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THE SANITARY SIGNIFICANCE OF THE FECAL AND ORAL STREPTOCOCCI IN WATER AND FOODS

by

STEVEN S. WEINSTEIN

B. S., Rensselaer Polytechnic Institute, 1964

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ABSTRACT

THE SANITARY SIGNIFICANCE OF THE FECAL AND ORAL STREPTOCOCCI IN WATER AND FOODS

BY

STEVEN WEINSTEIN

A study was undertaken to evaluate the use of the fecal and oral streptococci as indicator organisms of contamination of water and foods. Two major objectives were pursued.

The first was to determine to what extent the fecal streptococci occur apart from the intestinal tracts of humans and animals, and to determine whether the presence of these organisms in soil and plants could be ascribed to sources other than those arising through human or animal contamination. The results showed that the numbers of fecal streptococci in unfertilized and commercially fertilized soil fluctuated with the season. Greater numbers occurred in the summer months than in winter. The number of fecal streptococci correlated more closely with the numbers of the fecal coliforms than with the coliforms. It was noted in this study that apparently 30-40 per cent of the streptococci isolated from soil and plants could not be placed in any of the established species because of their divergency in the fermentation of certain sugars. These organisms fulfilled all the requisites for Sherman's enterococcus group and were designated _S. faecalis_-like and _S. faecium_-like. The _S. faecium_-like organisms occurred most frequently. In this study yellow pigmented streptococci were isolated from garden soil in the early spring and late

summer. Fecal streptococcus-like organisms were isolated from the exterior surfaces of corn, tomatoes, and green beans but rarely on the interiors parts of the vegetables. It was concluded from the distribution and types of fecal streptococci present in soil and on plants that their presence was most likely due to animal or insect contamination.

The second objective was to evaluate the use of *Streptococcus salivarius* and *Streptococcus mitis* as indicators of human pollution of water and foods. A new and improved medium for their isolation was developed. The medium is a mannitol-azide broth with the incorporation of penicillin and kanamycin. It was called penicillin-kanamycin MS broth. It was used to enumerate *S. salivarius* and *S. mitis* in human feces and foods while inhibiting the growth of all other organisms. The average number of oral streptococci in human feces was ten thousand per gram. However, when discharged into sewage or water, these organisms died out much too rapidly to be detected 24 hours later. Thus the failure to isolate *S. salivarius* and *S. mitis* in sewage led to the conclusion that these organisms would not be useful indicators of human fecal pollution of water.

The penicillin-kanamycin MS broth was used to test for the presence of the oral streptococci in frozen meat pies. *S. salivarius* and *S. mitis* were isolated from 71 per cent of the samples examined ranging in numbers from 7 to 17,500 per gram. It was also noted that when large numbers (over 1000 per gram) of fecal streptococci were encountered there was a corresponding increase in the number of oral

streptococci present. This suggested that the source of these organisms was due to both fecal and oral contamination.

An experiment was designed in an effort to trace one possible route by which *S. salivarius* and *S. mitis* may enter a food product. It was established that if these organisms come into contact with a surface such as a stainless steel counter they will remain viable for periods up to 72 hours. From this study it was concluded that these organisms have an excellent chance of being introduced into a food product by surface contaminated hands or materials. The presence of these organisms in foods would represent oral pollution by coughing and sneezing in addition to fecal pollution. Since neither *S. salivarius* nor *S. mitis* have been isolated from animals, the presence of these organisms in foods would represent pollution by the human rather than by both human and animal as is the case with *Escherichia coli* and with the fecal streptococci. Thus a sensitive testing methodology has been achieved which permits a more critical evaluation of handling procedures.

INTRODUCTION

The best single criterion by which the sanitary quality of water or food may be judged is the kind and numbers of bacteria that are present in it. One of the purposes of the bacteriological examination of water and foods is to ensure their freedom from human and animal excreta and therefore from organisms which may cause enteric infection such as the salmonellae and enteric viruses.

Generally, the determination of the presence or absence of fecal contamination has been based upon the use of indicator organisms of fecal contamination. These indicators of pollution have been bacterial organisms whose natural habitat is the intestinal tract of humans and animals. Therefore, their presence in food or water suggest fecal contamination.

The use of the coliform group of bacteria as indicators of fecal pollution of water has been a standard practice for nearly sixty years. Routine procedures for detection have been worked out and published in _Standard Methods for the Examination of Water and Wastewaters_, Twelfth Edition, 1965.

Although the coliforms are the current standard for assessing the sanitary quality of drinking water, several disadvantages have been cited in recent literature. One weakness of the coliform test is that it does not distinguish between human and animal contamination. Such differentiation would be valuable when the mode of contamination and its potential danger is to be judged. The coliforms have been

reported to multiply in certain waters which does not reflect a true indication of the amount of fecal pollution in a given water.

Due to the disadvantages cited above of using the coliforms as indicator organisms of fecal pollution of water, there has been a search for other indicator organisms to be used in place of, or in combination with the coliforms for determining the presence of fecal contamination in water. The fecal streptococcus group has been one of those groups. The natural habitat of these bacteria, like the coliforms, is the intestine of man and animals. The fecal streptococci offer certain advantages over the coliforms, in particular *Escherichia coli*, as indicator organisms for water in that they apparently never multiply in water, but disappear rather rapidly when added to streams or lakes (Leninger and McClesky, 1953). On the other hand, Mundt (1964) has claimed that the fecal streptococci are epiphytes or potential epiphytes on plants, and as a result, their presence in food or water may or may not indicate human or animal fecal pollution.

The oral streptococci, particularly *Streptococcus salivarius* and *Streptococcus mitis*, have been suggested by Appleman (1967, 1969) as possible indicators of human fecal contamination. Their natural habitat is the human mouth; however, some investigators have reported their presence in human feces (Sherman *et al*, 1943; Chapman, 1946; Kenner *et al*, 1961; and Appleman, 1967). Other than these reports, there has been only scant information of the presence of

these organisms in feces, sewage, water, or foods.

With this background information in mind, two major objectives were pursued in this investigation. The first was to determine to what extent the fecal streptococci occur apart from the intestinal tracts of humans and animals, and to determine whether the presence of these organisms in soil and plants could be ascribed to sources other than those arising through human or animal contamination. The second objective was to evaluate the use of the oral or viridans streptococci as indicators of fecal and oral pollution of water and foods.

REVIEW OF LITERATURE

Nomenclature and taxonomy of the fecal streptococci

There have been many studies on the classification of the enterococci since Thiercelin in 1899 first used the term "enterococcus" to designate a gram-positive diplococcus of intestinal origin. Andrews and Horder (1906) gave the name of "*Streptococcus faecalis*" to a group of nonhemolytic streptococci isolated from the human intestine.

A significant contribution to the classification of streptococci was made by Orla-Jensen (1919) who based his divisions on temperature limits of growth, tolerance of heat and sodium chloride, and fermentation characteristics. He identified two physiological types: (1) *Streptococcus faecium* and (2) *Streptococcus glycerinaceous* and *Streptococcus liquefaciens*. These species were heat-resistant streptococci of fecal origin that initiated growth at 10 and 45 C. *S. liquefaciens* differed from *S. glycerinaceous* only in its ability to liquify gelatin. In contrast to the *S. glycerinaceous* type, *S. faecium* fermented arabinose and seldom fermented glycerol or sorbitol. Orla-Jensen also noted the occurrence of streptococci from bovine feces that possessed unique physiological characteristics which differentiated them from other fecal streptococci. He proposed the name of *Streptococcus bovis* for these strains.

Dible (1921) reported that Andrews and Horder's "*S. faecalis*" was synonymous with Thiercelin's "enterococcus." He correlated morphology with fermentation reactions and

heat resistance. The mannitol-fermenting and heat-resistant strains were cocci occurring in pairs and in chains corresponding to the S. faecalis type, whereas the non-mannitol fermenting and heat sensitive strains were long chained streptococci corresponding to the Streptococcus salivarius type.

In 1937 Sherman published the first real definitive members of the genus Streptococcus. His scheme divided the genus into four divisions based on the ability to initiate growth at 10 and 45 C. He used the term "enterococcus" to designate a group of streptococci including hemolytic, non-hemolytic and gelatin-liquefying types. Sterman's enterococcus division excluded S. bovis and Streptococcus equinus, as these organisms did not initiate growth at 10 C or in broth containing 6.5 per cent sodium chloride. These streptococci were grouped in his "viridans" division. He considered the enterococcus group to share the following criteria; i.e., growth at 10 and 45 C, at pH 9.6, in 6.5% NaCl broth, in 0.1% methylene blue milk, survival at 60 C for 30 minutes and production of ammonia in peptone broth. According to his classification, the enterococcus group was comprised of four species namely, S. faecalis, Streptococcus zymogenes, S. liquefaciens, and Streptococcus durans. However, in a later study (1938), the close relationship of S. faecalis, S. liquefaciens, and S. zymogenes was noted. Varietal status was proposed for S. liquefaciens and S.zymogenes because these organisms differed from S. faecalis only in their proteolytic and hemolytic abilities. S. durans had many

differing physiological characteristics, and it was considered that this organism was distinctive at the species level. The name was appropriate in view of the organism's extreme tolerance to heat and desiccation. Sherman reported that the S. glycerinaceous of Orla-Jensen was synonymous to Andrews and Horder's S. faecalis. He also discounted the S. faecium species because all enterococci fermented glycerol. However, since Sherman's work there has been considerable evidence suggesting that S. faecium deserves recognition at the species level.

Niven, Smiley, and Sherman (1942) reported S. salivarius to be the predominating microorganism in most human throats. They reported that fecal samples from 15 persons yielded S. salivarius in numbers ranging from 1200 to 129 million per gram of feces. The medium used for isolation incorporated sucrose and sodium azide in a suitable nutrient agar. In 1943 Sherman, Niven, and Smiley stated that S. salivarius appeared to be a homogeneous and clearly defined species, especially marked by its ability to synthesize large amounts of polysaccharide from sucrose and to ferment both raffinose and inulin, and its inability to produce greening in blood agar. However, Streptococcus mitis was differentiated from S. salivarius by producing greening on blood agar, failing to ferment raffinose and inulin, and failing to synthesize polysaccharide from sucrose. Safford, Sherman, and Hodge (1937) also noted that unlike enterococci, S. salivarius did not grow at 10 C, was sensitive to heating at 60 C for 30 minutes, and did not produce ammonia from peptone.

Skadhauge (1950) investigated the ability of the enterococci to grow in a solid medium containing potassium tellurite (1:2500) and found that S. faecalis and its varieties, liquifaciens, and zymogenes were resistant to tellurite. The tellurite-sensitive strains included S. durans and S. faecium. Barnes (1956a) observed that the two species had different reduction and fermentation reactions. She found that, under appropriate conditions, S. faecalis reduced 2,3,5-triphenyltetrazolium chloride strongly, while S. faecium reduced this compound weakly or not at all. Barnes and Ingram (1955) and Barnes (1956b) reported that S. faecalis usually produces reduction, acid and coagulation in litmus milk in 24 hours, but that S. faecium reduces litmus milk much less rapidly and that sometimes acid production is the only reaction noted.

Deibel, Lake, and Niven (1957) further reported that S. faecalis was distinctive in that it utilized citrate, gluconate, and glycerol (anaerobically) as sources of energy. S. faecium strains gave opposite reactions. They found that the species, S. faecium, was quite distinct from S. faecalis with properties very similar to S. durans. They felt that the enterococci should be classified into distinct species with varieties in each respective species. S. zymogenes and S. liquefaciens would be considered varieties of S. faecalis, whereas S. durans should be considered a variety of S. faecium

Colobert and Morellis (1958) conducted a study of the homogeneity of the S. faecalis species by means of the

Adamsonian method. They concluded that S. faecalis was made up of the groups: S. faecalis proprium in one group and all the biotypes or varieties in the second group. They felt that the species S. faecalis determined according to Sherman's criteria consisted of a great number of biotypes.

There is much less controversy concerning the species status of S. bovis (Orla-Jensen, 1919) and S. equinus. S. bovis is clearly differentiated from the other species of fecal streptococci since it ferments both starch and inulin, fails to grow at pH 9.6, in 6.5% NaCl or at 10 C, and does not hydrolyze arginine. S. equinus is differentiated from S. bovis by its inability to hydrolyze starch.

Papavassiliou (1961) identified the majority of the strains he studied as S. faecalis, S. faecium, or S. bovis. Several strains either shared characteristics of two species or were completely atypical. He concluded that growth in the presence of potassium tellurite (1:2500) and in the presence of 6.5% NaCl and the fermentation of arabinose, glycerol, and raffinose were very important tests for the identification of the three species. The reduction tetrazolium, litmus milk, and fermentation of sorbitol were also helpful. However, he believed that the "pattern of reactions" was more important than single tests.

Hartsell and Caldwell (1961) reported that S. faecalis and its varieties were lyzed by a combination of lysozyme and trypsin, but not by lysozyme alone; S. faecium and S. durans were lyzed by lysozyme alone; and S. bovis was completely resistant to lysis. Chesbro (1961) tested the

sensitivity of the enterococci to lysozyme. The enterococci tested exhibited a spectrum of lysozyme sensitivity, with *S. faecalis* and its variety *liquefaciens* being the most resistant, while *S. faecium* and biotypes varied from partial to complete sensitivity. Data obtained by using these and other tests (Deibel, 1964a) have shown that *S. faecium* deserves status as a distinct species. Barnes (1964) and Whittenbury (1965) believe that the key differential tests are tellurite resistance and tetrazolium reduction.

Of great importance is the recent work of Deibel, Lake, and Niven (1963). They recognize only two species of fecal streptococci. The first is composed of *S. faecalis* (Andrews and Horder, 1906), which includes *S. glycerinaceous* (Orla-Jensen, 1919) and its varieties, *liquefaciens* and *zymogenes*. The second species is composed of *S. faecium* (Orla-Jensen, 1919) and its variety, *durans*.

Deibel (1964) noted that although all enterococci hydrolyze arginine, only *S. faecalis* and its varieties can utilize this amino acid as an energy source. Deibel and Niven (1964) also demonstrated that *S. faecalis* utilizes pyruvate as an energy source for growth.

Deibel (1964a) stated that in classifying the fecal streptococci "dependence must be placed on a spectrum of characteristics possessed by the strain in question, and its failure to comply in a few specific tests does not constitute sufficient grounds to negate speciation if it conforms with the overall species description. As in any taxonomic scheme, the occurrence of some transitional types

is to be expected." Deibel draws some generalizations from previous data concerning S. faecalis and S. faecium. S. faecium grows at a higher temperature, and tolerate heat to a greater extent than does S. faecalis. Also, S. faecium seems to be more tolerant to highly alkaline conditions (Chesbro and Evans, 1959). S. faecalis has a greater metabolic diversity than S. faecium.

Whittenbury (1965) concluded that of all the established tests, only reducing activity and potassium tellurite tolerance completely differentiated S. faecalis and S. faecium. The ability of S. faecalis to use citrate and malate as energy sources also differentiated these two species.

Raj and Colwell (1966) undertook a study of streptococci taxonomy by computer analysis. They recognized the fecal group as S. faecalis and varieties liquefaciens and zymogenes, S. faecium, S. durans, and so-called unclassifiable strains. The fermentation of raffinose by S. bovis seemed to be the only criterion which separated it from unclassifiable types. S. faecalis and varieties were differentiated from the other enterococci by the ability to reduce tetrazolium and by tolerance of 0.04% K-tell. Other enterococci showed three basic characteristics - slight or no ability to reduce tetrazolium, sensitivity to potassium tellurite, and lack of proteolytic activity. S. durans showed complete inability to utilize any of 8 sugars tested with the exception of melibiose fermented by one-half of the strains.

Hartman, Reinbold, and Saraswat (1966) in their excellent review of "Taxonomy of the Fecal Streptococci" defined the terms "enterococci," "fecal streptococci," "group D streptococci," and "*Streptococcus faecalis*." They included in the fecal streptococci all *Streptococcus* spp. consistently present in significant numbers in fresh fecal excreta, when adequate methods for detection of all types present are used. They included in the fecal streptococci the enterococcus group of Sherman, *S. bovis*, *S. equinus*, *S. salivarius*, and *S. mitis*. The latter 4 organisms were called the viridans group. The group D streptococci were composed of the enterococcus group and *S. bovis* and *S. equinus*.

Motility and Pigmentation

Graudal (1952, 1957) published reports on motile enterococci. He investigated the characteristics of 129 motile strains, predominantly of fecal origin, and found them all to be enterococci, including both *S. faecalis* and *S. faecium*. All strains contained the antigen characteristics of Lancefield Group D (1933) as well as thermostable O-antigens and thermolabile flagellar (H) antigens. Subsequent studies of the motile variety indicated that these organisms are widely distributed in nature and are not characteristic of any given environment.

Langston, Gutierrez, and Bauma (1960) described 11 motile strains of streptococci isolated from grass silage. Their results showed that these strains were similar to *S. faecium* based upon many of the properties already established.

They proposed the name of S. faecium var. mobilis, for these strains.

Hugh (1959) studied 6 strains of motile enterococci isolated from the oropharyngeal tonsil regions. In contrast to Langston et al (1960) and Graudal (1957), he considered the motile group D streptococci as another variety of S. faecalis.

Deibel (1964a) in his review of Group D streptococci pointed out that aside from their motility, motile strains were typical fecal streptococci, and the establishment of a varietal status (i.e., mobilis) has little taxonomic value and does not appear to be warranted.

A few investigators have reported finding yellow pigmented streptococci (Hannay, 1950; Graudal, 1957). Graudal isolated 4 strains from infant feces and 4 strains from seawater. All eight strains fulfilled the Sherman criteria. The 4 fecal strains fulfilled Sherman's criteria. The marine strains resembled S. faecium. Hannay isolated 3 strains from dairy cows which were identical to S. faecalis, except that they fermented raffinose.

Mundt and Graham (1968) reported the isolation of yellow streptococci from a variety of plants. These strains possessed the properties of the enterococci. They constituted a homogeneous group with properties of both S. faecalis and S. faecium, but because of unique attributes which set them apart from these species, they proposed the name S. faecium var. casseliflavus for these organisms.

<u>Media for isolation of the fecal streptococci</u>

Since the fecal streptococci have been suggested as indicators of fecal contamination of water and foods, efforts to develop media for the successful isolation, enumeration, and identification of the fecal streptococci have been many. A satisfactory medium for routine use should preferably be selective so that all other organisms present will be inhibited or suppressed.

One of the first selective media for the isolation of streptococci was described by Fleming (1932), who used tellurite to inhibit the growth of gram-negative bacteria. McKenzie (1941) proposed the use of thallium acetate as a third type of agent for the selective isolation of streptococci.

Hajna and Perry (1943) developed a medium, SF (*S. faecalis*) medium which was highly specific at 45.5 C for fecal streptococci. They reported that growth accompanied by an acid reaction, as shown by the change in color of the indicator in the medium, was almost complete evidence of the presence of fecal streptococci. The medium contained sodium azide and brom cresol purple as inhibitory agents.

White and Sherman (1944) devised a selective medium for the determination of fecal streptococci in milk. Their medium contained 325 units of penicillin combined with 0.03 per cent sodium azide. All fecal streptococci grew on this medium with the exception of *S. durans* which was partially inhibited. In 1946 Winter and Sandholzer modified the medium by doubling the concentration of penicillin and adding 0.001 per cent methylene blue.

Chapman (1944) reported the development of two solid media for the isolation of streptococci: Tellurite streptococcus medium and azide violet blue medium. The tellurite streptococcus agar contained crystal violet, trypan blue and sodium tellurite as inhibitory agents; the azide violet blue agar contained sodium tellurite, sodium azide, and crystal violet.

Chapman (1946) devised Mitis-Salivarius Agar for the isolation of streptococci from feces; while this medium was designed primarily for the isolation of _S. salivarius_, it was also found to support the growth of fecal streptococci.

A procedure for the primary isolation of enterococci developed by Winter and Sandholzer (1946) consisted of two parts: (1) A presumptive test in which the production of acid and growth turbidity in a dextrose azide medium after incubation at 45 C was interpreted as presumptive evidence of the presence of enterococci; and, (2) A confirmative test in which positive presumptive tests were confirmed by inoculation into a slantbroth preparation of a penicillin-sodium azide medium. Pin-point colonies on the slant, growth sediment in the broth, and a catalase-negative test were interpreted as confirmed evidence of enterococci.

Osterolenk and Hunter (1946) used the SF medium of Hajna and Perry to determine the distribution of fecal streptococci in animals. They found that this medium supported the growth of some non-streptococcus organisms. The addition of 6.5 per cent sodium chloride to the SF medium reduced the numbers of non-streptococci.

Wang and Dunlop (1951) found that the Winter-Sandholzer presumptive broth gave many false positives, and they modified the broth by increasing the protein content and by adding dipotassium phosphate. They compared the productivity of SF medium, the original Winter-Sandholzer Presumptive Broth, and the modified Winter-Sandholzer Broth. They found that SF medium was less productive than the Winter-Sandholzer Broth. They concluded that the SF medium was inhibitory to some strains of enterococci, and that enterococci were sometimes present in negative SF cultures.

Mallman and Seligman (1950) studied the use of lactose broth, Mallman's azide broth, Hajna and Perry's SF broth, and Rothe's Azide Dextrose Broth (1948) for the detection of fecal streptococci in water and sewage samples. They found that Rothe's medium had the greatest productivity, followed in decreasing order by Mallman's Azide Broth, Lactose Broth and SF Broth.

In 1953, Litsky, Mallman, and Fifield reported the development of confirmatory medium for use with a presumptive test utilizing Rothe's Azide Dextrose Broth. They found that a medium containing 0.02 per cent sodium azide and a 1:1,200,000 concentration of ethyl violet inhibited the growth of all gram-negative bacteria and all gram-positive bacteria other than enterococci. They reported that this medium was 100 to 1000 times as productive as the W-S method for the isolation and enumeration of enterococci in river water and sewage specimens. A slight modification of the medium was later introduced by Litsky, Mallman, and Fifield

(1955): the concentration of dextrose in the ethyl violet broth was reduced from 1.5 per cent to 0.5 per cent. Three loopfuls of inoculum from each positive presumptive tube were transferred to a tube of the confirmatory medium, Ethyl Violet Azide Broth (EVA). Incubation was at 37 C for 48 hours; the presence of enterococci was indicated by turbidity and usually by the formation of a purple "button" at the bottom of the tube.

Reinbold, Swern, and Hussong (1953) developed a selective plating medium for the isolation of the enterococci. The method was based upon the ability of the enterococci to utilize sodium citrate as a carbon source, to convert ditetrazolium chloride to a blue diformazan, and to grow in the presence of 0.01 per cent sodium azide. However, this medium permitted growth of lactobacilli.

Cooper and Ramadan (1955) compared three media (tetrathionate broth, potassium tellurite medium, and thallium acetate medium) for the direct isolation of fecal streptococci from human, cow, and sheep feces; they found that a potassium tellurite medium was successful in isolating streptococci from 97 per cent of the 30 specimens examined. The thallium acetate medium was equally as productive when animal feces were examined, but it failed to demonstrate the presence of enterococci in a number of specimens of human feces. The tetrathionate broth failed significantly in concentrating streptococci from sheep and bovine feces.

Slanetz, Bent, Bartley, (1955) modified Chapman's Mitis-Salivarius Agar Medium for the isolation of enterococci.

They used TTC and sodium azide as inhibitory agents, and found that this medium always gave higher estimates of enterococci sensities than did the tube-dilution procedure of Winter and Sandholzer (1946). They found that this medium inhibited practically all types of bacteria other than enterococci and gram-positive bacilli.

Barnes (1956) studied the reducing properties of many strains of fecal streptococci and noted that 2,3,5-triphenyltetrazolium chloride (TTC) was reduced rapidly by S. faecalis and its varieties, but not by S. faecium, S. faecium var. durans, or S. bovis. She used the differential properties of different species of streptococci to advantage in devising two thallous salt-containing media for selective enumeration and differentiation of S. faecalis and S. faecium. S. faecium and its variety durans reduce TTC poorly or not at all at PH 6.0 and produce while colonies. S. faecalis and its varieties grow well and reduce TTC strongly to an insoluble red triphenylformazan.

Slanetz and Bartley (1957) reported an improved medium for use with membrane filters for the enumeration of enterococci in water. The medium was called M-enterococcus Agar and contained 0.04 per cent sodium azide as an inhibitor and 0.01 per cent TTC as a differentiating agent. With this medium, they reported a ratio of enterococci to coliforms of 1.9:1 for the water samples, 1:1.7 for the sewage samples, 1:1.6 for fecal samples from humans, and 15:1 for fecal samples from animals.

Mossel, Van Diepen, and de Bruin (1957) used Packer's

crystal violet sodium azide blood agar (Packer, 1943), for the enumeration of fecal streptococci in foods. They found that this medium, used in pour plates at 36 C, gave satisfactory recovery of Lancefield group D Streptococci and completely inhibited the growth of 11 other species of aerobic and anaerobic food bacteria, including S. lactis.

Chesbro and Evans (1959) studied some factors affecting the growth of enterococci in highly alkaline media. They found that 0.05 M (0.53%) sodium carbonate and 0.05% Tween 80 facilitated isolation of S. faecium from fecal samples in a medium for the isolation of S. faecalis, sensu strictu. This medium was based on the ability of S. faecalis: To grow at 45 C following 4 hours resuscitation at 37 C, to ferment sorbitol, to decompose tyrosine, and to reduce TTC at pH 6.2.

Kjellander (1960) developed an Azide-Sorbitol Agar which was selective for fecal streptococci and which was also capable of differentiating streptococci according to their capacity to ferment sorbitol. Sodium azide (0.05%) and crystal violet (1:800,000) were the selective agents. An addition of 0.5% sorbitol and brom thymol blue as an indicator permitted differentiation of the sorbitol (+) and sorbitol (-) streptococci.

Croft (1959) reported the results of tests on 185 water samples for enterococci by two tube dilution methods and by a membrane filter procedure. These were Azide Dextrose (AD) Broth with Ethyl Violet Azide (EVA) Broth confirmatory, Buffered Azide Glycerol Glucose (BAGG) Broth with EVA

confirmatory and M-enterococcus Agar. AD-EVA and M-enterococcus Agars were found to give the same order of productivity. BAGG Broth gave a lower yield of enterococci in 70.8% of the water specimens tested.

Kenner, Clark, and Kabler (1961) reported a medium for the growth of fecal streptococci, which with minor modifications could be used in a multiple tube method, with a membrane filter procedure, or by the agar pour plate technique. The medium was called KF Streptococcal Medium. It contained, among other ingredients, 0.04 per cent sodium azide, 0.0015 per cent bromcresol purple, and 0.01 per cent TTC. The KF media as an MPN test and as a membrane filter method, was compared with M-enterococcus Agar (MF), BAGG Broth (MPN), and Dextrose Azide (DA) Presumptive Broth with confirmation in Ethyl Violet Azide Broth (EVA). The KF medium used either as an MPN test or as a membrane filter method yielded higher results in the recovery of the enterococci than by other comparative tests.

Splittstoesser, Wright, and Hucker (1961) made a comparative study of media for enumerating enterococci in frozen vegetables. The following presumptive media were investigated: _S. faecalis_ (SF) Medium (Hajna and Perry, 1943), Buffered Azide Glucose Glycerol (BAGG) Broth (Hajna, 1951), Enterococcus Presumptive (EP) Broth (Winter and Sandholzer, 1946), AD Broth (Rothe, 1948), and Thallous Acetate (TA) Broth (Barnes, 1956). Their results indicated that with most of the vegetable samples AD Broth yielded the maximal number of fecal streptococci.

Saraswat, Clark, and Reinbold (1963) made a comparison of 10 media commonly used for the detection and isolation of streptococci of sanitary significance in water and dairy products. To determine selectivity for enterococci, 29 non-enterococcus cultures were plated in the various media. Media were eliminated on the basis of low recovery, growth of non-enterococcus cultures and variation in the size and color of colonies. The medium selected, the Citrate Azide Medium of Reinbold, Swern, and Hussong (1953), was modified by increasing the azide concentration to 0.04 per cent in order to inhibit all organisms other than streptococci from growing on the medium.

Burkwall and Hartman (1964) examined 15 agar media for their yield, selectivity, readibility and simplicity of preparation for enumeration of enterococci in frozen foods. The thallium medium of Barnes was selected as the better of the high yield-fair selectivity type of medium and an Azide-Citrate medium of Reinbold appeared to be the better of the low yieldhigh selectivity type of medium. Sodium carbonate was found to increase recovery substantially when added to certain media, especially in the presence of 0.05% Tween 80.

Slanetz and Bartley (1964) compared the productivity and selectiveness of the KF medium (Kenner, Clark, and Kabler, 1960) and the M-enterococcus medium using the membrane filter technique. They reported that for shellfish water samples only 28 per cent of the colonies growing on membranes with the KF medium were fecal streptococci against 98.5 per cent confirmation on the M-enterococcus Agar membranes. They

concluded that the KF agar did not appear suitable for the selective detection of fecal streptococci in sea water.

Rose and Litsky (1965) developed an enrichment procedure for use with the membrane filter for the isolation and enumeration of fecal streptococci in water. The recovery procedure followed was an enrichment phase in peptone-yeast extract-casitone (PYC) broth followed by an incubation phase on M-enterococcus Agar. The recovery ratio on M-enterococcus Agar as compared to enrichment was 1:2.44, a notable increase with enrichment.

Hartman, Reinbold, and Saraswat (1966) reviewed all the literature on media and methods for isolation and enumeration of the enterococci. They pointed out that organisms vary in their ability to grow under one set of conditions, and no single medium or set of conditions is likely to result in selective recovery of all of the fecal streptococci in a sample containing quantities of other closely related bacteria. Preliminary enrichment may overcome some of these difficulties. They concluded that the three most logical selections for use in water at present are the DA-EVA combination for use with the MPN procedure, M-enterococcus Agar for the membrane filter procedure, and KF agar and Broth for MPN, membrane filter, or direct plating. Regardless of the method selected, isolates should be confirmed as fecal streptococci.

Smith and Bodily (1967) reported a Methylene Blue Azide Medium for isolation of enterococci. Direct streaking of MBA with saliva failed to produce any bacteria capable of

growing on MBA. However, 52 catalase-negative, gram-positive cocci from human feces isolated from MBA were classified as enterococci. The MBA medium was judged to be a highly selective medium with poor yields. It provided some means of separating the oral streptococci from the fecal streptococci.

Serology

The serological differentiation of fecal streptococci from other streptococci is based on the grouping scheme originated by Lancefield (1933). She classified 106 strains of hemolytic streptococci isolated from man, animals, milk, and cheese into five groups. The Group D Streptococci were a uniform group of 8 strains all derived from cheese. Lancefield and Hare, Hare and Maxted (1935) demonstrated that *S. faecalis* belonged to the serological group D. Smith, Niven, and Sherman (1938) took 76 cultures of *S. faecalis* var. *zymogenes* and found them all to belong to the Lancefield Group D. Sherman (1938) found *S. faecalis* var. *liquefaciens* and *S. durans* to possess the group D antigen. Skadhauge (1950) demonstrated the group D antigen in *S. faecium*.

When improved methods were used for the preparation of antisera and of extracts for precipitin tests, *S. bovis* was also found to have the group D antigen (Shattock, 1949). She used HCl extracts precipitated with ethanol for precipitation tests. She reported her group D substance to be protein in nature.

Skadhauge (1950) by means of the agglutination reactions was able to demonstrate heat stable O antigens and

heat labile K antigens among the S. faecalis and S. faecium strains and a capsular A antigen among the S. faecium groups in strains of human origin. S. faecalis and its varieties could be divided into at least four groups based on the O-antigens. In contrast, strains of S. faecium, S. faecium var. durans, and unclassified strains examined showed no O-antigen relationships on which grouping could be based.

Sharpe and Shattock (1952) serologically typed a number of fecal streptococci associated with neonatal diarrhea. By preparation of specific type sera 24 types of streptococci within Group D were designated on basis of agglutination tests. While there was no absolute correlation between serological type and physiological characteristics, a broad division could be made between S. faecalis and varieties on one hand and S. durans, S. bovis, and unclassified strains on the other.

Sharpe (1952) reported that S. faecium of group D shared a common type antigen with S. lactis of group N.

Perry and Briggs (1955) examined 68 strains of streptococci isolated from the rumen of 4 cows. All strains showed the physiological characteristics of S. bovis of group D. They found the presence of a common type antigen in streptococci of groups D and E. Perry, Newland, and Briggs (1958) found that of 37 strains of group D streptococci isolated from the rumen of calves, 10 S. bovis and one unclassified strain shared a common type antigen with group N.

Graudal (1957) studied the serology of motile

streptococci. He found them to contain the group D antigen. These motile strains also contained type-specific thermostable O-antigens and thermolabile flagellar antigens.

Sharpe and Fewins (1960) serologically typed 85 strains of S. faecium and unclassified group D strains isolated from canned hams and pig intestines. Seventy-seven of the isolates were distributed amongst 15 types and 4 subtypes.

Smith and Shattock (1962), and Fuller and Newland (1963) found that streptococci isolated from horse feces possessed the Group D antigen. These streptococci corresponded to the description of S. equinus (Andrews and Horder, 1906). S. equinus was more refractile to serological grouping than S. bovis.

Medrek and Barnes (1962) found that by varying the composition of the growth medium, the optimum yield of group D antigen was obtained when the organisms were grown in an unbuffered medium containing 5-10 g glucose per liter where the ultimate pH of the culture was 4.0 to 4.2. In a buffered medium containing low glucose concentration the final pH value of the culture remained above 6.0 and poor yields of group D antigen in the acid extract were obtained with S. faecium, S. durans, and S. bovis.

Many workers have investigated the location of the group D antigen. Elliott (1959, 1960) reported that the group D antigen was not located in the cell wall but located wholly or in part between the cytoplasmic membrane and the cell wall. He thought that the group D substance was a glucose-containing carbohydrate possibly dextran-like in nature.

Jones and Shattock (1960) using gel-diffusion tests showed that the group D antigen was serologically identical in _S_. _faecalis_, _S_. _faecium_, _S_. _durans_, and _S_. _bovis_. They found that the antigen was located in the residue (cell contents) after the cell-wall fraction had been removed.

Shattock and Smith (1963) examined spheroplasts, protoplasts, and L-forms from group D streptococci for the presence of the group antigen. Their results indicated the absence of the group D antigen in the cytoplasm. They concluded that since they knew it was not an integral part of the cell wall, it must be located between the cytoplasmic membrane and the cell wall.

Smith and Shattock (1964) investigated the cellular location of antigens in Streptococci of Groups D, N, and Q. The absence of group D antigen from the cell walls and protoplasts of Group D Streptococci lead to the conclusion that this antigen was located between the protoplast membrane and the cell wall. The Group Q strains examined possessed the Group D antigen in the cell contents although these strains did not physiologically correspond to any establishd group D species.

Shockman and Slade (1964) confirmed previous investigations by finding that the major portion of the group D antigen was closely associated with the protoplast membrane of _S_. _faecalis_. Hijmans (1962) also reported the absence of the group D antigen in L-phase variants of group D streptococci.

Barnes (1964) studied the distribution of serological

types amongst 367 strains of fecal streptococci which were identified by physiological tests as S. faecium, S. durans or unclassified strains. Strains of the same serological type showed a diversity of fermentation reactions, so that organisms which could be identified as S. durans showed a common type antigen with S. faecium strains.

Wicken, Elliott, and Baddiley (1963), Elliott (1962, 1963), and Armstrong et al (1959) studied the identity of the Group D antigen. The intracellular teichoic acids from 2 strains of group D streptococci were purified and their serological and chemical properties examined. Both compounds were shown to react with group D antiserum. They were shown to be polymers of glycerol phosphate containing a high concentration of glucose and bearing alanine residues on the sugar. It was concluded that the Group D substance was a glycerol teichoic acid in the form of a polymer of glucosyl glycerophosphate.

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  alanyl - glycosyl - glycerol
                          1
                      O = P - OH
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They indicated that this intracellular teichoic acid was located between the cell wall and the protoplast membrane.

Ecology and sanitary significance of fecal streptococci

I. Presence of fecal streptococci in human and animal feces

The coliforms have been utilized in this country for measuring pollution because they are of enteric origin, survive

longer than enteric pathogens, which they outnumber in the intestinal tract, and can be easily isolated and identified by relatively simple bacteriologic techniques. However, there has been a continuing interest in the use of the fecal streptococci as indicators of fecal pollution.

Houston (1910) attached sanitary significance to the fecal streptococci because of their abundance in human feces, their absence from large bodies of pure water, and their inability to multiply under such conditions. He concluded that their presence would indicate recent fecal contamination and therefore, might be a better indicator of pollution than the presence of coliforms.

Winter and Sandholzer (1946) reported that while fecal streptococci were present in all samples of human and animal feces tested, these organisms were never found in virgin soils or in soils from wooded areas. These results were generally confirmed by Mallman and Litsky (1951), and Medrek and Litsky (1957).

Cooper and Ramadan (1955) attempted to distinguish human pollution from animal pollution by means of the fecal streptococci. They concluded that typical *S. faecalis* indicated human origin and that a starch-positive *S. bovis* indicated animal origin.

In France, Buttiaux (1958) examined feces of humans, swine, cows, and sheep for the presence of fecal streptococci. He found that *S. faecalis* and varieties are more frequent in man than in animals, but do exist in animals. *S. faecium* was always present in sheep; it was frequent in

cows, swine, and also in man.

The incidence of fecal streptococci in cattle, swine, and sheep was reported by Kjellander (1960). In general, *S. bovis* constituted the predominant *Streptococcus* in the feces of these animals. The incidence of *S. faecium* and *S. faecalis* was lower, and approximated 10 per cent of the total streptococcal flora.

Bartley and Slanetz (1960) made a study of over 5000 strains of streptococci isolated from water, sewage, and feces of human beings and animals. Typical *S. faecalis* was isolated from human feces, but not from animal feces except in one case from a dog. Thus, they considered this organism to be chiefly of human origin. Types designated as *S. faecalis* var. *liquefaciens* were also prevalent in human feces. In general, *S. bovis* was the predominant species of streptococci found in the feces of cows. From the swine feces tested, only *S. faecium*, *S. equinus*, and certain *S. faecalis* biotypes were identified. Bartley and Slanetz noted that while typical *S. faecalis* may not always be the predominant type of *Streptococcus* in human feces, it is not found in the feces of most domestic animals. Thus, its presence in water would indicate contamination of human origin. The presence of *S. bovis* in a water sample would indicate bovine or ovine pollution.

Kenner, Clark, and Kabler (1960) investigated the streptococci present in the feces of human beings and of a few domestic animals. They found the enterococcus and *S. salivarius* groups predominating in human feces; bovine species

showed appreciable quantities of the *S. bovis* and *S. equinus* organisms. Based on their results they considered the fecal streptococci to consist of the enterococcus group *S. mitis-salivarius* group, *S. bovis* group, *S. equinus* group, and an atypical group closely resembling the enterococcus group, but with one or more differences in critical biochemical tests.

Mundt (1963) studied the occurrence of enterococci in animals in a wild environment, where the influence of man was negligible. Enterococci were recovered from the feces of 71 per cent of 216 mammals, 86 per cent of 70 reptiles, and 32 per cent of 22 birds sampled in the Great Smoky Mountains National Park. *S. faecalis* occurred most frequently in the feces of wild animals.

II. Presence of fecal streptococci in plants and in soil

Sherman (1937) noted the common occurrence of enterococci on plants. He reported that *S. faecalis* and *S. liquefaciens* types were rather common. Mundt, Johnson, and Khatchikian (1958) examined leaves, flowers, and shoots of plants grown in cultivated and uninhabited areas for enterococci and coliform bacteria. They reported that "enterococci" were isolated from 58.5 per cent of 106 samples including 63 plant species, and coliform bacteria were isolated from 76 per cent of these samples. Mundt (1961) reported enterococci from 27 per cent of the flowers sampled and 6.8 per cent of the buds from the same plants. Totals of 34, 32.2, and 10.4 per cent, respectively, of the flowers from non-agricultural, agricultural, and grass plants contained enterococci. Mundt (1963) described the occurrence of enterococci on plants from

the Great Smoky Mountains. Enterococci were obtained from 14.2 per cent of 2169 flowers and 3.4 per cent of 440 samples of leaves, buds, shoots, fruits, and seeds, but he found no evidence of plant specific species or variants of the enterococci.

Mundt, Coggin, and Johnson (1962) attempted to determine whether S. faecalis is a potential epiphyte on plants. The occurrence in seed or soil, movement from the seed and the emergent parts, and the ability to reproduce on the growing plant are criteria of bacterial plant epiphytes. S. faecalis var. liquefaciens established commensal growth on each of 5 plants. The organisms, increased in numbers on the plants equally as well as did the control bacteria.

There is general agreement that enterococci are not native to the soil, and their presence in soil samples represents contamination from either animal or plant sources. Mallman and Litsky (1951) investigated the survival of enteric organisms in various types of soil. Coliforms were found to persist in soil for long periods of time. The enterococci were found to die out rapidly in the soil. They felt that the enterococci would be a better indicator of public health hazards from sewage in soils and on vegetables than the coliforms.

Medrek and Litsky (1960) investigated 369 undisturbed soil samples for the presence of coliforms and enterococci. Seventy-three per cent of the samples contained coliform bacteria and 1.4 per cent yielded typical E. coli. Enterococci were found in 2.2 per cent of the 360 samples examined.

They thought that there appeared to be a close relationship between the presence of the enterococci and E. coli.

Eaves and Mundt (1960) investigated the distribution and characterization of streptococci from insects. The numbers of enterococci were divided about equally between those identified as being or resembling S. faecalis, S. faecium and an intermediate group. The lack of any pattern in distribution and the random occurrence of the streptococci suggests that they are present as the result of contact between the insect and the streptococci in the environment.

Geldreich, Kenner, and Kabler (1964) studied the occurrence of coliforms, fecal coliforms, and streptococci on vegetables and insects. Their results indicated that typical coliforms of the warm-blooded animal gut accounted for a relatively small percentage of the organisms associated with vegetables and insects. Of the streptococci found on vegetables and in insects, S. faecalis constituted the majority of all strains located.

Mundt (1964) reviewed the sanitary significance of streptococci from plants and animals. Throughout the literature S. faecalis has been the preferred and only indicator of pollution. However, Mundt contends that S. faecium occurs commonly in the human, and in some instances it has been the only enterococcus isolated from human stools. He maintains that in view of the ubiquity of both species in man and animals, it does not seem logical to restrict oneself to S. faecalis as the indicator organisms of pollution, or to make a distinction between the human and the animals with

respect to origin.

With regard to plants, Mundt (1964) reported that they occur extensively on some species of plants, not at all on others, and at random on the remainder. Except for corn, they occur rarely on grasses and cereals. The enterococci occurring on plants may stem directly as the result of contamination from animal wastes. Mundt points out in his review that insects play an important role in the transfer of the bacteria from animals wastes to flowers.

III. Presence of fecal streptococci in water

From ecological studies conducted to date, it may be surmised that the chief source of fecal streptococci is the intestines of man and animals. Thus, the occurrence of these bacteria in water infers the presence of fecal contamination.

In comparing the fecal streptococci to the coliforms as indicators of pollution, Savage and Wood (1917) showed that when both coliforms and fecal streptococci were placed in a tank of water containing small amounts of organic matter, the fecal streptococci were uniformly and rapidly eliminated whereas the coliforms persisted for long periods of time and in some cases actually increased in numbers.

Using the method of Winter and Sandholzer (1946) for the enumeration of fecal streptococci, Lattanzi and Mood (1950) reported a ratio of 63 to 1 of *Escherichia coli* to enterococci in water samples from the harbor at New Haven, Connecticut.

Leininger and McClesky (1963) examined various surface waters for incidence of coliforms and enterococci. They found that the difference between relatively clean and recently polluted water was more strikingly shown by the presence and number of enterococci.

Litsky, Mallman, and Fifield (1955) concluded, from a study of the presence of _E. coli_ and the fecal streptococci in fresh water, that when _E. coli_ was present, 80 per cent of the samples also contained the fecal streptococci. They suggested that "the enterococci be taken out of the realm of stepchildren and given their legitimate place in the field of sanitary bacteriology as indicators of sewage pollution."

Burman (1961) studied the coliform-streptococcus ratio in various water sources and noted a more rapid decrease in coliforms, and a marked tendency was observed for the ratio to approach unity. Fecal streptococci were not detected in water known to be free from fecal contamination.

Slanetz, Bartley, and Metcalf (1964) evaluated the comparative efficiency of coliforms, fecal coliforms, and fecal streptococci as indicators of the sanitary quality of shellfish waters. They reported the ratio of coliforms to fecal streptococci in water samples to be 10:1 and of _E. coli_ to fecal streptococci 2.4:1.

Bacterial Indicators of Fecal and Oral Pollution in Foods

Many workers in the food industry have proposed the fecal streptococci as satisfactory indicators of plant sanitation. In considering the fecal streptococci and the coliforms as indices of fecal contamination, a number of

investigators (Lochhead and Jones, 1936; Parr, 1936 and 1938) have demonstrated that the coliform organisms in fecal material, broth cultures, or in frozen foods, show a decrease in numbers during initial freezing and in the first few weeks of storage. In contrast, the fecal streptococci have been reported to be exceedingly resistant to low temperatures and to adverse conditions (Dible, 1921; Sherman, 1937).

Burton (1949) examined 376 specimens of commercially frozen vegetables for fecal contamination by use of a presumptive coliform test and a fecal streptococcus test (S.F. medium). Seventy specimens originally containing both fecal streptococci and coliforms were stored at -20C for at least one year; after this storage period, 89 per cent were positive for fecal streptococci, while only 60 per cent were positive for coliforms. Burton concluded that the fecal streptococci were the superior indicators in frozen foods.

Allen and Fabian (1954) tested the viability of fecal streptococci and coliform bacteria in 12 foodstuffs with pH values ranging from 2.8 to 6.7. They concluded that *S. faecalis* survived longer than did *E. coli* in foods with a pH of 3.5 or lower, but that there was no apparent difference in the viability of fecal streptococci and *E. coli* in less acid foods.

Larkin *et al* (1955) has compared the viability of *E. coli* with that of the fecal streptococci stored at 0°F for more than 200 days. The numbers of fecal streptococci remained constant, whereas the numbers of *E. coli* decreased significantly during storage.

In 1959, Buttiaux recommended that Group D Streptococci be used as sanitary indicators of fecal contamination in foods. He said their specificity as an index of fecal contamination was high since their enumeration was easy with the selective media available.

Kereluk and Gunderson (1959) examined 188 frozen meat pies for total number of bacteria, coliforms, fecal streptococci, and staphylococci. Ninety-three per cent had total counts under 100,000 per gram and seventy-five per cent had total counts under 25,000 per gram. The fecal streptococci were isolated consistently and in greater numbers than the coliforms. They concluded that a proposed standard of 100,000 bacteria per gram was reasonable and would not cause any hardships on the producers.

In the identification of 307 isolates of fecal streptococci from frozen chicken, turkey, beef, and tuna meat pies Kereluk (1959) reported *S. faecalis*, *S. faecalis* var. *liquefaciens*, and *S. faecium* were the predominant types isolated.

Kereluk and Gunderson (1959b) stored *E. coli* and fecal streptococci in sterile chicken gravy at -6F. At the end of 481 days, the coliform count decreased significantly whereas the fecal streptococcus count remained constant. They concluded that the fecal streptococci were a more reliable indicator for frozen food than coliforms since they survived frozen storage better.

The oral streptococci (*S. salivarius* and *S. mitis*) have been isolated from the mouths of almost all humans but

have not been detected in the fecal material of any of the domestic animals examined (Appleman and Lewis, 1967). In 1969 Appleman and Belcher examined frozen and unfrozen food-stuffs for these organisms. They succeeded in isolating oral streptococci from 44 per cent of the samples using a 3 tube MPN of Mitis-Salivarius Broth with confirmation to Mitis-Salivarius Agar. They concluded that when these organisms are detected in foods they are indicative of contamination from human sources.

Contamination of food products from the air has been given very little consideration. Labots (48) and Cerna (5*) have reported on air-borne bacteria counts in dairy plants in foreign countries. Labots (48) used a slit air sampler and reported counts of 510 bacteria per cubic foot. Using the sedimentation technique, Cerna (5*) revealed that counts ranged from 1 to 500 colonies per petri dish per 10 minutes of exposure. They concluded that counts were effected by: (a) the presence of workers in the given area, (b) the number of workers in the area, and (c) the activity of the workers.

Heldman _et al_ (1964) used a slit air-sampler to determine the numbers of air-borne bacteria, yeasts and molds in dairy product packaging areas. The mean air-borne bacteria count for all food packaging areas investigated was below 6 per cubic foot. This was a low count when compared to the only other reported value for a dairy plant by Labot of 510 bacteria per cubic foot. From the authors' observations during test periods and results they concluded that

several factors may contribute to air-borne microorganism counts: (a) workers in the area, (b) moving parts of packaging machines, (c) ventilation systems (d) movement of materials, and (e) opening and closing of doors. The workers may act as a source of air-borne contamination by talking, coughing, and sneezing.

MATERIALS AND METHODS

Specimens Examined for Numbers and Types of Fecal and Oral Streptococci

A. Fecal Streptococci

Feces of animals and humans, raw sewage from the Durham, Exeter, and Derry, New Hampshire waste treatment plants, soils, vegetables, and various water samples were examined for numbers and types of fecal streptococci. The animals included cows, sheep, rabbits, and dogs. The soils were obtained from a fallow field, a garden, a densely wooded area, and an orchard. The vegetables tested included corn, tomatoes, and green beans collected from garden soil. Water samples included fresh water from the UNH reservoir and estuarine waters.

B. Oral Streptococci

Feces of animals and humans, raw sewage from the Durham and Dover, New Hampshire waste treatment plants, dairy products and frozen meat pies from retail outlets, and various surfaces such as table tops, cutting boards, and floors were examined for the presence of *S. salivarius* and *S. mitis*.

Preparation of samples

Animals: Fresh fecal material was collected in sterile containers at the UNH barns. The fecal material of rabbits and dogs were collected in the animal quarters of UNH in the case of the rabbits and locally in the case of the dogs. The fecal material in all cases was serially diluted

in sterile phosphate buffered distilled water (pH 7.2) containing glass beads.

Humans: The fecal material was collected in sterile containers from volunteers at the University. Samples were prepared as with animal feces.

Soils: Preparation of soil samples involved the collection of 10-20 grams of topsoil and lower layers to a depth of approximately 6 inches. Dilutions of 1-5 and 1-10 were made in sterile phosphate buffer water.

Vegetables: The exterior parts of the samples were examined as follows:

Corn husks: The husks of 2 ears of corn made up each sample. After weighing the husks, they were introduced into a Waring Blendor which contained the appropriate amount of dilution water to make a 1-5 dilution. The material was blended until a fine suspension was obtained.

Green beans and tomatoes: The surfaces of 10 pods made up each sample. The pods were washed in as many milliliters of sterile phosphate buffer as the sample weighed. Then 1.0, 0.5, and 0.1 ml portions were plated out in M-enterococcus agar. The same procedure applied to the tomatoes with each tomato representing one sample.

The interior parts of the vegetables were examined as follows:

Corn kernels: After the husks were removed, kernels from the cob of each ear were aseptically removed until a 25 gram sample was obtained. The sample was put into 225 ml of sterile dilution water and emulsified in a Waring Blendor

until a fine suspension resulted.

Green beans and tomatoes: The green beans and tomatoes were dipped in NaOCl (200 ppm), rinsed thoroughly in sterile water and dried with sterile towels. They were then weighed and introduced into a Waring Blendor and emulsified in the appropriate amount of dilution water to make a 1-5 dilution.

Primary isolation media

The following are the compositions of two selective media used in this investigation for the detection of fecal streptococci:

1. BBL M-enterococcus Agar (Slanetz and Bartley, 1957)

per liter of distilled water

tryptose	20.0 g
yeast extract	5.0
glucose	2.0
dipotassium phosphate	4.0
sodium azide	0.4
TTC	0.1
Agar	10.0

pH 7.2

2. Difco KF Medium (Kenner, Clark and Kabler, 1961)

per liter of distilled water

proteose peptone No. 3	10.0 g
yeast extract	10.0
NaCl	5.0
sodium glycerol phosphate	10.0
maltose	20.0
lactose	1.0
sodium azide	0.4
Na_2CO_3	0.636
brom cresol purple	0.015
TTC	0.1
Agar	20.0

pH 7.2 - 7.3

Procedures employed in isolation of fecal streptococci

For the animal and human fecal samples, one gram of fresh feces was added to 99 ml of sterile phosphate buffered distilled water containing glass beads. This was shaken until a uniform suspension was produced. Appropriate quantities of the suspension were filtered through membrane filters and cultivated on M-enterococcus and KF agars. The streptococcal densities were reported as colony counts per gram (wet weight) of feces for each group of samples.

For the soil samples and vegetables, preparations were carried out as outlined before. Then pour plates in triplicates were made using M-enterococcus and KF agars. Densities were reported as colony counts per gram of sample.

The water and sewage samples were filtered through membranes in portions varying from one ml to 100 ml depending on the amount of pollution expected. The membranes were then placed on M-enterococcus and KF agars, in 50 mm petri dishes and incubated at 37 C for 48 hours. Streptococcal densities were reported as number of streptococci per 100 ml of sample.

Confirmatory procedures used for identifying fecal streptococci

For each sample between 15 and 40 colonies were cultivated on brain heart infusion slants by incubation at 35 C for 24-48 hours. A gram stain was made and the following confirmation procedures were carried out:

1. Growth in brain heart infusion broth at 45.5 C in two days.

2. Catalase test - a negative catalase test indicating

a positive test for streptococci.

3. Growth in 40 per cent bile in three days at 35

.

Colonies that fulfilled the above 3 criteria and showed gram-positive cocci in chains or pairs were confirmed as fecal streptococci.

Biochemical tests used for classification of fecal streptococci

When colonies were confirmed by the above procedures as being fecal streptococci, they were then further characterized as to species or type of fecal streptococci. The following procedures or tests permitted differentiation of the various species within the group itself:

1. Tellurite tolerance - This test best separated *S. faecalis* and varieties from *S. faecium* and varieties. The following technique was used: A trypticase-glucose medium containing 1:2500 concentration of potassium tellurite were prepared and streaked with the 24 hour broth culture of the test strain and incubated at 37 C for 48 hours. The tellurite-resistant strains produced jet-black colonies and the tellurite-sensitive strains did not grow.

2. Reactions in litmus milk as observed at 24 hours - Results were recorded as R = reduction, A = acid, C = clot, Cu = curd, and P = peptonized.

3. Reaction in skim milk containing 0.1 per cent methylene blue at 24 hours. Results were recorded as for litmus milk.

4. Growth in 6.5 per cent sodium chloride in brain

heart infusion broth after 48 hours.

5. Growth in brain heart infusion broth adjusted to pH 9.6 in three days. For this approximately 5 ml of a 38 per cent solution of $Na_3PO_4 \cdot 12 H_2O$ were added to 100 ml of medium to achieve pH 9.6.

6. Production of ammonia from Arginine broth after 48 hours at 37 C.

7. Gelatin liquefaction - This test was used for the identification of _S. faecalis_ var. _liquefaciens_. Gelatin tubes were incubated for 48 hours at 37 C and then put into the refrigerator at 4 C for 30 minutes. Tubes were shaken to see if liquefaction took place.

8. Hemolysis - Blood agar plates containing 5 per cent sheep blood were incubated at 37 C for 48 hours and recorded for the type of hemolysis.

9. Fermentation tests - All strains were tested for the ability to ferment certain sugars, which have been found useful for differentiating the species and types within the fecal streptococcal group. These sugars included arabinose, glycerol, raffinose, sorbitol, inulin, melibiose, melezitose, sucrose, mannitol, and salicin. The following method was used: To phenol red broth base, which contains phenol red as an indicator, 0.5-1.0 per cent of the desired sugar was added. The media was dispensed in 3 ml amounts and autoclaved at 10 lbs/10 min with the exception of arabinose which had to be filter sterilized. Each tube was inoculated with one drop of a 24 hour broth culture and incubated at 37 C. The tubes were read after 48 hours by changes in reaction of the

indicator.

10. Starch hydrolysis - Starch agar plates were streaked with a 24 hour culture of the test strain. After incubating for 24 hours at 37 C, the plate was flooded with a solution of Lugol's iodine. The plate was examined for clearing around the colonies. This test was used for the identification of _S. bovis_, which hydrolyzes starch.

11. Growth in ethyl violet azide broth (EVA) - A ll strains were inoculated into EVA and incubated for 48 hours at 37 C after which tubes were examined for the production of a purple or white button at the bottom of the tube.

12. Citrate reaction - All strains were examined for the ability to utilize potassium citrate as a sole carbon source. Strains were inoculated into citrate medium which contained a Durham tube. After 48 hours at 37 C tubes were examined for gas production (CO_2) in the Durham tube being evidence of a positive citrate test. This test separates _S. faecalis_ and varieties from _S. faecium_ and varieties.

13. TTC reduction - In a tryptose-glucose medium at pH 7.0 _S. faecalis_ and varieties form bright red colonies with no periphery due to the strong reduction of 0.01% TTC. _S. faecium_ and varieties form pink colonies or violet centers with white peripheries due to partial reduction of TTC.

14. Motility test - Isolates were stabbed into motility media and incubated for 48 hours at 37 C.

Motility Media (grams/liter): Gelatin, 15.0 g; agar, 0.5 g; brain heart infusion, 12.5 g; K_2HPO_4, 1.0 g; TTC, 0.01%.

TABLE 1

Selective Agar Media for Isolation of Fecal Streptococci

Medium	Selective and differential agents and conditions	Reference
M-enterococcus (BBL)	0.04% NaN_3, 0.01% TTC	Slanetz and Bartley (1957)
KF (Difco)	0.04% NaN_3, 0.0015% brom cresol purple, 1.0% sodium glycerophosphate 0.0636% sodium carbonate, 0.01% TTC	Kenner _et al_ (1961)
KF (BBL)	Same as above without brom cresol purple	Kenner _et al_ (1961)
Azide-sorbitol	0.05% NaN_3, 0.000125% crystal violet, 0.00008% brom thymol blue, 0.5% sorbitol	Kjellander (1960)
Mitis-Salivarius	0.002% potassium tellurite 0.00008% crystal violet 0.0075% trypan blue	Chapman (1944)
Modification A of Mitis-Salivarius Agar	Same as above plus 0.04% NaN_3, 0.01% TTC	Modification of Chapman (1944)
PYC enrichment Broth	Nonselective Peptone, yeast extract casitone	Rose and Litsky (1965)
Tween-carbonate	0.04% NaN_3, 0.05% Tween 80, 0.2% sodium carbonate, 0.01% TTC	Burkwall and Hartman (1964)
Methylene blue-Azide	0.02% methylene blue, 0.005% NaN_3	Smith and Bodily (1967)
Brain heart infusion	0.04% NaN_3, 0.01% TTC. Incubate at 45 C for 48 hours.	Experimental medium
Brain heart infusion	0.04% NaN_3, 0.01% TTC. Incubate at 37 C for 48 hours.	Experimental medium

Comparison of Media for the Isolation and Enumeration of Fecal Streptococci in Water

Samples of raw sewage and water from Little Bay were collected. Samples were serially diluted and appropriate portions were filtered through membranes. The membranes were placed on each test medium and were incubated at 35 C for 48 hours. Colonies were counted and subcultured on brain heart infusion slants for confirmation of fecal streptococci. The identification tests for confirmation were as stated previously. Agar media that have been examined in the present study are listed in Table 1.

The primary criteria used in the evaluation of the various media tested were the yields or comparative counts and the selectivity of the medium.

Rapid Method for the Qualitative and Quantitative Determination of S. faecalis and varieties in Water

The technique devised was an MPN procedure using citrate-azide broth as the medium. The broth has the following composition: meat extract, 0.5%; peptone, 0.5%; yeast extract, 0.5%; sodium citrate, 3.0%; Tween 80, 0.05%; sodium azide, 0.4%. The initial pH is 8.0. The medium is autoclaved for 5 minutes at 10 pounds or just enough to force the broth up the Durham tubes. Each tube contains 10 ml. of broth. Ninety-two strains of fecal streptococci were tested for the ability to utilize citrate incorporated with azide. Also _E. coli_, _A. aerogenes_, _Kl. pneumoniae_, _S. typhimurium_, _B. subtilis_, _S. lactis_, _Pr. vulgaris_, _S. citreus_, _S. aureus_, and

Ps. aeruginosa were tested since they are commonly found in sewage and water.

Samples of water and sewage were inoculated as follows: 10.0, 1.0, 0.1 ml of water were inoculated into five repicate tubes each of medium. The broth was double strength for the 10 ml inoculums. In the case of sewage 1.0 ml of the appropriate dilutions were inoculated into five replicate tubes of citrate-azide broth. Tubes were incubated at 35 C for 48 hours, after which they were read as positive if gas appeared in the Durham tubes. Confirmation of S. faecalis and varieties were made by streaking each positive tube on potassium tellurite agar. Black growth was recorded as positive confirmation. Both positive and negative tubes were also streaked on azide-blood to isolate colonies. Colonies were picked and biochemically identified and then reinoculated into citrate-azide broth.

Survival studies of fecal streptococci and E. coli in garden soil

The organisms used in this study included S. faecalis, S. faecium, a motile pigmented streptococcus (563), a nonmotile pigmented streptococcus (564), and E. coli. The organisms were grown in 50 ml of T-soy-BH broth for 24 hours to contain approximately 10^{8-9} cells per ml. The cultures were centrifuged, washed twice in 0.85% NaCl, and suspended in phosphate buffered water (pH 7.2) in spray bottles. Soil plots measuring 1.125 square feet were seeded with 50 ml of suspension. After 15 minutes elapsed a zero hour sample of the respective organisms was taken. This was done by

collecting 5 random soil samples consisting of both top and subsurface soil samples were then diluted appropriately in buffer water and put on a shaker for 15 minutes to obtain an even suspension. The fecal streptococci were assayed by pour plates of M-enterococcus agar. *E. coli* was assayed by the EC MPN method. The per cent survivors were calculated and plotted on a graph versus time.

Serological studies

The main objectives of this investigation were as follows: (1) To determine the relationship between the antigens of *S. faecalis* and varieties and *S. faecium*. (2) To determine the serological relationship between fecal streptococci isolated from nature (including non-fertilized soils) and those isolated from human feces.

TABLE 2

Organisms used for serotyping:

Species	No. strains	Source
S. faecalis	4	Human feces
S. faecalis	1	ATCC 9790
S. faecalis	1	Tomatoes
S. faecalis	12	Non-fertilized soil
S. faecalis var. *liquefaciens*	2	Human feces
S. faecalis var. *zymogenes*	1	UNH stock
Biotypes I-IV+	4	UNH stock
S. faecium	2	Human
S. faecium	1	USC K6A (ATCC)*
S. faecium	1	USC R55 (ATCC)*
S. faecium var. *durans*	1	UNH stock
S. faecium-like (yellow)	5	Non-fertilized soil
S. faecium	2	Green beans
S. faecium-like (no pigment)	5	Non-fertilized soil
S. avium A	1	R. H. Deibel
	Total - 43	

*These strains ordered from ATCC came back with these designations.

+These strains are defined according to Bartley and Slanetz (1960)

Preparation of antisera in rabbits

Cultures for vaccines were grown for 24 hours at 37 C in 500 ml of modified Todd-Hewitt Broth. The organisms were collected by centrifugation, washed twice in NaCl (0.85% w/v) and resuspended in NaCl (0.85% w/v) to give an optical density equivalent to McFarland No. 3. Then 30 ml of suspension were sonicated for approximately 1-1½ hours, after which the resulting suspension was centrifuged at 10,000 g for 20

minutes to remove cellular debris. The supernate was tested for the Group D antigen by the gel-diffusion method using commercially prepared Group D antiserum. If results were positive, the supernate was used for inoculation of rabbits.

Injection schedule

Vaccines were injected at 3 day intervals intravenously starting with two doses of 0.5 ml and followed by 1.0 ml doses. The number of injections required to produce good antiserum varied from rabbit to rabbit. Usually one series of 5 injections produced good antiserum.

Gel-diffusion technique

Mansi's (1957) modification of the double gel-diffusion method of Ouchterlony (1953) was used. The antiserum (c. 0.1 ml) was contained in a center well and antigen extracts (c. 0.1 ml) were dispensed in six wells arranged equidistantly in a hexagon around the well containing antiserum; the reaction was allowed to proceed at room temperature, and plates were inspected daily for 7 days. Lines developed usually within 2-3 days.

Development of a Selective Medium for the Isolation of S. salivarius and S. mitis

A. Utilization of Various Substrates by S. Salivarius, S. mitis, S. faecalis, and S. faecium

Each of the above organisms was inoculated into two tubes each of the following media (a third tube served as a blank control):

(1) Brain Heart Infusion + 6.5% NaCl

(2) Mannitol 1.0%
Peptone 0.5
Y. extract 0.5

(3) NO. (2) + 6.5% NaCl

(4) NO. (2) + 4.0% NaCl

(5) NO. (2) + 2.0% NaCl

After 4 hours incubation at 35 C, sucrose was added to give a final concentration of 5.0% to one of the two inoculated tubes. Nothing was added to the second. The tubes were further incubated at 35 C for 24 hours and examined for turbidity.

B. The Effect of Penicillin and Kanamycin on S. faecalis

An eighteen hour culture of *S. faecalis* was diluted to approximately 10^3 organisms per ml. One ml of this dilution was inoculated into Brain Heart Infusion Broth tubes containing concentrations of penicillin from 1 unit per ml to 500 units per ml plus 40 ug per ml of kanamycin. The effect of penicillin alone, penicillin and kanamycin, and kanamycin alone was to be determined. After 4 and 6 hours incubation at 35 C the broth tubes were diluted and pour plates were made in BHI Agar with the incorporation of 2000 units per ml of penicillinase. The plates were incubated at 35 C for 48 hours and counted.

C. The Effect of Penicillin and Kanamycin (60 ug/ml) on the Growth of S. faecalis and S. salivarius in a Mannitol Broth Medium

Composition of Medium	Grams per liter
Proteose peptone NO. 3	5
Yeast Extract	5
Mannitol	10

Sodium Chloride	20
Sodium azide	0.4
Brom Cresol purple	0.015
pH 7.2	

The above medium with the addition of penicillin (1000 u/ml) and kanamycin (60 ug/ml) will later be referred to as Penicillin-Kanamycin MS Broth (PKMS). 9.8 ml of the above medium was dispensed into test tubes and the tubes were autoclaved at 15 lbs./10 min. The tubes were divided into 3 equal sets. Within each set the concentration of penicillin ranged from zero to 500 u/ml. Kanamycin was added at a concentration of 60 ug/ml to every tube. One ml of a 10^4 dilution of a 24 hour culture of S. faecalis was inoculated into each tube of the first set. One ml of a 10^4 dilution of S. salivarius was inoculated into the second set; and one ml of both organisms was inoculated into the third set. The tubes were incubated for 6 hours at 35 C. At this time penicillinase was added to give a final concentration of 2000 u/ml. Also at this time 5.0 ml of the following broth was added to each tube:

	%
Sucrose	16.0
Peptone	.3
Y. extract	.3

This gave a final concentration of 5.0% sucrose in the medium. The tubes were further incubated for 42 hours at which time each tube was serially diluted and plated in BHI Agar. The plates were incubated at 35 C for 48 hours and counted.

Recovery of S. Salivarius from Raw Sewage

One ml of a 24 hour culture of S. salivarius 9759

was inoculated into 99 ml of raw sewage from the Durham, N.H. waste treatment plant. Serial dilutions of the 24 hour culture were plated in BHI Agar to determine the number of organisms per ml added. After standing for 10 minutes serial dilutions of the inoculated sewage were made and 1.0 ml of each dilution was inoculated into a 5 tube Most Probable Number (MPN) series of Penicillin-Kanamycin MS Broth. Also the number of fecal streptococci was determined by the membrane filter technique placing the membranes on M-enterococcus Agar. The MPN series was incubated for 6 hours at 35 C at which time penicillinase and sucrose broth was added. After further incubating for 42 hours all tubes showing turbidity (yellow) were subcultured on Mitis-Salivarius Agar for confirmation of *S. salivarius*.

Isolation and Enumeration of Oral Streptococci from Raw Sewage

Samples of sewage were collected from the Durham and Dover, N. H. plants. These samples were not inoculated with *S. Salivarius*. The same procedure as above was followed.

Isolation and Enumeration of S. salivarius and S. mitis from Human Fecal Material

Penicillin-Kanamycin MS Broth was compared with Sucrose-azide Agar (Sherman *et al*, 1943) and with Mitis-Salivarius Broth (Appleman, 1967) for the enumeration of *S. salivarius* and *S. mitis* in human fecal samples. One gram of feces was added to 99 ml of sterile phosphate buffered water containing glass beads. This was shaken until a uniform suspension of the fecal material was produced. Serial

Survival Studies of S. salivarius, S. faecalis, and S. faecium in Filter Sterilized Raw Sewage and Fresh Water

Twenty-four hour cultures of the above organisms isolated from human feces were centrifuged and washed three times in sterile phosphate buffered water (pH 7.2). The cells were resuspended in buffered water. One ml of each suspension was inoculated into duplicate flasks containing 99 ml of filter sterilized raw sewage (pH 7.5) and UNH reservoir water (pH 7.0). Controls of buffered water were also inoculated. Each flask was serially diluted and plated out in BHI Agar at zero time and various times afterward. The flasks were held at 20 C. After incubation of the plates at 35 C for 48 hours, counts were made and a plot of Log NO. Survivors versus Time was made. The slope of the like (k) was the number of hours it took to kill 90% of the organisms or one Logfold. The graphs are an average of 3 trials each of sewage and fresh water.

The Survival of Fecal and Oral Streptococci in Raw Sewage after Inoculating with Human Fecal Samples

To determine the survival rates of fecal and oral streptococci in sewage, a one gram sample of fecal material was inoculated into 99 ml of filter sterilized sewage. At zero time the initial number of fecal streptococci was determined by the MF technique using M-enterococcus Agar. The initial MPN of oral organisms was determined using PKMS Broth and confirming on Mitis-Salivarius Agar. Phosphate buffered water was used as a control. After 24 and 48 hours the number of fecal streptococci and oral streptococci were determined as above.

The Bacteriological Examination of Dairy Products and Frozen Meat Pies for Coliforms, Fecal and Oral Streptococci and Staphylococci

The commercially produced dairy products and frozen meat pies used in this investigation were purchased at retail markets in Durham, Dover, and Portsmouth, N. H.

Samples of sour cream, cottage cheese, and frozen meat pies were obtained by using a stainless steel spatula which was previously sterilized by dipping into alcohol and then flaming. A 50 gram sample was placed into a sterile Waring Blendor and 450 ml of sterile phosphate buffered water was added to make a 1-10 dilution by weight. The samples were blended for 2 minutes at high speed and serial dilutions were made from this suspension.

The Most Probable Number method was used for the enumeration of coliforms, fecal and oral streptococci. The MPN was obtained by adding 1.0 ml of the appropriate dilutions to series of 5 replicate Lauryl Sulfate Broth tubes, Enterococcus Broth tubes, and PKMS Broth tubes for the coliforms, fecal streptococci, and the oral streptococci respectively. Also Mitis-Salivarius Broth was compared with the PKMS Broth in the enumeration of the oral streptococci. Coliforms were confirmed in Brilliant Green Lactose Bile Broth, fecal streptococci were confirmed as usual, and oral streptococci were confirmed on MS Agar.

Tellurite-Glycine Agar was used to determine the presence of staphylococci.

The Effect of Various Storage Temperatures on the Survival of S. salivarius in Chicken Meat Pies

Chicken meat pies were bought in Portsmouth, N. H. from a retail outlet. A 24 hour culture of *S. salivarius* isolated from foods was centrifuged and washed three times in phosphate buffered water and resuspended in buffered water. Twenty-one pies were inoculated with 1.0 ml of the suspension with a sterile syringe and needle (18 gauge) in the geometric center of the pie. The pies were divided into 3 groups. One group was frozen at -100°F, the second at 0°F, and the third group was held at 41°F (refrigerator temp). The initial number of organisms inoculated was determined before freezing using PKMS Broth and confirming on MS Agar. Then the pies were sampled at various intervals of time to determine the number of survivors.

Recovery of S. salivarius from the Air after Aerosolizing with a 24 Hour Culture

S. salivarius isolated from foods was incubated for 24 hours in 300 ml of BHI Broth. The culture was centrifuged and washed in phosphate buffered water, and then resuspended in 150 ml of buffered water. This suspension was placed in a spray bottle and sprayed into a small enclosed room. After 30 minutes, 10 cubic feet of air was drawn through an Andersen Air Sampler with the aid of a vacuum pump. The medium used was Mitis-Salivarius Agar. Then hourly samples were obtained and the plates were incubated at 35 C for 48 hours at which time the number of *S. salivarius* organisms was recorded. Also swab plates were made by swabbing the walls, floor, and door knobs and streaking on MS Agar.

Survival of Oral Streptococci on Stainless Steel Surfaces

To determine how long *S. salivarius* and *S. mitis* can persist on surfaces the following experiment was performed. Small stainless steel cylinders were immersed in saliva which was pooled from 10 people. After 10 minutes the cylinders were removed and placed on sterile filter paper in a petri dish to drain and were then transferred to another petri dish and dried at 35 C for 30 minutes. Three of the cylinders were each placed in 9.0 ml of sterile phosphate buffered water. The tubes were shaken to remove any organisms on the cylinders and then serially diluted. Spread plates were made on Mitis-Salivarius Agar. The plates were incubated at 35 C for 48 hours and the initial number of *S. salivarius* and *S. mitis* organisms were counted.

The remaining cylinders were placed in a desiccator jar which was equilibrated with .01 M NaCl at 30 C overnight. At various intervals of time the cylinders were removed and sampled for oral streptococci. A plot of Log NO,. Survivors versus Time was made.

Detection of S. salivarius and S. mitis on Food Preparation Surfaces in the UNH Dining Halls

To determine whether *S. salivarius* and *S. mitis* were present on food preparation surfaces in dining halls, contact agar plates containing MS Agar plus 0.02 per cent sodium azide were pressed onto various surfaces where food was being prepared in 3 different dining halls. The plates were then incubated in the usual way and then examined for the presence of *S. Salivarius* and *S. mitis*. Numbers were expressed as number of organisms per contact plate.

RESULTS

Comparison of selective media for the isolation of fecal streptococci in sewage and water

Efforts to develop media for the successful isolation, enumeration, and identification of the fecal streptococci have been extensive. However, as pointed out by Hartman et al, 1966, a critical assessment of a number of selective media was warrented to determine their specificity and quantitative ability. The results of a comparison of 11 media for the enumeration of fecal streptococci in raw sewage are reported in TABLE 3. The M-enterococcus Agar was used as the standard for the comparison of the other 10 media tested.

Of 11 media tested the M-enterococcus, KF (Difco), KF (BBL), and PYC media were the most satisfactory when isolating fecal streptococci from raw sewage. The above media were 90-100 per cent selective for fecal streptococci. Although the Mitis-Salivarius Agar, Methylene Blue Azide Agar, and Brain Heart Infusion medium yielded more streptococcus-like colonies than the M-enterococcus medium, they all had many colonies that were not streptococci. When 0.04 per cent sodium azide was added to the Mitis-Salivarius Agar (NO. 5), it's specificity was increased to 95 per cent; however, the yield of fecal streptococci decreased to 89 per cent as compared to the M-enterococcus medium. The Tween-Carbonate medium showed good specificity but an inferior yield when compared to the M-Enterococcus medium.

TABLE 3

Comparison of the Efficiency of 11 Selective Media in Detecting Fecal Streptococci In Sewage Using the MF Technique

Medium	Source of sample	No. of samples	Av. per cent yield of Streptococcus -like organisms on 11 test media	Av. per cent confirmed fecal streptococci	Per cent yield times confirmed equals Av. No. fecal streptococci per sample
M-enterococcus (BBL)	Raw sewage	40	100	99	99
KF (Difco)	Raw sewage	40	111	97	107
Azide-sorbitol	Raw sewage	15	97	70	68
Mitis-Salivarius	Raw sewage	33	675	10	67
MS Agar, 0.04% NaN_3, 0.01% TTC	Raw sewage	23	89	95	84
PYC	Raw sewage	16	110	90	99
Tween-CO_3	Raw sewage	22	70	90	63
Methylene blue	Raw sewage	8	280	25	70
BHI plus 0.01% TTC at 45 C	Raw sewage	5	560	10	56
BHI plus 0.01% TTC plus 0.04% NaN_3 at 37 C	Raw sewage	16	50	92	46
KF (BBL)	Raw sewage	4	105	95	100

In TABLE 4 the results of a comparison of 9 selective media for the isolation of fecal streptococci from estuarine waters are reported. Both KF Agars yielded much higher counts of streptococcus-like colonies than did the M-enterococcus medium but they were only 32-41 per cent selective for fecal streptococci. The contaminating organisms on the KF media were mainly micrococci, gram-positive and gram-negative rods. The PYC enrichment procedure produced yields comparable to the M-enterococcus medium; however, the PYC enrichment medium was not as selective as the M-enterococcus medium. Therefore, for the enumeration of fecal streptococci in estuarine waters, the KF Agar and the PYC enrichment media do not appear suitable. The remaining media performed about the same as when raw sewage was used as the source of fecal streptococci. Thus the results indicated that while one of several media might be used in isolating fecal streptococci from sewage with equal efficiency, the M-enterococcus medium was the superior medium when isolating fecal streptococci from estuarine waters.

TABLE 4

Comparison of the Efficiency of 9 Selective Media in Detecing Fecal Streptococci in Estuarine Waters Using the MF Technique

Medium	No. of samples	Av. per cent yield of streptococcus-like colonies on 9 test media	Av. per cent of confirmed fecal streptococci	Per cent yield times per cent confirmed equals per cent fecal streptococci per sample
M-enterococcus (BBL)	25	100	99	99
KF (Difco)	25	225	32	72
KF (BBL)	5	190	41	78
Azide-sorbitol	6	122	67	82
Mitis-Salivarius	5	625	10	62
MS Agar plus 0.04% NaN_3, 0.01% TTC	5	75	82	61
PYC	6	113	88	99
Tween-CO_3	6	91	93	85
Methylene blue	6	310	21	65

Rapid method for the qualitative and quantitative determination of S. faecalis and varieties in water

The M-enterococcus agar has previously been found by Slanetz and Bartley (1957) and other investigators to support the growth of all types of streptococci commonly inhabiting the intestinal tract of man and animals. This includes such organisms as *S. bovis*, *S. faecalis*, and varieties *liquefaciens* and *zymogenes*, biotypes, and *S. faecium* and its variety *durans*. Bartley and Slanetz maintained that while *S. faecalis* may not be the predominant type of streptococci in human feces, it is not generally found in the feces of domestic animals. Thus, its presence in water would indicate contamination of human origin. Therefore, from the standpoint of origin of contamination it seemed desirable to develop a rapid method to quantitatively test for *S. faecalis* and varieties *liquefaciens* and *zymogenes* in water. For the selective isolation of S. faecalis and varieties, 3 factors were taken into account. The first was that all fecal streptococci can tolerate 0.04 per cent sodium azide; the second was that all fecal streptococci can grow at an alkaline pH; and, the third was that only *S. faecalis* and varieties can utilize citrate as the sole source of energy as opposed to *S. faecium* and atypical biotypes.

The results of the preliminary study to investigate the ability of fecal streptococci and other organisms commonly found in polluted water to utilize citrate incorporated with sodium azide is in TABLE 5.

Of the 42 strains of *S. faecalis* and varieties tested, 41 showed positive reactions in citrate-azide

TABLE 5

Types of Fecal Streptococci and Other Enteric Organisms Utilizing Citrate as an Energy Source

Organisms	No. of strains tested	No. of strains utilizing citrate	No. confirmed on K-tell
S. faecalis and varieties	42	41	41
S. faecium	14	0	
S. faecium-like	44	2	1
E. coli	3	0	
A. aerogenes	3	0	
Kl. pneumoniae	1	1	1
S. typhimurium	1	1	1
B. subtilis	1	0	
S. lactis	1	0	
Pr. vulgaris	1	1	1
S. citreus	1	0	
S. aureus	1	0	
Ps. aeruginosa	1	0	

broth as evidenced by gas in the Durham tubes. All 41 strains tolerated 0.04% potassium tellurite as evidenced by black growth on potassium tellurite agar. None of the S. faecium strains showed positive results in the medium. Two S. faecium-like strains gave positive results in this medium, one of which grew on K-tell agar. Of the other strains tested Kl. pneumoniae, S. typhimurium, and P. vulgaris were positive. The numbers of these organisms, however, in water would be insignificant when compared to the numbers of S. faecalis and varieties.

The efficiency of this medium was next tested for the detection of S. faecalis in raw sewage, oxidation pond effluent, and fresh water. The results of this work appears in TABLE 6.

Using the most probable number (MPN) procedure, the citrate-azide broth was virtually 100 per cent selective for S. faecalis and varieties. However, after identifying the fecal streptococci on the M-enterococcus agar, it was found that a much larger number of S. faecalis and varieties were isolated by the membrane filter technique. Therefore, one could not achieve a quantitative estimation of S. faecalis and varieties but could qualitatively determine if S. faecalis and varieties were present.

One of the main objectives of this research was to determine to what extent the fecal streptococci occurred apart from the intestinal tracts of human and animals, and to ascertain whether the presence of these organisms in water, soil, and plants could be ascribed to sources other than

TABLE 6

Most Probable Numbers of *S. faecalis* and Varieties in Water and Sewage Using a Citrate-Azide Broth at pH 8.0(*)

Sample No.	Source	MPN estimate *S. faecalis* and var./100 ml sample	No. colonies identified from azide-blood plates	% that were *S. faecalis* and var.	MPN x % = No. *S. faecalis* + var.	No. total fecal strep on M-enterococcus agar (MF technique)	% that were *S. faecalis* and var.	No. fecal strep x % *S. faecalis* and var. = No. *S. faecalis* + var.
1	Exeter Raw sewage	54,000	20	100.0	54,000	720,000	40.0	288,000
2	Exeter pond #2	9,200	15	100.0	9,200	129,700	31.5	40,800
3	Derry pond #4	790	5	100.0	790	33,000	38.6	12,400
4	Exeter Raw sewage	2,800	16	94.0	2,640	140,000	34.0	47,500
5	Exeter pond #2	2,300	12	91.5	2,100	126,800	32.3	40,300
6	Exeter pond #3	330	14	100.0	330	12,400	30.0	3,700
7	Exeter pond #4	130	10	100.0	130	6,100	32.5	1,980
8	Squamscott River	130	12	100.0	130	22,900	12.0	2,750

(*) Modification of Whittenbury (1965).

those arising through human or animal contamination. As a preliminary study to the occurrence of fecal streptococci in soil and plants, an examination of the types of fecal streptococci present in feces in humans and animals and in domestic sewage was performed. The purpose of this investigation was to determine what types of fecal streptococci are present in the above sources as compared to those present in nature.

The results of this study on the types of fecal streptococci isolated from the feces of humans and animals and from raw sewage are tabulated in TABLE 7. Sixty-five per cent of the strains examined from 21 human volunteers were S. faecalis, 14 per cent were S. faecium, 9 per cent were S. faecalis var. liquefaciens, 7 per cent were S. faecium var. durans, and 7 per cent were atypical or biotypes. No S. bovis or S. faecalis var. zymogenes were present in human feces.

In sewage, of 321 strains identified, 30 per cent were S. faecalis, 10 per cent were S. faecium, 6 per cent were S. faecium var. durans, and 10 per cent were S. faecalis var. liquefaciens. In contrast to human feces, a larger proportion of fecal streptococci were identified as atypical.

In cows and sheep, S. faecalis accounted for only 6 per cent of the number of strains examined. S. faecium accounted for 46 per cent in cows and 76 per cent of the total number of strains in sheep. Fecal streptococci were isolated only rarely in rabbits but when present, S. faecium

TABLE 7

Types of Fecal Streptococci Isolated from Sewage and From Human and Animal Feces by Using the Membrane Filter Procedure

		Source of Specimen					
		Human	Raw sewage	Cows	Sheep	Rabbits	Dogs
No. specimens examined		21	30	15	10	10	10
No. strains studied		204	321	50	63	15	17
S. faecalis	No. of strains	133	96	3	4	0	5
	%	65	30	6	6	0	29
S. faecium	No. of strains	28	32	23	48	9	3
	%	14	10	46	76	60	18
S. faecalis var. *liquefaciens*	No. of strains	17	32	0	2	0	0
	%	9	10	0	3	0	0
S. faecalis var. *zymogenes*	No. of strains	0	2	0	0	0	0
	%	0	0.6	0	0	0	0
S. faecium var. *durans*	No. of strains	12	19	0	3	2	0
	%	7	6	0	5	13	0
S. bovis	No. of strains	0	0	16	2	0	0
	%	0	0	32	3	0	0
Atypical or Biotypes	No. of strains	14	140	8	4	4	9
	%	7	44	16	6	27	53

was the predominating type. These results indicate that when S. faecalis is isolated, this most likely indicates human pollution. When S. faecium is isolated it most likely indicates animal pollution, although it does not preclude human contamination.

Physiological Characteristics of Streptococci Isolated in This Investigation

In the past, speciation of the fecal streptococci has been confusing although recently Hartman, Reinbold, and Saraswat (1966) have reviewed the problem and as a result a taxonomic scheme is emerging.

In the present investigation fecal streptococci were isolated from human and animal feces, raw sewage, and water. Organisms from these sources were classified into the following groups: S. faecalis and varieties liquefaciens and zymogenes, S. faecium and its variety durans (Hartman et al, 1966), S. bovis, and atypical strains. The key features used in differentiating these organisms are in TABLE 8. These characteristics were compiled from the investigation of more than 700 strains of fecal streptococci.

Based on the criteria used by Sherman, the enterococcus group comprises only 2 species and respective varieties. These are S. faecalis and varieties liquefaciens and zymogenes and S. faecium and its variety durans. The S. bovis-S. equinus organisms do not fulfill all of the Sherman criteria.

S. faecalis is well defined; its varieties liquefaciens and zymogenes differ from it only in the ability of

TABLE 8

Biochemical Characteristics of the Fecal Streptococci Isolated from Human and Animal Feces and Sewage Excluding Various Biotypes

Biochemical Reactions No. Strains	*S. faecalis* (341)	*S. faecalis* var. *liquefaciens* (82)	*S. faecalis* var. *zymogenes* (11)	*S. faecium* (278)	*S. faecium* var. *durans* (36)	*S. bovis* *S. equinus* (18)
*Growth at 10 & 45 C	+	+	+	+	+	-/+
*Growth at pH 9.6	+	+	+	+	+	-
*Growth in 6.5% NaCl	+	+	+	+	+	-
*Growth in 0.1% MB	+	+	+	+	+	+
*Growth in 40% Bile	+	+	+	+	+	+
*Resists 60 C/30 min	+	+	+	+	+	-
*NH_3 from arginine	+	+	+	+	+	-
Tolerates 0.04% K-tell	+	+	+	-	-	-
Strong TTC red.	+	+	+	-	-	-
Strong red. in litmus	+	+	+	-	-	-
Gelatin liquefaction	-	+	-/+	-	-	-
Beta-hemolysis	-	-	+	-	+/-	-
Arabinose	-	-	-	+	-	-
Glycerol	+	+	+	-	-	-
Raffinose	-	-	-	+/-	-	+
Sorbitol	+	+	+	-	-	-
Inulin	-	-	-	-	-	+
Nelibiose	-	-	-	+	+	-
Melezitose	+	+	+	-	-	-
Sucrose	+	+	+	+/-	-	+
Mannitol	+	+	+	+	-	-
Starch hydrolyzed	-	-	-	-	-	+/-
Utilization of citrate	+	+	+	-	-	-

*Sherman criteria

the former to liquefy gelatin and the latter to produce beta-hemolysis on blood agar. S. faecium is also well defined. The main feature distinguishing it from S. faecalis is its sensitivity to 0.04 per cent potassium tellurite. S. faecium also differs in its weak reduction of litmus milk, and the fermentation of arabinose and melibiose.

Although S. faecium var. durans is closely related to S. faecium, it differs in its inability to ferment arabinose and mannitol. Some strains also produce beta-hemolysis on blood agar.

The reduction of triphenyltetrazolium chloride (TTC) is another striking characteristic that distinguished S. faecalis and varieties from S. faecium and its variety. S. faecalis and varieties reduce TTC strongly at pH 6.0 while S. faecium and its variety reduce TTC weakly or not at all. At pH 7.2 on tetrazolium glucose agar S. faecalis and varieties has a deep red center and an off-white periphery, while S. faecium and varieties, and S. bovis are an off-white or pale pink colony with no periphery.

There are many other lesser important tests useful for distinguishing between S. faecalis and S. faecium such as fermentation tests, and the utilization of citrate and pyruvate as energy sources.

In the S. bovis-S. equinus group the fermentation abilities are weak. S. bovis may be differentiated from S. equinus by the ability of S. bovis to hydrolyze starch.

Numbers and Types of Fecal Streptococci Present in Soil

Soils that were not fertilized with animal manure

or subject to fecal contamination from domestic wastes were next tested for the presence of fecal streptococci in an attempt to establish whether this group could be found outside the animal body in nature. The soils included garden soil fertilized only with commercial fertilizer and soil from a grassland area which has remained fallow for a number of years. TABLE 9 shows the average number of fecal streptococci isolated per gram of soil from these two areas over a two year period.

In the samples of garden soil, the numbers of fecal streptococci averaged 19.5 per gram in the spring of '66, increased in numbers to 143 per gram in the summer months, and then decreased in numbers until the following spring. The same cycle took place in the samples of grassland soil, though the numbers were much lower than those in the garden soil. These results are better illustrated in Figure 1.

Besides these two areas, a densely wooded, non-inhabited area was investigated for the presence of fecal streptococci and of 35 soil samples, no fecal streptococci were recovered. This indicates that in non-inhabited areas, fecal streptococci are absent.

In conjunction with this study we compared the KF agar (Difco) with the M-enterococcus agar (BBL) for the recovery of fecal streptococci in soil. The results are in TABLE 10.

TABLE 9

Numbers of Fecal Streptococci in Garden Soil Fertilized with Commercial Fertilizer and in Non-Fertilized Soils as Determined by Plating in M-Enterococcus Agar

Garden Soil[a]			Grassland[b]		
Month	No. samples	Av. No. fecal streptococci per gram	Month	No. samples	Av. No. fecal streptococci per gram
April - June '66	15	19.5	April - June '66	15	1.82
July - Aug.	30	143.0	July - Aug.	30	89.0
Sept. - Oct.	24	119.0	Sept. - Oct.	17	73.4
Nov. - Jan. '67	12	12.0	Nov. - Jan. '67	12	5.0
Feb. - Mar.	10	2.0	Feb. - Mar.	10	1.0
April - June	52	23.5	April - June	32	1.0
July - Aug.	20	127.0	July - Aug.	20	47.0
Sept. - Oct.	15	91.0	Sept. - Oct.	15	25.0
Nov. - Jan. '68	10	17.0	Nov. - Jan. '68	10	2.0

a = Fertilized with ammonium citrate

b = Non-fertilized, fallow grassland

FIGURE 1

Numbers of fecal streptococci per gram of soil vs. season as determined with M-enterococcus agar

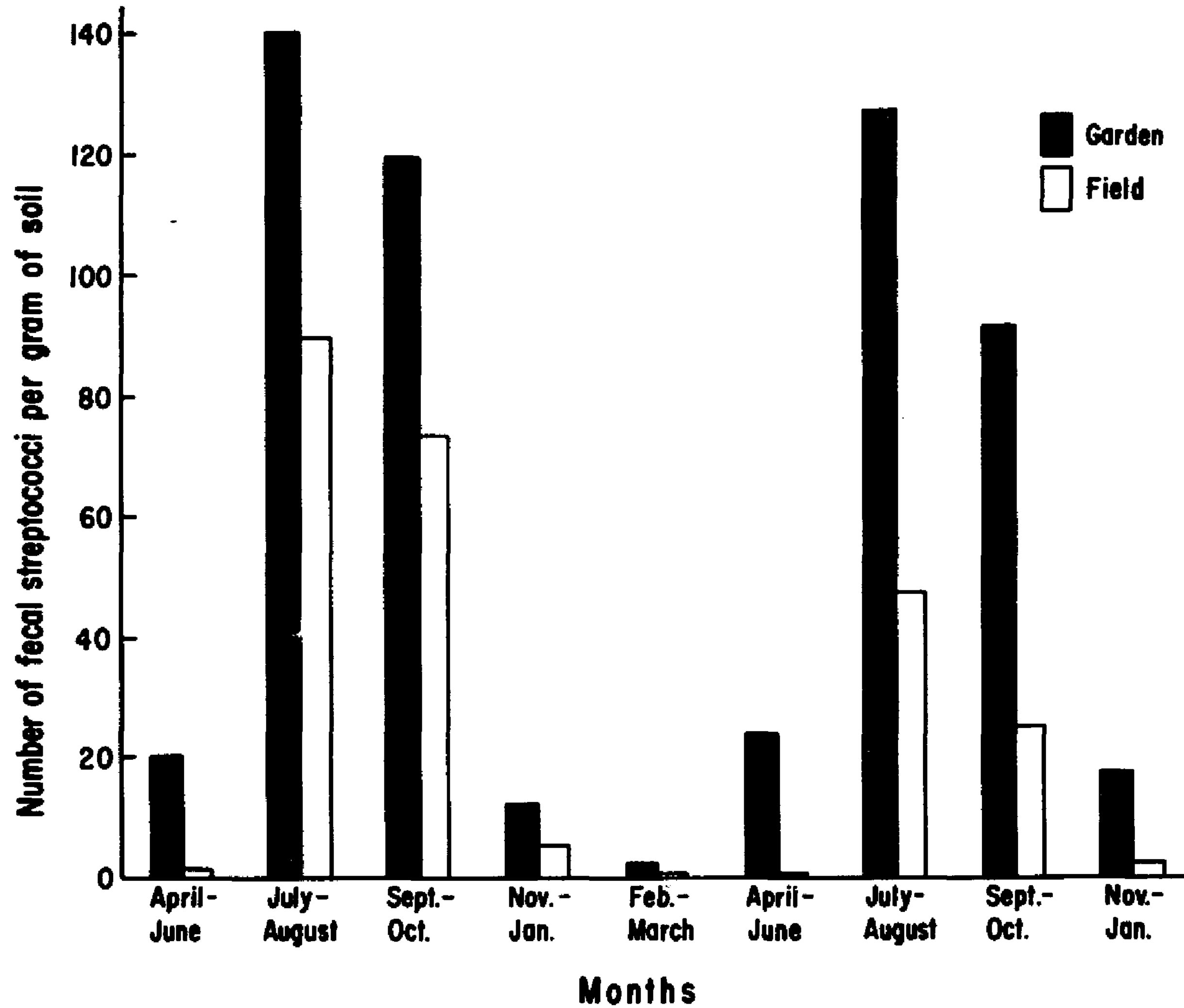


TABLE 10

Numbers of Fecal Streptococci in Soil as Compared by M-Enterococcus and KF Agars

Source	No. of samples	M-enterococcus agar (BBL) Av. No. streptococci/g	KF Agar (Difco) Av. No. strep/g
Grassland soil	17	77.5	70.5
Garden soil	30	109.0	96.5

The enterococcus medium was slightly more productive in detecting fecal streptococci than the KF medium. However, the KF medium is at a distinct disadvantage when sampling soil by the pour plate method in that the color of the medium is a dark purple which makes reading the plates very difficult. Therefore, for soil work, the M-enterococcus agar is preferable.

The next step was to determine what types of streptococci were present in these soils. The results of this study appear in TABLE 11. Of 103 strains identified, 5.8 per cent were _S_. _faecalis_, 7.7 per cent as _S_. _faecalis_-like, 7.7 per cent as _S_. _faecalis_ var. _liquefaciens_, 29.1 per cent as _S_. _faecium_, and 49.5 per cent as _S_. _faecium_-like. The _S_. _faecalis_-like and _S_. _faecium_-like organisms fulfilled all the requisites for enterococci and they represent strains differing only in minor respects from the recognized species of enterococci. These deviations are shown in TABLE 12. The essential features that differentiate the established species of

TABLE 11

Types of Fecal Streptococci Isolated from Soil

<table>
<tr><th>Sample No.</th><th>Source of soil</th><th>No. colonies characterized</th><th colspan="2"><u>S. faecalis</u></th><th colspan="2"><u>S. faecalis</u>-like</th><th colspan="2"><u>S. faecalis</u> var. <u>liquefaciens</u></th><th colspan="2"><u>S. faecium</u></th><th colspan="2"><u>S. faecium</u>-like</th></tr>
<tr><td>1</td><td>Garden</td><td>8</td><td colspan="2">2</td><td colspan="2">2</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">4</td></tr>
<tr><td>2</td><td>Garden</td><td>2</td><td colspan="2">0</td><td colspan="2">2</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td></tr>
<tr><td>3</td><td>Garden</td><td>10</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">10</td></tr>
<tr><td>4</td><td>Garden</td><td>6</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">4</td><td colspan="2">2</td></tr>
<tr><td>5</td><td>Garden</td><td>6</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">2</td><td colspan="2">4</td></tr>
<tr><td>6</td><td>Garden</td><td>10</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">4</td><td colspan="2">6</td></tr>
<tr><td>7</td><td>Grassland</td><td>10</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">2</td><td colspan="2">4</td><td colspan="2">4</td></tr>
<tr><td>8</td><td>Grassland</td><td>12</td><td colspan="2">0</td><td colspan="2">1</td><td colspan="2">1</td><td colspan="2">3</td><td colspan="2">7</td></tr>
<tr><td>9</td><td>Grassland</td><td>8</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">2</td><td colspan="2">4</td><td colspan="2">2</td></tr>
<tr><td>10</td><td>Grassland</td><td>11</td><td colspan="2">21</td><td colspan="2">2</td><td colspan="2">0</td><td colspan="2">4</td><td colspan="2">4</td></tr>
<tr><td>11</td><td>Grassland</td><td>7</td><td colspan="2">3</td><td colspan="2">1</td><td colspan="2">0</td><td colspan="2">2</td><td colspan="2">1</td></tr>
<tr><td>12</td><td>Grassland</td><td>13</td><td colspan="2">0</td><td colspan="2">0</td><td colspan="2">3</td><td colspan="2">3</td><td colspan="2">7</td></tr>
<tr><td colspan="2" rowspan="2">Total</td><td rowspan="2">103</td><td>No.</td><td>%</td><td>No.</td><td>%</td><td>No.</td><td>%</td><td>No.</td><td>%</td><td>No.</td><td>%</td></tr>
<tr><td>6</td><td>5.8</td><td>8</td><td>7.7</td><td>8</td><td>7.7</td><td>30</td><td>29.1</td><td>51</td><td>49.5</td></tr>
</table>

TABLE 12

Biochemical Reactions of *S. faecalis*, *S. faecium* and Atypical Strains of STREPTOCOCCI Isolated from Soil

Biochemical reaction	*S. faecalis*	*S. faecalis*-like			*S. faecium*	*S. faecium*-like		
Subgroups		*1	2	3		1	2	3
K-tellurite tolerance	+	+	+	+	-	-	-	-
Reduction of TTC (strong)	+	+	+	+	-	-	-	-
(partial)	-	-	-	-	+	+	+	+
(none)	-	-	-	-	-	-	-	-
Fermentation of								
arabinose	-	-	-	+	+	+	+	+
glycerol	+	-	+	+	-	-	+	+
raffinose	-	-	+	+	-	-	+	+
sorbitol	+	+	-	-	-	+	-	-
melibiose	-	-	-	-	+	+	-	-
melezitose	+	-	+	-	-	-	+	-

*These patterns represent the most common reactions of the atypical strains present in soil.

streptococci from the enterococcus-like organisms are fermentation patterns. In this investigation many strains of streptococci have been isolated which closely resemble S. faecalis and S. faecium. In many cases strains that had a yellow pigment were isolated that were identical to S. faecium except for this pigment. The pigmented streptococci were recovered in the spring and late summer chiefly in cultivated garden soil. They were occasionally found in grassland areas, never present in human or animal feces, sewage, and rarely in water. When found in the soil, their numbers ranged from 20 to 233 per gram of soil. TABLE 13 shows the physiological reactions of yellow streptococci isolated from soils. In general, they most closely resemble S. faecium because of the lack of the ability to tolerate tellurite and to reduce TTC strongly. Most strains of yellow streptococci fermented arabinose, all strains fermented melibiose and did not ferment melezitose, The above pattern is typical of S. faecium. Approximately 40 per cent of these pigmented streptococci were motile.

To further evaluate the usefulness of the fecal streptococci, including the pigmented strains, as indicators of recent fecal pollution in soil, it was necessary to determine the relative survival rates of S. faecalis, S. faecium, a pigmented motile strain, a pigmented nonmotile strain, and E. coli in the soil. The results of this experiment appear in TABLE 14.

After 16 days, the per cent survival for S. faecalis was 0.06 per cent, S. faecium 0.48 per cent, the motile

TABLE 13

Biochemical Reactions of Strains of Yellow Streptococci Isolated From Soils

Biochemical reactions	Types of Strains					
	1	2	3	4	5	6
Potassium tellurite tolerance	+	-	-	-	-	-
Reduction of TTC - strong	-	-	-	-	-	-
- partial	+	+	+	+	+	+
Production of NH_3 from arginine	+	+	-	+	+	+
Motility	-	-	+	+/-	-	+/-
Fermentation of arabinose	+	+	-	+	+	+
Glycerol	-	-	+	+/-	-	+/-
Raffinose	-	-	-	+	+	+
Sorbitol	-	-	+	-	+	+
Melibiose	+	+	+	+	+	+
Melezitose	-	-	-	-	-	-
Utilization of citrate	-	-	+	(-/+)	-	(-/+)
No. of Strains (80)	10	1	3	10	36	20
Per cent	12.5	1.25	3.75	12.50	45.00	25.00

TABLE 14

Survival of Fecal Streptococci and *E. coli* in Garden Soil

Day	*S. faecalis* Number/g	*S. faecalis* % Surv.	*S. faecium* Number/g	*S. faecium* % Surv.	Pigmented motile Strain 563 Number/g	Pigmented motile Strain 563 % Surv.	Pigmented Nonmotile Strain 564 Number/g	Pigmented Nonmotile Strain 564 % Surv.	*E. coli* Number/g	*E. coli* % Surv.
0	5.7×10^7	---	4.3×10^6	---	1.3×10^7	---	5.2×10^6	---	3.5×10^8	---
1	1.6×10^7	28.00	3.4×10^6	79.00	8.8×10^6	67.70	4.7×10^6	90.50	3.5×10^7	10.00
2	3.6×10^6	6.30	9.1×10^6	211.00	2.6×10^7	200.00	9.0×10^6	173.00	9.2×10^7	262.00
3	1.2×10^6	2.10	2.2×10^6	51.00	8.7×10^6	67.00	6.0×10^6	115.50	3.5×10^7	10.00
4	1.3×10^6	2.28	1.6×10^6	37.20	4.0×10^6	30.80	4.7×10^6	90.00	2.8×10^7	8.00
5	1.3×10^6	2.28	7.6×10^5	17.70	6.5×10^6	50.00	3.5×10^6	67.30	2.8×10^7	8.00
6	1.4×10^6	2.46	6.8×10^5	15.80	6.0×10^6	46.20	4.0×10^6	77.00	4.5×10^6	1.29
9	2.0×10^5	0.35	4.1×10^5	9.50	1.1×10^6	8.45	4.2×10^6	80.80	7.9×10^6	2.26
11	4.7×10^4	0.08	3.9×10^5	9.05	1.8×10^6	13.80	3.8×10^6	74.00	3.5×10^6	1.00
13	4.2×10^4	0.07	1.2×10^5	2.79	7.0×10^5	5.40	1.0×10^6	19.30	3.5×10^6	1.00
16	3.7×10^4	0.06	2.1×10^4	0.48	4.8×10^5	3.70	8.0×10^5	15.40	9.2×10^5	0.26

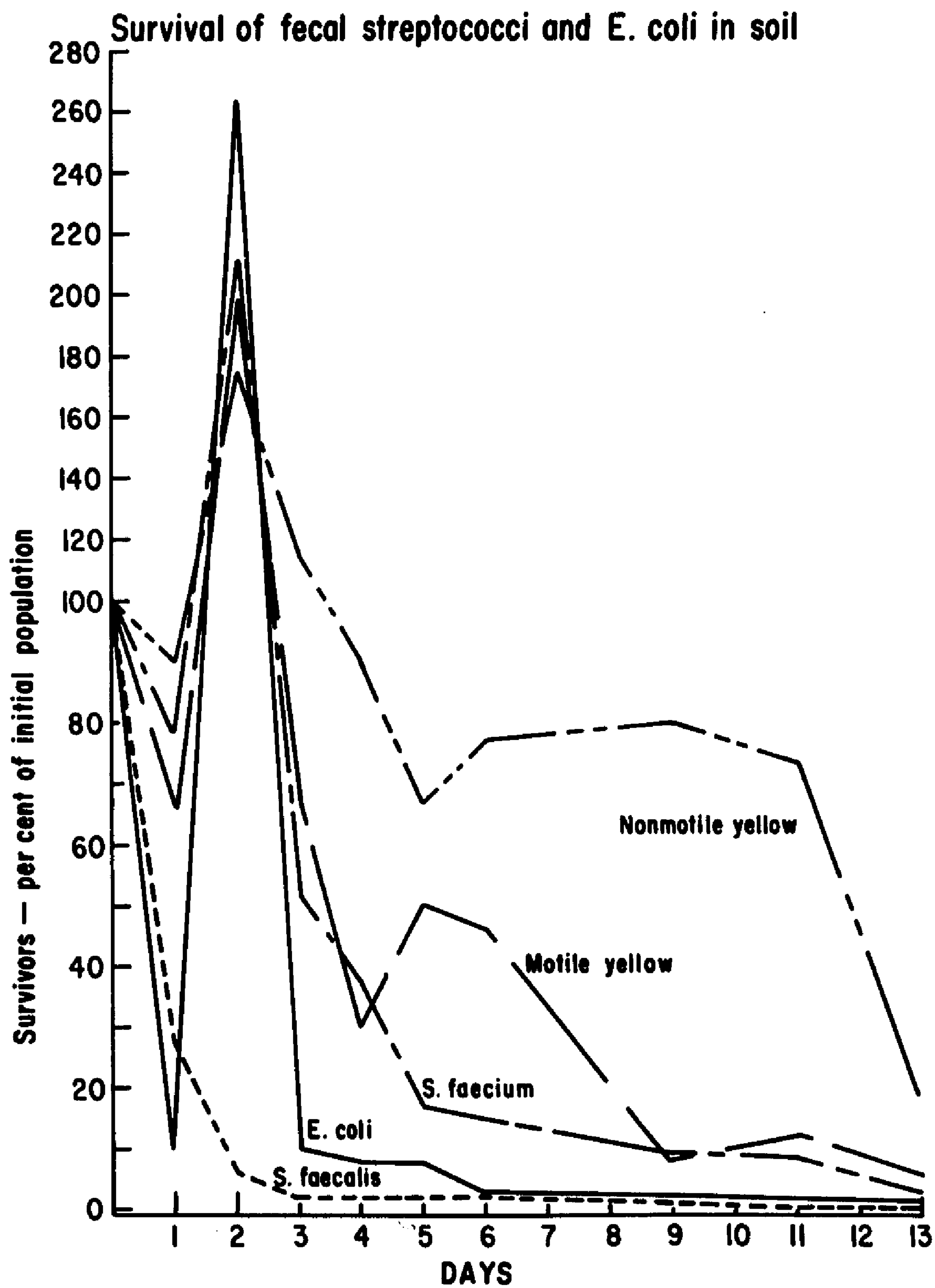
Note: Fecal streptococci count by pour plates of M-enterococcus agar. *E. coli* count by MPN elevated temperature test (EC).

pigmented streptococcus 3.70 per cent, the nonmotile pigmented organisms 15.40 per cent, and *E. coli* 0.26 per cent. These results showed that the two pigmented strains of streptococci had a longer survival time in soil than any of the other test organisms. *S. faecalis* was the only organism tested that did not show an increase in numbers anytime during the 16 days. This is illustrated in FIGURE 2. These results indicated that *S. faecalis* would be the best indicator of recent pollution in soil. The yellow streptococci apparently were able to remain viable in the soil for quite some time.

As to the origin of these pigmented streptococci, they were not isolated uniformly throughout the garden area but only at localized sampling areas. This suggested local contamination by either animals or insects or both. A number of burrowing holes in field soil were sampled and although streptococci were present none were pigmented. They have been previously found in insects such as the army worm, bean worm, aphid, and flies by Bartley (unpublished). Although this investigator did not consider insects in this study, the evidence suggests that they are a likely possibility.

Simultaneously, with sampling soils for fecal streptococci an investigation of the occurrence of these organisms on plants grown in the same soil was carried out. The objective was to determine if the same types of streptococci that were present in soil were found in plants or were the streptococci present in plants different from those present in the soil. The results appear in the

FIGURE 2



following tables.

The numbers of fecal streptococci per gram of corn husks and the types of streptococci identified are given in TABLE 15. Fecal streptococci were present in every sample tested ranging from 7-52 per gram. Of 111 strains identified, 3.6 per cent were _S. faecalis_, 17.1 per cent were _S. faecalis_ var. _liquefaciens_, 38.8 per cent were _S. faecium_, and 40.5 per cent were _S. faecium_-like. These results show that the same types of fecal streptococci present in the soil were present on the plants. When the interior parts, i.e., kernels, of the corn samples were examined it was found that there were no fecal streptococci present in any sample.

Tomatoes and green beans were examined for the presence of fecal streptococci both on the exterior and in the interior of the above vegetables. The results appear in TABLE 16.

Fecal streptococci were present on the exterior surfaces of 50% of the tomatoes sampled. The numbers ranged from 4-20 per ml of surface washings. Only one tomato was found to yield fecal streptococci in the interior portions. The results with the green beans were similar. Forty per cent of the 10 samples tested had fecal streptococci present. However, these organisms occurred in greater numbers on the green beans than on the tomatoes. Fecal streptococci were detected on the interior parts of 40% of the green beans tested. The identification of the streptococci isolated from tomatoes and green beans showed that the

TABLE 15

Numbers and Types of Fecal Streptococci Isolated From Corn Husks That Were Grown in Garden Soil Fertilized Only With Commercial Fertilizer

Sample No.	Source	No. fecal strep per gram	No. isolates identified	*S. faecalis*	*S. faecalis*-like	*S. faecalis* var. *liquefaciens*	*S. faecium*	*S. faecium*-like
1	Garden soil	25	12	0	0	0	6	6
2	Garden soil	15	7	3	0	1	3	0
3	Garden soil	38	15	0	0	3	6	6
4	Garden soil	52	23	0	0	5	8	10
5	Garden soil	10	8	0	0	4	2	2
6	Garden soil	13	7	0	0	2	2	3
7	Garden soil	32	5	0	0	2	3	0
8	Garden soil	9	6	1	0	0	2	3
9	Garden soil	12	9	0	0	1	3	5
10	Garden soil	28	7	0	0	0	3	4
11	Garden soil	7	4	0	0	0	2	2
12	Garden soil	17	8	0	0	1	3	4
				No. / %	No. / %	No. / %	No. / %	No. / %
	Total		111	4 / 3.6	0 / 0.0	19 / 17.1	43 / 38.8	45 / 40.5

Note: Isolation was achieved with pour plates of M-enterococcus agar.

TABLE 16

Numbers of Fecal Streptococci Isolated From Tomatoes and Green Beans Grown in Garden Soil Fertilized Only with Commercial Fertilizer

Sample No.	Source	No. fecal streptococci per ml in tomato washings	No. fecal streptococci per gram on interior of tomato	Sample No.	Source	No. fecal streptococci per ml in green bean surface washings	No. fecal streptococci per gram on interior of green beans
1	Tomatoes	20	0	11	Green beans	0	0
2	Tomatoes	0	0	12	Green Beans	0	0
3	Tomatoes	0	0	13	Green Beans	200	20
4	Tomatoes	8	0	14	Green Beans	150	28
5	Tomatoes	4	0	15	Green Beans	0	0
6	Tomatoes	0	0	16	Green Beans	30	5
7	Tomatoes	0	0	17	Green Beans	45	12
8	Tomatoes	12	4	18	Green Beans	0	0
9	Tomatoes	0	0	19	Green Beans	0	0
10	Tomatoes	7	0	20	Green Beans	0	0

Note: Plate counts on M-enterococcus agar.

majority were *S. faecium* and *S. faecium*-like. In summary, the numbers and types of fecal streptococci present in corn, tomatoes, and green beans show that fecal streptococci were present on all of the corn samples tested and in 40-50% of the tomatoes and green beans samples. This suggests that tomatoes and green beans are not natural hosts for fecal streptococci, otherwise, they would have been detected more often. Fecal streptococci were rarely found on the interior parts of the samples tested. The types present on these vegetables were very similar to those found in the soil.

The results of the soil and plant studies indicated that the majority of types of streptococci were quite similar to those isolated from the intestinal tract of animals, i.e., *S. faecium* or *S. faecium*-like.

Concurrent with the soil and plant studies, storm-water drainoff was collected from a fallow field in an effort to determine the numbers and types of fecal streptococci being washed into the estuarine waters of Little Bay. A comparison was made with the coliforms and fecal coliforms. TABLE 17 shows that the numbers of coliform was much greater than either the fecal coliforms or the fecal streptococci, indicating that many of these coliforms were of a non-fecal variety. It should also be noted that the average number of fecal streptococci approximated the average number of fecal coliforms. This suggested that the streptococci entering the estuarine environment via storm-water drainoff most likely originated from warm-blooded animals. In the identification of fecal streptococci, 31.0 and 39.8 per cent

TABLE 17

Comparison of Numbers of Fecal Streptococci, Coliforms, and Fecal Coliforms Isolated From Storm-Water Drainoff From Non-Fertilized Soil by the Membrane Filter Technique

Sample No.	No. coliforms per 100 ml on M-Endo agar	No. fecal coliforms per 100 ml on mfc pads at 44.5 C	No. fecal strep per 100 ml on M-enterococcus agar	No. colonies of fecal streptococci identified	*S. faecalis*	*S. faecalis* var *liquefaciens*	*S. faecalis*-like	*S. faecium*	*S. faecium*-like
1	542	184	221	18	2	1	1	6	8
2	128	98	84	12	0	0	3	3	6
3	470	274	180	18	2	0	0	6	10
4	324	74	92	9	1	0	0	3	5
5	640	268	250	20	6	2	2	6	4
6	726	162	130	10	0	2	4	0	4
7	428	68	90	10	2	0	0	4	4
8	364	49	60	6	2	0	0	4	0
	Av. = 452/ 100 ml	Av. = 147/ 100 ml	Av. = 138/ 100 ml		# 15 % 14.5	# 5 % 4.85	# 10 % 9.75	# 32 % 31.0	# 41 % 39.8

were identified as S. faecium and S. faecium-like respectively.

A comparison was also made of the numbers of coliforms, fecal coliforms, and fecal streptococci in soil from a fallow field, a garden, and a densely wooded area. The main objective was to correlate the occurrence of the fecal coliforms with the fecal streptococci. If these two groups are of intestinal origin only, then they would either be both absent or both present. If, however, the fecal streptococci found in the soil are not of intestinal origin, then they might be expected to be found in areas where fecal coliforms are not found. The results of this study appear in TABLES 18, 19, and 20.

The results from the grassland samples show that the fecal coliforms were not present in any of the sampling areas, whereas the coliform group were observed throughout this study. Fecal streptococci were present in 4 of the 12 samples in very low numbers. Thus, there was a much closer correlation between the fecal coliforms and the fecal streptococci than between the coliforms and the fecal streptococci.

TABLE 19 summarizes the results in garden soil. Except for a few samples there is a close correlation between the numbers of fecal streptococci and the numbers of fecal coliforms.

In TABLE 20, the absence of fecal coliforms indicate that the coliforms were of a non-fecal variety. The absence of the fecal streptococci also indicate that they are not present in soil that is not polluted by warm-blooded animals.

TABLE 18

Comparison of Numbers of Coliforms, Fecal Coliforms, and Fecal Streptococci in Non-Fertilized Grassland Soil

	Coliforms/gram	Fecal coliforms/gram	Fecal strepto-cocci/g
Sample No.	Confirmed MPN	EC MPN	Pour-plates Enterococcus agar
1	9,200	0	20
2	40	0	0
3	20	0	0
4	220	0	0
5	130	0	0
6	78	0	0
7	20	0	0
8	230	0	20
9	330	0	0
10	130	0	10
11	230	0	0
12	78	0	20

TABLE 19

Comparison of Coliforms, Fecal Coliforms, and Fecal Streptococci in Garden Soil Fertilized with Commercial Fertilizer

	Coliforms/ gram	Fecal coliforms/ gram	Fecal streptococci/ gram
Sample No.	Confirmed MPN	EC MPN	pour-plates Enterococcus agar
1	2,400 +	0	18
2	790	0	36
3	1,300	0	90
4	490	0	414 (pigmented)
5	5,400	110	882
6	9,200	170	45
7	3,500	36	81
8	2,200	20	18
9	9,200	0	18
10	1,100	68	27
11	1,700	490	18
12	110	8	27
13	5,400	3,500	105 (pigmented)
14	790	0	20
15	330	0	233 (pigmented)
16	330	0	150
17	2,400	2,400	10
18	490	20	110
19	1,300	20	60
20	9,200	78	100
21	230	20	25
22	640	20	24
23	790	230	50 (pigmented)
24	16,000	5,400	30 (pigmented)
25	9,200	78	40
26	16,000	20	50
27	3,500	0	20
28	16,000	20	0
29	5,400	0	0
30	5,400	0	15

TABLE 20

Comparison of Coliforms, Fecal Coliforms, and Fecal Streptococci in Soil From a Densely Wooded Forest Area

Sample No.	Coliforms/gram Confirmed MPN	Fecal coliforms/gram EC MPN	Fecal streptococci/gram Pour-plates M-enterococcus agar
1	230	0	0
2	330	0	0
3	230	0	10
4	330	0	0
5	330	0	0
6	310	0	0
7	230	0	0
8	330	0	0
9	460	0	0
10	230	0	0

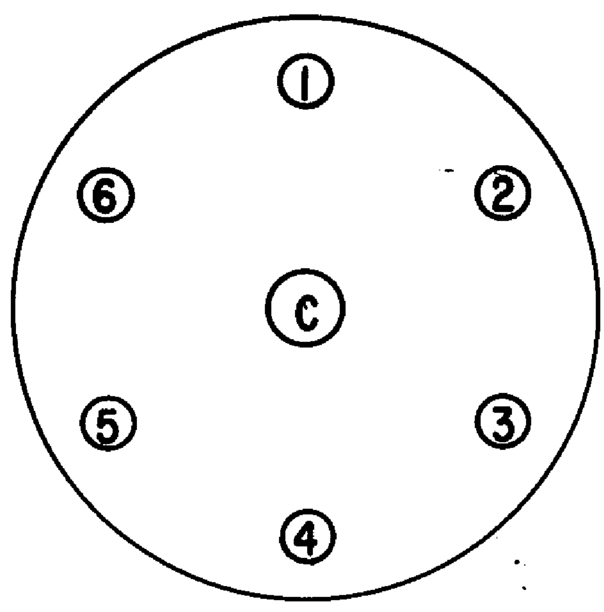
Serology of the fecal streptococci

It has been shown in this investigation that strains of streptococci isolated from soil and plants differ from the established species by one or more biochemical reactions. An attempt was made to determine if these *S. faecalis*-like and *S. faecium*-like organisms were serological Group D streptococci. The method of using disrupted organisms brings out the somatic O antigens as reported by Jones and Shattuck (1960) and Sharpe and Fewins (1960). Therefore, a second objective was to determine if the somatic antigens of the *S. faecalis*-like and *S. faecium*-like organisms were similar to those of *S. faecalis* and *S. faecium*. I found that by using disrupted cells as the inoculum in rabbits, that antiserum was produced against *S. faecalis* and *S. faecium* to titers of 1-320 as demonstrated by the gel diffusion test. The following figures illustrate the results of the serological studies.

FIGURE 3 shows that there was no reaction observed when normal rabbit serum was cross-reacted with the above antigenic preparations of the test strains of Group D streptococci.

FIGURE 4 shows the presence of the Group D antigen when antigen preparations of *S. faecalis*, *S. faecium* and *S. faecalis* var. *liquefaciens* were allowed to react with commercially prepared Group D antiserum. *Streptococcus pyogenes* (Group A), *Streptococcus agalactiae* (Group B), and *Streptococcus lactis* (Group N) showed no reaction.

FIGURES 5 and 6 show the relationship between the



C = Normal rabbit serum

1 = S. *faecalis* antigen

2 = S. *faecium* antigen

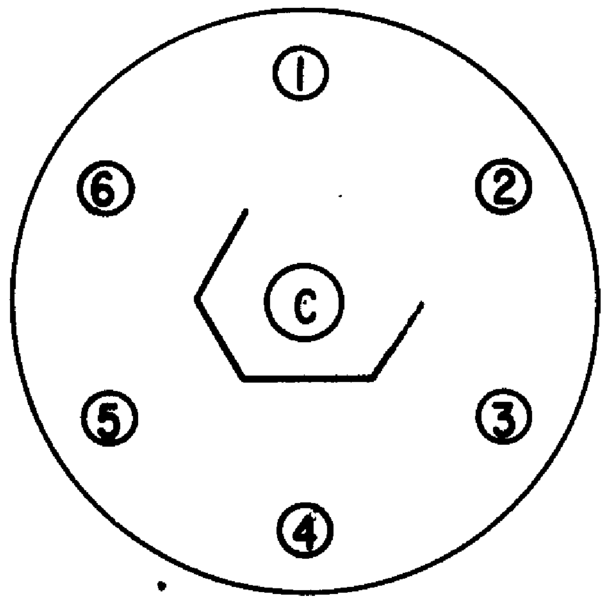
3 = S. *faecalis* var. *liquefaciens* antigen

4 = S. *faecalis* var. *zymogenes* antigen

5 = S. *faecalis* var. *durans* antigen

6 = Commercial Group D antigen

FIGURE 3



C = Common Group D antiserum

1 = S. *pyogenes* antigen

2 = S. *agalactiae* antigen

3 = S. *lactis* antigen

4 = S. *faecalis* antigen

5 = S. *faecium* antigen

6 = S. *faecalis* var. *liquefaciens* antigen

FIGURE 4

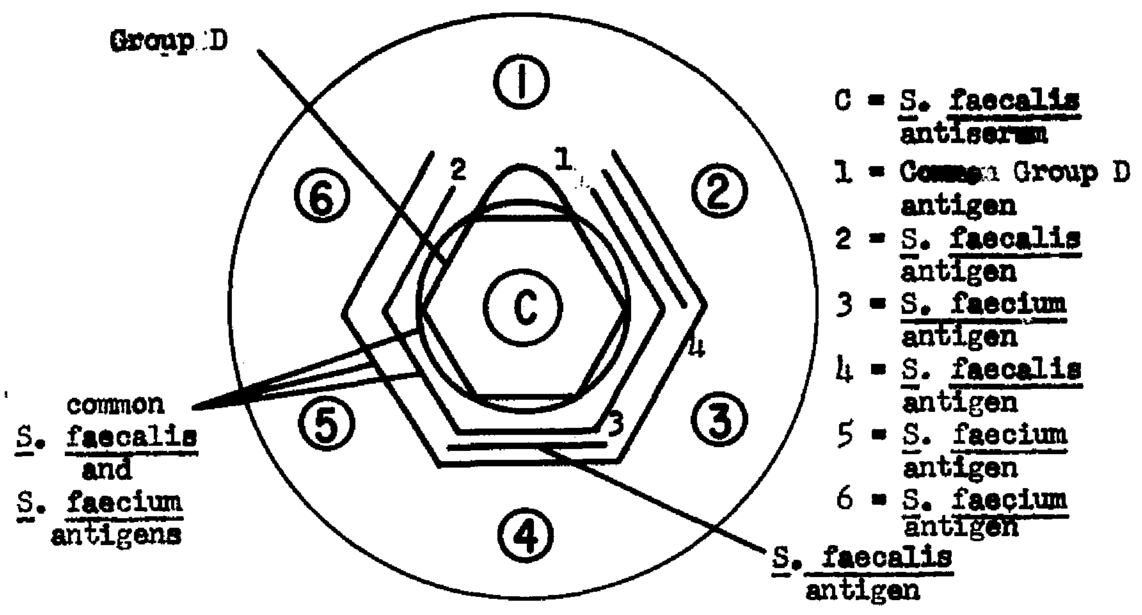


FIGURE 5

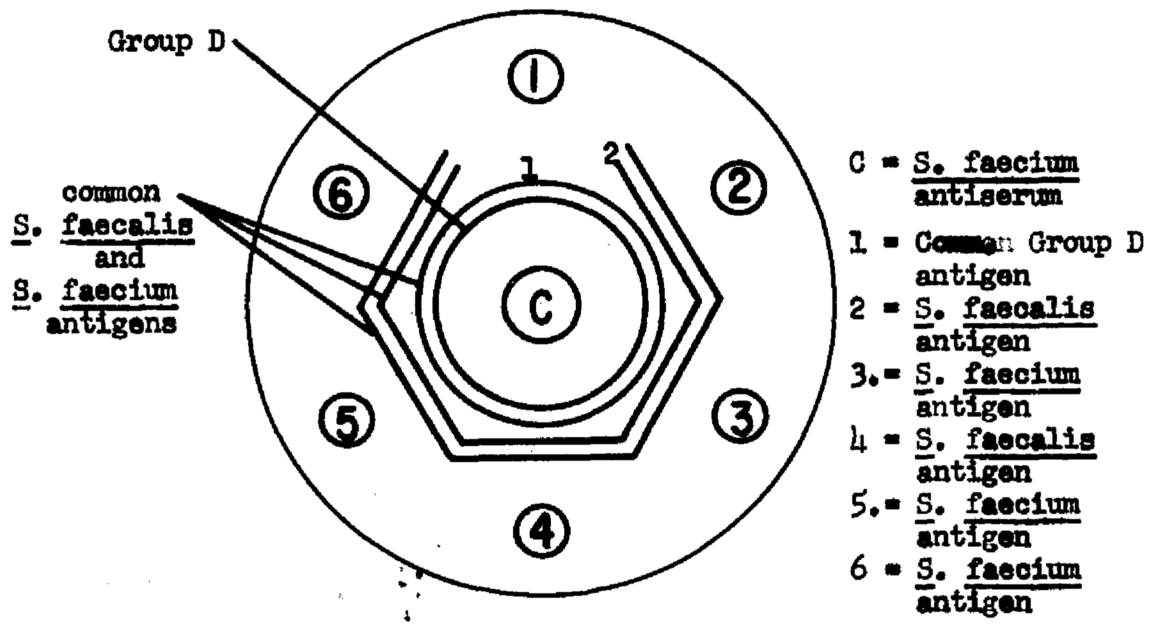


FIGURE 6

antigenic structures of S. faecalis and S. faecium. In FIGURE 5 antigen preparations of S. faecalis and S. faecium were allowed to react with antiserum to S. faecalis produced in rabbits. It can be seen that S. faecalis and S. faecium are similar in antigenic structure with the exception of one antigen present in S. faecalis that is not present in S. faecium. In FIGURE 6 the antigens of S. faecalis and S. faecium were allowed to react with antiserum to S. faecium produced in rabbits. This result supports the results of FIGURE 5 in that it shows S. faecalis and S. faecium to have 3 antigens in common besides the Group D antigen. The fourth antigen present only in S. faecalis did not shop up in FIGURE 6 because antiserum to S. faecium was used.

In FIGURE 7 antigen preparations of S. faecalis and biotype I were reacted with antiserum to S. faecalis. The antigens have been numbered 1, 2, 3, and 4 for convenience. These two organisms have antigens 1 and 4 in common with antigens 2 and 3 present only in S. faecalis.

FIGURE 8 shows the relationship between the antigens of S. faecalis and biotype II. Biotype II and S. faecalis have antigens 2 and 4 in common. Antigens 1 and 3 are present in S. faecalis but not biotype II.

FIGURE 9 illustrates the relationship between the antigens of S. faecalis and biotype III. These 2 organisms have antigens 1, 2, and 4 in common while antigen 3 is present only in S. faecalis.

FIGURE 10 shows that biotype IV and S. faecalis have antigens 2, 3, and 4 in common with antigen 1 present

S. faecalis Biotype I

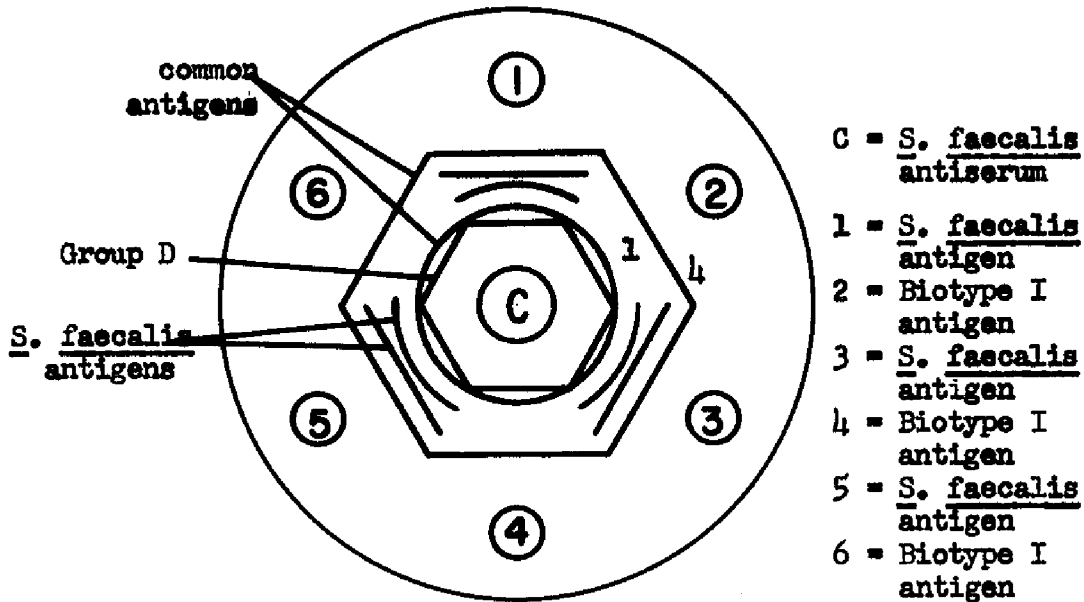


FIGURE 7

S. faecalis Biotype II

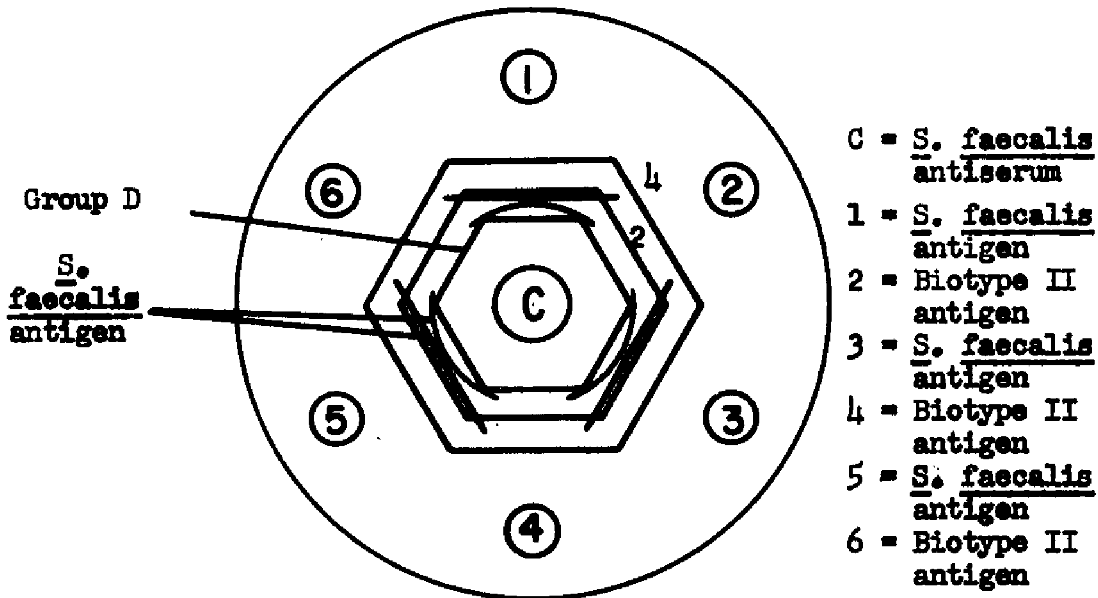


FIGURE 8

S. faecalis Biotype III

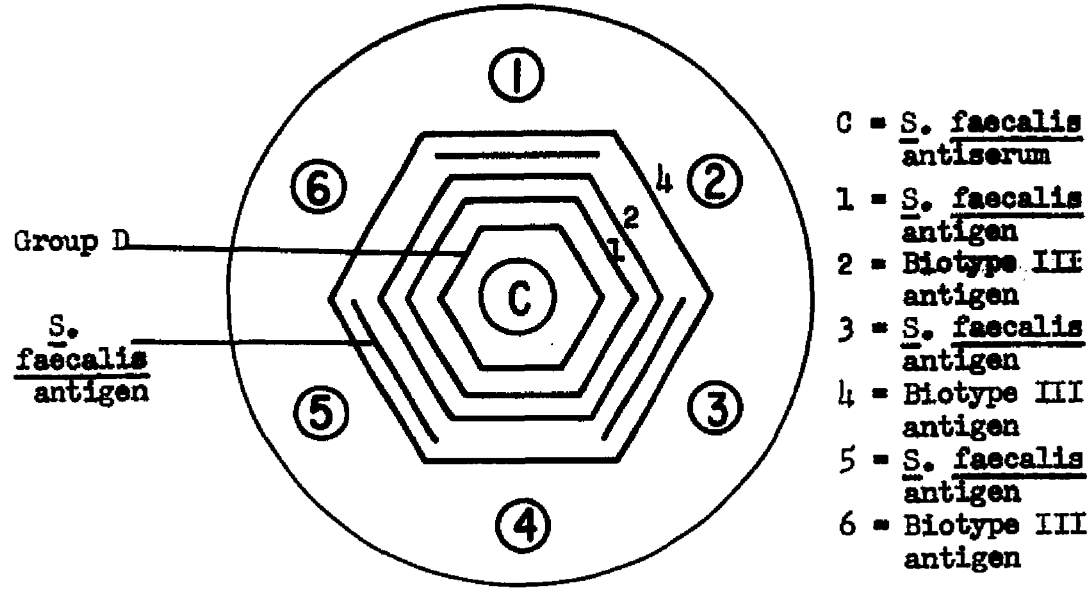


FIGURE 9

S. faecalis Biotype IV

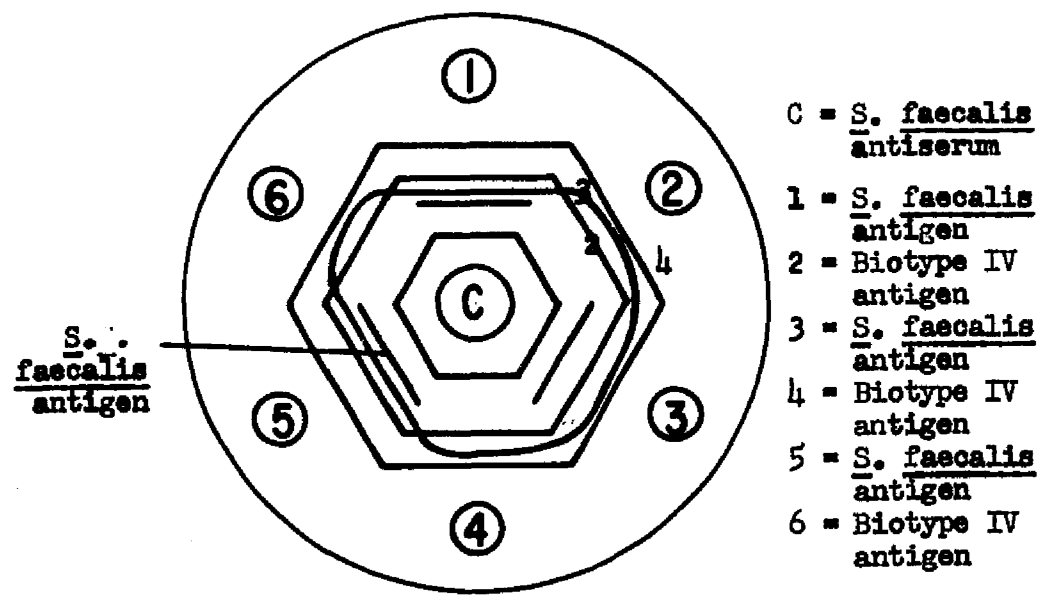


FIGURE 10

only in S. faecalis.

FIGURE 11 illustrates that *S. faecalis* and its variety liquefaciens have 3 antigens in common (1, 2, and 4), with antigen 3 unique to *S. faecalis*.

The following table summarizes the antigenic relationships among *S. faecalis*, *S. faecalis* var *liquefaciens*, *S. faecium*, and *S. faecalis* biotypes

TABLE 21

Number of Antigens Present in *S. faecalis* and Variety *Liquefaciens*, *S. faecium*, and *S. faecalis* Biotypes

Organism	No. antigens
S. faecalis	D, 1, 2, 3, 4
S. faecalis var. *liquefaciens*	D, 1, 2, 4
Biotype I	D, 1, 4
Biotype II	D, 2, 4
Biotype III	D, 1, 2, 4
Biotype IV	D, 2, 3, 4
S. faecium	D, 1, 2, 4

These results show that the biotypes, which differ from *S. faecalis* in fermentation reactions, also differ at the serological level by one or two somatic antigens as demonstrated by the gel diffusion method. *S. faecalis* var. *liquefaciens*, differing from *S. faecalis* in its ability to liquefy gelatin is also different serologically in that it

does not have antigen No. 3, which *S. faecalis* possesses. *S. faecium* is quite distinct from S. faecalis biochemically but serologically the only difference seems to be that it does not possess antigen No. 3, which is present in *S. faecalis*. Moreover, besides antigen No. 3 being present in *S. faecalis*, it was not detected in any other test strain except biotype IV. Therefore, this seems to be the one unique antigen that serves to distinguish *S. faecalis* from other fecal streptococci.

As part of the serologic studies, the *S. faecalis*-like and *S. faecium*-like strains isolated from soil and vegetables were examined for the presence of the Group D antigen. The results of this study appears in the following table.

TABLE 22

Number of S. faecalis-Like and S. faecium-Like Strains Isolated from Soil and Vegetables Possessing the Group D Antigen

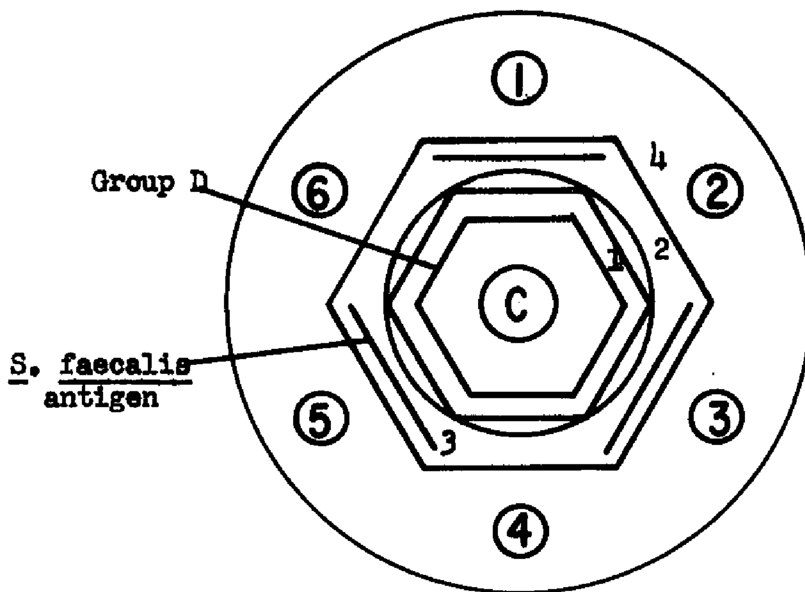
Type of strepto-cocci	No. strains tested from soil	No. giving positive Group D rxn	No. strains tested from vegetables	No. giving positive Group D rxn
S. fae-calis-like	16	16	4	3
S. fae-cium-like	9	8	7	7
Pigmented S. fae-cium-like	4	4	0	0

FIGURES 12 and 13 are examples of the majority of serologic findings when antigen preparations of *S. faecalis*-like strains were allowed to react with antiserum to *S. faecalis* and *S. faecium*, respectively. FIGURE 12 shows the relationship between *S. faecalis* and a *S. faecalis*-like strain isolated from tomatoes. This strain possessed the Group D antigen and one of the antigens present in *S. faecalis*. FIGURE 13 shows the relationship between an unpigmented strain identified as *S. faecium*-like with typical *S. faecium*. The strain isolated from tomatoes has the Group D antigen but does not have any of the other 3 antigens with *S. faecium*.

FIGURES 14 and 15 are illustrations of pigmented *S. faecium*-like organisms isolated from garden soil. Antigens of these strains were reacted with antiserum to *S. faecium*. FIGURE 14 shows that although this strain possesses the Group D antigen, it does not have any other antigens in common with *S. faecium*. FIGURE 15 shows a strain sharing the Group D antigen and also 2 of the other 3 *S. faecium* antigens. This is an example of a pigmented strain that is similar to typical *S. faecium*.

These serological results support the ecological and biochemical results in that these *S. faecium*-like and *S. faecalis*-like organisms isolated from soil and plants are similar to typical strains isolated from feces but yet differ by fermentation reactions at the biochemical level and by one or two antigens at the serological level.

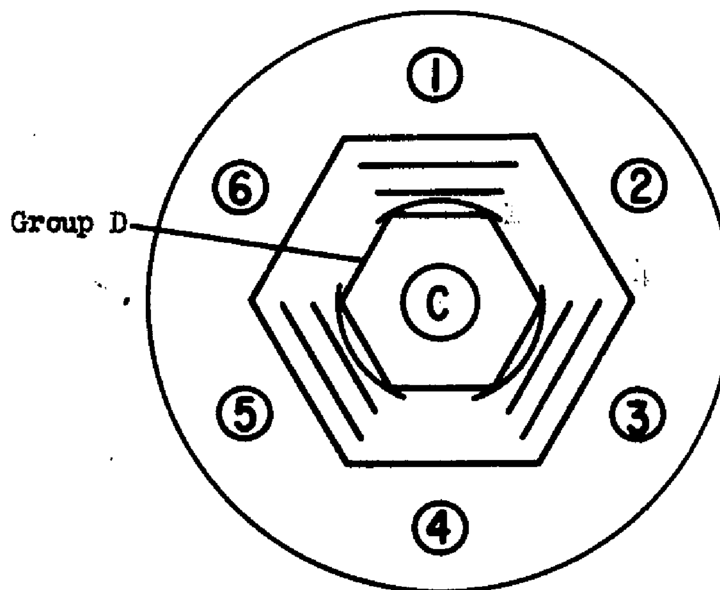
S. faecalis var. liquefaciens



C = S. faecalis antiserum
1 = S. faecalis antigen
2 = S. faecalis var. liquefaciens antigen
3 = S. faecalis antigen
4 = S. faecalis var. liquefaciens antigen
5 = S. faecalis antigen
6 = S. faecalis var. liquefaciens antigen

FIGURE 11

S. faecalis-like



C = S. faecalis antiserum
1 = S. faecalis antigen
2 = S. faecalis-like antigen
3 = S. faecalis antigen
4 = S. faecalis-like antigen
5 = S. faecalis antigen
6 = S. faecalis-like antigen

FIGURE 12

Source: Tomatoes

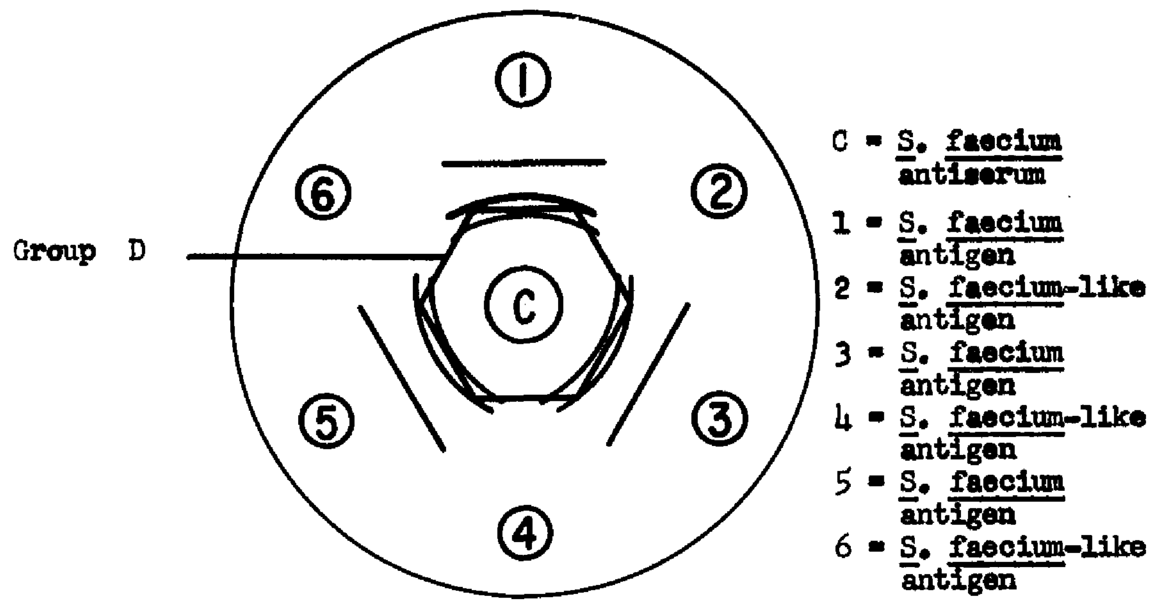


FIGURE 13

Source: Tomatoes (ext.)

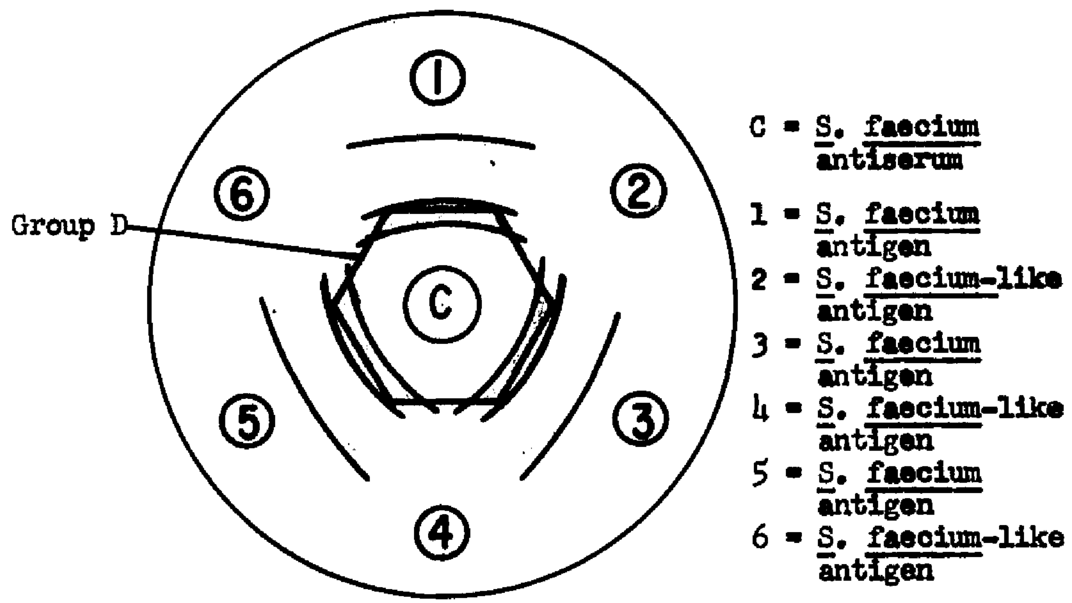


FIGURE 14

Source: Soil

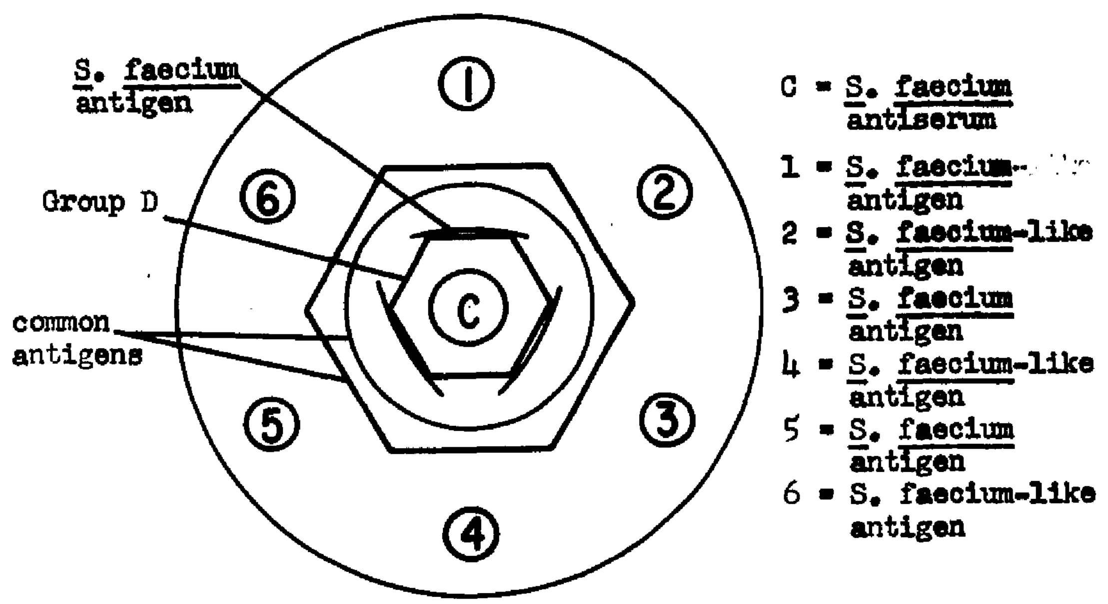


FIGURE 15

Source: Soil

The Sanitary Significance of the Oral Streptococci in Water and Foods

This investigation has indicated so far that the fecal streptococci and the fecal coliforms have been more indicative of fecal contamination than the coliforms. Many of the coliforms seemed to be of non-fecal origin. However, none of these groups presents a clear-cut distinction between contamination of human origin versus that of animal origin. Both the coliforms and the fecal streptococci have been isolated from the intestinal tract of both humans and animals. It has been reported by Appleman (1967) that the oral streptococci (*S. salivarius* and *S. mitis*) have been isolated from human feces but not from animals. Therefore if these organisms were isolated from water or foods it would be indicative of human pollution. The main problem with this group has been the lack of a good selective medium for their isolation. All media used up to the present for their isolation permits the growth of the fecal streptococci as well as the oral streptococci. The fecal streptococci have been shown to greatly outnumber the oral streptococci in fecal material and therefore might be inhibitory to the latter's isolation.

This background information leads to the second objective of this study which was to develop a selective medium for the enumeration of *S. salivarius* and *S. mitis* in feces, water, sewage, and foods.

The development of an improved medium for the isolation of the oral streptococci is based upon a technique

in which penicillin is incorporated into a broth whose composition is such that the fecal streptococci are allowed to metabolize for a period of 6 hours while the oral streptococci are dormant. This will allow the penicillin to destroy the fecal streptococci while causing no damage to the oral streptococci. After 6 hours penicillinase (2000 y/ml) and sucrose (final cnc @ 5%) are added to the broth tubes. At the end of 48 hours of incubation at 35 C the tubes showing turbidity are streaked to Mitis-Salivarius Agar as a confirmed test.

Utilization of Various Substrates by *S. salivarius*, *S. mitis*, *S. faecalis*, and *S. faecium*

TABLE 23 shows the results of an experiment designed to find a suitable substrate and proper conditions so that the fecal streptococci would be allowed to metabolize and the oral group would not.

In the BHI and mannitol broth plus 6.5 per cent NaCl, the oral strains were apparently irreversibly damaged by the salt. In the mannitol plus 2.0 per cent NaCl the fecal streptococci grew whereas the oral strains did not metabolize unless the sucrose was added. This was the first step in the development of a selective medium for the isolation of the oral streptococci.

The Effect of Penicillin and Kanamycin on the Growth of *S. faecalis*

The next step was to determine what concentration of penicillin was the most effective in killing *S. faecalis*

TABLE 23

Growth of the Fecal and Oral Streptococci in Various Media after 24 hrs Incubation at 35 C with and without the addition of sucrose after 4 hrs Incubation

Broth Medium	*S. faecalis*			*S. faecium*			*S. salivarius* 9759			*S. salivarius*			*S. mitis* 9811		
	1[*]	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Brain Heart Infusion + 6.5% NaCl	-[a]	+	+	-	+	+	-	-	-	-	-	-	-	-	-
Mannitol	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+
Mannitol + 6.5% NaCl	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-
Mannitol + 4.0% NaCl	-	+	+	-	+	+	-	-	+/-	-	-	+/-	-	-	-
Mannitol + 2.0% NaCl	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+

* 1 - Blank Control
2 - No Sucrose Added
3 - Sucrose Added

[a] + : Growth as evidenced by turbidity
- : No Growth

in a 6 hour incubation period. In connection with this study it had been reported by Hewitt et al (1965) that the bactericidal activity of penicillin was increased if kanamycin was added. TABLE 24 shows the effects of penicillin and kanamycin on S. faecalis.

With penicillin alone, S. faecalis remained viable. With penicillin plus kanamycin, S. faecalis was almost completely nonviable at 25 units/ml of penicillin and above, after 6 hours of incubation at 35 C. These results confirmed those of Hewitt in that penicillin and kanamycin have a synergistic effect.

The next test was designed to assay the penicillin technique in a mannitol broth. Sodium azide (0.04%) was added for the purpose of inhibiting Gram negative organisms, in particular the coliforms. The results of that test appear in TABLE 25.

S. faecalis was completely rendered nonviable by the penicillin and kanamycin while in the control tube they numbered 10^8 per ml. S. salivarius numbered 10^6 in both the tubes containing penicillin and those without the penicillin. The results of this experiment showed that this medium was capable of selectively killing S. faecalis while allowing S. salivarius to grow. Later this medium was slightly altered by the addition of tryptose and proteose peptone NO. 3.

Recovery of S. salivarius from Raw Sewage

A. From Sewage Seeded with S. salivarius

TABLE 24

The Effect of Penicillin and Kanamycin on the Growth of *S. faecalis*

Penicillin units/ml	# org/ml on BHI Agar after 4 hours Incubation/35 C		# org/ml on BHI Agar after 6 hours Incubation/35 C	
	Penicillin only	Penicillin + Kanamycin[a]	Penicillin only	Penicillin + Kanamycin
1	6.0×10^4	3.0×10^4	4.8×10^4	3.0×10^2
5	5.6×10^4	4.0×10^4	4.3×10^4	0
10	3.6×10^4	6.1×10^3	1.2×10^4	20
25	4.0×10^4	1.0×10^3	3.0×10^4	0
50	5.0×10^4	1.1×10^2	2.7×10^4	0
100	2.8×10^4	10	2.3×10^4	0
500	6.8×10^5	20	7.5×10^5	0
Control (0)	6.4×10^6	---	6.4×10^7	---

a - Kanamycin at 40 ug/ml
Note: Kanamycin alone had no effect on the growth of *S. salivarius* and *S. faecalis*.

TABLE 25

The Effect of Penicillin and Kanamycin (60ug/ml) on the Growth of *S. faecalis* and *S. salivarius* in Mannitol Broth

Penicillin units/ml	No. Organisms per ml after 48 hrs/35 C		
	S. faecalis	*S. salivarius* 9759	*S. faecalis* & *S. salivarius*
50	0	5.6×10^6	2.7×10^6
100	0	4.9×10^6	1.6×10^6
500	0	6.1×10^5	6.6×10^5
0	3.8×10^8	7.3×10^6	

The Penicillin-Kanamycin MS Broth was next tested in recovering S. salivarius from raw sewage. The purpose of this experiment was to determine if this medium could detect S. salivarius in raw sewage if present in large numbers.

In TABLE 26 the results show that the numbers of S. salivarius organisms recovered using the penicillin technique were on the average 1 log lower than the initial numbers present. The PKMS broth was found to kill 99.9 per cent of the fecal streptococci. Thus, these results proved that this method can recover S. salivarius from sewage with relatively good efficiency.

B. From Raw Sewage

Since S. salivarius was recovered from seeded sewage using Penicillin-Kanamycin MS Broth the next step was to attempt to enumerate these organisms in raw sewage. In 6 samples of sewage (3 from Durham and 3 from Dover, N.H.) S. salivarius and S. mitis were not isolated from any sample. This indicated that either the numbers of oral streptococci discharged into sewage may be too low to detect by this method or these organisms may be extremely sensitive to the extraenteral environment and therefore die out rapidly.

Survival Studies of S. salivarius, S. faecalis, and S. faecium in Filter Sterilized Sewage and Fresh Water

To determine whether S. salivarius dies out rapidly in sewage and fresh water, survival studies were performed in the labatory. The results of those studies appear in FIGURES 16 and 17.

TABLE 26

Recovery of Known Numbers of *S. salivarius* Organisms added to Untreated Sewage Using Penicillin-Kanamycin MS Broth and Confirming on Mitis-Salivarius Agar

Sample No.	Initial No.[a] of *S. salivarius* per ml added	Number of *S. salivarius* per ml recovered (MPN)	Initial No. of fecal streptococci per ml on M-enterococcus Agar	Number of fecal streptococci recovered using PKMS Broth
1	8.6×10^5	7.9×10^4	3.4×10^6	2.2×10^3
2	7.4×10^5	8.0×10^4	1.7×10^5	6.3×10^2
3	1.8×10^6	4.5×10^5	2.5×10^5	1.4×10^2
4	8.1×10^5	7.9×10^4	4.7×10^5	1.3×10^2
5	2.1×10^6	1.8×10^5	3.8×10^4	2.4×10^2

A - Pour Plates of BHI Agar

FIGURE 16

Log Survival versus Time in Filter Sterilized Sewage at 20C (average of 3 trials)

1 *S. faecalis*

2 *S. faecium*

3 *S. salivarius*

4 *S. faecium* in Phosphate Buffered Water

5 *S. salivarius* in Phosphate Buffered Water

6 *S. salivarius* in sterilized sewage plus 0.5% Sucrose

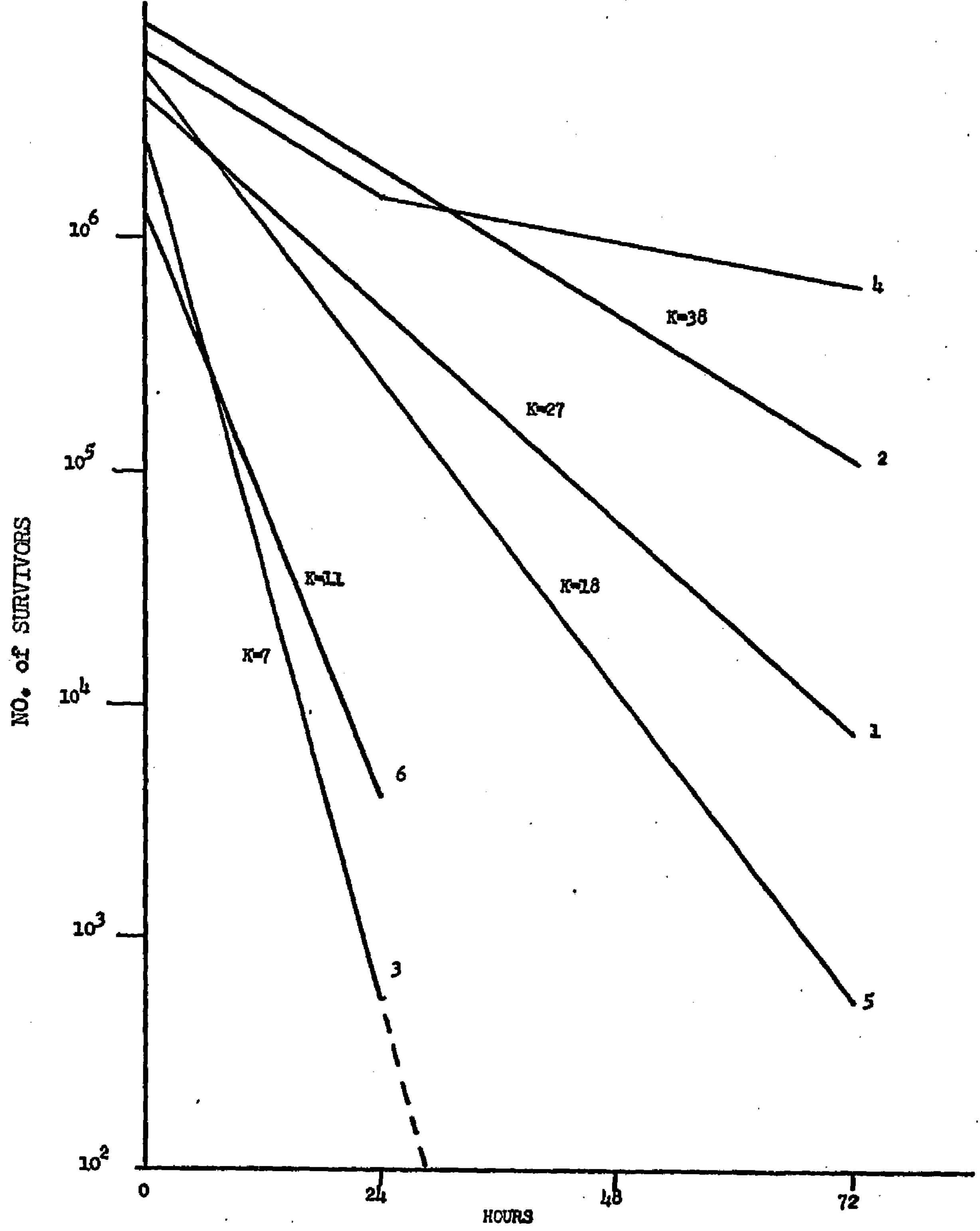

NO. of SURVIVORS
10^6
10^5
10^4
10^3
10^2
K=38
K=27
K=18
K=11
K=7
4
2
1
6
3
5
0
24
48
72
HOURS

FIGURE 17

Log Survival versus Time in Filter Sterilized Reservoir Water at 20C (average of 3 trials)

1 _S. faecalis_

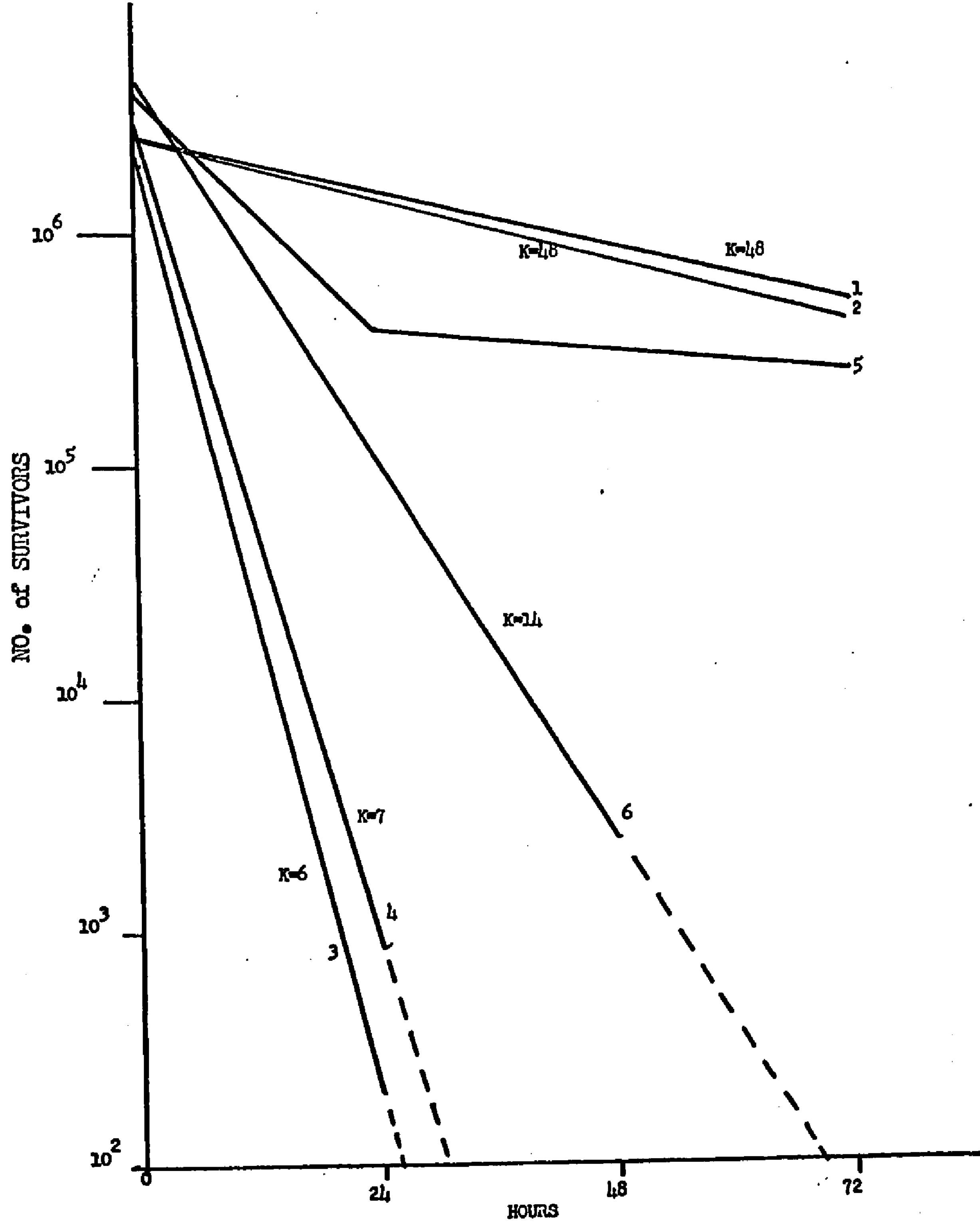
2 _S. faecium_

3 _S. salivarius_

4 _S. salivarius_ in sterilized reservoir water plus 0.5% Sucrose

5 _S. faecalis_ in phosphate buffered water

6 _S. salivarius_ in phosphate buffered water


NO. of SURVIVORS
10^6
10^5
10^4
10^3
10^2
0
24
48
72
HOURS
K=48
K=48
1
2
5
K=14
6
K=7
K=6
4
3

In raw sewage *S. salivarius* decreased in numbers by 3-4 logs in 24 hours. The K value or the number of hours required for the organism to decrease by 1 log was 7. When this value is compared with those of *S. faecalis* and *S. faecium*, it can be seen that the latter two organisms would serve as better indicators of fecal pollution than would *S. salivarius*. Because of the rapid die-off of *S. salivarius*, it would not be present when many pathogens might be present. This is not the case with the fecal streptococci. It can also be noted that in the buffered water control, *S. salivarius* died out rapidly when compared to *S. faecium*. Also the effect of 0.5 per cent sucrose in the sewage did not have any effect on the survival rate of *S. salivarius*.

The results in fresh water were similar. Examing the K values, it took about 8 times as many hours to kill 90 per cent of the fecal streptococci as it took to destroy *S. salivarius*. Therefore, the results of these two studies indicated that *S. salivarius* undergoes a 3-4 log decrease within 24 hours in both sewage and fresh water.

The Survival of Fecal Streptococci and Oral Streptococci in Raw Sewage after Inoculating with Human Fecal Samples

The previous results showed that *S. salivarius* had a rapid die-off rate in sewage and water. If the average fecal sample contains no more than 10^4 oral organisms per gram, then within 24 hours these organisms would not be detected in sewage. In TABLE 27 the results of a study are summarized in which fecal samples were inoculated into

TABLE 27

The Survival of Fecal and Oral Streptococci in Raw Sewage After Inoculating with Human Fecal Samples

SAMPLE NO.	Hours	No. Fecal streptococci per gm. on M-enterococcus agar (MF)	MPN per gm. of Viridans org. in PKMS Broth	Phosphate Buffered Water	
				No. fecal strep per gm. on enterococcus agar	MPN per gm. of Viridans org. in PKMS Broth
1	0	3.2×10^5	4.9×10^4	3.9×10^5	4.3×10^4
	24	3.5×10^5	0	4.1×10^5	6.3×10^2
	48	6.8×10^4	0	7.2×10^4	3.3×10^2
2	0	7.3×10^5	3.3×10^4	6.9×10^5	2.3×10^4
	24	7.1×10^5	0	6.5×10^5	2.6×10^2
	48	5.3×10^4	0	7.8×10^4	1.7×10^2
3	0	1.8×10^6	1.7×10^3	3.5×10^6	2.2×10^3
	24	2.1×10^6	0	3.9×10^6	0
	48	8.0×10^5	0	7.2×10^5	0

sewage to determine the initial numbers of oral streptococci and how many could be detected after 24 hours.

The results indicate that the initial number of oral streptococci in a fecal sample ranged from 10^3 to 10^4. After 24 hours these organisms could not be detected. Therefore the numbers of oral streptococci in a fecal sample are not large enough to detect in sewage or water within 24 hours of their discharge due to their rapid die-off rate.

Isolation and Enumeration of S. salivarius and S. mitis from Human Feces

With the development of the penicillin broth for the isolation of *S. salivarius* and *S. mitis* it was of interest to compare this technique with previous methods in order to establish whether this was truly a superior medium. The results of this study are summarized in TABLE 28.

Of the three methods, the Sucrose-Azide pour plates was the least efficient in isolating the oral streptococci from fecal samples. The MPN in Mitis-Salivarius Broth with confirmation to the same agar was superior to the Sucrose-Azide plates but less efficient than the MPN method using penicillin broth with confirmation to Mitis-Salivarius Agar. In the penicillin broth the fecal streptococci were largely destroyed and therefore did not compete with the oral streptococci for nutrients. Using the PKMS broth the average number of *S. salivarius* organisms approximated 10^4 per gram of fecal material. Also, a number of fecal samples from cows (8) and dogs (10) were examined for the presence of S. salivarius and S. mitis. In no sample was either

TABLE 28

Comparison of the Numbers[a] of Fecal and Oral Streptococci in Human Feces as Determined by 3 Methods

Sample No.	Sucrose-Azide Agar Pour Plate			Mitis-Salivarius Broth (MPN			Penicillin-Kanamycin MS Broth (MPN)		
	Fecal Strep	*S. salivarius*	*S. mitis*	Fecal Strep	*S. salivarius*	*S. mitis*	Fecal Strep	*S. salivarius*	*S. mitis*
1	1.2×10^{5}	0	0	2.6×10^{4}	7.9×10^{3}	2.1×10^{3}	7.8×10^{2}	7.9×10^{4}	6.3×10^{3}
2	5.6×10^{4}	5.0×10^{3}	0	6.3×10^{4}	6.3×10^{2}	0	3.3×10^{2}	7.0×10^{2}	0
3	1.5×10^{7}	0	0	7.9×10^{6}	0	0	9.4×10^{2}	1.7×10^{4}	1.2×10^{2}
4	6.2×10^{6}	0	0	2.7×10^{6}	7.0×10^{3}	0	4.6×10^{1}	4.9×10^{4}	0
5	4.7×10^{6}	0	0	2.3×10^{5}	9.2×10^{3}	2.7×10^{4}	5.4×10^{2}	2.7×10^{4}	7.0×10^{4}
6	5.7×10^{3}	2.8×10^{2}	0	6.3×10^{3}	4.9×10^{2}	0	0	2.2×10^{4}	1.1×10^{2}
7	7.3×10^{4}	1.7×10^{2}	0	1.4×10^{5}	3.1×10^{3}	7.0×10^{2}	0	4.6×10^{3}	0
8	8.3×10^{7}	0	0	5.4×10^{6}	1.3×10^{4}	1.4×10^{2}	9.2×10^{2}	7.0×10^{4}	0

[a] - All numbers based on wet weight of samples (#/gm).

organism detected which confirms the findings of other workers in that *S. salivarius* and *S. mitis* are present only in the intestinal tract of humans.

TABLE 29 shows the biochemical reactions of strains of *S. salivarius* and *S. mitis* isolated from human fecal material. The oral streptococci do not fulfill the Sherman criteria as the fecal streptococci do. *S. Salivarius* may be differentiated from *S. mitis* by the ability of *S. salivarius* to produce polysaccharide when cultured on 5.0 per cent sucrose agar.

The Bacteriological Examination of Foods for Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci

As indicated in the introduction, if it is possible to develop a methodology that is sufficiently sensitive to detect *S. salivarius* and *S. mitis* in foods an invaluable tool will have been gained in detecting human contamination. It has been shown that these organisms are absent in animal fecal material and present in human feces. In addition these organisms are present in large numbers (10^6 per ml) in saliva and would be an aid in detecting human oral pollution. With this in mind an examination was made of dairy products and frozen meat pies for the presence of *S. salivarius* and *S. mitis* as well as coliforms, fecal streptococci, and staphylococci.

TABLE 30 summarizes the results of the examination of dairy products. Except for a few samples, the dairy products were free of all indicator organisms.

TABLE 31 shows the results of the examination of

TABLE 29

Biochemical Characteristics of _S. salivarius_ and _S. mitis_ Isolated from Human Feces

Test	Result	_S. salivarius_		_S. mitis_	
		No. Strains reacting/ tested	% Reacting	No. Strains reacting/ tested	% Reacting
Polysaccharide Syn.	+	35/35	100	0/19	0
Blood Agar		35/35	100	13/19	68
Gelatin Liq.	-	35/35	100	19/19	100
Tolerance to 6.5 % NaCl	-	35/35	100	19/19	100
Tolerance to 0.1% MB Milk	-	35/35	100	19/19	100
Therman Resistance 60 C/30 min.	-	35/35	100	19/19	100
Growth at 45 C	-	28/35	80	19/19	100
Ferm: Sucrose	+	35/35	100	19/19	100
Maltose	+	35/35	100	19/19	100
Raffinose	+	35/35	100	10/19	53
Salicin	+	35/35	100	6/19	32
Arabinose	-	35/35	100	19/19	100
Mannitol	-	35/35	100	19/19	100
Sorbitol	-	35/35	100	19/19	100
Xylose	-	35/35	100	19/19	100
Glycerol	-	35/35	100	19/19	100
Melibiose	-	35/35	100	19/19	100
Melezitose	-	35/35	100	19/19	100
Rhamnose	-	35/35	100	19/19	100
Inulin	+	18/35	52	15/19	79
Lactose	+	35/35	100	16/19	84
Starch Hyd.	-	35/35	100	19/19	100
Motility	-	35/35	100	19/19	100

TABLE 30

The Bacteriological Examination of Dairy Products for Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci (No. Organisms Per Gram)

Sample No.	Product	Total Count	Coliforms (MPN)	Fecal Strep. (MPN)	Staphylococci Tellurite-Glycine Agar	Viridans Strep. (MPN) MSB[a]	Viridans Strep. (MPN) PKMS Broth
1	Sour Cream	9280	0	0	0	0	0
2	"	7560	"	"	"	"	"
3	"	6740	"	"	"	"	"
4	"	7400	"	"	"	"	"
5	"	8240	"	"	"	"	"
6	"	9320	"	"	"	"	"
7	"	6350	"	"	"	"	"
8	"	7620	"	"	"	"	"
9	"	8460	"	"	"	"	"
10	"	11500	"	"	"	"	"
11	"	9760	"	"	"	"	"
12	"	7560	"	"	"	"	"
13	"	8970	"	"	"	"	"
14	"	9480	"	"	"	"	"
15	Cottage Cheese	11400	"	26	"	"	"
16	"	10700	"	32	"	"	"
17	"	12700	"	0	"	"	"
18	"	13200	"	"	"	"	"
19	"	12800	"	"	"	"	"
20	"	11900	"	26	"	"	"
21	"	10200	"	0	"	"	"
22	"	690000	33	920	110	"	"
23	"	46000	49	1600	0	"	"
24	"	230000	70	2200	130	"	"
25	Sour Cream	6000	2	109	0	"	"
26	"	4000	14	130	"	"	"
27	Butter-milk	9600	0	0	"	"	"
28	"	10200	"	"	"	"	"
29	"	11200	"	"	"	"	"
30	"	10800	"	"	"	"	"

[a] MSB = Mitis-Salivarius Broth

TABLE 31

Bacteriological Examination of Frozen Chicken Meat Pies for Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci (No. Organisms Per Gram)

Sample No.	Total Count	Coliforms (MPN)	Fecal Strep (MPN)	Staphylococci Tellurite-Glycine Agar	*S. salivarius* MPN MS Broth	*S. salivarius* (MPN) PKMS Broth	*S. mitis* MS Broth (MPN)	*S. mitis* (MPN) PKMS Broth
1	7,900	33	490	0	55	278	0	0
2	3,300	11	63	0	4	22	0	0
3	3,700	5	49	0	4	26	0	0
4	1,200	8	240	0	17	46	0	0
5	2,650	14	221	0	43	79	0	0
6	41,000	5	700	0	26	70	0	0
7	27,000	2	130	500	0	0	0	0
8	45,000	79	1,300	400	278	345	79	141
9	210,000	94	109,000	200	9,180	16,000	175	542
10	75,600	43	54,200	0	5,420	9,200	0	0
11	54,000	34	9,180	100	2,400	5,420	172	278
12	67,500	26	16,000	400	3,450	5,420	0	0
13	240,000	79	141,000	600	5,420	17,500	0	0
14	47,000	43	16,000	0	542	1,750	141	542
15	36,000	22	348	0	0	0	0	0
16	28,000	17	172	200	0	0	0	0
17	13,800	920	46	0	0	0	0	0
18	22,500	160	46	100	0	0	0	0
19	13,300	348	33	0	0	0	0	0

frozen chicken pies. _S. salivarius_ was isolated from almost every sample and _S. mitis_ was detected in some samples. The penicillin technique was superior to the Mitis-Salivarius Broth in enumerating the oral streptococci from the meat pies. It should also be noted that where the fecal streptococcus count showed a marked increase in samples 8-14, there was a corresponding increase in the number of _S. salivarius_ organisms. This suggested that the source of the _S. salivarius_ organisms was of fecal origin rather than oral because of the large numbers of fecal streptococci also encountered.

The examination of turkey and beef pies (TABLES 32 and 33) revealed that while _S. salivarius_ was present in many samples, the numbers were not nearly as high as in the chicken pies. In the turkey pies there were large numbers of coliforms and fecal streptococci in the same samples with small numbers of oral streptococci. This was in contrast to the results of the chicken pies where large numbers of fecal and oral streptococci were isolated with small numbers of coliforms. Also the PKMS Broth was able to detect small numbers of oral streptococci whereas the Mitis-Salivarius Broth was not.

TABLE 34 presents a summary of the correlation of the presence of coliforms, fecal streptococci, oral streptococci, and staphylococci in meat pies. _S. salivarius_ and/or _S. mitis_ were isolated from 46 per cent of the frozen meat pies examined using Mitis-Salivarius Broth and from 71 per cent of the samples examined using the PKMS broth. These results therefore show that the Penicillin-Kanamycin MS

TABLE 32

Bacteriological Examination of Frozen Turkey Meat Pies for Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci (No. Organisms per Gram)

Sample No.	Total Count	Coliforms (MPN)	Fecal Strep (MPN)	Staphylococci Tell-Gly Agar	S. salivarius MS-Broth (MPN)	S. salivarius (MPN) PKMS Broth	S. mitis MS-Broth (MPN)	S. mitis (MPN) PKMS Broth
1	900	0	109	0	0	45	0	0
2	990	0	33	0	0	7	0	0
3	7,300	0	0	0	0	0	0	0
4	3,420	5	221	0	0	63	0	0
5	179,000	17,200	116,000	0	0	8	0	0
6	214,000	34,800	54,200	0	0	14	0	0
7	163,000	24,000	54,200	0	0	20	0	0
8	78,000	79	790	200	0	39	0	0
9	81,000	348	1,720	100	49	172	0	0
10	32,600	27	920	0	70	34	0	0
11	44,800	31	542	0	33	130	0	0
12	16,200	1,600	3,480	400	17	141	27	94
13	61,300	920	542	0	0	79	0	0
14	19,400	22	13	0	0	0	0	0

TABLE 33

Bacteriological Examination of Frozen Beef Meat Pies for Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci (No. Organisms Per Gram)

Sample No.	Total Count	Coliforms (MPN)	Fecal Strep (MPN)	Staphylococci TG Agar	*S. salivarius* MS Broth (MPN)	*S. salivarius* (MPN) PKMS Broth	*S. mitis* MS Broth (MPN)	*S. mitis* (MPN) PKMS Broth
1	1,500	0	5	0	0	21	0	0
2	1,250	0	0	0	0	0	0	0
3	1,320	0	33	0	7	7	0	0
4	1,470	0	26	0	0	0	0	0
5	1,390	0	34	0	0	0	0	0
6	78,000	5	230	0	0	23	0	0
7	23,000	13	23	0	6	7	0	0
8	12,900	13	49	0	12	31	0	0
9	17,500	7	63	0	0	26	0	0
10	33,400	70	141	0	23	109	0	0
11	21,500	0	17	0	0	0	0	0
12	20,000	0	26	0	0	0	0	0

TABLE 34

Correlation of the Presence of Coliforms, Fecal Streptococci, Oral Streptococci, and Staphylococci in Frozen Meat Pies

Product	No. Tested	Coliforms (MPN)		Fecal Strep (MPN)		Staphylococci TG Agar		Viridans Strep MS-Broth (MPN)		Viridans Strep (MPN) PKMS Broth	
		No. Positive	%	No. Positive	%	No. Positive	%	No. Positive	%	No. Positive	%
Chicken	19	19	100	19	100	8	42	13	68	13	68
Turkey	14	11	78	13	92	3	21	4	28	12	85
Beef	12	5	41	11	91	0	0	4	33	7	58
Total	45	35	77	43	95	11	24	21	46	32	71

Broth is superior to the Mitis-Salivarius Broth in enumerating oral streptococci from frozen meat pies.

The Effect of Various Storage Temperatures on the Survival of S. salivarius in Chicken Meat Pies

If S. salivarius and S. mitis are to be used as indicators of human pollution in foods, then it is of interest to determine what the effects of storage at low temperatures has on their viability after certain periods of time. The results of that study is in TABLE 35.

After 28 days of storage at -100°F, 0°F, and 41°F, the results show that S. salivarius remains viable at low temperature storage. This is important if these organisms are to be used to demonstrate the original sanitary quality of the product.

Recovery of S. salivarius from the Air after Aerosolizing with a 24 Hour Culture

One of the most likely routes by which S. salivarius may enter a food product is via the air from a cough or sneeze from the workers in a food processing plant. TABLE 36 shows the results of a study to determine how long S. salivarius may be detected in the air and on surfaces following inoculation with a spray bottle into an enclosed room (10' x 3' x 10').

Thirty minutes after inoculation, 99 organisms per 10 cubic feet of air were recovered. After one hour, none could be recovered. However, S. salivarius was isolated from the surfaces of the surrounding walls, floor, and

TABLE 35

The Effect of Various Storage Temperatures on the Survival of S. salivarius in Chicken Meat Pies (No. Organisms/Pie)

Days In Storage	MPN PKMS Broth	MPN PKMS Broth	MPN PKMS Broth
	-100 F	O F	(Refrigerator T) 41 F
0	92,000,000*	54,000,000*	54,000,000*
1	54,000,000	16,000,000	54,000,000
3	54,000,000	16,000,000	54,000,000
7	54,000,000	16,000,000	34,000,000
10	9,200,000	1,300,000	3,400,000
14	5,400,000	1,300,000	2,780,000
21	3,480,000	1,090,000	2,210,000
22	3,330,000	1,090,000	1,400,000

* Average of 3 pies for each determination

TABLE 36

Recovery of *S. salivarius* from the Air at Various Times After Aerosolization with *S. salivarius*

Time of Sampling After Inoculation	# *S. salivarius* per 10 cubic ft. air on MS* agar	Presence of *S. salivarius* on Swab Plates of MS Agar
30 min.	99	+
1 hr.	1	+
2 hr.	0	+
3 hr.	0	+
5 hr.	0	+
24 hr.	0	+

* MS = Mitis-Salivarius

door of the room 24 hours after inoculation. This suggested that if these organisms are present on a food processing surface via a cough or sneeze, then this surface is a potential source of oral contamination for the next 24 hours.

Persistence of S. salivarius and S. mitis on Stainless Steel Surfaces

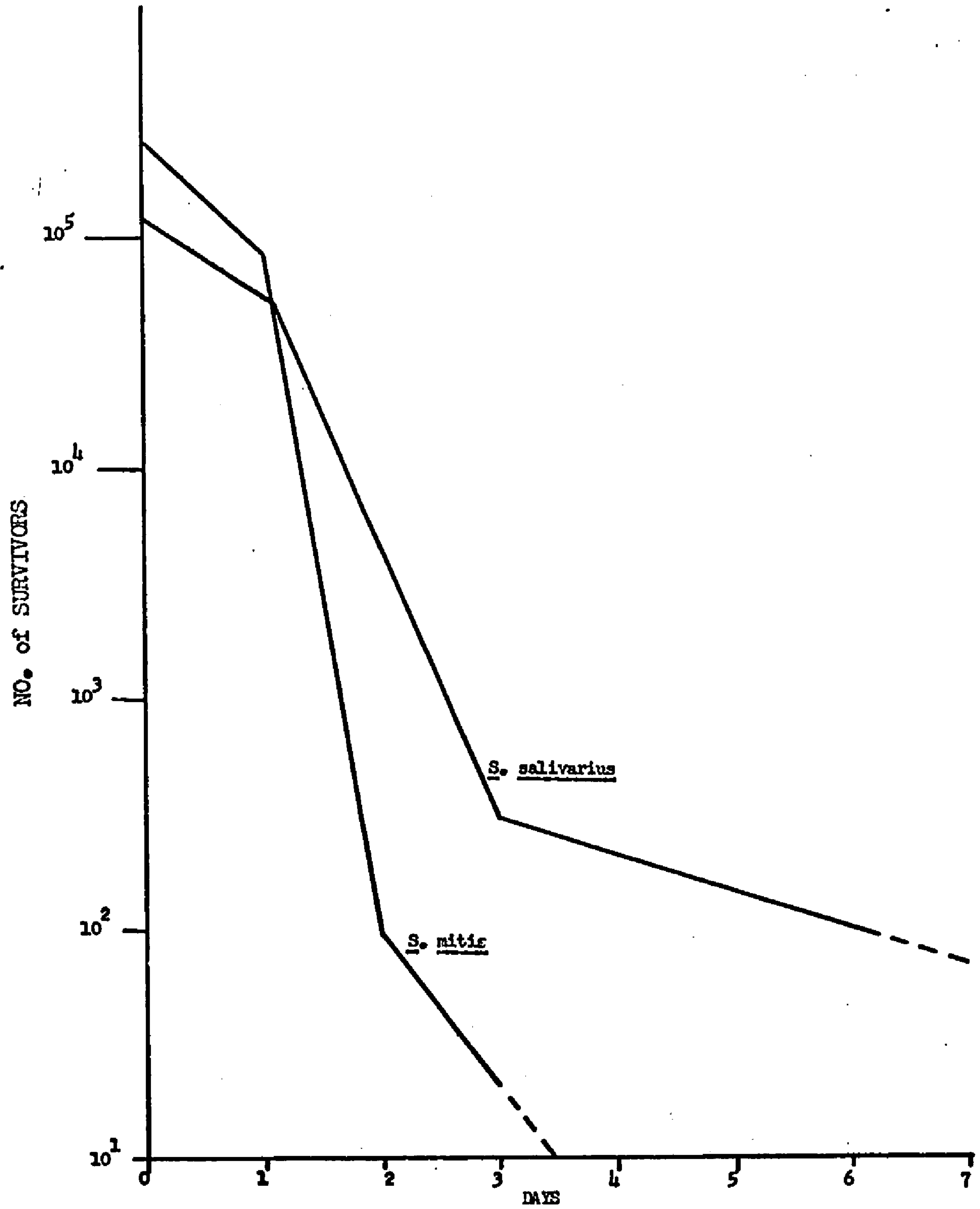
The object of this study was to determine how long the oral streptococci will remain viable once coming into contact with a surface such as might be encountered in a food processing plant. FIGURE 18 shows the results of this study.

Both *S. salivarius* and *S. mitis* were present initially in numbers approximating 100,000 organisms per ml of diluent. After 24 hours approximately 40,000 and 90,000 organisms could still be detected on the stainless steel cylinders. After 48 hours 4000 *S. salivarius* organisms per ml were still detected while the number of *S. mitis* organisms dropped off to about 100 per ml.

The significance of this data is that if the oral streptococci are introduced onto a surface where food is processed or packaged by a cough, sneeze, or saliva contaminated hands, then this becomes one possible route by which these organisms may enter a food product. Another route is by fecally contaminated hands. The presence of the oral streptococci by themselves presents no health hazard. However, if they are present either by oral or fecal contamination, then the possibility exists that pathogenic streptococci (*S. pyogenes*), viruses, or enteric pathogens

FIGURE 18

Survival of *S. salivarius* and *S. mitis* on Stainless Steel Surfaces



NO. of SURVIVORS
10^5
10^4
10^3
10^2
10^1
S. salivarius
S. mitis
0
1
2
3
4
5
6
7
DAYS

might also be present

Enumeration of S. salivarius and S. mitis on Food Preparation Surfaces in UNH Dining Facilities

TABLE 37 summarizes the results in an attempt to isolate oral streptococci from food preparation surfaces in three UNH dining halls using Mitis-Salivarius Agar with the addition of 0.04% NaN_3.

TABLE 37

Enumeration of S. salivarius and S. mitis* on Food Preparation Surfaces in UNH Dining Facilities using MS Agar plus 0.04% NaN_3

Dining Hall	NO. Samples	NO. Samples Showing Presence of *S. salivarius*	Aver. # *S. salivarius* per 50 Samples
Stillings	50	16	2
Huddleston	50	6	Less than 1
Memorial Union	50	10	1

* *S. mitis* was not detected in any sample from all three dining halls

The above preliminary survey indicated that *S. salivarius* was present in small numbers in a minority of samples examined. More work has to be performed in this field before any valid conclusions may be made.

DISCUSSION

The objectives of the investigation described in this thesis were twofold. The first was to evaluate the use of the fecal streptococci as indicators of fecal pollution and to determine whether these bacteria could be used to distinguish between contamination from human and animal sources. It was also sought to establish whether these organisms might be of non-fecal origin. The second objective was to determine whether the oral streptococci of the mouth, namely *S. salivarius* and *S. mitis* may also be used as indicators of human fecal pollution. It is known that these organisms are present in the feces of man and absent in the feces of animals.

In pursuing the first objective, a number of fecal streptococcal strains from different sources were examined as to their cultural, biochemical, and serological properties. As a preliminary study, a variety of selective media were compared in an effort to determine their relative efficiencies in the enumeration of fecal streptococci from sewage and water. The results showed that there is no single medium that is equally satisfactory for the isolation and enumeration of fecal streptococci present in diverse environments. The KF medium developed by Kenner *et al* (1960) was 97 per cent selective for fecal streptococci in sewage as compared to 99 per cent using M-Enterococcus Agar. However the KF medium was only 72 per cent selective for fecal streptococci in salt water as compared to 99 per cent using M-Enterococcus

Agar. Burkwall and Hartman (1963) modified the enterococcus medium by the addition of Tween 80 (0.05%) and sodium carbonate (0.2%) based on Chesbro and Evans (1959) work indicating that Na_2CO_3 and Tween 80 facilitated isolation of *S. faecium* from fecal samples in highly alkaline broth. They found that this medium was superior to the enterococcus medium when isolating fecal streptococci from frozen foods. When the Tween-carbonate medium was compared to the enterococcus medium in the isolation of fecal streptococci from sewage and estuarine waters, the results indicated that it was 90 and 93 per cent selective for fecal streptococci in sewage and salt water respectively. However the Tween-carbonate medium yielded only 70 per cent of the number of streptococci that the M-Enterococcus Agar did. Rose and Litsky (1965) reported that enrichment on peptone-yeast extract-casitone saturated pads with subsequent transfer to enterococcus medium improved the yields of fecal streptococci when isolated from water by the membrane filter procedure. In testing this medium the yields were increased approximately 10 per cent over those of the enterococcus medium; however, the selectivity decreased about 10 per cent. Therefore, there were about the same number of fecal streptococci on the PYC enriched membranes as on the enterococcus medium but there were some organisms that were not fecal streptococci on the former medium. A medium was designed for the selective enumeration of *S. faecalis* and varieties in polluted waters using citrate as the sole carbon source. Deibel, Lake, and Niven (1963) reported that the utilization of citrate as

an energy source is characteristic of _S. faecalis_; _S. faecium_ was unable to grow with this compound as the only energy source. Saraswat, Clark, and Reinbold (1963) developed their citrate-azide agar medium for the detection of enterococci in dairy products. However, their medium supported the growth of _S. faecium_ and other fecal streptococci. My results showed that the citrate-azide broth at pH 8.0 was 95-100 per cent selective for _S. faecalis_ and varieties _liquefaciens_ and _zymogenes_. Although it did not yield as many _S. faecalis_ organisms as the enterococcus medium, one could use this method for a quick qualitative estimation of _S. faecalis_ in a water sample. While _S. faecalis_ has not always been reported to be the predominant species of streptococci present in human feces, it is not present in domestic animals except dogs, to any large extent. Thus the presence of _S. faecalis_ in a water sample would indicate the probability of human fecal contamination as opposed to animal.

In pursuing the first objective an attempt was made to determine to what extent the fecal streptococci occurred in nature. Could the fecal streptococci isolated from soil, water, and plants be ascribed to human or animal fecal pollution or did they exist, as Mundt (1964) suggested, in an epiphytic relationship with plants? Also a study was performed to determine the correlation, if any, among the numbers of fecal streptococci, the coliforms, and the fecal coliforms, in environments considered to be relatively free from fecal pollution.

The results of the survey of the types of fecal

streptococci present in humans, animals, and sewage were similar to those of other workers. The fecal streptococci were always isolated from the feces of humans and animals. The results showed that S. faecalis and varieties liquefaciens and zymogenes were present in human feces to a much greater extent than in animals. S. faecalis accounted for 65 per cent of 204 strains of streptococci isolated from human feces while accounting for only 6 per cent in the fecal material of cows and sheep. S. faecium accounted for 46, 76, and 60 per cent of the strains of streptococci in cows, sheep, and rabbits respectively, while only comprising 18 per cent of the streptococcal population in humans. This data lends itself to suggest that S. faecalis is the predominant streptococcus in omnivores while S. faecium is the predominant streptococcus in herbivores. An interesting study might be carried out to characterize the streptococcal population in the intestinal tract of human vegetarians, to determine if S. faecium is the predominant streptococcus present.

In this investigation it was reported that the numbers of fecal streptococci present in soils that have not been manured and on plants grown in these soils fluctuated with the season. There was an increase in numbers from 10^1 per gram of soil in the spring to 10^2 per gram in summer. In the colder months the occurrence of these organisms were negligible. This is very closely correlated with the seasonal increase in the activity of animals, birds, and insects in the areas of fields and gardens. Also, the absence of the fecal coliforms and the fecal streptococci in densely wooded

areas indicated that these organisms were not present in soil which is not polluted by warm-blooded animals. Medfrek and Litsky (1960) reported that of 369 soil samples taken where chances of pollution were remote, 73 per cent contained coliforms, 1.4 per cent yielded *E. coli*, and 2.2 per cent yielded fecal streptococci. Our results were similar in that of 10 samples taken from a densely wooded area, 100 per cent showed the presence of coliforms as evidenced by the confirmed MPN test. Fecal coliforms were absent from all samples and fecal streptococci were present in 1 of 10 samples.

Streptococci resembling fecal streptococci were isolated from husks and exterior surfaces of green beans and tomatoes but only rarely on kernels of corn and the interior parts of beans and tomatoes. On the beans and tomatoes the fecal streptococci were randomly distributed in that they were present on some plants and not on others. This suggests that the fecal streptococci are not normal inhabitants of these plants but seem to be more of a transient resident, possibly being transferred to these plants by insects and small animals. The occurrence of these organisms on the interior parts of these plants might indicate broken surfaces allowing the entrance of bacterial organisms. These results are in contrast to Mundt's (1964) in that fecal streptococci were not invariably present on plants in an agricultural environment. He reported the presence of *S. faecalis* on beans, corn, and cabbage. I have not found this to be true.

For the most part, *S. faecium* and *S. faecium*-like organisms were the predominating streptococci isolated from

soil and plants throughout this study. Barnes and Ingram (1955) reported that *S. faecium* was associated solely with the hog intestine, but this study has shown that this organism is a normal inhabitant of the intestinal tract of other animals and man as well.

In some cases isolates that had a lemon-yellow pigment were found that were similar to *S. faecium* except for this pigment. Pigmented streptococci have been reported by Graudal (1957a, 1957b), Hannay (1950), and Mundt (1968). Graudal correlated motility with pigment. He considered motile streptococci to be composed of two sub-groups, pigmented and non-pigmented streptococci. I have found that while the great majority of motile streptococci were pigmented, only 45 per cent of the pigmented streptococci were motile. Hannay (1950) reported that 3 strains of yellow streptococci isolated from dairy cows were similar to *S. faecalis*, except for the fermentation of raffinose. Graudal (1957b), on the other hand, isolated 4 strains from infants' feces and found them to be similar to *S. faecium*. Mundt (1968) has isolated pigmented streptococci from many types of plants and reported that these strains appear to be a normal resident of plants and is apparently rarely associated with the human or with animals. He concluded that while the yellow strains were similar to both *S. faecalis* and *S. faecium*, there existed certain characteristics that were peculiar to the pigmented strains. He proposed the name of *S. faecium* var. *casseliflavus* for these strains. In my study the great majority of pigmented streptococci were found to ferment arabinose and

melibiose, which is characteristic of *S. faecium*. Besides, these two sugars this group is apparently an active one physiologically because several other sugars were attacked, notably raffinose, sucrose, salicin, and sometimes sorbitol. The fact that raffinose was fermented indicates that these strains do possess peculiar characteristics that *S. faecium* and *S. faecalis* do not have. In contrast to Mundt's findings, I found the pigmented streptococci to produce ammonia from arginine. Also, in contrast to Mundt's report, the majority of pigmented strains were sensitive to potassium tellurite (0.04%) and did not reduce TTC strongly. During this investigation pigmented streptococci were not isolated from the feces of humans or animals, or from raw sewage. Thus these pigmented streptococci do not apparently inhabit the digestive tract of man and domestic animals. The question of the origin of pigmented streptococci has not been completely solved. While Mundt reports that these strains exist in an epiphytic relationship with plants, the data that I have gathered is not extensive enough to support or oppose Mundt's findings. However, my data suggest that one possible source of these bacteria is insects. The yellow strains were not found in animal burrows, although non-pigmented streptococci were present in localized areas within garden soil suggests that they might be associated with localized contamination by insects, animals or bird droppings. To confirm this hypothesis, more work would have to be carried out with many species of insects to prove or disprove this hypothesis.

The occurrence of the pigmented strains, the *S. faecalis*-like and *S. faecium*-like strains in nature raises the question of what constitutes a species difference among bacteria. All of the above organisms were found to fulfill Sherman's criteria for the enterococcus group. These strains closely resembled *S. faecalis* and *S. faecium*. They were found to deviate from the established species in one or more fermentation tests. Deibel (1964) contends that in "All identification procedures, dependence must be placed on a spectrum of characteristics possessed by the strain in question, and its failure to comply in a few specific tests does not constitute sufficient grounds to negate speciation if it conforms with the overall species description. As in any taxonomic scheme, the occurrence of some transitional types is to be expected." Therefore, in this study the majority of strains isolated from nature would be designated as *S. faecium* if we choose to ignore minor fermentation differences. These strains were very similar to those present in animal feces which suggests that the presence of these organisms in soil and on plants represents fecal pollution of animal origin. Further studies are needed to fully elucidate the origin of the pigmented streptococci. Survival studies in the soil have shown that *S. faecalis* dies out quite rapidly while *S. faecium* and the pigmented streptococci die out more slowly. However these streptococci do not remain viable in high numbers for long periods of time as you might expect if they existed in epiphytic relationship. It has been concluded that since the great majority of

streptococci isolated from soil and plants were quite similar to those present in animal feces, it is reasonable to assume that they represent fecal contamination from animals.

In an effort to determine if *S. salivarius* and *S. mitis* are good indicators of human fecal pollution, it was necessary to first develop selective media for their isolation. Up until the present the weak point of all studies of these oral streptococci has been the lack of a good medium for recovery.

A broth medium was developed using mannitol as the only substrate and penicillin, kanamycin, and sodium azide as inhibitory agents. In the enumeration of oral streptococci from fecal samples and foods, this medium was capable of causing the bacteriolysis of the fecal streptococci population, while at the same time inhibiting the growth of coliforms and other Gram negatives.

The Penicillin-Kanamycin MS broth was compared with previously used media for the enumeration of *S. salivarius* and *S. mitis* in human and animal fecal material. It was found to be superior to Sucrose-azide Agar (Sherman *et al*, 1943) and to Mitis-Salivarius Broth (Appleman and Lewis, 1967). *S. salivarius* was isolated from every human fecal sample examined (11) while *S. mitis* was detected in 4 of 11 samples. The average number of *S. salivarius* organisms per gram was 10,000. The oral streptococci could not be isolated from the fecal material of cows and dogs. This confirmed other workers results in that these organisms are present only in human fecal material and therefore

represent an excellent marker for human pollution.

Both S. salivarius and S. mitis could not be isolated from raw sewage. It was subsequently found that these organisms undergo a rapid die-off in sewage and water. There was an average decrease of 3-4 logs in 24 hours. Therefore if the average inoculum of oral streptococci in fecal material is 10^4 and there is a 4 log decrease in sewage or water within 24 hours, then these organisms would not be isolated from sewage or water. Furthermore, at the time of sampling the sewage or water, it is quite probable that the organisms were discharged into the system 12-24 hours earlier. In contrast, the fecal streptococci would still be present in large numbers while the oral streptococci are absent.

Two of the prime requisites of a good indicator of fecal pollution are that the indicator organisms must always be present when pathogens are present and it must be more resistant towards the action of the environment in the water than the pathogens. S. salivarius and S. mitis do not fulfill either of the above criteria. Therefore, these organisms would not be reliable indicators of fecal pollution of water.

If, however, S. salivarius and S. mitis could be detected in foods then this would represent pollution by the human rather than by either human or animal sources as is the case with E. coli and with the fecal streptococci. This would then permit a more critical evaluation of handling procedures. It was subsequently found that S. salivarius

and/or S. mitis were isolated from 46 per cent of the frozen meat pies examined using Mitis-Salivarius Broth and from 71 per cent of the samples using Penicillin Broth. Appleman (1969), using a 3-tube MPN with Mitis-Salivarius Broth recently reported isolating S. salivarius and/or S. mitis from 44 per cent of the number of frozen foods he examined. It has been shown that the PKMS broth is the superior of the two media in isolating and enumerating the oral streptococci from frozen foods. It was also noted that in general when large numbers of fecal streptococci were encountered, large numbers of oral streptococci were also present. This indicates that the source of contamination was probably of fecal origin. It is recognized, however, that the presence of S. salivarius and S. mitis in a food product could represent oral pollution by coughing or sneezing or handling of materials with saliva contaminated hands in addition to fecally contaminated hands. There was also noted an interesting relationship between the numbers of coliforms, fecal streptococci, and oral streptococci in chicken and turkey pies. In chicken pies large numbers of fecal streptococci and oral streptococci were present in the same samples along with small numbers of coliforms. In the turkey pies there were large numbers of the fecal streptococci and coliforms with small numbers of oral streptococci. It might be speculated that S. salivarius and S. mitis might be present in substantial numbers in the intestinal tracts of chickens. Kenner, Clarke, and Kabler (1960) have reported the absence of these organisms in chickens. However, they were using KF media which supports

the growth of the fecal streptococci which may have inhibited the growth of the oral streptococci. Therefore it may be worth while to reexamine the intestinal flora of a number of animals using the PKMS Broth to determine if these organisms are present to any extent.

In an effort to further trace the route by which S. salivarius and S. mitis may enter a food product an experiment was set up to determine how long can S. salivarius be isolated from the air after an aerosol is produced of these organisms. The results showed that these organisms could be detected only up to 1 hour after the aerosol is produced. However, in a second experiment to determine how long S. salivarius and S. mitis persist on surfaces, it was found that these organisms remained viable up to 72 hours after inoculation. The implications are obvious. If these organisms are introduced into the air by an aerosol in a food processing plant, they will settle on the food preparation surfaces and will survive for 2-3 days thereafter. Therefore these organisms have an excellent chance of being introduced into the food product by surface contaminated hands or materials.

If the oral streptococci gain entry into a food product before the freezing process, results have indicated that they are capable of surviving the freezing process and will remain viable for long periods of time upon low temperature storage without any significant decrease in numbers. Several investigators (Gunderson and Rose, 1948; Hartsell, 1951, and Woodburn and Strong, 1960) have reported that the Salmonellae and staphylococci remain viable in

frozen foods for several months so it is important that the indicator organism survive for long periods of time without undergoing any decrease in numbers if it is to be used to evaluate the original sanitary quality of the food. It has been reported by Kereluk and Gunderson (1959) that *E. coli* decreases rapidly in numbers during low temperature storage while the fecal streptococci remain approximately constant. The oral streptococci apparently are quite similar to the fecal streptococci in this respect. Therefore, the fecal and oral streptococci would seem to be a more reliable indicator of the original sanitary quality of the frozen food than *E. coli* would be.

This investigation has shown that the oral streptococci would be a valuable aid in assessing the sanitary quality of frozen foods. They are a definite marker for the presence of human pollution. They have not been isolated from the fecal material of any animal. They can also be used to distinguish between oral and fecal contamination. If the food is orally polluted it will contain the oral streptococci but not the fecal streptococci. If the food was fecally polluted then it would most likely contain both groups. Thus a sensitive testing methodology has been achieved to evaluate the sanitary quality of frozen meat pies.

There still remains certain unanswered questions concerning the source of contamination in frozen meat pies. At what points along the line of processing packaging are these organisms entering the foodstuff? It would also be

of interest to ascertain whether these organisms have a chance to multiply in the foodstuff before the samples are frozen for market.

SUMMARY

This research was undertaken in an attempt to evaluate the use of the fecal and oral streptococci as indicator organisms of contamination of water and foods by humans and animals.

In comparing several selective media used for the detection of fecal streptococci in water it was found that the M-Enterococcus Agar (BBL) was superior to 10 other agar media tested. This medium was reported to be 99 per cent selective for fecal streptococci while the others tested supported the growth of many non-streptococcus organisms. Concurrent with the above study a broth medium was developed to qualitatively detect the presence of *S. faecalis* and varieties *liquefaciens* and *zymogenes* in water.

One of the major objectives of this study was to determine to what extent the fecal streptococci occurred in soils that were not fertilized with manure and in vegetables grown in these soils. The numbers of fecal streptococci present in the soil were much more closely correlated with the numbers of fecal coliforms than with the coliforms. Approximately 30-40 per cent of the streptococci isolated from the soil and plants could not be placed in any of the established species because of their divergency in the fermentation of certain sugars. These organisms fulfilled all the requisites for Sherman's enterococcus group and were designated as *S. faecalis*-like and *S. faecium*-like. These organisms were reported to possess the Group D antigen as demonstrated by

the gel diffusion method using disrupted cells as the antigens. This was reacted with Group D antiserum produced in rabbits against S. faecalis and S. faecium. Yellow-pigmented streptococci were isolated from garden soil in the early spring and late summer. These organisms were very similar to S. faecium.

Fecal streptococci were isolated from the exterior surfaces of corn, tomatoes, and green beans but rarely on the interior parts of the vegetables. It was concluded from the data on the numbers and types of streptococci present in soil and on plants that they most probably originated from animal or insect sources.

In the evaluation of the use of S. salivarius and S. mitis as indicators of human pollution of water and foods, an improved medium and method was developed for their isolation and enumeration. It is a mannitol-azide broth with the incorporation of penicillin and kanamycin. It was used to enumerate the oral streptococci in feces and foods while inhibiting the growth of all other organisms. The number of oral streptococci present in feces averaged 10^4 per gram using the PKMS broth as the isolation medium. Using Mitis-Salivarius Broth and Sucrose-azide Agar they numbered 10^3 and 10^2 respectively. Survival studies on the oral streptococci in sewage and fresh water showed that these organisms died out extremely rapidly thus limiting their use as indicators of human fecal pollution of water.

The examination of frozen meat pies revealed the presence of S. salivarius and S. mitis in 71 per cent of the

samples examined using PKMS Broth and in only 41 per cent of the samples using Mitis-Salivarius Broth. In general, when large numbers of fecal streptococci were present, large numbers of oral streptococci were also present suggesting that the source of contamination was of fecal origin.

Results also showed that *S. salivarius* and *S. mitis* remained viable for periods up to 72 hours after coming into contact with stainless steel surfaces. This suggested that surface contamination of food processing counters is one route by which these organisms may enter a food product.

In a preliminary survey of food preparation areas in the University of New Hampshire kitchen facilities, it was found that *S. salivarius* was present on surfaces such as cutting boards and stainless steel counters in small numbers (averaging 2 per plate). More work in this area is needed however before any valid conclusions could be made.

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