APPLIED MICROBIOLOGY, May 1972, p. 960-965

Vol. 23, No. 5
Printed in U.S.A.

Susceptibility of Cheese and Yoghurt Starter Bacteria to Antibiotics

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Received for publication 14 February 1972

Eight single-strain lactic streptococci, three commercial cheese starters, and six lactic acid bacteria isolated from yoghurt were examined for their susceptibility to penicillin, cloxacillin, tetracycline-hydrochloride and streptomycin. The ranges of the antibiotics causing 50% inhibition of the bacteria were (μg/ml): penicillin, 0.009 to 0.20; cloxacillin, 0.24 to 2.50; tetracycline, 0.09 to 0.60; and streptomycin, 0.35 to 13.0. The average concentrations required to cause 50 and 100% inhibition of the cheese starters were (μg/ml): penicillin, 0.12 and 0.26; cloxacillin, 1.91 and 3.9; tetracycline-hydrochloride, 0.13 and 0.36; and streptomycin, 0.59 and 2.06. All the cocci were about equally susceptible to tetracycline, and all organisms were more resistant to cloxacillin than penicillin. The yoghurt isolates were more resistant to streptomycin and more susceptible to penicillin than the cheese starters. The 2,3,5-triphenyltetrazolium chloride test, using *Streptococcus thermophilus* BC as assay organism, does not detect low levels of streptomycin in milk. However, it is useful in detecting cloxacillin residues.

Residues in milk, resulting from the use of antibiotics in mastitis therapy, may be an important cause of slowness in the development of lactic acid during cheese and yoghurt manufacture. Because of this effect, penicillin residues in milk have been implicated as causal agents of pasty body and low acidity in cheese by Whitehead and Lane (15).

Information on the susceptibilities of various cheese and yoghurt starters to penicillin is abundant in the literature. Marth and Ellickson (7) have compiled information concerning the levels of various antibiotics which cause partial or complete inhibition of different lactic acid bacteria. For certain antibiotics, notably streptomycin, little detailed information was available. Since then, Feagan (3) has reported the levels of chlortetracycline, oxytetracycline, and streptomycin required to cause a 30% reduction in acid production of various cheese starters. Contrasting results for the susceptibility of yoghurt cultures to streptomycin have been reported by Holec and Klimeš (5) and Nikolov (10). According to O. H. Langley (*personal communication*), the semisynthetic penicillin, cloxacillin, is increasingly used in Ireland in mastitis therapy, but to our knowledge no report of the susceptibility of cheese or yoghurt starters to cloxacillin is available.

The present report therefore examines the susceptibility of several cheese and yoghurt starters to antibiotics which are commonly used in mastitis therapy. Additionally, it points out certain anomalies associated with the detection of streptomycin.

MATERIALS AND METHODS

Cultures. In addition to three commercial cheese cultures, the following pure cultures were used: *Streptococcus lactis* C2, C6, C10, and M1W1; *S. cremoris* AC7, AC11, C3, and KH; *S. thermophilus* BC, Y1, and Y3; *Lactobacillus bulgaricus* NYL2 and Y4; and *L. lactis* BYL1.

Strain M1W1 was from the culture collection of W. E. Sandine. Strain BC was from Unigate Central Laboratory. Strains Y1, Y3, and Y4 were from the culture collection maintained at the National Dairy Research Centre and had been isolated from yoghurt cultures. The remainder of the strains were from the culture collection of M. L. Speck.

All cultures were routinely transferred in autoclaved (5 min at 121 C) 10% reconstituted nonfat milk solids (NFMS) using 1% inocula. Incubation temperatures were 21 C for the commercial cultures, 25 C for the single-strain cheese starters, and 37 C for the yoghurt cultures.

Antibiotics. The four antibiotics used were dissolved as follows: (i) benzyl penicillin (Crystapen, Glaxo) in sterile phosphate buffer, *p*H 6.0 (4); (ii) streptomycin sulphate (Glaxo) and (iii) cloxacillin (Orbenin, Beecham) in sterile water; (iv) tetracy-

cline-hydrochloride (Pfizer) in 0.01 M HCl. The potencies of the streptomycin and tetracycline-hydrochloride were 745 and 980 units/mg, respectively. The concentration of each antibiotic in its respective diluent was such that the addition of 0.5 ml to the milk resulted in the desired final antibiotic concentration.

Measurement of growth. The growth response of the individual cultures after incubation was determined by titrating the lactic acid produced in duplicate 10-ml volumes of culture (in NFMS) with 0.11 N NaOH to *p*H 8.3 using a Radiometer *p*H stat. All values are reported as per cent developed lactic acid (i.e., the per cent lactic acid of similar volumes of milk containing no inoculum was substracted from the per cent lactic acid determined).

Sensitivity determination. To duplicate sterile test tubes containing 0.5 ml of different levels of antibiotic was added 9.5 ml of previously cooled 10% NFMS containing 1% (v/v) of the culture under study. The test tubes were incubated at 30 C (cheese cultures) or 37 C (yoghurt cultures) until the desired development of lactic acid was reached (0.30 to 0.40%) in a control tube containing no added antibiotic. All test tubes were removed and cooled in an ice bath, and the contents (plus two successive 5-ml volumes of distilled water used to rinse each test tube) were titrated.

Semilogarithmic dosage response curves were constructed relating log of the antibiotic concentration to per cent developed lactic acid. The concentration of antibiotic required to give 50% inhibition was estimated by halving the titration value obtained in the absence of antibiotic and determining the concentration of antibiotic equivalent to it from the graph.

RESULTS

The purpose of the assay method was to determine the concentration of the antibiotic being tested which inhibited acid production in lactic acid bacteria to a specified (50 or 100%) degree. Since the amount of lactic acid produced by a culture depends on the incubation time, the sensitivity of one culture (*S. cremoris* C3) to different levels of penicillin was measured in samples at various times of incubation (Fig. 1). In the presence of levels of penicillin above 0.09 μg/ml, a certain amount of growth occurred early in the incubation period, before virtual inhibition was apparent. Low concentrations of penicillin decreased the rate of growth slightly, but their primary effect was to decrease the total amount of lactic acid produced.

To determine the exact concentration of antibiotic causing a specified degree of inhibition, the data of Fig. 1 were transformed to the form shown in Fig. 2. For the sake of clarity, only data for incubation periods of 4.5, 5.5, 6.5, and 7.5 hr are shown, but similar curves were obtained from the data after 5, 6, and 7 hr of

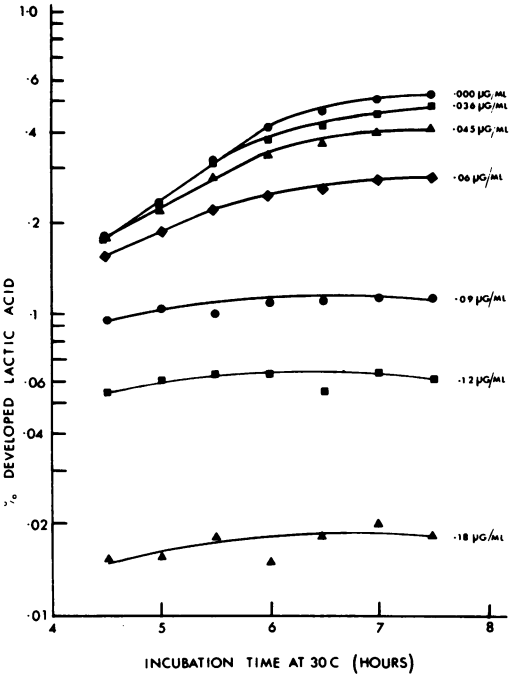

FIG. 1. *Effect of different concentrations of penicillin on growth in milk of S. cremoris C3.*

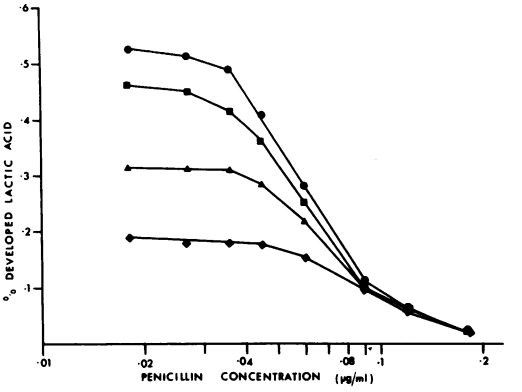

FIG. 2. *Effect of incubation time on the response of S. cremoris C3 to penicillin. Incubation times (h) are 4.5 (◆), 5.5 (▲), 6.5 (■), and 7.5 (●). Data derived from Fig. 1.*

incubation. From Fig. 2, the concentration of penicillin causing 50% inhibition of developed lactic acid was determined for each of the incubation times. As is apparent in Table 1, the greater the amount of growth which occurred, the lower the penicillin concentration required to cause 50% inhibition. Because this effect could influence comparisons between cultures, all subsequent tests were incubated until the developed lactic acid in milk containing no

TABLE 1. *Effect of incubation time on the concentration of penicillin required to cause 50% inhibition of Streptococcus cremoris C3*

Incubation time (hr)	Per cent developed lactic acid in absence of penicillin	Penicillin concn required to cause 50% inhibition (μg/ml)
4.5	0.183	0.093
5.0	0.230	0.085
5.5	0.320	0.073
6.0	0.410	0.069
6.5	0.465	0.064
7.0	0.508	0.063
7.5	0.530	0.062

antibiotic reached a value between 0.3 and 0.4%.

Representative dosage-response curves (relating log antibiotic concentration to per cent developed lactic acid) of *S. cremoris* KH and *S. thermophilus* BC to the four antibiotics are shown in Fig. 3 and 4, respectively. Strain BC was much more sensitive to penicillin and cloxacillin than strain KH, which was more sensitive to streptomycin. Both organisms appeared to have the same sensitivity to tetracycline.

The level of the four antibiotics which caused 50 and 100% inhibition of all the lactic acid bacteria tested is shown in Table 2. All the values for the 50% level of inhibition fell on the linear portion of the relevant dosage-response curves. In general, the levels causing 100% inhibition were more than twice those causing 50% inhibition. The single-strain lactic streptococci and the commercial cheese starters showed little variation in the concentration of each antibiotic causing 50% inhibition. Comparing the antibiotics on a weight basis, these strains were most sensitive to penicillin and tetracycline, less sensitive to streptomycin, and least sensitive to cloxacillin, requiring approximately 17 times less penicillin than cloxacillin to cause the same level of inhibition. In general, the same order of sensitivity was obtained for the yoghurt bacteria, except that these strains were much more sensitive to cloxacillin than streptomycin.

The thermophilic streptococci were about 10 times more sensitive to penicillin than the lactic streptococci and commercial cultures. The former were also the most resistant to streptomycin, of the organisms tested, being 5 times more resistant than the lactobacilli and 20 times more resistant than the lactic streptococci and commercial cultures. All organisms tested were more sensitive to penicillin than cloxacillin.

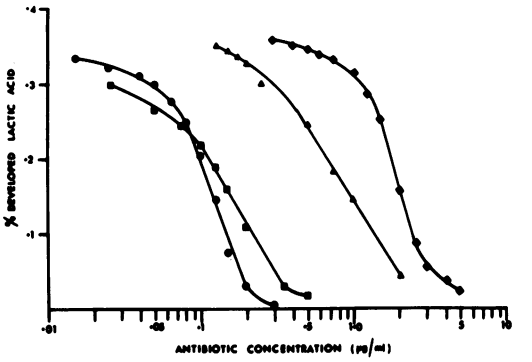

FIG. 3. Dosage-response curves of *S. cremoris KH to penicillin* (●), *tetracycline* (■), *streptomycin* (▲), *and cloxacillin* (◆).

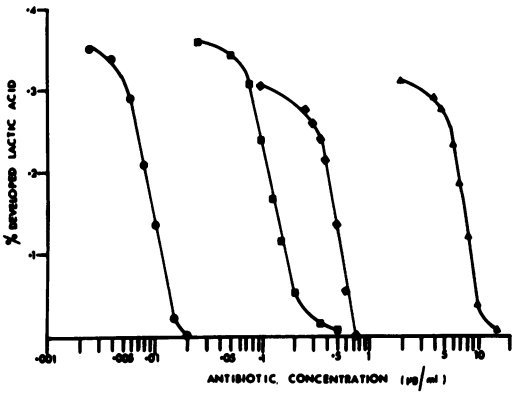

FIG. 4. *Dosage-response curves of S. thermophilus BC to penicillin* (●), *tetracycline* (■), *streptomycin* (▲), *and cloxacillin* (◆).

DISCUSSION

The method used for determining the quantity of antibiotic required to cause a specified degree of inhibition depended on the acid produced in the absence of antibiotic. Since low levels of penicillin had no measurable effect on acid production over short incubation periods and had an increasingly inhibitory effect over long incubation periods (Fig. 1), it is not surprising that the value obtained for the concentration of penicillin causing 50% inhibition depended on the extent of bacterial growth (Table 1).

An interesting result was that all lactic acid bacteria tested were inhibited to the same extent by tetracycline. The lactic streptococci and commercial cultures were equally sensitive to penicillin and more resistant to streptomycin. Reasons for these findings must remain the subject of speculation but presumably are connected with the mechanism of action of the relevant antibiotic.

TABLE 2. *Concentration of antibiotics (μg/ml) required to cause 50% and 100% inhibition of several lactic acid bacteria*[a]

	Penicillin		Cloxacillin		Tetracycline-hydrochloride		Streptomycin	
	50%	100%[b]	50%	100%	50%	100%	50%	100%
Streptococcus lactis								
C2	0.13	0.23	2.50	4.3	0.21	0.65	0.66	>3.0
C6	0.12	0.26	2.10	3.9	0.09	0.28	0.35	2.0
C10	0.09	0.26	1.60	4.0	0.13	0.40	0.41	1.9
M1W1	0.15	>0.30	2.42	>5.0	0.15	0.30	0.71	>2.0
S. cremoris								
AC7	0.13	0.25	2.05	3.6	0.11	0.30	0.84	>2.0
AC11	0.13	0.27	1.72	3.7	0.16	0.40	0.58	2.0
C3	0.07	0.30	1.16	2.2	0.12	0.36	0.52	1.9
KH	0.11	0.21	1.82	4.6	0.15	0.38	0.74	>2.0
Commercial culture								
1	0.20	>0.30	2.20	5.0	0.13	0.35	0.45	1.6
2	0.09	0.30	1.78	3.9	0.09	0.29	0.74	3.0
3	0.10	0.27	1.62	4.0	0.10	0.30	0.53	2.0
Average	0.12	0.26	1.91	3.9	0.13	0.36	0.59	2.1
S. thermophilus								
BC	0.009	0.015	0.47	0.70	0.13	0.30	7.30	12.5
Y1	0.007	0.009	0.34	0.48	0.20	0.50	11.20	19.0
Y3	0.010	0.015	0.46	0.70	0.24	0.44	13.00	21.0
Lactobacillus lactis BYL1	0.024	0.07	0.24	0.50	0.60	>2.0	2.29	8.0
L. bulgaricus								
NYL2	0.026	>0.15	0.31	>1.0	0.34	2.0	1.60	6.6
Y4	0.035	>0.15	0.26	1.0	0.39	>1.0	4.45	>8.0

[a] Values preceded by the sign > were the highest concentration tested for the particular organism.

[b] One hundred per cent inhibition is the minimum concentration of antibiotic required to give 0.025% developed lactic acid.

Streptomycin has been reported (6) to interfere with protein synthesis by binding to the 30*S* ribosomal subunit thus causing faulty translation of messenger ribonucleic acid (RNA). Perhaps streptomycin is less tightly bound to the ribosomes of thermophilic streptococci than to those of the lactic streptococci. It is also possible that the lactic streptococci may be more permeable to streptomycin than the thermophilic streptococci. Anaerobiosis has been reported by Davis et al. (2) to decrease the potency of streptomycin so that an adequate explanation may be that the thermophilic streptococci produce more anaerobic conditions in milk than the lactic streptococci and thus are less affected by streptomycin. It is interesting, therefore, to note that Vakil and Shahani (12) found that reactions of aerobic metabolism (lactose dehydrogenase) were much more affected by several antibiotics (including streptomycin) than those of anaerobic metabolism (β-galactosidase) in *S. lactis* UN so that the sensitivity of a culture to a particular antibiotic may depend on the relative importance of one type of metabolism over the other.

Davis et al. (2) also report that tetracycline interferes with the binding of charged transfer RNA to the ribosomes. This step in protein synthesis may be equally sensitive to tetracycline in all lactic acid bacteria, thus accounting for the present results. Such a possibility implies that lactic acid bacteria are equally permeable to tetracycline. Vakil and Shahani (13) found that the activities of the later enzymes of glycolysis were much more susceptible to inhibition by the tetracyclines (10) than the earlier ones. Adenosine triphosphate production is therefore affected, and, if the adenosine triphosphate-producing enzymes of the different lactic acid bacteria are equally sensitive to inhibition by tetracyclines, similar susceptibility levels would be found as in the present study.

Three of the cheese cultures used in the present study were also tested by Feagan (3), who used a reduction in acid production of 30% as a measure of inhibition. His results are in agreement with the present results with one exception. He reports that 0.5 μg of

streptomycin/ml causes a 30% reduction in acid production in strain C10, whereas in the present study this value was 0.22 μg/ml. The difference, while small, reflects a different methodology since his calculations are based on results obtained after a particular incubation time which was shown to affect the results in the present study.

Most of the cheese cultures studied by Feagan (3) were inhibited to the same extent by oxytetracycline and chlortetracycline in the range 0.05 to 0.10 μg/ml. All the cheese cultures of the present study, with the exception of strain C2, showed a 30% reduction in acid production in the presence of 0.05 to 0.10 μg of tetracycline/ml, bearing out the point that there is generally little difference in the sensitivity of a particular organism to the different tetracycline congeners (14). Sixteen of 32 cultures studied by Richards and Kennedy (11) were also sensitive to 0.1 μg of chlortetracycline/ml. The main reason for using tetracycline in the present study was that it is more stable than chlortetracyline or oxytetracycline (2). In this connection, Martin and Harper (8) have shown that addition of either of the latter two antibiotics to milk resulted in an immediate loss of activity, which was only slightly affected by subsequent cold storage. Thus, absolute values for the sensitivity of an organism to antibiotics are probably relevant only to the particular test medium.

The present results for the yoghurt cultures are also in fair agreement with those of Nikolov (10), who found that streptomycin levels of 5 to 7 μg/ml reduced the biochemical activity of yoghurt cultures, but contrast with those of Holec and Klimeš (5), who found that 0.38 μg/ml caused a decrease in acidity of 5° SH (0.11% lactic acid), a value which was, except for strain NYL2, without effect on the yoghurt isolates. In the case of strain NYL2, a decrease of 10% in acid production was caused by this level of streptomycin. These differences may be explained by interstrain variation, although in the present study interspecies variation was much greater than interstrain variation. As these authors studied the mixed yoghurt cultures, another possibility is that the nutritional factor(s) involved in the yoghurt symbiosis may affect the sensitivity of the individual cultures to antibiotics. This is unlikely since Cogan and Fox (1) found little effect of a growth stimulant (yeast extract) on the sensitivity of *S. thermophilus* BC to penicillin.

It has been suggested by Feagan (3) that milk for cheese making should not contain more than 0.1 IU of penicillin/ml (0.06 μg/ml). This concentration significantly affected the acid production of several lactic streptococci in the present study.

The 2,3,5-triphenyltetrazolium chloride (TTC) reduction test (9) using *S. thermophilium* BC as the assay organism is a method commonly used in detecting residual antibiotics in milk. Since this organism is much more resistant than cheese starter to streptomycin, a false sense of well being could easily obtain in deciding, as a result of the TTC test, that milk is free of antibiotic residues. This organism is five times more susceptible to cloxacillin than are cheese starters so that the TTC method is useful in detecting cloxacillin residues in milk for cheese making.

In the manufacture of yoghurt, streptomycin residues in the milk should be less of a problem since yoghurt starters are fairly resistant. However, at high levels of this antibiotic the balance between cocci and rods could be upset, since they are not equally sensitive, resulting in a poor quality product. Because of the sensitivity of yoghurt cultures to cloxacillin, and especially penicillin, milk for yoghurt manufacture should be monitored for these residues with great care.

ACKNOWLEDGMENTS

The author wishes to express his appreciation for the excellent technical assistance of P. Thornhill and F. Drinan and to S. Condon for critical review of the manuscript.

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