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Steven G. Morrow

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Abstract

THE EFFECTS OF CERTAIN ORAL BACTERIA ON THE IN VITRO GROWTH OF *Bacteroides gingivalis*

by

Steven G. Morrow

The effect of nine gram positive and gram negative cocci and bacilli, which are indigenous to the oral cavity, on the in vitro growth of *Bacteroides gingivalis* was examined in this study. A pure culture of *B. gingivalis* was obtained from the American Type Culture Collection. The nine oral test organisms were *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium hofmanii*, *Lactobacillus acidophilus*, *Branhamella catarrhalis*, and *Escherichia coli*. The effect on the growth of *B. gingivalis* of the nine test organisms was evaluated on a solid medium by use of a satellite phenomenon study and in a liquid medium by use of an optical density study. Incubation was conducted under an-

aerobic conditions for seven days at 30°C.

The sterile filtrates of trypticase soy broth which was used as the culture medium during incubation and growth of the nine provider organisms were evaluated for the presence of menadione by use of high pressure liquid chromatography.

The results of the satellite phenomenon study indicate that B. gingivalis (ATCC #33277) requires menadione (vit K) for in vitro growth. Of the nine organisms tested, the gram positive cocci were more efficient "provider" organisms than the gram positive and gram negative bacilli.

The results of the optical density study also indicate that B. gingivalis (ATCC #33277) requires menadione for in vitro growth. Of the nine provider organisms tested, only S. mutans demonstrated a significant ($p<0.05$) increase in the growth of B. gingivalis as compared to the positive control.

The high pressure liquid chromatography analysis indicates that of the nine provider organisms tested, only S. mutans produced a substance into its growth environment which was similar in molecular structure and polarity to menadione.

B. gingivalis is an important pathogen in oral-facial infections. It requires menadione for growth. The pathogenicity of *B. gingivalis* appears to be the result of a synergistic mixed infection which contains facultative gram positive cocci, especially *S. mutans*, which synthesize menadione or a similar substance, thus making it available at the site of infection as a needed growth requirement.

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THE EFFECTS OF CERTAIN ORAL BACTERIA ON THE IN VITRO GROWTH OF *Bacteroides gingivalis*

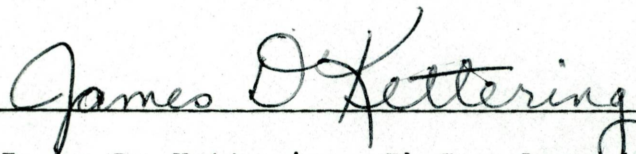
by

Steven G. Morrow

A Thesis in Partial Fulfillment
of the Requirements for the Degree
Master of Science in Microbiology

September 1986

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality as a thesis for the degree Master of Science.



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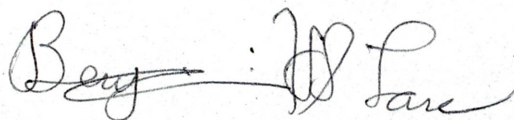

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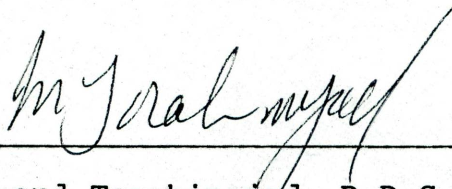

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INTRODUCTION

The contribution of microorganisms to pulpal and periapical pathology has been well established in the literature (Smith et al., 1958; Kakahashi, et al., 1965; Korzen, 1974). Recent studies indicate an increasing awareness of the role of gram-negative obligate anaerobes in the pathogenesis of the pulp and periapical tissues (Keudell, et al., 1982; Svetcov, et al., 1983). These anaerobes correspond closely to the types of organisms found in both early and late stages of periodontitis (Slot, 1977; Darwish and Socransky, 1978).

Members of the genus *Bacteroides* (gram negative obligate anaerobes) have been recovered with a high degree of frequency from teeth with necrotic pulps and periapical inflammatory disease. Also, these organisms always seem to occur as components of a mixed infection with gram positive facultative organisms being present. (Sundquist, 1976; Svetcov, et al., 1983).

Bacteroides are unique in that they are rarely pathogenic as a pure infection (MacDonald, et al., 1963; Takazoe, et al., 1971) and they require a specific "growth factor" which they are not able to synthesize (Lev, 1958; Gibbons and MacDon-

ald, 1960). *Bacteroides melaninogenicus* has been directly associated with pulpal and periapical pathology especially those cases which demonstrate clinical symptoms such as pain, swelling and/or sinus tract formation (Sundquist, 1976; Griffee, et al., 1960; Williams, et al., 1983; Yoshida, et al., 1987).

The purpose of this study was to test, in vitro, the effect of certain indigenous oral bacteria on the growth of *B. melaninogenicus* (*gingivalis*).

LITERATURE REVIEW

I Microbial Flora of The Oral Cavity

The oral cavity supports one of the most concentrated and varied microbial populations of any area of the human body. During the birth process the child has its first encounter with microbial organisms. The normal flora of the female genital tract includes lactobacilli, corynebacteria, micrococci, coliforms, streptococci, yeasts and protozoa. At the end of three months all mouths will be found to support a microbiota. After one year, streptococci, staphylococci, veillonella and neisseria can be isolated from all mouths. Actinomyces, nocardia, lactobacilli and fusobacteria can be cultured from one-half of the mouths. Bacteroides, leptotrichiae, corynebacteria and coliforms can be isolated in less than half of the mouths (Burnett and Schuster, 1978).

During childhood the bacterial population increases and in adolescence spirochetes and *B. melaninogenicus* are present in all mouths. In the adult, gram positive facultative and anaerobic cocci account for 59% of the cultivable organisms present in the saliva. Gram-negative facultative and anaerobic cocci comprise 17% of the oral flora, while gram nega-

tive facultative and anaerobic rods account for 7%. The gram positive rods, both facultative and anaerobic, constitute 17% of the organisms found in the adult oral cavity (Sokransky, 1971). The interactions between these organisms, their virulence, the local environment and individual host resistance factors produce a wide range of effects varying from health to disease.

II Importance of Bacteria in Pulpal and Periapical Pathology.

W.D. Miller in 1894 was the first to identify bacteria in diseased pulp tissue. Since that time several investigators have demonstrated the role of bacteria as etiologic agents in pulpal and periapical pathology. Kakahashi and associates (1965) studied the effect of exposing the pulp tissue of germ-free and conventional rats to the oral environment. The results of this study showed no pathological changes occurring in the exposed pulp or periapical tissues in the germ-free animals. However, in the conventional rats, pulp exposure resulted in necrosis and periapical pathology. They concluded that the presence of a microbial flora was the major determinant in pulpal and periapical pathology of rodents.

Many authors have claimed that microorganisms are the predom-

inant cause of periapical inflammation (Burkett, 1938; Appleton, 1950; Grossman, 1960; Burnett, 1962; Ostander and Crowley, 1966; Smith, 1968). Smith (1958) in an attempt to correlate bacteriologic findings with radiographic evidence of periapical pathology concluded that many of the organisms isolated from infected root canals were potent pathogens capable of producing periapical disease.

Balick (1972) studied the relationship between bacteria and periapical inflammation. He concluded that microorganisms are the causative factor for inflammation seen in the periapical tissues.

Korzen, et al., (1974) studied the effect of microorganisms on the periapical region of conventional and gnotobiotic rats monoinfected with S. mutans. They concluded, after histologic examination, that the inflammatory reaction to a mixed infection was much greater than to a monoinfection with S. mutans. Also, this study indicated that the severity of the inflammatory response of the pulp and periapical tissues can be related to the quantity of microorganisms within the root canal and the length of time the tissues are exposed to them.

Moller, et al., (1981) reported on a study in which the pulps of 78 teeth in nine monkeys were aseptically necrotized.

Twenty-six of the pulp chambers were kept bacteria free by immediately sealing the access cavity. Fifty-two of the teeth were infected by exposure to the indigenous oral flora for seven days before sealing. The results were recorded clinically, radiographically, and microbiologically at the beginning of the study and at the end (6 months). The final examination also included histological recordings.

The 26 initially noninfected teeth were all sterile at the final sampling, indicating that there was no contamination by either oral or hematogenous route. It was shown that noninfected necrotic pulp tissue did not induce inflammation in the apical tissues. By contrast, the 52 teeth with infected pulp tissue showed inflammatory reactions in the apical tissues clinically in 12 cases (23%) and radiographically in 47 cases (90%). Facultatively anaerobic streptococci, coliform rods and obligately anaerobic bacterial strains were most frequently found. All infected teeth showed histologic evidence of strong inflammatory reactions in the periapical tissues even when there was no clinical or radiographic evidence present.

Fabricius (1982) reported on a study in which chronic periapical disease was induced in monkeys by inoculating the root canals of teeth with bacteria in pure and mixed cul-

tures. The mixed infections showed the greatest capacity to induce apical periodontitis. No apical periodontitis could be detected in those teeth which the inoculated bacteria were not reisolated at the end of the study (6 months).

III Portals of Entry of Bacteria into the Root Canal System

Since microorganisms have been shown to play an important role in the etiology of pulpal and periapical disease, the manner in which the microbes gain entrance into the root canal system is of importance.

Dental Caries

Gibbons (1964) in studies using germ-free animals demonstrated that bacteria are essential for the production of caries. Morse (1976) stated that the most common way for bacteria to reach the pulp is through direct extension of dental caries.

Dentinal Tubules

The presence of bacteria in dentinal tubules has been seen by many investigators (Scott, 1954; Chirnside, 1958; Parish, 1963; Fusayama, 1966). The experimental passage of microorganisms through the dentinal tubules has been described by

Bender, et al., (1959). Chirnside (1961) also demonstrated that bacteria were capable of invading the dentin of pulpless teeth exposed to the oral environment. He concluded that if the odontoblastic processes degenerate following pulp necrosis and the dentin is exposed to the oral environment, the tubules are liable to bacterial invasion.

Periodontal Ligament

In a study of intact teeth with necrotic pulps, MacDonald (1957) obtained 38 positive anaerobic and aerobic cultures in 46 teeth examined. It was postulated that these organisms reached the pulp space from the oral cavity via the lymphatics and blood vessels of the periodontium. Hampp (1957) isolated oral spirochetes from the pulps of traumatically devitalized teeth. He concluded that this finding strongly suggested the forcing or invasion of these organisms from the gingival sulcus into the periodontal ligament and into the pulp via lateral channels or by a lymphatic apical route.

Grossman (1967) placed a readily identifiable test microorganism into the gingival sulci of dogs' and monkeys' teeth, traumatized the teeth and later recovered the same organisms from the pulp. He concluded that during trauma, blood vessels in the periodontal ligament were opened, providing a

pathway through which the microorganisms could reach the pulp.

Hematogenous Route

Robinson and Boling (1941), Burke and Knighton (1960), Smith and Tappie (1962), and Gier and Mitchell (1968) have reported the localization of blood-borne bacteria in areas of inflammation, a process called anachoresis. Transient bacteremias can thus localize in an injured pulp whose impaired circulation cannot effectively cope with or eliminate the invading organisms.

IV Flora of the Root Canal

Various media and techniques have been used in numberous studies on the pulpal microbial flora. The numbers and types of microorganisms isolated in these studies have varied according to the method of obtaining the sample, the types of culture media used, the use of aerobic or anaerobic culture conditions and the methods used to identify the various microorganisms.

Despite the influence of these factors on the variety of microorganisms detected, most studies have shown that the predominant microbial group present in infected root canals con-

sists of facultative anaerobic alpha-hemolytic streptococci. However, recent studies using obligate anaerobic techniques have shown that obligate anaerobes are important and may be the predominant species (Morse, 1980 in: Pathways of the Pulp).

Two early investigators of the bacteriology of the dental pulp were Henrici and Hartzell (1919). The results of their study showed that 65% of the strains isolated from the root canal were streptococci, 20% were staphylocci, and the remaining strains were corynebacteria, gram negative rods and yeasts. Grossman and Christian (1952) reported on a study of the microorganisms recovered from 1,000 infected teeth. They found 77.5% were gram positive cocci 5.5% were gram negative rods and 16.9% were yeasts. Winkler and Van Amerongen (1959) published the results of over 4,000 root canal cultures. Their results showed 79% gram-positive cocci (streptococci and staphylococci), 6.9% lactobacilli, 4% corynebacteria and 2% gram-negative rods. Their conclusion was that streptococci were the most serious pathogens present and the other organisms were chance contaminants. The studies of Henrici and Hartzell, Grossman and Christian, Winkler and Van Amerongen all had one factor in common, their culture technique did not include conditions favorable to the recovery of obligate anaerobes.

Kantz and Henry (1974) obtained samples from 24 teeth with necrotic pulps and cultured these using a method to maintain anaerobiasis during collection and transportation. These investigators were successful in identifying Actinomyces, Bacteroides, Campylobacter, Eubacterium, Fusobacterium, Peptococcus, and Veillonella. One hundred four of the 377 bacterial colonies recovered (27%) were obligate anaerobes.

Wittgow and Sabiston (1975) examined the bacterial flora of the root canals of 40 intact incisor teeth which demonstrated pulp necrosis (no response to the electric pulp tester). Upon entrance into the pulp chamber, four teeth were observed to have vital pulps. Thirty-six of the teeth had necrotic pulps; thirty-two (89%) yielded positive cultures; thirty-one (97%) of which yielded obligate anaerobes. All culturing and strain identification was done using the procedures described in the Virginia Polytechnic Institute Anaerobe Manual (Haldeman and Moore, 1972). Goodman (1977) studied the bacterial flora of the root canals of fifty-five teeth with necrotic pulps. Stuarts transport solution (Callabs, North Hollywood, California) was used as a holding solution to transport the sample to the laboratory. Fifty-five positive cultures were obtained in this study. All 55 cases contained at least one obligate anaerobe and one case contained four. The genus

which was recovered with the highest frequency was *Bacteroides*.

Sundquist (1976) reported on an extensive study of the bacterial flora of the root canals of 32 teeth with necrotic pulps as a result of trauma. Prereduced and anaerobically sterilized chopped meat glucose broth was used to transport the sample to the laboratory. All samples were incubated at 37°C, both aerobically and anaerobically. Eighteen of the 32 teeth sampled yielded positive cultures. Periapical inflammation with bone resorption was evident by radiographic examination on all 18 teeth which demonstrated positive cultures. No tooth without apical inflammatory disease yielded a positive culture. Seven of the 18 cases with periapical inflammatory disease demonstrated clinical symptoms of acute inflammation (pain and/or swelling). All seven of these cases yielded mixed infections from the root canal of which *B. melaninogenicus* was a component. Sundquist concluded from the results of his study that acute inflammation in the periapical region is induced by combinations of bacterial strains and that the presence of *B. melaninogenicus* among these strains is essential for the inducement.

V Bacterial Flora of the Periapical Area

It has been shown that in cases of pulpal disease, pathogenic microorganisms can be found in the root canal system and that this system can act as a pathway for these microbes to reach the periapical area. Several investigators have examined the bacterial status of the periapical area.

Hedman (1951) utilized a canula and culture wire to obtain bacterial cultures from the periapical areas of human teeth with necrotic pulps. The canula and culture wire were inserted through the tooth to reach the periapex. His results showed that 68.5% of the cases had viable bacteria in both the root canals and the periapical areas and 8.5% had bacteria in the root canals but not in the periapical areas. Twenty-three percent showed no bacteria in either location. Due to the experimental methods utilized in this study, these findings have been challenged by other investigators.

Shindell (1961) used a canula and culture wire technique similar to Hedman's in human teeth with necrotic pulps and periapical disease. His results showed only 5% of the cases to be positive for bacterial contamination in the periapical area. He therefore concluded that most areas of periapical pathology are sterile, and that the few cases that contain

bacteria are of little statistical significance.

Grossman (1959) studied cultures of areas of periapical pathology obtained from surgical specimens of root resection. He took four to five cultures during each procedure and incubated them aerobically in brain heart infusion broth. One hundred fifty cases were studied. Reliable data were available in 104 cases, and of these, 85.3% were negative for bacterial growth. However, it must be noted that no attempt was made to recover anaerobic bacteria.

Winkler and associates (1972) studied areas of periapical pathology on 15 human teeth by using a modified gram tissue stain. The teeth were extracted along with the attached periapical lesion. The tissues were decalcified and stained with hematoxylin and eosin as well as the Johns Hopkins modified bacterial stain. Bacteria were found uniformly dispersed throughout the lesions in slight to moderate concentrations in most cases. However, no determination of the viability of these organisms was made.

Langeland and associates (1977) reported on a histopathologic and histobacteriologic study of 35 surgical specimens of areas of periapical pathology. These investigators used hematoxylin and eosin and Brown and Brenn stain on the decal-

cified and fixed specimens. Only one case was reported where bacteria were present in the necrotic pulp and in the area of periapical pathology.

Tronstad et al., (1986) reported on a study which examined for the presence of bacteria in periapical inflammatory lesions. The microbiological sampling took place in conjunction with periapical surgery. Samples were taken from the soft tissue of the lesion and from scrapings of the surface of the root tip. Moller's UMGA III transport medium was used and incubation was conducted for seven days in an anaerobic chamber. Various members of bacteroides, peptostreptococcus, and actinomyces species were identified. The authors stated that this study clearly showed that anaerobic bacteria are able to survive and maintain an infectious disease process in periapical tissues.

VI Importance of Anaerobes in Infectious Disease.

Anaerobic bacteriology has been in a renaissance period of development for the last 10 to 15 years, and with it came a new approach to the clinical relevance of infections caused by anaerobes. Improvements in technology have permitted more difinitive bacteriologic and clinical studies which continue to emphasize the importance of anaerobes in human

er bacteria. *Bacterioides* species appear to cause infections more often than the other bacteria in this group. This organism forms part of the normal anaerobic flora of the oral cavity (Balows, 1972).

MacDonald, et al., (1963) reporting on a study of the pathogenesis of mixed anaerobic infections of mucous membranes, stated that *Bacteroides* regularly are components of mixed infections involving mucous membranes. *Bacteroides melaninogenicus* is particularly important. When mixed bacterial suspensions, which were obtained from samples of the human gingival crevice and contained *B. melaninogenicus*, were inoculated subcutaneously into guinea pigs, typical transmissible infections were regularly produced. Only minor, atypical non-transmissible lesions occured when *B. melaninogenicus* was eliminated from the inoculum. The addition of *B. melaninogenicus* to mixtures incapable of producing significant lesions restored them to full pathogenicity in 24 to 26 animals. They concluded that the primary pathogen in mixed infections produced by flora of the gingival crevice of man is *B. melaninogenicus*.

Socransky and Gibbons (1965) conducted a study of the infectivity of pure cultures and mixtures of organisms. Bacterial samples were obtained from the debris of human periodon-

tal pockets and injected subcutaneously into guinea pigs. When a suspension containing all organisms isolated with the exception of B. melaninogenicus was assayed for infectivity, it was found that none of 24 guinea pigs displayed an infective response. The addition of strain K110 of B. melaninogenicus to the previously assayed suspension resulted in a mixture which was uniformly infective.

In an attempt to determine which organisms played an essential role in this infection, the investigators selected five gram positive and five gram negative strains which were representative of the groups present in the original mixture. The 10 organisms tested in mixture without the addition of strain K110 of B. melaninogenicus produced an infective response in only one of 12 animals. However, the addition of strain K110 of B. melaninogenicus to the 10 organisms restored uniform infectivity.

The five gram negative organisms proved uniformly noninfective even with the addition of strain K110 of B. melaninogenicus. The five gram positive organisms were noninfective without B. melaninogenicus but produced infections in seven of eight animals when strain K110 of B. melaninogenicus was added.

Suspensions were prepared in which each of the gram positive organisms was individually deleted. All of the suspensions were infective in the presence of strain K110 of *B. melaninogenicus* except the one from which a gram positive coccobacillus had been deleted. The gram positive coccobacillus alone was noninfective, but in combination with strain K110 of *B. melaninogenicus* infections were produced in nine of ten guinea pigs tested. However, *B. melaninogenicus* was unable to produce infection by itself in any of the twelve animals tested. This gram positive organism was identified as a facultative coccobacilus which produced alpha hemolysis on blood agar plates and demonstrated biochemical characteristics which placed in between the enterococci and viridans group.

Lev (1958) demonstrated that rumen strains of *Fusiformis nigrescens (B. melaninogenicus)* required menadione or other analogues of vitamin K for growth. Gibbons and MacDonald (1960) reported that *B. melaninogenicus* required both hemin and a "growth factor" for cultivation. The "growth factor" necessary could be supplied by the filtrate of a 48-hour culture of *S. aureus*, menadione, or any naphthalene derivative possessing an oxygen, hydroxyl, or carboxyl group in the alpha position. It was also demonstrated that whole saliva, when centrifuged to remove bacteria and debris and

sterilized with ethylene oxide, also provided the necessary "growth factor". Sundquist (1976) reported that B. melaninogenicus was recovered in mixed cultures from all teeth in his study which had necrotic pulps and acute periapical inflammation with pain and/or swelling present. He concluded that acute inflammation of the periapical region is induced by specific combinations of bacterial strains and that the presence of B. melaninogenicus among these strains is essential for inducement.

Griffee and associates (1980) reported on a study in which they looked at the relationship of B. melaninogenicus to the symptoms associated with pulpal necrosis. Pain and/or sinus tract formation was used as a measure of acute periapical inflammatory disease. Twelve of the 33 teeth in this study demonstrated positive cultures of B. melaninogenicus, of these, 11 (92%) presented with pain and/or sinus tract formation. The remaining 21 teeth were negative for B. melaninogenicus. Seven of these 21 (33%) presented with pain and/or sinus tract formation. The investigators concluded that anaerobes are important in the pathogenisis of endodontic lesions, that B. melaninogenicus is an important pathogen in the development of symptoms commonly associated with pulpal necrosis and long term endodontic failures may be the result of persistence of B. melaninogenicus in pulpal or periapical

areas.

Williams and associates (1983) reported on a study of bacteriological cultures of aspirates of 10 acute (fluctuant swelling) dental abscesses of endodontic origin. Sampling was done by syringe aspiration. Seventy percent of the isolates were either obligate anaerobes or microaerophilic. Anaerobic bacteria were cultured from nine of the ten aspirates. *Bacteroides* species were recovered from eight of the ten with *B. melaninogenicus* being the most predominant.

Yoshida, et al., (1987) reported on a study in which they compared the bacterial contents of infected root canals in clinical cases of acute apical inflammation with exudation and in cases of chronic apical periodontitis. They reported that bacteroides and peptococcus species were commonly found in clinically acute cases while notably missing in clinically chronic cases.

RESEARCH QUESTION

Does *B. melaninogenicus* (*gingivalis*)* require menadione (vitamin K) for in vitro growth? Will certain indigenous oral bacteria supply the needed growth requirement for *B. melaninogenicus* (*gingivalis*) when grown in vitro in/on a medium lacking menadione (vitamin K)?

*Classic literature has used the nomenclature *B. melaninogenicus* to denote a group of obligately anaerobic gram negative non spore-forming bacilli which formed black pigmented colonies on blood agar plates. The current literature uses a more specific species nomenclature.

HYPOTHESES

Hypothesis I: *B. melaninogenicus* (*gingivalis*) requires menadione (vit K) for in vitro growth.

Hypothesis II: Certain indigenous oral bacteria will support the in vitro growth of *B. melaninogenicus* (*gingivalis*) when innoculated on/in a medium lacking menadione (vit K).

Hypothesis III: Certain indigenous oral bacteria will produce menadione (vit K) when grown in vitro and the production of menadione by these bacteria will enhance the in vitro growth of *B. melaninogenicus* (*gingivalis*).

Null Hypothesis: *B. melaninogenicus* (*gingivalis*) does not require menadione (vit K) for in vitro growth and certain indigenous oral bacteria do not produce menadione and will not effect the in vitro growth of *B. melaninogenicus* (*gingivalis*) when grown on/in a medium lacking menadione (vit K).

MATERIALS AND METHODS

I. Materials:

Microorganisms:

A. Gram negative obligate anaerobe: (Figure 1)

Bacteroides gingivalis (ATCC#33277)

B. Gram negative and gram positive indigenous oral bacteria:

1. *Streptococcus mitis*
2. *Streptococcus mutans*
3. *Streptococcus faecalis*
4. *Staphylococcus epidermidis*
5. *Staphylococcus aureus*
6. *Corynebacterium hofmanii*
7. *Lactobacillus acidophilus*
8. *Branhamella catarrhalis*
9. *Escherichia coli*

Culture Media:

A. Solid:

1. Enriched trypticase soy agar (ETSA). ATCC medium #1257.

Ingredient	Amount
Trypticase soy agar	40.0 g
Yeast extract	1.0 g
Agar	4.0 g
KNO_3	0.5 g
Sodium lactate 60% syrup	1.3 ml
Sodium succinate	0.5 g
Sodium formate	0.5 g
Hemin solution (see below)	1.0 ml
Distilled water	924.0 ml

Autoclave the above solution at 121°C for 15 minutes. Cool to 55°C. Aseptically add the following freshly prepared, filter sterilized solutions in the order listed.

Ingredient	Amount
Menadione solution (see below)	2.0 ml
4% cysteine $HCl.H_2O$	10.0 ml
0.5% dithiothreitol (DTT)	10.0 ml
10% glucose	10.0 ml

1% sodium formate . . . . 2.0 ml

4% Na_2CO_3 . . . . 10.0 ml

Defibrinated sheep blood. . . . . 30.0 ml

This medium solidifies very quickly and should be maintained at 50-55°C while dispensing.

Hemin Solution

Dissolve 1.12 g KOH in 100 ml distilled water. Add 100 ml 95% ETOH. Add 200 mg hemin.

Menadione Solution

Menadione . . . . 50.0 mg

95% ETOH. . . . . 50.0 ml

Distilled water . . . . 50.0 ml

Dissolve menadione in ethanol, then add water and filter sterilize.

2. ETSA minus menadione (ETSA-K)

B. Liquid:

1. Enriched trypticase soy broth. (ETSB) ATTC medium #1257 with trypticase soy broth.

2. ETSB minus menadione (ETSB-K)

3. Trypticase Soy Broth

II. **METHODS**:

Culture Method:

All culture media were prereduced for 24-48 hours. Incubation was done anaerobically at 30°C in Gas-Pak anaerobic jars (Figure 2) for seven days.

Experimental Method:

A. Growth study using satellite phenomenon:

A pure culture of *B. gingivalis* (ATCC #33277) was activated with 5 ml of ETSB-K medium.

A sample of this culture was streaked on 3 ETSA and 3 ETSA-K plates. These were used for positive and negative controls. Twenty-seven ETSA-K plates were streaked with the same culture of *B. gingivalis* (ATCC #33277).

Forty-eight hour cultures in trypticase soy broth of the nine indigenous oral test bacteria were obtained from stock cultures in the Microbiology Department. A satellite phenomenon study was conducted by cross-streaking three of the ETSA-K plates previously innoculated with the B. gingivalis culture with each of the nine oral test bacteria, which were used as provider organisms. These plates were incubated for seven days. The resulting colonial growth was examined and the growth supporting capability for B. gingivalis of the provider organisms was evaluated.

B. Growth study using optical density:

Forty-eight hour cultures of the 9 indigenous oral test bacteria in typticase soy broth were obtained from stock cultures in the Microbiology Department. After incubation the cultures were centrifuged and the supernatant was filter sterilized (Acrodisc Filter No. 4184, Gelman Instrument Company). Ten ml. of ETSB was placed into each of three test tubes to be used for the positive control. Ten ml. of ETSB-K was placed into each of three test tubes to be

used for negative control. Nine ml. of ETSB-K was placed into 27 test tubes to be used to evaluate the growth of B. gingivalis. One ml. of the sterile filtrate of each of the nine provider organisms isms was added to the 27 tubes containing the ETSB-K medium, resulting in three tubes each with 1 ml. for each of the nine provider organisms. These tubes were divided into three groups (Sample A, B, and C) each containing a positive control tube, a negative control tube, and nine experimental tubes.

All tubes were inoculated with 0.05 ml. of a sample of B. gingivalis obtained in the following manner. A 7 day culture of B. gingivalis (ATCC #33277) in 10 ml of ETSB was centrifuged and the supernatant discarded. The cells were washed 3 times with freshly boiled and cooled 0.067 m phosphate buffer (pH 7.2) containing 0.1% (v/v) sodium thioglycolate. The cells were then suspended in a volume of buffer equal to the original volume.

Growth of B. gingivalis was measured turbidimetrically at 690 nM using a spectrophotometer (Spectronic 21, Bausch and Lomb). Optical density readings were taken at 24 hour intervals for 7 days.

The growth supporting capability for *B. gingivalis* of the nine facultative oral test organisms was evaluated by measuring the effect of the different sterile filtrates from the provider organisms on the growth of *B. gingivalis*.

C. High pressure liquid chromatographic (HPLC) analysis for the presence of vitamin K.

High pressure liquid chromatographic analysis was performed to determine the presence of and measure the quantity of vitamin K in each of the nine ster-sterile filtrates which were used in the optical density study.

Preparation of HPLC samples:

Ten ml of each sterile filtrate were extracted using 10 ml of hexane. The extraction process was repeated three times and the hexane extracts were pooled. The pooled hexane extracts were evaporated to dryness in a Rotavap (Rotavapor R110, Brinkman Instrument Co., Westbury, NY). The residue was redissolved in 1.5 ml of methanol.

In addition to the sterile filtrates of the nine provider organisms, a sample of 10 ml of ETSB-K culture medium was also prepared for HPLC analysis in the same manner. A standard solution containing 0.5 mg/ml of menadione was prepared and used as the standard (positive control).

Method of HPLC analysis:

Five µl of the sample to be tested was injected into a Waters Bondapak C_{18} reverse phase analytical column (Millipore Corporation, S. San Francisco, CA). An elution solvent containing a mixture of methanol/ethanol/water (37/57/8) was used to elute the column. Pressure and flow rate were maintained at 2,000 psi and 1.5 ml/min by the use of Rabbit-HP pressure pumps (Rainin Instrument Co., Woburn, MA). Detection of vitamin K was achieved by the use of an Isco Variable Wavelength Absorbance Detector (Isco Instrument Co., Lincoln, NE) set at 254 nm. The quantity of vitamin K in each sample was determined by the use of a Shimadzu C-R3A Chromatopac Integrator (Shimadzu Corp., Kyoto, Japan).

D. Statistical analysis

An analysis of variance suitable to a split-plot factorial design with one within-subjects factor (days-7 levels) and one between-subjects factor (treatment-11 levels) was conducted on the data obtained from the optical density study. T-tests were also used to evaluate the interaction between days and treatment conditions on these data.

RESULTS

I. Results of Satellite Phenomenon Study:

A. Positive control: (ETSA and *B. gingivalis*)

Heavy growth of black pigmented colonies (Figure 3).

B. Negative control: (ETSA-K and *B. gingivalis*)

No growth (Figure 4).

C. *S. mitis*: ETSA-K, *B. gingivalis* with *S. mitis* as provider organism.

Abundant growth of black pigmented colonies extending to the edge of the plate. Growth was inhibited adjacent to the test organism. (Figure 5)

D. *S. mutans*: ETSA-K, *B. gingivalis* with *S. mutans* as provider organism.

Heavy growth of black pigmented colonies over the entire surface of the plate. Growth was inhibited adjacent to the test organism. (Figure 6)

E. *S. faecalis*: ETSA-K, *B. gingivalis* with *S. faecalis* as provider organism.

Growth of small colorless colonies over the entire surface of the plate with increased heavier growth adjacent to the test organism. No inhibition of growth was noted adjacent to the test organism. (Figure 7)

F. *S. epidermidis*: ETSA-K, *B. gingivalis* with *S. epidermidis* as provider organism.

Heavy growth of black pigmented colonies over the entire surface of the plate with heavier concentrated growth adjacent to test organism. No inhibition of growth was noted adjacent to the test organism. (Figure 8)

G. *S. aureus*: ETSA-K, *B. gingivalis* with *S. aureus* as provider organism.

Heavy growth of black pigmented colonies over entire surface of the plate. No inhibition of growth was noted adjacent to the test organism. (Figure 9)

H. *C. hofmanii*: ETSA-K, *B. gingivalis* with *C. hafmanii* as provider organism.

Growth of small colorless colonies over entire surface of the plate. No inhibition of growth was noted adjacent to the test organism. (Figure 10)

I. *L. acidophilus*: ETSA-K, *B. gingivalis* with *L. acidophilus* as provider organism.

Moderate growth of black pigmented colonies adjacent to the test organism with isolated black pigmented colonies growing as much as 25 mm away from the test organism. (Figure 11)

J. *B. catarrhalis*: ETSA-K, *B. gingivalis* with *B. catarrhalis* as provider organism.

Light growth of black pigmented colonies mixed in with the growth of the test organism. No growth occured away from the test organism. (Figure 12)

K. *E. coli*: ETSA-K, *B. gingivalis* with *E. coli* as provider organism.

Heavy growth of light pigmented colonies adjacent to and extending as much as 10 mm away from the test organism. No inhibition of growth adjacent to the test organism was noted. (Figure 13)

II. **Results of Optical Density Study:**

An analysis of variance suitable to a split-plot factorial design with one within-subjects factor (days - 7 levels) and one between-subjects factor (experiment - 11 levels) was conducted on these data. Significant effects were found for both days, $F(6,132) = 139.69$, $MSe = .006$, $p < .01$; experiment, $F(10,22) = 11.51$, $MSe = .247$, $p < .01$; and the interaction between days and experiment, $F(60,132) = 6$, $MSe = .006$, $p < .05$.

Tables One to Three and figures 14-16 show the daily optical density readings for the two controls and the nine experimental groups of sample A, B, and C. Table Four and figure 17 show the daily mean optical density readings for the control and experimental groups of the three test samples. Table Five shows the significant mean optical density readings ($P < .05$) with the positive control.

As can be seen in Table Four, with successive days there was increased growth of *B. gingivalis* as demonstrated by increased optical density values. Comparisons (with orthogonal *t*-tests) between mean optical density values of all cultures combined revealed significant ($p < .05$) differences between day one and day two, day two and day three, day three and day four, and day six and day seven. In all instances, the mean optical density values for all cultures combined was greater on the subsequent day.

With respect to experimental conditions, a reference to table five will show that with the exception of experimental group two and the negative control condition, there were no significant ($p<0.05$) differences between the experimental conditions and the positive control. The positive control did show a higher optical density than the negative control and experimental group two had a higher optical density than did the positive control ($p<0.05$). Further pairwise comparisons of the mean optical density values of the experimental groups revealed that all experimental groups had significantly higher optical density values ($p<0.05$) than the negative control. Furthermore, group two had the highest optical density values of all experimental groups.

To understand the significance of the interaction between

days and experimental conditions, preplanned comparisons between daily means of the optical density values for the control and experimental conditions were conducted using t-tests. Relative to the positive control, experimental condition one (S. mitis) had a significant difference ($p<0.05$) in optical density on day six and seven. Experimental condition two (S. mutans) had a significant difference ($p<0.05$) in optical density on all seven days. Experimental conditions three (S. faecalis) and four (S. epidermidis) had a significant difference ($p<0.05$) in optical density on days five, six, and seven. Experimental condition five (S. aureus) had a significant difference ($p<0.05$) in optical density on days four, five, six, and seven. Experimental conditions six (C. hafmanii) and eight (B. catarrhalis) had a significant difference ($p<0.05$) in optical density on all days except day one. Experimental condition nine (E. coli) had a significant difference ($p<0.05$) in optical density on days one and two. Experimental condition seven (L. acidophilus) showed no significant difference in growth of B. melaninogenicus on any of the seven days. Relative to the negative control, all daily comparisons with means revealed significantly greater ($p<0.05$) optical density in all experimental conditions. In general, the experimental conditions one, three, and eight produced greater optical density values relative to the positive control on the latter days of

the experimental conditions consistently produced greater optical density values than the negative control.

III. Results of HPLC Analysis:

The results of the HPLC analysis of the positive control, negative control, and the sterile filtrates of the nine provider organisms can be seen in Figures 18-31 and in Table Six. Four standard curves prepared by chromatography of a known amount of menadione (0.5 mg/ml) resulted in retention times of 5.255, 5.323. 5.292 and 5.35 minutes (Figs. 18-21). The mean retention time of the positive control samples was 5.30 minutes with a standard deviation of $\pm$ 0.08 minutes.

The chromatogram of trypticase soy broth (negative control) in which the nine provider organisms were cultured did not detect a component with a retention time within the standard deviation limits ($\pm$ .08 minutes) of the positive control (Fig. 22). Therefore, it was concluded that trypticase soy broth did not contain an amount of menadione detectable by this method.

The chromatograms of the sterile filtrates prepared from the 48 hour cultures of the nine provider organisms revealed that only *S. mutans* (Fig. 24) produced a component during the 48

hour culture period with a retention time that was within the standard deviation limits of the positive control (5.223 ±0.08 = 5.303). The sterile filtrate from the *L. acidophilus* (Fig. 29) revealed a component with a retention time very similar to the positive control (5.217±.08 = 5.297). Since the sterile filtrate samples were multicomponent solutions the chromatograph revealed all components that absorbed light at the wavelength (254 nm) for which the detector was set (Table Six).

DISCUSSION

Most investigators have found that pure cultures of indigenous oral bacteria lack infectious potential. Similarly, pure cultures of organisms isolated from naturally occuring mixed anaerobic infections are almost always noninfective. However, the entire bacterial complex consistantly displays pathogenic potential. These mixed anaerobic infections provide a clear-cut example of bacterial synergism in the production of infectious diseases.

It has been shown by a number of investigators (Sundquist, 1976; Griffee, 1980; Williams, 1983) that members of the *B. melaninogenicus* group of organisms have been consistantly recovered from symptomatic oral infections of endodontic origin. It was the purpose of this study to investigate the in vitro effect on the growth of *B. gingivalis* of nine indigenous oral bacterial and to attempt to establish a cause for any such effect.

The results of the satellite phenomenon study indicate that *B. gingivalis* requires menadione (vit K) for in vitro growth (Fig. 3 and 4). The gram positive provider organisms seemed to be more supportive of the growth of *B. gingivalis* in a

medium lacking the "growth factor", menadione, than the gram negative provider organisms. The *Streptococcus*, *Staphylococcus* and *Lactobacillus* test organisms all supported heavy to moderate growth of the *B. gingivalis* except *S. faecalis*. The production of colorless colonies in the presence of *S. faecalis* and *C. hafmanii* could possibly be the result of a lower concentration of menadione produced, thus resulting in a nutritional deficiency. The presence of a light growth of small pigmented colonies mixed in the growth of *B. catarrhalis* indicates that this organism is an inadequate producer of the needed "growth factor". The light pigmented colonies produced adjacent to the *E. coli* and extending only 10 mm into the agar could also indicate that this organism produced the "growth factor", but not in sufficient quantity to result in an optimal nutritional environment. The zones of inhibition of growth of *B. gingivalis* adjacent to some of the provider organisms could be the result of a competition by the two organisms for nutritional requirements.

The results of the optical density study also indicate that *B. gingivalis* requires menadione for in vitro growth (Table 4 and Figure 17). Both the gram positive and gram negative provider organisms facilitated the growth of *B. gingivalis* in this study. However, *S. mutans* was the only provider organism which influenced a significant ($<p<0.05$) increase in

growth of *B. gingivalis* when compared with the positive control on each of the seven days of the experiment.

The results of the HPLC analysis of the trypticase soy broth indicated that there was no detectable amount of menadione present in this culture medium. Therefore, any menadione present in a sterile filtrate of this medium following inoculation and incubation by a specific bacterium would be the result of that bacterium synthesizing menadione and releasing it as a by-product into the growth environment.

The HPLC analysis of the sterile filtrates from the nine indigenous oral provider organisms indicated that only *S. mutans* produced a substance into its growth environment which was similar in molecular structure and polarity to menadione. Other components of the sterile filtrates were detected at the 254 nm absorbance level. However, the retention time for none of these components was consistant with the retention time for the standard menadione solution.

SUMMARY AND CONCLUSIONS:

This study was conducted to test the in vitro effect of nine indigenous oral bacteria on the growth of B. gingivalis. A pure culture of B. gingivalis (ATCC#33277) and nine gram positive and gram negative indigenous oral bacteria commonly recovered from dental infections were used. The conclusions are: (a) The results of the satellite phenomenon study indicate that B. gingivalis requires menadione (vit K) for in vitro growth (fig. 3 and 4). In this study, the gram positive cocci were more effective provider organisms in stimulating the growth of B. gingivalis than the gram positive or gram negative bacilli. S. faecalis appeared to provide the least growth stimulation for B. gingivalis of the five gram positive cocci tested (fig. 5-13). (b) The optical density study also indicated that B. gingivalis required menadione (vit K) for in vitro growth (Table 4). Of the nine indigenous oral bacteria used in this study, only S. mutans, when used as a provider organism demonstrated a significant ($p<0.05$) increase in the growth of B. gingivalis (Table 5). (c) The results of the HPLC analysis of the sterile filtrates of the nine provider organisms indicated that only S. mutans produced a substance into its growth environment which was similar in molecular structure and polarity to menadione. This finding is consistant with the results of

the optical density study in which the sterile filtrate from the growth of S. mutans was the only one of the nine provider organisms which produced a statistically significant effect on the growth of B. gingivalis.

The results of this study support the findings of Socransky and Gibbons (1965) in that organisms of the B. melaninogenicus group are not pathogenic in a monoinfection but require a mixed infection for abcess formation. Also, some of these organisms require a "growth factor" that is provided by other bacteria, especially gram positive facultative cocci. This study also supports the findings of Gibbons and MacDonald (1960) that this "growth factor" can be supplied by menadione. Here, of the nine provider organisms tested, only S. mutans produced a substance similar to menadione and only the sterile filtrate of a 48 hour culture of S. mutans significantly influenced the growth of B. melaninogenicus.

Griffee (1960), Sundquist (1976), Williams (1983), and Yoshida (1987) have all reported that clinically acute infections of endodontic origin are mixed infections containing one or more anaerobes with a member of the B. melaninogenicus group being present. It is established medical practice that the primary treatment modality for anaerobic infections is surgical drainage and debridement (Balows, 1974). Since

acute infections of endodontic origin have been shown to be primarily anaerobic, their primary treatment modality should also be surgical drainage and debridement of the root canal system.

Systemic antibiotic therapy as the primary treatment modality for acute infections of endodontic origin is probably less than adequate and should be used only in support of drainage and debridement. Penicillin has been shown to be effective against most of the anaerobes present in acute endodontic infections (Sundquist, 1976). In cases of penicillin allergy, the usual drug of choice is erythromycin. Pharmacokinetic studies have shown, however, that erythromycin levels in mucosa may not be sufficient to attack many of the anaerobes but may attack most of the facultative bacteria (Ginsberg and Tager, 1980). Since acute infections of endodontic origin are usually mixed infections which seem to require facultative gram positive support bacteria, erythromycin may develop sufficient levels to sufficiently attack these support bacteria and adequately alter the local environment such that the use of the generally more toxic new generation antibiotics may not be necessary.

It is commonly accepted that only the gram negative coliform rods possess the capacity to synthesize vitamin K which is

needed for vitamin-K-dependent blood clotting factors especially Factor II (Platt, 1969). The results of this study indicate that some indigenous oral bacteria may also be capable of synthesizing vitamin K.

Figure 1. American Type Culture Collection culture number 33277 of *Bacteroides gingivalis*.

American Type Culture Collection
33277
BACTEROIDES
GINGIVALIS

Figure 2. BBL and Gas Pak Anaerobic Microbiology System. Beckman Dickinson and Company.

BBL®
GasPak®
Anaerobic System
DISPOSABLE HYDROGEN + CARBON DIOXIDE GENERATOR ENVELOPE
Each Envelope contains: 1 Piece Filter Paper, 1 Tablet Sodium Borohydride, 1 Tablet Sodium Bicarbonate plus Citric Acid
Instructions for Use
1. Cut off corner of envelope at dotted line and place the envelope in the GasPak® jar such that the printed side faces outward.
2. Add 10 ml. of tap or distilled water into the open corner of the envelope. Use a pipette or syringe, inserting only the tip into the envelope.
3. Close the GasPak® jar promptly according to manufacturers instructions.
This envelope is intended for use only in properly maintained and operated GasPak® systems.
For In Vitro Diagnostic Use
CAUTION: All usual precautions attendant to handling hydrogen gas should be observed when using this unit.
FOR PRECAUTIONS AND INSTRUCTIONS FOR USE CONSULT PACKAGE INSERT
Store in dry area at 2–30C
BBL Microbiology Systems
Becton Dickinson and Co.
Cockeysville, MD 21030

ETSA
B. GINGIVALIS

Figure 4. Negative control. *B. gingivalis* on ETSA-K medium incubated for seven days.

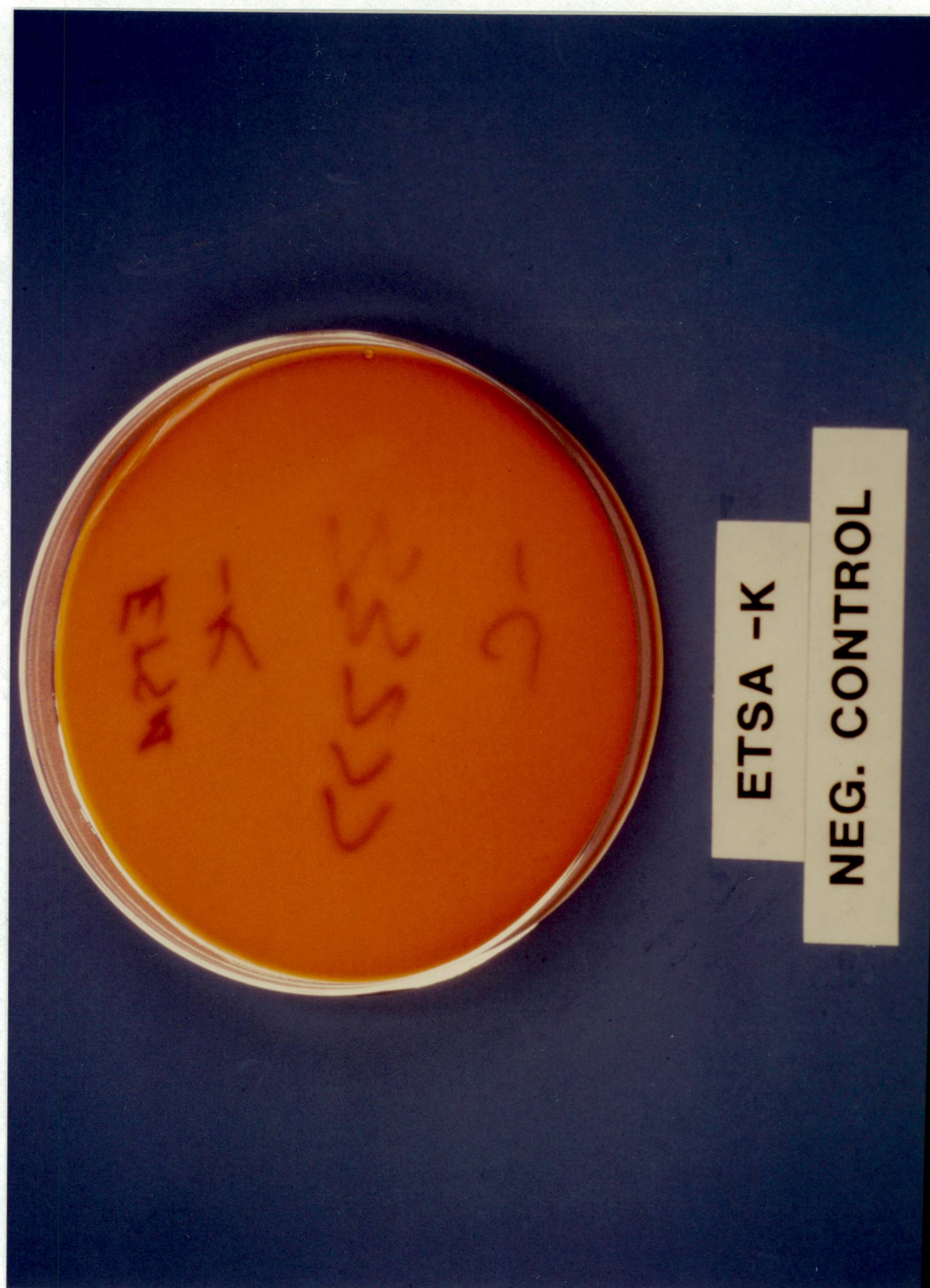

ETSA -K
NEG. CONTROL

Figure 5. B. gingivalis with S. mitis as provider organism on ETSA-K medium incubated for seven days.

ETSA -K
STREP. MITIS

Figure 6. B. gingivalis with S. mutans as provider organism on ETSA-K medium incubated for seven days.

ETSA -K
STREP. MUTANS

Figure 7. *B. gingivalis* with *S. faecalis* as provider organism on ETSA-K medium incubated for seven days.

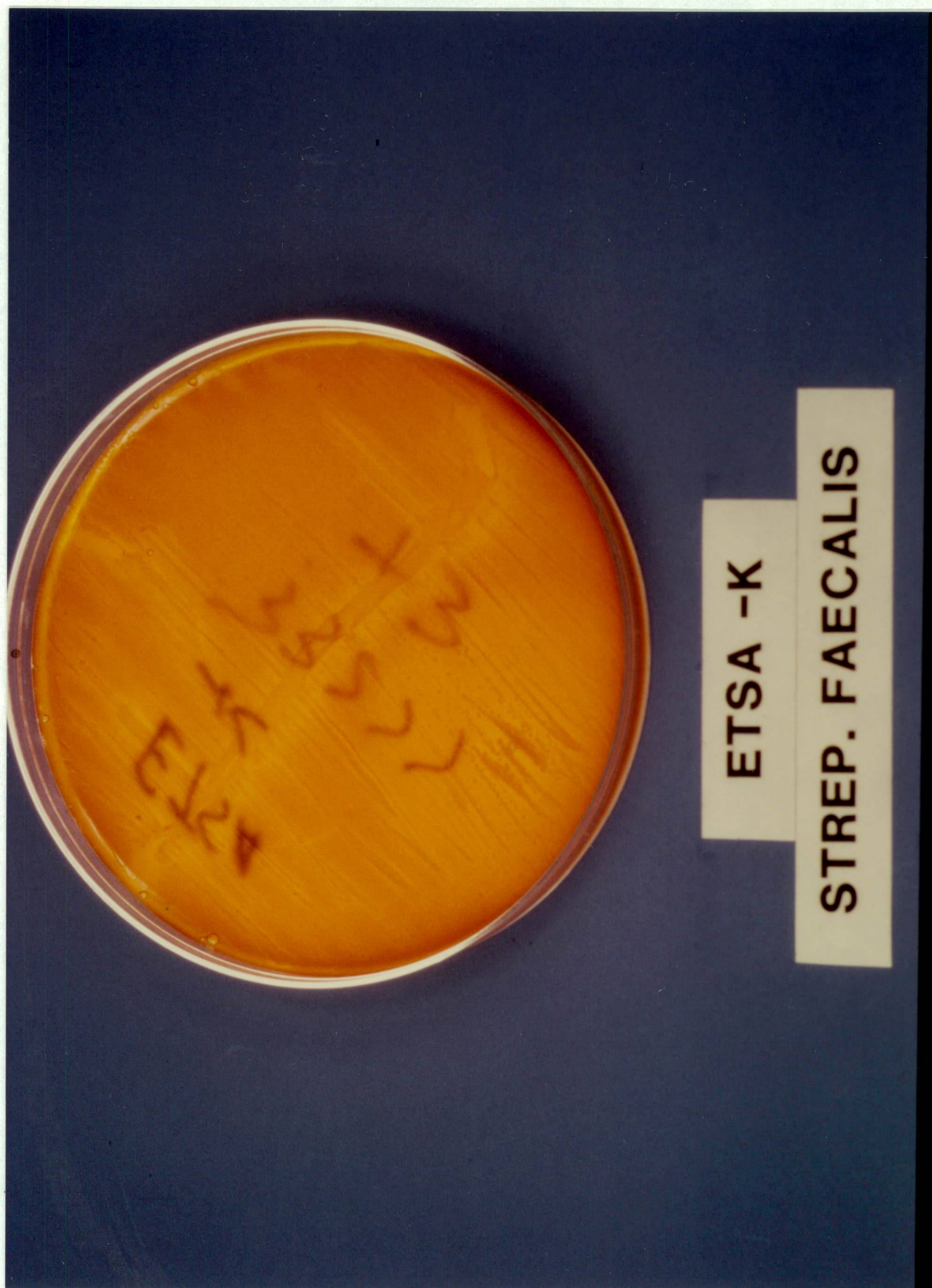

ETSA -K
STREP. FAECALIS

Figure 8. *B. gingivalis* with *S. epidermidis* as provider organism on ETSA-K medium incubated for seven days.

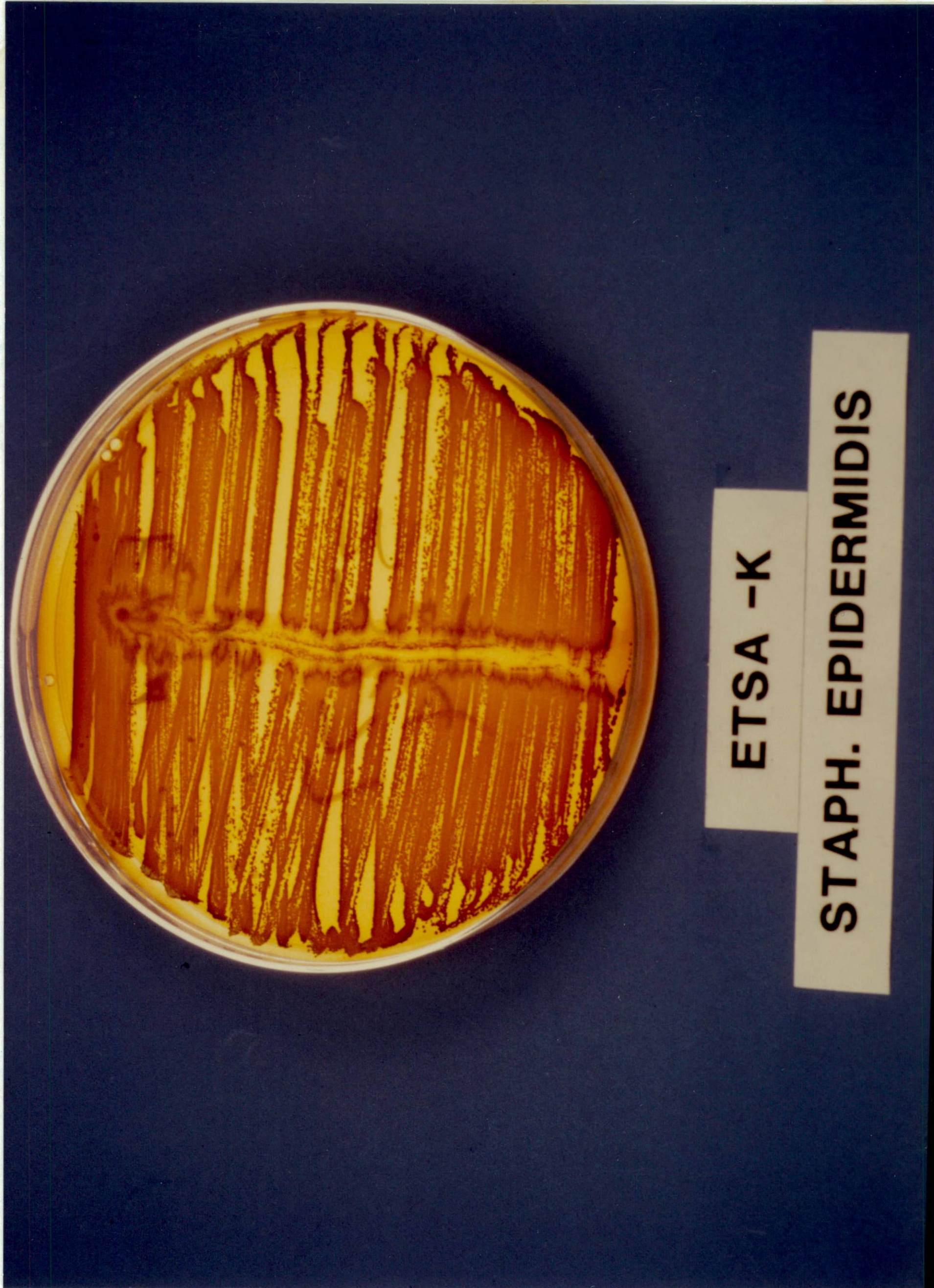

ETSA -K
STAPH. EPIDERMIDIS

Figure 9. _B. gingivalis_ with _S. aureus_ as provider organism on ETSA-K medium incubated for seven days.

ETSA -K
STAPH. AUREUS

Figure 10. _B. gingivalis_ with _C. hafmanii_ as provider organism on ETSA-K medium incubated for seven days.

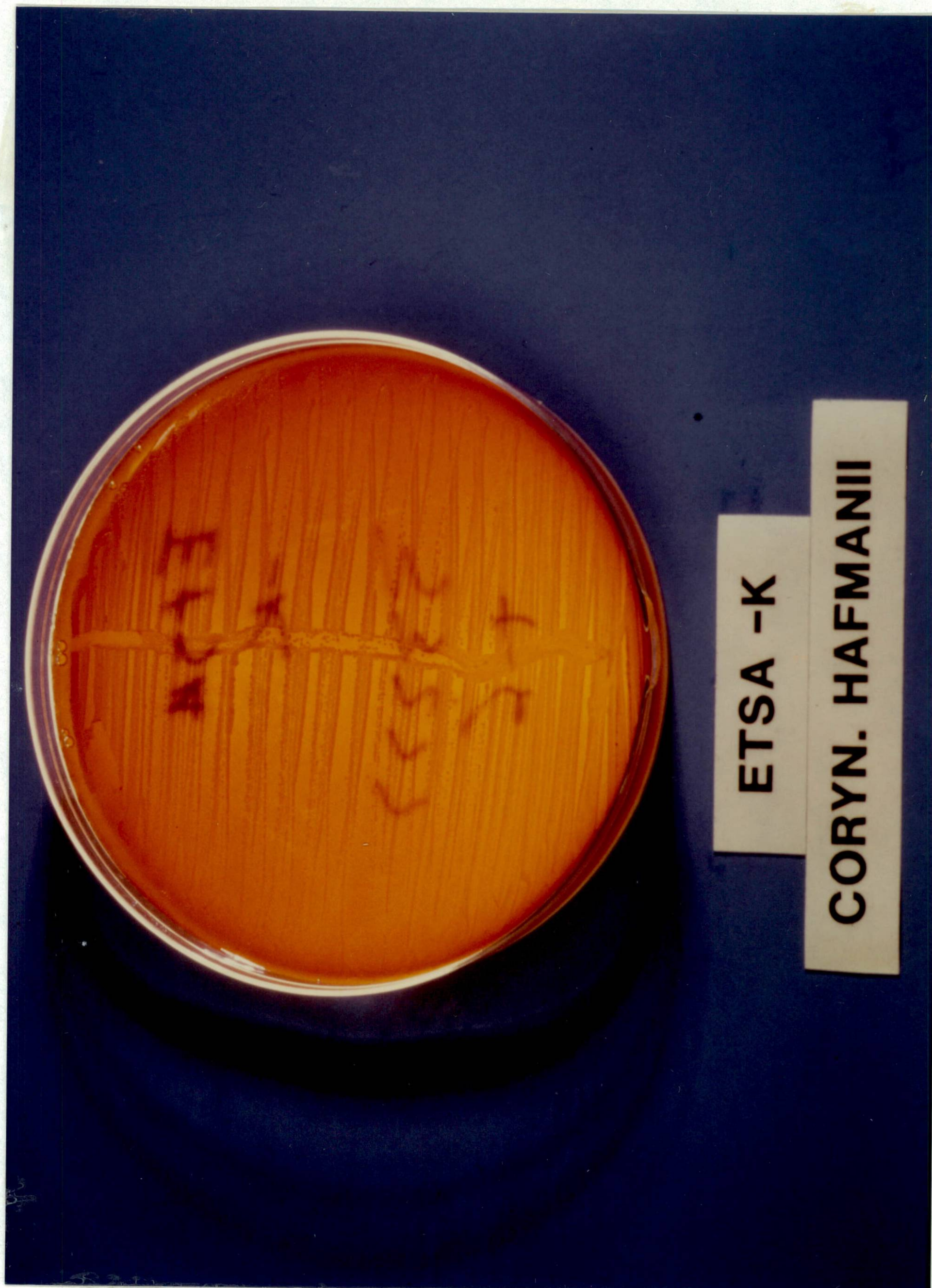

ETSA -K
CORYN. HAFMANII

Figure 11. *B. gingivalis* with *L. acidophilus* as provider organism on ETSA-K medium incubated for seven days.

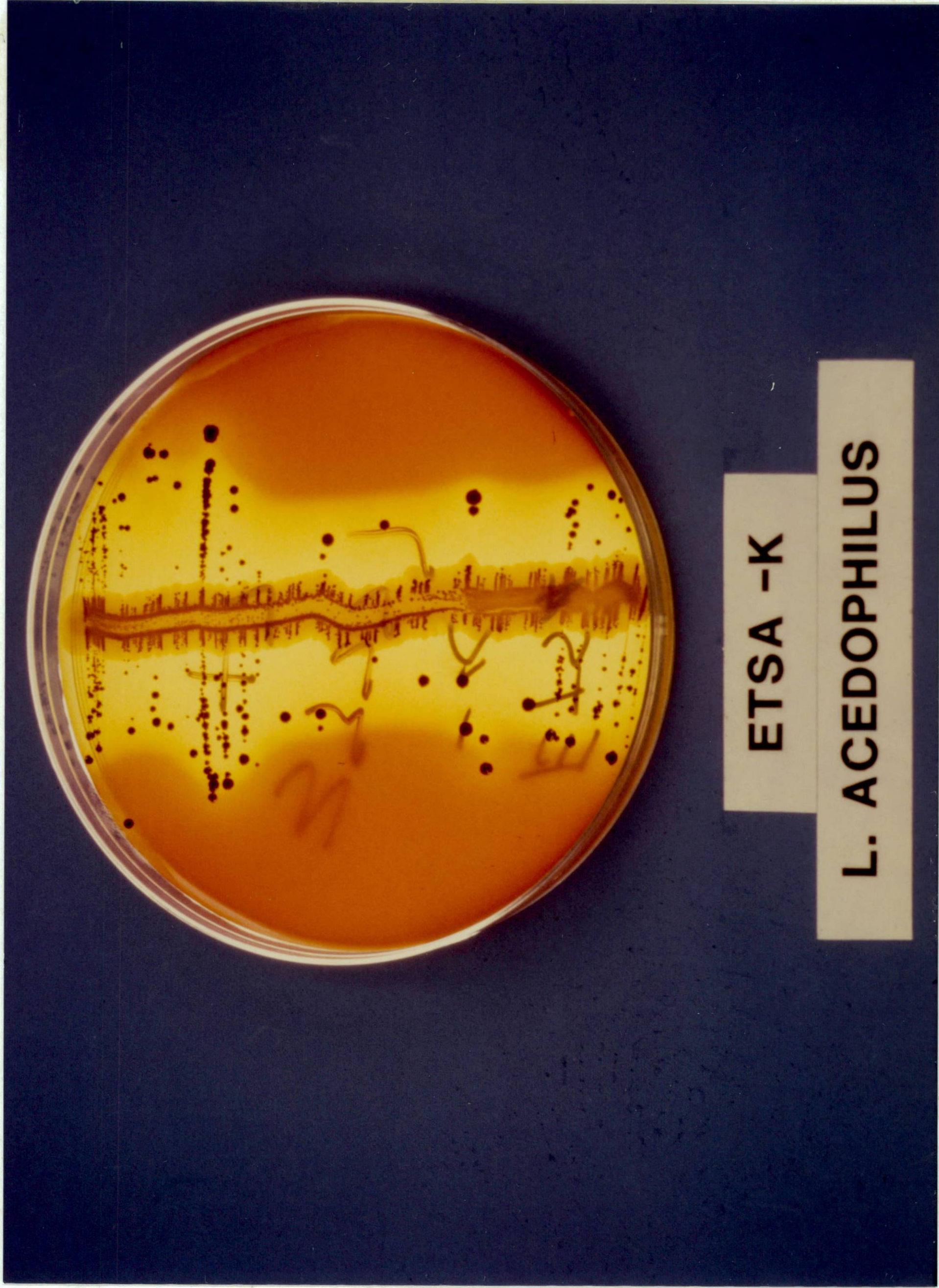

ETSA -K
L. ACEDOPHILUS

Figure 12. _B. gingivalis_ with _B. catarrhalis_ as provider organism on ETSA-K medium incubated for seven days.

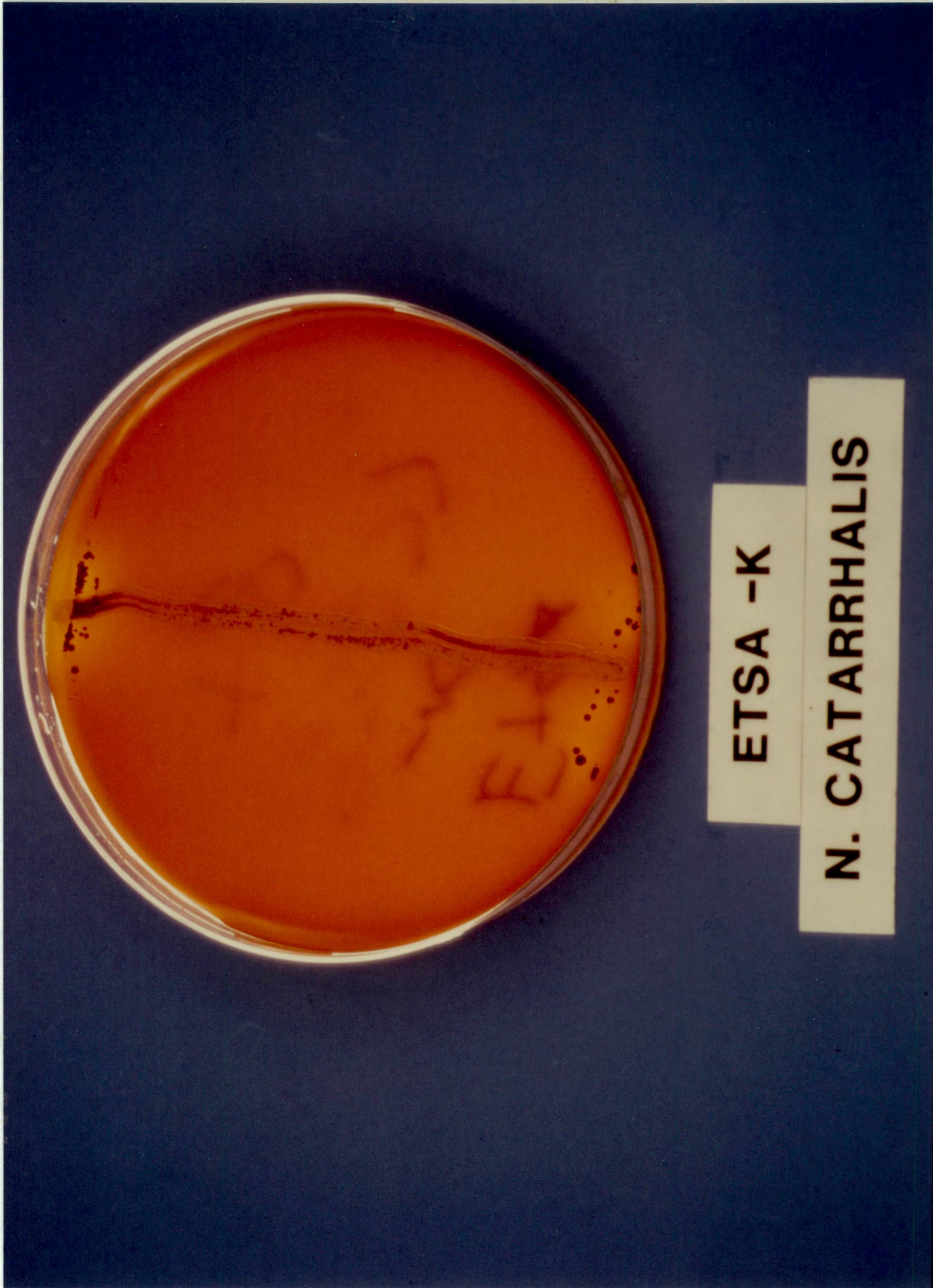

ETSA -K
N. CATARRHALIS

Figure 13. *B. gingivalis* with *E. coli* as provider organism on ETSA-K medium incubated for seven days.

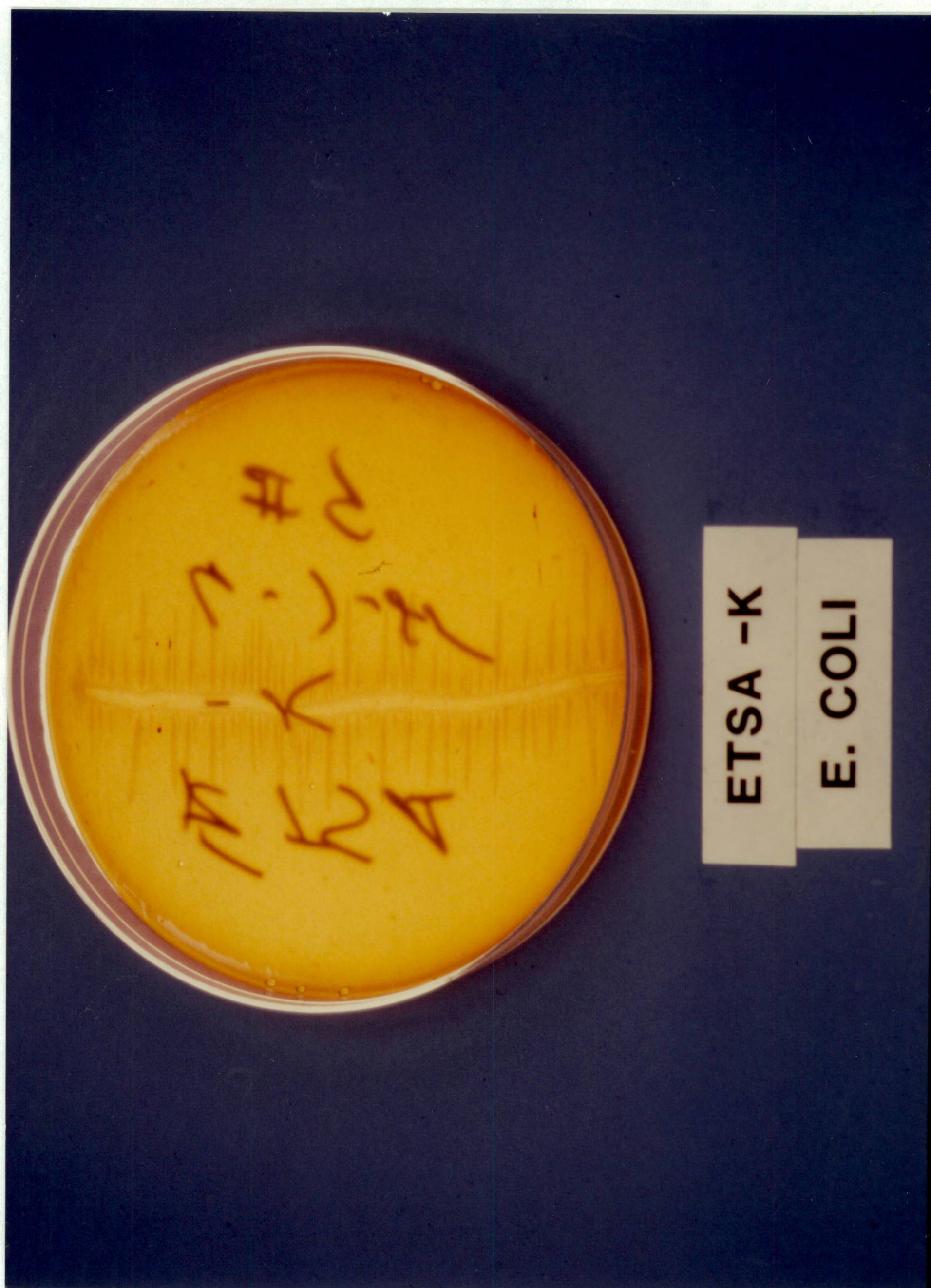
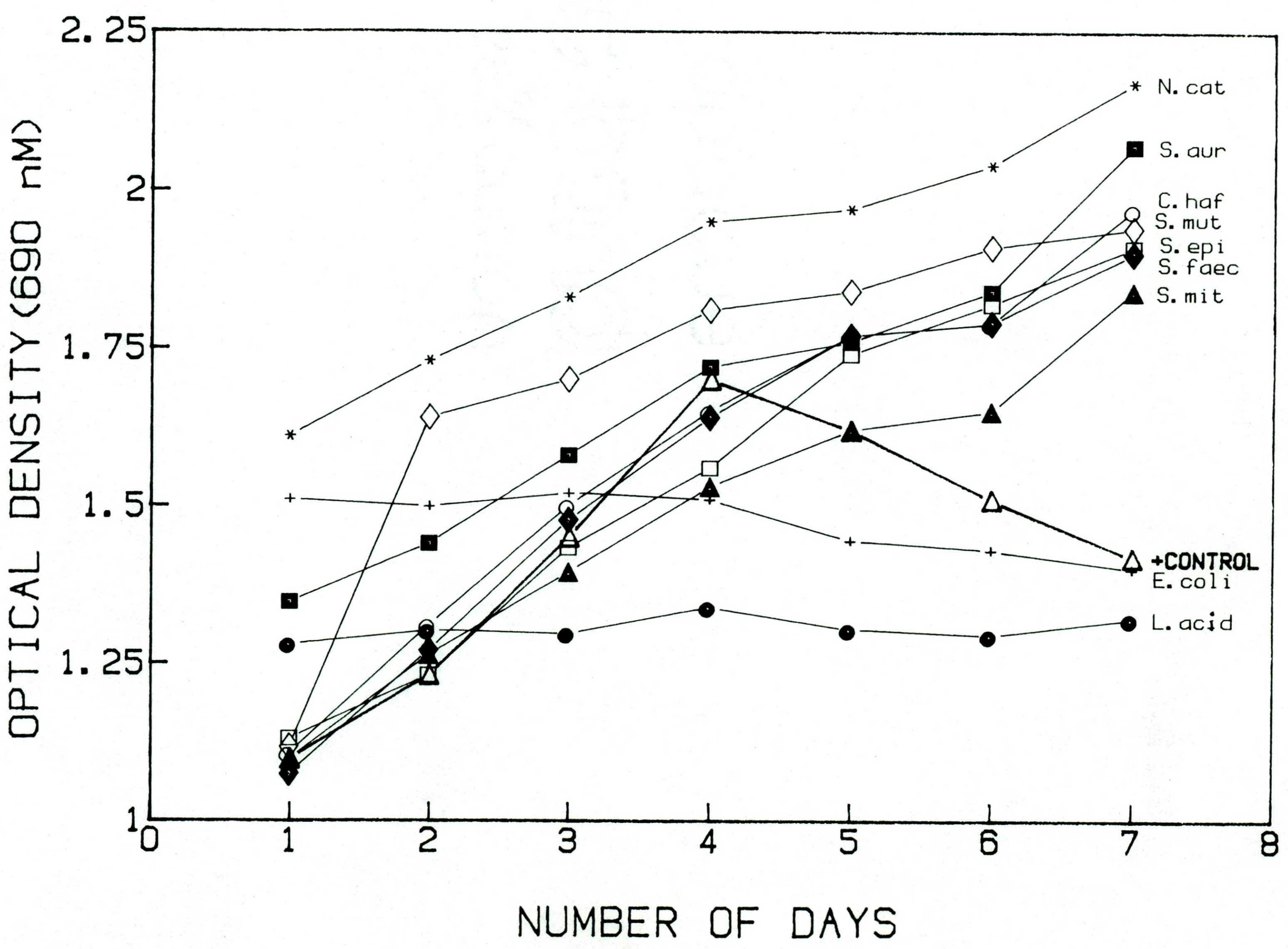

ETSA -K
E. COLI

Figure 14. Daily optical density readings for the growth of _B. gingivalis_ in ETSB for the positive control and nine experimental groups in ETSB-K for Sample A. Optical density readings for the negative control were less than 0.25.



OPTICAL DENSITY (690 nM)
2.25
2
1.75
1.5
1.25
1
0
1
2
3
4
5
6
7
8
NUMBER OF DAYS
N. cat
S. aur
C. haf
S. mut
S. epi
S. faec
S. mit
+CONTROL
E. coli
L. acid

+ CONTROL	1.098	1.231	1.450	1.700	1.620	1.510	1.418
- CONTROL	0.017	0.031	0.045	0.059	0.061	0.062	0.065
#1 S. mit	1.097	1.263	1.394	1.530	1.620	1.650	1.840
#2 S. mut	1.117	1.640	1.700	1.810	1.840	1.910	1.940
#3 S. faec	1.074	1.270	1.477	1.640	1.770	1.790	1.900
#4 S. epi	1.130	1.231	1.433	1.560	1.740	1.820	1.910
#5 S. aur	1.373	1.440	1.580	1.720	1.760	1.6840	2.070
#6 C. haf	1.106	1.310	1.500	1.650	1.770	1.790	1.970
#7 L. acid	1.270	1.302	1.297	1.339	1.304	1.298	1.321
#8 B. cat	1.610	1.730	1.830	1.950	1.970	2.040	2.171
#9 E. coli	1.510	1.499	1.520	1.510	1.445	1.430	1.407
DAY	1	2	3	4	5	6	7

TABLE 1: OPTICAL DENSITY READINGS FOR GROWTH OF *B. gingivalis* IN TRYPTICASE SOY BROTH FOR 7 DAYS IN EACH OF 11 TREATMENT GROUPS(SAMPLE A).

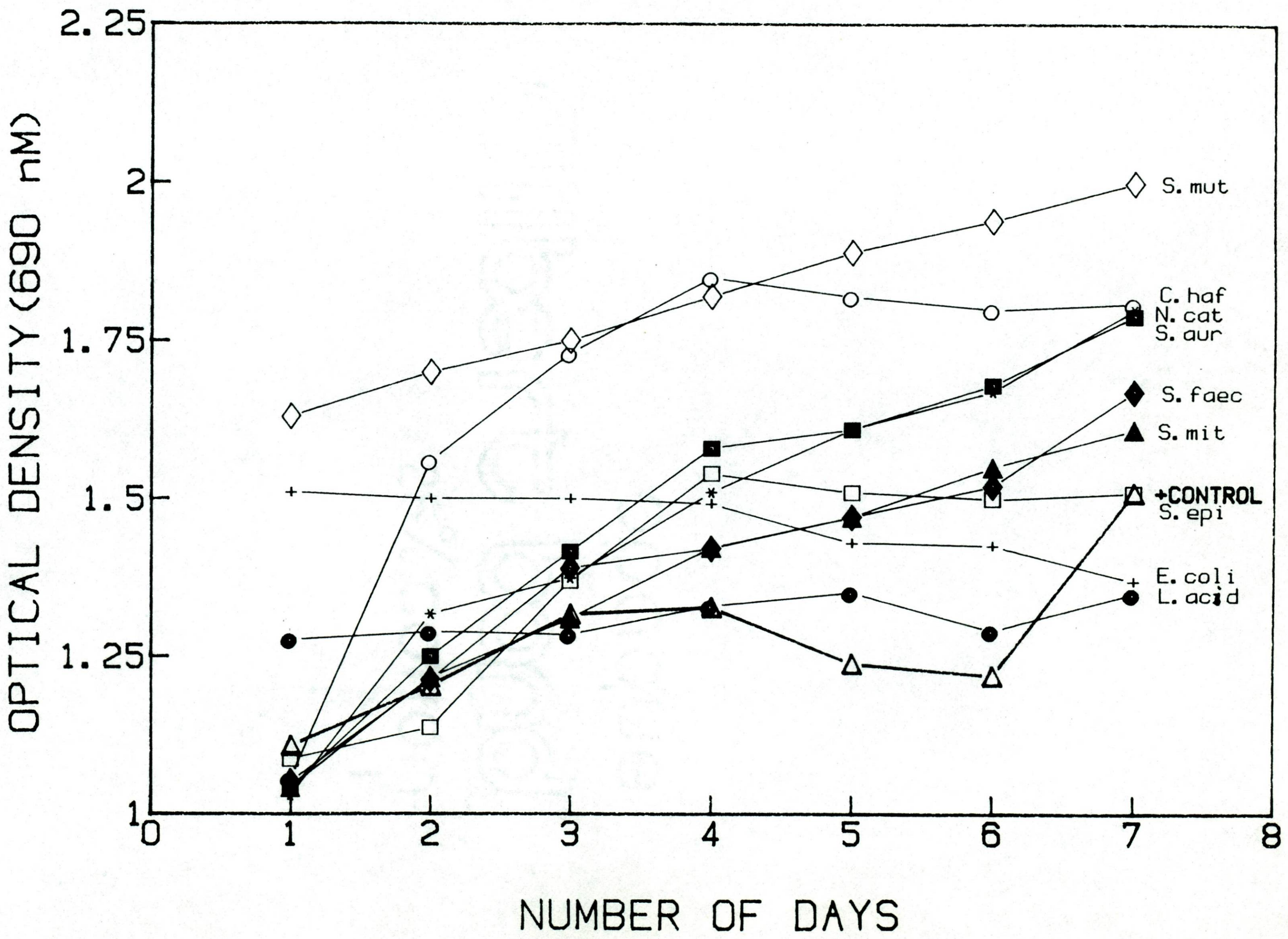
Figure 15. Daily optical density readings for the growth of *B. gingivalis* in ETSB for the positive control and nine experimental groups in ETSB-K for Sample B. Optical density readings for the negative control were less than 0.25.

OPTICAL DENSITY (690 nM)
2.25
2
1.75
1.5
1.25
1
0
1
2
3
4
5
6
7
8
NUMBER OF DAYS
N. cat
S. mut
S. faec
C. haf
S. epi
S. aur
+CONTROL
S. mit
L. acid
E. coli

DAY	1	2	3	4	5	6	7
+ CONTROL	1.285	1.310	1.486	1.520	1.530	1.540	1.540
- CONTROL	0.016	0.029	0.042	0.055	0.063	0.070	0.076
#1 S. mit	1.236	1.540	1.530	1.540	1.540	1.530	1.530
#2 S. mut	1.540	1.640	1.740	1.770	1.800	1.850	1.930
#3 S. faec	1.102	1.189	1.370	1.590	1.660	1.710	1.850
#4 S. epi	1.115	1.269	1.437	1.560	1.610	1.640	1.740
#5 S. aur	1.046	1.297	1.600	1.660	1.640	1.650	1.660
#6 C. haf	1.086	1.316	1.500	1.600	1.740	1.740	1.790
#7 L. acid	1.300	1.326	1.310	1.342	1.444	1.391	1.428
#8 B. cat	1.058	1.251	1.468	1.640	1.690	1.770	2.010
#9 E. coli	1.521	1.500	1.520	1.493	1.416	1.406	1.399

TABLE 2: OPTICAL DENSITY READINGS FOR GROWTH OF _B. gingivalis_ IN TRYPTICASE SOY BROTH FOR 7 DAYS IN EACH OF 11 TREATMENT GROUPS (SAMPLE B).

Figure 16. Daily optical density readings for the growth of *B. gingivalis* in ETSB for the positive control and nine experimental groups in ETSB-K for Sample C. Optical density readings for the negative control were less than 0.25.

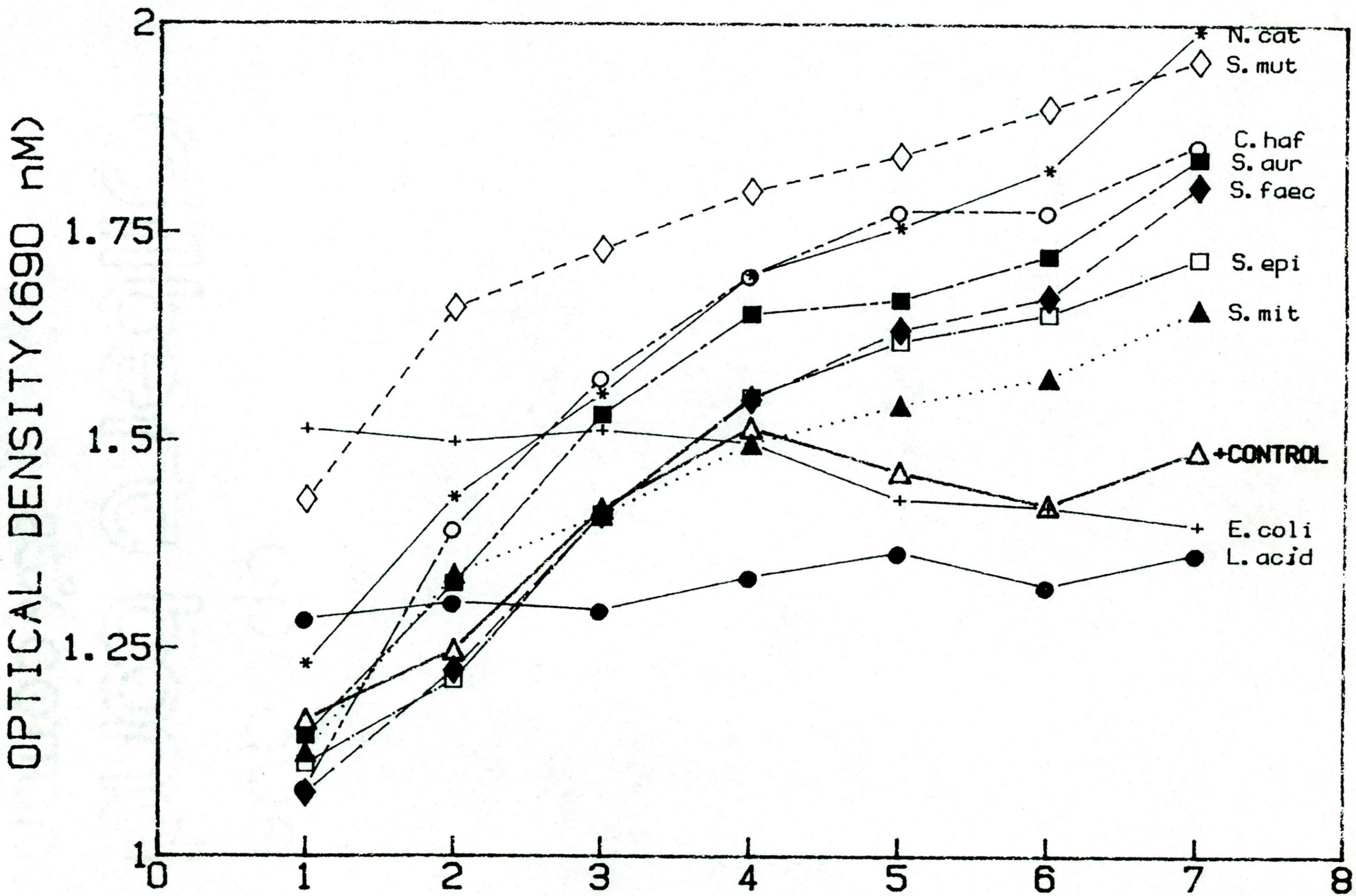


2.25
2
1.75
1.5
1.25
1
OPTICAL DENSITY(690 nM)
S. mut
C. haf
N. cat
S. aur
S. faec
S. mit
+CONTROL
S. epi
E. coli
L. acid
0
1
2
3
4
5
6
7
8
NUMBER OF DAYS

+ CONTROL	1.108	1.203	1.317	1.328	1.238	1.219	1.510
- CONTROL	0.073	0.088	0.094	0.105	0.105	0.106	0.107
#1 S. mit	1.042	1.216	1.310	1.422	1.471	1.550	1.610
#2 S. mut	1.630	1.700	1.750	1.820	1.890	1.940	2.000
#3 S. faec	1.051	1.213	1.390	1.421	1.471	1.520	1.670
#4 S. epi	1.087	1.137	1.370	1.540	1.510	1.500	1.510
#5 S. aur	1.041	1.250	1.416	1.580	1.610	1.680	1.790
#6 C. haf	1.056	1.560	1.730	1.850	1.820	1.800	1.810
#7 L. acid	1.277	1.289	1.285	1.330	1.352	1.291	1.350
#8 B. cat	1.029	1.317	1.374	1.510	1.610	1.670	1.800
#9 E. coli	1.510	1.500	1.500	1.492	1.430	1.426	1.390
DAY	1	2	3	4	5	6	7

TABLE 3: OPTICAL DENSITY READINGS FOR GROWTH OF *B. gingivalis* IN TRYPTICASE SOY BROTH FOR 7 DAYS IN EACH OF 11 EXPERIMENTAL GROUPS (SAMPLE C.

Figure 17. Mean optical density readings of Samples A, B, and C for the growth of *B. gingivalis* for the positive control and nine experimental groups. The optical density readings for the negative control were less than 0.25.



N. cat
S. mut
C. haf
S. aur
S. faec
S. epi
S. mit
+CONTROL
E. coli
L. acid
OPTICAL DENSITY (690 nM)
NUMBER OF DAYS
2
1.75
1.5
1.25
1
0
1
2
3
4
5
6
7
8

DAY	1	2	3	4	5	6	7	MEAN
+ CONTROL	1.164	1.248	1.418	1.516	1.463	1.423	1.489	1.389
- CONTROL	0.035	0.049	0.060	0.073	0.076	0.079	0.083	0.065
#1 S. mit	1.125	1.340	1.411	1.497	1.544	1.577	1.660	1.451
#2 S. mut	1.429	1.660	1.730	1.800	1.843	1.900	1.957	1.760
#3 S. faec	1.076	1.224	1.412	1.550	1.634	1.673	1.807	1.482
#4 S. epi	1.111	1.212	1.413	1.553	1.620	1.653	1.720	1.469
#5 S. aur	1.145	1.329	1.532	1.653	1.670	1.723	1.840	1.556
#6 C. haf	1.083	1.395	1.577	1.700	1.777	1.777	1.857	1.595
#7 L. acid	1.286	1.306	1.297	1.337	1.367	1.326	1.366	1.326
#8 B. cat	1.232	1.433	1.557	1.700	1.757	1.827	1.993	1.619
#9 E. coli	1.514	1.499	1.513	1.498	1.430	1.421	1.399	1.468
MEAN	1.129	1.269	1.382	1.472	1.500	1.503	1.590	

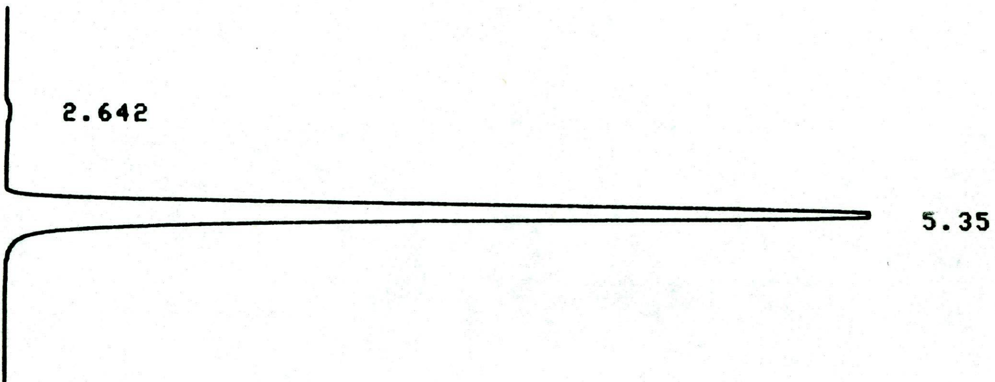
TABLE 4: MEAN OPTICAL DENSITY READINGS OF 3 SAMLES FOR GROWTH OF *B. gingivalis* IN TRYPTICASE SOY BROTH FOR 7 DAYS IN EACH OF 11 TREATMENT GROUPS.

+ CONTROL	1.164	1.248	1.418	1.516	1.463	1.423	1.489	1.389
- CONTROL	0.035	0.049	0.060	0.073	0.076	0.079	0.083	0.065
#1 S. mit						1.577	1.660	
#2 S. mut	1.429	1.660	1.730	1.800	1.843	1.900	1.957	1.760
#3 S. faec					1.634	1.673	1.807	
#4 S. epi					1.620	1.653	1.720	
#5 S. aur				1.653	1.670	1.723	1.840	
#6 C. haf		1.395	1.577	1.700	1.777	1.777	1.857	
#7 L. acid								
#8 B. cat		1.433	1.557	1.700	1.757	1.827	1.993	
#9 E. coli	1.514	1.499						
DAY	1	2	3	4	5	6	7	MEAN

TABLE 5: SIGNIFICANT MEAN OPTICAL DENSTY READINGS ($p < .05$ WITH THE POSTIVE CONTROL) FOR THE GROWTH OF *B. gingivalis* in TRYPTICASE SOY BROTH FOR 7 DAYS IN EACH OF 11 TREATMENT GROUPS.

Figure 18. High pressure liquid chromatogram of standard menadione solution (0.5 mg/ml).

START

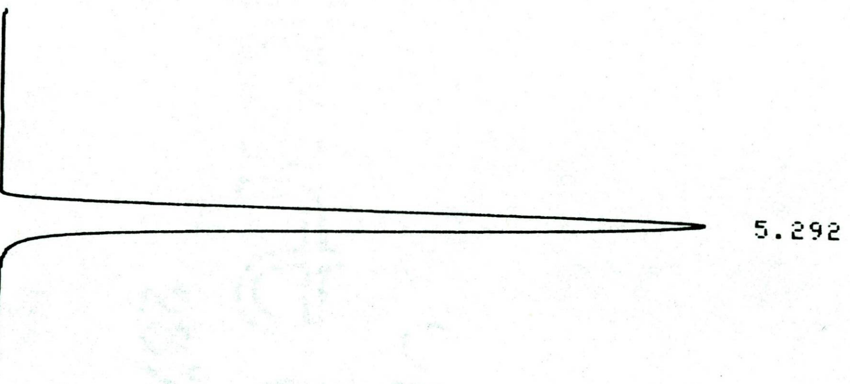


CHROMATOPAC C-R3A FILE 0
SAMPLE NO 0 METHOD 41
REPORT NO 1673

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	2.642	36592			0.2708	
2	5.35	13475795			99.7292	
	TOTAL	13512387			100	

Figure 19. High pressure liquid chromatogram of standard menadione solution (0.5 mg/ml).

START

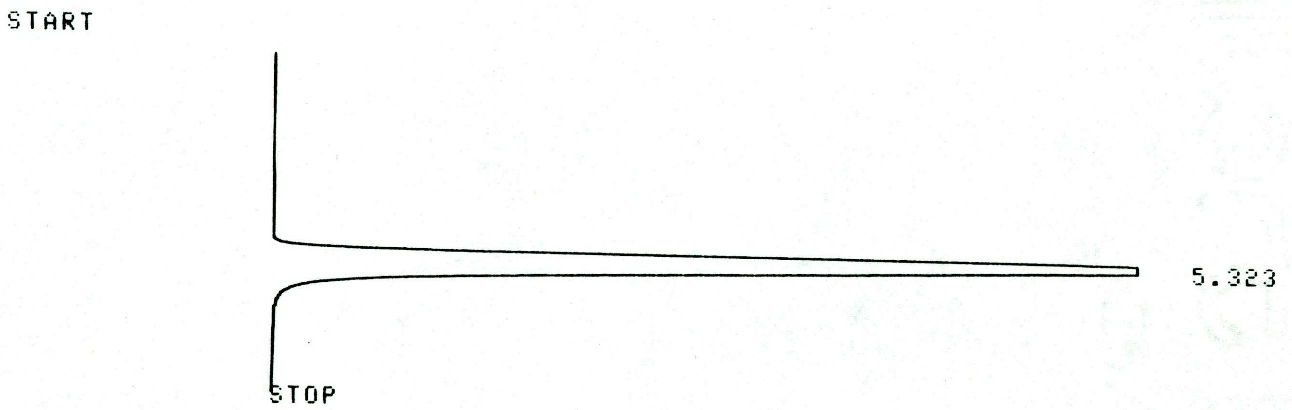


CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1663

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.292	12424937			100	

Figure 20. High pressure liquid chromatogram of standard menadione solution (0.5 mg/ml).



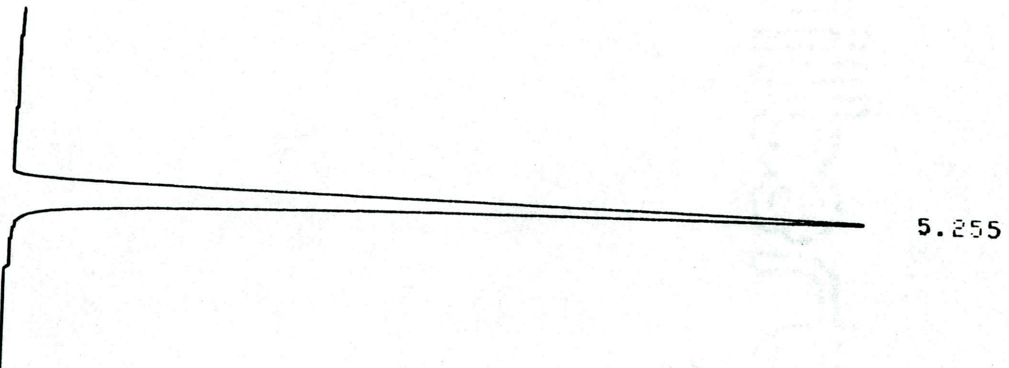
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1642

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.323	14876120			100	
	TOTAL	14876120			100	

Figure 21. High pressure liquid chromatogram of standard menadione solution (0.5 mg/ml).

START



CHROMATOPAC C-R3A

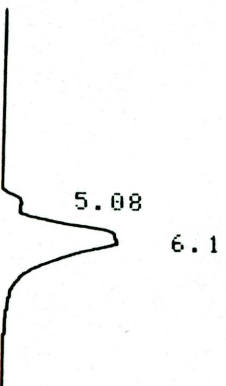
SAMPLE NO 0 FILE 0

REPORT NO 1662 METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.255	11261243			100	
	TOTAL	11261243			100	

Figure 22. High pressure liquid chromatogram of tryticase soy broth culture medium (negative control).

START



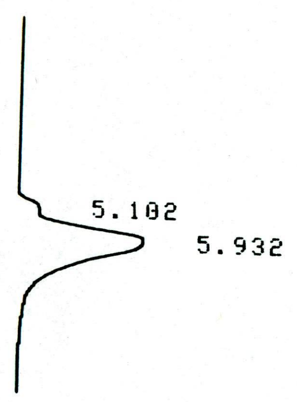
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1643

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.08	220998			5.5643	
2	6.1	3750730	V		94.4357	
	TOTAL	3971727			100	

Figure 23. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *S. mitis* in trypticase soy broth.

START



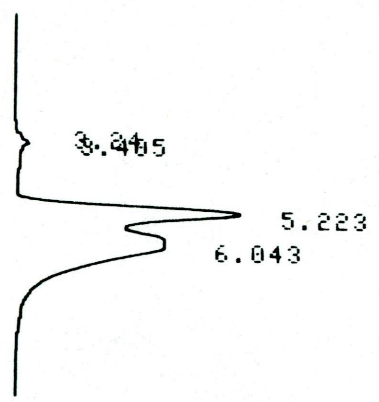
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1644

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.102	214686			4.8933	
2	5.932	4172693	V		95.1067	
	TOTAL	4387378			100	

Figure 24. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *S. mutans* in trypticase soy broth.

START



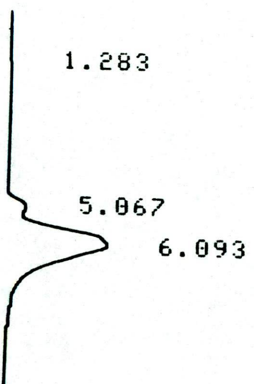
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1645

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	3.24	21654			0.2637	
2	3.405	62711	V		0.7636	
3	5.223	3263595			39.7374	
4	6.043	4864938	V		59.2353	
	TOTAL	8212898			100	

Figure 25. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *S. faecalis* in trypticase soy broth.

START



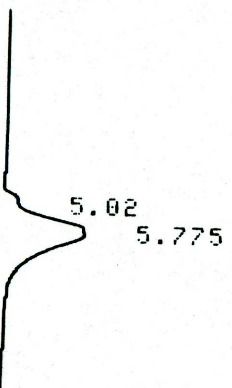
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1646

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.067	222483			6.2596	
2	6.093	3331798	V		93.7404	
	TOTAL	3554281			100	

Figure 26. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *S. epidermidis* in trypticase soy broth.

START



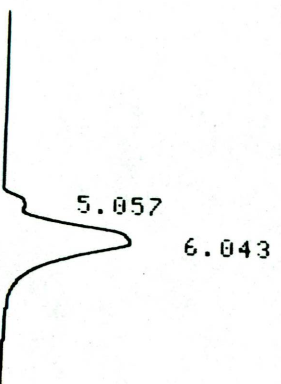
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1648

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.02	133108			4.7788	
2	5.775	2652285	V		95.2212	
	TOTAL	2785393			100	

Figure 27. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *S. aureus* in trypticase soy broth.

START



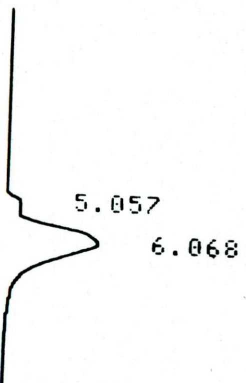
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1651

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.057	245765			5.5474	
2	6.043	4184528	V		94.4526	
	TOTAL	4430292			100	

Figure 28. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *C. hafmanii* in trypticase soy broth.

START



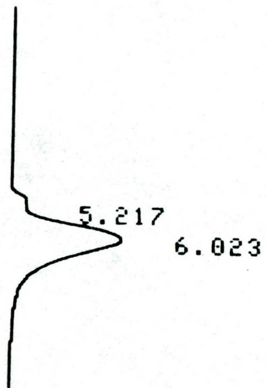
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1653

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.057	181170			5.6763	
2	6.068	3010530	V		94.3237	
	TOTAL	3191700			100	

Figure 29. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *L. acidophilus* in trypticase soy broth.

START



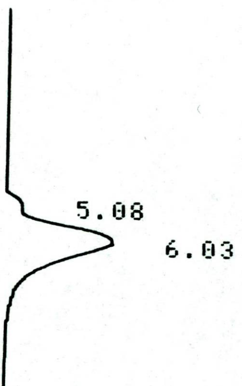
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1661

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.217	222868			5.8258	
2	6.023	3602704	V		94.1742	
	TOTAL	3825572			100	

Figure 30. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of _B. catarrhalis_ in trypticase soy broth.

START



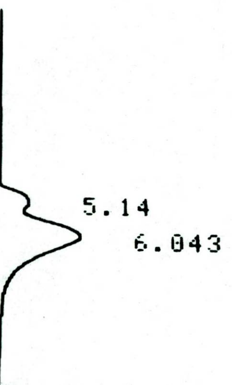
CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1667

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.08	129233			3.5337	
2	6.03	3527920	V		96.4663	
	TOTAL	3657153			100	

Figure 31. High pressure liquid chromatogram of sterile filtrate from 48 hour culture of *E. coli* in trypticase soy broth.

START



CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1670

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	5.14	458728			14.8283	
2	6.043	2634869	V		85.1717	
	TOTAL	3093596			100	

SAMPLE	RETENTION TIME (MIN.)	AREA	CONC (µg/ml)
+ Control	5.30	13009523	500.00
- Control	5.08	220998	12.74
	6.1	3750730	216.23
#1 S. mit.	5.1	214686	12.38
	5.932	4172693	240.56
#2 S. mut.	3.24	21654	1.25
	3.405	62711	3.62
	5.223*	3263595	188.15
	6.043	4864938	280.46
#3 S. faec.	5.067	222483	12.83
	6.093	3331798	192.08
#4 S. epi.	5.02	133108	7.67
	5.775	2652285	152.90
#5 S. aur.	5.057	245765	14.17
	6.043	4184528	241.24
#6 C. haf.	5.057	181170	10.44
	6.068	3010530	173.56
#7 L. acid.	5.217	222868	12.85
	6.023	3602704	207.7
#8 B. cat.	5.08	129233	7.45
	6.03	3727920	214.91
#9 E. coli	5.14	458728	26.45
	6.043	2634869	151.9

TABLE 6: High pressure liquid chromatography analysis of positive control, negative control and sterile filtrates of nine provider organisms (wave length = 254 nm, SD = 0.08 minutes, * = similar molecular structure and polarity to positive control).

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