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Utilization of Lactose, Glucose, and Galactose by a Mixed Culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in Milk Treated with Lactase Enzyme

VIRGINIA S. O'LEARY AND JOHN H. WOYCHIK*

Eastern Regional Research Center, Agricultural Research Service, Philadelphia, Pennsylvania 19118

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The mechanism responsible for an increased rate of acid production when yogurt starter cultures are grown in milk treated with lactase enzyme was investigated by studying carbohydrate utilization and acid development by a pure culture of *Streptococcus thermophilus* and a mixed yogurt starter culture consisting of *S. thermophilus* and *Lactobacillus bulgaricus*. In milk containing glucose, galactose, and lactose, glucose and lactose (but not free galactose) were fermented. Fermentation of lactose in control milk was accompanied by the release of free galactose, with the result that carbohydrate utilization was less efficient than in treated milk. This phenomenon also occurred when lactose was fermented by *S. thermophilus* in broth culture. Carbohydrate utilization by the mixed yogurt culture was more rapid when the lactose in milk was partially prehydrolyzed. Our results suggest that the more rapid acid development that took place when a mixed yogurt starter culture was grown in milk containing prehydrolyzed lactose was the result of a more rapid and efficient utilization of carbohydrate by *S. thermophilus* when free glucose in addition to lactose was available for fermentation. The evidence presented also suggests that uptake and utilization of glucose and lactose by *S. thermophilus* are different in broth and milk cultures.

A stimulation of acid production by lactic streptococci in milk cultures upon the addition of β-galactosidase to hydrolyze the milk lactose to glucose and galactose was reported by Gilliland et al. (4). They (4) suggested that optimum acid production by lactic streptococci does not take place in milk cultures due to an inability of the organisms to metabolize the lactose at maximum efficiency. Thompson and Gyuricsek (12) also observed accelerated acid development by dairy starter cultures in yogurt, buttermilk, and cottage cheese products manufactured from milk that had been pretreated with lactase enzyme.

The research reported here was undertaken to define the factors that control the rate of acid development in cultured dairy products derived from milk containing sugar either as lactose or as the free monosaccharides resulting from lactose hydrolysis. Carbohydrate utilization, total consumption, and efficiency of conversion to acid metabolites were studied by using typical yogurt starter culture organisms.

MATERIALS AND METHODS

Bacterial cultures. The yogurt culture used was a mixed starter consisting of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* ($LBST_4$) supplied by the Marschall Division of Miles Laboratories, Madison, Wis., in cryogenic form. Pure cultures of *S. thermophilus* ST_4 (Marschall Division of Miles Laboratories) were used in some studies. Cryogenic cultures, which were kept stored in liquid nitrogen, were allowed to thaw and then were inoculated into sterile skim milk using aseptic techniques. Transfers in sterile skim milk were made daily.

Preparation of lactase-hydrolyzed (LH) milk. Skim milk fortified with 4% (wt/vol) nonfat milk solids was incubated with 300 μg of Maxilact β-galactosidase per ml (Enzyme Development Corp.) to hydrolyze the lactose to glucose and galactose. Incubation was overnight at 4°C with constant stirring and resulted in hydrolysis of 70 to 75% of the lactose. Approximate sugar concentrations in the lactase-treated milk were 2.6% glucose, 2.3% galactose, and 2.0% lactose.

Fermentation of control and LH milks. Control and LH milks were pasteurized at 105°C for 15 min. This heat treatment inactivated the lactase enzyme. The milks were cooled to 43°C and then inoculated at a level of 2% with either the mixed yogurt starter culture or a pure culture of *S. thermophilus* that had previously been grown at least twice for 24 h at 30°C in sterile skim milk. Numbers of starter culture organisms in the inoculated milks at the beginning of the fermentation were determined by plate counts on Hansen's yogurt agar (11). There were no statis-

tically significant differences in inoculum levels of *S. thermophilus* and *L. bulgaricus* in the two milks. The inoculated milks were incubated in a water bath at 44°C, and acid development and sugar utilization were monitored during the incubation period.

Acid development. Acid development was determined by measuring pH during the fermentation period using a Radiometer pH meter, model 22 (Radiometer, Copenhagen, Denmark).

Sugar utilization in milk. Samples for sugar analyses were collected at hourly intervals during the fermentation period. One-milliliter samples of the milk cultures were placed in centrifuge tubes containing 1.0 ml of 1.0 M acetate buffer (pH 4.6) and well mixed in a Vortex mixer. Precipitated casein and bacterial cells were removed by centrifugation at 30,000 × *g* for 15 min at 4°C in a Sorvall Superspeed RC-2 centrifuge. The supernatants were used in the analyses for glucose, galactose, and lactose.

Sugar utilization in broth culture. Basal broth medium was prepared as described by Gilliland et al. (4). To the sterile basal medium was added 2% glucose and 2% lactose in the form of 20% solutions previously sterilized by membrane filtration (Millipore Corp., Bedford, Mass.), using a 0.22-μm pore size filter. Two milliliters of a 24-h culture of *L. bulgaricus* or *S. thermophilus* in Elliker broth was added to 100 ml of the sugar utilization medium. Flasks containing the inoculated broth were incubated in a 43°C water bath, and samples for glucose and lactose anaylses were withdrawn periodically during the incubation period.

Sugar analyses. Glucose was determined using the Salomon and Johnson reagent as described by Jasewicz and Wasserman (6), but with the following modifications. To 0.1 ml of sample containing 15 to 150 μg of glucose were added 2.0 ml of water and 1.5 ml of Salomon and Johnson reagent. The color was allowed to develop at room temperature for at least 1 h, and then the absorbancies of the solutions were read at 635 nm using a Zeiss model PMQ II spectrophotometer. Glucose concentrations were determined by comparison with a standard curve, which was prepared daily.

Lactose in milk was determined by measurement of the glucose released after hydrolysis with lactase as follows. A 3.0-ml portion of Maxilact lactase (Enzyme Development Corp.; 5 mg/3 ml) in 0.1 M phosphate buffer, pH 7.0, was added to 0.1 ml of sample and incubated for 3 h at 30°C. The enzyme reaction was terminated by placing the tubes in a boiling-water bath for 5 min, and the solutions were then clarified by centrifugation. One-tenth-milliliter portions of the supernatants were analyzed for glucose by the procedure previously described. The difference in the glucose concentrations determined before and after lactose hydrolysis was multiplied by 1.92 to give the actual lactose concentration. In the broth studies, lactose was determined directly using an Enzymax lactose-glucose analyzer (Leeds & Northrup, N. Wales, Pa.).

Galactose measurements were made by an enzymatic method utilizing galactose dehydrogenase. The method used was a modification of the procedure described in the Boehringer-Mannheim catalogue (3a). The assay medium consisted of 0.84 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.6; 0.05 ml of β-nicotinamide adenine dinucleotide, 5 mg/ml (Sigma Corp.); and 0.10 ml of sample and 0.01 ml of β-galactose dehydrogenase (Sigma Corp.) diluted to approximately 3 U/ml. The assay solutions were incubated at 25°C for 75 min, and then the absorbance due to the reduction of nicotinamide adenine dinucleotide was read at 340 nm using a Zeiss model PMQ II spectrophotometer.

All experiments were conducted in duplicate, and each experiment was repeated at least twice. Experimental data were analyzed statistically by computer, using standard statistical computer packages for Student's *t* test, analysis of variance, and linear regression.

RESULTS

Acid development. The curves presented in Fig. 1 illustrate the decrease in pH observed when *S. thermophilus* alone and *S. thermophilus* plus *L. bulgaricus* were grown in control and LH milks. Acid development was more

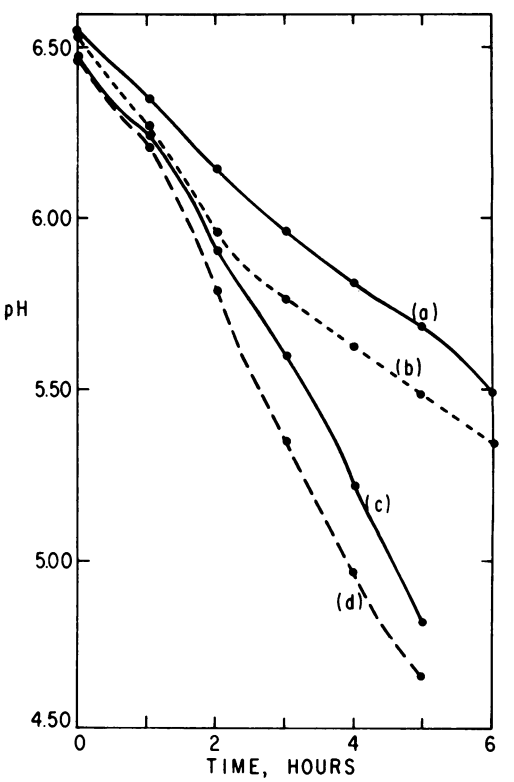

FIG. 1. *pH change in control and LH milks during fermentation by S. thermophilus alone and S. thermophilus plus L. bulgaricus. (a) Control milk, S. thermophilus; (b) LH milk, S. thermophilus; (c) control milk, mixed culture; (d) LH milk, mixed culture.*

rapid (statistically significant at $\alpha = 0.01$) in LH milk in both cases.

Sugar utilization patterns in control and LH milks. The disappearance of glucose, galactose, and lactose in LH milk during fermentation by the mixed culture is presented in Fig. 2. Sugar concentrations in the LH milk prior to inoculation were 2.64% glucose, 2.31% galactose, and 2.06% lactose. Glucose was utilized throughout the incubation period, whereas lactose utilization took place only up to 4 h, with the most rapid period of utilization occurring between 2 and 4 h. Free galactose was not utilized.

Utilization of glucose and lactose by a pure culture of *S. thermophilus* in LH milk is shown in Fig. 3. Both lactose and glucose were utilized by this organism in the 24-h incubation period. However, in a broth culture containing glucose and lactose, *S. thermophilus* utilized lactose only (Table 1). *L. bulgaricus*, on the other hand, utilized glucose preferentially.

Utilization of lactose by the mixed culture in control milk is presented in Fig. 4. As lactose was consumed during the first 4 h of incubation, galactose accumulated in the medium. Galactose levels increased from 0.01% at 0 h to 0.14% at the end of 4 h. The extremely low levels (less than 0.01%) of glucose present in the control milk remained relatively unchanged throughout the incubation period and are not included on the graph. Utilization of lactose by *S. thermophilus* in broth culture was also accompanied by the appearance of galactose in the medium. In one set of experiments, the catabolism of 0.74% lactose resulted in the accumulation of 0.35 to 0.40% galactose, indicating that virtually all the galactose produced from lactose through intracellular hydrolysis was released into the medium. In our experiments with the mixed culture, there was a decrease (statistically significant at $\alpha = 0.01$) in free galactose levels in control milk after 4 h of incubation, indicating that some of the galactose released into the medium as a result of lactose fermentation was utilized.

Lactose consumption in control milk and total carbohydrate metabolized in all forms by the mixed culture in control and LH milks are presented in Fig. 5. The actual amount of disaccharide metabolized in control milk was calculated by subtracting the galactose that accumulated in the medium from the lactose consumed. Carbohydrate was consumed more rapidly in the LH milk. In addition, the carbohydrate consumed in LH milk was metabolized more efficiently by the organisms.

The data for the amount of sugar metabolized versus decrease in pH for control and LH milks

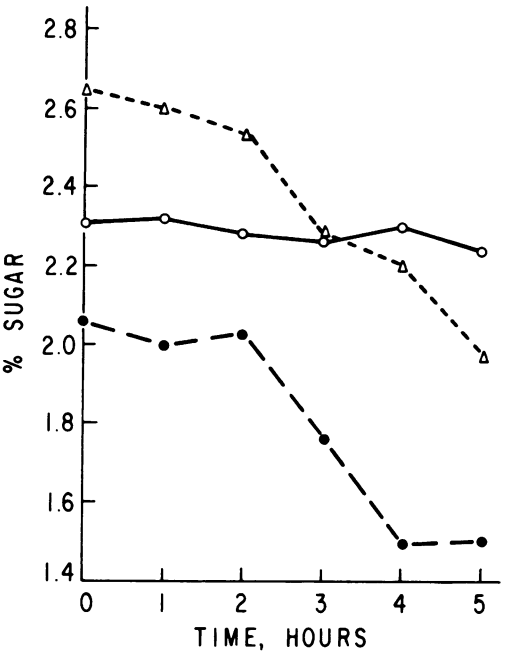

FIG. 2. *Utilization of glucose, galactose, and lactose by a mixed yogurt starter culture during growth and fermentation in LH milk. Symbols: ○, galactose; △, glucose; ●, lactose.*

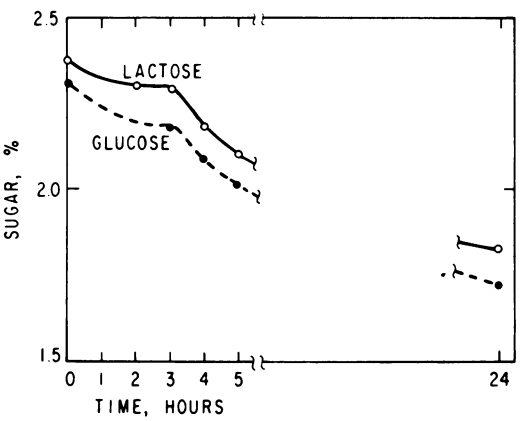

FIG. 3. *Utilization of glucose and lactose by S. thermophilus during growth and fermentation in LH milk.*

were subjected to linear regression analysis. There was a linear relationship between total sugar metabolized and decrease in pH, with linear correlation coefficients at the 95% confidence level of 0.903 for the control milk and 0.915 for the LH milk. Analysis of variance of the slopes of the two lines revealed no significant differences between the two slopes. These results indicate that an equivalent unit drop in

TABLE 1. *Utilization of glucose and lactose by pure cultures of S. thermophilus and L. bulgaricus in broth containing a mixture of the two sugars*

Time (h)	*S. thermophilus*		*L. bulgaricus*	
	Glucose (%)	Lactose (%)	Glucose (%)	Lactose (%)
0	2.33	1.89	2.31	1.91
8	2.39	1.71	2.17	1.85
12	2.29	1.37	2.16	1.81
16	2.29	1.27	2.17	1.82
24	2.29	1.15	1.61	1.60

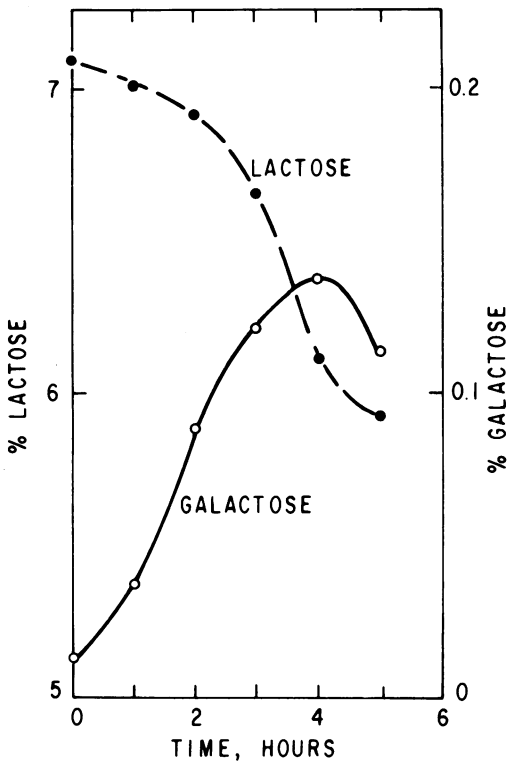

FIG. 4. *Carbohydrate utilization by a mixed yogurt starter culture during growth and fermentation in control milk.*

pH occurred when equal amounts of sugar were metabolized irrespective of whether they were supplied as the mono- or disaccharide form.

DISCUSSION

In a previous study (10) we reported that growth rates and patterns of growth of a mixed yogurt starter culture consisting of *S. thermophilus* and *L. bulgaricus* in milk pretreated with lactase enzyme were not significantly different than in control milk. We found that *S. thermophilus* began to multiply rapidly almost immediately after inoculation into the milk medium and was thus the predominant organism during the first few hours of incubation, whereas *L. bulgaricus* did not initiate rapid growth until 4 h of incubation, at which time *S. thermophilus* was in the stationary growth phase. Although glucose in LH milk disappeared throughout the incubation period, lactose utilization ceased after 4 h. These results indicate that *S. thermophilus* utilized both glucose and lactose, whereas *L. bulgaricus* utilized glucose exclusively. Neither organism utilized free galactose when glucose was present. A number of researchers (1, 5, 7-9) have reported that glucose inhibits microbial utilization of other carbon sources. This phenomenon is referred to as catabolite inhibition. McGinnis and Paigen (8) found that glucose inhibited the utilization of a wide number of sugars by *Escherichia coli*, including maltose, lactose, mannose, galactose, L-arabinose, and xylose. Catabolite inhibition by glucose has also been shown

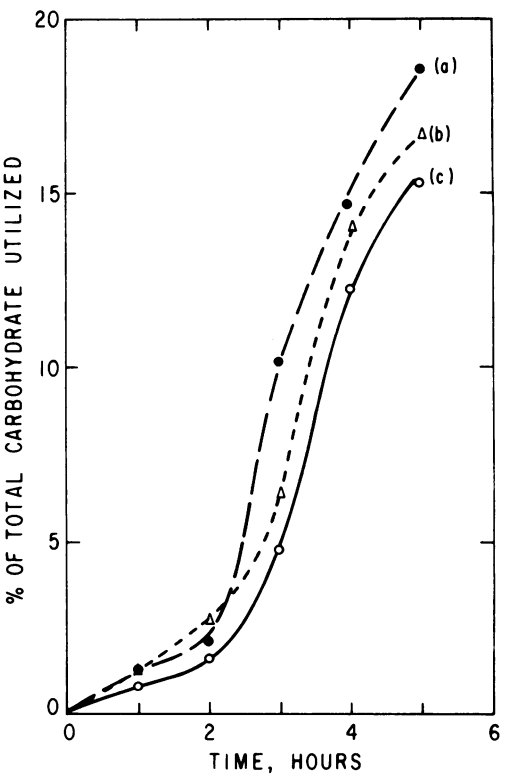

FIG. 5. *Percentage of lactose uptake in control milk and of total available carbohydrate utilized by a mixed yogurt starter culture during growth and fermentation in control and LH milks. (a) Percentage of carbohydrate utilized, LH milk; (b) percentage of lactose consumed, control milk; (c) percentage of carbohydrate utilized, control milk.*

to occur in the lactic acid bacteria. Hasan and Durr (5), for instance, demonstrated that glucose inhibited the utilization of galactose and lactose by preinduced cells of *L. plantarum*. McGinnis and Paigen (8) suggested that catabolite inhibition is a control mechanism that allows utilization of the most advantageous substrate when a mixture of carbon sources is available. According to *Bergey's Manual* (2), *S. thermophilus* preferentially ferments the disaccharides lactose and sucrose. We found this to be true in broth culture but not in milk. When a pure culture of *S. thermophilus* was grown in lactased milk, approximately equal amounts of glucose and lactose were metabolized, whereas in broth culture containing the two sugars only lactose was metabolized. It appears from these results that in broth culture lactose is preferentially utilized by *S. thermophilus* and may even, in fact, prevent the utilization of glucose, whereas in milk culture both sugars are fermented simultaneously. Possibly, milk contains some substance that facilitates utilization of glucose in the presence of lactose or represses lactose inhibition of glucose utilization.

When *S. thermophilus* ferments lactose, the glucose released through intracellular hydrolysis is catabolized, whereas little if any of the galactose is metabolized, but, rather, it is released into the medium. Gilliland et al. (4) also reported an accumulation of galactose during metabolism of lactose by *S. lactis* C_{10} in milk cultures. McGinnis and Paigen (9) reported that in *E. coli* glucose generated intracellularly produced catabolite repression. Their conclusions were based on experiments in which synthesis of mannose-metabolizing enzymes was repressed when lactose was added to an *E. coli* culture growing on mannose. Such an effect was not observed with *L. plantarum*, however, in similar studies conducted by Hasan and Durr (5). Our results indicate that glucose generated intracellularly during lactose hydrolysis by *S. thermophilus* may play a role in inhibiting the catabolism of galactose by this organism. Possibly, intracellular glucose represses synthesis of galactokinase or the enyzmes of the D-tagatose-6-phosphate pathway, both of which enzyme systems have been reported to be active in the metabolism of galactose by group N streptococci (3).

In control milk *L. bulgaricus* appeared to utilize galactose, which was released into the medium by *S. thermophilus*. Hasan and Durr (5) reported that galactose is a potent inducer of β-galactosidase in *L. plantarum*. Thus, uptake of galactose by *L. bulgaricus* in the absence of free glucose may be facilitated due to its possible role as an inducer of lactose utilization.

Sugar utilization by the mixed yogurt culture was more rapid in LH milk. This effect was the result of an increased rate of carbohydrate consumption by *S. thermophilus* and a more efficient catabolism of the carbohydrate consumed by the cells. The increased rate of carbohydrate consumption we observed seemed to be due to the ability of this organism to take up both glucose and lactose simultaneously in milk culture. Apparently neither sugar prevents the uptake of the other in milk. It is interesting to note that, although it is the glucose portion of lactose that is preferentially metabolized, when adequate free glucose is available for fermentation, utilization of lactose does not subside. The results we obtained with *S. thermophilus* may not, however, apply to dairy starters containing other lactic streptococci, since the ability to simultaneously ferment glucose and lactose is probably a relatively rare phenomenon.

Gilliland et al. (4) suggested that increased acid production by lactic streptococci in milk cultures in the presence of β-galactosidase might be due to conversion of a greater percentage of glucose than lactose to acid end products. We have previously shown (10) that LH yogurts having a pH of 4.6 contained more lactic acid than did control yogurts at the same pH. From these results we concluded that the ratios of acid metabolites produced by the starter culture organisms were different in the two milks. Alterations in the proportions of the various acid metabolites produced may in part be responsible for differences in the rates of pH decline noted during fermentation in control and LH milks. However, more rapid and efficient utilization of carbohydrate in LH milk would appear to be the major factor responsible for the more rapid pH decrease. The possibility that the metabolic process of lactose catabolism is slower than that of glucose also should not be discounted as a possible contributing factor.

ACKNOWLEDGMENTS

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