DEVELOPMENT OF THE DOUBLE-TUBE SYSTEM FOR THE CULTIVATION OF ANAEROBIC BACTERIA FROM FOODS

by

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INTRODUCTION

Estimation of the bacterial load present in a given food system is important to the food industry to ensure the quality and safety of food supplies. The widely used standard plate count method provides only aerobic bacterial counts of food systems. In recent years new practices in fermentation, processing, and preservation have created a need to monitor the presence and number of anaerobic bacteria in food. Some of these processing methods result in lowering the redox potential of the food; thus selecting for the growth of anaerobic microorganisms. Some anaerobes may cause undesirable changes in the food, and some are pathogenic.

The incidence of anaerobes (including Clostridium perfringens) at the poultry and meat packing plant level is well documented (Greensburg et al., 1966; Lillard, 1971, 1973; Smart, 1979). Other studies have shown the growth of anaerobes on cold stored red meat and poultry (Steinkraus and Ayres, 1964; Enfors and Molin, 1978, Enfors et al., 1979; Gill and Tan, 1980). These studies indicate that anaerobes are prevalent in many food systems, thus the need for routine monitoring of anaerobes along with aerobes.

A number of methods have been introduced to grow and subsequently count anaerobic bacteria. The most commonly used are the Brewer anaerobe jar (Brewer and Allgeier, 1966), Hungate roll tube system (Hungate, 1950, 1969), and the anaerobe glove box (Socransky et al., 1959; Aranki et al., 1969; Aranki and Freter, 1972). Much literature is devoted

to the relative strengths and weaknesses of each method. The disadvantages of all these methods are that they require specialized equipment and procedures to introduce gasses for displacement of O_2. Some of the procedures are time consuming and expensive to use on a routine basis.

Recently a commercial product called the "Lee tube" was introduced (Ogg et al., 1979). It consists of an inverted small glass tube attached inside a larger glass tube. Anaerobiosis is achieved by filling the cavity of the specialized tube with a media containing a reducing agent. The reducing agent binds any free oxygen present. Utilization of the Lee tube for anaerobic counts of food has not yet been reported.

Fung and Lee (1981) recently devised a double tube procedure in which a small tube is inserted into a larger tube, and anaerobiosis is achieved by a reducing agent in the media sandwiched between the two tubes. No gassing or special apparatus was necessary.

The purpose of this investigation was to perfect the double-tube system of Fung and Lee (1981) for anaerobic cultivation of bacteria from food systems, and to compare major anaerobic enumeration systems with the double-tube system.

LITERATURE REVIEW

As early as 1680 Leeuwenhoek demonstrated that some life forms, 'animalcules', could exist and grow in the presence of gasses other than oxygen (Dobell, 1960). Pasteur discovered anaerobiosis in 1861, and introduced the terms aérobes and anaérobes (Sonnenwirth, 1972). Since that time bacteria have been classified into several groups on the basis of their oxygen sensitivity and requirements (Smith, 1975). Obligate anaerobes (anaerobes) will not tolerate molecular oxygen at concentrations higher than about 8% for the less sensitive obligates (Loesche, 1969). Aerotolerant bacteria can tolerate limited amounts of oxygen, but grow better anaerobically. Facultative organisms have metabolic capability for growth in aerobic and anaerobic conditions. Microaerophiles do not grow well when exposed to full atmospheric oxygen. Obligate aerobes require molecular oxygen which serves as the final electron acceptor in their metabolism. There are organisms with wide variation of oxygen tolerance within each category.

Seventy-three years ago Runeberg stated that "one of the main reasons why our knowledge about the anaerobic bacteria, especially in human pathology, is not on the same plane with the knowledge of the living habits and importance of oxygen-tolerant bacteria comes from the technical difficulties in obtaining pure cultures and close study of the living habits of these bacteria always has given. Extensions of knowledge

of anaerobic bacteria have usually gone hand in hand with improvements of technical procedures in culture, and still do so" (Runeberg, 1908 as translated by Smith and Holdeman, 1968). Sonnenwirth (1972) has reiterated that this was still the major problem in the field of anaerobic microbiology. Smith (1975) also states that this "reflect to some extent, the difficulties that inevitably result when an obligate aerobe, such as man, attempts to work with obligate anaerobes".

The purpose of investigating the double-tube anaerobic system is to help accelerate the advancement of anaerobic technology. In order to place this system in its proper perspective a review of the development of anaerobic technology, from its inception to the current time, is necessary.

I. Early Developments.

A. Boiling

Boiling was first used by Pasteur as a means of removing oxygen from the culture medium (Hall, 1929). This is the simplest method of removing free oxygen. Oxygen removal occurs because gasses have a reduced solubility at boiling temperatures, and the escaping water vapor has a vacuum effect on dissolved oxygen. Boiling alone is not adequate to reduce oxygen tension for surface cultivation, or allow for growth of strict anaerobes.

B. Vacuum

Early attempts of oxygen removal involved drawing a vacuum on a sealed container. Pasteur developed the first vacuum process for growing anaerobes (Hunziker, 1902). This

process involved simply drawing a vacuum on a flask of warm culture broth by means of a long constricted neck (see Appendix IV for illustration). In 1887 Roux modified this method by allowing the media container to be sealed better, and inoculation was done under a more anaerobic condition (Hunziker, 1902).

Following a different concept, Novy constructed a glass cylinder with a rubber ring on a broad rim top (Appendix IV). Petri dishes were placed into the cylinder, and a bell jar was placed on the top rim. Vacuum was then drawn on the cylinder via the bell jar (Hunziker, 1902). The glass cover and ground glass stopcock were found to break or jam, so they were later replaced with brass covers equipped with two small metal pinch-cocks (Gage and Spurr, 1913).

The use of a vacuum alone proved to be impractical. It was found to be difficult to construct an air tight chamber (Hall, 1929). The methods of removing air (mercury aspirators, water aspirators, various air pumps) were not able to give a complete vacuum. It is now known that the strictist obligate anaerobes are inhibited by as little as 0.5% oxygen (Loesche, 1969). In addition, prolonged incubation under a vacuum resulted in dehydration of the cultivating medium (Hall, 1929).

C. Inert gasses

The initial use of an inert gas for anaerobiosis is attributed to Pasteur (Hunziker, 1902), who bubbled either carbon dioxide or hydrogen gas into the media. This method drove off free oxygen in a similar manner as boiling. Hauser and Liborius developed one of the first apparatuses for passing

gas (hydrogen was preferred) into inoculated media (Hunziker, 1902). Hueppe, and later Fraenkel in 1888, modified this system by using a flask or test tube and a rubber stopper containing a glass tube immersed in the media (Appendix IV). When saturation of the media with gas occurred the tubes were fused (Hunziker, 1902). For solid media Blucher, in 1890, introduced a simple method in which the tube of agar was inverted into a beaker of water and glycerol. Gas was passed through a curved rod into the tube (Hunziker, 1902).

Most of the gassing methods at that time were effective only with broth or melted agar. For surface plating Kitasto, in 1889, and Roth, in 1893, used a flask with flattened sides and two openings for gassing (Hall, 1929). Jones (1916) developed a method using a turned iron base with two openings for gas introduction, and a trough on the top for a petri dish. The petri dish was sealed with paraffin, and the oxygen flushed out with hydrogen. McLeod (1913) and Spray (1930) developed a self-contained anaerobe dish (Appendix IV). The bottom of Spray's method was a deep glass dish with a ridge down the middle. On one side 40% pyrogallic acid was placed, and on the other, 20% NaOH. A standard 100 mm petri dish bottom was inverted and sealed to the top of this bottom dish. The components in the bottom dish were mixed, and a reducing gas of uncertain composition was produced.

D. Anaerobic jars

Liborius, in 1886, combined evacuation and hydrogen gassing in a bell jar. The jar was held down by means of a clamp on a rubber ring, and provided room for several

plates at once (Hall, 1929). Novy's jar (discussed above) was adapted for use with an inert gas (Hall, 1929). Kedrowski, in 1885, designed a simpler container with perforations to permit the flowing of gas prior to sealing the container (Hunziker, 1902).

McIntosh and Fildes (1916) purposed a method that removed oxygen by the oxidation of hydrogen. This oxidation reaction was based on a principle for reduction, by a catalyst, developed by Laidlaw (1915), and known as the Laidlaw principle. The catalyst used was modified by Eggerth and Gagnon (1933), and Zinsser and Bayne-Jones (1934). The catalyst was a platinized, or palladinized asbestos, wrapped in a piece of wire gauze, and activated by heating.

At this point the major problem of anaerobic jars was adapting the catalyst to the system so that it was not exposed to oxygen, resulting in an explosion. Simultaneously Brown (1921, 1922) and Fildes and McIntosh (1921) modified current systems in an attempt to remedy this problem. Brown wrapped the catalyst in fine nichrome, and activated it by means of electric current. Boëz (1927) applied evacuation of the jar before adding the inert gas (hydrogen), reducing the chance of explosion, and increasing the speed of reduction. Weiss and Spaulding (1937) redesigned the jar lid structure so that it was adaptable to virtually any sturdy jar, and not limited to use with only specifically designed jars.

Brewer (Brewer and Brown, 1938; Brewer, 1939) developed what was possibly the most practical anaerobic system for

surface cultivation at the time. Brewer used a modification of the Brown jar (Appendix IV), and eliminated the electrical wire passing through hydrogen by not passing it into the jar, but rather directly into a brass sealed heating element. This allowed the lid to be placed flat on a table without possible damage to the electrical or gas connections. This newly designed lid could be directly connected with a conventional type plug to 110 volts (unlike its predecessors), and the need for a hydrogen cylinder, reducing valve, generator, and rheostat were eliminated.

At the time of World War II anaerobic systems were still not versatile enough. The U.S. Army researchers, in an attempt to develop a practical anaerobic system, incorporated carbon dioxide into the Brewer anaerobic jar (Evans et al., 1948) since hydrogen and electricity were not readily available in all parts of the world at that time. This modification allowed anaerobic bacterial cultivation in field work.

A different method for obtaining anaerobiosis in a sealed jar was recommended by Turner et al. (1963). This method involved placing moistened oats in a desiccant jar, placing a stand over the oats, and placing a candle on top of the stand. On this stand were also placed stacked petri dishes. The candle was lit and the lid air tightly placed on the top. Vedamuthu and Reinbold (1967) used this method to grow and enumerate pure cultures of Propionibacterium.

E. Chemical reducing agents

Reduction of the oxygen in the media was conceived as a method to simplify the cultivation of anaerobes. One of the earliest forms of reducing agents was by use of respiration activities of aerobic bacteria (Hunziker, 1902). Pasteur claimed that aerobic bacteria were able to use all the free oxygen in the medium, and thus developed an oxygen free environment for the anaerobes to grow in. Kedrowski attributed the workability of this method to by-products of the aerobes that form a suitable environment for anaerobes (Hunziker, 1902).

Roux developed a procedure using mixed cultures to achieve anaerobiosis. The anaerobic bacteria was placed between two layers of nutrient agar, and Bacillus subtilis placed upon the surface. To isolate and remove the anaerobic bacteria in this system, the bottom of the tube had to be broken (Hunziker, 1902). This method received limited attention through the years, primarily because the use of mixed cultures was able to serve only as an indirect method for studying anaerobes. Later, Smith and Hungate (1958) cultivated a methane-producing anaerobe on agar media by growing Escherichia coli in the medium, which was then killed prior to inoculation with the anaerobe. Porter (1948) reported that this method can yield a redox potential (Eh′) of -400 mV (see Appendix I for a discussion of Eh′).

Using another approach, Librius, in 1886, observed that glucose stimulated anaerobic growth (Hunziker, 1902). This reducing sugar served as the most important reducing agent for a number of years (Hall, 1929). For some time researchers

debated if carbohydrate provided more to the system than just fermentable compounds. Theobald Smith showed that glucose had reducing properties, as well as nutritive value, for anaerobic growth (Hall, 1929). The reducing properties of glucose were unknown for many years, but it is now known that reductone compounds (strong reducing compounds) derived from glucose were responsible for reduction (Hodge and Osman, 1976). Novy, in 1893, determined that litmus, gelatin, and glucose had valuable reducing ability (Hall, 1929).

Kitasato and Weil, in 1890, attempted to find a stronger reducing agent than glucose (Hunziker, 1902). They found that sodium formate was possibly a better agent, but this compound was never extensively used. They also found that pyrogallol was a good reducing agent. However pyrogallol at 0.5% and above was found to inhibit bacterial growth. Despite this, alkaline pyrogallol became a popular reducing agent in anaerobic cultivation till the middle of this century. It was used both as a component of the cultivating "medium" as well as a compound placed in a closed container with the inoculated plates. Buchner, in 1888, placed alkaline pyrogallol inside a tube system developed by Esmarch (Esmarch roll cultures; discussed below), and placed plates in bell jars with pyrogallol as the reduction agent (Hall, 1929). After that development, much of the research on anaerobes was done with alkaline pyrogallol as the reducing agent for binding molecular oxygen (Hall, 1929; Hunziker, 1902).

Hammerl, 1901, and Rivas, 1902, studied several sulfur containing compounds, such as Na_2S, K_2S, and NH_4SH as reducing

agents. Their results were somewhat inconclusive, but did show that using just a reducing agent was not adequate to promote surface growth of obligate anaerobes. By 1925, Hosoya determined that L-cysteine hydrochloride worked better than any previously used reducing agents including L-cysteine, thioglycollic acid, and sodium sulfate. Hosoya and Kishino, 1925, found that cysteine in combination with sulfuretted hydrogen or sodium sulfide was an even more effective reducing agent (Hall, 1929). Quastel and Stephen (1926) confirmed Hosoya's results with L-cysteine, and attributed its reducing ability to the SH group in the cysteine molecule.

Sodium thioglycolate in a solid media was first introduced by Brewer (1940). The natural reducing ability of cooked meat media was first described by Robertson (1915). Both of these cultivating methods are still currently used.

F. Deep agar layers

The principle that a deep layer of heated agar would remain reduced for a reasonable period of time, due to the slow reabsorption of oxygen into the media, has been known for over a century. Hesse, in 1885, and Liborius, in 1886, recommended preparing a flask or tube of 5-20 cm of agar, and inoculating it just prior to solidification. Inoculation could be done on the agar once it had solidified, at which point another layer of agar was placed on top to facilitate anaerobiosis (Hunziker, 1902). Esmarch, in 1886, developed a method in which the tube containing melted agar, inoculated with bacteria, was hand-rolled horizontally so that the agar formed a layer on the inner wall of the glass tube. Gelatin

was then poured into the tube cavity thereby expelling oxygen from the cavity, and forming an anaerobic environment (Hunziker, 1902). Prior to the turn of the century, Schill and Marpmann independently modified Esmarch's roll cultures by placing a smaller sterile tube into the agar before solidification, and then hand-rolled the apparatus in ice water (Hunziker, 1902; Hall, 1929).

Following this concept Miller et al. (1939) developed what has since been called the Prickett tube. They used an oval tube with broad sides, allowing for better visibility of the colonies. The agar was prereduced by heat, and poured into the tube. It was inoculated, and a layer of agar containing methylene blue was used to fill the neck of the tube (Appendix IV). This method is still used in some laboratories at this time.

Another concept was to place a liquid or solid overlayer on the media to physically displace the oxygen. Pasteur placed oil on top of agar in tubes to facilitate anaerobiosis. Kitasato was the first to use paraffin as an overlaying agent. Kasparec, in 1896, developed a long neck flask which he sealed with paraffin. When the media was heated the layer of paraffin would rise, expelling oxygen as it does, to the top of the neck. This removed the contact of hot paraffin from the surface of the media, and reduced any detrimental effect of hot paraffin on bacteria. The media was then inoculated by piercing the paraffin layer with a needle or loop containing bacteria. Liquid paraffin could then be poured over the solid paraffin thus sealing the paraffin layer, but preventing con-

tact of the hot paraffin with the bacteria (Hunziker, 1902). Rosenthal, 1902, used lanolin, Nicolle used vaseline (Hall, 1929), and Hall (1915) used mineral oil as an overlaying agent, but with limited success.

Koch placed sterile mica blades on top of inoculated media in a petri dish, leaving no air space between the mica and agar (Hunziker, 1902). Sanfelice modified this method by placing the smaller section of the petri dish squarely onto the medium in the larger section (Hall, 1929). Brewer (1942) designed a petri dish lid specifically for this purpose (Appendix IV). Brewer also used a reducing agent in the media which was not standard procedure for these types of oxygen exclusion methods prior to that time.

G. Oxidation-reduction indicators

Little literature is devoted to the early development of indicators of reduction (Eh'). Use of indicators seems to have been developed more as an after thought than as a field of their own. Kitasato and Weyl, during their studies with reducing agents (discussed above), found that sodium sulphonate (indigo blue), while not satisfactory as a reducing agent, was a good indicator of oxidation (Hunziker, 1902). Litmus was recommended by Cohen as an indicator (Hunziker, 1902), and methylene blue was first reported as an indicator by Smith (1893). Brewer adopted methylene blue for use in an indicator paper strip (Brewer et al., 1966), allowing for convenient measurement of oxidation with the Brewer anaerobe jar.

More sensitive indicators have been introduced recently.

Among the more widely used ones are: resazurin (Pittman, 1946; Hungate, 1950), which is now probably the most commonly used indicator; phenosafranine (Bryant, 1963), which usually requires the presence of a palladium chloride catalyst to be totally reduced; and benzyl viologen, which has a desirable sensitivity to oxygen, but is toxic at workable concentrations (Hungate, 1969). A thorough discussion of oxidation-reduction indicators is presented in Appendix III.

A. Anaerobic jars

Brewer and Allgeier (1966) developed an anaerobic jar that utilized both H_2 and CO_2. Their design used a catalyst of 50 alumina pellets coated with 0.5% palladium used previously by Heller (1954), which did not require heating to function, and hence called a "cold" catalyst. This catalyst was active at room temperature, and was a major advancement in self-contained anaerobic systems. Brewer and Allgeier (1966) also utilized a disposable hydrogen generator. The generator, based on earlier designs by Brewer and co-workers (1955, 1965), used a sodium borohydride tablet and a citric acid - sodium bicarbonate tablet in a convenient package. These two tablets, activated by addition of water to the package produced H_2 and CO_2 gas respectively in the jar.

This system required no vacuum, or electric current since H_2 binds O_2 when catalized by palladium ("cold" catalyst), and CO_2 promotes the growth of anaerobic bacteria. With minor variation, depending on the organism being cultivated, Watt (1971) and Collee et al. (1972) had good success growing common

anaerobes encountered in a clinical specimen with this jar.

With the Brewer and Allgeiers method as the basis, many companies (BBL-Dickinson and Company, Cockeysville, MD; Oxoid Inc., Columbia, MD) have developed commercial production of anaerobic jars and accessories. Seip et al. (1976) determined that the BBL jar (GasPak) achieved -100 mV in one hour, and -300 mV within two hours. The commercially produced anaerobic jars are now standard equipment in most clinical and food laboratories where anaerobes are cultivated.

The major disadvantages of this system are 1) the bacterial cultivation plates must be prepared under aerobic conditions; 2) the jars remain aerobic for at least 15 - 30 minutes after closing, which may effect the more strict anaerobes; and 3) from personal observations the failure rate of the jar to achieve anaerobiosis is about 1 in 5.

B. Anaerobic glove box

To eliminate the major problems of the anaerobic jar, the anaerobic chamber (glove box) was subsequently developed. These chambers are kept continuously anaerobic, and have space for inoculating the plates in an anaerobic environment. Socransky et al. (1959) first described and utilized a glove box specifically for inoculating plates under anaerobic conditions (incubation was done in a Brewer jar). They demonstrated the advantage of anaerobic inoculation over aerobic or 'bench' inoculation.

Several other glove box structures have been developed since Socransky's method. Rosebury and Reynolds (1964) constructed a metal box with an air lock. Most of the oxygen

was removed by a long period of evacuation followed by the use of 10% H_2 in N_2 passed over a palladium catalyst. Drasar (1967) used a perspex constructed cabinet with glove ports, and an airlock. Removal of oxygen was done by burning a spirit lamp inside the chamber, and pumping the cabinet atmosphere through alkaline pyrogallol. The chamber was continuously flushed with 5% CO_2 in N_2. Lee et al. (1968) constructed a fiber glass glove box made anaerobic by an N_2 filled balloon inside the chamber. Gordon and Dubos (1970) used a similar structure, making it anaerobic by a long period of continuous N_2 flow. All these structures, and others designed in the same period, were expensive, rigid (usually metal), and fairly complex to operate. In addition, little comparative analysis with other anaerobic methods has been reported.

Aranki et al. (1969) introduced a clear flexible vinyl plastic chamber with a built in incubator. The O_2 tension was maintained at 5-10 ppm, and the Eh′ of a broth placed inside was measured at -280 mV. Media with cysteine hydrochloride reached this mV level quicker, but could not achieve a lower oxidation-reduction (O-R) potential. The addition of a thin layer of agar with palladium black was found to allow for a final Eh′ of -290 mV (reduction of indigo carmine usually took place within two days). They also found that the removal of the chamber catalyst (palladium-coated alumina) allowed the O_2 concentration to increase at a rate of 1 ppm/minute. Anaerobic bacteria counts obtained in this system were highly comparable with direct microscopic counts. The conclusion was made that the chamber they used matched, or excelled the roll

tube technique of Hungate (1950, 1966) in respects of Eh', O_2 tension, and isolating efficiency.

A modification of the rigid structure design was done by Leach et al. (1971). The anaerobic box was rigid in structure, but had glove ports and a viewing window of 0.5 inch perspex. The chamber was kept anaerobic simply by a steady flow of CO_2 into the box, which due to its heavier specific gravity expelled the oxygen out of the bottom portion of the chamber (where the plates were inoculated and incubated). This design also allowed items to be placed in and taken out of the chamber through the top. The partial pressure of O_2 inside the box was reported as less than 0/1 mm Hg. This method was also stated as less expensive and easier to operate than Aranki's system.

Aranki and Freter (1972) determined that to isolate extremely strict anaerobes in the glove box, the use of a palladium chloride catalyst and a reducing agent (cysteine was suggested) are required in the media. This had been done earlier (Aranki et al., 1969) in a two step procedure, but Aranki and Freter (1972) devised a simpler one step method that keeps the water insoluble catalyst sufficiently dispersed in the media to prevent sedimentation. Also the use of cysteine (or any adequate reducing agent) and palladium was recommended in all plates used in this chamber.

Phenosafranine was determined by Aranki and Freter (1972) to be sensitive enough to oxygen to be of value in anaerobic chambers. A properly functioning chamber is indicated when phenosafranine becomes colorless, or shows a marked decrease

of color, within a two day period.

The conditions of the glove box were compared with those of the roll-streak tubes (Cato et al., 1970) and Brewer jar comparisons showed the glove box to be as reliable as the GasPak and roll tube for cultivation of anaerobes normally encountered in clinical studies.

More recently Edwards and McBride (1975) and Cox and Herbert (1978) constructed a glove box which allowed for the growth of methanogenic bacteria. Methanogenic bacteria are among the most sensitive anaerobes (Morris, 1975), and require techniques designed to prevent any introduction or exposure of oxygen. The chambers designed by Edwards and McBride (1975) and Cox and Herbert (1978), were equipped with Ultra Low Oxygen Chambers (ULOC) inside. The ULOC was made of an anaerobic jar with palladium catalyst and silica gel inside, and wrapped with rubber tubing through which warm water was circulated to keep the ULOC at a desired temperature. Inoculated petri dishes were placed inside the ULOC where the oxygen concentration was maintained at 0.0001% (well below the upper 0.001% limit reported by Edwards and McBride (1975) as being inhibitory to methanogens).

The primary disadvantages of the anaerobic glove box are 1) high initial cost; 2) operating expenses (continuous use of gas and electricity); 3) maintenance cost, i.e. cost of replacing and repairing the chamber and accessories; 4) a period of operation training is required; 5) leakage, operator error, or mechanical malfunction can allow oxygen to enter

the chamber; 6) length of operator time; 7) moisture build-up inside the glove box, especially when a large number of plates are inside.

C. Roll tube

Hungate (1947, 1950) developed a method in which inoculated tubes of medium were flushed with CO_2, sealed with rubber stoppers, cooled, and hand spun so that at least the top inside half of the tube was covered with a thin layer of agar. This method was similar to Esmarch's roll cultures, except that the CO_2 flushing allowed the center cavity to be reduced so that the addition of gelatin, as Esmarch did, was unnecessary.

This method was found to allow growth of strict anaerobes better than any method at that time (Hungate, 1950). Hungate and others have since modified and improved this method to allow the growth of a wide variety of strict anaerobes (Bryant, 1953, 1963; Eller, 1971; Kistner, 1960; Holdeman and Moore, 1972; Hungate, 1966, 1969; Hungate et al., 1966). The system has been modified to allow the growth of the methanogenic bacteria also (Smith, 1966; Paynter and Hungate, 1968).

The basic design of the roll tube system includes gassing the tubes by means of a needle that fits over the lip of the tube, and passes CO_2 gas into the tube while bacteriological manipulations are made. One popular system that was designed for the procedure is referred to as the VPI Anaerobic Culture System (Holdeman et al., 1977). This apparatus has a platform that enables the tubes to be set upright. Bent needles are suspended above, and release CO_2 into the tubes to flush

air out. Media (3-4 ml) can then be added, after which the tubes can be inoculated. With practice a technician can safely stopper the tube without allowing oxygen to enter. The tubes are then spun on electric spinners until the media solidifies as a thin layer on the inside walls of the tube.

Media preparation is a critical aspect of the roll tube procedure since, unlike the glove box, the media cannot be held anaerobically for a period of days to achieve optimum O-R potential. The media is boiled initially, and cooled while an oxygen-free gas (usually CO_2) is bubbled into it. At this point the media pH should be adjusted, then the reducing agent added. The exposure of the reducing agent to oxygen should be kept to a minimum since excessive amounts of oxidized reducing agent may be toxic to some fastidious anaerobes (Holdeman et al., 1977). The media is then sealed air tight, and autoclaved. Agar may be added before the initial boiling, or prior to autoclaving. Roll tube techniques are discussed in detail by Hungate (1969), Bryant (1972), and Holdeman and Moore (1972).

Disadvantages of the roll tube system include 1) time consuming; 2) requires skilled technicians; and 3) from personal observations failure rate to achieve proper anaerobiosis is 10% of the tubes.

D. Lee tube

A method for the cultivation of all but the most strict anaerobes was developed by Ogg et al. (1979). This method in principle is similar to other deep agar shakes. The 'Lee tube' as it is commercially named, is a double-walled tube

which forms a thin cylinder of solid medium between the inner and outer walls. Anaerobiosis is achieved initially by boiling and autoclaving the medium, then addition of a reducing agent, and the slow reabsorption of oxygen from the head space back into the solidified media. The glass cylinder, forming the inner wall, makes the medium layer thin enough for easy observation of colonies.

Lee tubes were also effective for allowing growth of Pectinatus cervisiae, an organism which has proven difficult to grow on solid media (Lee et al., 1981).

The Lee tube method has the advantage of ease of operation, and relative low cost (no gas or special equipment). The disadvantages of the system include 1) difficulty to obtain isolates in the tube; 2) difficulty of cleaning, and 3) extreme fragility.

MATERIALS AND METHODS

I. Development of the Double-tube System

Fung and Lee (1981) developed a double-tube system consisting of a glass test tube (150 mm x 10 mm O.D.) inside a larger glass test tube (150 mm x 15 mm O.D.) sealed by a rubber stopper. Using Anaerobic agar (Difco), anaerobiosis was achieved by the deoxygenating effect of heat during sterilization and subsequent binding of any remaining free oxygen by a reducing agent (sodium thioglycolate) in the media. Anaerobiosis was maintained due to the extremely slow absorption of oxygen from the head space into the medium. The rubber stopper and tape seal effectively excluded air from entering the system.

The inner tube of the double-tube system causes the agar to form a thin layer in the space between the test tubes. This agar layer was thick enough for growth of organisms, but thin enough to allow visibility of the colonies for counting.

The dimensions of the glass tubes used by Fung and Lee (1980) are not standard size tubes in the United States, so the new double-tube system was redesigned at Kansas State University using common sized tubes. A screwcap tube, 25 mm (O.D.) x 150 mm (Pyrex #9825), served as the outer tube, and a 16 mm (O.D.) x 150 mm (Pyrex #9800), test tube with a lip was used as the inner tube. For best fit, the bottom of the inner tube was heated and flattened. This gave the tube a

flat bottom and shortened it approximately 2 mm. Shortening the inner tube allowed the lip of the tube to rest squarely on the tip of the outer tube.

The inner tube can be removed, allowing isolates to be taken from the double-tube in the same manner as a roll tube (use of a Pasteur pipet instead of a wire needle was found more suitable). When the inner tube was flattened for a better fit, if the bottom was flattened to the point where it increased in diameter it caused the inner tube to disrupt the agar as it was removed. Increasing the agar concentration in the medium to 39 was found to be best for holding the media integrity as the inner tube was removed. Agar concentrations higher than 3% caused cloudiness in the medium.

Medium was prepared by boiling (to reduce it), and after adjusting the pH to 6.8 - 7.0, approximately 25 ml of medium was added to the outer tube. The screwcaps were tightly placed on the outer tubes before autoclaving. The inner tubes were sterilized separately in a wire basket wrapped in aluminum foil. After autoclaving, the outer test tube containing the medium, was tempered at 45 - 50^{o}C at which point they were ready for inoculation. Aliquots of diluted bacterial cultures or food suspensions (also small sections of solid foods) were quickly introduced to the melted agar in the outer tube. The screwcap was replaced, and the tube was slowly inverted twice to disperse the sample. The inner tube was then aseptically placed into the larger tube using flame sterilized forceps. Inserting the inner tube into the outer tube forced the agar to form a thin film between the cavity

of the two tubes, creating an anaerobic film for bacterial growth. After the cap was screwed securely, the assemblage was dipped in ice water to accelerate cooling and setting of the agar.

II. Anaerobic Systems Tested.

The Lee tube (Simplee Inc., Denver, CO), BBL GasPak anaerobe jar system (Baltimore Biological Laboratories, Division of Becton, Dickinson and Company, Cockeysville, MD), Forma Scientific Anaerobe System (glove box) model 1024 (Mallinckrodt Inc., Marietta, Ohio), and the Hungate roll tube (Bellco Glass Inc., Vineland, NJ; VPI system, Virginia Polytech, VG) were used as comparative systems in testing the effectiveness of the double-tube system in growing pure cultures of anaerobes, and anaerobes from food systems. All commercial systems were performed according to the manufacturer's recommendations.

III. Anaerobic Cultivation Media Tested.

A. Brain Heart Infusion (BHI) - Supplemented:

Brain Heart Infusion Broth (Difco) .... 37 g

Calf Brains	7%
Beef Heart	86%
Proteose peptone	3%
Bacto Dextrose 07%	
Sodium Chloride	2%
Disodium phosphate 09%	

yeast extract (Difco) ..... 5 g

resazurin solution (0.1%) ..... .1 ml
distilled water ..... 1000 ml

Boil the above compounds together, then cool.

The remaining compounds are then added:

hemin solution	10 ml
Vitamin K_1	0.2 ml
L-cysteine HCL (U.S. Bio)	0.5 g

To make solid medium, agar (Difco) was added at a 3% level for the double-tubes, 2% for the roll tubes, and 1.5% for petri dishes. The agar was added before the first boiling, if the medium is to be placed into the tubes prior to autoclaving, or just before autoclaving if the medium was not autoclaved in the tubes.

The pH was adjusted to 6.8 - 7.0 (final pH) immediately after the reducing agent was added. Some media undergo pH change during autoclaving, and must be readjusted to pH 6.8 - 7.0 after autoclaving. This can be done by two methods 1) determine if the change of pH units is consistent and adjust the pH prior to autoclaving accordingly (example: if it consistently rises 0.5 pH units, adjust the pH to 6.3 - 6.5 prior to autoclaving), 2) the media can be autoclaved in a large sealed flask and then the pH aseptically adjusted prior to dispensing into the tubes.

B. Medium 10:

glucose	0.15 g
yeast extract	0.05 g
trypticase	0.2 g
hemin solution	1.0 ml
mineral 1 solution	3.8 ml
mineral 2 solution	3.8 ml
volatile fatty acid mix	0.31 ml
resazurin (0.1%)	0.1 ml
distilled water	50.0 ml

reducing agent ..... 0.05 g (0.05%)

For solid medium, 3% agar was added for the double-tubes, 2% for roll tubes, and 1.5% was added for petri dishes.

If CO_2 is required by the bacteria being cultivated in the double-tube, 5 ml of sterile 8% Na_2CO_3 solution can be added after autoclaving, or 0.4 g of Na_2CO_3 added before autoclaving. This was not necessary for the bacteria used in this study.

The pH was adjusted as before.

Hemin solution:

KOH	0.28 g
ethanol (95%)	25 ml
hemin	100 mg
distilled water	up to 100 ml total volume.

Mineral 1 solution:

K_2HPO_4	6 g
distilled water	100 ml

Mineral 2 solution:

KH_2PO_4	0.6 g
$(NH_4)_2SO_4$	0.6 g
NaCL	1.2 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$CaCl_2 \cdot 2H_2O$	0.16 g
Distilled water	100 ml

Volatile fatty acid mix (VFA):

acetic	17 ml
propionic acid	6 ml
N-butyric acid	4 ml
N-valeric acid	1 ml
isovaleric	1 ml
isobutyric acid	1 ml
DL-a-methylbutyric acid ...	1 ml

The compositions of the above media were obtained from Holdeman et al. (1977), and Caldwell and Bryant (1966).

C. Anaerobic agar (Difco):

Bacto casitone	20 g
sodium chloride	5 g
Bacto dextrose	10 g
Bacto agar	20 g
sodium thioglycolate (Difco)	2 g
Bacto methylene blue	0.002 g
Distilled water	1 L

Agar concentrations were the same as before. The pH of this media is commercially buffered to 7.2 at 25°C.

All media for the roll tube system was CO_2 flushed, and maintained under CO_2 as specified by the Hungate roll tube procedure (Hungate, 1969). Media for all other methods was not flushed or handled under CO_2.

IV. Test Cultures.

The following cultures were tested in the double-tube system, and other "conventional" anaerobic systems to compare the effectiveness of the double-tube system as an anaerobic system.

1) Butyrivibrio fibrisolvens strain 49 (ATCC 27208)
2) Clostridium perfringens strain 214-D (obtained from H. Walker, Iowa State University)
3) Clostridium sporogenes strain MC-25 (Midwest Culture Service)
4) Eubacterium limosum (ATCC 8486)
5) Megasphaera elsdenii strain B159 (ATCC 17752)
6) Streptococcus bovis strain 7H4 (ATCC 15351)

The negative control (aerobe) used was:

7) Pseudomonas fragi strain 2F36 (obtained from S. Hartsell, Purdue University)

Stock cultures of 2, 3, and 4 were kept in reduced BHI-supplemented broth. Stock cultures of 1 and 6 were kept in reduced Medium 10. All anaerobic stocks were kept in test tubes with rubber closure screw-on caps, and stored at 4 - 5° C. The cultures were routinely subcultured using a 22-gauge needle following a syringe method described by Macy et al. (1972).

The negative control (aerobic) stock culture was kept at 4 - 5° C and routinely subcultured on nutrient agar slants.

V. Method of Counting Colonies from the Anaerobic Systems Used.

A. Hungate roll tube

There is no standard method for counting colonies in roll tubes, and thus no method for statistical estimation for counts. A common way to count the colonies is to mark the tube into halves, or fourths, and count each section. Placing the roll tube against a contrasting background, or lighted field, may help facilitate counting. A tube containing more than 300 colonies was considered too numerous to count (Holdeman et al., 1977).

B. Double-tube

The double-tube is counted in a similar manner as the Hungate roll tube. A paper roll of a contrasting color can be inserted into the inner tube to facilitate counting. However, use of an oblique light was found to be the best.

Occasionally agar breakage (excessive gas production),

or spreaders (excess motility) may cause problems in counting colonies. The agar breakage can be controlled by reducing the amount of fermentable sugars present in the medium, and (or) counting the CFU's when they first appear, prior to medium breakage, and then recounting them at the end of the incubation period. Excess motility can be dealt with by again counting the colonies when they first appear, marking the position of each colony prior to the formation of spreaders, and again at the end of the incubation time. When spreaders did appear they were counted as one colony forming unit (CFU). Nontouching spreaders were considered separate CFU's.

C. Lee tube

The Lee tube is counted much like the double-tube. Ogg et al. (1979) recommended inserting a paper roll into the bottom opening to facilitate counting.

D. Petri dishes in anaerobic systems

All petri dishes were counted by the method outlined in Standard Methods (APHA, 1978).

VI. Effect of Reducing Agents in the Double-tube System.

Sodium thioglycolate (Difco), L-cysteine·HCL (U.S. Biochemical Corporation), Dithiothreitol (Sigma), and L-cysteine·HCL + $Na_2S \cdot 9H_2O$ (Fisher) (see Appendix II) were individually added at a 0.5% concentration to 6 double-tubes containing BHI-supplemented agar. Eubacterium limosum was diluted in BHI-supplemented dilution blanks, and inoculated into the double-tubes containing one of the four reducing agents. The tubes were incubated at 37°C for 72 hours. The same procedure was repeated with Butyrivibrio fibrisolvens strain 49

using Medium 10 agar with one of the four reducing agents, and incubated at 37° C for 48 hours. Colonies in each tube were counted after incubation.

The experiment was repeated once.

VII. Estimation of Oxidation-Reduction Potential in the Double-tube System.

Sodium thioglycolate, L-cysteine·HCL, Dithiothreitol, and L-cysteine·HCL + $Na_2S \cdot 9H_2O$ were added individually at 0.05% to double-tubes containing BHI-supplemented agar. One of the oxidation-reduction indicators; methylene blue (Sigma), resazurin (Allied Chemical), indigo carmine (Sigma), nile blue (Sigma), cresyl violet acetate (Sigma), phenosafranine (sigma), and benzyl viologens (Sigma) at 0.001%, was introduced individually to double-tubes in duplicate, each containing one of the reducing agents, and then autoclaved. The pH of the media was adjusted to 7.0, the inner tubes placed inside, and the system stored at 30°C.

Color change of the indicators in the double-tubes were compared to that of an oxidized control double-tube (no reducing agent and excessive exposure to oxygen) containing the same O-R indicator. Comparisons were made at 15-30 minutes, 24 hours, and 48 hours after the medium set in the tubes.

VIII. Evaluation of Anaerobic Cultivation of the Double-tube Using Pure Cultures.

A. Clostridium sporogenes

Clostridium sporogenes MC-25 was freshly grown in cooked meat media. The culture was diluted with phosphate buffer, and inoculated, using a simple random sample design, into

double-tubes, Lee tubes, and petri dishes all containing Anaerobic Agar (Difco). One set of the petri dishes were placed into the Forma anaerobic chamber, and another set into BBL GasPaks. The plates and tubes were incubated at 37°C for 48 hours and colonies counted and statistically compared.

B. Pseudomonas fragi

Pseudomonas fragi strain 2F36, a strict aerobe, was freshly grown in Nutrient broth, and 1 ml (undiluted) was inoculated into six double-tubes containing reduced Nutrient agar (Difco) with L-cysteine·HCL. The tubes were incubated at 32°C for 72 hours before growth was ascertained to determine if an aerobe could grow in the double-tube system.

C. Aerobic-anaerobic growth of isolates from the double-tube system

Samples obtained from broiler drumsticks (standard swab method) were cultivated in the double-tube containing BHI-supplemented agar at 35°C for 48 hours. One hundred isolates were obtained from these double-tubes and cultivated aerobically and anaerobically. Anaerobic cultivation was done by inoculating the isolates individually into vented test tubes containing BHI-supplemented broth with L-cysteine·HCL. The test tubes were then placed into the Forma anaerobic chamber, and incubated at 35°C for 48 hours. Aerobic cultivation was done by inoculating the isolates individually into vented test tubes containing BHI-supplemented broth with no reducing agent and incubated aerobically at 35°C for 48 hours. The same individual isolates were used for aerobic and anaerobic cultivation, and aerobic and anaerobic growth of the same

isolate was compared and recorded.

Megasphaera elsdenii and Butyrivibrio fibrisolvens were grown in the double tube containing Medium 10 agar at 39°C for 48 hours. Thirty isolates of each were incubated aerobically and anaerobically in Medium 10 broth as before.

D. Comparison of five pure cultures grown in the double-tube, Hungate roll tube, and Forma anaerobic chamber

Cultures of Streptococcus bovis, Clostridium perfringens, and Eubacterium limosum were freshly grown in rubber closure screw-on cap test tubes containing reduced BHI-supplement broth. Cultures of Megasphaera elsdenii, and Butyrivibrio fibrisolvens were freshly grown in rubber closure screw-on cap test tubes containing Medium 10 broth. Cultures were diluted in BHI-supplemented or Medium 10 dilution blanks, and inoculated into 12 double-tubes, Hungate roll tubes, or petri dishes (inside the Forma anaerobic chamber) in a non simple random sample design, i.e. the inoculating samples were not drawn from the same dilution blank(s). BHI-supplemented agar, and Medium 10 agar were used in the anaerobic systems to cultivate the representative bacteria accordingly. All systems were incubated at 39°C for 48 hours (24 hours for Cl. perfringens and 72 hours for E. limosum), after which the counts of each organism for each system was recorded and then statistically compared.

The experiment was repeated once.

IX. Practical Application of the Double-tube System.

A. Comparative analysis of counts obtained from ground beef

Fifty grams of frozen ground beef (obtained from the

Animal Science Dept., KSU) was thawed for approximately 24 hours at 4 - 5°C, and placed into a bottle containing 100 ml of sterile distilled water. The ground beef and water were mixed by vigorous hand shaking. The mixture was then diluted in sterile phosphate buffer. Double-tubes, Lee tubes, and petri dishes (15 of each) containing Anaerobic Agar (Difco) were inoculated using a simple random sample design (all inoculating samples were drawn from the same dilution blanks). The petri dishes were then placed into BBL GasPaks. All plates and tubes were incubated at 35°C for 48 hours, and colonies counted and statistically compared.

The experiment was repeated once.

B. Comparative analysis of counts obtained from broiler drumsticks

Eighteen broiler drumsticks were divided into three lots of six drumsticks each, and placed individually into cryovac bags (type B620, size 8" x 20", std. gauge). One lot was vacuum-packaged (vacuum drawn on bag to .95Kp/cm^2 where 1.0Kp/cm^2 equals 1 atm.), another was flushed with carbon dioxide (vacuum drawn to .95Kp/cm^2, then reevacuated with CO_2 to .80-.75Kp/cm^2 vacuum, and sealed), and the third lot was flushed with nitrogen gas (same method as CO_2 flushing). Packages were stored at 4 - 5°C.

The drumsticks were sampled every 2-3 days over an 11-12 day period, using the standard swab method (9 cm^2 template), for anaerobic bacteria growth. Phosphate buffer served as the diluent.

Six double-tubes and six petri dishes containing BHI-

supplemented agar were inoculated with the diluted drumstick sample using a simple random sample design. The petri dishes were placed into the Forma anaerobic chamber. All plates and tubes were incubated at 35°C for 48 hours. Counts were obtained and statistically compared.

The experiment was repeated once.

C. Use of the double-tube system in monitoring Vacuum-packaged ground beef:

Four types of ground beef were used in this study. Type 1 was raw material from a Comitrol flake cutting machine. Type 2 was lean beef trim, 1/2" followed by an 1/8" grind. Type 3 was 1/2" ground, vacuum-packaged for 4-6 days, then 1/8" ground. Type 4 was 1/2" ground, vacuum-packaged for 4-6 days, then 1/8" ground twice. One-half of all four ground beef types were mixed for 5 minutes under a 28" Hg vacuum. The remainder were mixed without a vacuum. All ground beef was then vacuum-packaged in approximately 1 lb. amounts in Saran coated Surlyn film with a BiVAC packaging machine.

Samples were taken for bacterial counts on preparation day (same day that 1/8" grind was performed on types 2, 3, and 4). All packages were then stored in the dark at 3°C for 2-3 days, then displayed in an open top, Hussman display case with GE natural fluorescent lighting (1076 Lumens/m^2), and an air discharge temperature of -5 to -4°C product temperature was less than 4°C). Samples for bacterial analysis were taken on days 0, 7, 14, and 21 of display. One hundred grams of each ground beef sample were mixed with 300 ml sterile distilled water in a Lab Blender 400 Stomacher

(Sharpe and Jackson, 1972) for 2 minutes, and further diluted using phosphate buffer. Double-tubes, containing BHI-supplemented, and petri dishes, containing Standard Plate Count Agar (Difco), were inoculated with the diluted ground beef sample. The petri dishes were incubated aerobically at 32°C for 48 hours, and the double-tubes were incubated at 35°C for 48 hours before making aerobic and anaerobic viable cell counts, respectively.

D. Use of the double-tube system in monitoring Vacuum-packaged and nonvacuum-packaged ground beef.

Lean beef trim from 1/2" followed by an 1/8" grind was used. The trim was accumulated, and ground (1/2" plate) at 2°C, but then exposed to 10°C for 4 hours prior to mixing. One-half was mixed under vacuum conditions (28" Hg), and the remainder was mixed without vacuum conditions. One-half was vacuum-packaged in approximately 1 lb. samples in Saran coated Surlyn film, the remainder wrapped, without vacuum, in oxygen permeable polyvinylchloride (PVC).

Samples for bacterial analysis were obtained on preparation day (same day the 1/8" grind was performed). The PVC samples were displayed as before on preparation day. The vacuum-packaged samples were stored in the dark at 3°C for 2-3 days before placing on display. Samples for bacterial analysis were taken from the PVC packages on day 3 and 5 of display, and from the vacuum-packaged samples on day 5 and 14 of display.

Standard aerobic plate counts, and double-tube counts were obtained in the same manner as before.

X. Methods of Statistical Analysis.

The comparative data was computer analyzed by the following procedures (SAS User's Guide, 1979):

Duncan's Multiple Range Test at alpha = 0.05;

General Linear Model Procedure (GLM), from which P values were obtained;

GLM using the model X=REP TRT TRT(REP) METHOD METHOD(TRT*REP)/ SOLUTION to eliminate replication effect on the sample mean;

GLM using the model LS=REP TRT TRT(REP) METHOD/SOLUTION to eliminate the replication effect on the sample variance;

Univariate analysis to determine data distribution.

RESULTS AND DISCUSSION

I. Effect of Reducing Agents on Bacterial Growth in the Double-tube System.

Costilow (1981) reported the Eh′ of the sodium thioglycolate to be less than -100 mV, L-cysteine·HCL; -210 mV, dithiothreitol; -330 mV, and L-cysteine·HCL + $Na_2S \cdot 9H_2O$; -510 mV. These reducing agents were chosen in this study because they are the most commonly used, and least likely to be toxic (sodium thioglycolate is toxic to certain organisms). They are also relatively simple to use and store (see Appendix B).

A. Effect on Eubacterium limosum and Butyrivibrio fibrisolvens

Table 1 shows that Eubacterium limosum growth in the double-tube was unaffected by different reducing agents. The upper Eh′ limit value of Bacteriodes fragilis, an organism similar to E. limosum (Eggerth, 1933), was found to be +150 mV at pH 7 (Vennesland and Hanke, 1940). The E. limosum test culture used was found to have a definite sensitivity to exposure to atmospheric oxygen for a 2 hour period, and in media with oxidized resazurin (-50 mV or above, see Appendix C). This would be expected since the Eo′ value of all four agents is below -50 mV.

Table 1

Effect of reducing agents on growth of Eubacterium limosum and Butyrivibrio fibrisolvens in the double-tube system.

Reducing agent:	Test Organisms		Reported E_o' of reducing agent:
	E. limosum	B. fibrisolvens	
Sodium thioglycolate	3.6×10^8 a	4.4×10^6 b	-100 mV
L-cysteine·HCL	2.5×10^8 a	7.5×10^6 a,b	-210 mV
Dithiothreitol	4.0×10^8 a	2.6×10^7 a	-330 mV
Cysteine·HCL + $Na_2S \cdot 9H_2O$	4.6×10^8 a	9.7×10^6 a	-571 mV

All means are an average of counts from 12 duplications. E. limosum was cultivated in BHI-supplemented and B. fibrisolvens in Medium 10. All means denoted by the same subscript are not significantly different at alpha = 0.05 level.

Table 1 shows that Butyrivibrio fibrisolvens grew best in the presence of the more negative Eh′ potential reducing agents. There was found to be a significant difference between growth with sodium thioglycolate, and dithiothreitol or cysteine + Na_2S. Cysteine·HCL appears to fall in the middle, showing no significant difference in allowing growth from the other reducing agents. Hungate (1969) reported that any exposure to free oxygen has a definite toxic effect on Butyrivibrio fibrisolvens. Loesche (1969) reported that oxygen concentrations above 0.5% are inhibitory to B. fibrisolvens. So these results are expected considering the Eo′ of each reducing agent.

It is shown then, that any of the four reducing agents in the double-tube system will allow growth of an obligate anaerobe. But for the best growth of a more strict obligate anaerobe, dithiothreitol or L-cysteine·HCL + $Na_2S \cdot 9H_2O$ should be used, although L-cysteine·HCL alone may be satisfactory.

II. Estimation of the Oxidation-Reduction Potential in the Double-tube System.

Since the double-tube system (Fig. 1) is a sealed environment, direct measurements of the O-R potential by means of a millivolt meter was impossible without affecting the O-R of the system. Therefore colorimetric estimation was used. Oxidation-reduction dyes were chosen that would give a gradual increase in molecular oxygen sensitivity. Those chosen were methylene blue (99% reduced at -49 mV), resazurin (-111 mV), indigo carmine (-185 mV), nile blue (-202 mV), cresyl violet acetate (-227 mV), phenosafranine (-312 mV), benzyl viologens

(-419 mV) (see Appendix C for a discussion on indicators).

The results of this experiment are shown in figures 2 through 4. These show that sodium thioglycolate, cysteine, dithiothreitol, and cysteine + Na_2S reduced all the O-R indicator dyes, except phenosafranine and benzyl viologen. This indicated that the system had at least an Eh´ of -227 mV with these reducing agents. When dithiothreitol and cysteine + Na_2S were used a marked color decrease of phenosafranine occurred. This is significant since, in the more sophisticated anaerobe glove box (Aranki et al., 1969; Aranki and Freter, 1972) a palladium catalyst in the media was necessary to achieve reduction of phenosafranine color.

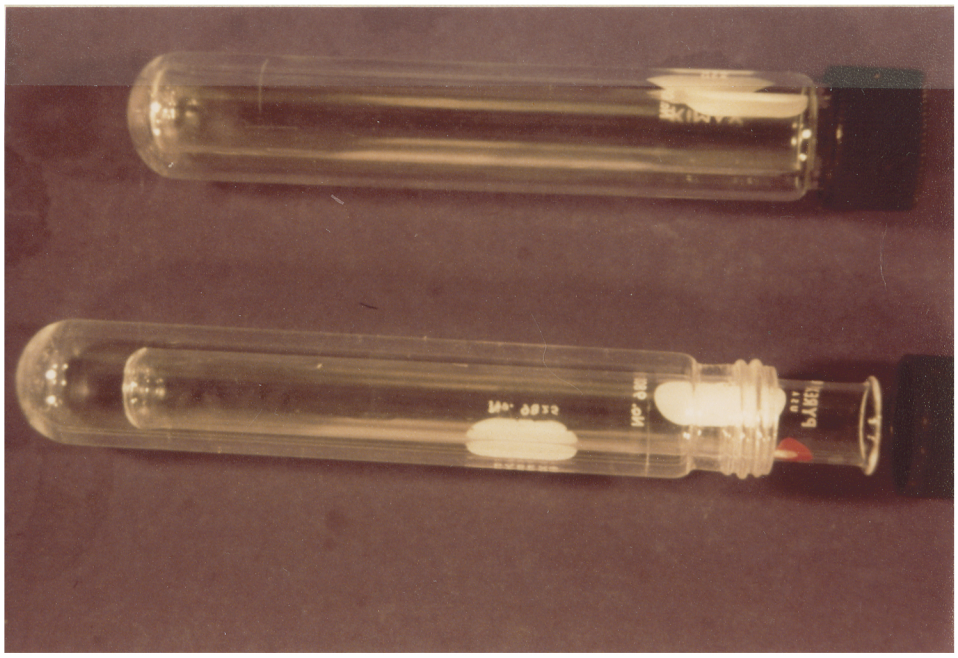
All tubes were found to achieve a reduced color within 15 minutes after the agar set, except for phenosafranine which continued to reduce in color for 24 hours. In addition, control tubes containing methylene blue became colorless within two hours; with resazurin became colorless within 24 hours; and with indigo carmine a marked color decrease occurred within 24 hours. This indicated that the double-tube system, even without a reducing agent or careful handling, can achieve an Eh´ of less than -100 mV.

Tubes with benzyl viologen showed no color development (indicating reduction) over a period of 48 hours. The exact concentration of benzyl viologen needed for a proper color development was unknown though.

From the colorimetric change observed it can be surmised that dithiothreitol to cysteine + Na_2S will produce an Eh´ in the double-tube of at least -227 mV, and probably closer to

Figure 1. The Double-tube anaerobic system.

Figure 2. Resazurin in the Double-tube system. Reducing agents in the media are (r to l); Control, Sodium thioglycolate, L-cysteine·HCL, Dithiothreitol, L-cysteine·HCL + $Na_2S \cdot 9H_2O$.



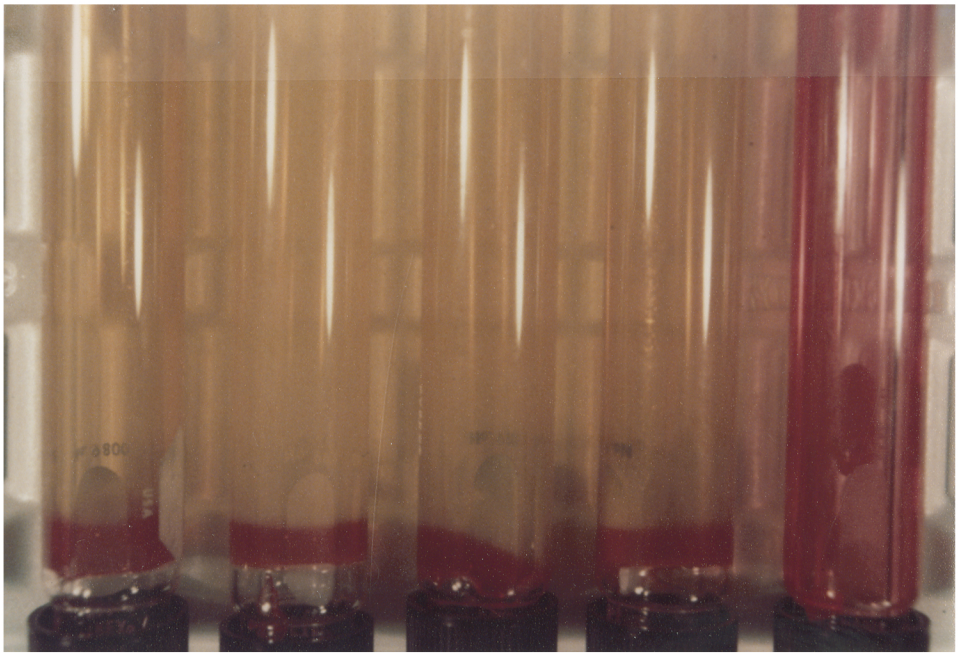
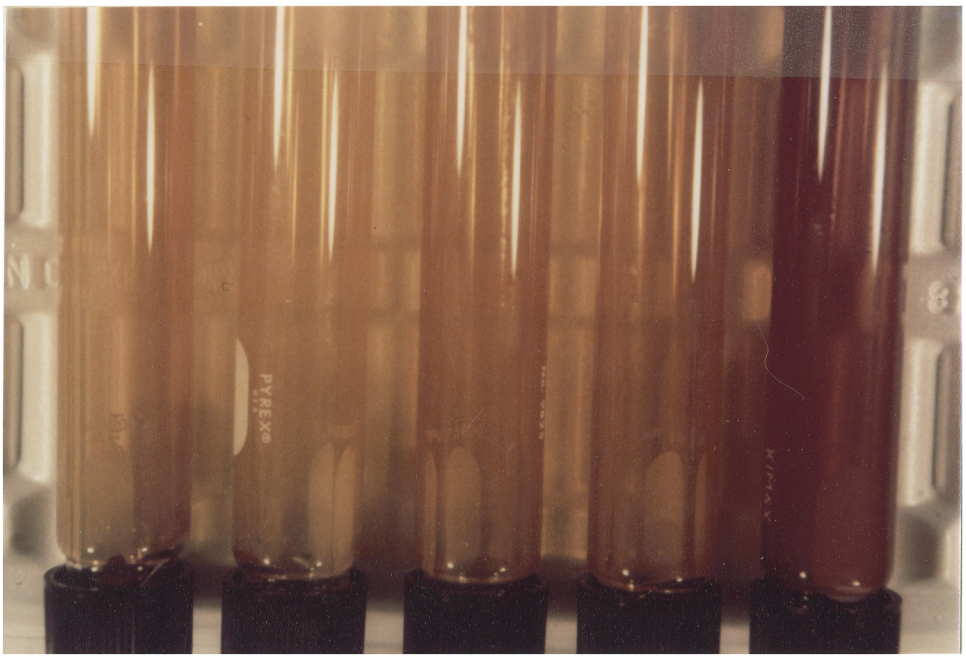


Figure 3. Cresyl violet acetate in the Double-tube system. Reducing agents in the system are (l to r); Control, thioglycolate, L-cysteine·HCL, Dithiothreitol, L-cysteine·HCL + $Na_2S \cdot 9H_2O$.

Figure 4. Phenosafranine in the Double-tube system. Reducing agents in the system are (t to b); L-cysteine·HCL + $Na_2S \cdot 9H_2O$, Control, Dithiothreitol.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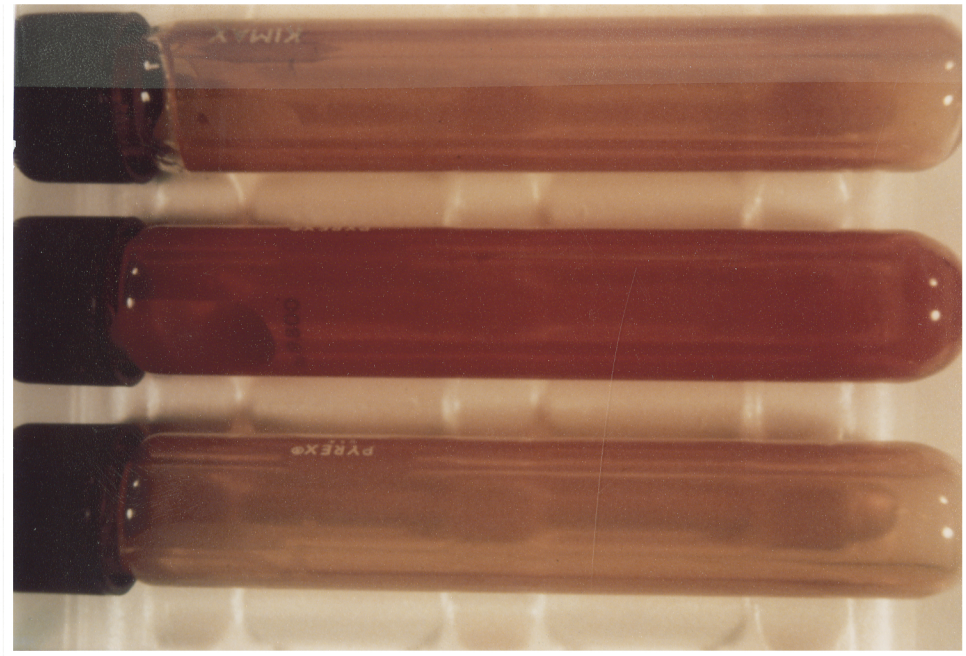
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Table 2

Comparison of counts of Clostridium sporogens obtained from four anaerobic systems.

Method	Mean	No. of observations
Double-tube	1.2×10^7 a	29
Lee tube	1.1×10^7 a	22
Forma anaerobic chamber	9.1×10^6 a	16
BBL GasPak	4.6×10^6 b	19

The same subscript denotes the sample means are not significantly different at the alpha = 0.05 level. Anaerobic agar (Difco) was used in all anaerobe systems.

-300 mV due to the marked decrease of phenosafranine color. Sodium thioglycolate and cysteine·HCL also produced an Eh´ of at least -227 mV in the double-tube since they discolorized cresyl violet.

III. Evaluation of the Double-tube System With Pure Cultures.

A. Clostridium sporogenes

Table 2 indicates that there was no statistically significant difference between the mean counts of Clostridium sporogenes from the double-tube compared with those from the Lee tube or Forma anaerobic chamber.

The counts obtained from the BBL GasPak system were found to be significantly less than those from the other three systems. This is probably due to the fact that the anaerobic jars did not function well during the course of this study, and anaerobiosis was not achieved in all GasPaks initially. Some jars even lost anaerobiosis during the incubation period allowing even some mold growth. This occurred with new BBL GasPaks, despite continual changing and heating of the catalyst.

Clostridium sporogenes is reported to be a moderate anaerobe (Fredette et al., 1967) with an established upper limit Eh´ for growth of +145 to +150 mV at pH 7 (Hanke and Katz, 1943; Hanke and Bailey, 1945). Therefore the double-tube was shown to be comparable to other methods in growing a moderate anaerobe.

B. Pseudomonas fragi

In order to determine if aerobic bacteria can grow in the double-tube system, an obligate aerobe, Pseudomonas fragi, was inoculated into the system. Except for a few colonies

on the top surface of the agar, no growth was observed after three days of incubation. Therefore aerobes apparently will not grow in this system.

C. Aerobic-anaerobic growth of isolates from the double-tube system.

Of 100 isolates obtained from the double-tubes used to cultivate samples of broiler drumsticks, all grew both aerobically and anaerobically. Thus there is no evidence of aerobic organisms growing in the double-tubes inoculated with the drumstick samples.

Isolates of Megasphaera elsdenii and Butyrivibrio fibrisolvens were found to all grow anaerobically, but none grew aerobically. This is as expected since both are obligate anaerobes. Therefore the double-tube system can be used to grow obligate anaerobes.

D. Comparative growth of five pure anaerobic cultures

Five pure cultures of anaerobes with three levels of oxygen sensitivity based on groups established by Loesche (1969) were studied. Group I with a maximum growth at oxygen partial pressures up to 20%, consisted of Clostridium perfringens and Streptococcus bovis. Clostridium perfringens is considered to have an oxygen sensitivity between groups I and II, with an upper Eh′ growth limit of +160 mV at pH 7 (Hanke and Bailey, 1945; Barnes and Ingram, 1956) Eubacterium limosum and Megasphaera elsdenii were considered as group II, with maximum growth at oxygen partial pressure (pO_2) less than 3%. Eubacterium limosum was placed into this group because of the observed sensitivity of the test strain, and a similar organism,

Bacteriodes fragilis (Eggerth and Gagnon, 1945), was placed into this group by Loesche (1969). Megasphaera elsdenii was determined by Loesche (1969) to be in the range of group II sensitivity. Butyrivibrio fibrisolvens was considered to be in group III sensitivity range, with a maximum growth at pO_2 less than 0.5% (Loesche, 1969).

With cultures of different oxygen sensitivities the anaerobiosis of the double-tube was compared with the Hungate roll tube and the Forma anaerobic chamber. Table 3 shows that no statistically significant differences existed between the mean counts of all three systems on growing any of the five anaerobes ($P = .67$). The difference of means and variances of the two replications of each organism ($P = .0001$) was taken into account in this statistical evaluation, as shown in the General Linear Model (GLM) in table 3. In addition, no significant difference was found between the variances of counts from the three systems for any of the cultures ($P = .08$). Again the difference of variance between the two replications ($P = .026$) was accounted for in this statistical analysis.

This comparison demonstrates that the double-tube is capable of allowing the growth of test organisms from all three groups of O_2 sensitivity and is comparable to the Hungate roll tube and the Forma anaerobic chamber. As reported by Loesche (1969) B. fibrisolvens did not grow well with a pO_2 more than 0.3%, and M. elsdenii with a pO_2 more than 1.0%. These organisms grew well in the double-tube system, which further demonstrated the capability of the system.

Table 3

Comparison of means from five pure cultures of anaerobic bacteria obtained from three anaerobic systems.

Bacteria	Anaerobic System		
	Double-tube	Roll tube	Forma chamber
Clostridium perfringens	2.0×10^7	2.2×10^7	2.3×10^7
Streptococcus bovis	6.6×10^7	7.5×10^7	7.1×10^7
Eubacterium limosum	2.1×10^7	2.4×10^7	1.5×10^7
Megasphaera elsdenii	2.2×10^4	2.2×10^4	1.8×10^4
Butyrivibrio fibrisolvens	9.8×10^7	8.9×10^7	1.0×10^8

No statistically significant difference was observed ($P > 0.05$) by use of the General Linear Model procedure.

General Linear Models Procedure

Source	DF	Type IV S.S.	F value	P value
REP	1	7515.8798	51.38	0.0001
TRT	4	451579.1317	770.08	0.0001
TRT*REP	4	74271.7063	126.66	0.0001
Method	2	118.7923	0.41	0.6672
Method (REP*TRT)	18	6669.5749	2.53	0.0007

Table 3
(con't)

REP	1	0.39943	5.90	0.0259
TRT	4	114.44105	422.5	0.0001
TRT*REP	4	1.31283	4.85	0.0079
Method	2	0.38603	2.85	0.0841

General Linear Model used to eliminate replication error for comparisons of mean and variance. REP = replication, TRT = treatment (bacteria type).

IV. Practical Application of the Double-tube System.

A. Comparison of anaerobic counts obtained from ground beef.

The double-tube system was tested against the Lee tube and BBL GasPak system in obtaining anaerobic counts from ground beef. The results and statistical comparisons of this study are recorded in table 4. There was no significant difference between counts obtained from all three methods.

B. Comparisons of counts obtained from atmosphere modified packaged broiler drumsticks.

For a further demonstration of the capability of the double-tube in cultivating anaerobes from foods, broiler drumsticks, stored in atmosphere modified packages, were sampled. Modified storage atmospheres can induce the growth of a wide variety of anaerobes and facultatives such as Lactobacillus plantarum and Clostridium in food stores under CO_2 conditions, and Aeromanas hydrophilia in foods stored under N_2 conditions (Enfors and Molin, 1978; Enfors et al., 1979).

Tables 5-7 show there was no significant difference between the means and statistical regression lines of anaerobic counts of the drumsticks obtained from the double-tube and Forma anaerobic chamber system over a period of 11-12 days. This indicates that the double-tube system is as effective as the Forma anaerobic chamber in enumerating anaerobes from drumsticks under these experimental conditions.

C. Use of the double-tube in monitoring anaerobic growth of vacuum-packaged ground beef.

Because the double-tube proved highly favorable for

Table 4

Comparison of counts of ground beef obtained from three anaerobic systems.

Method	Mean	No. of observations
Lee tube	4.2×10^3	30
Double-tube	3.9×10^3	30
BBL GasPak	3.9×10^3	30

Sample means were not significantly different ($P > 0.05$).

BHI - supplemented medium was used in all anaerobe methods.

Table 5. Anaerobic counts of vacuum-packaged broiler drumsticks.

Anaerobic method	Sample	Anaerobic counts (CFU/cm^2) after storage					
		0 days	2 days	4 days	7 days	9 days	11 days
Double-tube system	1)	$3.3x10^6$	$1.6x10^7$	$3.2x10^7$	$1.9x10^8$	$1.1x10^7$	$1.2x10^8$
	2)	$9.6x10^1$	$1.9x10^2$	$1.7x10^4$	$5.6x10^4$	$9.6x10^4$	$1.1x10^4$
Forma anaerobic chamber system	1)	$2.5x10^6$	$1.3x10^7$	$2.2x10^7$	$6.5x10^7$	$3.5x10^7$	$2.0x10^8$
	2)	$1.2x10^2$	$2.1x10^2$	$2.5x10^4$	$1.2x10^5$	$1.1x10^5$	$1.1x10^5$

All means are an average of 6 replications of tubes or plates per sample stored at 4-5°C. Analysis of sample means and computed regression lines was done by General Linear Model procedure. No significant difference was found between sample means ($P = 0.07$).

Table 6. Anaerobic counts of CO_2 flushed broiler drumsticks.

		Anaerobic counts (CFU/cm^2) after storage					
		0 days	2 days	4 days	7 days	9 days	11 days
Anaerobic method	Sample						
Double-tube system	1)	$1.0x10^4$	$2.8x10^5$	$3.3x10^5$	$2.0x10^5$	$2.2x10^6$	$3.0x10^7$
	2)	$1.2x10^3$	$8.3x10^3$	$1.1x10^4$	$3.2x10^4$	$6.7x10^3$	$2.6x10^5$
Forma anaerobic chamber system	1)	$1.4x10^4$	$3.3x10^5$	$3.7x10^5$	$2.8x10^5$	$7.8x10^5$	$3.0x10^7$
	2)	$1.3x10^3$	$5.4x10^3$	$1.2x10^4$	$3.4x10^4$	$1.4x10^4$	$8.0x10^5$

All means are an average of 6 replications of tubes and plates per sample stored at $4-5^0C$. Comparative analysis of sample means and computed regression lines from each method was done by General Linear Model procedure. No significant difference was found between sample means ($P = 0.16$).

Table 7. Anaerobic counts of N_2 flushed broiler drumsticks.

		Anaerobic counts (CFU/cm^2) after storage					
		0 days	2 days	5 days	7 days	10 days	12 days
Anaerobic method	Sample						
Double-tube system	1)	$2.6x10^3$	$1.6x10^4$	$3.5x10^5$	$7.2x10^6$	$2.5x10^7$	$3.7x10^8$
	2)	$1.2x10^3$	$8.3x10^3$	$1.1x10^4$	$3.2x10^4$	$6.7x10^3$	$2.6x10^5$
Forma anaerobic chamber system	1)	$1.9x10^3$	$1.6x10^4$	$3.1x10^5$	$8.0x10^6$	$2.1x10^7$	$2.7x10^8$
	2)	$1.3x10^3$	$5.4x10^3$	$1.2x10^4$	$3.4x10^4$	$1.4x10^4$	$8.0x10^5$

All means are an average of 6 replications of tubes and plates per sample stored at 4-5°C. Analysis of sample means and computed regression lines was done by General Linear Model procedure. No significant difference was found between sample means ($P = 0.92$).

Table 8

Aerobic and anaerobic bacterial counts of vacuum-packaged ground beef.

Ground beef sample	CFU/g	
	SPC	Double-tube
Initial counts of varaible:		
1	2.4×10^4	1.3×10^4
2	2.2×10^4	7.1×10^3
3	3.8×10^2	5.4×10^2
4	1.1×10^3	1.1×10^3
Display day 0:		
1-A	3.4×10^5	3.0×10^5
1-B	1.5×10^5	4.5×10^4
2-A	9.5×10^5	4.4×10^3
2-B	1.6×10^2	2.4×10^3
3-A	3.5×10^3	1.6×10^3
3-B	2.4×10^2	3.2×10^2
4-A	6.4×10^2	5.4×10^3
4-B	3.6×10^3	3.4×10^2
Display day 7:		
1-A	5.6×10^5	4.8×10^6
1-B	4.0×10^6	2.0×10^6
2-A	5.4×10^4	2.4×10^4
2-B	8.4×10^4	1.1×10^5
3-A	1.7×10^5	1.3×10^5
3-B	3.2×10^5	2.7×10^5
4-A	5.4×10^5	4.2×10^5
4-B	4.0×10^5	9.4×10^5

Table 8
(con't)

Display day 14:		
1-A	$5.7x10^6$	$3.2x10^8$
1-B	$8.2x10^6$	$2.8x10^7$
2-A	$4.4x10^4$	$4.9x10^5$
2-B	$3.4x10^6$	$5.7x10^6$
3-A	$5.7x10^6$	$5.9x10^6$
3-B	$4.1x10^6$	$5.0x10^6$
4-A	$2.8x10^6$	$2.2x10^7$
4-B	$8.0x10^6$	$1.4x10^7$
Display day 21:		
1-A	$5.1x10^6$	$2.3x10^6$
1-B	$5.9x10^6$	$5.7x10^6$
2-A	$3.8x10^6$	$1.1x10^6$
2-B	$4.7x10^6$	$2.1x10^6$
3-A	$4.4x10^6$	$1.9x10^6$
3-B	$6.4x10^6$	$1.1x10^6$
4-A	$2.7x10^6$	$1.6x10^6$
4-B	$5.3x10^6$	$3.2x10^6$

Sample codes:

1- raw material from Comitrol flake cutting machine.

2- lean beef trim, 1/2" and 1/8" ground.

3- lean beef trim, 1/2" ground, vacuum-packaged, 1/8" ground.

4- lean beef trim, 1/2" ground, vacuum-packaged, 1/8" ground twice.

A- prepared under vacuum conditions.

B- prepared under normal atmospheric conditions.

growing food anaerobes it was used to monitor anaerobic growth in a study on vacuum-packaged ground beef. The results of this study are shown in table 8. The counts are those obtained from the double-tube and the standard plate count. Comparing the anaerobic counts from the double-tube to the aerobic counts, it appears that, regardless of mixing conditions and type of ground beef, the aerobic and anaerobic organisms grew at the same rate. This would indicate that, although the samples varied in counts from each other, the experimental conditions and samples did not promote dominance of either aerobic or anaerobic type organism.

D. Use of the double-tube in monitoring growth of anaerobic bacteria on vacuum-packaged and nonvacuum-packaged ground beef.

The double-tube was used to obtain anaerobic counts from vacuum-packaged and nonvacuum-packaged ground beef. The results shown in table 9 are counts obtained from the double-tube and standard plate count. These indicate that the aerobes grew better under a nonvacuum condition than under vacuum conditions. The vacuum conditions also had a more definite inhibition of all bacterial growth than did the nonvacuum conditions. No difference between the two mixing conditions of the samples was found.

V. Summary of Double-tube Performance.

The series of experiments indicated that the double-tube system is highly reliable in promoting growth of anaerobic and facultative pure cultures as well as those in food systems. The ability of the double-tube system to grow Butyrivibrio

Table 9

Aerobic and anaerobic bacterial counts of vacuum-packaged and non-vacuum packaged ground beef.

Ground beef sample	CFU/g	
	SPC	Double-tube
Preparation day:		
Initial count of variable 1	4.2×10^3	5.6×10^2
Initial count of variable 2	5.7×10^3	6.9×10^2
Display day 3 (PVC)		
1-A	8.8×10^4	6.1×10^3
1-B	8.4×10^4	1.2×10^4
2-A	1.0×10^5	5.0×10^3
2-B	1.1×10^5	6.0×10^3
Display day 5 (PVC)		
1-A	7.9×10^6	3.0×10^4
1-B	2.8×10^6	2.0×10^4
2-A	5.9×10^6	3.2×10^4
2-B	2.5×10^6	4.6×10^4
Display day 5 (VAC)		
1-A	3.8×10^5	4.2×10^4
1-B	4.2×10^5	3.6×10^4
2-A	2.3×10^5	4.4×10^4
2-B	5.4×10^5	4.6×10^4
Display day 14 (VAC)		
1-A	7.9×10^4	2.4×10^4
1-B	1.9×10^5	2.3×10^4
2-A	3.9×10^5	4.0×10^4
2-B	4.2×10^5	7.1×10^4

Table 9
(con't)

Sample code: VAC- vacuum packaged.

PVC- non-vacuum packaged.

1- mixed under vacuum conditions.

2- mixed under normal atmospheric conditions.

A- held at $2^{o}C$ prior to storage.

B- exposed to $10^{o}C$ for 4 hours.

fibrisolvens without using any gassing procedures to reduce the Eh´ of the media is unexpected since this organism is extremely sensitive to molecular oxygen. Thus the double-tube is capable of monitoring all anaerobes from common foods since anaerobes from food are less likely to be as stringent in anaerobic requirement as B. fibrisolvens. If the double-tube is used to grow CO_2 requiring anaerobes, such as those encountered in clinical bacteriology, formate, bicarbonate, or other such compounds can be added to the media. This is an area for future study.

This double-tube system, developed independently by Fung and Lee (1981), is somewhat similar to Marpmann's modification of the Esmarch roll cultures as described by Hall (1929) in using two tubes to achieve anaerobiosis. It differs from Marpmann's system in that the top is sealed by a screw cap on top of the inner tube with a lip, and the use of a reducing agent in the agar to achieve a low O-R potential. Marpmann used only heat to reduce the agar media, and a cotton or paraffin seal. The system also has not been reported as anaerobic. The double-tube maintains a proven reduced system (-227 mV to -300mV) and a proven anaerobic environment.

Advantages of the double-tube system are 1) it does not require gas flushing or incubating; 2) inexpensive; 3) does not require purchase of extra, or special equipment; 4) simple to use; 5) no dangers of explosions or fires; 6) allows for individual observation of the tubes during incubation; 7) anaerobiosis is not lost due to leaks (only a slight absorption occurs within 48 hours); 8) anaero-

biosis always occurs (the reducing mechanism will not fail); and 9) the removable inner tube allows isolates to be obtained, and makes the system easy to clean.

APPENDICES

Appendix I

Theoretical Concepts Attributing to Oxygen Sensitivity of Anaerobes.

A. Hydrogen peroxide.

The reason for the biological inhibition of molecular oxygen to anaerobic bacteria has been a subject of debate for almost a century. The first step in the solution came when Gottstein and Beijerincki, in 1893, discovered that many bacteria produce catalase (Hall, 1929). Lowenstein followed this observation in 1903 by showing that anaerobic bacteria did not produce catalase (Hall, 1929). The next step was the observation of Callow (1923), and McLeod and Gordon (1923a, 1923a) that this lack of catalase would allow the cell to be poisoned by peroxides. Hydrogen peroxides (H_2O_2) are formed by cells in a two-electron reduction step of oxygen (flavoproteins act as the catalyst) (Prins, 1977). Catalase and peroxidase (Heme-containing enzymes) remove peroxides (Prins, 1977). Since peroxides are known to be toxic to all living cells (Haugaard, 1968), the absence of catalase in anaerobes would explain their sensitivity to molecular oxygen. This became the basis of the hydrogen peroxide accumulation theory which, advocated by McLeod and Gordon (1923b, 1925b), was popular for many years.

B. Enzymes.

Novy (1925) opposed the H_2O_2 accumulation theory, and instead claimed that the differences of anaerobes and aerobes was due to their metabolic enzyme systems. This oppositional

viewpoint was further supported by the inability of studies to detect peroxide production by most anaerobes (McLeod and Gordon, 1925b; Whittenberry, 1964; Hall, 1929). Using Clostridium acetobutylicum it was found that NADH oxidase activity was greatly increased by the presence of oxygen (O'Brien and Morris, 1971). This group of enzymes convert NADH to NAD using oxygen as the hydrogen acceptor. NADH oxidase may therefore give the cell "reducing power" helping to eliminate free oxygen. Also the rate of NADH oxidation, at high amounts of oxygen, becomes greater than the rate which the bacteria can supply NADH. Thus the NADH metabolic functions are interrupted (Hentges and Maier, 1972).

Decker et al. (1970) studied the enzyme systems of various bacteria and concluded that aerobes depend on cytochrome oxidase in metabolism, and usually oxygenase for anabolism. Facultatives obtain energy by cytochrome oxidase (oxygen-dependent), and NADH regenerating enzymes (oxygen-independent), so that they can compensate for changes of anaerobic to aerobic by synthesizing new enzymes. And finally, anaerobes do not have oxygenase or cytochrome oxidase. This concept, though, does not offer an explanation for the observed variety of oxygen sensitivity found within bacteria (Fredette et al., 1967; Loesche, 1969). Decker et al. (1970) also did not account for the facultative bacteria, Lactobacillus sp., which do not possess a functional cytochrome oxidase system (McCord et al., 1971).

Recently a theory has grown in popularity which links the hydrogen peroxide accumulation theory with new knowledge

of the highly reactive superoxide (O_2^-). Bacteria produce superoxide by iron-sulfur proteins or low molecular weight electron carriers interacting with molecular oxygen (McCord et al., 1971).

Three types of superoxide dismutase have been isolated; dismutase containing copper-zinc, manganese, or iron (Hassan, 1980). E. coli, the most studied organism for the superoxide theory, contains the iron and manganese containing types (Hassan and Fridovich, 1979). The manganese containing enzyme has been found to be constitutive, and is even produced by the cell in anaerobic conditions (Hassan and Fridovich, 1979). The manganese containing enzyme can be induced, and is present only when the cell is growing aerobically. Some E. coli strains produce manganese dismutase that require the presence of only trace amounts of oxygen to induce their production (Hassan and Fridovich, 1979), and if manganese dismutase production is inhibited the E. coli cells dies when transferred from anaerobic to aerobic conditions (Hassan and Fridovich, 1977). The copper-zinc dismutase is generally found only in eukaryotic cells (Hassan, 1980).

Superoxide dismutase initiates reactions of superoxide ions to each other forming hydrogen peroxide. McCord et al. (1971) found that strict anaerobes lacked superoxide dismutase and, usually, catalase activity. Therefore, when exposed to molecular oxygen, the accumulation of O_2^- would indiscriminately react with cellular metabolites.

$$O_2 \xrightarrow[\text{proteins}]{\text{iron-sulfur}} O_2^- \xrightarrow[\text{dismutase}]{\text{Superoxide}}$$

oxygen superoxide

$$(O_2^- + O_2^- + 2H^+) \rightleftharpoons H_2O_2 + O_2$$

peroxide

$$\xrightarrow[\text{Peroxidase}]{\text{Catalase (or)}} H_2O + 1/2\ O_2$$

Further support of this theory came when it was shown that all aerobic bacteria that utilize cytochrome systems will produce both superoxide dismutase and catalase (Gregory and Fridovich, 1974). For the facultative Lactobacillus plantarum, no catalase production was found, but superoxide existed at a low, nontoxic level (McCord et al., 1971), and the H_2O_2 could all be eliminated by peroxidase as H_2O (Lehninger, 1975). The study results of L. plantarum may be found to apply to most facultatives that lack a functional cytochrome oxidase system. A correlation also has been found between the amount of superoxide dismutase present in an anaerobe and its sensitivity to oxygen (Tally, et al. 1977).

It is still not known though, if H_2O_2 and (or) O_2^- are present in all bacteria (Gordon et al., 1953; McCord et al., 1971). And some obligate anaerobes, such as Eubacterium limosum, produce superoxide dismutase (Hewitt and Morris, 1975; Smith, 1975; Morris, 1976), in relatively high amounts indicating that this theory must not cover the entire reason for anaerobic sensitivity.

C. Redox potential.

None of the above theories account for why different

obligate anaerobes have different oxygen sensitivities. Several other hypotheses have thus been purposed. The one most commonly referred to involves the redox potential (Eh′) of the media, indicating the reduction or oxidation of a system (see Appendix table A). The Eh′ is significant because in nature oxygen is mainly responsible for raising the O-R (redox) potential. Though high potentials can be caused by other oxidizing compounds in the laboratory, oxygen cannot be present in the media without raising the Eh′ (Hungate, 1969). The Eh′ value is obtained by direct measurement of the system with a potentiometer and platinum electrode, giving a value of volts (V) or millivolts (mV) (Hentges and Maier, 1972). The measured Eh′ can then be used to calculate the oxygen concentration of the system using the formula:

$$E_{(\text{oxygen})} = Eo_{(\text{oxygen})} + \frac{RT}{nF} \ln\left(\frac{\text{conc. of } O_2}{\text{conc. of reduced form of } O_2}\right)$$

Using this formula, the amount of oxygen present in one liter of water in one atmosphere pressure, at $30^{o}C$, is 1.48×10^{19} molecules (where 1 mole of oxygen contains 6.06×10^{23} molecules), and at an Eh′ of -330mV the oxygen concentration is 1.48×10^{-56} molecules/liter (Hungate, 1969).

This relationship between Eh′ and oxygen concentration then allows the measurement of the Eh′ of the cultivating media, and gain an insight as to its molecular oxygen concentration (pO_2). In addition, all anaerobes so far studied have been found to have an Eh above which they will not grow, at a given pH (Smith, 1975). Studies have also been done where

the Eh´ was not due to the oxygen concentration in the media, and a relationship of Eh´ and anaerobe growth was still found (Hentges and Maier, 1972). This would indicate that anaerobe growth is more than a function of the oxygen concentration, but must include the oxidation state of the cultivating environment.

D. Other anaerobic hypotheses.

Other hypotheses and conjectures for anaerobic sensitivity have also been developed. These include the oxidation of cofactors and enzymes containing sulfhydryl groups, and changes in the concentration of certain metabolites, caused by O_2, deranging important processes of metabolism (Prins, 1977) in addition to others (Morris and O'Brien, 1971). Again none of these ideas offer a complete explanation to anaerobiosis.

The reasons for anaerobic sensitivity to molecular oxygen are complex, and not well understood. A combination of several biological and biochemical factors is probably involved and still awaits elucidation. It is known that the oxidation state, as well as the amount of free oxygen, present in the environment are crucial factors effecting anaerobiosis.

Table A.

Redox potential abbreviations used, or related to those used, in these appendices (References: Jacob, 1970; Smith, 1975).

EH ... Standard hydrogen electrode potential (0 mV).

Eh´... Tendency of a solution to accept electrons; is pH dependent (usually measured at pH 7 at 30°C).

Eo ... Standard redox potential of a 50% reduced substance (based on standard hydrogen electrode).

Eo´... Standard redox potential of a 50% reduced substance at pH 7 (based on standard hydrogen electrode).

rH ... Negative log of the partial pressure of the gaseous hydrogen (pH_2).

Appendix II

Reducing Agents

Although the exact mechanisms leading to anaerobic behavior of anaerobes are not well understood, it is known that redox potential with respect to the presence of oxidizing agents (usually O_2) is critical. To lower the Eh´ to the point where anaerobes grow well, the oxidizing compounds present in the media must be reduced. Heat can be used to reduce them (removes (O_2), but to establish a low Eh´ (-150 mV or less) chemical reducing agents are added. Some common reducing agents are:

		Eo´ (mV) at pH 7
1)	Sodium thioglycolate	-100 or less (Costilow, 1981)
2)	L-cysteine·HCL	-210 (Cleland, 1964)
3)	Dithiothreitol	-330 (Cleland, 1964)
4)	H_2 + palladium chloride	-420 (Costilow, 1981)
5)	Titanium III·citrate	-480 (Costilow, 1981)
6)	Cysteine·HCL + $Na_2S \cdot 9H_2O$	-571 (Hungate, 1969)

The first three can be prepared in stock aqueous solutions (0.05%) placed under CO_2, and autoclaved. Palladium chloride with H_2 is made according to the method of Aranki and Freter, (1972). The fifth agent listed is made by adding 5 ml of a solution of titanium trichloride (15%) to 50 ml of 0.2M sodium citrate. This mixture is neutralized using saturated sodium bicarbonate, and sterilized by filtration. It is mixed with

media to a 0.5 - 2mM concentration, depending on what is desired (Zehnder and Wuhrmann, 1976). The last agent listed is made by adding 0.25g of $Na_2S \cdot 9H_2O$ to 10 ml of distilled water, adjusting to pH 10, and adding 0.25g of L-cysteine·HCL. Distilled water is then added to give a total volume of 20 ml. The solution is added to give a 0.05% total reducing agent concentration in the media (2 ml to 100 ml H_2).

Appendix III

Oxidation - Reduction Indicator Dyes

Some organic dyes change color under different oxidation-reduction (redox) states of a system, thus providing an indication of the redox potential (Eh′). These dyes generally exist as colored oxidized forms, and colorless reduced forms. The mechanism for the reduction of redox dyes by the cell involves the hydrogen-carrying dehydrogenases (now called Beta (acceptor) oxidoreductase). Some common indicators are:

	Eo′ (mV) at pH 7, 30°C
Methylene blue	+ 11
Indigo tetrasulphonate	- 46
Resazurin (resorufin)	- 51
Indigo trisulphonate	- 81
Indigo carmine	-125
Nile blue	-142
Indigo monosulphonate	-160
Cresyl violet	-167
Neutral blue	-192
1,5- Antraquinone sulphate	-200
Phenosafranine	-252
Benzyl viologens	-359

The values shown above are Eo′ which means that at this mV level the dye is 50% oxidized and 50% reduced (see table A). The potential range for the dye to be 99% oxidized to 99% reduced is 120 mV (2 electrons taken up), and 240 mV (4 electrons taken up). For example, indigo carmine with an Eo′ of -125

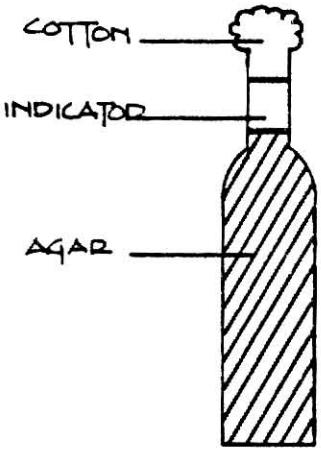
mV would be 99% oxidized at 065 mV, and 99% reduced at -185 mV.

Resazurin, a common indicating agent, functions differently from other indicating compounds because it undergoes an irreversible change to resorufin. Resorufin is actually the indicator dye with an Eo´ of -51 mV at pH 7. Benzyl viologens also functions differently than other indicators in that it is colorless when oxidized, and colored when reduced (Merick Index).

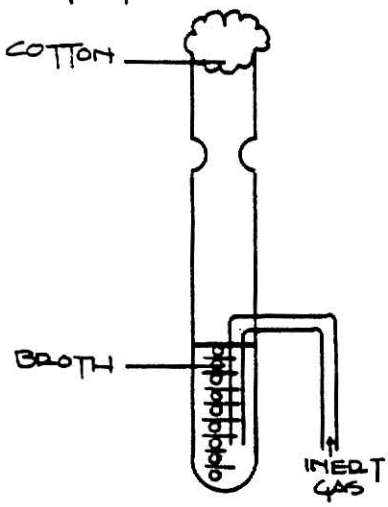
Reference: Jacob (1970).

APPENDIX IV

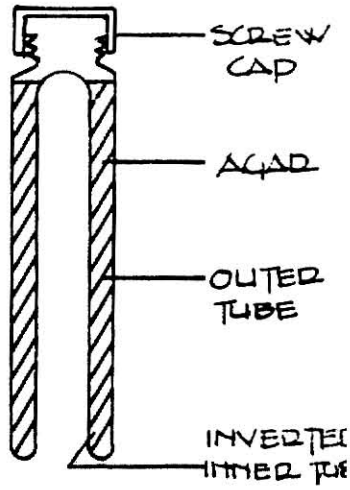
A. ANAEROBIC SYSTEMS — TUBES AND DISHES



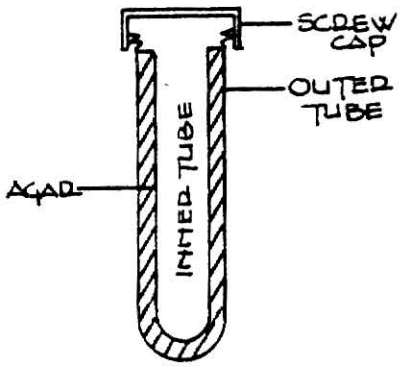
PRICKETT TUBE
(MILLER et. al., 1939)



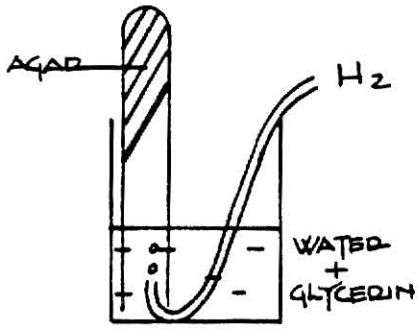
HAUSER AND LIBORIUS APPARATUS
(HUNZIKER, 1902)



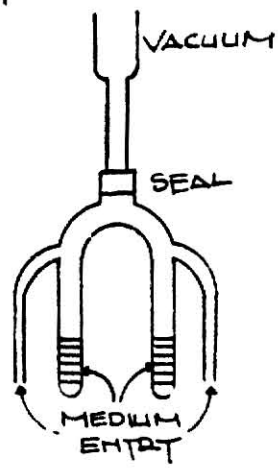
LEE TUBE
(OGY et al. 1979)



DOUBLE TUBE
(FUNG AND LEE, 1981)



BLUCHER'S APPARATUS
(HUNZIKER, 1902)



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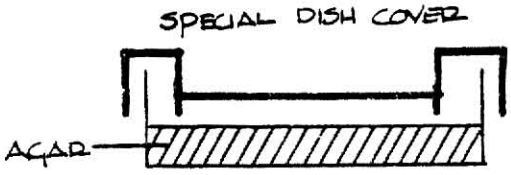
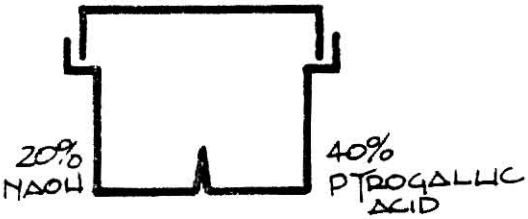
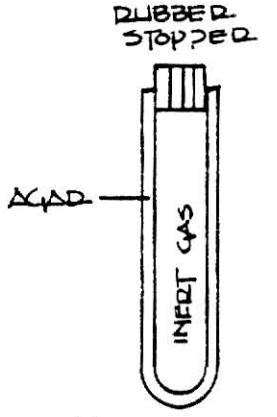
(HUNZIKER, 1902)



BREWER'S ANAEROBIC PETRI DISH
(BREWER, 1942)



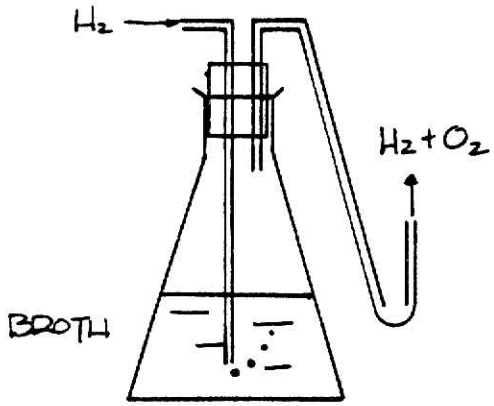
SPRAT CULTURE DISH
(SPRAT, 1930)



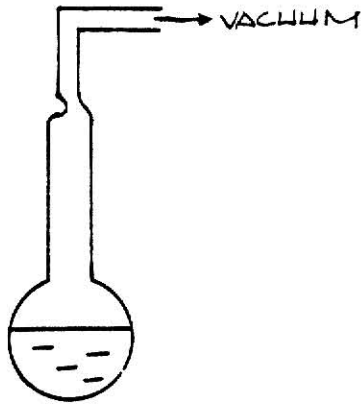
HUNGATE ROLL TUBE
(HUNGATE, 1969)

APPENDIX IV.

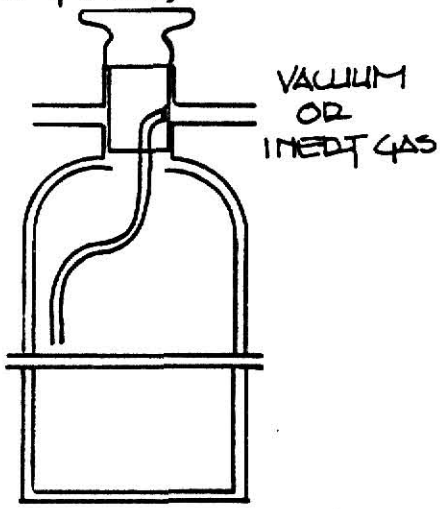
B. ANAEROBIC SYSTEMS — FLASKES & JARS

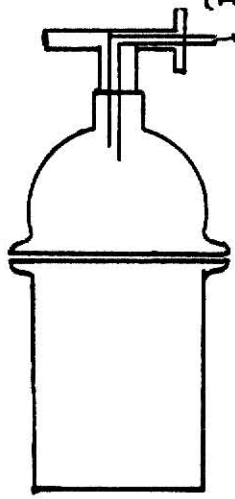


HUEPPE'S METHODS
(HUNZIKER, 1902)

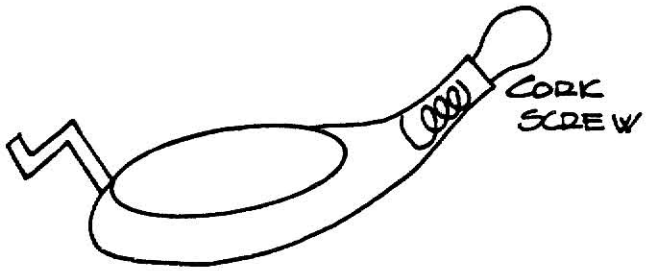


PASTEUR'S VACUUM FLASK
(HUNZIKER, 1902)

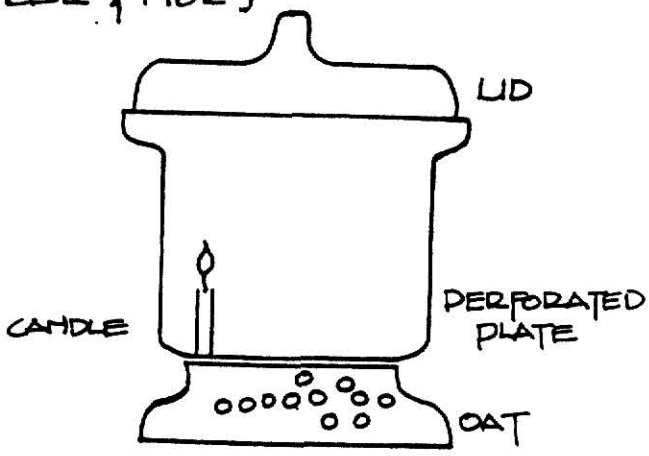




NOVY'S ANAEROBIC JARS
(HUNZIKER, 1902)



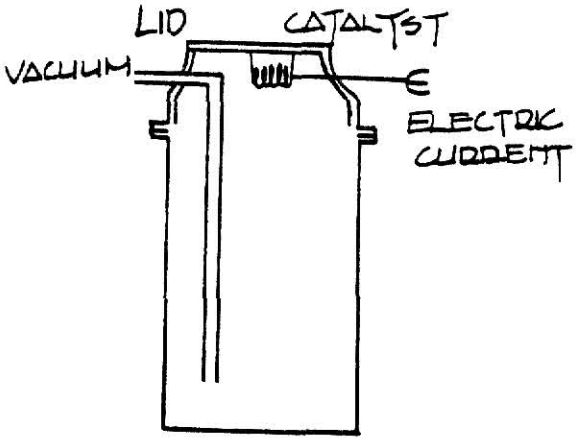
ROTH'S ANAEROBIC PLATE
(HUNZIKER, 1902)



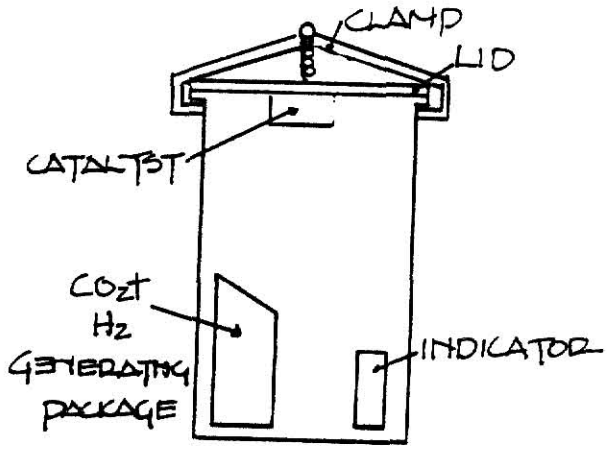
CANDLE OAT JAR
(VEDAMUTHU AND REINBOLD, 1967

APPENDIX IV

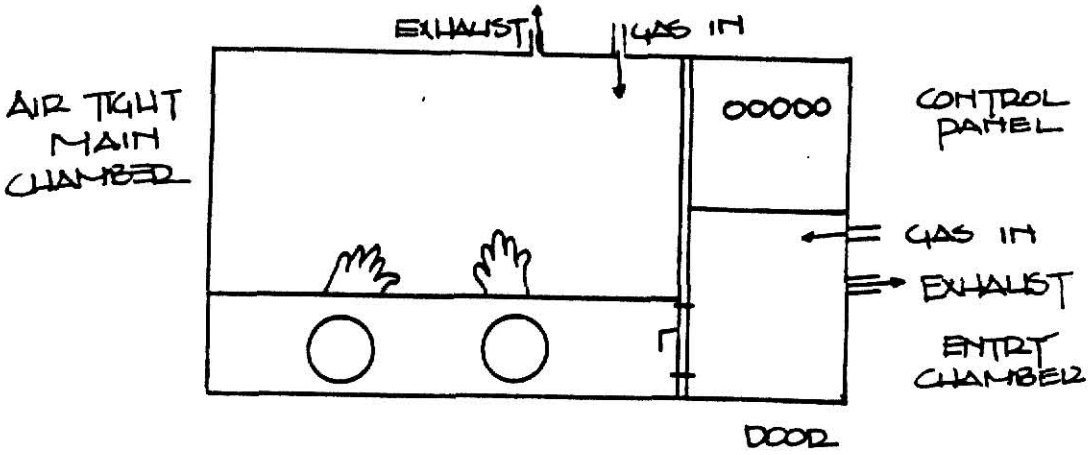
C. ANAEROBIC UNITS AND INSTRUMENTS



EVANS MODIFICATIONS OF BREWER'S ANAEROBIC JAR
(EVANS et. al., 1948)



BREWERS SELF CONTAIN ANAEROBIC JAR
(BREWER AND ALLGEIER, 1966



SIMPLIFIED ANAEROBIC GLOVE BOX
(LEACH et al., 1971)

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DEVELOPMENT OF THE DOUBLE-TUBE SYSTEM FOR THE CULTIVATION OF ANAEROBIC BACTERIA FROM FOODS

by

KEVIN LEE ANDERSON

B.S. Kansas State University, 1980

B.S. Kansas State University, 1980

AN ABSTRACT FOR A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

IN

FOOD SCIENCE

Department of Animal Sciences and Industry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1982

ABSTRACT

The Prickett tube method and plate count method using a BBL GasPak system are two common methods for monitoring anaerobic bacteria from foods (Speck, 1976). For strict anaerobes the procedures established by VPI Anaerobe Laboratory are generally used (Holdeman et al., 1977). Recently a preliminary double-tube anaerobic system for food bacteriology was reported by Fung and Lee (1981). The purpose of this investigation is to make a detailed study concerning the efficacy of this simple system for cultivation of strict anaerobes when compared with established anaerobic systems. Also this system is used to monitor anaerobic bacteria of foods using an established system as a reference.

The double-tube system is composed of a test tube (140 mm x 16 mm O. D.) with a lip inserted into a screw cap test tube (150 mm x 25 mm O.D.). The larger tube contains sterile melted anaerobic agar (25 ml) using L-cysteine·HCL as a reducing agent along with an inoculum of culture or food sample to be enumerated. The aseptic insertion of the smaller sterile inner tube forces the inoculated agar into a thin film between the walls of the two tubes. Anaerobiosis occurs due to the binding of molecular oxygen into the agar from the head space. The Eh´ of this system was determined colorimetrically, by a variety of oxidation-reduction indicators, to be about -227 mV to -300 mV depending on the reducing agent used in this system.

Comparative counts from the double-tube, Hungate's roll

tube, and the Forma anaerobic chamber showed that there was no statistically significant differences between these methods in enumerating strict obligate anaerobes such as Butyrivibrio fibrisolvens and Megasphaera elsdenii as well as Clostridium perfringens and Eubacterium limosum.

The double-tube system was found to be comparable with the BBL GasPak system in anaerobic viable count of ground beef ($P > 0.05$). It was also highly comparable with the Forma anaerobic chamber in counts obtained from vacuum-packaged broiler drumsticks ($P = 0.07$), CO_2 flushed packaged drumsticks ($P = 0.16$), and N_2 flushed packaged drumsticks ($P = 0.92$).

The double-tube method not only provided highly comparable counts of anaerobes to other established anaerobic methods, but it has the advantages of being simple to use, easy to clean, inexpensive for routine analysis, and when the inner tube is removed isolates of anaerobes can be recovered for further studies.