Effect of the Metabolism of Urea on the Acidifying Activity of *Streptococcus thermophilus*

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ABSTRACT

One of the main functions of *Streptococcus thermophilus* strains used in the dairy industry is the production of lactic acid. In cheese and fermented milk manufacturing processes, the pH evolution kinetics must be reproducible in order to ensure the good quality of the final products. The objective of the present study was to investigate the effect of the metabolism of urea on the acidifying activity of fast- and slow-acidifying strains of *S. thermophilus*. Milk treatment with a purified urease and utilization of the urease inhibitor fluoramide revealed that urea metabolism by *S. thermophilus* influences the pH evolution kinetics through 2 distinct means. First, ammonia production from urea tends to increase the pH. This effect is greater when lactic acid concentration is low due to a lower buffering capacity of milk. Second, urea metabolism also modifies growth and lactic acid production by *S. thermophilus*. Depending on the strains and the growth stage of the cultures, consumption of urea induces either a faster or a slower pH decrease. For the slow-acidifying strain RD678, suppression of urea metabolism by adding fluoramide decreased the time necessary to reach pH 6 by 195 min. This effect was less pronounced for the 2 fast-acidifying strains RD674 and RD677. These results show that urea metabolism may have a considerable influence on the acidifying properties of *S. thermophilus* strains.

(Key words: lactic acid bacteria, *Streptococcus thermophilus*, acidifying activity, urea)

INTRODUCTION

The lactic acid bacterium *Streptococcus thermophilus* is used in the manufacture of several kinds of cheeses and fermented milks, in which its main functions are

the production of lactic acid, aroma compounds, and exopolysaccharides. Lactic acid assists in milk coagulation and curd draining, imparts a fresh acid flavor to fermented milks, and helps to suppress the growth of pathogens and spoilage microorganisms.

In contrast to the other lactic acid bacteria used as starter cultures, *S. thermophilus* possesses a urease, which converts urea into ammonia and carbon dioxide (Juillard et al., 1988; Tinson et al., 1982). During the growth of *S. thermophilus* in milk, production of ammonia from urea slows down the pH decrease or induces a temporary increase of pH (Tinson et al., 1982; Spindler and Corrieu, 1989; Latrille et al., 1992). The presence of urease is not essential for the growth of *S. thermophilus*, as a naturally occurring urease-deficient strain has been described (Juillard et al., 1988).

Urea concentration in milk depends on several factors such as the bovine breed, the stage of lactation, the season, and the nitrogen balance of the diet (de Peters and Ferguson, 1992). Milk urea nitrogen can be used to estimate urinary nitrogen excretion and the target range for milk urea concentration of Holstein cows is between 1.4 and 1.9 mM (Kohn et al., 2002). Variations of the urea content of milk affect the pH evolution kinetics during the manufacture of cheeses and fermented milks when *S. thermophilus* strains are used. As a consequence, several important sensory properties of the dairy products may be affected. For example, a high urea content in milk may lead to Reblochon cheeses with a higher moisture content (Martin et al., 1997).

Despite its implications in cheese making, the metabolism of urea by *S. thermophilus*, and especially its effect on the pH evolution kinetics during the fermentation of milk, has been seldom investigated. In this study, we aimed to characterize the effect of urea on the acidifying activity of fast- and slow-acidifying *S. thermophilus* strains. The first type of strains is often used in the manufacture of yogurt and hard cheeses, whereas the latter type is often used for the production of soft cheeses.

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MATERIALS AND METHODS

Strains

Streptococcus thermophilus RD672, RD674, RD677, and RD678 were obtained from Rhodia Food (Dangé Saint Romain, France). RD672 and RD678 are slow-acidifying strains of *S. thermophilus*, whereas RD674 and RD677 are fast-acidifying strains. *Streptococcus thermophilus* CNRZ407 was obtained from the collection of the Institut National de la Recherche Agronomique (Jouy-en-Josas, France).

Measurement of the Acidifying Activity

After cultivation for 24 h at 37°C in 5 mL of M17 broth (Terzaghi and Sandine, 1975), the strains were inoculated at 2% (vol/vol) in 5 mL of reconstituted skim milk (100 g/L; Elle et Vire, Condé-sur-Vire, France) that had been sterilized for 10 min at 110°C. The cultures were incubated for 24 h at 37°C and inoculated at 1% in 150 mL of reconstituted skim milk (100 g/L; Elle et Vire, Condé-sur-Vire, France) that had been heated for 10 min at 90°C. The culture flasks were then placed in a water bath at 37°C and the pH was continuously measured during 15 h using a CINAC apparatus (Ysebaert, Frépillon, France). The acidification rate (dpH/dt) was calculated from these data and expressed as pH units/min (Spinnler and Corrieu, 1989). The urease inhibitor fluorofamide (Kenny, 1983) (N-[diaminophosphinyl]-4-fluorobenzamide; ICN, Orsay, France), was used at a final concentration of 10 μ M. A concentrated stock solution of this compound was prepared at 400 μ M and added to milk after sterilization by filtration (0.22 μ m). All experiments were done in triplicate.

Measurement of Growth and Biochemical Analyses

Absorbance of the milk cultures after 15 h of growth was measured by clarification of the medium (Levata-Jovanovic and Sandine, 1996). The sample was diluted (1/10, vol/vol) with a solution of ethylenediaminetetraacetic acid (EDTA) at 2 g/L whose pH was adjusted to 12.5 with sodium hydroxide (10 M), and absorbance was then read at 405 nm. The difference between the absorbance of the sample and that of noninoculated milk treated in the same conditions was calculated.

Ammonia, urea, and lactic acid were assayed using enzymatic kits (Boehringer, Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. Before the assays, the samples were centrifuged for 20 min at 21,000 $\times$ g and the supernatants were recovered. Carbon dioxide was measured using a previously described method (Monnet et al., 2002).

Milk Titration

Titration curves were established by measuring the pH after addition of lactic acid (500 g/L) in 60- μ L increments to a conical flask containing 250 mL of milk at 37°C. The milk (which was not inoculated with *S. thermophilus*) was continuously stirred and acid was added approximately every 30 s to allow for equilibrium (Lucey et al., 1993). Titration was also done after treatment of milk with 1.36 U/mL of jack bean urease (Sigma, Saint-Quentin-Fallavier, France) for 3 h at 37°C. During the experiment, there was no significant loss of ammonia and carbon dioxide by volatilization. All experiments were carried out in triplicate.

RESULTS

Effect of Urea Hydrolysis on the pH of Milk

To evaluate the impact of urea hydrolysis on the pH, we treated reconstituted skim milk (initial pH was 6.52) with urease, as described in Materials and Methods. This treatment resulted in a pH increase of 0.28 unit (U). Urea hydrolysis thus has a considerable effect on the pH of milk. During urea consumption by *S. thermophilus*, lactic acid is also produced, which decreases the pH. As the buffering capacity of milk depends on the pH (Lucey et al., 1993), it is possible that the effect of urea hydrolysis on pH is not the same when it occurs early during cultivation as when it occurs late. To investigate this effect, increasing amounts of lactic acid were added to milk and to urease-treated milk, as described in Materials and Methods (Figure 1A). The difference of pH between the 2 samples reflects the effect of urea hydrolysis at different concentrations of lactic acid (Figure 1B). This difference decreased dramatically when the concentration of lactic acid increased, and at a concentration of 10 g/L, it was only of about 0.06 U. This is the consequence of an increase of the buffering capacity of milk in the presence of lactic acid. It is possible that the buffering properties of milk in titration experiments are not strictly equivalent to those of milk fermented by *S. thermophilus*. Indeed, increase of the buffering capacity at low pH may be due to solubilization of colloidal calcium phosphate (Lucey et al., 1993), which may differ according to the acidification rate (acidification is faster in titration experiments than in growth experiments). However, the difference of pH between milk and urease-treated milk in titration experiments is probably a good indication of the impact of urea hydrolysis by *S. thermophilus*. Indeed, if there is a slight difference between the buffering properties of milk in titration experiments and those in *S. thermophilus* cultures, this difference should be the same when milk is treated or not with urease.

Figure 1. Effect of lactic acid addition on the pH of milk (pH_M) and of urease-treated milk (pH_U) (A), and evolution of the value of $pH_U - pH_M$ (B).

Effect of the Metabolism of Urea on the Acidifying Activity of *S. thermophilus*

The milk titration experiments with lactic acid described previously suggest that, when urea is consumed late during the growth of *S. thermophilus* (high lactic acid concentration), the decrease in the acidification rate due to the production of ammonia will be lower than when it is consumed early (low lactic acid concentration). However, the effect of the metabolism of urea on milk acidification may not be limited to the effect of ammonia on the pH. Indeed, consumption of urea by

S. thermophilus may also modify the rate of lactic acid production. This might occur, for example, if the metabolism of urea significantly affects the growth of *S. thermophilus*. One way to evaluate this effect would be to compare milk cultures in the presence and in the absence of an urease inhibitor. Acetohydroxamate is a widely used urease inhibitor (Andrews et al., 1984), but it was unsatisfactory for the present investigation, as it did not completely inhibit the consumption of urea by growing *S. thermophilus* cells, even at a concentration of 5 mM. This was not the case for the substrate analog fluorofamide, which was used at a final concentration of 10 μM . In order to establish that fluorofamide acts only on urea hydrolysis, it was necessary to check that addition of this compound had no effect during cultivation of *S. thermophilus* in a medium devoid of urea. For this purpose, strains RD672, RD674, RD677, and RD678 were grown in urease-treated milk. In these experiments, addition of fluorofamide did not change growth and acidification (data not shown). Furthermore, in milk that was not treated with urease, fluorofamide had no effect on the growth and acidifying activity of the urease-deficient strain CNRZ407 described by Juillard and coworkers (1988). It can thus be concluded that in milk, fluorofamide has no effect on growth and acidification of *S. thermophilus*, except the effect resulting from the inhibition of urease.

In the absence of fluorofamide (i.e., when the strains are able to use urea), *S. thermophilus* RD674 and RD677 were more acidifying than strains RD672 and RD678 (Figure 2). Indeed, after 15 h of growth, their pH was lower than 4.5, whereas it was higher than 5.0 for the other strains. Furthermore, growth of these strains was higher (Table 1). In the absence of fluorofamide, there was a temporary decrease of the acidification rate between 160 and 250 min during the growth of strain RD672, as the dpH/dt value increased (Figure 2). For strain RD678, there was even a temporary increase of pH between 150 and 230 min. For the 2 other strains, no temporary increase of pH or of dpH/dt could be observed at the beginning of the growth of the cultures, but there was a quasi-stagnation of dpH/dt between 100 and 150 min. For the 4 strains, milk urea (3.1 mM) was completely consumed during the growth of the cultures.

Depending on the strains, addition of fluorofamide (urea consumption by the strains is then inhibited) had varying effects on the pH evolution kinetics. For strain RD672, a stimulation of the pH decrease was observed, especially between 200 and 600 min (Figure 2). This stimulating effect was higher for strain RD678, whereas it was lower for strain RD674. In the case of strain RD677, addition of fluorofamide stimulated the pH decrease until 200 min, and the opposite was subse-

Figure 2. Evolution of the pH and its first time derivative (dpH/dt) during the growth of *S. thermophilus* RD672, RD674, RD677 and RD678 in milk. pH in the presence (---) and in the absence (—) of flurofamide, and dpH/dt in the presence (— · —) and in the absence (· · ·) of flurofamide.

Table 1. Effect of the urease inhibitor flurofamide on growth and lactic acid production by *S. thermophilus*. Strains were grown for 15 h in reconstituted skim milk.^{1,2}

Strain	Flurofamide	Growth (absorbance at 405 nm)	Lactic acid (g/L)
RD672	—	1.72	3.22
	+	2.32 ³	2.92 ³
RD674	—	6.37	6.88
	+	5.78 ³	6.17 ³
RD677	—	6.90	6.20
	+	6.60 ³	5.69 ³
RD678	—	1.66	2.85
	+	1.42 ³	2.04 ³

¹The values are means of three repetitions. Standard errors were always lower than 5%.

²Urea concentration in reconstituted skim milk was 3.1 mM.

³Values were different from those in absence of flurofamide ($P < 0.05$).

Figure 3. Evolution of the difference of pH between culture experiments of *Streptococcus thermophilus* RD672 (— · — · —), RD674 (— — —), RD677 (· · · · ·) and RD678 (— · — · —) in the presence (pH_{+Flu}) and in the absence (pH_{-Flu}) of fluoroamide, as a function of pH_{+Flu} . The difference of pH between milk (pH_M) and urease-treated milk (pH_U) as a function of pH_M (data from milk titration experiments with lactic acid presented in Figure 1) is also shown (—).

quently observed. The temporary increase (strains RD672 and RD678) or stagnation (strains RD674 and RD677) of dpH/dt that was observed at the beginning of the culturing time in the absence of fluoroamide (when strains are able to use urea), did not occur when urea consumption was inhibited. Similar pH curves were obtained when culture experiments (in the presence or in the absence of fluoroamide) were repeated (at all cultivation times, difference of pH between repeated cultures was always lower than 0.04 U for strains RD672, RD674, and RD677, and 0.09 U for RD678).

Figure 3 shows the curves relating the difference of pH between the culture experiments carried out in the presence and in the absence of fluoroamide, as a function of the pH of the culture experiments in the presence of fluoroamide. These curves characterize the effect of the metabolism of urea on the acidifying activity of *S. thermophilus*. The curve representing the difference of pH between milk and urease-treated milk as a function of pH (data from milk titration experiments with lactic acid presented in Figure 1) is also shown. It represents the direct effect of urea hydrolysis on pH (i.e., via the effect of ammonia and carbon dioxide production on pH) and will be named hereafter “titration curve.” The curves obtained for strains RD672, RD674, and RD677 have a similar profile, showing three distinct phases. During the first phase, from pH 6.6 to about 6.0 to 5.8, they tend to increase and to come close to the titration

curve. This phase corresponds to the consumption of urea by *S. thermophilus*. At the end of this phase, nearly all urea (>95%) had been consumed. During the second phase, the 3 curves are close to the titration curve, especially for strains RD672 and RD674. At this growth stage of the cultures, the impact of the metabolism of urea on the acidifying activity of *S. thermophilus* was thus equivalent to the direct effect of ammonia and carbon dioxide on the pH. In the third phase, the 3 curves tend to decrease and to move away from the titration curve. This may be explained by a higher production of lactic acid by *S. thermophilus* in the absence of fluoroamide (i.e., when strains are able to use urea). Indeed, if urea consumption by *S. thermophilus* also increases its production of lactic acid, the pH-increasing effect of urea consumption (via production of ammonia) would thus be compensated for by the pH-decreasing effect due to increased lactic acid production. Measurements of lactic acid concentration at the end of growth of the cultures confirmed that urea consumption (cultivation in the absence of fluoroamide) increases lactic acid production (Table 1). The curve corresponding to strain RD678 (Figure 3) was similar to those of the three other strains, except that it was higher than the titration curve from pH 6.0 to 5.5. This may be explained by a temporary inhibition of lactic acid production by this strain in the absence of fluoroamide (i.e., when the strain is able to use urea). For strains RD674, RD677, and RD678, addition of fluoroamide decreased growth after 15 h, whereas the reverse was observed for strain RD672 (Table 1).

DISCUSSION

The objective of the present study was to investigate the effect of the metabolism of urea on the acidifying activity of *S. thermophilus*. The urease present in this bacterium converts urea into ammonia ($pK = 9.24$) and carbon dioxide, which combines with water to form carbonic acid ($pK = 6.35$). As the production of ammonia is twice that of carbonic acid and the pH of milk is much lower than the pK of ammonia, urea hydrolysis tends to increase the pH. Our results show that this effect is much higher when it occurs at low lactic acid concentration (i.e., in conditions equivalent to the beginning of growth), than at high lactic acid concentration (i.e., in conditions equivalent to the end of growth). Consequently, it is expected that for fast-acidifying strains and for strains that consume urea late during growth of the culture, the effect of the metabolism of urea on the evolution of pH is limited. It is interesting to note that variations of acidifying activity due to urea consumption by *S. thermophilus* are known to occur especially in cheese-manufacturing processes in which acid-

ification is slow. Furthermore, the effect of urea consumption on the evolution of pH (which was evaluated by comparing culture experiments in the presence and in the absence of fluorofamide) during the growth of the 2 slow-acidifying strains used in the study, was greater than for the fast-acidifying strains.

However, the effect of the metabolism of urea on the acidifying activity of *S. thermophilus* cannot be attributed to the sole effect of ammonia and carbon dioxide on pH. Indeed, experiments with the urease inhibitor fluorofamide enabled us to show that urea consumption also affects lactic acid production by *S. thermophilus*. For 3 of the 4 strains investigated, urea consumption increased lactic acid production, which tends to counteract the pH-increasing effect of urea hydrolysis. This may be linked to a stimulation of growth by urea, but not only, as no increased growth was observed for strain RD672. For the fourth strain (RD678), urea consumption considerably reduced the decrease in pH during part of growth of the culture. This effect is probably the result of a temporary decrease in lactic acid production. The present study also shows that the effect of urea metabolism on the acidifying activity of slow-acidifying *S. thermophilus* strains may be very different depending on the strain. For example, for *S. thermophilus* RD678, the metabolism of urea was responsible for an increase of 195 min of the time necessary to reach pH 6 (Figure 2), whereas this increase was 65 min for strain RD672.

In order to better understand the effect of urea on the growth and acidifying activity of *S. thermophilus*, it would be interesting to investigate all the physiological consequences of the metabolism of urea. For example, it is possible that some ammonia and carbon dioxide produced from urea may be used for the synthesis of cellular components during the growth of *S. thermophilus* in milk. Urea consumption may also have an effect on the intracellular pH of *S. thermophilus*.

CONCLUSIONS

Utilization of the urease inhibitor fluorofamide makes it easy to characterize the effect of urea on pH evolution kinetics during the growth of *S. thermophilus* in milk. Depending on the strains and the growth stages of the

cultures, consumption of urea induces, either a faster or a slower pH decrease. We suggest that selection of strains used in cheese-manufacturing processes should include characterization of the effect of urea. This will be useful for limiting the variations of pH evolution kinetics due to the consumption of urea by *S. thermophilus*.

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