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An Evaluation of Antibiotics Upon Selected Dairy Organisms

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AN EVALUATION OF ANTIBIOTICS UPON SELECTED DAIRY ORGANISMS

by

Roy Brog

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Roy Brog

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INTRODUCTION

This study was designed to answer a three-fold purpose. Primarily, it has endeavored to measure the inhibitory effect of three antibiotics (potassium pencillin, dihydrostreptomycin, and oxytetracycline) on lactic acid producing organisms. The lactic acid-producing organisms include Streptococcocus lactis, Streptococcus thermophilis, and Lactobacillus bulgaricus.

The determination of an effective dose of a given antibiotic which will inhibit 50 percent of the lactic acid-producing organism ($E.D._{50}$) will be established. A 50 percent cut in acid development will certainly lead to an inferior product.

The secondary purpose has related to the detection of inhibitory substances (antibiotics) in milk. What is the possibility of detecting the concentration of antibiotic which correlates to the $E.D._{50}$ established in the first phase of the problem?

The third purpose embraces a study of the sensitivity of tests for detecting inhibitory substances.

REVIEW OF LITERATURE

History and definition of antibiotics

Since the beginning of life, contagious and lethal organisms have been shed into the soil from sick vertebrae and invertebrae. Bacteriologists have pondered and studied for clues which would explain the disappearance of the organisms. Theories have been put forth to explain this phenomena. Dr. Louis Pasteur demonstrated in 1877 that the anthrox organism could be killed in cattle by placing certain soil-borne saprophytic organisms into the surface wound. Bacteriologists were quick to discover that certain organisms produced substances antagonistic to certain disease pathogens. These inhibitory substances are elaborated by microorganisms to protect themselves against the unfavorable influence of other organisms in a situation called antibiosis. The term antibiotic is derived from antibiosis, "anti" meaning against and "biotic" meaning life processes (15).

Zinsser (40) defines an antibiotic as a chemical compound derived from or produced by living organisms, which is capable, in small concentration, of inhibiting and/or destroying the life processes of other microorganism. Several hundred antibiotic agents have been isolated from various sources. Penicillin, streptomycin, chloramphenicol, tetracycline, oxytetracycline, chlortetracycline, polymyxin, bacitracin, and neomycin are among the more prominent antibiotics.

Penicillin is derived from Penicillium notatum, streptomycin is derived from Streptomyces griseus and unlike penicillin is bactericidal.

Oxytetracyc-line was developed by the careful screening of a tremendous number of species of fungi (40).

Synthesized antibiotics have been shown to be equally effective and identical in every respect to the natural substance (40).

Broad spectrum antibiotics

Broad spectrum antibiotics are those antibiotics which are effective against both gram positive and gram negative organisms. Penicillin is noted for its effectiveness against gram positive organisms and streptomycin is unique against gram negative and acid-fast organisms (40). Streptomycin's chief shortcoming is its toxicity to man. The most serious damage being to the eighth cranial nerve; deafness is sometimes produced (40).

Chloramphenicol is a broad spectrum antibiotic effective against rickettsiae and some virus as well as gram positive and negative organisms. Tetracycline is another broad spectrum antibiotic, as is the related compounds, oxytetracycline and chloretetracycline which are sold commercially under the trade names terramycin and aueromycin respectively.

About 150 antibiotics have been prepared in purified form but only a few of these have been sufficiently nontoxic to be of use in medical practice (40).

Origin and chemical structure

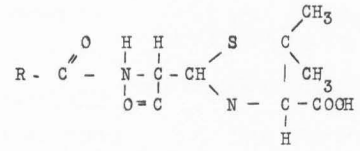
The three antibiotics pertinent to this thesis were selected in view of their non-related chemical structures. Penicillin, streptomycin, and the tetracyclines are not chemically related.

Penicillin

Penicillin is produced by various strains of Penicillium notatum and Penicillium chrysogenum. Fleming is credited with the discovery of test tube penicillin in 1929 (40). No new strain has been discovered which exceeds the penicillin-producing ability of the original Fleming mold for surface culture methods. However, a strain was found that was superior to all other in producing penicillin for submerged culture methods. This outstanding strain of penicillin-producing mold was discovered growing on a cantaloupe rind found in a garbage can (15).

Jones (15) portrays the following table on the different fractions of penicillin known. There are four natural penicillins listed (F, G, X, and K) and two synthetic ones, (O and V).

The Formula and Names of Some Important Penicillins



The General Formula for the Pencillins

R equals:	Chemical Names	American Names
(a) C_2H_5. CH = CH. CH_2-	(2 pentenylpenicillin)	Penicillin F
(b)	(benzylpenicillin)	Penicillin G
(c) HO	(hydroxy benzyl penicillin)	Penicillin X
(d) CH_3 $(CH_2)_6$-	(n-heptyl penicillin)	Penicillin K
(e) CH_2 = $CHCH_2SCH_2$-	(Allylmercaptomethyl penicillin)	Penicillin O
(f)	(phenoxymethyl penicillin)	Penicillin V

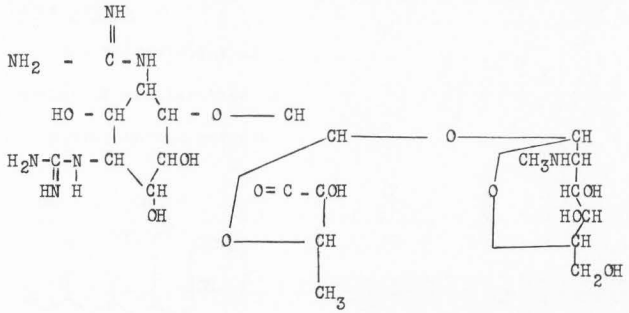
In their early work with penicillin, the Oxford workers used a purely arbitrary unit of potency to measure the concentration of penicillin. This unit became known as the Oxford Unit (O.U.) and has now been accepted as the International Unit. The International Standard Unit for penicillin has been defined as that amount of activity present in 0.6 microgram of the international pure crystallin standard sodium salt of Penicillin G; one milligram contains 1,667 Oxford Units.

Streptomycin

Streptomycin is a potent antibiotic, isolated from Streptomyces griseus by Waksman and associates in 1943 (40). Only certain strains of Streptomyces griseus produce the antibiotic. Streptomycin is a valuable therapeutic agent because it is effective against gram-negative bacteria and in that respect supplements other drugs which are active against gram-positive bacteria, such as, penicillin (15).

The production of streptomycin involves the aerobic, pure-culture growth of Streptomyces griseus in huge fermentation tanks by the submerged growth method. Streptomycin is adsorbed from the growth medium by use of carbon, after which the streptomycin is harvested by elution from the carbon (15).

Streptomycin is a glycoside hydrolyzed by acid into streptidine and streptobiosamine. Streptobiosamine yields streptose and N-methyl-l-gly-cosamine by a simple break of an oxygen bond. Streptomycin is a basic substance produced commercially as a white powder in the hydrochloride, the sulfate, or the double calcium chloride salts. The free base and the salts are freely soluble in water or insoluble in organic solvents.



Streptomycin (15)

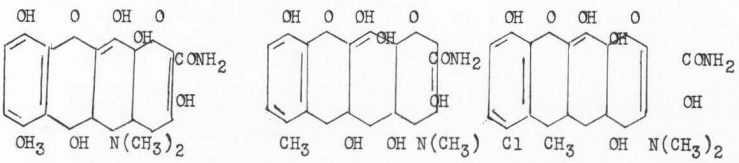
After the formula of Streptomycin Sulfate was revealed, it was discovered that the free carbonyl group could be reduced by catalytic hydrogenation, converting streptomycin into Dihydrostreptomycin Sulfate. Dihydrostreptomycin has antibacterial activity comparable to that of streptomycin and apparently is more stable. At least two fractions of streptomycin can be isolated from cultural media: streptomycin A, the less active streptomycin B (mannisidostreptomycin). Commercial production is largely of streptomycin A, of which the potency on a weight basis is about five times that of streptomycin B (15).

Streptomycin can be assayed bacteriologically or chemically. The potency of pure streptomycin is expressed in the metric system, although originally a biological "S" unit was stated. One microgram of pure streptomycin base is equalivalent to one "S" unit. One thousand "S" units of streptomycin base is the amount of streptomycin that will inhibit the growth of a standard strain of Escherichia coli in one liter of nutrient medium. The activity of commercial streptomycin is stated in milligrams of grams of streptomycin base (15).

Dihydrostreptomycin is essentially equal to streptomycin in its bacteriostatic action for most of the common bacteria (15).

Tetracycline

The tetracycline antibiotics are usually considered together because of similarities of origin, chemical structure, pharmacology, and antibacterial activity.



Tetracycline Oxytetracycline Chlortetracycline

The tetracyclines are called broad-spectrum antibiotics because they exhibit a wide range of antibacterial activity which overlaps the antibacterial spectra of penicillin, streptomycin, and chloramphenicol (15). In addition, the tetracyclines will act against a few microorganisms unaffected by other antibiotics, e.g., the rickettsiae and certain of the large viruses belonging to the psittacosis group in animals and to the lymphogranuloma venereum group in man. The tetracycline antibiotics are relatively potent against most gram positive bacteria, but less active against gram-negative organisms (15).

Only rapidly growing organisms are affected by the tetracyclines. Multiplication can be inhibited by small amounts of tetracycline, but higher concentrations are required to kill the microorganisms (40). The antibacterial activity of the tetracyclines is influenced to only a minor degree by the presence of serum, blood, and the number of bacterial activities of the different fractions of penicillin are inversely proportional to the degree of binding by the serum proteins (15). Penicillin G. X. and dihydro-F are about 50 percent bound in normal plasma and thereby lose about one-half of their antibacterial

activity. Since about 90 percent of penicillin K is bound by the serum proteins, its antibacterial activity is proportionately reduced.

Mechanisms

Much is known regarding the mechanisms through which antibiotics perform their bacteriostatic and bacteriocidal effects; however, the exact manner in which penicillin influences bacterial metabolism has not been demonstrated. In general, the antibacterial action of penicillin arises from its ability to inhibit the metabolic activities vital to the bacterial cell. Krampitz and Werkman (1947) (15) demonstrated that penicillin inhibits the synthesis of ribonucleic acid by rapidly growing bacteria. Penicillin blocks the systhesis of the cell wall, thus penicillin appears to block the assimilation of protein essential for growth and reproduction by the bacterial cell. Man's body cells lack combining groups for penicillin and therefore our body cells are not vulnerable to penicillin.

Streptomycin seems to interfere with one or more of the cellular enzyme systems essential for bacterial cell division. Acetyl coenzyme A cannot enter the citric acid cycle. Bacterial growth continues during the action of streptomycin but cell division is inhibited resulting in tremendously elongated cells (15). Man's body cells are not readily penetrated by streptomycin.

Little is known about the antimicrobial mechanism of the tetracyclines. These antibiotics may interfere with the bacterial protein synthesis of the rapidly growing and reproducing bacterial cell. Concentrations of chlortetracycline inhibiting bacterial growth will markedly reduce the conversion of glutamate into cell protein by the bacterium. Higher concentrations of chlortetracycline also interfere

with the accumulation of glutamate within the cell. It has been suggested without much evidence that chlortetracycline interferes with the oxidative metabolism of the bacterial cell by inhibition of acetate metabolism (15).

Reference regarding the mutually synergistic and antagonistic actions of antibiotics can be cited (19, 40, and others). This data for the most part is based largely upon laboratory research using barely therapeutic concentrations of antibiotics. The same antagonism and synergisms seem not to operate significantly at the higher dosage levels of antibiotics employed clinically. The possibility of antibiotic antagonisms becoming significant during treatment of a patient appears slight. Antibiotic synergism is difficult to demonstrate clinically, although the additive action of two antibiotics can be observed occasionally (15).

Synergism and antagonism

The antibiotics have been divided into two groups:

1. Penicillin streptomycin, bacitracin, and neomycin.
2. Chloramphenicol, chlortetracycline, oxytetracycline and erthromycin.

The sulfonamides behave like group two antibiotics. Members of group one are frequently synergistic with each other but never antagonistic. Members of group two show only an additive effect. The addition of a group one antibiotic to a member of group two may result in either synergism or antagonism. Resistance of the pathogen provides the major justification for combined therapy of diseases caused by a single species of organism (40).

Effect of antibiotic concentration

No direct relationship exists between the various antibiotics with regards to their effect on a particular organism; by the same token, each organism reacts differently to individual antibiotics. To further complicate any concept of generalties, a selected organism may show variation when treated by a chosen antibiotic, e.g. penicillin inhibits bacterial multiplication at low concentrations and kills rapidly at high concentrations. The antibacterial action of penicillin, also the tetracyclines, is highest during the period of the greatest bacterial multiplication (log phase) (15).

The literature is saturated with effects and persistence of specific antibiotics in blood, broth, milk, and urine (4, 5, 11, 16, 19, 29, 32, and 34).

The time of physical contact between the organism and the antibiotic is vital. Antibiotics perform their usefulness by affecting the metabolic transformations (15, and 28).

The medium, the pH, the temperature, and the vehicle can and do affect the net results of any specific antibiotic on a given organism (35, and 36). The underlying principle behind all antibiotic action is the chemical union between the basic chemical structure of the antibiotic with that of the organism being inhibited. The antibiotic must pass, in most instances, through the cell wall of the organism. If a binding group in the medium exerts greater affinity to the antibiotic than does the reacting metabolite (s), the antibiotic will be required in higher concentration and/or in longer intervals of time for identical results. In other words, it is a chemical reaction (series of chemical reactions) which can be accelerated, retarded, or even inhibited by other chemical combinations which show greater

affinity than the antibiotic-metabolite combination (28). A classic example of pH effects can be demonstrated using streptomycin. Streptomycin is much more antibacterial in a slightly alkaline than acid pH; it is 20 to 80 times more active against gram-negative bacilli at a pH of 8 than at 5.8. For this reason, alkali is administered to carnivora and omnvora during streptomycin therapy for urinary tract infection. The increase in locat acidity resulting from tissue damage may explain the failure of streptomycin to control bacteria in circumscribed tissue lesions (15).

METHODS FOR DETECTING INHIBITORY SUBSTANCES

No direct method for detecting antibiotics in milk is presently known. The term "inhibitory substances" has been coined which includes bacteriophage, pesticides, antibiotics, chlorine and iodophors, etc. Bacteriophage is inactivated at 80° C for ten minutes. Standard procedure for milk being assay for inhibitory substances includes the heat treatment. This automatically identifies bacteriophage as the causative agent perchance it is present.

A differentiation test is available which distinguishes whether inhibition resulted from antibiotics or from santizers (9). Tests which are positive for inhibitory substances and negative for bacteriophage are inoculated with one ml of two percent glucose solution plus a charge from a suspension of baker's yeast. The tubes are incubated for an additional six hours and checked for production of gas. Santizers, if present, inhibit yeast and hence gas is not produced. If antibiotics are causing the inhibition, yeast will reproduce in the presence of antibiotic and gas production will be evident (9).

Following is a summary of tests available to test milk for inhibitory substances.

Direct morphological examination

The morphological examination or direct microscopic examination is based on the principle that when a culture is inhibited by any foreign substance, there will be fewer cells per field as compared with the control when observed under the microscope; also, there will

be morphilogical changes present such as swelling or elongation of the individual cells. Streptococcus cremoris is reported to produce rod-shaped cells under the influence of 0.05 units penicillin per ml (21).

Dye reduction tests

This group of tests include the use of such dyes as Methylene blue, resazurin, and triphenyltetrazolium chloride. The scientific principle is the actively growing bacteria reduce certain dyes with resulting color changes. When inhibitory substances are present in the medium, the organisms will not grow, the dye will not be reduced, and the color will not change.

Acidity tests - coagulation tests

Acidity and coagulation tests are recommended highly (8, and 21) in the literature. The principle involved is to compare the milk being tested with a control. Any sample tested which reflects a marked inhibition in acidity after innoculation and incubation is considered adulterated by an inhibitory substance (s). The coagulation test is incubated longer until the control is coagulated. The sample which do not coagulate are adulterated.

Plate methods

Cylinder plate method, disc assay, and reversible disc assay may be classified as agar plat methods or simply plate methods. All agar plate methods have two items in common; (a) the petri plate, and (b) an agar medium.

The cylinder-plate method is carried out by placing small, sterile stainless steel cups or glass cups (cut from glass tubing) on the surface of the seeded agar. One of the cylinders is filled with a standard

solution for control and the others are filled with the unknown. The petri plate, loaded with the cylinders, is then incubated for a given time and temperature. The area (zone) around the cylinder which microbial growth does not occur is measured and compared with the zone of a standard solution (21).

The disc assay utilizes the seeded agar which is contained within a petri plate, the same as the cylinder-plate method. In the place of the cylinder, a paper disc is substituted which is impregnated (saturated) with the standard solution prior to placing it on the seeded agar. Other discs are saturated in the unknown and placed on the identical petri plate in other positions around the plate. After incubation at given time and temperature, the plates are removed and inspected for zones of inhibition (1, and 24).

The reversible disc assay utilizes and seeded agar medium in which the agar medium lacks an essential nutrient. The essential nutrient is supplied by the milk. The milk diffuses around the disc which results in a zone of growth around any disc wherein is not found an inhibitory substance. Any disc which does not produce a zone of growth equivalent to the control is considered as containing an inhibitory substance, hence the name reversible disc assay (2).

Turbidity tests

Marth (21) mentions a turbidimetric method to assay for antibiotics which was suggested by Berridge and Barret but has not been applied to milk. Serial dilutions of the test antibiotic were added to log-phase cultures of Streptococcus agalactiae. After thirty minutes of incubation, turbidity determinations were made and compared to previously

established standard. The turbidimetric method was found applicable for assays of penicillin, streptomycin, chlortetracycline and gramicidin (21).

Nitrate test

The principle involved in the nitrate test is the ability of an active growing culture of Micrococcocus pyogenes var. aureus.[1] to transform nitrate to nitrite. In the presence of an inhibitory substance the transformation is not effected and the decreased nitrite production can be measured colormetrically and compared with a control. Mattick (23) gives the chemistry involved in developing the color. The diasotization of sulfanilic and the subsequent coupling with alpha naphthylamine hydrochloride is responsible for the production of the color. The test can be completed in two hours (23).

Fluorescent test

The fluorescent approach calls for the addition of fat-soluble fluorescein (Fluoral) and uranine, as a "marker" to the antibiotic preparations intended for intramammary infusion. The marker can be detected visually in the milk for 48 hours after treatment and with the use of ultraviolet light, the "marker" can be detected for 96 hours. The "marker" is nontoxic to the treated animals and does not affect milk production. Statistical analysis of the data showed a close correlation between the excretion of marker and antibiotic from the treated udders (10).

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[1] Culture No. 254 Department of Bacteriology, University of Connecticut.

PROCEDURE

The first problem was to determine the concentration necessary for the test antibiotic to inhibit 50 percent of the organisms.

Selection of antibiotics

Antibiotics were selected because of their dissimilar chemical structure. The three antibiotics were: Potassium penicillin, dihydrostreptomycin and oxytetracycline. The antibiotics were supplied by the Veterinary Science Department of Utah State University.

Selection of organisms

Three dairy organisms were selected, Streptococcus lactis, Streptococcus thermophilus, and Bacillus bulgaricus. The organisms were secured from the American Type Culture Collection, 2029 M Street, N.W., Washington 6, D.C.

Selection of method

The method selected to establish a 50 percent inhibition determination was the plate counting technique. A modified Elliker's medium was used to foster the growth of the three different organisms. Elliker's medium is composed of the following ingredients:

Tryptone	20 g
Gelatin	2.5 g
Glucose	5.0 g
Lactose	5.0 g
Sucrose	5.0 g
Yeast Extract	5.0 g
NaCl	4.0 g
Sodium Acetate	1.5 g
Agar	15.0 g
Calcium Carbonate	4.0 g
Brom Cresol Purple	--Trace

Water to make 1000 ml and bring to boil. Autoclave 15 minutes at 121° C. Final pH = ± .1.

Technique

Standard solutions of the individual antibiotics were formulated and the culture dilutions were made in buffered, sterile water (distilled), the final dilution being made into sterile, inhibitory-free, skim milk. The Elliker's medium was autoclaved and pipetted into sterile test tubes each tube being filled with a predetermined amount of medium. The filled tubes were again autoclaved and immediately placed in a water-bath at 45° C. The following sequence of events then took place:

1. One ml of skim milk containing the organism was pipetted into a sterile petri plate.
2. The test tube full of Elliker's medium was poured into the plate, and at the same time the antibiotic was added and the plate was swirled until complete mixing was evident. The Elliker's medium, the antibiotic solution, and the culture in each case equaled exactly 12 ml.
3. The plates were allowed to solidify and then inverted and incubated at their optimum temperatures until the controls showed distinct growth.
4. The plates were counted and tabulated. Plates were made in replicas of five for statistical significance.

Results

After three months of experimentation the procedure was abandoned for the following reasons:

1. The results were not definite. On five identical replicas, three might show 30-40 colonies or more (same as control) while the other two would show no growth at all. The reason for the inconsistency was not determined.

2. Elliker's medium is not the same as milk and it may be that equal concentration of antibiotic would react differently in the two media.

The standard titratable acidity test was substituted for the plate counting procedure in establishment of an E.D.$_{50}$ (Effective dose which will inhibit 50 percent of the organisms).

Calculation of inhibition

Standard, sterile, inhibitory-free, milk solution, equally spaced in log concentration of the respective antibiotics, plus appropriate controls were inoculated with the test organism and the developed acidity of the controls was compared to the developed acidity in the standard solutions. The percent inhibition was calculated after the following formula:

1. Control prior to incubation, minus control after incubation, equals developed acidity, equals 100 percent.

2. Standard solution's developed acidity equals X percent.

   X minus 100 equals percent inhibition in standard solution.

Example	Percent
Total acidity in control after two hours of incubation	= .45
Total acidity in control prior to incubation	= .32
Developed acidity in the control	= .13 or 100
Total acidity in standard solution after two hour incubation	= .44
Total acidity in control prior to incubation	= .32
Developed acidity in standard solution	= .12 or 92

Therefore:

.13 : 100 percent
.12 : X

x = 92 percent 100 - 92 = 8 percent inhibition

The $E.D._{50}$ formula states: The two closest percentages of inhibition nearest to 50 percent, one on either side of 50 percent is used to calculate the $E.D._{50}$.

The general formula for $E.D._{50}$ is:

$$\frac{\text{50 - the inhibition percentage closest to, but lower than 50\%}}{\text{The inhibition percentage closest to, but higher than 50\% - the percentage of inhibition closest to, but lower than 50\%}} = x$$

Add x to the exponent of the log concentration lower than 50 percent and look-up the antilog of this number. The antilog = $E.D._{50}$.

An actual $E.D._{50}$ calculation follows:

Concentration in Logs	Percent acidity in duplicate I	II	Percent inhibition
Control	.45	.45	0
10^{-3}	.45	.45	0
10^{-2}	.45	.45	0
10^{-1}	.43	.45	8
10^{-0}	.38	.38	54
10^{1}	.36	.36	69

Therefore: $\frac{50 - 8}{54 - 8} = \frac{42}{46} = .91$

Take the .91 and add it to the exponent of the log concentration which was immediately lower than the 50 percent inhibition concentration. In the above example it would be 10^{-1}; 10^{-1} + .91 added to the exponent equals $10^{-1.91}$. With the use of a log book, look-up the antilog of $10^{-1.91}$ which equals .81. .81 = the $E.D._{50}$. .81 units of

the above antibiotic (penicillin) will inhibit 50 percent of the acid production in particular culture (Streptococcus lactis).

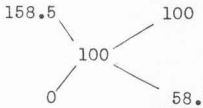
After establishing the $E.D._{50}$ for Streptococcus lactis, Streptococcus thermophilus and Lactobacillus bulgaricus in each of the three antibiotics, namely: penicillin, dihydrostreptomycin, and oxytetracycline, the sensitivity of two basic tests for inhibitory substances were applied in these $E.D._{50}$ concentrations. The identical concentrations were used in the detection test, as were used in establishing the $E.D._{50}$ concentrations.

The dye reduction test was used in connection with Streptococcus thermophilus, Streptococcus lactis, Lactobacillus bulgaricus and Bacillus steareothermophilus. The agar plate method was used in connection with Bacillus subtilus and Bacillus stearothermophilus.

The purpose of these tests was to establish whether or not concentration of the antibiotics were detectable which correlated to the $E.D._{50}$.

Standardization of antibiotic

Potassium penicillin contained 1585 units of penicillin per milligram (mg) in the powder form. Therefore, 0.1 gram (100 mg) was carefully weighed to the closest 0.0001 of a gram. To this 0.1 gram was added sufficient distilled water to make 100 ml. The liquid dilution had a concentration of 1585 units per ml. Ten ml of the 1585 units per ml, plus 90 ml distilled water gave a new dilution of 158.5 units per ml. To obtain a solution which contained 100 units per ml the following calculation was performed: (Pearson square)



$\frac{58.5}{158.5}$ = 100%; what percent is 100?

100 is 63% when solving for x
58.5 is 37%

Therefore, 63 ml of penicillin dilution containing 158.5 units per ml was added to 37 ml of distilled water to obtain a solution which contained 100 units of penicillin per ml.

The 100 unit concentration was next diluted to 10, 1, 0.1, 0.01, and 0.001, respectively.

The dihydrostreptomycin and the oxytetracycline came in a liquid form in units of micrograms/ml. The basic formula listed above was used in making the standard solutions.

Dye reduction assays

Neal and Calbert (25) procedure was used as the standard for running the dye reduction test. Log concentrations were assayed using four different organisms: Streptococcus lactis, Streptococcus thermophilus, Lactobacillus bulgaricus, and Bacillus steareothermophilus (each were run in duplicate).

Agar plate assays

Filter paper discs (0.5 inch No. 740-ES&S) were used for added sensitivity (7). These discs were dipped carefully, with sterile dry forceps, into each dilution of antibiotic, starting with the concentration in a systematic sequence so that there would be no carryover (contamination) from the forceps. The discs were run in triplicate using two different organism, Bacillus subtilis, and Bacillus

stearothermophilus. Pictures of the results are shown under results and discussions.

Source of organisms

Strains of four different organisms were obtained from the American Type Culture Collection (ATCC); 2029 M Street, N.W.; Washington 6, D. C. The following strains were procured:

Bacillus stearothermophilus	7953
Streptococcus lactis	6963
Lactobacillus bulgaricus	7994
Streptococcus thermophilus	7952

Bacillus stearothermophilus 7953 gave excellent results; however, the other strains did not develop sufficient acid, even after several transfers, and organoleptic tests proved them to be unnatural in odor and flavor and they were discarded.

The Streptococcus lactis which was used throughout this research was Strain K supplied by Utah State University, Dairy Department.

The Lactobacillus bulgaricus and Streptococcus thermophilus was supplied by the Cache Valley Dairy Association.

The Bacillus subtilis was supplied by the Bacteriological Department, Utah State University.

RESULTS AND DISCUSSIONS

The $E.D._{50}$ results for Streptococcus lactis, Streptococcus thermophilis and Lactobacillus bulgaricus gives an indication as to the sensitivity of the organisms when treated with antibiotics.

Streptococcus lactis sensitivity

Strain K from Utah State University showed an eight percent inhibition at 0.1 units of penicillin per ml; 1.0 unit/ml penicillin showed 54 percent inhibition. The $E.D._{50}$ of Streptococcus lactis under the influence of potassium penicillin was 0.81 units/ml of penicillin.

Dihydrostreptomycin on the same organism showed an $E.D._{50}$ at 10.0 micrograms/ml.

Oxytetracycline showed an $E.D._{50}$ at 1.10 micrograms/ml on the same organism.

Streptococcus thermophilus sensitivity

$E.D._{50}$ for Streptococcus thermophilis under the influence of Potassium penicillin, dihydrostreptomycin, and oxytetracycline is 0.04 units/ml, 4.78 micrograms/ml and 0.23 micrograms/ml respectively.

Lactobacillus bulgaricus sensitivity

$E.D._{50}$ for Lactobacillus bulgaricus under the influence of Potassium penicillin, dihydrostreptomycin, and oxytetracycline is 45.0 units/ml, 0.79 micrograms/ml and 5.89 micrograms/ml respectively.

A comparison of the effects of penicillin, dihydrostreptomycin, and oxytetracycline are shown graphically below:

Note also the short-perpendicular line on each graph. This line indicates the threshold concentration (lowest concentration detectable) using the TTC and/or the disc assay method.

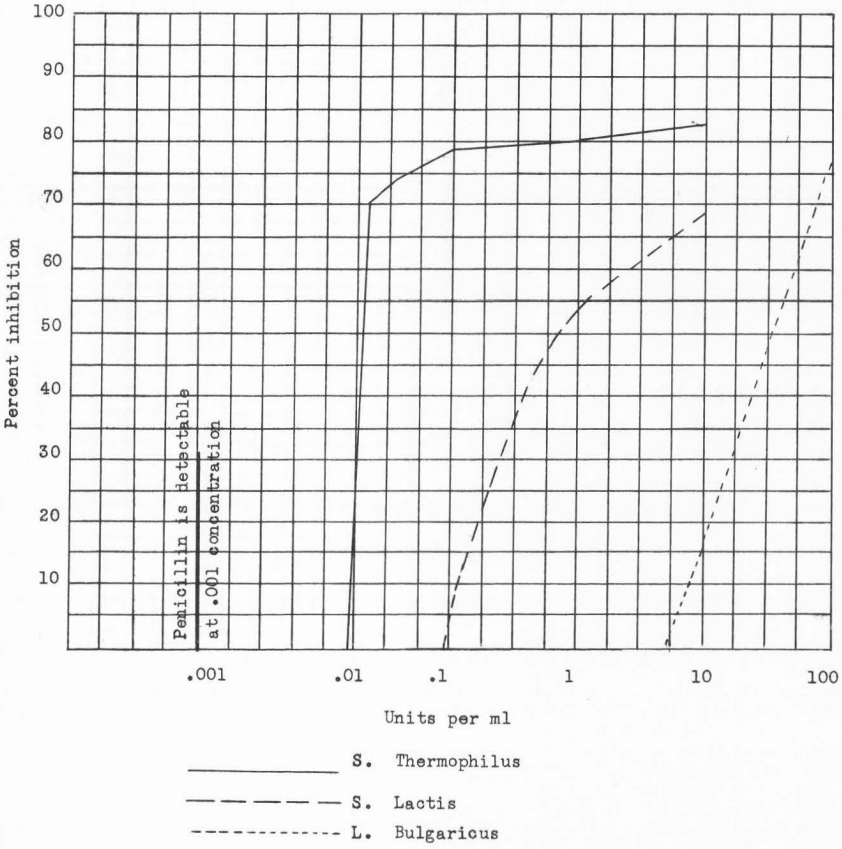


Figure 1. The effect of potassium penicillin on three dairy organisms.

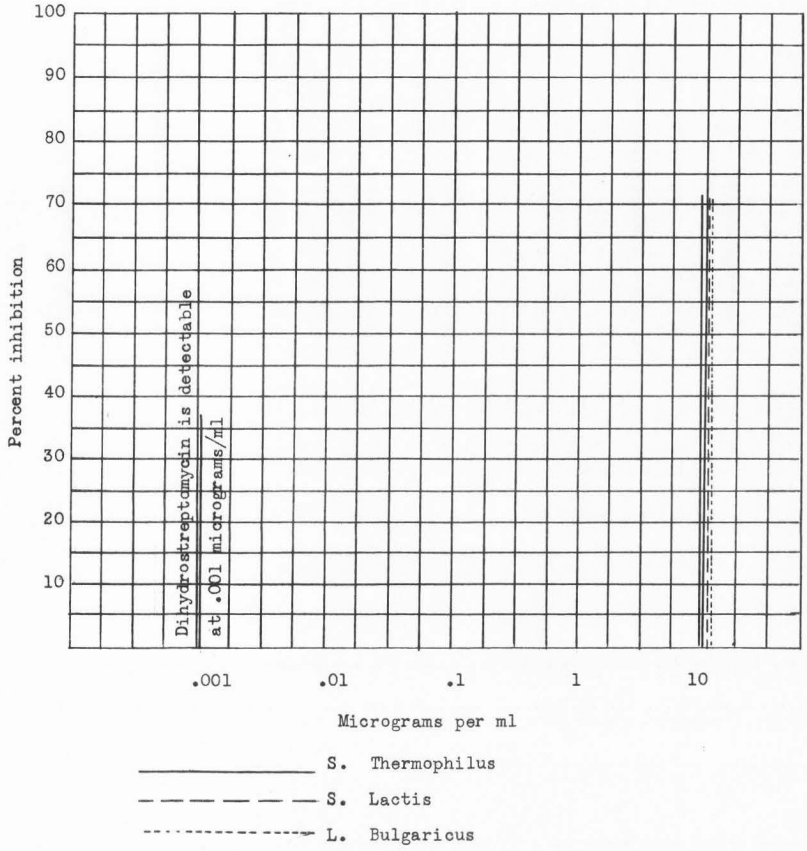


Figure 2. The effect of dihydrostreptomycin on three dairy organisms.

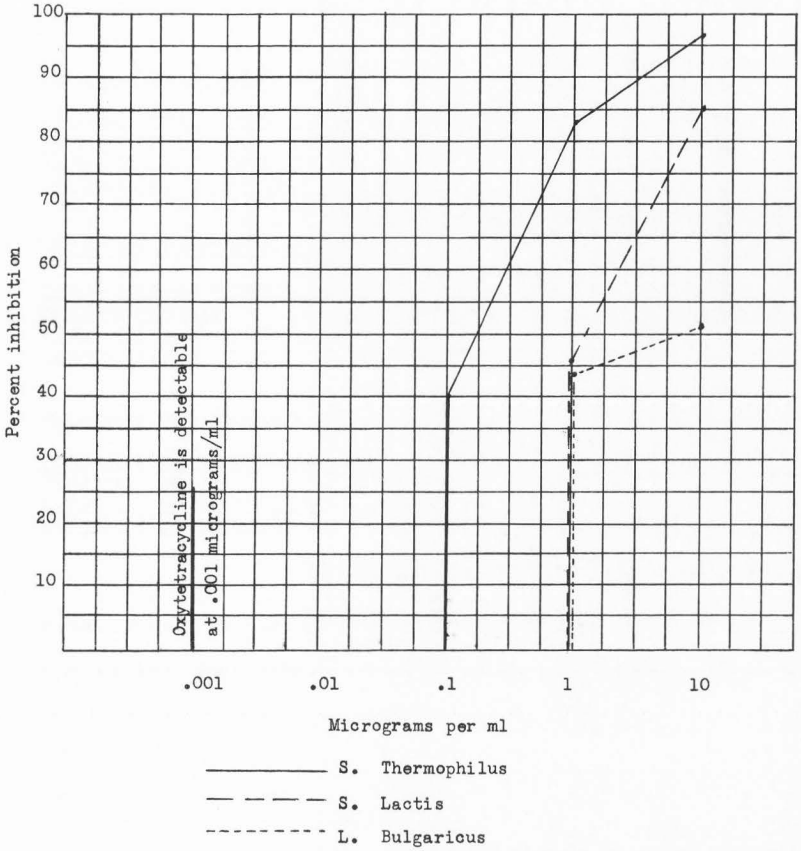


Figure 3. The effect of oxytetracycline on three dairy organisms.

The following tables give the threshold concentration of antibiotic detectable using different organism in conjunction with the disc assay and the TTC test.

Pencillin (units/ml)	Disc Assay	TTC
Bacillus stearothermophilus	.001	.001
Bacillus subtilis	.01	--
Streptococcus thermophilus	----	.03
Lactobacillus bulgaricus	----	.06

Dihydrostreptomycin (micrograms/ml)	Disc Assay	TTC
Bacillus stearothermophilus	0.1	.001
Bacillus subtilis	2.5	---
Streptococcus thermophilus	----	10.
Lactobacillus bulgaricus	----	2.5

Oxytetracycline (micrograms/ml)	Disc Assay	TTC
Bacillus stearothermophilus	1.0	.001
Bacillus subtilis	.01	---
Streptococcus thermophilus	----	.13
Lactobacillus bulgaricus	----	1.0

It is interesting to note that the same organism would be inhibited in the TTC test to yield a lighter pink than the control but would not show a zone of inhibition on the agar plate method until a higher concentration of the antibiotic was used. This is indeed peculiar; however, when zones did appear on the agar plates they were decisive and easily identified as can be seen by the following photos.

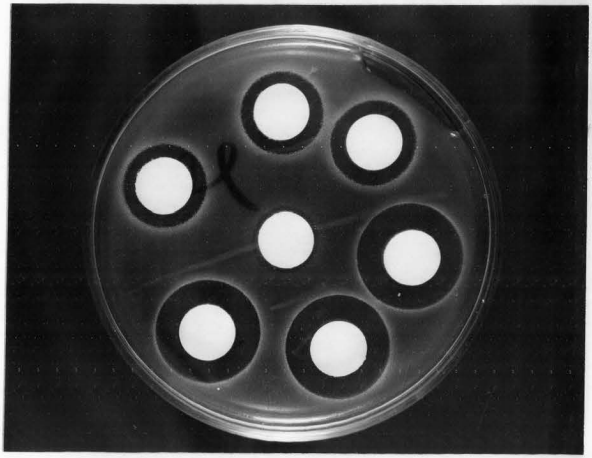


Figure 4. The effect of penicillin on agar seeded with Bacillus subtilis.

The discs are in triplicate except for the control which is found in the center.

Penicillin in two concentrations:

Three on top = 0.1 unit/ml
Three on bottom = 1.0 unit/ml

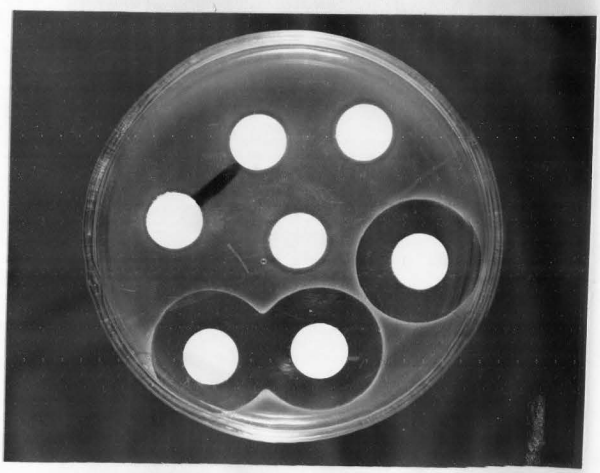


Figure 5. The effect of streptomycin and penicillin on agar plate seeded with Bacillus subtilis.

Three upper discs = Streptomycin .001 microgram/ml
Center disc = Control
Three lower discs = Penicillin 10.0 units/ml

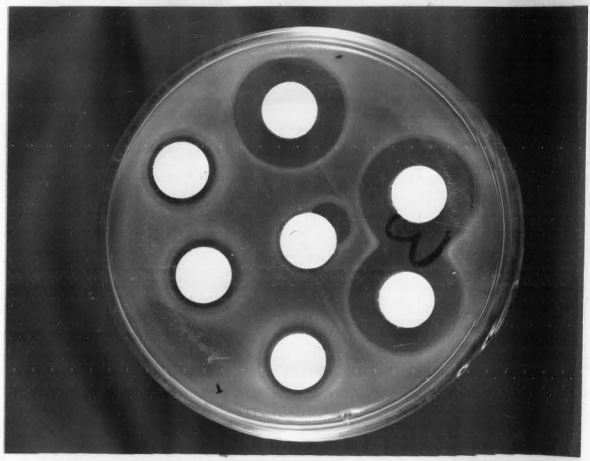


Figure 6. The effect of oxytetracycline on agar plate seeded with Bacillus subtilis.

Three upper right discs = Oxytetracycline 10 microgram/ml
Center = Control
Three lower left = Oxytetracycline 1 microgram/ml

Note positive edge on control, probable contamination from forceps!

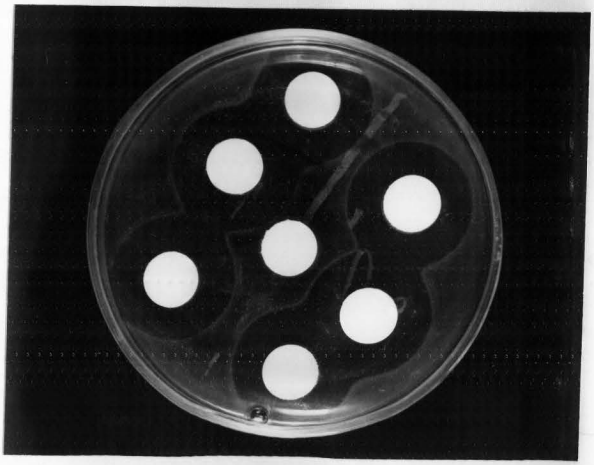


Figure 7. The effect of penicillin on agar plate seeded with Bacillus steareothermophilus.

Center = Control
Three upper left = Penicillin .08 units/ml
Three lower right = Penicillin .06 units/ml

SUMMARY

$E.D._{50}$ concentrations for three antibiotics (penicillin, dihydrostreptomycin and oxytetracycline) were established for three dairy organisms. The $E.D._{50}$ levels are:

	Penicillin Units/ml	Dihydro-streptomycin Micrograms/ml	Oxytetra-cycline Micrograms/ml
Streptococcus lactis	0.81	10.00	1.10
Streptococcus thermophilus	0.04	4.78	0.23
Lactobacillus bulgaricus	45.00	7.90	5.89

The disc assay and the TTC (2-3-5, Triphenyltetrazolium chloride) test, and dye reduction test were then applied to the $E.D._{50}$ concentrations to determine whether these concentrations were detectable. The $E.D._{50}$ concentrations were easily detectable using the test organisms Bacillus steareothermophilus in either the disc assay or the TTC method. Bacillus subtilis (disc assay method) gave excellent protection except in the case of Streptococcus thermophilus in the presence of oxytetracycline. Streptococcus thermophilis and Lactobacillus bulgaricus offered only partial protection in conjunction with the TTC test.

The following table is a summary of the threshold concentrations as they pertained to each test using the different organisms:

Bacillus steareothermophilus

Lowest detectable concentration (threshold value)

	Penicillin	Streptomycin	Oxytetracycline
2-3-5 Triphenyltetrazolium chloride (TTC)	.001	.001	.001
Disc assay	.001	.1	1.0

Bacillus subtilis

Lowest detectable concentration (threshold value)

	Penicillin	Streptomycin	Oxytetracycline
Disc assay	0.01	2.34	0.01

Streptococcus lactis

Lowest detectable concentration (threshold value)

	Penicillin	Streptomycin	Oxytetracycline
$E.D._{50}$	0.81	10.00	1.10

Streptococcus thermophilus

Lowest detectable concentration (threshold value)

	Penicillin	Streptomycin	Oxytetracycline
2-3-5 Triphenyltetrazolium chloride (TTC)	0.03	10.00	1.00
$E.D._{50}$	0.04	4.78	0.23

Lactobacillus bulgaricus

Lowest detectable concentration (threshold value)

	Penicillin	Streptomycin	Oxytetracycline
2-3-5 Triphenyltetrazolium chloride (TTC)	0.06	2.34	1.00
$E.D._{50}$	45.00	7.9	5.89

CONCLUSIONS

The $E.D._{50}$ was not significant. The $E.D._{0}$, and $E.D._{75}$ was found to be within extremely narrow limits of antibiotic concentration. The $E.D._{50}$ tends to become less meaningful when slight increase in antibiotic concentration mark major percent changes in inhibition.

The following conclusions were made:

1. The sensitivity of the assay is highly dependent upon the organisms.
2. Bacillus steareothermophilus is the most sensitive test organism which was used in this project. Its sensitivity was excellent in either the disc method or the TTC test.
3. Bacillus steareothermophilus is sufficiently sensitive for routine testing for penicillin, dihydrostreptomycin, and oxytetracycline in dairy plants. Bacillus subtilis although not as sensitive will detect all combinations used in this thesis except Streptococcus thermophilus in the presence of oxytetracycline.
4. Streptococcus thermophilus, Lactobacillus bulgaricus, and Streptococcus lactis are not sensitive enough to use as test organisms.

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APPENDIX

TTC Using Bacillus Steareothermophilus

Penicillin	I	II	III
Control	++	++	++
.001	+	+	+
.005	+	+	+
.01	+	+	+
.03	+	+	+
.06	+	+	+
.08	+	+	+
.1	+	+	+
1.0	+	+	+
10.0			

Streptomycin			
Control	+++	+++	+++
.001	+	+	+
.002	+	+	+
.006	+	+	+
.01	+	+	+
.1	+	+	+
1.0	+	+	+
2.34	-	-	-
10.0	-	-	-

Oxytetracycline			
Control	+++	+++	+++
.001	++	++	++
.005	++	++	++
.01	++	++	++
.10	+	+	+
.13	+	+	+
1.0	++	+	+
10.0	+	+	+

Key

+++ No inhibition - Red
++ Slight inhibition - Pink (dark)
- Complete inhibition - White

Two hours incubation time.

TTC Using Streptococcus Thermophilus

Penicillin	I	II	III
Control	+++	+++	+++
.001	+++	+++	+++
.005	+++	+++	+++
.01	+++	+++	+++
.03	+	+	+
.06	-	-	-
.08	-	-	-
.1	-	-	-
1.0	-	-	-
10.0	-	-	-

Streptomycin			
Control	+++	+++	+++
.001	+++	+++	+++
.002	+++	+++	+++
.006	+++	+++	+++
.01	+++	+++	+++
.1	+++	+++	+++
1.0	+++	+++	+++
2.34	+++	+++	+++
10.0	++	++	++

Oxytetracycline			
Control	+++	+++	+++
.001	+++	+++	+++
.005	+++	+++	+++
.01	+++	+++	+++
.10	+++	+++	+++
.13	-	++	++
1.0	-	-	-
10.0	-	-	-

Key

+++ No inhibition - Red
++ Slight inhibition - Pink (dark)
+ Definite inhibition - Pink (light)
- Complete inhibition - White

Two hours incubation time.

TTC Using Lactobacillus Bulgaricus

Penicillin	I	II	III
Control	+++	+++	+++
.001	+++	+++	+++
.005	+++	+++	+++
.01	+++	+++	+++
.03	+++	+++	+++
.06	++	++	++
.08	++	++	++
.1	++	++	++
1.0	++	++	++
10.0	+++	++	++
Streptomycin			
Control	+++	+++	+++
.001	+++	+++	+++
.002	+++	+++	+++
.006	+++	+++	+++
.01	+++	+++	+++
.1	+++	+++	+++
1.0	+++	+++	+++
2.34	++	++	++
10.0	-	-	-
Oxytetracycline			
Control	+++	+++	+++
.001	+++	+++	+++
.005	+++	+++	+++
.01	+++	+++	+++
.10	+++	+++	+++
.13	+++	+++	+++
1.0	++	++	++
10.0	+	+	+

Key

+++	No inhibition - Red
++	Slight inhibition - Pink
+	Definite inhibition - Pink (light)
-	Complete inhibition - White

Two hours incubation time.

Disc Assay Using Bacillus Subtilus

Penicillin	Control	I	II	III
.001	-	-	-	-
.005	-	-	-	-
.01	-	1.	1.	1.
.03	-	2.	2.	2.
.06	-	3.	3.	3.
.08	-	3.5	3.5	3.5
.1	-	4.	4.	4.
1.0	-	5.	5.	5.
10.0	-	8.5	8.5	8.5
Streptomycin				
.001	-	-	-	-
.002	-	-	-	-
.006	-	-	-	-
.01	-	-	-	-
.1	-	-	-	-
1.0	-	-	-	-
2.34	-	1.	1.	1.
10.0	-	3.	3.	3.
Oxytetracycline				
.001	-	-	-	-
.005	-	-	-	-
.01	-	.5	.5	.5
.10	-	1.	1.	1.
.13	-	1.	1.	1.
1.0	-	3.	3.	3.
10.0	-	6.	6.	6.

Key

- No zone
Numbers = Periphery differential between disc and zone. (In millimeters)

Six hours incubation time.

Disc Assay Using Bacillus Subtilus

Penicillin	Control	I	II	III
.001	-	1	1	1
.005	-	5	5	5
.01	-	7	7	7
.03	-	8	8	8
.06	-	9	9	9
.08	-	9	9	9
.1	-	10	10	10
1.0	-	12	12	12
10.0	-	15	15	15
Streptomycin				
.001	-	-	-	-
.002	-	-	-	-
.006	-	-	-	-
.01	-	-	-	-
.10	-	1	1	1
1.0	-	3	3	3
2.34	-	4	4	5
10.0	-	8	8	8
Oxytetracycline				
.001	-	-	-	-
.005	-	-	-	-
.01	-	-	-	-
.10	-	-	-	-
.13	-	-	-	-
1.0	-	1	1	1
10.0	-	5	5	5

Key

- No zone

Numbers = Periphery differential between disc and zone. (In millimeters)

Six hours incubation time.

Direct morphological examination

1. Heat the milk samples and the control to 80° C. for three minutes.
2. Cool to 37° C. and inoculate with a fresh culture of Streptococcus thermophilus. Use a 1: 10 dilution of the Streptococcus thermophilus in tempered antibiotic-free skim milk.
3. Incubate the inoculated samples at 37° C. for 90 minutes.
4. Prepare slides of the control and of the unknowns using methylene blue or the Newman-Lampert method and examine microscopically.

Interpretation

Inhibitory substances are present in any sample which causes cell distortion or enlargement and/or has a clump count per field which was 50 percent less than the control.

Dye reduction tests[1]

The classic and most widely used dye reduction test is the 2,3,5-Tripenyltetrazolium chloride test by Neal and Calbert (25).

1. Place 10 ml of raw milk to be tested into a sterile test tube equipped with a screw cap or rubber stopper.
2. Pasteurize at 80° C. for five minutes.
3. Cool to 37° C. and inoculate with 1 ml of a 1:1 dilution of a 12 to 14 hour active starter culture. Dilute the starter culture with tempered sterile skim milk that is known to be free of inhibitory substances.

[1] Note: The 2,3,5-Triphenyltetrazolium chloride may be purchased from the following companies; Nutritional Biochemical Corp., Cleveland, Ohio; Paul Lewis Laboratories, Inc., Milwaukee, Wisconsin; Dajac Laboratories, Inc., Leominster, Massachusetts; and Van Water and Rogers, Salt Lake City, Utah.

4. Stopper tube and invert twice to mix culture and milk, then incubate in a 37° C. water bath for two hours.

5. Following incubation at 0.3 ml of a 1:25 solution of TTC reagent. Stopper and invert twice to mix. The TTC reagent can be prepared with distilled water and stored under refrigeration.

6. Return sample to the 37° C. water bath for an additional thirty minutes.

7. After the second incubation period remove samples and compare color with a control sample that is known to be free of inhibitory substances.

Interpretation

Any sample distinctly lighter in color (less red) than the control is considered to contain inhibitory substances. A sample expressing strong inhibition will develop no color. (Avoid exposure to light during the performance of the test.)

Acidity-coagulation test (8)

1. Propagate a culture of Streptococcus thermophilus routinely at least three times per week in sterile (slightly brown) 10 percent reconstituted nonfat milk.

2. Pipette 10 ml of each sample of raw milk to be tested into a sterile culture tube. Screw-cap, 16 by 125 mm., culture tubes are recommended. This size of tube does not float when partly filled with 10 ml of milk.

3. A sample of raw milk known not to contain inhibitory substances should be used as a control; however, this has been

found unnecessary if several samples are tested at one time. The negative samples serve as controls.

4. Pasteurize samples at 143° F. for 30 minutes. The culture-tube caps should be tight and the tubes submerged. The time required for the temperature of samples to reach 143° F. varies with the size of the samples, etc. If the test conditions are sufficiently standardized, a come-up time may be used routinely.

5. Cool samples immediately by setting them in tap water. Ice water should be used if the samples are not to be inoculated immediately.

6. Dilute fresh Streptococcus thermophilus culture by adding one part sterile 10 percent reconstituted nonfat milk. Shake the dilute culture and add two ml to each sample. Shake each sample so that sample and culture will be thoroughly mixed.

7. Incubate inoculated samples at 104° F. for three hours and titrate with 1/10 N NaOH and phenphthalein. (Acidity Method).

8. Incubate inoculated samples at 104° F. for six hours or until control is coagulated. (Coagulation Method).

Interpretation

Any sample which has developed less acidity than the control contains inhibitors. The inhibitors usually encountered are antibiotics (8).

Plate methods

1. Agar medium which has the following composition is used: 1000 ml distilled water, 11.5 grams agar, 10 g yeast extract,

20 g trypticase, and 0.5 g glucose and autoclave at 121° C. for 15 minutes.

2. A 17 hour culture of Bacillus stearothermophilus which has been fostered on the broth (same ingredients as above with the exception of the agar) is used to seed the cooled agar. The cells should be evenly distributed throughout the medium. Bacillus stearothermophilus is cultured at 550°- 60° C.
3. Six ml of the agar are added to each flat-bottomed petri dish.
4. Sterile 0.5 inch discs are saturated with heat-treated milk samples and placed onto the agar surface by the use of sterile forceps.
5. Filter paper circles are inserted under the plate covers.
6. The plates are incubated at 55°-60° C.
7. At the end of two hours the plates are removed and examined for zones of inhibition.

The Bacillus stearothermophilus can be used in the TTC test for added sensitivity.

Nitrate test

1. Nutrient agar slant streaked with Micrococcus pyogenes var. aureus and incubated at 37° C. for 24 hours is used as the test organism.
2. The test organism is removed from the agar slant by means of a wire loop, and suspended in nutrient broth. The broth is incubated for 14 hours.
3. Ten ml of the suspected milk samples are pipetted into a 6 x 5/8 inch test tube. Ten ml of an inhibitory-free skim milk is pipetted into another test tube. To this

control and to the suspected samples are added 0.1 ml of 20 percent sodium nitrate solution and five ml of the suspension of organisms.

4. Shake thoroughly and incubate for 90 minutes at 37° C.
5. After incubation, one ml of 15 percent trichloroacetic acid solution is added to each test tube. The tubes are shaken and allowed to stand for one minute.
6. The contents of the tubes are filtered through No. 40 Whatman filter paper.
7. Five ml of the filtrate and five ml of nitrite-free distilled water are next pipetted into a 6 x 5/8 inch test tube and placed in an ice-water bath.
8. Add one ml of sulfanilic acid solution and allow to stand for five minutes.
9. The Klett-Summerson colorimeter reading is used to obtain a standardized value as shown in the following formula:

$$\frac{\text{Colorimeter reading of suspected sample}}{\text{Colorimeter reading of control}} \times 100 = \text{standardized value}$$

Interpretation

Samples which show less color intensity than the control contain inhibitors.

Sensitivity of tests

The sensitivity of the tests previously discussed are dependent upon a variety of factors. Some of the important factors which cause variation in sensitivity when assaying milk for inhibitory substances are:

1. Type of organism used for the assay
2. pH of the medium
3. Nutrients in the medium
4. Thickness or depth of nutrient again in petri dish
5. Number of virile organism in seeded agar medium
6. Time and temperature of incubation
7. Phase of organism (lag. log. stationery, or death)
8. Type of antibiotic or combination of antibiotic in the milk
9. Amount of the inhibitory substance present
10. Size (diameter) and thickness of discs
11. Amount of oxygen and sunlight (ultriviolet) present
12. Number of discs used (two, one on top of another gives greater sensitivity.)

The literature has several conflicting claims for the sensitivity of the different tests (13, 14, 28, and others). The multiplicity of factors mentioned above, coupled with the different laboratory equipment and different technicians, working with organisms having the same name and morphology, but reacting differently, not to mention the problems of dilutions, age of test organism and stability of the antibiotics, makes it understandable why differences in sensitivity occur throughout the literature.

No single organism has been discovered which is equally sensitive to the different antibiotics. The Food and Drug Administration recommends different organisms when assaying for different antibiotics, in other words, if one desires to assay for antibiotics, he should assay for each one separately, which makes it impractical for the technician

who is not operating in a research laboratory. Following is a list of antibiotics with the recommended assay organisms (21).

Penicillin	Sarcina lutes
Streptomycin	Bacillus subtilis
Tetracycline	Bacillus cereus var. mycoides
Bacitracin	Micrococcus flavus
Polymixin	Brucella bronchiseptica
Erythromycin	Sarcina lutea

Summary of sensitivity

The author has made a study of the literature in an attempt to develop a guide which would show approximately how small of a concentration of antibiotic could be detected using one of five popular assaying procedures (Reduction of Dyes, Agar Plate, Microscopic Examination, Nitrate Test, and Acidity-Coagulation Test). Following is the summary showing the lowest concentration detectable by each test. Where two or more concentrations are given the claims are from different authors, publication and/or using different organisms, and/or modifications of the basic test.

Dye reduction tests

Penicillin (units per ml) 0.3, 0.2, 0.05, 0.04, 0.025, 0.02, 0.01, and 0.005.
Chlortetracycline (micrograms/ml) 0.2 and 0.03.
Chlorine (PPM) 30.
Iobac (PPM) 30.
Quaternary Compounds (PPM) 10.
Iosan (PPM) 8.
Tetracycline (micrograms/ml) 0.05
Aureomycin (micrograms/ml) 0.03, 0.2, 0.1, and 0.05.
Streptomycin (micrograms/ml) 4.0, 0.7, 0.6, and 0.5.
Neomycin (micrograms/ml) 0.8.

Direct morphological examination

Penicillin (units per ml) 0.05, 0.025, and 0.015.
Tetracycline (micrograms/ml) 0.15
Bactracin (micrograms/ml) 0.01
Chloramphenicol (micrograms/ml) 0.75
Iodophors (PPM) 40.0

Acidity-coagulation tests

Penicillin (units per ml)	0.02
Aureomycin (micrograms/ml)	0.50
Dihydrostreptomycin (micrograms/ml)	6.50
Terramycin (micrograms/ml)	0.70
Tetracyn HCL (micrograms/ml)	0.90

Agar plate tests[1]

Penicillin (units per ml)	0.05, 0.02, 0.01, and 0.005.
Streptomycin (micrograms/ml)	0.75
Quaternary Ammonium (PPM)	50.

Nitrate test

Penicillin (units per ml)		0.1
Chlortetracycline	(PPM)	0.5
Streptomycin	(PPM)	1.0
Oxytetracycline	(PPM)	0.9
Bactracin	(PPM)	10. units/ml

The sensitivity of the above mentioned tests have no practical value unless a tolerance can be established for other organisms which is greater than the tolerance shown for the test organisms. This means, that if the test organisms show negative for inhibitory substances, the organisms which are used to make fermented products such as cheese, starter, buttermilk, and yogurt, must be able to function normally in the same concentration which yielded the negative test. Around this problem, the following thesis is oriented.

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[1] The cylinder plate method has been adapted for use as the official procedure by the Food and Drug Administration Laboratories (21).