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Citric Acid Metabolism in Hetero- and Homofermentative Lactic Acid Bacteria

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The effect of citrate on production of diacetyl and acetoin by four strains each of heterofermentative and homofermentative lactic acid bacteria capable of utilizing citrate was studied. Acetoin was quantitatively the more important compound. The heterofermentative bacteria produced no acetoin or diacetyl in the absence of citrate, and two strains produced traces of acetoin in its presence. Citrate stimulated the growth rate of the heterofermentative lactobacilli. Acidification of all heterofermentative cultures with citric acid resulted in acetoin production. Destruction of accumulated acetoin appeared to coincide with the disappearance of citrate. All homofermentative bacteria produced more acetoin and diacetyl in the presence of citrate than in its absence. Citrate utilization was begun immediately by the streptococci but was delayed until at least the middle of the exponential phase in the case of the lactobacilli.

The requirement of citrate for the production of diacetyl and acetoin is well recognized in certain species of lactic acid bacteria, especially *Streptococcus lactis* var. *diacetylactis* and *Leuconostoc cremoris* (citrovorum) (13, 14), but the relationship has not been studied in detail for other lactic acid bacteria. However, Christensen and Pederson (4) reported that homofermentative but not heterofermentative lactic acid bacteria produced increased levels of diacetyl when citrate was added to the medium. In addition, several workers have reported acetoin and/or diacetyl production by lactobacilli in media containing either citrate or malate, but whether the acids were being utilized simultaneously during growth was not measured (2, 4, 7, 8, 15, 18). Citrate utilization appears to be widespread among lactobacilli as Fryer (10) reported that 19 out of 25 strains of *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus brevis* utilized citrate in the presence of a fermentable carbohydrate. *Leuconostoc cremoris* when grown in milk utilizes citrate under both neutral and acidic conditions but only produces diacetyl and acetoin under the latter conditions (5). This does not appear to have been studied for other heterofermentative lactic acid bacteria.

The present investigation was carried out to clarify the relationship existing between citrate utilization and diacetyl and acetoin production during growth of certain homofermentative and heterofermentative lactic acid bacteria.

MATERIALS AND METHODS

Cultures. The heterofermentative cultures used were *Lactobacillus viridescens* 7-7, *Lactobacillus fermenti* D1, *Leuconostoc lactis* S3 and *Leuconostoc paramesenteroides* 9-1, whereas the homofermentative ones were *Lactobacillus plantarum* G1 and P5 and *S. lactis* var. *diacetylactis* 1816 and DNCW4. With the exception of P5 (obtained from M. E. Sharpe, NIRD, Shinfield, Reading, England) and 1816 (obtained from C. Daly, Oregon State University, Corvallis) all cultures were isolated from different samples of milk and cheese and were classified according to the procedures of Sharpe et al. (23). They were routinely maintained in MRS broth (17), modified by the omission on Tween 80 and sodium acetate, and with the glucose concentration reduced to 1.0% (MMRSC).

Experimental procedure. The media with and without citrate (MMRSC and MMRS) were inoculated with 1% of the culture understudy and incubated at 30 C. A sample was withdrawn periodically (generally at 1- or 2-h intervals) during growth and subjected to the various analyses.

In the case of the heterofermentative bacteria, additional experiments were carried out under acid conditions. One liter of MMRSC, inoculated with 0.1% of the respective culture, was incubated overnight (16 h) at 30 C. After division into 2 volumes of 500 ml each, citric acid and sufficient sterile 1 M NaOH to keep the pH within ±0.1 units of the control were added to one of the flasks. The citric acid added ranged from 1.50 to 1.73 mg/ml, which compares with 1.58 mg/ml in MMRSC. The contents of each flask were sampled periodically during incubation for the various analyses.

Analyses. Growth was measured either by titrat-

ing 10 ml of culture to pH 8.3 with 0.11 N NaOH, correcting for the inherent acidity of the medium, and expressing results as the percentage of lactic acid developed or by determining the increase in absorbance at 600 nm. Citrate, diacetyl, and acetoin were measured by the procedures of Marier and Boulet (19) and Walsh and Cogan (26, 27), respectively. In the estimation of citrate, the broth plus trichloroacetic acid was centrifuged at 5,000 × *g* for 10 min instead of being filtered before analysis.

RESULTS

Development of medium. Sodium acetate and Tween 80 at the concentrations present in MRS had no effect on the rates of growth of strains D1 and 7-7 and hence were omitted from the medium. The other cultures were not tested but grew quite well in the modified medium as measured by turbidity. The presence of Mn^{2+} at concentrations as low as 2 μg/ml stimulated growth of all strains except strain DNCW4 (Table 1). Concentrations up to 15 μg/ml had little additional effect. The normal concentration of Mn^{2+} in MRS, MMRS, and MMRSC is 12.3 μg/ml as Mn^{2+}. Citrate also stimulated growth (see below) but the response to Mn^{2+} was much greater, especially in the case of the homofermentative lactobacilli (viz., strains P5 and G1). However, more growth was obtained when both compounds were present.

Heterofermentative bacteria. The presence of citrate had no effect on the growth rate of the leuconostocs, although the final amount of acid produced was somewhat higher in the case of citrate-grown cells. However, citrate increased the growth rate and total acid produced by the heterofermentative lactobacilli, reducing the generation times from 153 and 162 min to 120 and 96 min for strains 7-7 and D1, respectively. Citrate dissimilation began immediately on incubation in all four cultures and was utilized at a faster rate than acid was produced. Data for strain 7-7 are shown in Fig. 1.

Acetoin was not produced in the absence of citrate, but strains S3 and 7-7 produced maxima of 1.8 and 10.6 μg/ml, respectively, in the presence of citrate (Table 2). Production of acetoin

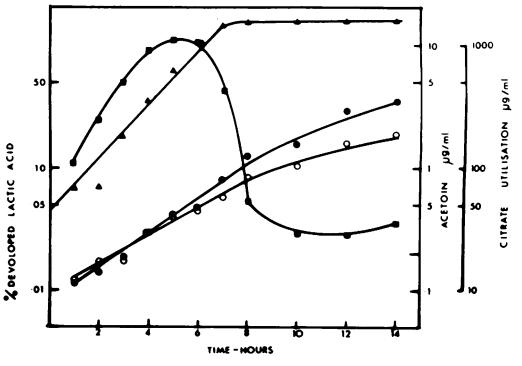

FIG. 1. *Lactic acid production, acetoin production and citric acid utilization by Lactobacillus viridescens 7-7 in the presence and absence of citrate. Symbols: ○, acid production-citrate; ●, acid production +citrate; ■, acetoin production +citrate; ▲, citrate utilization. No acetoin was produced in the absence of citrate, and no diacetyl was produced under either condition.*

TABLE 1. *Effect of Mn^{2+} and citrate on the growth of different lactic acid bacteria in MRS without Tween 80 and sodium acetate*

Culture	μg of Mn^{2+}/ml			
	0		2	
	−[a]	+	−	+
Lactobacillus viridescens 7-7	0.4[b]	1.0	1.0	2.6
Lactobacillus fermenti D1	0.7	1.1	1.0	3.9
Leuconostoc lactis S3	0.9	1.1	2.2	2.6
Leuconostoc paramesenteroides 9-1	0.8	0.9	2.7	3.3
Lactobacillus plantarum P5	1.1	1.2	4.1	5.3
Lactobacillus plantarum G1	1.3	1.5	4.1	5.8
S. lactis var. *diacetylactis* DNCW4	2.0	2.4	1.7	2.4

[a] −, Absence of citrate; +, presence of 1.58 mg of citrate per ml.

[b] Absorbancy at 600 nm. MMRS and MMRSC without Mn^{2+} were inoculated with 0.2% of the respective culture (grown in MMRSC), divided into 10-ml volumes to which was added Mn^{2+} (as $MnSO_4$) to give the desired final concentration. After incubation for 18 h at 30 C, the absorbance was read at 600 nm.

TABLE 2. *Maximum amounts of diacetyl and acetoin produced by the different cultures in the presence and absence of citrate*[a]

Culture	Acetoin (μg/ml)		Diacetyl (μg/ml)		Recovery (%)[b]
	−[c]	+	−	+	
Lactobacillus viridescens 7-7	0.0	10.6	0.0	0.0	3.0
Lactobacillus fermenti D1	0.0	0.0	0.0	0.0	0.0
Leuconostoc lactis S3	0.0	1.8	0.0	0.0	0.5
Leuconostoc paramesenteroides 9-1	0.0	0.0	0.0	0.0	0.0
Lactobacillus plantarum P5	7.3	116	0.0	2.1	31.2
Lactobacillus plantarum G1	30.0	148	0.7	1.9	39.5
Streptococcus lactis var. *diacetylactis* 1816	9.1	286	0.0	3.2	78.5
Streptococcus lactis var. *diacetylactis* DNCW4	3.5	245	0.0	6.1	68.2

[a] 1.58 mg/ml.

[b] Percentage of citrate recovered as diacetyl + acetoin, assuming 2 mol of citrate → 1 mol of diacetyl + acetoin + 2,3-butylene glycol.

[c] −, Absence of citrate; +, presence of citrate.

when it occurred did not parallel citrate utilization or growth, whereas destruction of the acetoin produced coincided with the disappearance of most of the citrate from the medium. Data for strain 7-7 are shown in Fig. 1. Little or none of the citrate utilized by any heterofermentative culture during growth under neutral conditions was recovered as diacetyl plus acetoin (Table 2).

The results for acetoin production under acid conditions are shown in Fig. 2. Before addition of citric acid the percentages of developed lactic acids were 0.32, 0.40, 0.41, and 0.39 in the case of strains 7-7, D1, S3, and 9-1, respectively, corresponding to pH values of 4.4, 4.4, 4.2, and 4.2. Little or no growth (less than half a generation) occurred in the controls (i.e., absence of citrate) during this series of experiments. The maximum amounts of acetoin produced in the presence of citrate ranged from 3.5 to 290 μg/ml in the case of strains D1 and S3, respectively. Under neutral conditions these strains had produced maxima of 0 and 1.8 μg/ml of acetoin (Table 2). The remaining strains, 7-7 and 9-1, produced similar amounts of acetoin (12 μg/ml) under acid conditions compared with 10.6 and 0 μg/ml, respectively, under neutral conditions. The lactobacilli utilized citrate much more rapidly than the leuconostocs, and once most of the citrate was utilized destruction of acetoin (and diacetyl in the case of strain 9-1) occurred.

Increasing the amount of citric acid added under acid conditions increased the maximum amount of acetoin produced by all cultures. This is shown for strain 7-7 in Fig. 3. Destruction of the accumulated acetoin coincided with the disappearance of most of the citrate from the medium. On continued incubation of strain 7-7 a small but definite increase in acetoin production occurred. Little of the citric acid utilized under acid conditions by strain 7-7 was recovered as acetoin plus diacetyl (Table 3).

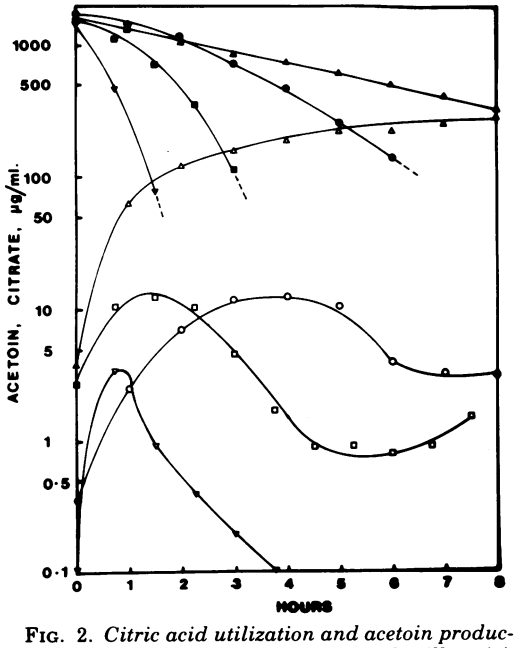

FIG. 2. *Citric acid utilization and acetoin production by Lactobacillus fermenti D1, Lactobacillus viridescens 7-7, Leuconostoc paramesenteroides 9-1, and Leuconostoc lactis S3 under acid conditions. Citrate utilization.* ▼, *D1;* ■, *7-7;* ●, *9-1;* ▲, *S3. Acetoin production:* ▽, *D1;* □, *7-7;* ○, *9-1;* △, *S3. Cultures were preincubated for 16 h before addition of citric acid.*

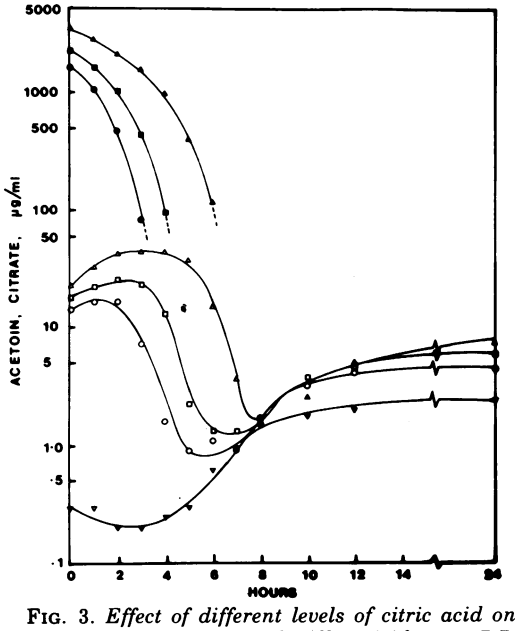

FIG. 3. *Effect of different levels of citric acid on acetoin production by Lactobacillus viridescens 7-7. Acetoin production:* ▽, *control;* ○, *8.49 mM citrate;* □, *11.11 mM citrate;* △, *17.88 mM citrate. Citrate utilization:* ●, *8.49 mM;* ■, *11.11 mM;* ▲, *17.88 mM. The culture was preincubated for 16 h before addition of citric acid.*

TABLE 3. *Maximum concentrations (mM) of diacetyl and acetoin produced and citric acid utilized by lactobacillus viridescens 7-7 under acid conditions*

Initial level of citric acid added	Citric acid utilized	Diacetyl produced	Acetoin produced	Citrate recovered (%) as diacetyl + acetoin[a]
8.49	6.08	0.01	0.19	6.6
11.11	5.86	0.01	0.29	10.2
17.88	12.87	0.02	0.50	8.1

[a] Assuming 2 mol of citrate → 1 mol of diacetyl + acetoin + 2,3-butylene glycol.

This was also true of strains D1 and 9-1. In contrast, all of the citrate was recovered as diacetyl plus acetoin in the case of strain S3.

Diacetyl was not produced by any of the cultures during growth in the presence or absence of citrate. Under acid conditions, little diacetyl (<1 μg/ml) was produced by the lactobacilli, whereas the leuconostocs produced maxima of 9 and 23 μg/ml in the case of strains 9-1 and S3, respectively.

Homofermentative bacteria. The presence of citrate had no effect on the rate of growth of any homofermentative bacteria although larger final acidities were obtained in all cases. Citrate utilization by the streptococci began immediately on incubation and proceeded at a faster rate than acid production. In contrast, citrate utilization by the lactobacilli did not begin until toward the end of the exponential phase of growth when the pH had fallen to about 4.8. A comparison of strains 1816 and P5 is shown in Fig. 4.

The homofermentative bacteria produced no diacetyl and small amounts (<10 μg/ml) of acetoin in the absence of citrate except for strain G1, which produced 30 μg of acetoin/ml and 0.7 μg of diacetyl/ml (Table 2). Much greater amounts of both compounds were produced in the presence of citrate. Acetoin was quantitatively the more important compound, and more was produced by the streptococci than the lactobacilli (Table 2). In the lactobacilli, acetoin production paralleled growth in the presence and absence of citrate and continued into the stationary phase in the presence of citrate. In contrast, in the streptococci, acetoin production paralleled citrate utilization but not growth (Fig. 4). In the presence of citrate, destruction of the accumulated acetoin (and diacetyl) by the streptococci coincided with the disappearance of citrate from the medium. Destruction was also evident in the absence of citrate and coincided with the entry into the stationary phase. No destruction was evident in the case of the lactobacilli. Data for strains 1816 and P5 are shown in Fig. 4.

DISCUSSION

That acetate had no effect on growth of strains D1 and 7-7 is not surprising since lactic acid bacteria do not have an acetate requirement except in the absence of lipoic acid, which is a normal constituent of yeast extract (6, 25). The effect of Mn^{2+} on growth agrees with the results of Evans and Niven (9) for *Lactobacillus viridescens* and leuconostocs since identified as pediococci (12). The present data would suggest that stimulation by Mn^{2+} is a general property of lactic acid bacteria, especially homofermentative lactobacilli.

In all strains, except the heterofermentative lactobacilli, citrate stimulated the total amount but not the rate of growth obtained. This may be a reflection of the buffering capacity of cit-

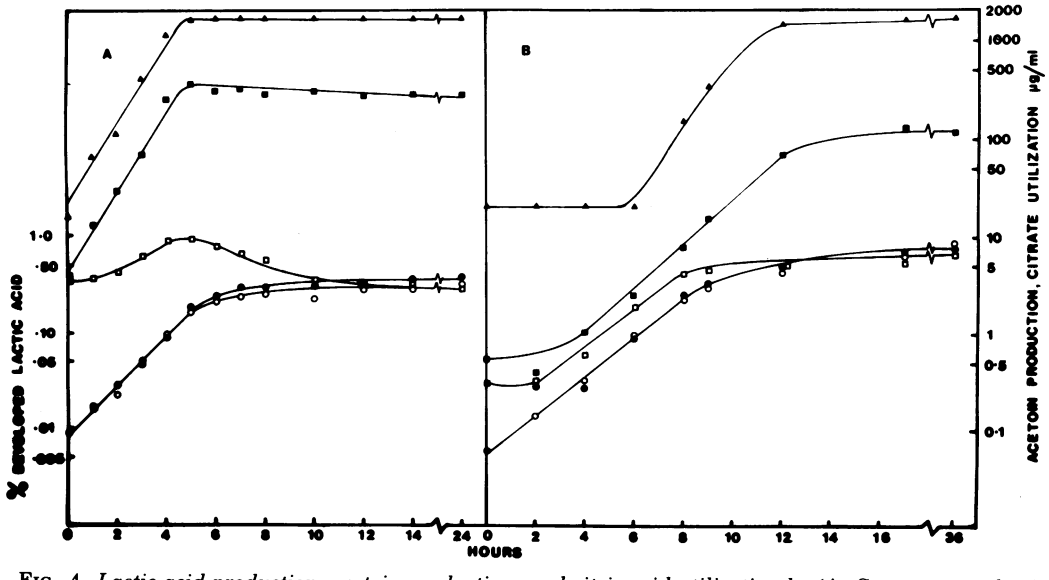

FIG. 4. *Lactic acid production, acetoin production, and citric acid utilization by (A) Streptococcus lactis var. diacetylactis 1816 and (B) Lactobacillus plantarum P5 in the presence and absence of citrate. Symbols: ○, acid production −citrate; ●, acid production +citrate; □, acetoin production−citrate; ■, acetoin production +citrate; ▲, citrate utilization.*

rate or more likely of the retention of higher pH values by the CO_2 produced from citrate. The present results are in direct conflict with the findings of Harvey and Collins (14), who found that citrate stimulated the growth rate of *S. lactis* var. *diacetylactis* DRCI. The stimulation of the growth rate of the heterofermentative lactobacilli by citrate suggests that it is acting as an energy source. However, this is not true since no growth (of any strain) occurred in the absence of glucose and presence of citrate. It is possible that in the absence of citrate the rate of formation of pyruvate is rate limiting in the heterofermentative lactobacilli and that the function of citrate is to act as an additional source of pyruvate. An alternative explanation may be that of Harvey and Collins (14), who felt that citrate serves as a source of carbon for the synthesis of some essential cell constitutent that, in the absence of citrate, is synthesized more slowly from some other compound of the medium.

Considerable differences were found between acetoin and diacetyl production and citrate utilization in homofermentative and heterofermentative lactic acid bacteria. These findings may have taxonomic implications, since there are fundamental differences between the two types of bacteria, e.g., in carbohydrate metabolism. In general the heterofermentative bacteria produced little if any acetoin in the presence or absence of citrate, whereas the homofermenters produced large amounts (>100 μg/ml) in the presence and small amounts in the absence of citrate.

The findings with the homofermenters agree with the results of Harvey and Collins (14), who reported that significant amounts of acetoin were only formed by *S. lactis* var. *diacetylactis* in the presence of citrate. These workers suggested that in the presence of citrate acetoin formation acts as a detoxification mechanism to remove excess pyruvate not required for cell synthesis. In the absence of citrate little or no acetoin is produced (14) since all the pyruvate produced from carbohydrate is required to regenerate oxidized nicotinamide adenine dinucleotide (NAD) to allow glycolysis to proceed (13). The heterofermentative rods and cocci produced acetoin from citrate under acid conditions and utilized citrate with little or no production of acetoin under neutral conditions. The reason(s) for these apparently contradictory results are obscure, but similar findings have been reported for *Leuconostoc cremoris* growing in milk (5).

It is possible to speculate on the differences in the recoveries of citrate as diacetyl and acetoin in the case of the homofermentative and heterofermentative bacteria. In the case of the former, the remainder of it may be recovered as 2,3-butylene glycol which was not measured in the present study. A more plausible alternative in the case of the heterofermentative bacteria is that citrate is being utilized in some other metabolic pathway during growth (perhaps lipid synthesis), and that it is only in the absence of growth (acid conditions) that diacetyl and acetoin are produced to any great extent. In this connection, Branen and Keenan (1) found that more diacetyl-acetoin and less growth was produced by *Lactobacillus casei* in agitated cultures.

Destruction of accumulated acetoin is probably a consequence of the activity of reduced nicotinamide adenine dinucleotide-linked acetoin reductase. A reduced nicotinamide adenine dinucleotide-linked diacetyl reductase has been reported in *S. lactis* var. *diacetylactis* (22), whereas Bryn et al. (3) have shown that both activities are carried out by a single enzyme in *Aerobacter aerogenes*. We have found both activities in the two strains of *S. lactis* var. *diacetylactis* used in the present study (unpublished data). Destruction of acetoin when it occurred coincided with the disappearance of citrate, implying that its rate of formation is faster than its rate of destruction. It is also possible that the presence of citrate represses the formation of the acetoin-degrading enzyme(s).

The nonutilization of citrate by the homofermentative lactobacilli until late in the exponential phase is a peculiar finding. It is unlikely to be due to the need for inducible enzymes since the inocula were grown in the presence of citrate and induction is usually very rapid, e.g., requiring only 90 s in the case of the β-galactosidase of *Escherichia coli* (16). It may be due to passive transport of citrate as was found for *Lactobacillus casei* (1).

Citrate "fermenting" streptococci and leuconostocks have been implicated as causal agents of a defect in cottage cheese characterized by floating curd (21) and in Cheddar cheese characterized by open texture (20, 24). CO_2 production from citrate is presumed to be the cause of the defect. Several of the strains used in the present study were isolated from samples of Cheddar and Edam cheese showing evidence of abnormal gas formation. If one assumes that acetoin production is also a measure of CO_2 formation, then all lactic acid bacteria capable of utilizing citrate would be potential defect producers. The heterofermenters utilize citrate under both neutral and acid conditions but only produce acetoin under acid conditions (pH 4.4).

Therefore, whether CO_2 is produced at the pH of cheese (pH 5.2) must be determined specifically if one is to implicate the heterofermentative bacteria as causal agents of the defect. In this connection, Fryer et al. (11) have suggested that the use of starters containing *S. lactis* var. *diacetylactis* should not lead to blowing of cheese by citrate "fermenting" homofermentative lactobacilli since all or most of the citrate will be utilized before final wrapping (i.e., during manufacture and pressing). The present data suggest that even in the presence of citrate, homofermentative lactobacilli will not cause the defect unless they reach near maximum numbers in the ripening cheese.

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ADDENDUM

Four strains of citrate-utilizing *Lactobacillus casei*, C2, C6, A148C (obtained from M. E. Sharpe) and C2 (isolated by us), also did not utilize citrate until late in the exponential phase of growth, suggesting that this is a general characteristic of citrate-utilizing homofermentative lactobacilli.

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