

GAS PRODUCTION BY ASSOCIATED SWISS CHEESE BACTERIA¹J. E. HUNTER² AND W. C. FRAZIER

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SUMMARY

Gas production in skimmilk cultures was markedly stimulated and inhibited by interactions of the late gas-formers with some but not all of the Swiss cheese lactic flora. The starter lactobacilli were markedly stimulatory for both *Clostridium tyrobutyricum* and *Propionibacterium shermanii*. *Streptococcus thermophilus* was very stimulatory for the clostridium but without effect on the propionic, whereas *Streptococcus faecalis* generally had little effect on either gas-former. The nisin-producing strain of *Streptococcus lactis* was markedly inhibitory for the clostridium but without much effect on the propionic, whereas the ordinary strain of *S. lactis* had little effect on either. Gas production was stimulated when the clostridium and propionic were grown together. The stimulatory and inhibitory properties of the skimmilk cultures for the late gas-formers were at least in part dialyzable, heat- and acid-stable substances. The stimulatory activity of *Lactobacillus helveticus* was shown to be correlated with metabolic products rather than with the number of cells in the lactic culture.

The appearance and numbers of eyes in Swiss-type cheese depend on the timing, rate, and duration of the gas-producing bacterial fermentations in the cheese mass (2). Normal eye formation has been attributed to the fermentation of lactate by members of the genus *Propionibacterium* (7, 13). Eyes rough in appearance, too large, and too numerous, as well as splitting and cracking of the cheese, are late gas defects resulting from the production of excessive amounts of gas after the curd has lost its elasticity late in the ripening process. Late gas defects have been associated with fermentation of lactate by butyric acid bacteria (1, 10) and propionic acid bacteria (6). Van Beynum and Pette (12) named the lactate-fermenting butyric anaerobes *Clostridium tyrobutyricum*.

In the manufacture of Swiss cheese a number of populations of lactic acid bacteria may have grown and declined by the time the gas-forming propionics and clostridia appear (3, 4). Swartling and Lindgren (9) reported that growth and acid formation of species of *Clostridium* were promoted when grown with coliforms, streptococci, leuconostocs, lactobacilli, propionics, and *Bacillus subtilis*. Swiss cheese starter bacteria found stimulatory included *Streptococcus thermophilus*, *Lactobacillus lactis*, and *Propionibacterium* spp. The present work was undertaken as an investigation of the interactions of the lactics, propionics, and clostridia found in Swiss-type cheese.

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MATERIALS AND METHODS

Gas-producing fermentations were followed by means of the gasometer shown in Figure 1. All fermentations were carried out in either fresh or reconstituted skimmilk from bulk sources. Gas evolved from the fermentation in Test Tube A (Figure 1) displaced an acid-salt solution (5% H₂SO₄, 20% Na₂SO₄) from the 10-ml. pipette, B, into the reservoir, C. Changes in the volume of gas were read on the pipette with the fluid in the pipette and reservoir at the same level. Readings were corrected for changes in temperature or pressure by comparison with a gasometer attached to an uninoculated control.

Portions of skimmilk were subjected to 48-hr. fermentations by the following lactic acid bacteria:

Lactic acid bacteria	Developed titratable acidity ^a
	(%)
<i>Streptococcus thermophilus</i> Strain MC	0.48
<i>Lactobacillus bulgaricus</i> Strain Gere A	1.77
<i>Lactobacillus helveticus</i> Strain H-80	2.12
<i>Lactobacillus lactis</i> Strain 39a	1.08
<i>Streptococcus faecalis</i> Strain DK	0.31
<i>Streptococcus lactis</i> Strain X-13 (nisin)	0.28
<i>Streptococcus lactis</i> Strain Rogers	0.31

^a Titratable acidity of uninoculated control subtracted.

An uninoculated portion of the original skimmilk was refrigerated while the lactic fermentations were taking place. The first four organisms listed are commonly used Swiss cheese starter bacteria, whereas *Streptococcus faecalis* is a heat- and salt-tolerant milk organism that can grow at the temperature of cheese in the press. *Streptococcus lactis* is commonly present in raw milk delivered to cheese factories and may include nisin-producing strains (represented here by Strain X-13) such as have been used to inhibit butyric acid fermentations in cheese.

At the end of the incubation period, the developed titratable acidity of each culture was determined and sufficient DL-lactic acid was added to all samples, including the unfermented control, to bring the titratable acidity up to 2.5% and the pH down to about 3.5. The pH was adjusted back to 5.75 by the careful addition with mixing of a slurry of Ca(OH)₂. By this procedure, all cultures had some DL-lactic acid present and the total lactate concentration of all cultures was about equal. As soon as the pH was adjusted to 5.75 each culture was chilled in ice to stop further lactic fermentation. When the pH of all cultures and the control had been adjusted, they were sterilized by autoclaving at 120° C. for 15 min. The pH of each culture was checked following autoclaving and where necessary readjusted to pH 5.75 by the careful aseptic addition of Ca(OH)₂.

Secondary gas-forming fermentations by *Propionibacterium shermanii* 31C (from J. M. Sherman) and *Clostridium tyrobutyricum* S-12 (isolated from

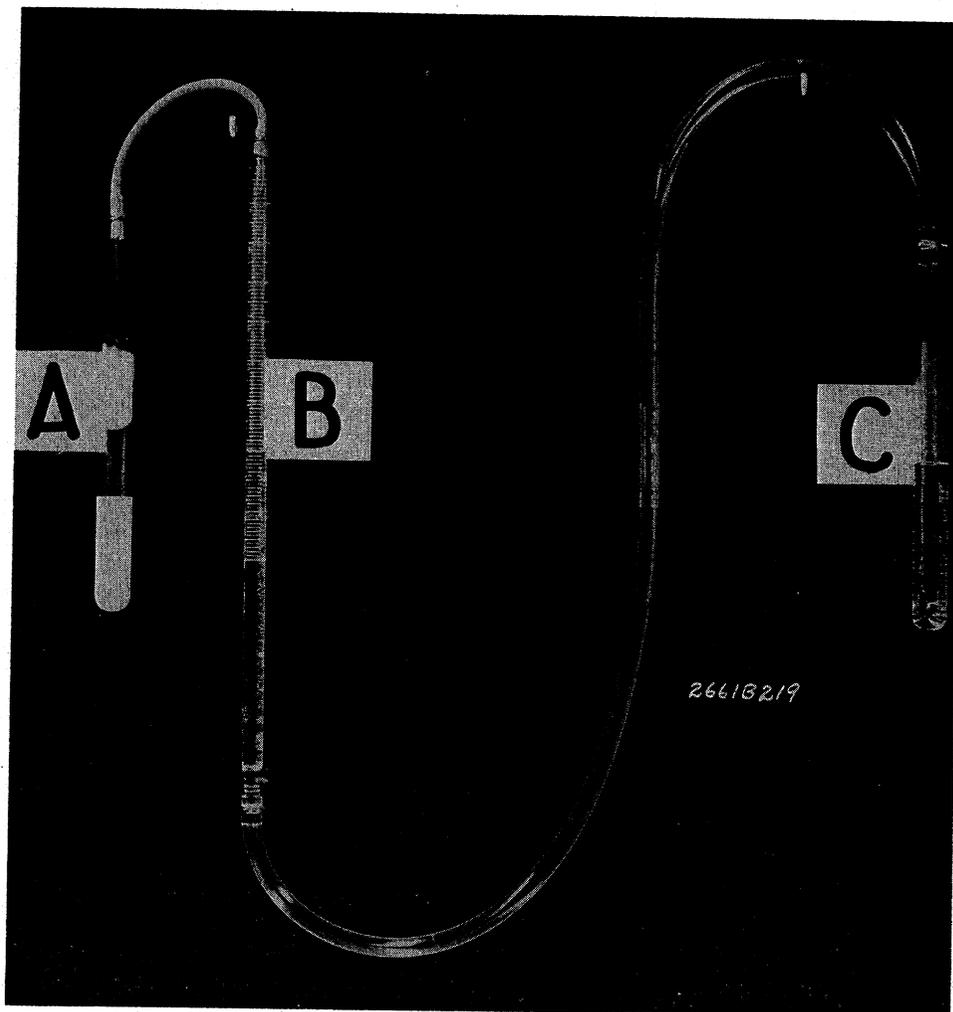


FIG. 1. Gasometer for test tube-sized fermentations. A. Fermentation tube. B. Ten-milliliter pipette containing displacement fluid. C. Displacement fluid reservoir.

Swiss cheese) were followed in each of the lactic fermented milks. The clostridial and propionic inocula for the secondary gas-forming fermentation were grown in APT broth, harvested while in the logarithmic phase of growth by centrifugation, and washed twice by resuspension in a pH 5.75 buffer containing $M/30$ KH_2PO_4 , 0.1% peptone, and 0.05% sodium thioglycolate. Concentration of cells in the inoculum was adjusted so that 0.3 ml. contained 10^7 clostridium cells or 10^8 propionic cells, and this quantity was placed in the bottom of a sterile cotton-plugged test tube. Ten milliliters of the freshly autoclaved and cooled lactic milk cultures were immediately transferred by pipette to the previously labeled tube containing the inoculum. The cotton plugs in the culture tubes were clipped off, and the butt end was forced down into the neck of the

culture tube; the rubber stoppers bearing the hose connections were inserted and the stoppered tubes placed in a vacuum desiccator. The atmosphere was replaced with 90% N₂ and 10% CO₂ by evacuation and flushing (11). The gasometer headspaces were filled with nitrogen gas by raising the reservoir of displacement fluid until the entire pipette was filled and then forcing down the level of displacement fluid with a stream of high-purity nitrogen. The fermentation tubes were removed from the vacuum desiccator and attached to gasometers.

Secondary gas-forming fermentations were run in triplicate on each lactic milk culture. Cumulative measurements of gas evolution were made, and the arithmetic mean of the three replicates plotted for purposes of comparison of the length of lag periods and maximum rates of gas production. For purposes of statistical comparison the three replicates were plotted separately and individual values representing lag and maximum rate of gas production taken from the graphs. The lag period was defined as the time from inoculation required for gas production to become exponential. The largest amount of gas produced during any 10-hr. period was considered in calculating the maximum rate of gas production.

Dialysates were prepared by immersion of a cellulose dialysis tubing bag of distilled water into each lactic milk culture and milk control. Dialysis was allowed to proceed for three days in the refrigerator with frequent swirling. The bags were carefully withdrawn and rinsed with distilled water, and the contents were removed and used as the cell-free dialysates of the lactic cultures.

The statistical significance of differences in gas production was determined by means of the F test (8). The 0.05 level of probability was taken as statistically significant and the 0.01 level as highly significant.

RESULTS

Effect of initial lactic fermentation on subsequent gas-producing fermentations. Gas production by *Clostridium tyrobutyricum*. Gas production by *C. tyrobutyricum* in skimmilk fermented by various lactics was compared to gas production in skimmilk which had not undergone a lactic fermentation (Figure 2). The four Swiss cheese starter bacteria (*L. helveticus*, *L. bulgaricus*, *L. lactis*, and *S. thermophilus*) markedly stimulated gas production by the clostridium. *L. helveticus* and *L. bulgaricus* were particularly effective, both in shortening the lag and in increasing the rate of gas production.

Gas production by the clostridium was not significantly altered by *S. lactis* Strain Rogers or *S. faecalis*, with the exception of a 100-hr. lengthening of the lag period in the *S. faecalis*-fermented milk.

Skimmilk in which *S. lactis* X-13, a nisin-producing lactic strain, had grown was markedly inhibitory for the clostridium, lengthening the lag time appreciably and suppressing the gaseous fermentation.

Gas production by Propionibacterium shermanii. Portions of the lactic-fermented skimmilks prepared for the previous experiment were inoculated with 10⁷ cells of *P. shermanii* per milliliter of milk. The procedures for inoculation

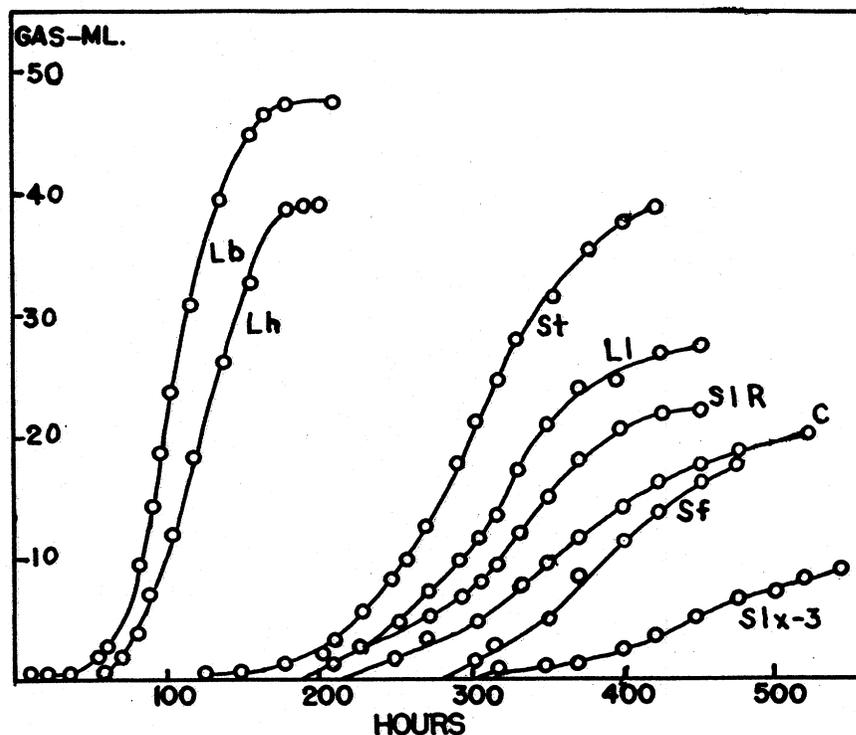


Fig. 2. Gas production by *C. tyrobutyricum* at 30° C. and pH 5.75 in reconstituted skim milk fermented by various lactics. Key: Lb, *L. bulgaricus*, Lh, *L. helveticus*, St, *S. thermophilus*, LI, *L. lactis*, SIR, *S. lactis* Strain Rogers, C, control, Sf, *S. faecalis*, SIX-13, *S. lactis* Strain X-13.

and gas measurement were the same as for *C. tyrobutyricum*. Of the various lactics employed only *L. bulgaricus* had a statistically significant effect on the maximum rate of gas production by the propionic (Figure 3). A significant shortening of lag in gas production by the propionic was also brought about by an initial fermentation by *L. bulgaricus* and, in addition, by *L. helveticus*, *S. lactis* Strain Rogers, and *S. lactis* X-13. There was no evidence of inhibition of the propionic by the nisin-producing *S. lactis* X-13.

Gas production by Clostridium tyrobutyricum and Propionibacterium shermanii in mixed fermentation. Mixed fermentations by *C. tyrobutyricum* and *P. shermanii* were run in portions of the same lactic milk cultures used in the preceding experiments. These portions were inoculated simultaneously with 10^6 cells of *C. tyrobutyricum* and 10^7 cells of *P. shermanii* per milliliter, and gas evolution was followed in the gasometer (Figure 1). Results are shown in Tables 1 and 2.

A consistent stimulation in gas production was noted in all of the mixed butyric-propionic fermentations, as compared to the separate fermentations. The degree of stimulation varied markedly, depending on which lactic had fermented the skim milk initially. In fermented milk already quite favorable

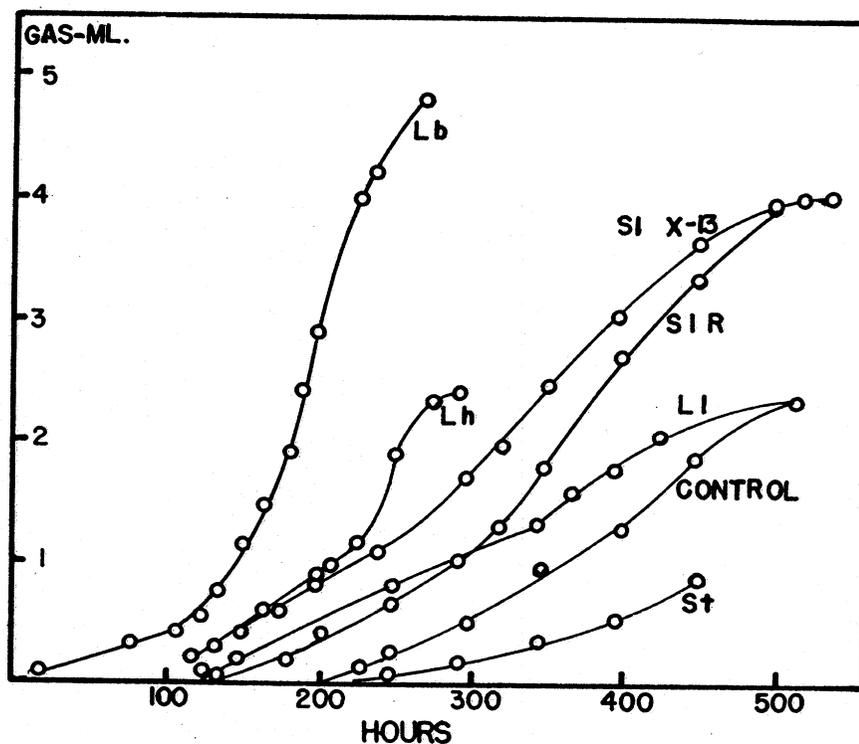


Fig. 3. Gas production by *P. shermanii* at 30° C. and pH 5.75 in reconstituted skim milk fermented by various lactics. Key: Lb, *L. bulgaricus*, Lh, *L. helveticus*, SIX-13, *S. lactis* Strain X-13, SIR, *S. lactis* Strain Rogers, LI, *L. lactis*, St, *S. thermophilus*.

TABLE 1

Effect of associative growth on lag in gas production at pH 5.75 and 30° C. by *C. tyrobutyricum* and *P. shermanii* in skim milk fermented by various lactics*

Lactic organism	Length of lag time in initiation of gas production		
	<i>C. tyrobutyricum</i>	<i>C. tyrobutyricum</i> and <i>P. shermanii</i>	<i>P. shermanii</i>
	(hr.)		
<i>L. helveticus</i>	50**	47-*	123*
<i>L. bulgaricus</i>	35**	27-**	65*
<i>L. lactis</i>	187	120	120
<i>S. thermophilus</i>	127*	127	258
<i>S. faecalis</i>	282*	** 94 **	285
<i>S. lactis</i> Strain Rogers	119	101	132*
<i>S. lactis</i> X-13	376**	105	114**
None—control	170	142-*	220

* Significant difference ($0.05 > P > 0.01$) from the control.

** Highly significant difference ($P < 0.01$) from the control.

**— Compared to *C. tyrobutyricum* fermentation of the same lactic fermented milk.

—** Compared to *P. shermanii* fermentation of the same lactic fermented milk.

* The lactic fermented milks were autoclaved prior to inoculation with the gas-formers.

TABLE 2

Effect of associative growth on maximum rate of gas production at pH 5.75 and 30° C. by *C. tyrobutyricum* and *P. shermanii* in skimmilk fermented by various lactics*

Lactic organism	Maximum rate of gas production		
	<i>C. tyro-</i> <i>butyricum</i>	<i>C. tyro-</i> <i>butyricum</i> and <i>P.</i> <i>shermanii</i>	<i>P. sher-</i> <i>manii</i>
	(ml/10 hr.)		
<i>L. helveticus</i>	4.3**	7.4*	0.20
<i>L. bulgaricus</i>	4.0**	11.2*	0.49**
<i>L. lactis</i>	3.9**	7.1	0.10
<i>S. thermophilus</i>	2.6**	6.7**	N.D.
<i>S. faecalis</i>	1.1	5.5**	0.14
<i>S. lactis</i> Strain Rogers	1.5	3.8*	0.17
<i>S. lactis</i> X-13	0.64	0.77	0.14
None—control	0.93	5.1**	0.09

N.D. Not determined. Mixed fermentation compared to clostridium fermentation alone.

* Significant difference ($0.05 > P > 0.01$).

** Highly significant difference ($P < 0.01$). Separate fermentations compared to the control. Mixed fermentation compared to the sum of the separate butyric and propionic fermentations of the same milk.

* The lactic fermented milks were autoclaved prior to inoculation with the gas-formers.

to the butyric fermentation, gas production in the mixed fermentation was stimulated to a relatively lesser degree than in unfermented milk or milk fermented by less favorable lactics. Increase in rate of gas production in the mixed over the separate fermentations was more than additive in all lactic-fermented milks, except for the butyric-inhibiting *S. lactis* X-13 milk.

Effect of extent of lactic fermentation on gas production. The various lactics used in the preceding experiments fermented milk to widely differing developed titratable acidities, with the more acidogenic lactics tending to be more stimulatory to subsequent gaseous fermentations. A flask culture of *L. helveticus* in skimmilk was sampled periodically as the lactic acid fermentation proceeded. Titrations of developed acidity and direct microscopic counts were made on each sample, which was then chilled in ice water and stored at 4° C. until the parent fermentation was complete. At that time, sufficient lactic acid was added to all samples to bring their titratable acidities up to the maximum 2.8% achieved at the end of the fermentation. All samples were adjusted to pH 6.0 with $\text{Ca}(\text{OH})_2$ and autoclaved 15 min. at 120° C. Gas evolution following inoculation with *C. tyrobutyricum* was followed with the gasometer. Carbon dioxide evolution by 10^9 cells of *P. shermanii* suspended in 3.2 ml. of each sample was followed manometrically with a Warburg apparatus.

The extent of the lactic fermentation had a pronounced effect on the subsequent gas-producing propionic and butyric fermentation (Table 3). Rates of gas production by both *C. tyrobutyricum* and *P. shermanii* were progressively increased as the developed titratable acidity of the lactic culture was increased. A plot of maximum rate of gas production (Figure 4) by the clostridium against per cent titratable acidity developed by *L. helveticus* revealed a linear relationship. Direct microscopic counts indicated that the number of lacto-

TABLE 3
Effect of the extent of the lactic fermentation by *L. helveticus* on the rate of gas production by *C. tyrobutyricum* and *P. shermanii* at pH 6.0 and 30° C.^a

Titratable acidity developed by <i>L. helveticus</i> (%)	Rate of gas production	
	<i>C. tyrobutyricum</i>	<i>P. shermanii</i>
	(ml/10 hr. ^b)	(μ l/hr. ^c)
None—control	0.80	11
0.15	1.1	20
0.55	1.9	24
1.18	2.2	27
2.81	4.7	51

^a All samples adjusted to 2.81% titratable acidity with lactic acid prior to adjustment to pH 5.75 with Ca(OH)₂.
^b Gas measured in gasometer (Figure 1).
^c Gas measured in Warburg apparatus.

bacillus cells reached a maximum at about 1% developed titratable acidity and remained fairly constant for the duration of the fermentation. The stimulatory effect of the *L. helveticus* fermentation on gas production by *C. tyrobutyricum* was correlated with the products or effects of energy metabolism of the lactobacilli, but apparently not with the number of lactic cells present in the fermented skimmilk. Since the concentration of lactic acid was adjusted to approximately the same level throughout the experiment, the stimulatory effect was not caused by the quantity of lactic acid.

Gas production in dialysates of lactic skimmilk culture. Dialysates were prepared simultaneously under identical conditions from portions of each of the pH-adjusted lactic cultures used in the experiments shown in Figures 2 and 3. The dialysates were crystal-clear and whey-colored, and did not form a precipitate when trichloroacetic acid was added. Rate of gas production by *P. shermanii* and *C. tyrobutyricum* in the various dialysates is shown in Table 4. Rates of gas production in the culture dialysates were determined by means of a Warburg apparatus and were compared to the rate in the dialysate of the

TABLE 4
Rate of gas production by *C. tyrobutyricum* and *P. shermanii* at 30° C. and pH 6.0 in dialysates of various lactic milk cultures

	Dialysate— μ l. of gas/10 min.							
	Control	H-80	Gere A	39a	Mc	DK	Rogers	X-13
<i>C. tyrobutyricum</i> S-12	14.9	23.7	23.0*	12.0	21.7	8.1	9.8	1.9**
<i>P. shermanii</i> 31C	3.4	6.2**	5.7**	4.3	3.6	3.6	3.2	3.2

* Significantly different rate from in the control (0.05 level of P).

** Highly significant different rate from in the control (0.01 level of P).

Key to milk cultures from which the dialysates were prepared:

Control—Unfermented reconstituted skimmilk
H-80—*L. helveticus* H-80
Gere A—*L. bulgaricus* Gere A
39a—*L. lactis* 39a
Mc—*S. thermophilus* Mc
DK—*S. faecalis* DK
Rogers—*S. lactis* Strain Rogers
X-13—*S. lactis* X-13

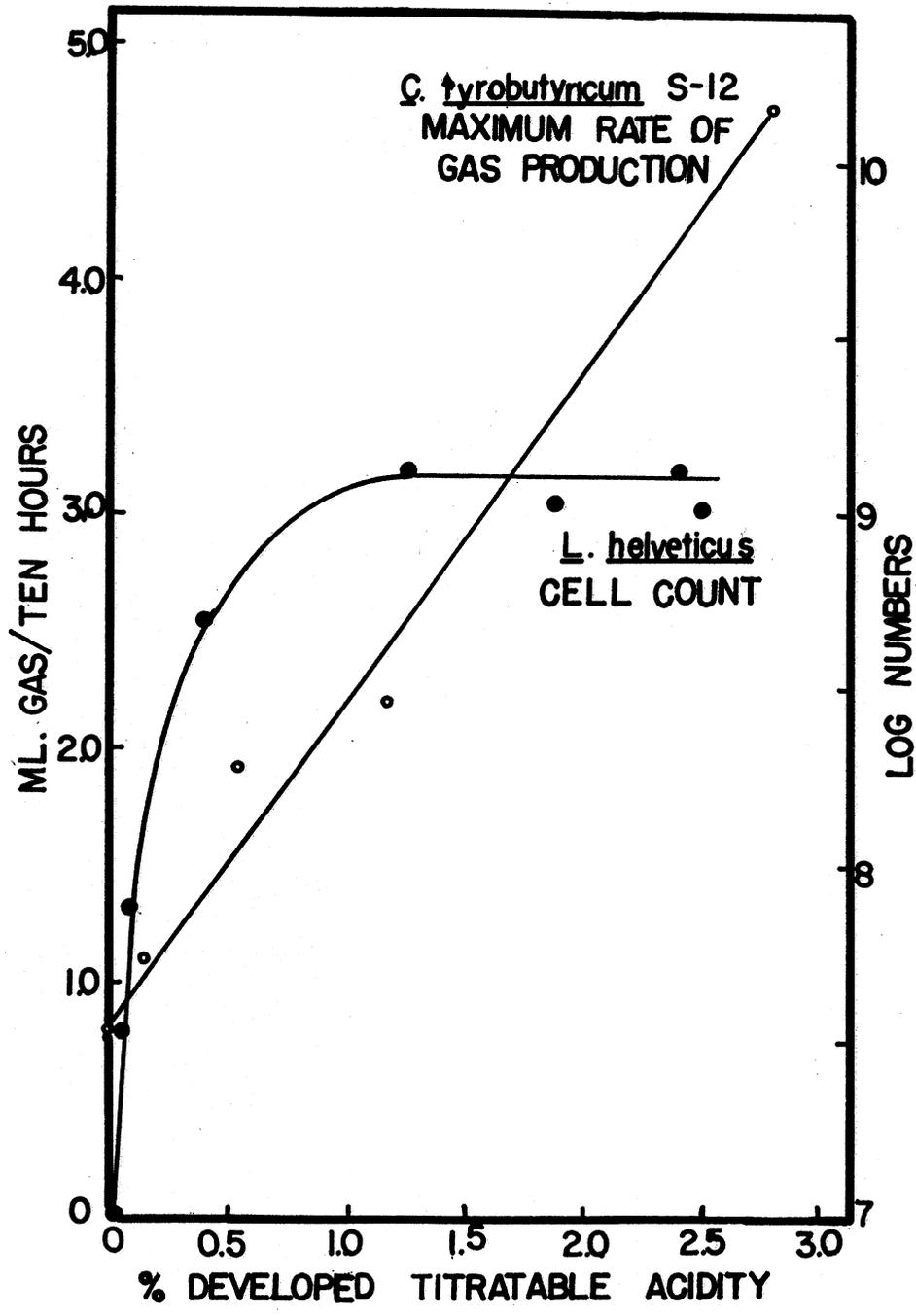


FIG. 4. Maximum rate of gas production by *C. tyrobutyricum* in relation to cell numbers and acid production of *L. helveticus*.

unfermented control milk. Gas production by *C. tyrobutyricum* was stimulated in dialysates of *L. helveticus*, *L. bulgaricus*, and *S. thermophilus*, and was inhibited by the dialysate of *S. lactis* X-13. No significant differences were found in the rate of gas production between the dialysates of the other cultures and the unfermented milk. These results agree closely with those for the complete culture, with the single exception of *L. lactis*. This organism was stimulatory in skimmilk culture, but its dialysate was without significant effect on the rate of the clostridial fermentation.

The rate of gas production by *P. shermanii* (Table 4) was stimulated in the dialysates of *L. helveticus* and *L. bulgaricus*, but there was no significant difference in rate between dialysates of the other cultures and of the unfermented control. This also agrees closely with the results shown with whole skimmilk cultures.

DISCUSSION

In the sequence of bacterial populations occurring in Swiss cheese, the gas-producing bacteria become active relatively late in the curing process. By that time, large numbers of bacteria of various types have already grown in the cheese and may have an effect on gas production by the propionics and butyries. Present results indicate that the various lactic populations differ markedly in their effect on gas production by clostridia and propionics, with the normal Swiss cheese starter bacteria being particularly stimulatory for the butyric fermentation.

The complexity of the interrelationships existing between Swiss cheese bacteria was illustrated by the mixed butyric-propionic fermentation in milk that had been fermented by lactic acid bacteria. The mixed fermentation generally produced gas earlier and at a faster rate than the separate propionic and butyric fermentations; however, the basic stimulatory or inhibitory effect of the initial lactic fermentation remained.

The identity of the stimulatory and inhibitory substances in the lactic milk cultures is unknown, although it is established that they are heat- and acid-stable and of small enough molecular weight to diffuse through a dialysis membrane. The dialysate would provide a good starting material for future attempts to identify the active substances, since it is free of the colloidal and particulate matter present in the lactic milk cultures.

The origin of the stimulatory material in the milk fermented by the starter bacteria is of interest. It is possible to postulate two sources for the stimulant. The material may be a metabolic product or it might be associated with the release of lactic cell contents upon autolysis. The cell contents include enzymes which may modify the milk before heating, so as to make it more stimulatory for the gas-formers. In the present work it was shown that the stimulatory effect continued to increase in a linear manner with increase in acid production for a considerable period after the number of lactic cells had stopped increasing (Figure 4), but autolysis was not extensive. Thus, it seems unlikely that the release of cell contents was the main source of stimulant but, rather, that the stimulatory material was related to the metabolic products of the lactic organism.

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