The critical role of urease in yogurt fermentation with various combinations of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*

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ABSTRACT

The proto-cooperation between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* relies on metabolite exchanges that accelerate acidification during yogurt fermentation. Conflicting results have been obtained in terms of the effect of the *Strep. thermophilus* urease and the NH₃ and CO₂ that it generates on the rate of acidification in yogurt fermentation. It is difficult to perform a systematic study of the effects of urease on proto-cooperation because it is necessary to distinguish among the direct, indirect, and strain-specific effects resulting from the combination of the strains of both species. To evaluate the direct effects of urease on proto-cooperation, we generated 3 urease-deficient mutants ($\Delta ureC$) of fast- and slow-acidifying *Strep. thermophilus* strains and observed the effects of NH₃ or CO₂ supplementation on acidification by the $\Delta ureC$ strains. Further, we examined 5 combinations of 3 urease-deficient $\Delta ureC$ strains with 2 CO₂-responsive or CO₂-unresponsive strains of *L. bulgaricus*. Urease deficiency induced a shortage of ammonia nitrogen and CO₂ for the fast- and slow-acidifying *Strep. thermophilus* and for the CO₂-responsive *L. bulgaricus*, respectively. Notably, the shortage of ammonia nitrogen had more severe effects than that of CO₂ on yogurt fermentation, even if coculture with *L. bulgaricus* masked the effect of urease deficiency. Our work established (1) that urease deficiency inhibits the fermentative acceleration of proto-cooperation regardless of the *Strep. thermophilus* and *L. bulgaricus* strain combinations, and (2) that urease is an essential factor for effective yogurt acidification.

Key words: urease, proto-cooperation, yogurt fermentation

INTRODUCTION

Yogurt is a traditional fermented food that dates back to 6,000 BCE (Weerathilake et al., 2014). Yogurt is made from milk fermentation by a coculture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, 2 lactic acid bacteria (**LAB**) commonly consumed by humans. Proto-cooperation exists between these 2 LAB (Tamime and Robinson, 2007); however, some of the interactions between them are ill defined. Today, our understanding of proto-cooperation is that *L. bulgaricus* proteolyzes casein with its surface PrtB protease, providing AA and peptides to *Strep. thermophilus* that, in turn, provides formic acid (Veringa et al., 1968) and carbon dioxide (Galesloot et al., 1968; Driessen et al., 1982) to *L. bulgaricus*. The growth rate of each species and, consequently, the milk acidification rate are boosted by these metabolite exchanges in cocultures compared with monocultures of each species.

The analysis of proto-cooperation during yogurt fermentation is complex because it is difficult to (1) distinguish between direct and indirect effects of proto-cooperation on bacterial growth in cocultures in milk, and (2) have the appropriate controls. Regarding controls, milk monocultures of *Strep. thermophilus* or *L. bulgaricus* must be supplemented with AA and formate, respectively, to prevent nutrient limitation. Moreover, because the efficiency of proto-cooperation depends on the combination of *Strep. thermophilus* and *L. bulgaricus* strains, using several strains of each species must be considered before drawing general conclusions about proto-cooperation. To avoid the problem of nutritional limitation in monocultures, we supplemented the milk with (1) peptides that result from the digestion of casein for *Strep. thermophilus* monocultures, and (2) sodium formate for *L. bulgaricus* monocultures. These culture conditions, called pseudo-cocultures, allowed us to analyze the effects of factors other than limited peptide and formate on growth and acidification. For instance, we found that dissolved oxygen (**DO**) has an inhibitory effect on proto-cooperation by showing that DO concen-

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tration in milk had to decrease to almost 0 mg/kg to allow active acidification by the industrial starter LB81 (comprising *Strep. thermophilus* 1131 and *L. bulgaricus* 2038; Horiuchi et al., 2009). Using pseudo-cocultures, we also established that Nox, the NADH oxidase of *Strep. thermophilus* 1131, was an essential factor for efficient proto-cooperation as it was the major oxygen-consuming enzyme in the coculture with *L. bulgaricus* 2038 and strongly contributed to the decrease of DO in milk (Sasaki et al., 2014).

Aeration with N₂ gas was performed to reduce DO in the milk medium before fermentation. We showed that flushing N₂ gas into milk before fermentation shortened the acidification time for both *Strep. thermophilus* 1131 monoculture and coculture with *L. bulgaricus* 2038 (Horiuchi and Sasaki, 2012).

The urease enzyme (urea amidohydrolase, EC 3.5.1.5) catalyzes hydrolysis of urea into ammonia and carbon dioxide. *Streptococcus thermophilus* is the only LAB to display significant urease activity (Iyer et al., 2010). In *Strep. thermophilus*, the urease cluster has 8 open reading frames, *ureI* (urea transport homologous), *ureABC* (structural genes), and *ureEFGD* (accessory genes) (Mora et al., 2004).

Effective acidification of *Strep. thermophilus* and *L. bulgaricus* coculture is important for yogurt fermentation. Effective acidification may prevent the outgrowth of contaminants and decrease the production cost of the fermentation process. Urease produced by *Strep. thermophilus* is thought to provide CO₂ to *L. bulgaricus* and stimulate its acidification; however, there are conflicting reports regarding the role of urease in yogurt fermentation. Arioli et al. (2017) showed that urea hydrolysis by *Strep. thermophilus* urease increased the bacterial population in the coculture of *Strep. thermophilus* CNRZ385 and *L. bulgaricus* MIM91. In contrast, Mora et al. (2004) reported that when *Strep. thermophilus* DSM 20167^T wild type or its derived urease-deficient mutant A16 were cocultured with *L. bulgaricus* ATCC 11842^T in the presence of urea, the acidification rate was higher with the urease-deficient mutant than with the wild-type strain. They proposed that ammonia generated by *Strep. thermophilus* urease had a buffering effect that reduced the rate of pH decrease and decreased the acidification rate of the *Strep. thermophilus*–*L. bulgaricus* coculture. Because both studies reached different conclusions and only used 1 or 2 strains in culture, it is impossible to draw a general conclusion on the importance of urease. In this work, we performed a more systematic analysis with several strain combinations of *Strep. thermophilus* and *L. bulgaricus* to determine the role of urease for this economically important coculture. We used 3 fast- and slow-acidifying strains of *Strep. thermophilus*

plus their *ureC*-deficient mutants (Δ *ureC*), and 7 *L. bulgaricus* strains, 4 that were stimulated by CO₂ (CO₂-responsive) and 3 CO₂-unresponsive strains, as well as 5 strain combinations for cocultures. This experimental setting allowed us to clarify the role of urease in yogurt fermentation.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Table 1 shows the strains and plasmids used in this work. Strains included 3 strains of *Strep. thermophilus*: ATCC19258^T, ST1131, and ST499; and 7 strains of *L. bulgaricus*: ATCC11842^T, LB2038, LB494, LB496, LB497, LB536, and LB600. *Streptococcus thermophilus* ST1131 and ST499 and *L. bulgaricus* LB494, LB496, LB497, LB2038, LB600, and LB536 were obtained from the Food Research and Development Center, Meiji Dairies Corporation (Tokyo, Japan). These strains have been used in the commercial production of yogurt and their acidification is much faster than that of type strains.

Streptococcus thermophilus and *L. bulgaricus* strains were cultured at 37°C for 16 to 18 h in 1/2 M17 broth (Becton, Dickinson and Co., Franklin Lakes, NJ) supplemented with 1% lactose and 1/2 de Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Co.), respectively. After storage at 4°C up to 7 h, the cultures were inoculated at 1% (vol/vol) in fresh medium and cultured at 37°C for 16 to 18 h. These cultures were then used for inoculation of the various milk media.

Monocultures of *Strep. thermophilus* or *L. bulgaricus* were cultured in skim milk medium supplemented with 0.1% peptides that result from the digestion of casein (CE90GMM, Nippon Shinyaku Ltd., Kyoto, Japan) or 1 mM sodium formate (Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively, and inoculated at a final optical density (OD) value at 660 nm (OD₆₆₀) of 0.05. The coculture of *Strep. thermophilus* and *L. bulgaricus* was inoculated at a 1:1 ratio with a final OD₆₆₀ of 0.05 in skim milk medium with 0.4 g/L urea (Wako Pure Chemical Industries Ltd.) when necessary. The skim milk medium contained 10% (vol/vol) skim milk powder and was sterilized by heating at 95°C for 2 min and immediately cooled to 45°C to prevent formate production by autoclave treatment. Gas flushing with N₂ was performed to decrease CO₂ and dissolved oxygen in the skim milk medium. The N₂ gas was filtered with a 0.45-μm cellulose acetate filter, and flushing was performed as previously described (Horiuchi et al., 2009).

Monocultures and cocultures were carried out at 43°C under 4 conditions in skim milk medium: (1) without N₂ flushing, (2) with N₂ flushing (15 min), (3) with

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype	Reference
<i>Streptococcus thermophilus</i>		
ATCC19258 ^T	Slow-acidifying strain	Mora et al. (2004)
ST1131	Fast-acidifying strain	Sasaki et al. (2014)
ST499	Fast-acidifying strain	This study
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>		
ATCC11842 ^T	CO ₂ -unresponsive	Mora et al. (2004)
LB2038	CO ₂ -responsive	Sasaki et al. (2014)
LB494	CO ₂ -responsive	This study
LB496	CO ₂ -unresponsive	This study
LB497	CO ₂ -responsive	This study
LB536	CO ₂ -responsive	This study
LB600	CO ₂ -unresponsive	This study
Plasmid		
pG ⁺ host6	Em ^r , ¹ thermo-sensitive replicon	Maguin et al. (1996)
pG ⁺ urC69	pG ⁺ host6 containing a deleted <i>ureC</i> fragment, Em ^r	This study

¹Erythromycin-resistant.

N₂ flushing and supplemented with 30 mM NaHCO₃ (4) with N₂ flushing and supplemented with 30 mM NaHCO₃ and 10 mM (NH₄)₂SO₄. Plastic test tubes were used for cultures without N₂ flushing and tightly closed glass tubes were used after N₂ flushing.

Measurement of Acidity of Fermentation

Acidity was measured by titrating a 9-g milk sample against 0.1 N NaOH using phenolphthalein as an indicator. Acidity (%) is calculated by using the following formula:

$$\text{Acidity (\%)} = 0.1 \text{ N NaOH titer (mL)} \times 0.1 \times \text{factor},$$

where factor was measured by titrating 0.1 N NaOH we made against 0.1 mol/L hydrochloric acid (Wako Pure Chemical Industries Ltd.) using phenolphthalein as an indicator. The acidification rate was determined by the acidity increase per minute in a milk sample.

Construction of *ureC* Knockout Mutants of *Strep. thermophilus*

The construction of the *ureC* knockout mutants ($\Delta ureC$) of *Strep. thermophilus* ATCC19258^T, ST1131, and ST499 was based on the method described by Mora et al. (2004). According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), the sequence of the urease cluster is highly conserved among 8 strains of *Strep. thermophilus*. The *ureC* gene of ST1131 was amplified with primers LAB099 and LAB102 and cloned (Figure 1).

The 693-bp in-frame deletion of *ureC* was obtained by PCR using primers LAB099 and LAB100 to obtain fragment 1 first and then primers LAB101 and

LAB102 to amplify fragment 2. Then, primers LAB099 and LAB102 were used to generate a 1,026-bp fragment combining fragments 1 and 2 (Figure 1). This 1,026-bp fragment was inserted at the *Xba*I and *Xho*I sites of pG⁺host6 (thermo-sensitive replicon, Appligene, Pleasanton, CA) to generate the pG⁺urC69 plasmid. The pG⁺urC69 plasmid was first electroporated into *Lactococcus lactis* IL1403; then, plasmid DNA was then prepared and electroporated into *Strep. thermophilus* ATCC19258^T, ST1131, or ST499. The expected gene replacements were induced via the double crossover events as previously described (Biswas et al., 1993). The *ureC* knockout mutants of *Strep. thermophilus*

Figure 1. Schematic representation of the *Streptococcus thermophilus ureC* gene. The *ureC* gene of *Strep. thermophilus* ST1131 was used to generate a 1,026-bp fragment with a 693-bp in-frame deletion from nucleotides (nt) 412 to 1104. The vertical arrows represent the codons encoding histidine residue H138, H140, H223, H250, and H324. The small black rectangle indicates the location of the active site (nt 961 to 981). The primers (horizontal arrows) used to generate the deletion were LAB 099 (nt 1 to 25), LAB 100 (nt 398 to 411 and nt 1105 to 1118), LAB101 (nt 1105 to 1118 and nt 398 to 411), and LAB102 (nt 1695 to 1719). LAB 099 = GACCTCGAGATGAGTTTAAAAATGGATCGTGAAG; LAB100 = TCACCGACACGTCCGAGGTCAATACCAC; LAB101 = GTGGTATTGACCTCGGACGTGTCGGTGA; LAB102 = GCGTCTAGATTAGAACAAAGAAATAACGTTGAGAC.

ATCC19258^T, ST1131, and ST499 were validated by sequencing the *ureC* genes or by PCR to confirm the 693-bp deletion (data not shown) and were designated $\Delta ureC19258^T$, $\Delta ureC1131$, and $\Delta ureC499$, respectively.

Measurement of CO₂ and NH₃ Produced by *Strep. thermophilus* Wild Type and $\Delta ureC$

Wild-type (WT) *Strep. thermophilus* and $\Delta ureC$ derivative cultures were used to inoculate at a final OD₆₆₀ of 0.05 in skim milk supplemented with 0.1% casein digested peptides and 0.4 g/L urea and, only for CO₂ measurement, flushed with N₂ gas for 30 min. These cultures were transferred to tightly closed glass bottles without headspace for CO₂ measurement or to plastic test tubes for NH₃ measurement and incubated at 43°C. After 6 h of cultivation, the CO₂ concentration of the medium was determined by a glass electrode method using a CO₂ concentration meter (CGP-31, DKK-TOA, Tokyo, Japan). For NH₄-N measurement, sample and deproteinization agent (Wako Pure Chemical Industries, Ltd.) were mixed and centrifuged at 15,133 × *g* for 10 min at 4°C. The supernatant was filtered with a 0.22-μm cellulose acetate filter and used for NH₄-N measurement by ammonia test (Wako Pure Chemical Industries Ltd.). The calibration curve using NH₄Cl ranged from 1 to 8 mg/L NH₄-N for WT and 1 to 4 mg/L NH₄-N for $\Delta ureC$.

Urease Assay

Cells were centrifuged at 6,726 × *g* for 5 min at 4°C, washed 3 times in 50 mM sodium phosphate buffer (pH 7.0), suspended in the same buffer, transferred to a screw-cap tube containing glass beads (0.105–0.125 mm) in an amount equivalent to the cell suspension weight, and then disrupted at 6.5 m/s for 30 s using a Fast Prep-24 instrument (MP Biomedicals, San Diego, CA). The homogenate was centrifuged at 10,509 × *g* for 15 min at 4°C, and the supernatant was used for the urease assay. Total protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976) using BSA as a standard. Urease activity was determined by measurement of released NH₄-N as follows: 250 μL of supernatant containing 1 to 4 μg of protein for WT strains or 5 to 16 μg of protein for $\Delta ureC$ strains was mixed with 250 μL of 100 mM urea solution and incubated at 37°C for 30 min. After incubation, 250 μL of the solution was transferred into a 2.0-mL tube containing deproteinization agent (Wako Pure Chemical Industries, Ltd.) and mixed. This mixture was centrifuged at 15,133 × *g* for 10 min at 4°C, and the supernatant was filtered with 0.22-μm

cellulose acetate filter. Total NH₄-N concentrations in the filtered supernatant were determined by using ammonia test (Wako Pure Chemical Industries Ltd.). The calibration curve using NH₄Cl ranged from 1 to 8 mg/L NH₄-N. Urease activity was determined as the amount of released NH₄-N for 1 min per 1 μg of protein.

Measurement of Urea in Skim Milk

Measurement of urea in skim milk was based on a previously described method (Food Safety and Standards Authority of India, 2015). First, 5 mL of skim milk sample and 5 mL of 24% trichloroacetic acid were mixed and centrifuged at 15,133 × *g* for 10 min at 4°C. The supernatant was filtered with filter paper; then, 900 μL of filtered supernatant and 900 μL of *p*-dimethyl amino benzaldehyde solution (1.6 g of *p*-dimethyl amino benzaldehyde dissolved in 100 mL of ethanol and 10 mL of concentrated HCl added) were mixed and left standing for 10 min at room temperature. Then, optical density was measured at 420 nm. The amount of urea in the skim milk sample was calculated based on a standard curve.

Statistical Analysis

Statistical analysis was performed by using the analysis tool of Excel 2007 (Microsoft Corp., Redmond, WA). A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Characterization of 3 WT *Strep. thermophilus* Strains and Urease Knockout Derivatives

We generated urease knockout mutants ($\Delta ureC$) for the 3 *Strep. thermophilus* strains studied in this work; that is, ST1131, ST499, and ATCC19258^T, using double crossover gene replacement leading to an in-frame deletion of 693 bp (Figure 1) in the *ureC* gene. Because this 693-bp region contained the codons for the 5 histidine residues (H138, H140, H250, H223, and H324) required for nickel and substrate binding and the presumed active site MVCHHLD (AA 321 to 327; Figure 1, Mora et al., 2004), we predicted that the deleted gene product would be inactive. However, to confirm our prediction, WT and $\Delta ureC$ strains were characterized for urease activity and CO₂ and NH₃ production (Table 2). In addition, we monitored the biomass obtained with monocultures cultured in supplemented skim milk or 1/2 M17 broth supplemented with 1% lactose (Table 2).

Table 2. Urease activity, CO₂ and NH₃ production, and counts (cfu) of *Streptococcus thermophilus* wild type and urease-deficient mutants ($\Delta ureC$) isogenic strains¹

Item	Urease activity ² (mg/L·min·μg of protein)	CO ₂ ³ (mg/L)	NH ₄ -N ⁴ (mg/L)	Count (cfu)
Wild type				
ATCC19258 ^T	2.0 ± 0.65	5.0 ± 0.57 × 10 ²	2.5 ± 0.08 × 10 ²	1.5 ± 0.45 × 10 ⁸
ST1131	1.0 ± 0.15	4.6 ± 0.36 × 10 ²	2.2 ± 0.25 × 10 ²	6.0 ± 2.0 × 10 ⁹
ST499	1.9 ± 0.40	5.6 ± 0.71 × 10 ²	2.1 ± 0.15 × 10 ²	3.4 ± 0.85 × 10 ⁸
Urease-deficient mutants ($\Delta ureC$)				
$\Delta ureC$ 19258 ^T	ND ⁵	7.3 ± 1*	ND	1.7 ± 0.95 × 10 ^{7*}
$\Delta ureC$ 1131	ND	6.4 ± 1*	ND	1.1 ± 0.77 × 10 ^{8*}
$\Delta ureC$ 499	ND	7.6 ± 2*	ND	2.4 ± 0.76 × 10 ^{7*}
Medium ⁶	— ⁵	ND	2.7	—

¹The plus/minus values show SD of the results from at least 3 independent experiments.²Amount of released NH₄-N in 1 min per μg of protein.³Amount of CO₂ in the culture after cultivation (6 h).⁴Amount of NH₄-N in the culture after cultivation (6 h).⁵ND = below detection threshold; — = not performed.⁶Uninoculated skim milk supplemented with urea (0.4 g/L).*Numbers with asterisks are significant ($P = 0.05$) vs. wild type.

The urease activity of ST1131, ST499, and ATCC19258^T was 1.0, 1.9, and 2.0 mg/L·min·μg of protein, respectively, whereas the activity of their knockout derivatives ($\Delta ureC$ 1131, $\Delta ureC$ 499, and $\Delta ureC$ 19258^T) was below the detection limit. We also determined CO₂ and NH₃ production levels in these *Strep. thermophilus* cultures. The concentrations of CO₂ in the cultures of ST1131, ST499, and ATCC19258^T were 4.6 × 10², 5.6 × 10², and 5.0 × 10² mg/L, respectively, whereas CO₂ was not detectable in the medium, which was flushed with N₂ gas before incubation. In the $\Delta ureC$ 1131, $\Delta ureC$ 499, and $\Delta ureC$ 19258^T cultures, CO₂ concentrations were 6.4, 7.6, and 7.3 mg/L, respectively, showing a decrease to at least 1.5% of the cultures of their isogenic WT counterparts (Table 2).

In uninoculated skim milk medium after N₂ gas aeration, the concentration of NH₄-N was 2.7 mg/L. The concentrations of NH₄-N in the ST1131, ST499, and ATCC19258^T cultures were 2.2 × 10², 2.1 × 10², and 2.5 × 10² mg/L, respectively, whereas NH₄-N could not be detected in any of the $\Delta ureC$ cultures.

Because inactivation of urease was confirmed for all $\Delta ureC$ strains at both the molecular and phenotypic levels, counts of *Strep. thermophilus* WT and $\Delta ureC$ strains were determined to evaluate the effect of urease deficiency on growth. The counts (cfu/mL of culture) were always lower with $\Delta ureC$ strains compared with their WT counterparts albeit with some variation. The counts were 6.0 × 10⁹ cfu for ST1131 and 1.1 × 10⁸ cfu for $\Delta ureC$ 1131 (i.e., decrease to 1.8% of WT), 3.4 × 10⁸ cfu for ST499 and 2.4 × 10⁷ cfu for $\Delta ureC$ 499 (i.e., decrease to 7.1% of WT), and 1.5 × 10⁸ cfu for ATCC19258^T and 1.7 × 10⁷ cfu for $\Delta ureC$ 19258^T (i.e.,

decrease to 11.3% of WT). These data showed that urease deficiency significantly limited the proliferation of all 3 *Strep. thermophilus* strains.

Effect of NaHCO₃ and (NH₄)₂SO₄ Supplementation on Acid Production of UreC Knockout Strains

The acidification curves of *Strep. thermophilus* WT strains and their $\Delta ureC$ derivatives were monitored in skim milk supplemented with urea (0.4 g/L) and casein digested peptides, and flushed with N₂ to eliminate the dissolved CO₂ (Figure 2). Based on the observed acidification rates, we distinguished (1) the fast-acidifying strains ST1131 and ST499, which required 3 h to reach 0.5% acidity, and (2) the slow-acidifying strain ATCC19258^T, which required 9 h. However, for all 3 strains, urease deficiency led to a dramatic decrease in acidification rates (Figure 2).

To evaluate whether CO₂ or NH₃ provided by the urease activity affected the acidification rate of *Strep. thermophilus* strains, NaHCO₃ and (NH₄)₂SO₄ were supplemented as sources of CO₂ and NH₃ to WT and $\Delta ureC$ monocultures. For all 3 WT strains, NaHCO₃ and (NH₄)₂SO₄ supplementation mostly did not stimulate acidification (data not shown). For the 2 $\Delta ureC$ strains ($\Delta ureC$ 499 and $\Delta ureC$ 1131), NaHCO₃ supplementation slightly accelerated the acidification rates, whereas supplementation with NaHCO₃ and (NH₄)₂SO₄ nearly recovered the acidification rates of $\Delta ureC$ 499 and $\Delta ureC$ 1131 similar to that of their WT counterparts. The effect of the double supplementation on the growth of $\Delta ureC$ 19258^T remained limited.

Figure 2. Acidification kinetics in wild type (WT) and urease-deficient ($\Delta ureC$) *Streptococcus thermophilus* monocultures of (A) ST1131 and $\Delta ureC$ 1131, (B) ST499 and $\Delta ureC$ 499, and (C) ATCC19258^T and $\Delta ureC$ 19258^T. The WT strains were cultured in skim milk flushed with N_2 (○), and the $\Delta ureC$ were cultured in skim milk flushed with N_2 (●), with $NaHCO_3$ supplementation (▲) or with $NaHCO_3$ and $(NH_4)_2SO_4$ supplementation (■). Acidity (%) corresponds to the amount of acid produced by *Streptococcus thermophilus* in the skim milk medium. Error bars show SD of the results from at least 3 independent experiments.

Effect of N_2 Flushing and $NaHCO_3$ and $(NH_4)_2SO_4$ Supplementation on Acid Production of *L. bulgaricus* Strains

Seven strains of *L. bulgaricus* (LB494, LB496, LB497, LB2038, LB536, LB600, and LB ATCC11842^T) were cultured in skim milk with or without N_2 flushing to evaluate their CO_2 requirement for growth and acidification (Figure 3). Flushing with N_2 decreased the amount of CO_2 present in the skim milk medium from 2.5 mg/L to less than 1.49 mg/L.

Strains LB497, LB494, LB2038, and LB536 appeared CO_2 -responsive because N_2 flushing of the culture medium significantly decreased their acidification rates, whereas addition of $NaHCO_3$ (30 mM) restored acidification (Figure 3A, B, C, and D). In contrast, neither N_2 flushing nor $NaHCO_3$ supplementation modified the acidification rates of LB496 and LB600 (Figure 3E and F). Finally, for ATCC11842^T, a faint stimulatory effect on acidification rate was observed after both N_2 flushing and $NaHCO_3$ supplementation (Figure 3G). These

data established that LB496, LB600, and ATCC11842^T required no CO_2 or less than 1.49 mg/L CO_2 in skim milk medium for growth and acidification; we designated such strains “ CO_2 -unresponsive.”

The effects of NH_3 supplementation were also tested on *L. bulgaricus* acid production. Supplementation with $(NH_4)_2SO_4$ did not affect acidification rates of the 7 studied strains (Figure 3). This result established that the *L. bulgaricus* strains did not require more than 2.7 mg/L NH_4 -N in skim milk for growth.

Coculture with CO_2 -Responsive and CO_2 -Unresponsive *L. bulgaricus* Strains

We selected the *L. bulgaricus* strains LB536 (CO_2 -responsive, Figure 4A) and LB496 (CO_2 -unresponsive, Figure 4B) for cocultures with ST1131 and $\Delta ureC$ 1131 in skim milk supplemented with 0.4 g/L urea and flushed with N_2 gas. Regardless of the *L. bulgaricus* strain, urease deficiency of $\Delta ureC$ 1131 caused a severe decrease (closed circles) in the acidification rate of the

Figure 3. Acidification kinetics of 7 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains in various growth media. The *L. bulgaricus* strains were cultured at 43°C in skim milk medium without (◇) or with (●) N₂ flushing and supplementation with NaHCO₃ (▲), or with NaHCO₃ and (NH₄)₂SO₄ (■). Acidity (%) corresponds to the amount of acid produced by *L. bulgaricus* in the skim milk medium. Error bars show SD of the results from at least 3 independent experiments.

Figure 4. Acidification kinetics of cocultures of *Streptococcus thermophilus* ST1131 or ST1131 urease-deficient mutants ($\Delta ureC1131$) with either *Lactobacillus delbrueckii* ssp. *bulgaricus* (A) LB536 (CO_2 -responsive) or (B) LB496 (CO_2 -unresponsive). Cocultures were performed at $43^\circ C$ in the skim milk medium flushed with N_2 (○, ●), supplemented with $NaHCO_3$ (▲), or with $NaHCO_3$ and $(NH_4)_2SO_4$ (■). The open symbol (○) indicates the coculture of *Strep. thermophilus* wild type (WT) with *L. bulgaricus*. Closed symbols (●, ▲, and ■) indicate the coculture of urease-deficient mutants ($\Delta ureC$) with *L. bulgaricus*. Acidity (%) corresponds to the amount of acid produced by *Streptococcus thermophilus* and *L. bulgaricus* in the skim milk medium. Error bars show SD of the results from at least 3 independent experiments.

cocultures compared with the cocultures involving WT ST1131 (open circles).

To evaluate the effects of CO_2 and NH_3 provided by urease activity, we monitored the acidification rate of $\Delta ureC1131$ with each of the *L. bulgaricus* strains in medium supplemented with $NaHCO_3$ with or without $(NH_4)_2SO_4$. The effects of CO_2 and NH_3 supplementation on acidification rates differed between the CO_2 -responsive and CO_2 -unresponsive *L. bulgaricus* strains cocultured with $\Delta ureC1131$. In the coculture of $\Delta ureC1131$ with the CO_2 -responsive LB536 strain, supplementation with both $NaHCO_3$ and $(NH_4)_2SO_4$ accelerated the acidification rate. In the coculture of $\Delta ureC1131$ with the CO_2 -unresponsive LB496 strain, only $(NH_4)_2SO_4$ supplementation stimulated the acidification rate, whereas $NaHCO_3$ supplementation had no effect, as expected. These observations established that $(NH_4)_2SO_4$ supplementation accelerated the acidification in cocultures with the $\Delta ureC$ *Strep. thermophilus* strain regardless of CO_2 -responsiveness of *L. bulgaricus* co-starter.

Cocultures of Fast- or Slow-Acidifying *Strep. thermophilus* Strains with CO_2 -Responsive *L. bulgaricus*

To evaluate whether the effect of urease deficiency on acidification in yogurt fermentation depended on the

acidification rate of the *Strep. thermophilus* strain used as a co-starter, 3 WT strains of *Strep. thermophilus* and their $\Delta ureC$ derivatives were cocultured with the CO_2 -responsive LB2038 strain in skim milk medium supplemented with 0.4 g/L urea and flushed with N_2 gas (Figure 5).

The time to reach 0.7% acidity was 4 h in the coculture of LB 2038 with the fast-acidifying strains (ST1131 and ST499) but 10.5 h in the coculture with the slow-acidifying strain ATCC19258^T. However, with all tested strain combinations, the urease deficiency of *Strep. thermophilus* decreased the acidification rates: the time required to reach 0.7% acidity increased 1.5- to 2-fold compared with that of cocultures with the WT *Strep. thermophilus* counterparts and LB2038.

Both $NaHCO_3$ and $(NH_4)_2SO_4$ supplementation accelerated acidification of the cocultures of $\Delta ureC$ strains with *L. bulgaricus* 2038, except for $\Delta ureC19258^T$, in which supplementation with $NaHCO_3$ and $(NH_4)_2SO_4$ did not have a significant effect.

DISCUSSION

In this study, we reexamined the effect of urease on acidification rates during yogurt fermentation. There are conflicting reports (Mora et al., 2004; Monnet et al., 2005; Arioli et al., 2009, 2017) regarding the effects of urease on acidification rates in monocultures and

Figure 5. Acidification kinetics of the coculture of *Streptococcus thermophilus* ST1131/ $\Delta ureC$ 1131, ST499/ $\Delta ureC$ 499, and ATCC19258^T/ $\Delta ureC$ 19258^T with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB2038. The cocultures were performed in the skim milk medium with N₂ (○, ●), supplemented with NaHCO₃ (▲) or NaHCO₃ and (NH₄)₂SO₄ (■). The open symbol (○) indicates the coculture of *Streptococcus thermophilus* wild type (WT) with *L. bulgaricus*. Closed symbols (●, ▲, and ■) indicate the coculture of urease-deficient mutants ($\Delta ureC$) with *L. bulgaricus*. Acidity (%) corresponds to the amount of acid produced by *Streptococcus thermophilus* and *L. bulgaricus* in the skim milk medium. Error bars show SD of the results from at least 3 independent experiments.

cocultures of *Strep. thermophilus* and *L. bulgaricus*. We explain these discrepant reports as follows: (1) nitrogen starvation and formate deficiency encountered by *Strep. thermophilus* and *L. bulgaricus* monocultured in milk medium, respectively, did not allow for the effect of urease to be studied under normal physiological conditions; and (2) the limited number of *L. bulgaricus* and *Strep. thermophilus* strains and combinations that were used did not account for the diversity of strain phenotypes. We addressed these 2 points in the current work. First, we supplemented skim milk medium with casein peptides for *Strep. thermophilus* monocultures and with sodium formate for *L. bulgaricus* monocultures to prevent starvation during assessment of the effect of urease deficiency on acidification rates. Second, we used several strains of each LAB species. For instance, we used fast- and slow-acidifying *Strep. thermophilus* strains to construct isogenic *ureC*-deficient mutants ($\Delta ureC$). We also characterized the CO₂ response of several *L. bulgaricus* strains and used CO₂-responsive and CO₂-unresponsive strains. Based on our data, we then used 5 strain combinations for cocultures. Together, our data demonstrated that urease is an essential factor for effective proto-cooperation and yogurt fermentation.

Effect of Urease Deficiency on CO₂ and NH₃ Production by *Strep. thermophilus* Strains

Urease deficiency resulted in dramatic reduction of CO₂ and NH₃ production, regardless of the strain (Table 2). As expected, we confirmed that urease is the main provider of CO₂ and NH₄-N in *Strep. thermophilus* cultures. However, between 6.4 and 7.6 mg/L of CO₂ was detected in the $\Delta ureC$ cultures, whereas CO₂ was not detected in uninoculated (control) skim milk (Table 2), indicating that other enzymes such as carbonate dehydratase provided a small amount of CO₂ that can be measured in the absence of urease (Sieuwerts et al., 2010). Conversely, NH₄-N was not detected in any $\Delta ureC$ cultures, although a trace amount was found in uninoculated milk. This observation indicated that the milk NH₄-N was consumed by the $\Delta ureC$ strains during their growth. For cultures of WT strains, 210 to 250 mg/L of NH₄-N was measured. These values were below the predicted 359 mg/L of NH₄-N based on the urea concentration in skim milk supplemented with 0.4 g/L urea (total urea concentration was 633 mg/L). These results indicated that *Strep. thermophilus* consumed part of the NH₃ provided by urease and other enzymes such as glutamate dehydrogenase.

Effect of Urease Deficiency on the Growth of *Strep. thermophilus*

For all 3 strains of *Strep. thermophilus*, urease deficiency severely decreased acidification rates and bacterial counts; counts of $\Delta ureC$ strains were 10% that of WT strains (Table 2) in the skim milk medium with (Figure 2) or without N₂ flushing (data not shown). These results revealed that urease was important for the growth of both fast- and slow-acidifying strains. However, Pernoud et al. (2004) did not observe significant delay in acidification and growth (OD₄₀₅) of *Strep. thermophilus* RD672 in the presence of flurofamide, a urease inhibitor. We propose that the effect of urease deficiency was underestimated because the *Strep. thermophilus* monoculture did not grow efficiently due to nitrogen starvation (lack of supplementation with peptides; Horiuchi and Sasaki, 2012).

To evaluate whether CO₂ or NH₃ provided by urease affected acidification by *Strep. thermophilus*, we added NaHCO₃ or (NH₄)₂SO₄ as sources of CO₂ or NH₃ to $\Delta ureC$ monocultures. Supplementation with (NH₄)₂SO₄ significantly accelerated the acidification of *Strep. thermophilus*, whereas supplementation with NaHCO₃ had only a slight or no effect (Figure 2). Among the tested strains, stimulation by (NH₄)₂SO₄ supplementation was limited in the $\Delta ureC19258^T$ culture. It may be that the NH₄ transporters are deficient in that strain. Despite this specific case, our results established that urease is important for the growth of *Strep. thermophilus*, mainly through NH₃ supply. It is noteworthy that (NH₄)₂SO₄ significantly accelerated acidification, even though the medium was supplemented with casein digested peptides, which provided at least 18 types of AA, including glutamate and aspartate that could constitute sources of ammonia nitrogen sources. This observation indicated that the requirement of *Strep. thermophilus* for ammonia nitrogen cannot be easily substituted by AA nitrogen. Monnet et al. (2005) reported that NH₃ supplied by urease could contribute to glutamine synthesis. In agreement, Arioli et al. (2007) reported that urease transcription was activated upon nitrogen limitation and was repressed by glutamate, glutamine, and NH₃. Our results also support that NH₃ provided by urease is important for AA and nucleic acid metabolism going through glutamine and carbamoyl phosphate in *Strep. thermophilus*.

Effect of CO₂ and NH₃ on Acidification by *L. bulgaricus*

Although the *L. bulgaricus* genomes exhibit some decarboxylase genes and they may produce CO₂, the N₂ flushing of the milk medium revealed that CO₂ respon-

siveness of *L. bulgaricus* varied among strains (Figure 3). Out of the 7 studied *L. bulgaricus* strains, 4 strains (LB497, LB494, LB2038, and LB536) required CO₂ for effective acidification under N₂ flushing and 3 strains (LB496, LB600, and ATCC11842^T) did not. The former result (Figure 3A, B, C, and D) strongly suggests that the decarboxylase genes in the strains' genomes were not able to provide enough CO₂ for optimal growth. Bringel and Hubert (2003) reported that *Lactobacillus plantarum* required CO₂ for the biosynthesis of pyrimidine; this may also be the case for the *L. bulgaricus* CO₂-responsive strains.

Regarding ammonia, Arioli et al. (2017) reported that in the absence of carbon source, NH₃ provided by urease brought the intracellular pH of *L. bulgaricus* MIM91 to an alkaline value and allowed a better homeostasis of the strain metabolic activity. Hence, they suggested that NH₃ provided by urease had positive effects on *L. bulgaricus*, although ammonia supplementation did not increase the intracellular pH of *L. bulgaricus* grown in skim milk medium. Our study establishes that (NH₄)₂SO₄ supplementation did not increase the acidification rates of any of the 7 *L. bulgaricus* strains. These results strongly suggest that NH₃ provided by urease did not directly affect the growth of *L. bulgaricus* strains in milk.

We conclude that the urease of *Strep. thermophilus* directly affected the acidification of CO₂-responsive *L. bulgaricus* strains through the provision of CO₂, whereas NH₃ provided by urease activity had no direct effect on *L. bulgaricus* growth.

Function of Urease in Yogurt Fermentation

In monocultures (Figure 2), the time required to reach 0.4% acidity increased by 2.5- to 4-fold due to urease deficiency but increased by only 1.6- to 2-fold in cocultures.

The first observation is that delay in acidification was stronger in *Strep. thermophilus* monocultures than in cocultures with *L. bulgaricus*. It could be that *L. bulgaricus* provided a limited amount of ammonia nitrogen to *Strep. thermophilus* in coculture and that *L. bulgaricus* contributed to the acidification and partly masked the effect of urease deficiency. With all 5 combinations of *Strep. thermophilus* and *L. bulgaricus*, the urease deficiency of *Strep. thermophilus* significantly decreased the acidification rate (Figures 4 and 5). However, the mode of action differed between CO₂-responsive and CO₂-unresponsive *L. bulgaricus* strains. As shown in our work, with CO₂-responsive strains (such as LB536, Figure 4A), the use of the $\Delta ureC1131$ strain led to a decreased acidification rate, which could be overcome by the supplementation with NaHCO₃ and (NH₄)₂SO₄.

In cocultures of CO₂-unresponsive *L. bulgaricus* strains (such as LB496, Figure 4B) with $\Delta ureC1131$, the acidification rate was stimulated only by (NH₄)₂SO₄ supplementation and not by NaHCO₃ addition.

We showed in this study that urease deficiency affected the acidification of all tested protococooperations regardless of the strains' phenotypes in terms of acidification speed for *Strep. thermophilus* or CO₂-responsiveness for *L. bulgaricus*. The effects of urease deficiency are mediated (1) by a shortage of ammonia nitrogen, which affects all *Strep. thermophilus* strains, and (2) by a shortage in CO₂, which only affects the growth of CO₂-responsive *L. bulgaricus* strains. Consequently, the main effect of urease deficiency is mediated through the ammonia nitrogen limitation rather than CO₂ shortage. Our data established that urease is essential for effective protococooperation and yogurt acidification by *Strep. thermophilus* and *L. bulgaricus*.

REFERENCES

- Arioli, S., G. Della Scala, M. C. Remagni, M. Stuknyte, S. Colombo, S. Guglielmetti, I. De Noni, E. Ragg, and D. Mora. 2017. *Streptococcus thermophilus* urease activity boosts *Lactobacillus delbrueckii* ssp. *bulgaricus* homolactic fermentation. *Int. J. Food Microbiol.* 247:55–64.
- Arioli, S., C. Monnet, S. Guglielmetti, C. Parini, I. De Noni, J. Hogenboom, P. M. Halami, and D. Mora. 2007. Aspartate biosynthesis is essential for the growth of *Streptococcus thermophilus* in milk, and aspartate availability modulates the level of urease activity. *Appl. Environ. Microbiol.* 73:5789–5796.
- Arioli, S., P. Roncada, A. M. Salzano, F. Deriu, S. Corona, S. Guglielmetti, L. Bonizzi, A. Scaloni, and D. Mora. 2009. The relevance of carbon dioxide metabolism in *Streptococcus thermophilus*. *Microbiol.* 155:1953–1965.
- Biswas, I., A. Gruss, S. D. Ehrlich, and E. Maguin. 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. *J. Bacteriol.* 175:3628–3635.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
- Bringel, F., and J. Hubert. 2003. Extent of genetic lesions of the arginine and pyrimidine biosynthetic pathways in *Lactobacillus plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. casei*: prevalence of CO₂-dependent auxotrophs and characterization of deficient. *Appl. Environ. Microbiol.* 69:2674–2683.
- Driessen, F. M., F. Kingma, and J. Stadhouders. 1982. Evidence that *Lactobacillus bulgaricus* in yogurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. *Neth. Milk Dairy J.* 36:135–144.
- Food Safety and Standards Authority of India. 2015. Pages 11–13 in *Manual of Methods of Analysis of Foods (Milk and Milk Products)—A Laboratory Manual*. Food Safety and Standards Authority of India, Ministry of Health and Family Welfare, Government of India, New Delhi, India.
- Galesloot, T. E., F. Hassing, and H. A. Veringa. 1968. Symbiosis in yoghurt. I. Stimulation of *Lactobacillus bulgaricus* by a factor produced by *Streptococcus thermophilus*. *Neth. Milk Dairy J.* 22:50–63.
- Horiuchi, H., N. Inoue, E. Liu, M. Fukui, Y. Sasaki, and T. Sasaki. 2009. A method for manufacturing superior set yogurt under reduced oxygen conditions. *J. Dairy Sci.* 92:4112–4121.
- Horiuchi, H., and Y. Sasaki. 2012. Short communication: Effect of oxygen on symbiosis between *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *J. Dairy Sci.* 95:2904–2909.
- Iyer, R., S. K. Tomar, T. Uma Maheswari, and R. Singh. 2010. *Streptococcus thermophilus* strains: Multifunctional lactic acid bacteria. *Int. Dairy J.* 20:133–141.
- Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss. 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J. Bacteriol.* 178:931–935.
- Monnet, C., D. Mora, and G. Corrieu. 2005. Glutamine synthesis is essential for growth of *Streptococcus thermophilus* in milk and is linked to urea catabolism. *Appl. Environ. Microbiol.* 71:3376–3378.
- Mora, D., E. Maguin, M. Masiero, C. Parini, G. Ricci, P. L. Manachini, and D. Daffonchio. 2004. Characterization of urease genes cluster of *Streptococcus thermophilus*. *J. Appl. Microbiol.* 96:209–219.
- Pernoud, S., C. Fremaux, A. Sepulchre, G. Corrieu, and C. Monnet. 2004. Effect of the metabolism of urea on the acidifying activity of *Streptococcus thermophilus*. *J. Dairy Sci.* 87:550–555.
- Sasaki, Y., H. Horiuchi, H. Kawashima, T. Mukai, and Y. Yamamoto. 2014. NADH oxidase of *Streptococcus thermophilus* 1131 is required for the effective yogurt fermentation with *Lactobacillus delbrueckii* ssp. *bulgaricus* 2038. *Biosci. Microbiota Food Health* 33:31–40.
- Sieuwerds, S., D. Molenaar, S. A. F. T. Van Hijum, M. Beerthuyzen, M. J. A. Stevens, P. W. M. Janssen, C. J. Ingham, F. A. M. De Bok, W. M. De Vos, and J. E. T. Van Hylckama Vlieg. 2010. Mixed-Culture transcriptome analysis reveals the molecular basis of mixed-culture growth in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. *Appl. Environ. Microbiol.* 76:7775–7784.
- Tamime, A. Y., and R. K. Robinson. 2007. *Yoghurt—Science and Technology*. 3rd ed. Woodhead Publishing Limited, Cambridge, UK.
- Veringa, H. A., T. E. Galesloot, and H. Davelaar. 1968. Symbiosis in yoghurt. II. Isolation and identification of a growth factor for *Lactobacillus bulgaricus* produced by *Streptococcus thermophilus*. *Neth. Milk Dairy J.* 22:114–120.
- Weerathilake, W. A. D. V., D. M. D. Rasika, J. K. U. Ruwanmali, and M. A. D. D. Munasinghe. 2014. The evolution, processing, varieties and health benefits of yogurt. *Int. J. Sci. Res. Publ.* 4:1–10.