysis on homolactic fermentation.

4. Conclusions

During milk fermentation, *S. thermophilus*, *L. delbrueckii*, and most LAB are faced with constantly changing environmental stimuli and stresses, which can affect cellular physiology. These predictable environmental changes include pH variations, the limitation of nutrient availability, and the accumulation of toxic metabolites (*i.e.*, lactic acid) formed by the fermentation process. Exposure to low pH for a long period of time causes an arrest of growth, a dramatic reduction of glycolytic fluxes and a progressive loss of viability (Cotter and Hill, 2003; Hutkins and Nannen, 1993; Siegumfeldt *et al.*, 2000). Urease activity, which is an enzymatic reaction that is known to be a stress response that is activated to counteract acidic environmental pH in a number of bacteria, has been described as a potential metabolic regulatory mechanism for energetic metabolism in the dairy bacterium *S. thermophilus*. In *S. thermophilus*, urease increases the overall change in enthalpy that is generated by microbial metabolism as a consequence of increased glycolytic flux (Arioli *et al.*, 2010). Outside of the 'selfish' utility of urease for cells harbouring this enzymatic activity, the cooperative relevance of urease in an ecological context in which different microbial species share the same environment has been poorly investigated. According
to the results presented here and on the basis of previous observations (Arioli et al., 2010; Monnet et al., 2005), urease production should be considered an altruistic cooperative trait, which is costly for urease-positive individuals but provides a local benefit because other individuals can take advantage of the release of ammonia. However, the effect of ammonia molecules on the lactose consumption and lactic acid production of *L. delbrueckii* subsp. *bulgaricus* MIM91 in milk cannot be directly linked to the variation of pH in because intracellular alkalization was detected only in saline solution in absence of carbon source.

Unlike other described cooperative behaviours, such as those involving siderophores (Griffin et al., 2004), urease first provides a benefit to the individual that harbours the enzymatic activity and then provides a local benefit. In this study, we report the first evidence that the urease activity of *S. thermophilus* affects the physiology of this species during milk fermentation. These findings put a new light in the interactions that occur between the two species in the yogurt consortium, but also between a urease-positive species and the other urease-negative lactic acid bacteria in milk environment. In this context, urease activity should be taken into account as a metabolic booster for the optimal growth of lactic acid bacteria in milk. In every microbial cell, the modulation of cellular catabolism arises from multiple overlapping regulatory mechanisms and from metabolic feedback into regulatory networks. In this context, within the yogurt consortium, urease-dependent ammonia could directly affect kinetic parameters of enzymes involved in homolactic fermentation of both *S. thermophilus* and *L. delbrueckii*. The boost of lactic acid production that is triggered by urease in this microbial consortium occurs when *S. thermophilus* show the maximum urease activity (i.e. in the early exponential growth phase, Mora et al., 2005) to support their “selfish” energetic behaviour (i.e., rapid sugar consumption and lactic acid production) (Mora et al., 2013).

From an ecological point of view, we should note that microbes do not live as single species in natural environments but co-exist with other microbial species. Therefore, the adaptation process
of a defined environmental niche appears to be strongly influenced by the metabolic relationships that exist among different species within the community. The data presented here still do not clarify the role/s of ammonia molecules in the microbial metabolism. The increase of lactic acid production in *L. delbrueckii* could be the consequence of a trophic interaction modulated by ammonia released by *S. thermophilus* urease activity. In microorganisms, carbon and nitrogen metabolisms are largely interconnected, and glycolytic intermediates are also precursors of molecules involved in nitrogen metabolism. Nevertheless, the genome of *L. delbrueckii* highlights the intrinsic evidence for specialization through loss of function provided by the complete absence of a large number of enzymes involved in the biosynthesis of amino acids, as a consequence of the species adaptation to the protein-rich milk environment (van de Guchte *et al.*, 2006). However, the publically available genomes of *L. delbrueckii* revealed two enzymes involved in ammonia utilization, glutamine synthase and asparagine synthetase, but a direct link between their activities and the carbon metabolism is far from clear.


ACKNOWLEDGEMENTS

We thank Françoise Rul, INRA Jouy-en-Josas France, for providing *S. thermophilus* CNRZ 385.

We acknowledge Dr Stefano Amalfitano Istituto di Ricerca sulle Acque (IRSA-CNR) Monterotondo Rome Italy, for his support on flow cytometry applications. This work was partially supported by Sacco srl, Cadorago, Italy.
FIG 1 Graphic representation of the molecular interactions that play key roles in the mutualistic behaviour of the yogurt consortium. The effect of *S. thermophilus* urease on urea hydrolysis and the hypothetical role of the NH$_3$ released by this enzyme on the pH$_{in}$ of *L. delbrueckii* are shown in red.
FIG 2 The effect of ammonia on variations in the pH\textsubscript{in} and pH\textsubscript{ex} of the urease-negative A16(\Delta ureC3) strain of \textit{S. thermophilus} (A) and \textit{L. delbrueckii} MIM91 (B). The density diagrams (upper and lower left panels) show the FSC of cells in the presence and absence of the addition of 5 mM NH\textsubscript{3} vs. the green (cFSE) fluorescence levels. T0 and T1 density plots of the \textit{S. thermophilus} population before and after 15 min of incubation at 42°C, respectively. The frequency histograms (lower right panels) show the increasing green fluorescence that occurs due to NH\textsubscript{3}-dependent intracellular alkalisation. The horizontal and vertical line refers to the average cFSE fluorescence of the cell population at T0.
FIG 3 The effect of *S. thermophilus* CNRZ385 urease activity on the pH\textsubscript{in} of the urease-negative A16(ΔureC3) strain of *S. thermophilus* (A) and *L. delbrueckii* MIM91 (B). The density diagrams show the green (cFSE) fluorescence levels of the cells in the presence and absence of the addition of 2.5 mM urea vs. FSC. T0 and T1 density plots of *S. thermophilus/L. delbrueckii* mixed populations before and after 15 min of incubation at 42°C, respectively. Gate 1, cFSE-labelled cells of urease-negative *S. thermophilus* A16(ΔureC3). Gate 2, unlabelled cells of urease-positive *S. thermophilus* CNRZ385. Gate 3, cFSE-labelled cells of *L. delbrueckii* MIM91. pH\textsubscript{in} and pH\textsubscript{out} are indicated.
FIG 4 Variations in pH_{in} and pH_{out} and L-lactic acid production in urease-positive *S. thermophilus* CNRZ385 during incubation at 42°C in urea-free reconstituted skimmed milk in the presence and absence of 2.5 mM urea and 5 mM NH_{3}. The amount of L-lactic acid is reported as the average of three determinations ± SEM.
FIG 5 Variations in pH_{in} and pH_{out} and D-lactic acid production in *L. delbrueckii* MIM91 during incubation at 42°C in urea-free reconstituted skimmed milk in the presence and absence of 5 mM NH$_3$. The amount of D-lactic acid is reported as the average of three determinations ± SEM.
FIG 6 Dynamics of metabolite pools in *L. delbrueckii*, as determined by in vivo NMR during incubation at 42 °C in solution A. Time course of (1-\(^{13}\)C\(^{-}\)Glc\(^{-}\)) lactose consumption and (1-\(^{13}\)C\(^{-}\)) lactic acid formation in *L. delbrueckii* MIM91. A) Each spectrum represents 5 min of accumulation. The spectra after 10, 15 and 25 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) \(\beta\)-\(\alpha\)-(1-\(^{13}\)C\(^{-}\)Glc\(^{-}\)) lactose; (2) \(\alpha\)-(1-\(^{13}\)C\(^{-}\)Glc\(^{-}\)) lactose; (3) (1-\(^{13}\)C\(^{-}\)) lactic acid. The metabolite concentrations (B, C) were measured in *in vivo* \(^{13}\)C NMR experiments using EdCs that were activated with 14 mM (1-\(^{13}\)C\(^{-}\)Glc\(^{-}\)) lactose (white symbols) or 14 mM (1-\(^{13}\)C\(^{-}\)Glc\(^{-}\)) lactose and 5 mM NH\(_3\) (black symbols). The black arrows refer to spectra shown in A. The error bars represent the SEM.
FIG 7 pH-dependent glucose consumption (grey circles) and lactic acid production (white triangles) in EdCs of *L. delbrueckii* MIM91 that were treated with 100 mM of the uncoupler gramicidine. The error bars represent the SEM.
FIG 8 Variation of cFSE fluorescence, pH_{out}, and D/L-lactic acid production in *S. thermophilus* CNRZ385/L. *delbrueckii* MIM91 mixed cultures during incubation at 42°C in urea-free reconstituted skimmed milk in the presence and absence of 2.5 mM urea or 5 mM NH₃. The amount of D/L-lactic acid is reported as the average of three determinations ± SEM.
FIG 9 Population size of *S. thermophilus* CNRZ385/*L. delbrueckii* MIM91 mixed cultures after 3 h and 6 h of growth at 42°C in urea-free reconstituted skimmed milk in the presence and absence of 2.5 mM urea. (A) Flow cytometry total cell count. (B) Selective count of viable *S. thermophilus* CNRZ385. (C) Selective count of viable *L. delbrueckii* MIM91. The error bars represent the SEM. Statistically significant differences were determined by an unpaired Student’s t test (*, P < 0.05; **, P < 0.01).
Fig. 6S1

Kinetics of lactose metabolism in *L. delbrueckii*, as determined by *in vivo* NMR. Time course of \((1^{13}C\text{Glc})\)-lactose consumption and \((1^{13}C)\)-lactic acid formation in *L. delbrueckii* MIM91 in absence (A) and in presence of NH\(_4\)Cl 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. \((1^{13}C\text{Glc})\)-lactose was added at time zero and each spectrum acquired during the indicated interval. (1) \(\beta\)-(1\(^{13}\text{C}\text{Glc})\)-lactose, (2) \(\alpha\)-(1\(^{13}\text{C}\text{Glc})\)-lactose; (3) \(1^{13}\text{C}\)-lactic acid.
The effect of ammonia and NH₄Cl on the pH-dependent variations of CFSE fluorescence of *L. delbrueckii* MIM91. The frequency histograms show the increasing green fluorescence that occurs due to NH₃ (A) or NH₄Cl (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.

**Fig. S2**