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R. B. Maxcy
University of Nebraska-Lincoln

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RESEARCH PAPERS

INFLUENCE OF SURFACE ACTIVE AGENTS ON SOME LACTIC STREPTOCOCCI [1]

R. B. MAXCY

Department of Dairy Science, University of Nebraska, Lincoln

SUMMARY

Fatty acids and other surface active agents were used to clarify the relationship of surface activity to the inhibition of lactic streptococci. There was complete inhibition of lactic streptococci in media where surface activity exceeded a critical level. The effectiveness of inhibition by fatty acids, nonionic surface active agents, anionic surface active agents, decyl alcohol, growth medium, and temperature variation was directly related to the surface activity. The surface active agents created an unfavorable environment at most concentrations, rather than acting as a specific inhibitory substance to which the organisms were capable of adapting and thereafter resuming normal growth. The variety of chemical compounds and conditions that produce a common physical effect indicate the inhibition involves physical phenomena at the bacterium:menstruum interface. Results indicate that surfactants are important in the inhibition of lactic streptococci.

Fatty acids are present in freshly drawn milk, and the concentration may increase considerably as naturally occurring hydrolytic products of milk fat. Free fatty acids are common constituents of many dairy products and appear on soiled dairy equipment. The hydrolytic products are surface active and retard bacterial growth (3, 4, 11). Ayers, Rupp, and Johnson (2) showed lactic streptococci to be sensitive to certain surface active materials. Tarassuk and Smith (11) reported that free fatty acids in milk lowered the surface tension and retarded bacterial growth by creating an adverse environment. Costilow and Speck (3) worked with a milk medium to which a variety of fatty acids was added. They showed that inhibition was limited to fatty acids of intermediate chain length and attributed this effect to some unknown factor other than surface tension. Guirard, Snell, and Williams (5) and Kodicek and Worden (8) showed that certain long-chain fatty acids stimulated the growth of some of the lactobacilli. Hofmann et al. (7) showed some long-chain unsaturated fatty acids to be stimulatory under certain conditions to lactobacilli, and Tween 40 enhanced the stimulation. On the other hand, Tween 40 suppressed completely the growth-promoting effect of *cis*-7-tetradecenoic acid. The growth effects of *cis*-5-dodecenoic acid were markedly inhibited by Tween 40 and saturated fatty acids. Though the sensitivity of many microorganisms to free fatty acids is well recognized (9), the mechanism remains obscure.

One function in the mechanism of action of fatty acids likely involves adsorption on the cell wall (12). The adsorption is expected to alter the relationship of the cell to its environment, and the adsorption is particularly important in dilute media (10). Hays and Elliker (6) showed that synthetic surface active agents under acid conditions are effective sanitizing agents.

Though surface activity seems a logical effect of fatty acids, the excellent work of Costilow and Speck (3, 4) set a pattern of thinking in terms of free fatty acids being directly inhibitory to the metabolic process. Thus, the following work was undertaken to clarify the relation of fatty acids and surface activity in the inhibition of growth of lactic streptococci.

EXPERIMENTAL PROCEDURE

The lactic streptococci used in this work were *Streptococcus lactis* UN-C1, *Streptococcus lactis* (Nelson FD-56), and *Streptococcus cremoris* (courtesy of P. R. Elliker). Results for the three organisms were essentially the same; therefore, only the data for *S. lactis* UN-C1 will be given. Incubation was at 30 C, except in those experiments where temperature was a factor to be studied. Stock cultures were grown in litmus milk and held in the refrigerator after monthly transfers and incubation for growth.

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Prior to experimental use, the microorganisms were carried through at least two transfers in 1% micro inoculum broth (1 g of dehydrated micro inoculum broth (Difco) per 100 ml), which also served as the basal medium for the study of additives. Inoculation was made with a 2-mm loop into 5 ml of medium in 16 by 125 mm test tubes. The test medium contained approximately 60,000 cells per milliliter as determined by plate counts on control tubes.

For the inhibition of growth in milk, samples of 100 ml of commercial homogenized milk alone and with the additions to be studied were autoclaved in Erlenmeyer flasks. To each flask 1 ml of a 24-hr culture of the test organism was added. The resulting growth was determined after incubation by measuring the acid production, which could be determined with an accuracy of 0.01%.

Lauric-1-C-14 acid was obtained from Volk Radiochemical Company, Chicago. To follow the radioactivity, 1 ml of the samples of the cultures to be studied was dried in a planchet at 90 C and counted by means of a Baird Atomic General Purpose Multiscaler with a Thin Window Geiger Flow Counter.

The presence or absence of growth was normally detectable by the naked eye within less than 24 hr; however, the incubation period was extended to 96 hr for absolute evaluation. When quantitative data were needed, growth was determined turbidimetrically by using a Coleman Spectrophotometer Model 6A with a wave length setting of 640 mμ. The sterile uninoculated medium was used as the blank. Bacterial survival tests were made using standard plate count agar with incubation at 30 C.

Decyl alcohol and capric acid were obtained from Distillation Products Industries. Lauric acid was obtained from Nutritional Biochemicals Corporation. The surface active agents were Nacconol (an alkyl aryl sulfonate by Allied Chemical Industries, Inc.), Tig (a commercial liquid cleaner by the Diversey Corporation), Triton X-100 (a nonionic surface active agent by Rohm & Haas Company), Tweens (nonionic surface active agents by Atlas Chemical Industries, Inc.), and Ultrawet DS (an anionic surface active agent by Atlantic Refining Company).

The surface activity was determined by measuring the surface tension at the liquid:air interface. A duNouy tensiometer with a ring of 4 cm mean circumference was used, and the values are given in dynes per centimeter. The measurements were made at 25 C, except in those experiments where temperature was a factor to be studied. Each value reported represents an average of three measurements, and the range of the three trials was generally less than 0.5 dyne per centimeter. Measurements were made on sterile samples after overnight equilibration at approximately the same time that an identical sample was inoculated for growth studies.

RESULTS

Surface activity and inhibition in homogenized milk. Representative surface active agents for this work were tried in milk. A typical example of the results of an individual trial representing three replications related to each control is given in Table 1. When an alcoholic solution of capric acid to represent 0.1% capric acid and 1.0% ethyl alcohol in milk was used, the surface tension was reduced from 45.0 to 40.5 dynes per centimeter, and the acid production during subsequent incubation was reduced from 0.68% on the control to 0.59% on the test samples. When capric acid alone was incorporated into the milk, thereby eliminating the alcohol as a mutual solvent for capric acid and water, the surface tension was reduced from 45.0 to 41.7 dynes per centimeter, and the acid production during incubation was reduced from 0.68 to 0.61%. Thus, the presence of alcohol was not necessary, even though the concentration of capric acid was far in excess of solubility in the aqueous phase. Adsorption was indicated, as the capric acid was in the proximity of the cells for inhibition.

TABLE 1

Effect of capric acid and Nacconol on surface tension and inhibition of acid production by *S. lactis* in homogenized milk

Additive		Surface tension	Per cent titratable acidity	
Material	Per cent			
		(dynes per cm)	*(8 hr)*	*(24 hr)*
None	0	45.0	.37	.68
Capric acid	0.1	41.7	.32	.61
Capric acid + Ethyl alcohol	0.1 + 1.0	40.5	.31	.59
None	0	43.5	.40	.70
Nacconol	0.1	40.4	.38	.67

A common water soluble synthetic surface active agent, Nacconol, at the 0.1% level in homogenized milk, decreased the surface tension from 43.5 to 40.4 dynes per centimeter, and acid production by *S. lactis* during incubation was decreased from 0.70 to 0.67%.

Surface activity in dilute growth media. To maximize the effect of the surface active agents,

use of dilute media were explored. A medium of skimmilk solids or of micro inoculum broth was found satisfactory. Micro inoculum broth at a 1% concentration (1 g of dehydrated medium per 100 ml) was chosen as it provided a clear initial solution in which growth was measured turbidimetrically. The concentration of the fatty acids was always below the quantity that produced a cloudy solution and phase separation.

A variety of common, yet chemically different, surface active agents was tried in dilute micro inoculum broth. The comparative effectiveness of surface tension depression at various concentrations is presented in Figure 1.

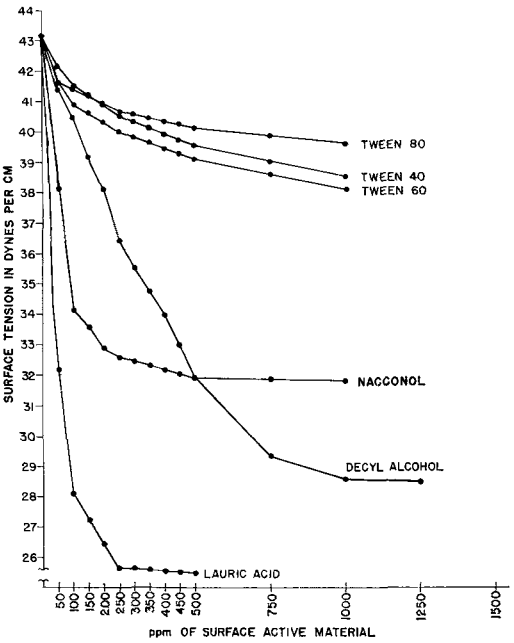

FIG. 1. Effect of varying concentrations of surface active materials on surface tension of a 1% micro inoculum broth solution.

Decyl alcohol was chosen because of the similarity of the apolar group to the intermediate fatty acids. Surface active agents in the dilute medium exhibited much the same pattern as in whole milk, but in a dilute medium a comparable concentration produced a much lower surface tension. Nacconol, decyl alcohol, and lauric acid each reduced the surface tension below 34 dynes per centimeter and each was shown to cause complete inhibition of growth. Even when the concentration of the Tweens was at the 0.1% level, the surface tension was between 38 and 40 dynes per centimeter, and this level was without marked inhibition of growth.

Relation of surface activity and the complete inhibition of S. lactis. A wide range of surface active agents including decyl alcohol, fatty acids, nonionic surface active agents, and anionic surface active agents was tried. With increasing concentrations a critical level was reached at which each surface active agent caused complete inhibition of the lactic streptococci. Representative data given in Table 2

TABLE 2

Surface tension depression in micro inoculum broth and the complete inhibition of growth of *S. lactis*

Material	Concentration	Range of surface tension
		(dynes per cm)
Capric acid	100-150 ppm	37-38
Decyl alcohol	200-250 ppm	35-36
Lauric acid	20- 30 ppm	35-36
Nacconol	100-125 ppm	33-34
Tig [a]	100-150 ppm	33-34
Triton X-100	120-140 ppm	33-34

[a] A commercial liquid cleaner by the Diversey Corporation, Chicago.

show that inhibition of growth is related to surface activity and relatively independent of the chemical nature of the surfactant.

Butyric acid was relatively noninhibitory and was essentially inactive in reducing the surface tension. The same was true for ethyl decanoate. Sodium stearate produced indefinite results, due to turbidity and the tendency of micelle formation at the surface.

To obtain information on the fate of the cells in the presence of capric acid in excess of the concentration known to provide total inhibition, 350 ppm of capric acid in 1% micro inoculum broth was used. An inoculum of *S. lactis* to provide 300,000 per milliliter at zero time in the medium to be tested was held at 25 C and standard plate counts made to determine the number of surviving cells for varying time intervals. Approximately 99.9% of the cells were dead after 1 hr and no viable cells could be found after 72 hr.

Combined effect of surfactants and growth media. To study the effects of combinations of surface active agents and growth media, selected levels of Nacconol were included, with varying concentrations of growth medium. A range of surface tension values between 32 and 36 dynes per centimeter was obtained (Figure 2). The lower limit for growth was approximately 34 dynes per centimeter. There was a variation of two or three dynes per centimeter between the different organisms with *S. lactis*

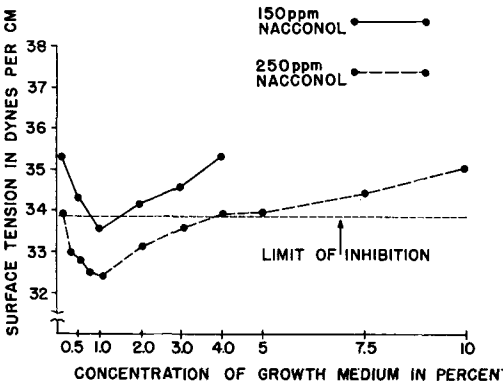

FIG. 2. Effect of varying concentrations of growth medium on surface tension and inhibition of growth of *S. lactis* in solutions containing Nacconol.

UN-C1 being able to tolerate the lowest surface tension. Any combination of Nacconol and growth medium that reduced the surface tension measurement below a critical level resulted in complete inhibition.

Effect of surface active agents on total growth. Trials were made to see if sublethal concentrations produced a lag in the onset of growth or a reduction in total growth over a prolonged period. Concentrations of capric acid, Nacconol, Triton X-100, and Ultrawet DS were chosen to approach the level of complete inhibition. The general inhibitory effect is shown in Table 3 by the results of a single trial representative of 14 individual experiments. Capric acid, for example, at 20 ppm retarded growth during the first 12 hr as well as limiting total growth. At higher concentrations there was marked retardation in the rate and in the total growth.

Inhibition of growth by surface activity influenced by temperature. Another approach toward providing the influence of surface activity in inhibition was through the use of varying concentrations of a surfactant in the medium and incubation for growth at 21, 30, and 37 C. Surface tension measurements were made at the temperature of incubation. An increase in the temperature caused an increase in surface activity and a decrease in the surface tension values (Figure 3). *S. lactis* grew when

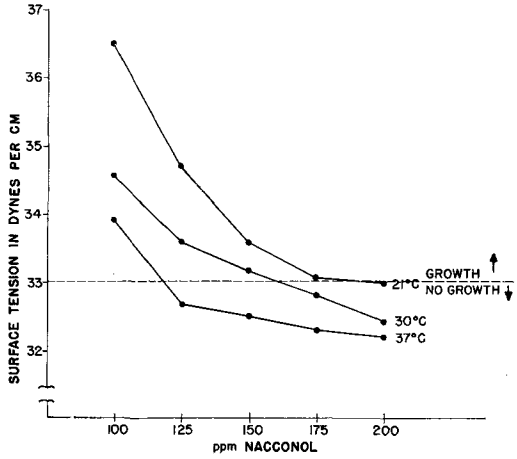

FIG. 3. Effect of temperature on surface tension and growth of *S. lactis* in 1% micro inoculum broth.

the surface tension measurements were approximately 33 dynes per centimeter or above, whereas there was complete inhibition when the values were below approximately 33 dynes. The data thus indicate the importance of surface activity in the inhibition of *S. lactis*.

Physical association of fatty acids and S. lactis. Work with the less water-soluble fatty acids, as exemplified by lauric acid and stearic acid, indicated the physical state of these compounds in the medium was of considerable importance. Beyond a certain level, which was not routinely exceeded in this work, phase inversion and small oil droplets appeared on the surface of the medium. When oil droplets existed, the surface tension measurements were abnormally low, but there was not a propor-

TABLE 3

Influence of capric acid and time of incubation on growth of *S. lactis* at 30 C in 1% micro inoculum broth

Capric acid	Per cent transmission						
	12 hr	24 hr	36 hr	60 hr	84 hr	108 hr	132 hr
(ppm)							
0	89	76	75	72	66	65	65
20	91	76	76	77	75	77	76
40	91	85	85	87	87	88	88
60	94	88	90	92	93	93	94
80	98	90	92	93	94	94	94
100	97	92	93	94	95	95	95
120	100	94	94	94	95	95	96

tionate effect on growth. The effectiveness of the fatty acids was dependent on dispersion throughout the medium, rather than an accumulation at the air:menstruum interface. It might logically be expected that the surface active agents were accumulating at the bacterium:menstruum interface.

To prove the accumulation of fatty acids with the bacterial cells, 20 ppm of lauric-1-C-14 acid was added to 1% micro inoculum broth. When *S. lactis* was grown for 48 hr in 5 ml of medium with the labeled lauric acid, and cells had settled, a 1-ml sample from the top of the test tube showed an average count of 5,095 per min, whereas a representative 1-ml sample from the remaining 4 ml showed an average count of 5,947 per min. There was no visible sedimentation on the uninoculated control. Likewise, centrifugation for 30 min at 325 g gave no indication of phase separation, as judged by distribution of radioactivity. Replication of the experiments showed essentially the same results.

Another approach was to add 5 ml of 50 ppm of labeled lauric acid in 1% micro inoculum broth to 5 ml of a 48-hr culture of *S. lactis* in 1% micro inoculum broth. The mixture was centrifuged for 30 min at 325 g to obtain visible sedimentation of the cells. A 1-ml sample from the top of the tube showed an average count of radioactivity of 3,950 per min, whereas a representative 1-ml sample from the bottom 4 ml showed a count of 7,221 per min. Replication of the experiment showed essentially the same results. Thus, the results indicated a rapid and close association of the fatty acids and the bacterial cells.

DISCUSSION

While the use of milk as a growth medium to study the effects of fatty acids and other surface active agents on the lactic streptococci is ideal, a milk medium is complex and the solids reduce the effectiveness of the surface active agents (1). There must be considerable interaction, since most of the fatty acids are sparingly soluble to completely insoluble. Complete inhibition or rapid destruction of lactic streptococci by surface active agents is not obtained in milk (3). With comparable levels of surface activity, the inhibitory effect of surface active agents in milk and in dilute media is very similar. Thus, the use of a dilute medium allows greater surface activity and greater inhibition to emphasize the importance of surface activity as a factor in inhibition.

While the results reported in this work with dilute media do not all agree with those of Costilow and Speck (3), there are some significant common findings. They found capric acid to be more effective than lauric acid in both surface tension depression and the inhibition of *S. lactis*. In the work reported here lauric acid was more effective both in surface tension depression and in inhibition of lactic streptococci in the dilute medium. The longer-chain fatty acids would be expected to give greater surface activity and inhibition in accordance with Traube's rule. Furthermore, the short-chain fatty acids, e.g., butyric acid, were without effect in dilute media or in milk. The sodium salts of long-chain fatty acids, e.g., sodium stearate, were without effect in milk and in the dilute medium where they did not remain in suspension.

All the above data indicate surface activity to be an important factor in inhibition of lactic streptococci. As judged by the close association of lauric acid with the cells, the surface active agents accumulate at the bacterium:menstruum interface rather than acting on the medium per se, as suggested by previous workers (2, 11). While the inhibitory activity is at the interface, it is expected that materials active at the bacterium:menstruum interface would also influence the surface tension of the medium. The importance of these phenomena is supported by the finding that the manipulation of the following factors could change the surface activity and change the degree of inhibition of growth of the microorganisms: (a) fatty acids, (b) nonionic surface active agents, (c) anionic surface active agents, (d) decyl alcohol, (e) growth medium concentration, and (f) temperature of incubation. The effect was in proportion to the surface tension depression.

The surface active agents created an unfavorable environment at most concentrations, rather than a specific inhibitory substance to which the organism was capable of adapting and thereafter resuming normal growth. The presence of adsorbed material might naturally be expected to alter the transfer process of nutrients into the cells. It is thus apparent that surface activity is an important consideration in evaluation of the quantity of growth.

The similarity of effect of fatty acids, nonionic surface active agents, anionic surface active agents, and decyl alcohol is quite surprising and can only lead to speculation on a yet-obscure explanation of the mechanism involved. The extreme sensitivity of the organism to all surfactants indicates some vital process is involved. Further work on an explanation of this mechanism is now under way.

ACKNOWLEDGMENT

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