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Methodology Development for the Study of the Anaerobic Bacterial Flora of Bovine Feces

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METHODOLOGY DEVELOPMENT FOR THE STUDY OF THE ANAEROBIC BACTERIAL FLORA OF BOVINE FECES

BY

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METHODOLOGY DEVELOPMENT FOR THE STUDY

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OF BOVINE FECES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Dr. Paul R. Middaugh, Thesis Adviser — Date

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INTRODUCTION

The intestinal bacterial flora of an animal has a considerable influence on the physiology of that animal. Some bacteria of the intestine are pathogenic and can initiate infections if they gain access to a susceptible individual. Some of the intestinal bacteria can produce potent carcinogens *in vitro* and can presumably do this also *in vivo*. Food passing through the alimentary tract of an animal is also chemically modified through the enzymatic action of the bacterial flora. The animal is also protected from some enteric infections through the antagonistic and competitive actions of the established organisms of the normal bacterial flora.

Most of the normal alimentary tract flora studies have been conducted with the human. The bovine rumen has also been a popular subject for bacterial flora studies. There have been, however, few studies made of the normal bacterial flora of the intestine of animals, especially the bovine. The studies that do exist are studies of the facultatively anaerobic bacteria of the flora or are studies of the anaerobic bacteria of the flora in which inferior methods of identification and/or anaerobiosis production were used.

Recent studies of the feasibility of refeeding animal wastes to utilize nutrients remaining in the wastes have not incorporated a sterilization step for the feces. The omission of a sterilization step is more energy-efficient than is the incorporation of such a

step. Unfortunately, little is known about the fecal flora of cattle and such refeeding plans may be beset with considerable difficulties. The organisms may be able to survive oxygen exposure well enough to be viable when the animal consumes the feces. If the organisms are pathogenic, they may be able to cause disease by entering the bloodstream through lacerations in the alimentary tract walls or by initiating an enteric infection. If the organisms are not pathogenic, they may still be able to cause problems by upsetting the normal rumen fermentation. Since such a small amount of data concerning the bovine fecal flora exists, the risk posed by refeeding bovine wastes can not be evaluated with any degree of accuracy. The purpose of this study is to investigate the normal bovine fecal flora and to evaluate possible hazards of refeeding animal wastes.

The objectives of this study are to develop media and methods for quantitatively cultivating the predominant organisms of the bovine bacterial fecal flora, to enumerate and identify the predominant bacteria of the bovine fecal flora, and to determine the die-off rate of the predominant bacteria of the flora when the organisms are exposed to air.

LITERATURE REVIEW

The first 3 steps in the study of the anaerobic bacterial flora of any sample are the selection of an appropriate method of producing anaerobiosis, the selection of an appropriate primary isolation medium, and the selection of a proper maintenance medium. The selection of the methodology for biochemical testing of the isolates and the selection of the experimental design, to allow enumeration of the various bacteria of the normal flora, are later steps to be undertaken after the first 3 steps.

The method used to produce anaerobic conditions is especially important in studies of the anaerobic bacterial flora of feces. The method used must produce anaerobic conditions good enough to allow the growth of the most oxygen sensitive bacteria present in the flora. The requirements for anaerobic conditions are most strict when the bacteria have been freshly isolated from the sample. The method used for initial isolation, therefore, must produce anaerobic conditions at least as good as methods used in subsequent work with the cultures. Unnecessary exposure of the cultures to atmospheric oxygen should be avoided at any time.

The nonselective primary isolation medium must be able to support the growth of as many of the bacterial species present in the sample as possible. In addition, it should be able to maintain the viability of each bacterial species for as long a period of time as possible to facilitate prolonged incubation of the

primary isolation cultures to allow the production of colonies by the more slowly growing organisms.

The maintenance medium must support the growth of as many members of the bacterial flora as possible. In addition, it must be able to support the growth of the organisms to the extent that they can be maintained indefinitely by serial subculture. It is also desirable that the organisms be able to be stored for as long a period of time as possible in the maintenance medium.

Methodology

Many different methods of achieving anaerobiosis have been used in the isolation, culture, and characterization of anaerobic bacteria. Each method has its own advantages and disadvantages.

Stoppered-tube methods. The stoppered-tube method makes use of culture tubes containing a prereduced anaerobically sterilized growth medium and a reducing atmosphere, sealed by rubber stoppers of various types. Two versions of the stoppered-tube method are in use at this time. The open-tube version was developed by Hungate (1950), improved by Moore (1966), and further improved by Holdeman and Moore (1972). Growth medium is reduced by boiling and adding chemical reducing agents, after which it is protected from oxygen exposure by a stream of oxygen-free gas. A thick-walled glass culture tube with a tapered neck is flushed with a stream of oxygen-free gas, during which the medium is added to the tube. After the medium is added and most of the air is flushed out of

the tube, the stopper is tightly seated into the tapered neck of the tube and is held in place only by friction. After the medium has been dispensed into the tubes, a metal clamp is used to hold the stoppers in the tubes during autoclaving. After autoclaving, the tube clamp is removed and the tubes are then ready for use. Alternatively, the prereduced medium may be autoclaved in a stoppered flask and then aseptically added to sterile tubes flushed with oxygen-free gas (Bryant, 1972). Cultural manipulations are performed with each culture tube under a stream of sterile oxygen-free gas. Inoculum is transferred from tube to tube using either an inoculating loop or a Pasteur pipette (Holdeman and Moore, 1975).

In the closed-tube method the medium, prereduced by boiling and adding chemical reducing agents, is dispensed into culture tubes as with the open-tube method. The culture tubes used are standard screw-capped culture tubes sealed with small butyl rubber septa. The septa are held in place in the ends of the culture tubes by standard screw caps, each cap having a hole drilled through its center. No clamp is needed to hold the septa in place during autoclaving because the screw cap performs this function. The hole in the screw cap allows inoculum to be passed through the rubber septum without opening the tube. Broth cultures may be transferred using glass syringes with small-gauge needles to transfer the inoculum through the rubber septa. Colonial growth from solid medium may be transferred by opening the tube and using a

modified Pasteur pipette to take up part of the colony while a stream of oxygen-free gas excludes air from the tube. An alternative method of transferring colonial growth makes use of a glass syringe with a bent, small-gauge needle to take up part of the colony. The needle is forced through the rubber septum without opening the tube, both speeding-up the process of subculturing colonies and preventing oxygen exposure of the culture (Macy et al., 1972).

Miller and Wolin (1974) modified the closed-tube method by using serum vials instead of the screw-capped tubes. Butyl rubber septa are used with the serum vials so as to reduce the rate of oxygen contamination of the growth medium. The septa are held in place using the standard metal caps used for serum vials. Medium preparation procedures are the same as those used by Holdeman and Moore (1975) and inoculation procedures are the same as those described by Macy et al. (1972).

Both the open-tube and closed-tube methods are reasonably rapid, but the closed-tube method reduces exposure of the cultures to oxygen to a greater extent. Although the use of Pasteur pipettes in conjunction with the open-tube method reduces oxygen exposure considerably, there is still too much exposure to oxygen to allow the growth of extremely oxygen-sensitive anaerobic bacteria such as the methanogenic bacteria. The use of a glass syringe along with the use of the closed-tube method reduces oxygen exposure to the extent that even the very oxygen-sensitive

bacteria can be cultivated (Macy et al., 1972) including the methane-producing bacteria from human feces (Nottingham and Hungate, 1968).

Anaerobic chamber methods. There are various methods of cultivating anaerobic bacteria which make use of an anaerobic chamber. This chamber may be constructed with either rigid or flexible walls. Stainless steel, other metals, thick acrylic plastic, and fiberglass have been used to construct rigid anaerobic chambers while thin vinyl plastic film is generally used in the construction of flexible anaerobic chambers (Aranki and Freter, 1972).

Rigid chambers have the disadvantages of being extremely expensive and difficult to flush air out of when initially set up. They do, however, have the advantage that the are relatively impermeable to oxygen, nitrogen, carbon dioxide, and hydrogen so the atmosphere within the rigid anaerobic chamber is more stable than that within a flexible chamber. Flexible anaerobic chambers have the disadvantages that they are more susceptible to mechanical damage than the rigid chambers and they are somewhat permeable to oxygen and chamber gases (Aranki and Freter, 1972), although the degree of permeability depends upon the type and thickness of the plastic film used in construction of the flexible chamber. The flexible chamber does have the advantages that it is generally less expensive than the rigid chamber and it is much easier to flush air out of a flexible chamber when it is initially set up

than it is to do the same with a rigid chamber (Aranki and Freter, 1972).

Cultural manipulations are performed using rubber gloves (Aranki and Freter, 1972) which should be of low gas permeability to minimize loss of the chamber atmosphere and contamination of it by atmospheric oxygen entering the chamber through the gloves. Short gloves are generally used with flexible anaerobic chambers. These are taped to rigid cuffs which are attached to sleeves made from the chamber material and continuous with the chamber walls. The alternative is the use of arm-length gloves directly attached to the chamber walls. This system is generally used with rigid-wall anaerobic chambers.

Materials are passed into and out of an anaerobic chamber through an airlock of some design. The usual airlock system consists of a chamber, generally cylindrical, attached to one end of the anaerobic chamber. Each end of the airlock is fitted with a door having an airtight seal and the airlock chamber itself is strong enough to allow partial evacuation without collapse (Aranki and Freter, 1972). A gas fitting and valve are necessary to allow evacuation of the airlock and replacement of the atmosphere with an anaerobic atmosphere. A vacuum-pressure gauge or manometer is also helpful to monitor the interior pressure of the airlock to prevent boiling of liquid media or collapse of the airlock due to excessive evacuation. When articles are to be brought into the chamber they are placed in the airlock, the airlock is repeatedly

evacuated and filled with the chamber atmosphere, and then the articles are brought into the chamber. When articles are to be removed from the chamber they are placed in the airlock, the inner door is closed, and the articles are taken out of the airlock.

If the atmosphere in the anaerobic chamber contains a high percentage of carbon dioxide, a simpler type of airlock may be used. A sliding door on the top of the anaerobic chamber is opened and articles are added to or removed from the chamber directly. The sliding door minimizes mixing of air with the chamber atmosphere when the door is opened. The relatively high density of carbon dioxide allows this type of airlock to operate (Leach et al., 1971).

An oxygen-free atmosphere is produced in the anaerobic chamber by either constantly flushing it with oxygen-free gas or by constantly recirculating the chamber atmosphere through an oxygen-removal apparatus. When the chamber is constantly flushed with oxygen-free gas, the oxygen-free gas is produced by either using an oxygen-removal device to remove traces of oxygen from gas obtained from tanks of compressed gas or by combusting a fuel in the presence of oxygen. The latter method is much more economical than the method using tanks of compressed gas but questions have been raised about the possible toxic effects of small amounts of carbon monoxide and various oxides of nitrogen, produced in the combustion of fuels, on anaerobic bacterial cultures (Koopman et al., 1973). When the chamber atmosphere is recirculated to

achieve anaerobiosis, a small amount of hydrogen is included in the atmosphere to act as the reducing agent. The atmosphere is blown through a bed of palladium catalyst pellets which catalyzes the reduction, by hydrogen, of any oxygen present to water. The production of anaerobiosis by the recirculation of the atmosphere is a more economical process than is the process of flushing the chamber constantly with oxygen-free gas because in the former process gas is added only when it is necessary to replace that lost in the operation of the airlock or lost by permeation through the gloves or chamber walls (Aranki and Freter, 1972).

Media to be used in an anaerobic chamber are reduced prior to use by placing the freshly-prepared media in the reducing atmosphere of the chamber until they become reduced. This reduction process is accelerated by adding various chemical reducing agents to the media. In addition, palladium chloride may be used in an overlay of agar plates. The palladium chloride used in this manner operates in the same manner as the palladium in the catalyst pellets used for the oxygen-removal apparatus. This overlay has been found to be necessary to allow the growth of surface colonies of extreme anaerobes (Aranki and Freter, 1972).

Since only trace amounts of oxygen are present in a properly functioning anaerobic chamber, it is not possible to use a flame to sterilize inoculating loops and the lips of culture tubes. Instead, a low-voltage power supply is used to heat a nichrome heating element which is used to sterilize the lips of culture

tubes. Inoculating loops are sterilized by using the wire portion to create a short circuit across the output of the low-voltage power supply. The wire then becomes a heating element itself and is sterilized (Aranki et al., 1969).

The major advantages of the use of an anaerobic chamber are that standard bacteriological glassware and techniques may be used. Stoppered culture tubes are not needed and, indeed, defeat the purpose of using an anaerobic chamber. Instead, standard straight-bore culture tubes with cotton stoppers, metal caps, or plastic caps are used for broth and slant cultures. Standard glass petri dishes are used for agar streak cultures. Operations are conducted within the anaerobic chamber, with the result being that the bacteria are not exposed to a high concentration of oxygen at any time during cultural manipulations. The cultures are also incubated within the chamber, with the result that they are not exposed to high oxygen concentrations at any time during their growth.

The major disadvantages of the use of an anaerobic chamber are that the gloves are clumsy to use, the humidity within the chamber causes difficulties, and the bacterial cultures may be exposed to slightly more oxygen during incubation than are cultures cultivated using the stoppered-tube method. The dexterity problems accompanying the use of rubber gloves may be practically overcome by experience in the use of the gloves. Also, gloves which fit more tightly cause fewer problems during cultural manip-

ulations than do gloves which fit loosely. Tight-fitting gloves, on the other hand, tend to be difficult to remove from the hands because the gloved hands perspire profusely in the warm anaerobic chamber. Talc sprinkled into the gloves makes the gloves somewhat easier to remove, but does not completely eliminate the problem. Thinner gloves may be used to increase manual dexterity at the expense of increased gas permeability. Thinner gloves allow both the inward diffusion of atmospheric oxygen and the outward diffusion of the chamber atmosphere. As a result, thin gloves tend to increase the oxygen concentration inside the chamber and increase the amount of gas needed to operate the chamber.

Water vapor within the chamber causes several problems in anaerobic chamber operations. The palladium catalyst pellets used in the oxygen-removal apparatus are readily inactivated in the presence of moisture. As a result, the pellets must be regenerated more frequently when the chamber atmosphere is saturated with water vapor than when the chamber atmosphere is anhydrous. When the chamber atmosphere is saturated with water vapor, moisture tends to condense on agar surfaces making it difficult to achieve isolation in agar streak cultures. In addition, the agar streak cultures often become overgrown by highly motile facultative or obligately anaerobic bacteria. Moisture also tends to condense on glass surfaces, with the result that contaminating organisms are often able to enter culture vessels.

Humidity within anaerobic chambers is generally controlled by

exposing pans of drying agent to the chamber atmosphere (Aranki and Freter, 1972). This drying agent must be replaced or regenerated at intervals which are dependent upon the amount of water vapor in the chamber. Since the drying agent is able to handle a limited amount of water vapor and ties up the water vapor relatively slowly, it is important to reduce the amount of water vapor entering the chamber atmosphere as much as possible.

Any oxygen entering the chamber is reduced to water vapor by hydrogen in the chamber atmosphere through the action of the palladium catalyst. As a result, a reduction of oxygen contamination of the chamber atmosphere by the use of gloves and chamber walls of lower gas permeability will reduce moisture levels in the chamber.

A considerable amount of moisture in the chamber is the result of evaporation of water from media within the chamber. This may be minimized by storing only needed media within the chamber and by storing the media in sealed plastic bags.

The anaerobic chamber method gives quantitatively similar results in studies of clinical isolates and fecal flora as does the stoppered-tube method. Aranki et al. (1969) found that when using a palladium black overlay, the recovery percentage of the mouse cecal flora was at least as great using the anaerobic chamber method as it was using the open-tube method of Hungate (1950). Dowell (1972) found that the anaerobic chamber method and the open-tube method of Hungate (1950) were approximately equal in effectiveness

when used for the isolation of anaerobes from clinical specimens and stated that the open-tube method is very good for quantitative studies of intestinal contents. In their studies of human fecal flora, Moore and Holdeman (1974) obtained an average viable count of 4.75×10^{11} bacteria per gram dry weight of fecal material using the open-tube method and Attebery et al. (1972) obtained an average viable count of 2.27×10^{11} bacteria per gram dry weight of fecal material using the anaerobic chamber method. This tends to indicate that the open-tube method is superior to the anaerobic chamber method with respect to the recovery of intestinal bacteria.

It is difficult to interpret the results of these comparison studies. Measurements of the oxygen tension in anaerobic chambers used by various workers reveals that the oxygen removal efficiency of these chambers varies widely. As a result, a comparison between the performance of a particular anaerobic chamber and that of the open-tube method may not be an indication of the relative performance of the two methods in general. In addition, different media are often used with the two methods. This adds another variable and the difference in media used may account for the performance differences rather than some inherent advantage of one method or the other. Finally, the bacteria vary with respect to their sensitivity to oxygen. If bacteria having a low sensitivity to oxygen are used in a study of this type, a method which results in less oxygen exposure for the bacteria or a higher degree of anaerobiosis achieved will not appear to perform

significantly better than an inferior method.

Anaerobic jar methods. Anaerobic jars provide another means of incubating bacterial cultures anaerobically. The jars themselves are usually of stainless steel, glass, or rigid plastic construction. The jar must be able to be sealed gas tight and must be able to withstand partial evacuation without breaking, collapsing, or losing the gas-tight seal.

Oxygen is eliminated from the atmosphere in the jar by a catalyst which catalyzes the reduction of oxygen, by hydrogen, to water (Dowell and Hawkins, 1974). An electrically-heated platinum-asbestos catalyst is used in the Brewer jar (Brewer, 1939). The possibility of an electrical malfunction causing sparking in the presence of both hydrogen and oxygen, as when the heating element is first activated in the operation of the jar, makes the Brewer jar a dangerous means of achieving anaerobiosis. A palladium-alumina room-temperature catalyst (Sutter et al., 1975) is used in the Torbal jar, the GasPak jar, and the disposable GasPak system. If care is made to remove any fine particles of pulverized catalyst pellets from the catalyst charge before use, there is practically no danger of explosion associated with use of this method of achieving anaerobiosis.

The anaerobic atmosphere inside the jar may be produced by either a self-contained gas generator or a tank of compressed gas (Dowell and Hawkins, 1974). A commercially available gas

generator system, the GasPak generator envelope (#70304, BBL), when activated produces hydrogen, to be used in the reduction of oxygen to water, and carbon dioxide (Dowell and Hawkins, 1974) which is stimulatory for, or required by, rumen bacteria (cited by Bryant, 1959) and most anaerobes in general (Finegold et al., 1974). When it is desired to use a tank of compressed gas as the source of the anaerobic nitrogen-carbon dioxide-hydrogen atmosphere, a tank of mixed gases is generally used. A gas regulator is used to reduce the tank pressure to a usable level and the gas is piped to the anaerobic jar through a system of tubing and valves.

Various mixtures of gases have been recommended by various workers. Sutter et al. (1975) recommended a gas mixture of 80-90% nitrogen, 5-10% carbon dioxide, and 5-10% hydrogen for the isolation of clinically-important anaerobic bacteria. Van Houte and Gibbons (1966), on the other hand, favored a gas mixture of 90% hydrogen and 10% carbon dioxide for the isolation of bacteria from human feces. Dowell (1975) preferred a gas mixture of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide for the isolation of clinically-important anaerobic bacteria. This is in agreement with the mixture recommended by Sutter et al. (1975).

When a self-contained gas generator envelope (GasPak) is used to achieve anaerobiosis, bacterial cultures are placed in the anaerobic jar along with a GasPak generator envelope, a packet of palladium catalyst pellets, and a redox indicator. The redox indicator may be a commercial indicator strip (#70504, BBL), the

methylene blue indicator recommended by Dowell and Hawkins (1974), or some other redox indicator or method of indicating redox potential. Water is added to the gas generator envelope and the jar is sealed (Dowell and Hawkins, 1974). The gas generator will produce enough hydrogen to reduce all of the oxygen in the jar, but evacuation of the jar before the gas generator begins to prcduce gas will reduce the amount of oxygen which must be reduced. When the amount of oxygen is reduced, less water is produced and moisture-related problems will likewise be reduced.

When a cylinder a mixed gases is used to produce the jar atmosphere, the anaerobic jar is connected to a three-way valve which allows a rapid change from a vacuum source to the source of mixed gases. The bacterial cultures are placed in the jar along with a packet of palladium catalyst pellets and a redox indicator. The jar is then sealed and connected to the three-way valve. Initially the jar is partially evacuated and then is refilled with the gas mixture. This process is repeated a total of three times, after which the jar connection is sealed and disconnected from the valve (Dowell and Hawkins, 1974).

When either the gas generator or the cylinder of mixed gases is used, no further action need be taken to produce anaerobiosis since the catalyst pellets operate without any activation, unlike the catalyst in the Brewer jar which must be electrically heated before it is able to catalyze oxygen reduction (Dowell and Hawkins, 1974). If the system is operating properly, the redox indicator

should show a low redox potential after overnight incubation.

Media used with the anaerobic jar method must not be exposed to oxygen for an extended period of time before use. For clinical specimens, media are either used the same day that they are prepared or they are stored in anaerobic jars for not more than two weeks before being used (Dowell and Hawkins, 1974). For the more oxygen-sensitive intestinal bacteria, media may be reduced in an anaerobic chamber for 48 hr before use and then may be inoculated in the chamber (Sutter et al., 1975). Reduced plates are probably superior to freshly-prepared plates because the reduced plates are at a low redox potential at the time of inoculation while the freshly-prepared plates are at a high redox potential initially. Exposure to the initially high redox potential of the freshly-prepared medium tends to inhibit or kill most obligately-anaerobic bacteria. The freshly-prepared medium is eventually reduced to a low redox potential, but damage to the bacteria occurs before substantial reduction of the medium is able to occur.

The anaerobic jar method has the advantages that ordinary bacteriological glassware and materials are used and ordinary bacteriological techniques are used. Standard straight-bore glass culture tubes with cotton stoppers, metal caps, or plastic caps may be used for tube cultures. Glass petri dishes may be used for streak cultures. Plastic equipment should be tested to be certain that it does not oxidize reduced medium and that toxic materials do not leach out of the plastic into the medium when the medium is

in contact with the plastic. Cultural manipulations are performed on an open laboratory bench so the awkwardness associated with the use of rubber gloves with an anaerobic chamber is avoided.

The two main disadvantages of the anaerobic jar method are that excessive amounts of moisture tend to collect inside of the jar and the bacteria are excessively exposed to oxygen during culture manipulations. Water vapor inside of the jar causes the same difficulties as in an anaerobic chamber. Moisture level control is provided by adding small packets of drying agent, such as silica gel, or by putting a piece of filter paper in the lid of each petri dish. The piece of filter paper should be just small enough to fit inside of the lid of the particular petri dish used and is held in place by drops of glycerin on the inner surface of the lid. Both the filter paper and the glycerin tend to absorb moisture and reduce humidity within the jar (Dowell and Hawkins, 1974). The bacteria are exposed to oxygen during the culture manipulations which are performed in the open air. In addition, it takes a certain amount of time for the oxygen in the jar to become reduced. As a result, the bacteria are exposed to toxic oxygen for a sufficient length of time to cause growth inhibition or complete death of the culture in some cases. When a cylinder of mixed gases is used to produce the atmosphere or when the jar is initially evacuated before the gas generator produces gas, the initial evacuation quickly removes most of the oxygen from the jar. As a result, anaerobic conditions are produced much more

rapidly than if a gas generator were used without initial evacuation (Dowell, 1975). Inactive catalyst prevents the production of anaerobic conditions so only fully active catalyst pellets should be used. The catalyst pellets are partially or fully inactivated by hydrogen sulfide, produced by bacterial cultures, and by moisture. In order to assure the production of anaerobic conditions within a reasonable time period, the catalyst pellets should be replaced each time the anaerobic jar is opened (Dowell, 1972). Deactivated catalyst pellets are regenerated by subjecting them to dry heat at 160-170 C for 2 hr (Dowell and Hawkins, 1974).

A modification of the anaerobic jar method, devised by Martin (1971), reduces exposure of the bacterial cultures to oxygen during cultural manipulations. When the unmodified anaerobic jar method is used for the incubation of a number of cultures, the cultures are exposed to air until all of the cultures have been inoculated and put into the jar, which is then processed normally. When the modified anaerobic jar method is used, inoculated cultures are immediately placed in the anaerobic jar which is being continuously flushed with oxygen-free carbon dioxide. When all of the cultures have been inoculated, the jar is processed normally.

Ellner et al. (1973) modified the method of Martin (1971) to include a medium reduction step. Plates of medium are placed in an anaerobic jar and incubated in the reducing atmosphere for at least 24 hr to reduce the medium. When the plates are to be used, the lid of the anaerobic jar is replaced by a loosely-fitting

vented lid connected to a source of oxygen-free carbon dioxide. A sufficient flow rate of the gas is provided to exclude air from the jar. Plates are removed from the jar as needed, immediately inoculated, and placed into a second empty anaerobic jar which is equipped the same as the first jar. When the second anaerobic jar is full, the loosely-fitting lid is replaced by the standard lid and the jar is then processed normally.

Method using chemically-reduced liquid media. Chemically-reduced liquid media may be used to provide the proper environment for the growth of anaerobic bacteria. The exclusive use of liquid media to produce pure cultures from a mixed bacterial flora does require the use of a slightly different approach than does the use of solid media. When solid media are used, a dilution series is used to dilute the sample so that at least one pour-plate from one of the dilutions will have a colony count of between 30 and 300 colonies. On the other hand, when liquid media are used, the dilution series is used to dilute the sample to the point that, on the average, less than one cell will be inoculated into each tube of liquid medium. In order to determine the amount of dilution necessary to achieve this, a direct microscopic count must be made of the sample. Also, in order to recover a reasonable proportion of a complex mixed flora, an extremely large number of tubes of media must be inoculated.

Chemically-reduced liquid media are similar to aerobic media

with the exceptions that a small amount of agar-agar is added to the medium to increase its viscosity and a small amount of a chemical reducing agent is added to reduce the redox potential of the medium. The viscosity increase caused by the addition of agar-agar minimizes mixing of the broth medium by convection currents in the tube. Because the tube contents do not circulate, the medium at the top of the tube vecomes oxidized first because of direct contact with air, while the medium at the bottom of the tube remains reduced. Oxidation of the medium proceeds from the top of the tube to the bottom of the tube, with the result that the medium at the bottom of the tube remains reduced for the longest period of time. Medium is dispensed into the tubes to a depth of 4-6 in to allow the production of good anaerobic conditions at the bottom of the tube and to increase the time needed for oxidation of the medium to progress from the top of the tube to the bottom of the tube. Cysteine-hydrochloride, sodium thioglycollate, sodium sulfide, and ascorbic acid are 4 of the many commonly used compounds used as reducing agents with this method.

The primary advantages of this method of cultivating anaerobic bacteria are that no special equipment is required either in media preparation or in handling of the cultures, no special equipment or techniques are necessary for incubation of the cultures, and standard bacteriological techniques are used. Media are prepared and dispensed aerobically as for standard

aerobic media. The cultures are also incubated aerobically which eliminates the need for anaerobic jars or chambers. Since standard bacteriological techniques are used and the cultures are manipulated on an open lab bench, a relatively large number of culture manipulations may be done in a relatively short period of time, which is desirable considering that a large number of tubes of media must be inoculated in a flora study. The only special consideration that must be made in cultural manipulations is that inoculations must be made into the medium at the bottom of the tube, which is the most highly reduced medium in the tube.

The primary disadvantages of this method are that a large volume of medium is needed for primary isolation, a large amount of hand labor is necessary (Bryant, 1959), there is too much exposure of the bacterial culture to oxygen during the cultural manipulations, and there is too much exposure of the medium to oxygen during incubation of the cultures. The first 3 disadvantages are due to the nature of the method and are not able to be remedied without changing the basic method. Exposure of the medium to oxygen during culture incubation causes the chemical reducing agent to become rapidly oxidized, with the result that the redox potential of the medium rises somewhat rapidly. In addition, the medium becomes toxic to anaerobic bacteria when excessively exposed to oxygen (Dowell, 1975), possibly because of hydrogen peroxide formed when medium components react with oxygen (Smith, 1975) or because of toxic properties of the oxidized form

of the reducing agent used (Moore and Holdeman, 1974). Exposure of the medium to oxygen may be minimized by the use of screw-capped culture tubes or eliminated by incubation of the cultures in anaerobic jars (Dowell and Hawkins, 1974). The latter solution, while being useful for the cultivation of bacteria too oxygen-sensitive to be cultivated using the unmodified method, does require an anaerobic jar and consequently does eliminate one of the advantages of this method.

Ringed plate method. Another method of cultivating anaerobic bacteria is the ringed plate method of Brewer. In this method, a thick layer of liquified solid medium containing a chemical reducing agent is poured into the bottom half of a standard glass petri dish. The liquified medium may be mixed with an inoculum and allowed to solidify, producing a pour-plate. Alternatively, the medium may be allowed to solidify after which an inoculum may be streaked for isolation on the center portion of the agar surface. The special top half of the petri dish, which has a raised ring molded on the inside, is placed down on the surface of the medium so that the ring seals against the agar surface and the gas space within the ringed area is inaccessible to air. The reducing agent in the medium reduces the medium in the area of the plate surrounded by the ring and the atmosphere in the gas space above this region of the medium surface (Brewer, 1942).

The advantages of using the ringed-plate method for cultivating

anaerobic bacteria are that standard bacteriological techniques are used, there is no need for expensive anaerobic jars, and the cultures may be inspected at any time during incubation without exposing the bacteria to air. In addition, this method produces a sufficient degree of anaerobiosis to reduce methylene blue and allow the growth of Clostridium perfringens, C. septicum, C. tetani, and C. novyi (Brewer, 1942). Although C. perfringens is able to grow at a redox potential as high as +160 mv at neutrality and C. septicum is tolerant of oxidized medium and small amounts of oxygen in the atmosphere, C. tetani is moderately sensitive to oxygen and C. novyi is highly sensitive to oxygen (Smith, 1975). Growth of the latter two organisms indicates that a good degree of anaerobiosis is being produced.

The disadvantages associated with the use of this method to cultivate anaerobic bacteria are that special petri dish lids are required, the method is not usable with liquid media, the bacteria are exposed to excessive amounts of air during culture manipulations, reduction of the medium and atmosphere in the gas space takes a certain amount of time, and the medium is exposed to excessive amounts of oxygen during incubation of the cultures. The first disadvantage requires that a monetary investment be made in specialized glassware which has no use other than in this method. The second disadvantage makes it necessary to use one of the other methods of achieving anaerobiosis to prepare broth cultures. The excessive exposure of the cultures to air limits the usefulness

of this method. Only anaerobic bacteria able to survive this amount of oxygen exposure can be cultivated using this method. The relative slowness of medium and atmosphere reduction also limits this method to use with relatively oxygen-tolerant or sporeforming anaerobic bacteria. The medium outside of the ring is directly exposed to the air so the reducing agent is rapidly oxidized, with a concurrent rise in redox potential of the medium and an increasing toxicity of the medium as for the chemically-reduced liquid medium method.

A number of other methods have been used in the past in studies of anaerobic bacteria. These methods are too numerous to mention and have only limited application to fecal flora studies. These early anaerobic methods are discussed in a review article (Hall, 1929).

Performance Of Methods

The open-tube method of achieving anaerobiosis has been used in many studies of anaerobic bacteria from a variety of sources, with good results. This method has been used to isolate oxygen-sensitive bacteria of the genera: Ruminococcus, Peptostreptococcus, Bacteroides, Fusobacterium, and Eubacterium (Bryant et al., 1958). In addition, this method has been used to cultivate 23-34% of the bacteria visible in a direct microscopic count of swine feces. This was accomplished using either a rumen fluid- or cecal extract-based medium (Salanitro et al., 1977). The open-tube method has also been used in the study of cattle intestinal flora. In this

case, the primary isolation medium based on intestinal fluid was used to cultivate between 1 and 10% of the bacteria from various regions of the intestine of cattle fed a high-roughage diet (Maki and Picard, 1965).

The anaerobic chamber method has been used to cultivate bacteria from the cecum of the mouse. This anaerobic chamber used palladium-alumina catalyst pellets in conjunction with atmosphere recirculation to create an anaerobic atmosphere. A recovery of 57-123% of the bacteria visible in a direct microscopic count was obtained using media containing either palladium black or palladium chloride (Aranki and Freter, 1972).

The anaerobic jar method has been used to cultivate bacteria from human feces. In this study, the Brewer anaerobic jar was used with blood agar plates. Less than 3% of the bacteria enumerated by a direct microscopic count were cultivated in this study (Van Houte and Gibbons, 1966).

The open-tube method has been compared with the ringed-plate method and the anaerobic jar method in the cultivation of bacteria from swine intestinal contents. GasPak gas generator envelopes were used to furnish the anaerobic atmosphere in the anaerobic jar. In this study, the open-tube method was found to give the highest recovery of bacteria from the swine intestine (Vervaeke and Van Nevel, 1972).

A study by Spears and Freter (cited by Hentges and Maier, 1972) compared the open-tube method with the anaerobic jar method

in the cultivation of bacteria from the mouse cecum. A recovery of 19.7-33.7% of the direct microscopic count was obtained using the open-tube method as compared to a recovery of only 1.1-5.8% using the anaerobic jar method.

The anaerobic chamber method has been compared with the anaerobic jar method in the cultivation of bacteria from the cecum of the mouse. A McIntosh anaerobic jar was used in the anaerobic jar method and an anaerobic chamber continuously flushed with oxygen-free gas produced by the combustion of natural gas was used in the anaerobic chamber method. A recovery of 17.7-37.1% of the direct microscopic count was obtained using the anaerobic chamber while a recovery of only 1.3-5.8% was obtained using the anaerobic jar (Koopman et al., 1973).

The open-tube method has been compared with the anaerobic chamber method in the cultivation of bacteria from human feces. In one study, by Moore and Holdeman (1974), a recovery of 4.75×10^{11} bacteria per gram dry weight of feces was obtained using the open-tube method. In a separate study, Attebery et al. (cited by Moore and Holdeman, 1974) succeeded in recovering 2.27×10^{11} bacteria per gram dry weight of feces using the anaerobic chamber method. Although it appears that the open-tube method offers superior recovery of human fecal bacteria, this apparent advantage may be due to an inefficient oxygen-removal apparatus. A properly functioning anaerobic chamber is capable of reducing media within it to a redox potential of from -250 to -300 mV (Koopman et al.,

1973), as compared to a redox potential of approximately -150 mV which was attained by media used by Hungate (cited by Koopman et al., 1973) to cultivate methane bacteria.

Types Of Primary Isolation Media

Two basic types of primary isolation media have been used in microflora studies. Selective media have been utilized in an attempt to isolate specific groups of bacteria from a mixed flora. Nonselective media, on the other hand, have been employed in an attempt to isolate as many types of bacteria from a mixed flora as possible.

Selective media are often used in microflora studies to attempt to simplify the process of determining the composition of the microflora. The assumption made is that any bacterial colonies appearing on a plate of selective medium are colonies of the specific organism the medium is selective for. If this were actually the case, the contribution of this particular organism to the total microflora could be easily determined by a simple plate-count procedure. In actual practice, though, selective media are imperfectly selective and tend to allow the growth of some organisms which are supposed to be inhibited and inhibit some strains of the organism which the medium is selective for. The latter problem is a continuing problem and results in erroneously low colony counts. The problem of growth of unwanted organisms, on the other hand, can be remedied. In some cases, the selective medium can also be made to be differential to separate the desired

organisms from the undesired organisms. The usual solution is to subject each colonial isolate to a series of tests to separate the organisms selected for from the other organisms. The use of a larger number of additional tests generally results in a more positive identification of the isolate in question.

Nonselective media are used in microflora studies in an attempt to eliminate the problem of imperfect selectivity of a selective primary isolation medium. The nonselective medium is designed to support the growth of as many groups of bacteria present in the microflora as possible. As a result, a considerable number of tests must be run on each colonial isolate to identify the organism and to allow the determination of the microflora composition.

Primary Isolation Media For Rumen Flora Studies

Many nonselective primary isolation media used in fecal flora studies were originally used in rumen flora studies. In some cases, the medium used in rumen flora studies was not modified when used in fecal flora studies. On other cases, the medium used in rumen flora studies was modified for use in fecal flora studies.

Rumen fluid-glucose-cellobiose agar (RGCA) has been a popular nonselective primary isolation medium for rumen flora studies. Bryant and Burkey (1953) developed RGCA medium specifically for rumen flora studies. RGCA contained glucose and cellobiose as carbon sources, ammonium sulfate as the nitrogen source, bicarbonate and phosphate buffer systems, resazurin as the redox indicator,

rumen fluid as a source of growth factors, mineral salts, cysteine hydrochloride as the reducing agent, and a 100% carbon dioxide atmosphere. The medium was first reduced by boiling and then the reducing agent was added to further reduce the medium. The open-tube method was used with this medium. Standard RGCA medium was used by Dehority (1963) in a study emphasizing cellulolytic rumen bacteria, Bryant et al. (1960) in a study of rumen bacteria from cattle fed a diet tending to induce bloating, and Bryant et al. (1958) in a study in which RGCA was found to give higher counts than the other medium used.

RGCA medium has also been modified in various ways. Bryant and Small (1956) modified RGCA by adding rumen fluid to give a final concentration of 40%. They used this modified RGCA to cultivate bacteria of the rumen flora, especially bacteria of the genus Butyrivibrio. In addition, they used this medium to isolate bacteria of the genera Succinivibrio and Lachnospira from rumen contents (Bryant and Small, 1956a). Bryant (1956) also used this modified RGCA to isolate Selenomonas ruminantium from the rumen. Bladen et al. (1961) modified RGCA by adding maltose as an additional fermentable carbohydrate. They used this medium to isolate bacteria of the bovine rumen flora, especially the ammonia-producing bacteria. They obtained a total recovery between 2×10^8 and 1×10^9 bacteria per gram wet weight of rumen contents. Maki and Foster (1957) modified RGCA by adding phytone and trypticase as additional nitrogen sources. With this medium they were able to

recover between 3 and 73% of the bacteria visible in a direct microscopic count, depending upon the diet the test animal was fed. Bryant et al. (1960) modified RGCA and used the modified medium for total aerobic bacterial counts of rumen contents. This medium was formulated as for standard RGCA except the bicarbonate buffer system and cysteine hydrochloride were omitted from the modified medium. In addition, the medium was not reduced by boiling so that the medium would be at a relatively high redox potential. The medium was dispensed aerobically into open petri dishes and used as any other aerobic medium. Grubb and Dehority (1976) modified RGCA medium by adding soluble starch as an additional fermentable carbohydrate. This medium, designated RGCSA, gave equal or significantly higher recovery of rumen bacteria than did the other two media tested.

RFM (rumen fluid medium) has also been used in some studies of rumen bacteria. The form of RFM used by Caldwell and Bryant (1966) contained glucose, cellobiose, and soluble starch as carbon sources; mineral salts; sodium sulfide and cysteine hydrochloride as reducing agents; phosphate and bicarbonate buffer systems; clarified rumen fluid as a source of volatile fatty acids and growth factors; and a 100% carbon dioxide atmosphere. The medium was prepared as for RGCA and the open-tube method was used. With this method and medium, members of the genera: Butyrivibrio, Succinivibrio, Selenomonas, Lachnospira, Succinimonas, Bacteroides, Borrelia, Eubacterium, Peptostreptococcus, Ruminococcus, and

Lactobacillus were isolated from rumen contents. In fact, RFM may be the best nonselective medium for the isolation of carbohydrate-fermenting rumen bacteria.

Caldwell and Bryant (1966) modified RFM and created a new medium, Medium 10. Medium 10 had the same basic ingredients as RFM with the exception that rumen fluid in RFM was replaced by a combination of trypticase, yeast extract, hemin, and a volatile fatty acid mixture in Medium 10. The fatty acid mixture contained acetic, propionic, butyric, isobutyric, n-valeric, isovaleric, and DL-α-methylbutyric acids. They compared Medium 10 with RFM and found that there was no significant difference in recovery of rumen bacteria between the 2 media. They felt, however, that Medium 10 was preferable for use with established cultures of rumen bacteria and for use when a more highly defined medium was desired. Although Caldwell and Bryant felt that RFM was the best non-selective medium for fermentative rumen bacteria, Grubb and Dehority (1976) found that RGCSA gave recoveries at least as great as did RFM and significantly greater than did Medium 10.

Bryant and Robinson (1961) developed a nonselective primary isolation medium quite similar to RFM. This medium, designated Medium 98-5, contained glucose, cellobiose, and soluble starch as carbon sources; ammonium sulfate as the nitrogen source; bicarbonate and phosphate buffer systems; resazurin as the redox indicator; cysteine hydrochloride and sodium sulfide as the reducing agents; mineral salts; and rumen fluid as a source of fatty acids

and growth factors. The medium was prepared in the same manner as RGCA and the open-tube method was used. Two different atmospheres were used in the stoppered tubes, a 100% carbon dioxide atmosphere and a 50% carbon dioxide-50% hydrogen atmosphere. Medium 98-5, using either atmosphere, was found to give approximately twice the recovery than was observed using RGCA in rumen flora studies.

Chung and Hungate (1976) developed an improved medium for the primary isolation of rumen bacteria using the open-tube method. Alfalfa hemicellulose and cellulose were obtained by extracting ground alfalfa with water. The solid was then treated with sodium hydroxide and potassium hydroxide after which it was neutralized with hydrochloric acid. A suspension of the solid was mixed with an equal volume of Medium 98-5, modified to contain 33% rumen fluid instead of the usual 40% rumen fluid. This medium gave approximately double the recovery of rumen bacteria that was obtained with either Medium 98-5 with 33% rumen fluid or Medium 10 with 33% rumen fluid.

Bryant et al. (1958) modified trypticase-soy agar and produced a nonselective primary isolation medium for rumen flora studies. Trypticase-soy agar was modified by omitting the sodium chloride and adding resazurin as a redox indicator, cysteine hydrochloride as a reducing agent, sodium carbonate as part of a bicarbonate buffer system, and both glucose and cellobiose as carbon sources. The medium was prepared and used as for the open-

tube method. This medium was found to be somewhat inferior to RGCA for the primary isolation of rumen bacteria. Trypticase-soy agar was also modified by the addition of a small amount of glucose and was used for the nonselective isolation of aerobic and facultatively anaerobic rumen bacteria.

Trovatelli and Matteuzzi (1976) developed a nonselective isolation medium designated TPG medium. TPG medium contained trypticase and phytone as nitrogen sources, glucose as a carbon source, yeast extract as a source of growth factors, mineral salts, a phosphate buffer system, and cysteine hydrochloride as the reducing agent. This medium was used with the open-tube method. With this medium they were able to obtain counts of as high as 10^9 bacteria per milliliter of rumen fluid, when the cattle were fed a diet high in carbohydrates. Most of the organisms isolated were tentatively identified as being bifidobacteria.

Bauman and Foster (1956) developed a nonselective liquid medium for use in the primary isolation of rumen bacteria. This medium, designated TS broth, contained trypticase, phytone, and dibasic ammonium phosphate as nitrogen sources; glucose and sodium citrate as carbon sources; a phosphate buffer system; resazurin as the redox indicator; cysteine hydrochloride as the chemical reducing agent; and a low concentration of agar-agar to increase the viscosity of the broth and allow the growth of certain anaerobes which need a small quantity of agar-agar for good growth. The medium was boiled long enough to drive off most of the dissolved

oxygen but not long enough to reduce the medium. Inoculation of the tubes of medium was carried out under a stream of nitrogen gas and screw-capped culture tubes were used to minimize exposure of the medium to oxygen. The only bacteria of the rumen flora isolated using this medium and method were 13 cultures of bacilli identified as being similar to Lactobacillus bifidus or L. parabifidus and 9 cultures of cocci similar to Pediococcus acidi-lactici and P. hennebergi.

Gall et al. (1947) developed two nonselective chemically-reduced liquid media for rumen flora studies. Both of the media were used in unstoppered tubes sealed by vaspar after inoculation. The media were heated to drive out dissolved oxygen, but were not treated in any other way to reduce the oxygen concentration or redox potential of the medium.

The skimmed milk medium contained cellulose and glucose as carbon sources; yeast extract as a source of growth factors; skimmed milk as a source of fermentable carbohydrate and nitrogen; a phosphate buffer system; and peptone, tryptone, and beef extract as nitrogen sources (Gall et al., 1947). Huhtanen et al. (1952) modified this medium by omitting the skimmed milk and adding a bicarbonate buffer system. Also, the tubes of medium were autoclaved with the vaspar seal in place. Huhtanen and Gall (1953) further modified the medium of Huhtanen et al. (1952) by mixing that medium with artificial saliva in a 5:4 (v/v) ratio.

The tomato juice medium contained tomato juice, glucose and

cellulose as carbon sources, meat infusion and tryptose as nitrogen sources, and a phosphate buffer system (Gall et al., 1947). This medium was not used in the other 2 studies the skimmed milk medium was used in.

Bacterial growth occurred in both unmodified media at the 10^{-11} dilution level (Gall et al., 1947) and in the medium of Huhtanen et al. (1952) at the same level. With the modified medium, Huhtanen and Gall (1953a) were able to isolate a variety of bacteria from rumen contents including lactic acid-utilizing bacteria and bacteria resesembling members of the genera Propionibacterium, Fusobacterium, Lactobacillus, and Veillonella. The saliva medium was able to support the growth of curved bacilli from the rumen and was able to achieve a redox potential of approximately -300 mV (Huhtanen and Gall, 1953).

The liquid media were used in tubes for the initial culture of rumen bacteria in these studies. Purification of the cultures was accomplished by using solid media in tubes (Gall et al., 1947) or by using the pour-plate procedure with the ringed-plate method (Huhtanen et al., 1952).

A wide variety of selective media have been used in rumen flora studies. Selective media have been used both to simplify the determination of the composition of the microflora and to isolate specific groups of bacteria which are of interest.

Dehority and Grubb (1976) developed a medium which could be used to selectively grow rumen bacteria able to utilize certain

substrates. This medium, designated RA medium, was based on RGCA medium but contained no carbohydrates. The medium was prepared as for RGCA with the exception that the ingredients, minus the sodium bicarbonate and cysteine hydrochloride, were pre-incubated for 7 days before autoclaving. This step was incorporated to increase the selectivity of the medium through the removal of undesired substrates present in the medium ingredients. The sodium bicarbonate and cysteine hydrochloride were aseptically added along with the desired substrate or substrates. When cellobiose, glucose, starch, and xylose were combined as substrates, the resulting medium was a good nonselective rumen flora medium. It was comparable to RGCSA medium in the cultivation of rumen bacteria.

Cellulose-digesting bacteria of the rumen are important in the nutrition of the ruminant and are among the predominant bacteria in the rumen. As a result, there have been many efforts to cultivate these bacteria and a number of different media have been used to attempt to isolate these organisms.

Cellulose broth was used by Bryant et al. (1958) in a study of rumen flora using the most-probable-number (MPN) method of determining viable counts of cellulolytic bacteria. Cellulose broth of a different formulation was used by Bryant and Burkey (1953) in their rumen flora studies and by Bryant et al. (1960) in their study of rumen bacteria in cattle fed a bloat-provoking diet.

Hungate (1950) developed a nonselective medium for cellulose-digesting bacteria grown using the open-tube method. The medium

contained resazurin as the redox indicator, cellulose suspension as the source of cellulose, cysteine hydrochloride as the chemical reducing agent, rumen fluid as a source of fatty acids and growth factors, bicarbonate and phosphate buffer systems, and mineral salts. He isolated Bacteroides succinogenes using this medium and the open-tube method. In another study, Hungate (1957) used this medium plus a feed-extract medium to isolate B. succinogenes, Streptococcus bovis, Ruminococcus flavefaciens, and R. albus from the bovine rumen. The feed-extract medium was a modification of the medium for cellulose-digesting bacteria. The rumen fluid, cellulose suspension, and some of the mineral salts of the cellulose medium were omitted for the feed-extract medium and extracts of hay, barley, and cottonseed meal were added.

Jarvis and Annison (1967) also developed a medium for the cultivation of cellulose-digesting bacteria. This primary isolation medium contained filter paper as the source of cellulose, rumen fluid and yeast extract as the sources of growth factors, mineral salts, phosphate and bicarbonate buffer systems, sodium sulfide and cysteine hydrochloride as the chemical reducing agents, and indigo disulphonate as the redox indicator. This medium was used in the study of the rumen flora of the sheep, using the open-tube method of achieving anaerobiosis.

Appleby (1955) used a selective medium to isolate proteolytic bacteria from the rumen of the sheep. The medium contained mineral salts; phosphate and bicarbonate buffer systems; resazurin as the

redox indicator; sheep rumen fluid as the source of fatty acids and growth factors; casein, and occasionally peptone, as the nitrogen source; and cysteine hydrochloride as the reducing agent. The open-tube method was used. The proteolytic bacteria isolated with this medium were all facultative anaerobes, with the exception of Clostridium sporogenes which was also isolated.

F-S medium was developed by Hamlin and Hungate (1956) to isolate bacteria of the rumen flora, especially the starch-digesting bacteria. The medium contained feed extract, soluble starch or corn starch as the source of starch, mineral salts, resazurin as the redox indicator, cysteine hydrochloride as the reducing agent, and a phosphate buffer system. The open-tube method was used with this medium. This medium and method were sufficient to cultivate rumen bacteria, including Bacteroides amylophilus, in counts ranging from 6×10^6 to 4×10^9 bacteria per milliliter of rumen fluid.

A selective medium for lipolytic bacteria was used by Hobson and Mann (1961) in studies of the rumen microflora of sheep, using the open-tube method of culturing anaerobic bacteria. The medium contained mineral salts, phosphate and bicarbonate buffer systems, linseed oil as the source of lipid, rumen fluid as the source of growth factors, cysteine hydrochloride as the reducing agent, and resazurin as the redox indicator. Lipolytic bacteria, in counts up to 10^8 bacteria per milliliter of rumen contents, were isolated using this medium.

Hobson and Mann (1961) also used a medium selective for glycerol-fermenting bacteria in their rumen flora studies. This medium contained mineral salts, yeast extract and rumen fluid as the sources of growth factors, glycerol as the carbon source, sodium acetate, bromocresol purple, phosphate and bicarbonate buffer systems, and cysteine hydrochloride as the reducing agent. The open-tube method was again used. Using this method, glycerol-fermenting bacteria, mainly members of the genus Selenomonas, were isolated from the rumen of the sheep.

Some selective media used in rumen flora studies have been used to attempt to isolate members of a specific taxonomic group. Some of the media have been used to cultivate a broad group of organisms such as the Enterobacteriaceae while other media have been used to cultivate members of a single genus such as Lactobacillus.

Lactobacilli have been isolated in these studies using a variety of media. These media include: Rogosa Lactobacillus Medium (Bryant et al., 1960), SL medium (Jensen et al., 1956), Briggs medium (Jensen et al., 1956), and fortified skim milk medium (Jensen et al., 1956).

Many other organisms in the rumen are of interest, although selective media for them often do not exist. Media for Quin's Oval (Orpin, 1972) and members of the genus Desulfovibrio (Howard and Hungate, 1976) are examples of selective media which have been developed for organisms of lesser importance in the rumen micro-

flora.

Nutritional Characteristics Of Some Rumen Bacteria

Most of the rumen bacteria are unable to grow in the complete absence of sodium and potassium ions, with the exception of Megasphaera elsdenii and Streptococcus bovis (Caldwell and Hudson, 1974). Ammonium ion and cysteine furnish the nitrogen required by most rumen organisms, while most rumen bacteria do not utilize amino acids and other nitrogen-containing compounds (Bryant and Robinson, 1961). Specifically, amino acids are required by Selenomonas ruminantium, Megasphaera elsdenii, Streptococcus bovis, Lachnospira multiparus, Succinivibrio dextrinosolvens, and a strain of Butyrivibrio fibrisolvens, while ammonium ion is required by Eubacterium ruminantium, Ruminococcus albus, R. flavefaciens, and one strain of B. fibrisolvens (Bryant and Robinson, 1963). Acetate is also required by many rumen bacteria, as is hemin (Bryant and Robinson, 1962). In addition, many of the rumen bacteria require at least one of the following volatile fatty acids: n-valeric, isovaleric, isobutyric, and 2-methylbutyric (Bryant and Robinson, 1961).

The cellulose-digesting bacteria, as a group, have been studied more than other bacteria of the rumen. Members of the genus Butyrivibrio were found to require carbon dioxide, cysteine, biotin, certain other amino acids, pyridoxal hydrochloride, and folic acid for growth (Gill and King, 1958). Specifically, Butyrivibrio fibrisolvens was found to be stimulated by an unknown

factor present in rumen fluid and absent in hog mucin (Gordon and Moore, 1961). Strains of Ruminococcus albus were found to need only 1 of 2 volatile fatty acids, isobutyric acid or 2-methylbutyric acid (Dehority et al., 1967). It has also been found that while all strains of R. albus could chemically attack both flax hemicellulose and corn hull hemicellulose, no strains could metabolize corn hull hemicellulose and only 1 strain could metabolize flax hemicellulose (Dehority, 1965). Ammonia is required as the sole nitrogen source of R. albus and high levels of amino acids are found to be inhibitory to this organism (Bryant and Robinson, 1961a). Ruminococcus flavefaciens was found to require a fermentable carbohydrate for growth, either cellulose or cellobiose but not glucose (Ayers, 1958). Cotton wool, filter paper, or powdered cellulose all were found to be good sources of cellulose (Sijpesteijn, 1951). In the study of Dehority (1965), it was found that of the strains tested all could chemically attack both flax hemicellulose and corn hull hemicellulose. However, only 2 strains could metabolize flax hemicellulose and no strains could metabolize corn hull hemicellulose (Dehority, 1965). R. flavefaciens also was found to require amino acids as a source of nitrogen, growth factors present in yeast extract, and carbon dioxide and/or bicarbonate ion for growth (Ayers, 1958). Dehority et al., (1967) found that their strains of R. flavefaciens required at least 1 of the following volatile fatty acids: isobutyric, isovaleric, and 2-methylbutyric. Both strains of R. flavefaciens

studied by Scott and Dehority (1965) were found to require biotin and be stimulated by Vitamin B_{12}. In addition it was found that R. flavefaciens showed improved growth in media in which Clostridium sporogenes had previously been grown (Sijpesteijn, 1951). Bacteroides succinogenes was found to require ammonia as its sole nitrogen source (Bryant and Robinson, 1961a). In addition, the strains studied by Dehority (1963) were found to require acetic, valeric, isobutyric, and isovaleric acid, while the strains studied by Dehority et al. (1967) were found to require only a straight-chain volatile fatty acid at least 5 carbons long and either 2-methylbutyric acid or isobutyric acid. The strains of B. succinogenes studied by Scott and Dehority (1965) were found to require biotin but did not require folic acid, nicotinamide, pantothenate, pyridoxine, riboflavin, or thiamine.

Primary Isolation Media For Fecal Flora Studies

RCM medium was used by a number of workers in the study of fecal bacteria. It is a nonselective medium and has been used in various forms in various studies. The medium was developed by Hirsch and Grinsted (1954) as a chemically-reduced liquid medium. It contained glucose, soluble starch, and sodium acetate as carbon sources; yeast extract as a source of growth factors; meat extract and peptone as nitrogen sources; cysteine as the reducing agent; and a small amount of agar-agar as a viscosity-increasing addition. RCM liquid medium was used by Willingale and Briggs (1955) in a study of porcine intestinal bacteria. The tubes of medium were

incubated aerobically and the MPN method of enumeration was used. Viable counts of approximately $1x10^9$ bacteria per gram of feces were obtained using this method and medium. The other studies in which RCM was used made use of solid RCM medium. Smith (1961) used solid RCM supplemented with blood in his study. The inoculated plates were incubated in an anaerobic jar with a hydrogen atmosphere containing a small amount of carbon dioxide. Van Der Wiel-Korstanje and Winkler (1970) modified RCM by adding horse blood and China blue. This nonselective, differential medium was used for the isolation of human fecal bacteria. Inoculated plates were incubated in an anaerobic jar having an atmosphere composed of hydrogen with a small amount of carbon dioxide. The medium was differential in that colony color allowed the differentiation of several groups of bacteria found in human feces. Plate counts of human feces gave total viable counts as high as $1x10^{10}$ bacteria per gram wet weight of feces, most of the isolates being members of the genera Bifidobacterium and Bacteroides. Peach et al. (1974) supplemented RCM with horse blood, glucose, and liver digest and used this medium for the isolation of non-sporulating bacteria from the feces of humans. Plates of the modified RCM medium were inoculated in an anaerobic chamber and incubated in an anaerobic jar. The jar contained a hydrogen atmosphere with a small amount of added carbon dioxide. Bacteria of the genera Fusobacterium, Bifidobacterium, Eubacterium, and Bacteroides were isolated in this study. Vervaeke and Van Nevel (1972) used RCM supplemented with

hemin to cultivate porcine cecal and colonic bacteria using the open-tube method, the ringed-plate method, and the anaerobic jar method. They obtained the highest viable counts, approaching $1x10^{10}$ bacteria per gram wet weight of cecum or colon contents, using the modified RCM medium with the open-tube method. In a similar study, Vervaeke et al. (1973) compared the recovery of RCM using the open-tube method and RCM using the ringed-plate method. Approximately equal recoveries were obtained with both methods in the cultivation of bacteria from the porcine small intestine. The open-tube method was found to be somewhat superior in the cultivation of bacteria from the porcine cecum and large intestine. Fuller and Lev (1964) modified RCM and produced a medium selective for Gram-negative obligately anaerobic bacteria from the porcine digestive tract. The RCM was modified by the addition of neomycin and crystal violet as selective agents and horse blood as a supplement. Plates of solid medium were incubated in anaerobic jars to provide anaerobic conditions for the organisms isolated, including members of the genera Veillonella, Peptostreptococcus, and Bacteroides, presumptively identified using the Gram stain.

Media based on trypticase-soy medium have also been used in fecal flora studies. Zubrzycki and Spaulding (1962) used human blood-supplemented trypticase-soy agar plates to cultivate human fecal bacteria. The plates were incubated in an anaerobic jar with an anaerobic atmosphere containing a small percentage of carbon dioxide. With this medium and method, they obtained an average

viable count of $1x10^9$ bacteria per gram wet weight of human feces. Mitsuoka et al. (1973) modified trypticase-soy agar by adding horse blood to it. They used this medium to isolate aerobic bacteria and lactobacilli from the intestinal contents of a number of animals. Spears and Freter (1967) found that sheep blood-supplemented trypticase-soy agar incubated in an anaerobic jar gave a lower recovery of mouse cecal bacteria than did modified Medium 98-5 used with the open-tube method. The atmosphere in the jar consisted of hydrogen with a small amount of carbon dioxide added. Aranki and Freter (1972) modified trypticase-soy agar by adding placenta powder, cysteine hydrochloride and palladium chloride as reducing agents, bicarbonate and phosphate buffer systems, glucose as a carbon source, yeast extract as a source of growth factors, menadione, and hemin. This medium, designated A II, was used in conjunction with an anaerobic chamber to cultivate mouse cecal bacteria. A II was further modified by Cadogan-Cowper and Wilkinson (1974) to produce a new medium, designated UA agar. UA agar had potassium nitrate and magnesium sulfate added to the ingredients in A II medium. UA medium, incubated in an anaerobic chamber, was used to cultivate members of the genera Fusobacterium and Bacteroides from a variety of human clinical specimens. In addition, extremely oxygen-sensitive bacteria, morphologically similar to members of the genus Treponema, were successfully isolated from porcine fecal specimens.

A thioglycollate-based medium was used by Wilbur et al. (1960)

to cultivate porcine intestinal bacteria. Pour-plates were incubated in desiccator jars which had been evacuated and filled with natural gas. Bacterial counts of over $1x10^9$ bacteria per gram wet weight of feces were obtained with this medium and method.

An intestinal fluid-based solid medium was developed by Maki and Picard (1965) for use in bovine intestinal flora studies. This medium was named ICGSA medium and was used with the open-tube method. ICGSA medium contained glucose, starch, and cellobiose as carbon sources; phosphate and bicarbonate buffer systems; mineral salts; resazurin as a redox indicator; centrifuge-clarified ileum contents; and cysteine as the reducing agent.

Eugon agar has also been used in intestinal flora studies. Maki and Picard (1965) used this medium in a study of the intestinal bacterial flora of cattle. Eugon agar plates incubated in an anaerobic jar were found to offer superior recovery of bovine intestinal bacteria as compared to ICGSA medium using the open-tube method. Spears and Freter (1967) modified Eugon agar by adding sheep blood to it. This medium was incubated in an anaerobic jar with a hydrogen-carbon dioxide atmosphere and was used to cultivate bacteria from the mouse cecum.

Spears and Freter (1967) used Medium 98-5 with the open-tube method to cultivate bacteria from the cecum of the mouse. This medium and method gave higher recoveries of mouse cecal bacteria than did either trypticase-soy-blood agar or Eugon-blood agar incubated in an anaerobic jar. Medium 98-5 gave recoveries as high

as 34% of the direct microscopic count of mouse cecal contents.

Medium 10 was used by Eller et al. (1971) in their study of human fecal bacteria. Total cultural counts of over $1x10^{11}$ bacteria per gram wet weight of feces were obtained using this medium with the open-tube method. Mitsuoka et al. (1973) also used this medium, with a modification of the open-tube method, to cultivate facultative anaerobes from the feces of a variety of animals.

Van Houte and Gibbons (1966) used modified heart infusion agar as a nonselective medium for the study of the microflora of human feces. Heart infusion agar was modified by adding menadione and horse blood to supply growth factors not present in the standard medium. With this medium and the anaerobic jar method they were able to cultivate an average of approximately $8x10^{9}$ bacteria per gram wet weight of feces as compared with an average direct microscopic count of approximately $3x10^{11}$ bacteria per gram wet weight of feces. Attebery et al. (1972) also used a blood-based medium in their human fecal flora studies.

Modified beef infusion agar was used by Weiss and Rettger (1937) with the anaerobic jar method to cultivate human fecal bacteria, especially members of the genus Bacteroides. The medium contained beef infusion agar, bovine blood as a source of various growth factors, glucose as a carbon source, peptone as a nitrogen source, and a phosphate buffer system. The anaerobic jar contained a hydrogen atmosphere and a palladium catalyst was used for oxygen

removal.

Zani et al. (1974) isolated bifidobacteria from porcine feces using a nonselective medium incubated in an anaerobic jar. The medium contained glucose as the carbon source, cysteine hydrochloride as the chemical reducing agent, yeast extract as a source of growth factors, mineral salts, phytone and trypticase as nitrogen sources, agar-agar, and a phosphate buffer system. The nitrogen atmosphere of the anaerobic jar contained a small amount of carbon dioxide.

Gossling and Slack (1974) used the open-tube method and a rumen fluid-based medium for cultivating human fecal bacteria, with the result being that 55% of the bacteria visible in a direct microscopic count were cultivated. The medium contained soluble starch, maltose, and glucose as carbon sources; menadione and sheep rumen fluid as sources of growth factors; mineral salts; cysteine hydrochloride as the reducing agent; resazurin as the redox indicator; and ammonium sulfate as a nitrogen source.

Aranki et al. (1969) used a nonselective primary isolation medium and the anaerobic chamber method to isolate obligately anaerobic bacteria from the cecum of the mouse. The isolation medium, designated ETSA medium, contained trypticase-soy broth, sodium carbonate for the control of pH, cysteine hydrochloride as the primary reducing agent, menadione and hemin as sources of growth factors, and agar-agar. Control plates with phenosafranin or indigo carmine redox indicating dyes were incubated with the

inoculated plates of ETSA medium and were used to monitor the redox potential of media incubated in the chamber. With this medium and method they were able to cultivate 10-43% of the bacteria visible in a direct microscopic count. When ETSA medium was used with a palladium black overlay, however, the recovery was increased to 20-46% of the bacteria visible in a direct microscopic count.

Attebery et al. (1972) studied the microflora of human feces using an anaerobic chamber for cultural manipulations and anaerobic jars for the incubation of cultures. The nonselective primary isolation medium used contained glucose, soluble starch, maltose, fructose, and sodium lactate as carbon sources; trypticase, thiotone, and phytone as nitrogen sources; yeast extract and menadione as sources of growth factors; liver digest; mineral salts; bicarbonate and phosphate buffer systems; sheep blood; cysteine hydrochloride and sodium thioglycollate as the chemical reducing agents; and resazurin as the redox indicator. Total cultural counts of approximately $1x10^{11}$ bacteria per gram dry weight of human feces were obtained using this medium.

Willingale and Briggs (1955) used Briggs liquid medium (Briggs, 1953), with a small amount of agar-agar added to increase the viscosity of the broth, in their study of porcine intestinal bacteria. This medium was used in aerobically-incubated tubes for the MPN enumeration of the total bacterial flora. Counts of approximately $1x10^{9}$ bacteria per gram of feces were obtained using this medium and RCM medium.

EG medium of Mitsuoka et al. (1973) was used in the isolation of anaerobic bacteria from the intestinal contents of man and animals. The steel wool method of producing anaerobiosis (Parker, 1955) was used with this medium. EG medium contained horse meat extract and proteose peptone as nitrogen sources, yeast extract and horse blood as sources of growth factors, soluble starch and glucose as carbon sources, cystine, a phosphate buffer system, Dow Antifoam B, agar-agar, and cysteine hydrochloride as the reducing agent. This medium and method were used to cultivate 6.6×10^{10} bacteria per gram wet weight of feces from a human fecal sample, 3.8×10^{10} bacteria per gram wet weight of feces from a canine fecal sample, and 2.2×10^{10} bacteria per gram wet weight of feces from a murine fecal sample.

A variety of selective media have also been used in fecal flora studies to isolate specific groups of bacteria. Many other selective media could be used, or could be adapted to be used, in fecal flora studies.

Lactobacillus selective medium was developed by Rogosa et al. (1951) for the selective isolation of lactobacilli. It contained mineral salts, agar-agar, yeast extract as a source of growth factors, trypticase as a source of nitrogen, a phosphate buffer system, glucose as a carbon source, Tween 80 as a source of fatty acids, acetic acid, sodium acetate, and ammonium citrate. This medium was also used by Attebery et al. (1972) in their study of human fecal bacteria.

Rogosa (1956) developed a selective medium for veillonellae. This medium contained streptomycin and basic fuchsin as the selective agents, sodium thioglycollate as the reducing agent, trypticase as the nitrogen source, yeast extract as the source of growth factors, sodium lactate as the carbon source, agar-agar, and Tween 80 as a source of fatty acids. Van Houte and Gibbons (1966) used spread-plates of this medium, incubated in anaerobic jars, to isolate veillonellae from human feces. The atmosphere in the jar was mainly hydrogen, with a small amount of carbon dioxide added.

Omata and Disraely (1956) developed a medium selective for fusobacteria. This medium contained human ascitic fluid, yeast extract as a source of growth factors, glucose as a carbon source, casitone as a nitrogen source, sodium chloride, crystal violet and streptomycin as selective agents, L-cystine, and agar-agar. Van Houte and Gibbons (1966) used this medium in their study of human fecal bacteria. The spread-plates were incubated in a hydrogen atmosphere, containing a small amount of carbon dioxide, in an anaerobic jar.

Attebery et al. (1972) used a variety of other selective media in their study of human fecal bacteria. These included rifampin blood agar, neomycin blood agar, kanamycin-vancomycin blood agar, egg yolk agar with neomycin, and Eugonagar containing maltose.

Van Houte and Gibbons (1966) also used a number of other selective media in their human fecal flora study. They used Mitis-

salivarius agar and horse blood-supplemented azide blood agar base for streptococci, EMB agar for coliforms, Rogosa SL agar for lactobacilli, and Staphylococcus medium 110 for bacteria of the genus Staphylococcus.

Maki and Picard (1965) used Bismuth Sulfite agar for the isolation of bacteria of the genus Salmonella from bovine intestinal contents. In addition, they used Mycosel agar for the isolation of fungi and EMB agar for the isolation of coliforms.

Bovine Fecal Flora Studies

Hartman et al. (1966) used the media and methods of Hartman et al. (1962) to study the ileal and fecal bacterial floras of calves. The calves were fed whole milk and the ileal and fecal samples were taken at various times after feeding, resulting in a wider range of bacterial counts than would have been expected had the samples been taken at the same time after feeding. Selective media were used to isolate groups of bacteria with no characterization of isolates being done. Average total aerobic bacterial counts ranged from 1.3×10^9 to 2.5×10^9 bacteria per gram wet weight of sample while average total anaerobic bacterial counts ranged from 7.9×10^8 to 1.6×10^9 bacteria per gram wet weight of sample. Of the specific groups of bacteria enumerated, lactobacilli comprised the largest group with average counts ranging from 1.0×10^9 to 3.1×10^9 bacteria per gram wet weight of sample. Coliforms made up the second-largest group with average counts ranging from 1.0×10^8 to 3.1×10^8 bacteria per gram wet weight of

sample. The smallest group of bacteria enumerated in this study was that of the streptococci. Average counts of streptococci ranged from 2.0×10^7 to 6.3×10^7 bacteria per gram wet weight of sample.

Maki and Picard (1965) obtained an average total anaerobic bacterial count of bovine colon contents of 2×10^7 bacteria per gram wet weight of colon contents. They used a variety of selective and nonselective media incubated under aerobic and anaerobic conditions to isolate the organisms making up the bovine colon bacterial flora. The most numerous group of organisms found was that of species of the genus Mucor, with an average count of 5×10^6 organisms per gram wet weight of feces. Species of the genus Sphaerophorus were slightly less numerous, with an average count of 2×10^6 organisms per gram wet weight of feces. Bacillus pumilus and species of Brevibacterium were third most numerous with an average count of 1×10^6 organisms per gram wet weight of feces. Escherichia coli was next with an average count of 6×10^5 organisms per gram wet weight of feces and Clostridium butyricum, with an average count of 4×10^5 organisms per gram wet weight of feces, followed Escherichia coli. Following C. butyricum was Streptococcus bovis with an average count of 3×10^5 organisms per gram wet weight of feces. Bacillus subtilis, species of Clostridium, and species of Streptomyces were less numerous with an average count of 1×10^5 organisms per gram wet weight of feces. Escherichia freundii, Streptococcus faecalis, and an unidentified Gram-positive

bacillus which was catalase positive, curved, and filamentous all had an average count of $1x10^4$ organisms per gram wet weight of feces. Following these three organisms was Candida krusei with an average count of $5x10^3$ organisms per gram wet weight of feces. Bacillus cereus and Brevibacterium lipolyticum were the least-numerous organisms found in this study, with an average count of $1x10^3$ organisms per gram wet weight of feces. In this study, 1-10% of the organisms visible in a direct microscopic count were isolated in culture.

Mitsuoka et al. (1973) used the plate-in-bottle method of producing anaerobiosis. This was a modification of the open-tube method in which special flat, square culture plates were incubated inside of large glass bottles which were stoppered and filled with an oxygen-free atmosphere. They also incubated some media aerobically and used the steel wool method of Parker (1955) to incubate other media anaerobically. Using these methods and a variety of selective and nonselective primary isolation media, they isolated and enumerated bacteria from bovine feces. Direct microscopic counts of the bovine feces ranged from $2.5x10^8$ to $7.9x10^9$ organisms per gram wet weight of feces. Cultural counts of bifidobacteria were in the range of $0\text{-}3.1x10^5$ organisms per gram wet weight of feces, counts of clostridia were in the range of $0\text{-}1.3x10^7$ organisms per gram wet weight of feces, counts of veillonellae were in the range of $0\text{-}7.9x10^6$ organisms per gram wet weight of feces, counts of catenabacteria were in the range of

$0\text{-}2.0\times10^{7}$ organisms per gram wet weight of feces, counts of peptostreptococci were in the range of $0\text{-}2.0\times10^{8}$ organisms per gram wet weight of feces, counts of streptococci were in the range of $5.0\times10^{3}\text{-}5.0\times10^{7}$ organisms per gram wet weight of feces, counts of lactobacilli were in the range of $1.6\times10^{2}\text{-}4.0\times10^{3}$ organisms per gram wet weight of feces, counts of staphylococci were in the range of $0\text{-}6.3\times10^{4}$ organisms per gram wet weight of feces, counts of corynebacteria were in the range of $1.3\times10^{5}\text{-}1.3\times10^{8}$ organisms per gram wet weight of feces, counts of molds were in the range of $7.9\times10^{2}\text{-}2.5\times10^{3}$ organisms per gram wet weight of feces, counts of yeasts were in the range of $0\text{-}5.0\times10^{2}$ organisms per gram wet weight of feces, and counts of spirochetes were in the range of $0\text{-}5.0\times10^{8}$ organisms per gram wet weight of feces. Counts of *Enterobacteriaceae* were in the range of $1.6\times10^{5}\text{-}6.3\times10^{7}$ organisms per gram wet weight of feces, counts of *Bacteroidaceae* were in the range of $2.0\times10^{7}\text{-}5.0\times10^{9}$ bacteria per gram wet weight of feces, *Spirillaceae* counts were in the range of $2.0\times10^{8}\text{-}2.5\times10^{9}$ bacteria per gram wet weight of feces, and counts of *Bacillus* were in the range of $1.6\times10^{6}\text{-}6.3\times10^{6}$ bacteria per gram wet weight of feces. Identification of these groups of organisms was done using a minimum of testing and should be considered as being only presumptive identification of each group.

MATERIALS AND METHODS

Source of Fecal Samples

All fecal samples were obtained from purebred Hereford steers kept in open pens. In the first 3 trials the animals sampled were fed a diet consisting of 6 lb of corn per animal per day plus as much hay as they would consume, which was greater than 12 lb per animal per day. The animal sampled in the fourth trial was fed a diet consisting of 18 lb of shelled corn per day, 1-5 lb of a 32% protein supplement per day, and as much silage as it would consume.

Sampling Procedure

The methods used for collecting and processing fecal samples were based upon the methods of Moore and Holdeman (1974). Samples were taken of fecal specimens on the ground, immediately after normal defecation. Fecal specimens having an abnormal consistency were rejected. A large, sterile, stainless steel spatula with a blade measuring 20x100 mm (#14-365B, Fisher Scientific Company) was used to repeatedly sample from the central region of the specimen. The upper region, which was directly exposed to atmospheric oxygen, and the bottom region, which was presumably contaminated with soil organisms, were avoided in this sampling so as to attempt to obtain a sample representative of the fecal mass immediately prior to defecation. An attempt was made to obtain a sample of between 100 and 200 g, which was large enough

to fill the sample bag used[1].

The sample bag used was a sterile Gono-Pak plastic sample bag (#B1018, Nasco). After the bag was filled with the fecal sample, the bag was squeezed to express as much air as possible in order to minimize exposure of the sample to atmospheric oxygen. The bag was sealed by whirling and tying before being transported, at ambient temperatures, to the laboratory. The sample was transported rapidly so that the total transport time was less than 30 min to minimize flora changes due to temperature factors[2] or oxygen exposure[3]. Moore and Holdeman (1974) found that transportation of human fecal samples at ambient temperatures of 20-30 C under a carbon dioxide atmosphere caused no statistically-significant changes in the bacterial flora within a time period of 48 hr.

At the laboratory the bag was opened aseptically and an oxygen-free carbon dioxide cannula was placed in the mouth of the bag in order to flush any remaining air from the bag. The bag was then reclosed and kneaded thoroughly in all directions to homogenize the fecal sample. This mixing of the sample was necessary due to the heterogeneity of fecal samples, as was found to be the case with human fecal samples (Moore and Holdeman, 1974).

One or more subsamples of approximately 1 g each, for bacteriological analysis, were taken while the sample bag was being flushed with oxygen-free carbon dioxide from a cannula. A micro-spatula with a blade measuring 8x55 mm (#S1569, Scientific

Products) was alcohol-sterilized in 70% (v/v) aqueous ethanol before being used to take the subsamples from the central region of the homogenized sample. These subsamples were immediately placed in anaerobic dilution tubes. The sample bag was kneaded after each subsampling.

After the subsamples for bacteriological analysis had been taken, subsamples of approximately 20 g each were taken for the determination of the moisture content of the sample. The subsamples were taken using a large spatula. As many subsamples were taken as allowed by the size of the original sample, with 6 being the maximum number of subsamples taken, with no kneading of the sample bag between subsamplings[4]. The subsamples were placed on drying plates for the determination of moisture content.

Procedure for Bacteriological Plating

The procedure for bacteriological plating was based upon that used by Moore and Holdeman (1974). Each subsample for bacteriological analysis was placed in a preweighed 9.0 ml gelatin dilution tube containing 6 glass beads (#G6000-4, 6 mm diameter glass beads, Scientific Products), using an alcohol-sterilized micro-spatula. During this operation the dilution tube was continuously flushed with oxygen-free carbon dioxide to exclude air. After addition of the subsample, the dilution tube was reweighed after the tube was stoppered. The weight of the dilution tube was subtracted from the weight of the dilution tube with the sample added to determine the wet weight of the fecal subsample.

This first dilution tube was designated the 10^{-1} dilution tube[5]. The 10^{-1} dilution tube was 10-fold serially diluted out to the 10^{-8} dilution using additional 9.0 ml gelatin dilution tubes. In each step of the dilution series the source tube was vigorously shaken vertically with the tube held in a vertical position[6]. A 1.1x1.1-1.0 ml pipette (#1258-22222, Bellco Glass Inc.) was used to transfer the 1.0 ml sample from the center of the source tube to the next 9.0 ml dilution tube in the series. This process was repeated for each tube in the series.

Four replicate roll-pour tubes were prepared from each dilution tube used as a source of inoculum. Before being used as a source of inoculum, the dilution tube was vigorously shaken to homogenize the contents before a 1x0.1 ml pipette (#1229-01010, Bellco Glass Inc.) was used to remove a 0.1 ml sample from the center of the dilution tube. The 0.1 ml sample was added to a roll-pour tube of primary isolation medium which had been previously liquified by steaming and allowed to cool, to a temperature just above the solidification temperature, in a 55 C incubator. After addition of the sample, the tube was restoppered, inverted gently 4 times to completely mix the contents, and rolled in a sink full of cold water to solidify the agar medium.

The tubes were incubated in a 37 C incubator for 5 days in a vertical position and then were stored at room temperature until the colonies were counted and subcultured. All of the colonies in a tube were counted using a modified 20X stereoscopic dissecting

microscope. The colony count per gram dry weight of feces was calculated after correction for sample size, dilution factor of the dilution tube used as the source of inoculum, and percentage of moisture in the fecal sample.

Preparation of Subsamples for Moisture Determination

Each subsample for moisture determination was removed from the plastic sample bag using a large spatula. Plastic 100x15 mm petri dish halves (#1001, Falcon) were previously weighed on an analytical balance (#1581, Cenco Instruments Corporation) and then placed on a triple-beam balance (#750-S, Ohaus Scale Corporation). Fecal material was removed from the sample bag and placed in a petri dish half until the subsample weighed approximately 20 g. The subsample was then spread evenly on the inside of the petri dish half, using the large spatula, and the petri dish half and subsample were then reweighed using the analytical balance.

Moisture Determination

Prepared subsamples were dried to constant weight in a vacuum oven at 70 C under a reduced pressure of 20 in Hg. After constant weight was achieved, the petri dish halves were reweighed using the analytical balance. The wet weight of the fecal subsample was determined by subtracting the weight of the petri dish half alone from the weight of the petri dish half plus the subsample contained in it, before the drying process had begun. The dry weight of the fecal subsample was determined by subtracting the weight of the petri dish half alone from the weight of the subsample plus petri

dish half, after the subsample had been dried to constant weight. The percentage of solids was calculated by dividing the dry weight of feces by the wet weight of feces and then multiplying the quotient by 100. These 3 calculations were done for each fecal subsample.

Direct Microscopic Clump Counts

The procedure for direct microscopic clump counts was adapted from the procedure used by Moore and Holdeman (1974). The 10^{-3} and 10^{-4} dilution tubes of each dilution series were sampled for the direct microscopic clump count. The dilution tubes were thoroughly agitated to homogenize the contents, after which duplicate 0.01 ml subsamples were removed from the center of each dilution tube sampled using a 0.2x0.001 ml pipette (#13-677D, Fisher Scientific Company). Each subsample was evenly spread over an area, bounded by a ring of Vaspar[7], on a clean microscope slide (#M6147, Scientific Products). The sample was evenly spread by tilting the slide and allowing gravity to spread out the smear. The smear was allowed to air dry, was heat-fixed, and was gently Gram-stained using the Kopeloff method[8]. Ten fields from each smear were examined, 2 fields from the edge of the smear and 8 fields from the center of the smear, as recommended by Moore and Holdeman (1974). The average clump count per field was calculated for each smear, smears containing less than 100 clumps per field being considered countable smears. Two separate counts were made of each countable smear. The direct microscopic clump count was

calculated, taking into consideration the average clump count per field, the microscopic factor, and the dilution factor of the dilution tube sampled[9]. Each count of each smear was then corrected for the size of the fecal subsample and the percentage of solids in the fecal sample[10].

Counting and Subculturing Colonies

A modified 20X stereoscopic dissecting microscope was used to observe and subculture colonies in culture tubes. Before colony counting or subculturing was attempted, a helical black line was drawn up the side of each roll tube to act as a reference line. The reference line was produced by placing the roll tube upright in a tube streaker (#7790-33333, Bellco Glass Inc.) and spinning the tube while the tip of a waterproof marking pen (Sharpie Marking Pen, Sanford's) was held against the tube and slowly moved upward. The rate at which the marking pen was moved upward was such that adjacent parts of the helical line were approximately 0.5 cm apart. As a result, the adjacent parts of the line were close enough together that they were both visible in the visual field of the dissecting microscope at the same time. As a result, the area of the culture medium between the lines was completely visible in a single microscope field.

Colonies were counted by placing the roll tube on the plastic stage of the microscope and focusing on the agar surface closest to the objective lens. Colonies on or within the agar were counted by starting at the bottom of the tube and following the helical

line upward, keeping adjacent parts of the helical line on opposite sides of the visual field of the microscope. A tally counter (#B4117, Scientific Products) was used to record the colony count, which was considered to be valid if the tube being counted had between 25 and 325 colonies. The colony count for each countable tube was then corrected for the size of the fecal subsample, the moisture content of the fecal sample, and the dilution factor of the dilution tube sampled[11].

Subculturing of colonies was done while the tube was on the plastic microscope stage. A flame-sterilized oxygen-free carbon dioxide cannula was hung over the neck of the roll tube after the tube stopper had been flame-sterilized and removed and after the roll tube had been placed on the plastic microscope stage. Care was taken to prevent the cannula from being forced into the agar, which could cause plugging of the cannula, resulting in the oxidation of the medium in the roll tube. Tubes of maintenance medium were placed under the carbon dioxide cannulas on the transfer apparatus after the tube stoppers had been flame-sterilized and removed.

Isolated colonies were picked using a flame-sterilized bent iron picking needle[12]. After the picking needle was used to pick a colony, it was used to stab-inoculate a tube of semisolid maintenance medium. The inoculated tubes of maintenance medium were incubated at 37 C until growth occurred.

After the cultures in the maintenance medium had grown, they

were further purified using a pour-tube procedure. In this procedure, a straight iron picking needle[13] was used to transfer a small amount of inoculum from a tube of semisolid maintenance medium to a roll-pour tube of liquified primary isolation medium. The roll-pour tube was then mixed by gentle inversion and rolled in a sink full of cold water to solidify the agar. After all roll-pour tubes had been prepared, they were incubated in a vertical postion at 37 C until growth occurred. Isolated colonies in the roll-pour tubes were picked, using a bent iron picking needle, and tubes of maintenance medium were stab-inoculated. The tubes of maintenance medium were incubated at 37 C until growth occurred and then were stored at room temperature as stock cultures.

Trial 1 - Test of Media Used in a Human Fecal Flora Study (Moore and Holdeman, 1974)

This initial study was done to determine the feasibility of the general method for determining the moisture content of fecal samples, the use of aluminum foil squares as drying plates, and the use of RGCA as the primary isolation medium. In this study, 4x4 in squares of aluminum foil (Reynolds Wrap) were used as sample plates for drying fecal subsamples to constant weight. The plates were weighed at various time intervals during the drying process in order to determine the time necessary to dry the fecal subsamples to constant weight. A single dilution series was used in this study, with the 10^{-5}-10^{-8} dilution tubes being sampled for the cultural count. A direct microscopic clump count, using the

10^{-3} and 10^{-4} dilution tubes, was also done and RGCA roll-pour tubes were used for primary isolation.

Trial 2 - Comparison of Primary Isolation Media

This study was done to compare the cultural recoveries of 5 different media. In this study, petri dish halves were used as sample plates for the determination of fecal moisture content and the plates were again weighed at various time intervals during the drying process. A single dilution series was again used and the 10^{-5}-10^{-8} dilution tubes were again sampled from for the cultural count. A direct microscopic clump count was done to compare with the cultural counts in determining the percentage recovery of each medium compared in this study. The 5 media compared were: RGCA (Moore and Holdeman, 1974), a modification of RFM of Caldwell and Bryant (1966), and a modification of Medium 10 of Caldwell and Bryant (1966) containing 10, 20, or 30% clarified rumen fluid. Four replicate tubes of each of the 5 media tested were prepared for each dilution tube sampled from.

Trial 3 - Use of a Double Dilution Series

This study was done to make use of 2 fecal subsamples and a double dilution series to increase the statistical sampling of the fecal sample and to allow for an assessment of both the heterogeneity of the homogenized fecal sample and the various sampling errors in the bacteriological plating process. In this study, fecal subsamples for the determination of fecal moisture content were dried for 15 days. Double subsamples and dilution series were

used for the cultural count and modified Medium 10 containing 30% rumen fluid was used as the primary isolation medium. The 10^{-6}-10^{-8} dilution tubes from each dilution series were sampled for the cultural count and no direct microscopic clump count was used in this trial. Sweet E broth was used as the maintenance medium. The primary isolation tube to be subcultured into maintenance medium was chosen at random from the group of countable roll-pour tubes. All of the colonies in the chosen tube were then subcultured into individual tubes of maintenance medium. After growth had occurred in the tubes of maintenance medium, Gram stains, using the Kopeloff modification of the Gram stain, were done of each tube showing visible turbidity.

Trial 4 - Sampling of a Feedlot Steer

This study was done to make use of a fecal sample from an animal fed in a commercial feedlot. This study was done as for trial 3 with the exceptions that sample plates containing fecal subsamples for the determination of fecal moisture content were again weighed at various time intervals during the drying process and semisolid modified Medium 10 containing 30% rumen fluid was used as the maintenance medium. Countable tubes derived from subsample A were separated from the countable tubes derived from subsample B. One tube from each group was then selected at random and all of the colonies in each of the chosen tubes were then subcultured into individual tubes of maintenance medium.

Statistical Analysis of Data

Statistical analyses of data were performed according to Mendenhall (1971). The arithmetic mean[14] was calculated for the values obtained in each trial for percent solids. In addition the small-sample standard deviation[15] and the 90% confidence interval[16], calculated using Student's t distribution, were determined. The arithmetic mean was calculated for the values obtained in each trial for the direct microscopic clump count. The small-sample standard deviation and the 90% confidence interval were also determined for the values of the direct microscopic clump count. In trials 1, 3, and 4 the arithmetic mean was calculated for the values of the corrected plate count. In addition, the small-sample standard deviation and the 90% confidence interval were also determined. In trial 2, these calculations were done separately for each of the 5 primary isolation media tested. In trial 1, the percentage recovery[17] of the primary isolation medium was calculated and in trial 2 this was done separately for each of the 5 primary isolation media tested. In trial 2, all possible pairs of the 5 primary isolation media tested were compared with respect to the mean corrected plate count using the t test for the difference between 2 means[18]. The hypothesis that the 2 means were equal was tested at both the 20 and 1% level of significance. In the maintenance medium tests in trials 3 and 4, the cultural recovery[19] of each medium was calculated. In trial 4, the cultural recovery of modified Medium 10 containing 30% rumen fluid was

separately calculated for each of the 2 sets of subcultures.

RESULTS

One of the principal objectives in this study was to find a primary isolation medium which would support the growth of a large proportion of the organisms present in bovine feces. After a suitable primary isolation medium was found, the search was begun for a suitable maintenance medium.

Trial 1 - Medium Used in a Human Fecal Flora Study

The squares of aluminum foil used in the drying of fecal subsamples for moisture content determination showed extensive corrosion at the end of the drying period. Table 1 shows the results of the moisture content determination. Drying was complete in 175.5 hr. A mean of 21.7% solids was obtained in this study, with a standard deviation of 0.544 and a 90% confidence interval of 21.25-22.15% solids. Table 2 shows the results of the direct microscopic clump count procedure. A mean of 4.15×10^9 bacteria per gram dry weight of feces was obtained using the direct microscopic clump count procedure, with a standard deviation of 9.15×10^8 and a 90% confidence interval of 3.07×10^9-5.23×10^9 bacteria per gram dry weight of feces. Table 3 shows the results of the colony count procedure. A mean of 2.0×10^8 bacteria per gram dry weight of feces was obtained using the colony count procedure, with a standard deviation of 4.1×10^7 and a 90% confidence interval of 1.51×10^8-2.47×10^8 bacteria per gram dry weight of feces. RGCA medium was used as the primary isolation medium in this study. It supported

Table 1. Trial 1 moisture determination

PLATE NUMBER	WEIGHT OF FECES ALONE (G) DRYING TIME (HR) 0	48	96	151	175.5	305.5	PERCENT SOLIDS
1	19.43	4.32	4.19	4.15	4.12*	4.16	21.2
2	23.38	5.59	5.40	5.33	5.31*	5.34	22.7
3	17.97	4.05	3.89	3.87	3.85*	3.87	21.4
4	17.69	4.06	3.92	3.89	3.86*	3.89	21.8
5	16.48	3.77	3.58	3.57	3.54*	3.56	21.5
6	10.86	2.65	2.35	2.34	2.32*	2.34	21.4

* Lowest value for dried weight.

Table 2. Trial 1 direct microscopic clump count[a]

SMEAR	COUNT NUMBER	AVERAGE COUNT PER FIELD[b]	DMCC[c]	CORRECTED DMCC[d]
A	1	43.6	1.91×10^{10}	2.98×10^{9}
A	2	60.6	2.65×10^{10}	4.14×10^{9}
B	1	76.5	3.34×10^{10}	5.21×10^{9}
B	2	62.7	2.74×10^{10}	4.28×10^{9}

[a] With a microscopic factor of 4.37×10^{5}, a reciprocal of the dilution tube sampled of 1×10^{3}, a fecal sample of 1.45 g wet weight, a sample size correction factor of 1.39, and a solids percentage of 21.7.

[b] Bacteria per microscopic field.

[c] Direct microscopic clump count.

[d] Direct microscopic clump count corrected for size of fecal sample and moisture content of fecal sample.

Table 3. Trial 1 colony counts[a] using RGCA primary isolation medium

DILUTION OF ROLL TUBE COUNTED	TUBE NUMBER	COLONY COUNT OF TUBE[b]	PLATE COUNT[c]	CORRECTED PLATE COUNT[d]
10^{-7}	1	129	1.29×10^{9}	2.01×10^{8}
10^{-7}	2	161	1.61×10^{9}	2.51×10^{8}
10^{-7}	3	95	9.5×10^{9}	1.5×10^{8}
10^{-7}	4	126	1.26×10^{9}	1.97×10^{8}

[a] With a reciprocal of the dilution tube sampled of 1×10^{7}, a fecal sample of 1.45 g wet weight, a sample size correction factor of 1.39, and solids percentage of 21.7.

[b] Colonies per tube.

[c] Colonies per g.

[d] Colonies per gram dry weight of feces.

the growth of 4.8% of the bacteria visible in a direct microscopic clump count.

Trial 2 - Comparison of Primary Isolation Media

The plastic petri dish halves used in the drying of fecal samples were not affected by the samples and did not appear to affect the samples. Table 4 shows the results of the moisture content determination. Drying was complete in 119 hr. A mean of 23.2% solids was obtained in the moisture determination procedure, with a standard deviation of 0.407 and a 90% confidence interval of 22.86-23.54% solids. Table 5 shows the results of the direct microscopic clump count procedure. A mean of 3.6×10^9 bacteria per gram dry weight of feces was obtained using the direct microscopic clump count procedure, with a standard deviation of 3.6×10^8 and a 90% confidence interval of 3.18×10^9-4.02×10^9. Table 6 shows the results of the colony count procedure using the 5 different primary isolation media. A mean of 1.1×10^9 bacteria per gram dry weight of feces was obtained in the colony count using RGCA as the primary isolation medium, with a standard deviation of 2.4×10^8 and a 90% confidence interval of 8.2×10^8-1.4×10^9 bacteria per gram dry weight of feces. A mean of 9.4×10^8 bacteria per gram dry weight of feces was obtained in the colony count using RFM as the primary isolation medium, with a standard deviation of 3.1×10^8 and a 90% confidence interval of 4.2×10^8-1.5×10^9 bacteria per gram dry weight of feces. A mean of 1.3×10^9 bacteria per gram dry weight of feces was obtained in the colony count using modified Medium 10 containing 10% rumen

Table 4. Trial 2 moisture determination

PLATE NUMBER	WEIGHT OF FECES ALONE (G) DRYING TIME (HR) 0	24	48	72	95	119	144	PERCENT SOLIDS
1	21.48	5.45	5.47	5.14	5.07	5.04*	5.05	23.5
2	21.22	5.65	5.25	5.06	4.99	4.96*	4.96	23.4
3	20.42	8.22	5.04	4.86	4.80	4.76*	4.77	23.3
4	21.34	8.48	5.16	5.01	4.94	4.92*	4.92	23.1
5	22.91	5.79	5.79	5.50	5.40	5.36*	5.36	23.4
6	20.94	5.29	4.91	4.81	4.72	4.69*	4.69	22.4

* Lowest value for dried weight.

Table 5. Trial 2 direct microscopic clump count[a]

SMEAR	COUNT NUMBER	AVERAGE COUNT PER FIELD[b]	DMCC[c]	CORRECTED DMCC[d]
A	1	39.8	1.74×10^{10}	3.5×10^{9}
A	2	39.2	1.71×10^{10}	3.4×10^{9}
B	1	46.6	2.04×10^{10}	4.1×10^{9}
B	2	37.6	1.64×10^{10}	3.3×10^{9}

[a] With a microscopic factor of 4.37×10^{5}, a reciprocal of the dilution tube sampled of 1×10^{3}, a fecal sample of 0.85 g wet weight, a sample size correction factor of 0.863, and a solids percentage of 23.2.

[b] Bacteria per microscopic field.

[c] Direct microscopic clump count.

[d] Direct microscopic clump count corrected for size of fecal sample and moisture content of fecal sample.

Table 6. Trial 2 colony counts[a] using 5 different primary isolation media

MEDIUM USED[b]	DILUTION OF ROLL TUBE COUNTED	TUBE NUMBER	COLONY COUNT OF TUBE[c]	RECIPROCAL OF DILUTION SAMPLED	PLATE COUNT[d]	CORRECTED PLATE COUNT[e]
1	10^{-8}	1	50	$1x10^{8}$	$5.00x10^{9}$	$1.3x10^{9}$
1	10^{-8}	2	33	$1x10^{8}$	$3.30x10^{9}$	$8.9x10^{8}$
1	10^{-8}	3	31	$1x10^{8}$	$3.10x10^{9}$	$8.3x10^{8}$
1	10^{-8}	4	45	$1x10^{8}$	$4.50x10^{9}$	$1.2x10^{9}$
2	10^{-7}	1	267	$1x10^{7}$	$2.67x10^{9}$	$7.2x10^{8}$
2	10^{-8}	1	30	$1x10^{8}$	$3.00x10^{9}$	$8.1x10^{8}$
2	10^{-8}	3	47	$1x10^{8}$	$4.70x10^{9}$	$1.3x10^{9}$
3	10^{-8}	1	58	$1x10^{8}$	$5.80x10^{9}$	$1.6x10^{9}$
3	10^{-8}	2	55	$1x10^{8}$	$5.50x10^{9}$	$1.5x10^{9}$
3	10^{-8}	3	27	$1x10^{8}$	$2.70x10^{9}$	$7.3x10^{8}$
4	10^{-7}	1	305	$1x10^{7}$	$3.05x10^{9}$	$8.2x10^{8}$
4	10^{-8}	1	45	$1x10^{8}$	$4.50x10^{9}$	$1.2x10^{9}$
4	10^{-8}	2	68	$1x10^{8}$	$6.80x10^{9}$	$1.8x10^{9}$
5	10^{-8}	1	59	$1x10^{8}$	$5.90x10^{9}$	$1.6x10^{9}$
5	10^{-8}	2	69	$1x10^{8}$	$6.90x10^{9}$	$1.9x10^{9}$
5	10^{-8}	3	61	$1x10^{8}$	$6.10x10^{9}$	$1.6x10^{9}$
5	10^{-8}	4	70	$1x10^{8}$	$7.00x10^{9}$	$1.9x10^{9}$

[a] With a fecal sample of 0.85 g wet weight, a sample size correction factor of 0.863, and a solids percentage of 23.2.

[b] 1=RCCA, 2=RFM, 3=Modified Medium 10 containing 10% rumen fluid, 4=Modified Medium 10 containing 20% rumen fluid, and 5=Modified Medium 10 containing 30% rumen fluid.

[c] Colonies per tube.

[d] Colonies per g.

[e] Colonies per gram dry weight of feces.

fluid as the primary isolation medium, with a standard deviation of $4.8x10^8$ and a 90% confidence interval of $4.9x10^8$-$2.1x10^9$ bacteria per gram dry weight of feces. A mean of $1.3x10^9$ bacteria per gram dry weight of feces was obtained in the colony count using modified Medium 10 containing 20% rumen fluid as the primary isolation medium, with a standard deviation of $5.0x10^8$ and a 90% confidence interval of $4.6x10^8$-$2.1x10^9$ bacteria per gram dry weight of feces. A mean of $1.8x10^9$ bacteria per gram dry weight of feces was obtained in the colony count using modified Medium 10 containing 30% rumen fluid as the primary isolation medium, with a standard deviation of $1.8x10^8$ and a 90% confidence interval of $1.6x10^9$-$2.0x10^9$. Table 7 shows the results of the comparison of colony counts of 5 different primary isolation media, using the t test for the difference between 2 population means. RGCA, RFM, and modified Medium 10 containing either 10 or 20% rumen fluid did not give significantly different recoveries at the 20% level of significance. On the other hand, colony counts using modified Medium 10 containing 30% rumen fluid were significantly higher than colony counts using the other 4 primary isolation media. The colony counts using modified Medium 10 containing 30% rumen fluid were superior at the 20% level of significance to the colony counts using modified Medium 10 containing either 10 or 20% rumen fluid and were superior at the 1% level of significance to the colony counts using either RGCA or RFM. Recovery percentages for the 5 media ranged from 26% with RFM to 50% with modified Medium 10 containing 30% rumen fluid.

Table 7. Trial 2 medium comparison using the t test for the difference between 2 population means.

MEDIUM #1[a]	MEDIUM #2[a]	SD_1^2 [b]	SD_2^2 [c]	n_1 [d]	n_2 [e]	SD_x^2 [f]	SD_x [g]	$mean_1$ [h]	$mean_2$ [i]	t	DF[j]	CV 20% [k]	CV 1% [l]	DIFF 20% [m]	DIFF 1% [n]
1	2	5.6×10^{16}	9.7×10^{16}	4	3	7.2×10^{16}	2.7×10^{8}	1.1×10^{9}	9.4×10^{8}	0.78	5	±1.5	±4.0	no	no
1	3	5.6×10^{16}	2.3×10^{17}	4	3	1.3×10^{17}	3.6×10^{8}	1.1×10^{9}	1.3×10^{9}	-0.73	5	±1.5	±4.0	no	no
1	4	5.6×10^{16}	2.5×10^{17}	4	3	1.3×10^{17}	3.6×10^{8}	1.1×10^{9}	1.3×10^{9}	-0.73	5	±1.5	±4.0	no	no
1	5	5.6×10^{16}	3.3×10^{16}	4	4	4.5×10^{16}	2.1×10^{8}	1.1×10^{9}	1.8×10^{9}	-4.7	6	±1.4	±3.7	yes	yes
2	3	9.7×10^{16}	2.3×10^{17}	3	3	1.6×10^{17}	4.0×10^{8}	9.4×10^{8}	1.3×10^{9}	-1.1	4	±1.5	±4.6	no	no
2	4	9.7×10^{16}	2.5×10^{17}	3	3	1.7×10^{17}	4.1×10^{8}	9.4×10^{8}	1.3×10^{9}	-1.1	4	±1.5	±4.6	no	no
2	5	9.7×10^{16}	3.3×10^{16}	3	4	5.9×10^{16}	2.4×10^{8}	9.4×10^{8}	1.8×10^{9}	-4.7	5	±1.5	±4.0	yes	yes
3	4	2.3×10^{17}	2.5×10^{17}	3	3	2.4×10^{17}	4.9×10^{8}	1.3×10^{9}	1.3×10^{9}	0	4	±1.5	±4.6	no	no
3	5	2.3×10^{17}	3.3×10^{16}	3	4	1.1×10^{17}	3.3×10^{8}	1.3×10^{9}	1.8×10^{9}	-2.0	5	±1.5	±4.0	yes	no
4	5	2.5×10^{17}	3.3×10^{16}	3	4	1.2×10^{17}	3.5×10^{8}	1.3×10^{9}	1.8×10^{9}	-1.9	5	±1.5	±4.0	yes	no

[a] 1=RGCA, 2=RFM, 3=modified Medium 10 containing 10% rumen fluid, 4=modified Medium 10 containing 20% rumen fluid, and 5=modified Medium 10 containing 30% rumen fluid.

[b] (Small-sample standard deviation for Medium #1)^2.

[c] (Small-sample standard deviation for Medium #2)^2.

[d] Number of measurements for Medium #1.

[e] Number of measurements for Medium #2.

[f] Pooled estimator of (standard deviation)^2.

[g] Pooled estimator of standard deviation.

[h] Arithmetic mean for Medium #1.

[i] Arithmetic mean for Medium #2.

[j] Degrees of freedom.

[k] Critical value (20% level of significance).

[l] Critical value (1% level of significance).

[m] Significant difference at 20% level of significance.

[n] Significant difference at 1% level of significance.

Trial 3 - Use of Double Dilution Series

Table 8 shows the results of the moisture content determination. Subsamples of the fecal sample in this trial were dried for 360 hr to constant weight. A mean of 17.8% solids was obtained in the moisture determination procedure, with a standard deviation of 0.265 and a 90% confidence interval of 17.58-18.02% solids. Table 9 shows the results of the colony count procedure. A mean of 5.6×10^9 bacteria per gram dry weight of feces was obtained using modified Medium 10 containing 30% rumen fluid, with a standard deviation of 1.9×10^9 and a 90% confidence interval of 4.2×10^9-7.0×10^9 bacteria per gram dry weight of feces, in the colony count procedure. Sweet E broth, when used as a maintenance medium, supported the growth of 61.1% of the isolates from the primary isolation tube. Most of the bacteria able to grow in Sweet E broth were Gram-negative bacilli. Most of them were vacuolated and morphologically similar to bacteria of the genus *Bacteroides*. A lesser number were helical in shape and morphologically resembled bacteria of the genus *Campylobacter*. A still smaller group of Gram-negative bacilli found were morphologically similar to bacteria of the genus *Fusobacterium*. A small number of Gram-positive cocci were also found to grow in the Sweet E broth. Some were morphologically similar to fecal streptococci while others were morphologically similar to bacteria of the genus *Staphylococcus*. A small number of Gram-positive bacilli were also found to grow in Sweet E broth. Some of these morphologically resembled bacteria of the

Table 8. Trial 3 moisture determination

PLATE NUMBER	WEIGHT FECES (G)[a] DRYING TIME (HR) 0	360	PERCENT SOLIDS
1	22.05	3.90	17.7
2	21.39	3.79	17.7
3	26.27	4.61	17.5
4	20.95	3.68	17.6
5	24.50	4.47	18.2
6	23.15	4.16	18.0

[a] Weight of feces alone.

Table 9. Trial 3 colony counts[a] using modified Medium 10 containing 30% rumen fluid

SUBSAMPLE	DILUTION OF ROLL TUBE COUNTED	TUBE NUMBER	COLONY COUNT OF TUBE[b]	RECIPROCAL OF DILUTION SAMPLED	PLATE COUNT[c]	CORRECTED PLATE COUNT[d]
A	10^{-8}	1	203	1×10^{8}	2.03×10^{10}	4.2×10^{9}
A	10^{-8}	2	211	1×10^{8}	2.11×10^{10}	4.4×10^{9}
A	10^{-8}	3	187	1×10^{8}	1.87×10^{10}	3.9×10^{9}
A	10^{-9}	1	31	1×10^{9}	3.10×10^{10}	6.4×10^{9}
B	10^{-8}	1	189	1×10^{8}	1.89×10^{10}	5.4×10^{9}
B	10^{-8}	3	199	1×10^{8}	1.99×10^{10}	5.7×10^{9}
B	10^{-9}	2	33	1×10^{9}	3.30×10^{10}	9.4×10^{9}

[a] With a fecal sample of 0.85 (subsample A) or 0.60 (subsample B) g wet weight, a sample size correction factor of 0.863 (subsample A) or 0.625 (subsample B), and a solids percentage of 17.8.

[b] Colonies per tube.

[c] Colonies per g.

[d] Colonies per gram dry weight of feces.

genus Lactobacillus while others may have been clostridia which do not sporulate readily. A small number of Gram-positive coryneform bacilli were also found to grow in Sweet E broth. These were morphologically similar to bacteria of the genus Propionibacterium and may be contaminants from the skin of the technician.

Trial 4 - Sampling of a Feedlot Steer

In this trial a Hereford steer being fed in a commercial small feedlot operation was sampled. Table 10 shows the results of the moisture content determination. Subsamples of the fecal sample in this trial were dried for 169 hr to constant weight. A mean of 23.5% solids was obtained in the moisture determination procedure, with a standard deviation of 0.383 and a 90% confidence interval of 23.05-23.95% solids. Table 11 shows the results of the colony count procedure. A mean of 1.2×10^9 bacteria per gram dry weight of feces was obtained in the colony count procedure using modified Medium 10 containing 30% rumen fluid as the primary isolation medium, with a standard deviation of 2.4×10^8 and a 90% confidence interval of 1.0×10^9-1.4×10^9 bacteria per gram dry weight of feces. Semisolid modified Medium 10 containing 30% rumen fluid was capable of supporting the growth of 100% of the isolates from the primary isolation tubes. Cultures of these isolates were not viable after 1 week of storage at room temperature.

Table 10. Trial 4 moisture determination

PLATE NUMBER	WEIGHT OF FECES ALONE (G) DRYING TIME (HR) 0	24.5	48.25	71.5	120	144.5	169	PERCENT SOLIDS
1	20.84	5.13	5.10	5.02	4.88	4.87*	4.89	23.4
2	22.11	5.51	5.44	5.39	5.14	5.14	5.13*	23.2
3	22.51	6.05	5.78	5.65	5.40*	5.41	5.41	24.0
4	21.06	6.92	5.09	5.05	4.88*	4.88	4.88	23.2

* Lowest value for dried weight.

Table 11. Trial 4 colony counts[a] using modified Medium 10 containing 30% rumen fluid

SUBSAMPLE	DILUTION OF ROLL TUBE COUNTED	TUBE NUMBER	COLONY COUNT OF TUBE[b]	RECIPROCAL OF DILUTION SAMPLED	PLATE COUNT[c]	CORRECTED PLATE COUNT[d]
A	10^{-8}	1	36	$1x10^{8}$	$3.60x10^{9}$	$1.3x10^{9}$
A	10^{-8}	2	40	$1x10^{8}$	$4.00x10^{9}$	$1.5x10^{9}$
A	10^{-8}	3	33	$1x10^{8}$	$3.30x10^{9}$	$1.2x10^{9}$
A	10^{-8}	4	39	$1x10^{8}$	$3.90x10^{9}$	$1.4x10^{9}$
B	10^{-8}	1	57	$1x10^{8}$	$5.70x10^{9}$	$8.8x10^{8}$
B	10^{-8}	4	66	$1x10^{8}$	$6.60x10^{9}$	$1.0x10^{9}$

[a] With a fecal sample of 0.62 (subsample A) or 1.62 (subsample B) g wet weight, a sample size correction factor of 0.644 (subsample A) or 1.53 (subsample B), and a solids percentage of 23.5.

[b] Colonies per tube.

[c] Colonies per g.

[d] Colonies per gram dry weight of feces.

DISCUSSION

Animals used in study. In the first 3 trials, control cattle used in a feed trial were used as the source of fecal samples. These animals were used because they were kept under carefully controlled constant conditions, were fed a carefully controlled diet, and were carefully monitored during their life. The diet fed these control animals was not, however, representative of diets which would be fed to animals in an actual feedlot operation. Since the intent of this study was to study the bovine fecal flora with respect to refeeding bovine wastes in a feedlot operation and the diet of the animal may influence its fecal flora, it was decided to include trial 4 in which the animal sampled was fed a diet used in a feedlot operation.

Moisture content of feces. It has been found that the moisture content of human fecal samples varies between groups of humans and between individual humans. As a result, it has been found to be necessary to determine the moisture content of human fecal samples in order that bacterial counts can be expressed in terms of bacteria per gram dry weight of feces. Bacterial counts of human feces must be expressed in this manner in order to compare counts made of different individuals and different groups of people (Moore and Holdeman, 1974). It was assumed in this study that this individual and group variation in fecal moisture content found in humans also

occurred in the bovine. With this in mind, all direct microscopic clump counts and cultural counts were expressed in terms of bacteria per gram dry weight of feces. The aluminum foil plates used in trial 1 were found to be unsatisfactory for drying fecal subsamples because of extensive corrosion. The chemical reaction occurring between the subsample and the sample plate would be expected to affect the weight of the subsample. With this in mind, the plastic petri dish halves were selected to replace the aluminum foil sample plates for trials 2-4 because of their light weight, large surface area, and chemical inertness. The subsample sizes used in the moisture determination were chosen to allow rapid drying of the subsample. In trial 1, the fecal material was spread to a thickness of approximately 0.125 in on each sample plate. This was found to allow fairly rapid drying but had the disadvantage of allowing a wide variation in the weights of the fecal subsamples. This problem was alleviated in trials 2-4 by using subsamples of a constant 20 g weight. When a 20 g fecal subsample was spread evenly within the petri dish half, the layer of fecal material was thin enough to dry to constant weight in a time period of 2 weeks. The subsamples were dried at a reduced pressure of 20 in Hg because that was the lowest pressure the particular vacuum oven used could safely withstand. Drying was done at a temperature of 70 C in order to prevent the formation of an impermeable crust at the top of the subsample. This crust, which tends to be formed at higher drying temperatures, greatly slows down the drying process by preventing

evaporation of moisture from the fecal material below the crust. The sample plates were weighed at various time intervals in trials 1 and 2 in order to determine the approximate amount of time required to dry a fecal subsample. The longest time, 175.5 hr in trial 1, was doubled to give a safety factor and the subsamples in trial 3 were dried for this length of time, which was approximately 2 weeks, and were then reweighed. In trial 4, the sample plates were again weighed at intervals because a different herd of animals was used and these animals were fed a different diet than the animals sampled in the first 3 trials. The herd and diet change could have changed the drying characteristics of the fecal material.

Direct microscopic clump count. Direct microscopic clump counts are necessary in fecal flora studies in order to determine the effectiveness of the culture medium and method of achieving anaerobiosis for the cultivation of fecal bacteria. Direct microscopic clump counts of fecal material are, however, extremely difficult to interpret. A considerable amount of fiberous and granular material is present in the smears. This material often looks much like bacteria. In addition, some precipitate, which may be mistaken for small cocci, from the Gram stain reagents may be present. These factors make direct microscopic clump counts unreliable to an unknown degree. Direct microscopic clump counts were done in trials 1 and 2 to determine the cultural recoveries of the media. Direct microscopic clump counts were not done in trials 3

and 4 because of the inherent unreliability of the counts. Vaspar rings were produced using a rubber "O" ring in the direct microscopic clump count procedure because this process is much faster, easier, and more reproducible than is the process of producing wax squares using a wax pencil and a ruler. The only change made in the procedure for direct microscopic clump counts was that the formulas for calculating the number of bacteria per milliliter of sample were changed to accomodate a different smear area.

Cultural count. The first assumption made about the bovine fecal bacteria was that the organisms present were nutritionally similar to organisms making up the human fecal bacterial flora. As a result, RGCA was the first medium used in an attempt to cultivate bovine fecal bacteria because it was the medium used in the human fecal bacterial flora study by Moore and Holdeman (1974). When RGCA was found to be a relatively poor medium for the cultivation of bovine fecal bacteria, an assumption was made that the organisms present were nutritionally similar to bovine rumen bacteria. As a result, 3 different media used in rumen flora studies were used in an attempt to cultivate bovine fecal bacteria. RGCA, in addition to being a medium used in a human fecal flora study, was also a medium used in rumen flora studies. It was used in trial 2 as a basis for comparison with the other media studied. RFM was selected as one of the media to be tested because it has been stated to be one of the best nonselective media for the isolation of

carbohydrate-fermenting rumen bacteria (Caldwell and Bryant, 1966). Medium 10 was selected as the third basic medium to be tested in trial 2 because Caldwell and Bryant (1966) found that it was one of the best media for established cultures of rumen bacteria. It was decided to use rumen fluid in place of the volatile fatty acid mixture used in Medium 10 because it has been proposed that certain unknown growth factors are present in rumen fluid and are not present in the volatile fatty acid mixture. It was decided to retain the trypticase, yeast extract, Vitamin K, and hemin in the formula for Medium 10 in an attempt to correct possible deficiencies of the particular batch of rumen fluid used. This was necessary because the composition of rumen fluid varies with the particular animal it is obtained from, the time after feeding the rumen fluid is collected, and the diet the animal is being fed. Three levels of rumen fluid were tested with the modified Medium 10 in order to determine the optimum level of rumen fluid for maximum recovery of organisms. The anaerobic salts solution of Holdeman and Moore (1975) was used instead of the salts solutions ordinarily used for RGCA, RFM, and Medium 10 in order to prevent the addition of another variable in the study. All of the media used in the study were prepared using this anaerobic salts solution.

Medium comparison. Since it was expected that the colony counts for the 5 media tested would be nearly the same, it was decided to use Student's t test to attempt to determine if any of the 5 media

were significantly superior, in terms of colony counts, to the other media. Four repetitions of each dilution tube sampled were initially done, this being the maximum number of roll-pour tubes of each medium 1 technician could prepare from each dilution tube in a reasonable length of time. It was expected that 1 of the 4 roll-pour tubes of each medium, prepared from each dilution tube sampled, would likely be unusable because of oxidation of the medium, breakage of the tube in a laboratory accident, lumps in the medium, an uneven distribution of colonies in the medium, or slippage of the agar film down into the bottom of the tube.

Gram stains. Gram stains were done of the cultures in Sweet E broth because this medium had a lower concentration of agar-agar in it than did the semisolid modified Medium 10 containing 30% rumen fluid. Stains penetrate very poorly in smears made from cultures in semisolid medium. As a result of this, many marginally Gram-positive organisms will stain Gram-negative when stained from a culture grown in semisolid medium. The Gram stain results of cultures from Sweet E broth should be interpreted with extreme caution. Initially, since this medium is unable to support growth of the organisms for more than 1 week, the cells stained may be dead or dying, or may be morphologically atypical because of nutritional deficiencies of the medium. In addition, a single Gram stain of a particular culture is an extremely undependable way of determining the true Gram stain reaction and morphology of an

organism, especially of anaerobic bacteria which are extremely pleomorphic even in a nutritionally proper medium. Many Gram stains should be done of each culture after various periods of incubation and in various media to get a true indication of the Gram reaction and morphology of the organism. Finally, because of the fact that anaerobic bacteria are so pleomorphic and many of the bacteria of the fecal flora may be unnamed and/or morphologically atypical for the genus they belong to, it is virtually impossible to identify a particular organism as being a member of a particular genus with any degree of accuracy using only the Gram stain results as the criteria for genus selection.

Moisture content determination. The moisture content of bovine feces varied between individual animals to a certain extent. It appears that individual variation is probably as great as variation produced by different diets. The overall average solids percentage for all subsamples of all 4 trials was 21.3% solids. This is very close to the value of 21.5% solids reported for human feces (Moore and Holdeman, 1974).

Discussion of results of direct microscopic clump counts. The direct microscopic clump counts in trials 1 and 2 did not differ greatly. The overall average direct microscopic clump count for trials 1 and 2 was 3.9×10^9 bacteria per gram dry weight of feces. This was 0.77% of the direct microscopic clump count for human

feces, as reported by Moore and Holdeman (1974). This considerable difference in direct microscopic clump counts between human and bovine feces tends to indicate that there may be considerable basic differences between the human and bovine fecal floras.

Discussion of results of cultural counts. The cultural counts in the 4 trials showed variation between individual animals. The individual variation is probably as great as the variation produced by different diets. The overall average cultural count, using modified Medium 10 containing 30% rumen fluid, for trials 2-4 was 3.2×10^9 bacteria per gram dry weight of feces. This was 0.67% of the cultural count for human feces, as reported by Moore and Holdeman (1974). This difference in cultural counts between human and bovine fecal samples may indicate that the human and bovine fecal floras may be considerably different.

Discussion of comparison of results between present and previous studies. The direct microscopic clump counts and the cultural counts obtained in this study can not be compared with the other bovine fecal flora studies because these other studies by Hartman et al. (1966), Maki and Picard (1965), and Mitsuoka et al. (1973) did not include a moisture determination step. Since the moisture content of the feces used in these other studies is unknown, no meaningful comparison can be made between the present study and these other studies.

Discussion of results of gram stains. If the Gram-stain identification of the Sweet E cultures are accurate, the distribution of bacterial types found in this study was approximately the same as that found by Mitsuoka et al. (1973). The study done by Mitsuoka et al. (1973), however, may be considerably in error because of the almost exclusive use of selective media, the use of only a limited number of further tests on the isolates, and the use of a means of producing anaerobiosis which does not rapidly eliminate the oxygen in the culture container. The large range of counts, in some cases by nearly a factor of 10^9, indicates that some problem may exist in the sampling or cultivating procedures. This large range of counts for many of the bacterial groups makes it nearly impossible to determine the composition of the bovine fecal flora. The study done by Mitsuoka et al. (1973) is consequently inadequate and can be greatly improved upon.

Discussion of objectives. Not all objectives of this study were fulfilled because of a lack of time. Media and methods for cultivating the bovine fecal flora were developed and the organisms were enumerated. A satisfactory medium for maintaining cultures of the isolates was not successfully developed nor was a medium for doing biochemical tests on the isolates. Media used for maintaining and biochemically testing rumen bacteria may be usable. The die-off rate of the predominant bacteria of the flora was not determined because it is important to know which organisms are dying at what

rate. Since identification of isolates was impossible for lack of proper maintenance and biochemical testing media, this last objective was not fulfilled.

CONCLUSIONS

1. The use of petri dish halves in conjunction with a drying oven is a usable method of drying fecal subsamples for the determination of fecal moisture content.

2. The amount of debris in fecal smears tends to limit the accuracy of the direct microscopic clump count to the extent that it should not be depended on except as an indication of culture medium efficiency.

3. Modified Medium 10 containing 30% rumen fluid is an acceptable primary isolation medium for the study of the bovine fecal flora. The recovery obtained with this medium, 50% of the direct microscopic clump count, compares favorably with recoveries obtained in other fecal flora studies (Moore and Holdeman, 1974).

4. Semisolid modified Medium 10 containing 30% rumen fluid is a poor maintenance medium because culture viability was lost in less than 1 week at room temperature. This is probably due to a nutritional deficiency of the medium or a buildup of toxic metabolic products. This medium, however, is vastly superior to Sweet E which was able to support the growth of only 61% of the isolates.

APPENDIX A - PREPARATION OF CLARIFIED RUMEN FLUID

The method of preparing clarified rumen fluid was based on the method used by Holdeman and Moore (1975). The source of unprocessed rumen fluid was a bovine rumen obtained from a local locker plant the same day the animal was slaughtered. The rumen was cut open on arrival at the laboratory and the contents were removed, a handful at a time, to a square of cheesecloth (grade 20, Curity Cheesecloth, The Kendall Company), 4 layers thick. The corners of the cheesecloth were brought together and the mass of rumen contents was thoroughly squeezed to express the fluid portion which was collected in a large plastic funnel leading to a 5 gal plastic jug. The fluid was then siphoned into large Erlenmeyer flasks with a capacity of at least 1 L each, filling them to approximately half of their capacity. These were stoppered with cheesecloth stoppers and autoclaved for 25 min at 121 C.

The autoclaved rumen fluid was partially clarified by centrifugation for 30 min at 6000 rpm (5875 x g) using the GSA rotor on a Servall Superspeed centrifuge (type SS-3, Ivan Sorvall, Inc.). The relatively clear supernatant fluid was decanted and subjected to a second centrifugation step.

In the second centrifugation step, the partially-clarified rumen fluid was centrifuged for 30 min at 9200 rpm (10228 x g) using the SS-34 rotor on the Servall Superspeed centrifuge. The clarified supernatant fluid was then decanted and put into 99 ml screw-

capped dilution bottles (#M8085-Bottle, Scientific Products) and was autoclaved for 25 min at 121 C. The bottles of sterile clarified rumen fluid were stored at 5 C until needed for the preparation of media.

APPENDIX B - MEDIUM FORMULAS

Primary Isolation Media

Ingredients	RGCA	RFM	M1010[a]	M1020[b]	M1030[c]
clarified rumen fluid[d] (ml)	150	200	50	100	150
glucose (Difco) (g)	0.124	0.25	0.25	0.25	0.25
cellobiose (Gibco) (g)	0.124	0.25	0.25	0.25	0.25
soluble starch (Matheson, Coleman, and Bell) (g)	0.25	0.25	0.25	0.25	0.25
ammonium sulfate (Fisher) (g)	0.5	0.5	0.5	0.5	0.5
resazurin solution[e] (ml)	2	2	2	2	2
distilled water (ml)	100	50	200	150	100
anaerobic salts solution[f] (ml)	250	250	250	250	250
L-cysteine-$HCl \cdot H_2O$ (Sigma) (g)	0.25	0.25	0.25	0.25	0.25
vitamin K-hemin solution[g] (ml)	5	5	5	5	5
$Na_2S \cdot 9H_2O$ (Mallinckrodt) (g)	-----	0.125	0.125	0.125	0.125
trypticase (BBL) (g)	-----	-----	1.0	1.0	1.0
yeast extract (Difco) (g)	-----	-----	0.25	0.25	0.25
agar-agar (Gibco) (g/tube)	0.2	0.2	0.2	0.2	0.2
ml broth per tube	11	11	11	11	11
final pH	7.0	7.0	7.0	7.0	7.0
autoclaving time (min at 121 C)	25	25	25	25	25

[a] Modified Medium 10 containing 10% rumen fluid.

[b] Modified Medium 10 containing 20% rumen fluid.

[c] Modified Medium 10 containing 30% rumen fluid.

[d] Prepared as per instructions in Appendix A.

[e] Prepared as per instructions in VPI Manual (Holdeman and Moore, 1975).

[f] Prepared as per instructions in VPI Manual.

[g] Prepared as per instructions in VPI Manual.

Maintenance Media

Ingredients	Sweet E[a]	ssM1030[b]
clarified rumen fluid[c] (ml)	150	150
arabinose (Sigma) (g)	0.5	-----
cellobiose (Gibco) (g)	0.5	0.25
fructose (Difco) (g)	0.5	-----
glucose (Difco) (g)	0.5	0.25
maltose (Sigma) (g)	0.5	-----
soluble starch (Matheson, Coleman, and Bell) (g)	0.5	0.25
sodium pyruvate (Calbiochem) (g)	0.8	-----
gelatin (Difco) (g)	1.5	-----
ammonium sulfate (Fisher) (g)	0.25	0.5
resazurin solution[d] (ml)	2	2
peptone (Difco)	0.25	-----
trypticase (BBL)	-----	1.0
yeast extract (Difco)	0.25	0.25
anaerobic salts solution[e] (ml)	250	250
distilled water (ml)	100	100
L-cysteine-$HCl \cdot H_2O$ (Sigma) (g)	0.25	0.25
$Na_2S \cdot 9H_2O$ (Mallinckrodt) (g)	-----	0.125
vitamin K-hemin solution[f] (ml)	5	5
agar-agar (Gibco) (g)	0.375	0.015[g]
ml broth per tube	3	10
final pH	7.0	7.0
autoclaving time (min at 121 C)	25	25

[a] Prepared as per instructions in VPI Manual (Holdeman and Moore, 1975).

[b] Semisolid modified Medium 10 containing 30% rumen fluid.

[c] Prepared as per instructions in Appendix A.

[d] Prepared as per instructions in VPI Manual.

[e] Prepared as per instructions in VPI Manual.

[f] Prepared as per instructions in VPI Manual.

[g] Grams of dry agar-agar added to tubes individually.

Gelatin Dilution Fluid for Dilution Tubes

gelatin (Difco)	1.0 g
anaerobic salts solution[a]	250 ml
resazurin solution[b]	2 ml
distilled water	250 ml
L-cysteine-HCl·H_2O (Sigma)	0.25 g

[a] Prepared as per instructions in VPI Manual (Holdeman and Moore, 1975).

[b] Prepared as per instructions in VPI Manual.

The gelatin dilution fluid was prepared as for other prereduced media. Six 6 mm diameter glass beads were added to each dilution tube, followed by 9.0 ml of dilution fluid. The 18 mm dilution tubes were then autoclaved for 15 min at 121 C. The final pH was 7.0.

APPENDIX C - MEDIA PREPARATION

Prereduced, anaerobically-sterilized media were produced by the method of Holdeman and Moore (1975). The media were produced in Erlenmeyer flasks having 24/40 standard taper ground glass female fittings. Flasks of 125 ml (#F4281-125, Scientific Products), 500 ml (#F4281-500, Scientific Products), and 1000 ml (#F4281-1L, Scientific Products) capacity were used to prepare batches of media 125, 500, or 1L in volume.

The glass boiling chimney consisted of a 250 ml round-bottomed boiling flask (#F4025-250, Scientific Products) which was modified by adding a male 24/40 standard taper ground glass fitting (#G9005-7, Scientific Products) to the bottom of the flask, opposite the neck of the flask. The ground glass fittings of both the flask and the chimney were smeared with a small quantity of Dow-Corning high vacuum grease (S9005-1-Grease, Scientific Products) before they were fitted together. Before the ground glass fittings were fitted together, a magnetic stirring bar (#S8305-15, Scientific Products) was added to the Erlenmeyer flask to allow stirring of the contents and the maintenance of an even boil. The flask and chimney were then fitted together before medium ingredients were added in order to prevent powdered or granular ingredients from fouling the mating surfaces and preventing the establishment of a good seal.

After the flask and chimney were joined, they were placed on a

magnetic-stirring hot plate (#H2402-1, Corning Model PC-351, Scientific Products) with a clamp on the back holding an 18 in long ring stand rod (S9158-3, Scientific Products) in a vertical position. Two clamps (#C5997-1, small, round, extension clamp, Scientific Products and #C6012-1, clamp holder, Scientific Products) were placed on the vertical rod to hold the chimney and prevent tipping of the flask and chimney.

Dry ingredients, excluding the reducing agent, were added to the flask through the chimney. After the dry ingredients had been added, the magnetic stirrer was activated to break up lumps and prevent the formation of new lumps when the liquid ingredients were added. The liquid ingredients were then added and were used to wash particles of the dry ingredients sticking to the chimney and flask walls down into the flask. Slow addition of the liquid ingredients reduced the formation of lumps and the stirring bar was allowed to break up most of the lumps before the hot plate heat control was turned to the maximum heat position.

When the medium began to boil, the hot plate was turned off, while the stirrer was still activated, until the rate of boiling was not excessive and then the heat level was regulated to cause the medium to boil up into the lower half of the chimney. A 2-holed number 5 rubber stopper (#R5160-5, Scientific Products) was then inserted into the upper tube of the chimney. One hole in the stopper had a 2 in section of 6mm outside diameter glass tubing (#G6050-6, Scientific Products) inserted into it to condense some

of the water vapor which would otherwise be lost from the medium. The other hole in the stopper had a single gas cannula from the medium preparation gas manifold inserted through it. A gentle stream of oxygen-free nitrogen, a flow rate of approximately 300 ml per min, was flowing through the cannula to sweep out oxygen driven out of the medium and to prevent the entrance of atmospheric oxygen into the chimney.

The medium was allowed to boil for 20 min, if the batch was 500 ml or less in volume, or 25 min, if the batch was 1000 ml in volume. While the medium was boiling, the acid and alkali solutions for pH adjustment (Holdeman and Moore, 1975) were steamed in a steam utensil sterilizer (type 20x20x24, #T-085-075, American Sterilizer Company) for 10 min to drive out dissolved oxygen so that the medium would not become oxidized when the pH was adjusted.

After the prescribed period of boiling, the oxygen-free nitrogen flow was discontinued and a gentle flow of oxygen-free carbon dioxide, a flow rate of approximately 300 ml per min, was substituted. The flask and chimney were removed from the hot plate as a unit and were placed in an ice bath. After the medium ceased boiling, the chimney was removed from the flask and the cannula was inserted into the flask after removal from the chimney. The cannula was inserted into the flask below the level of the medium so that the carbon dioxide would bubble through the medium and cause it to become saturated with carbon dioxide. At this time a

dial thermometer (model #2261, 0-180F, Weston) was also inserted into the flask to monitor the cooling of the medium to room temperature.

When the medium had cooled to room temperature, the dial thermometer was removed from the flask and the chemical reducing agent was added to the medium. A combination pH electrode (#476051, Corning Glass Company) was then inserted into the flask while carbon dioxide was still being bubbled through the medium. The pH meter had been previously standardized in the range of pH 4 to pH 7 using commercial buffers. The flask was then placed on a magnetic stirrer (#14-511-2, Fisher Scientific Company) which was adjusted to give a slow stirring rate so that the stirring bar would not hit the pH electrode. Pasteur pipettes 9 in long (#1276, Bellco Glass Inc.) were used to add the acid or alkali solutions, either 5N HCl or 8N NaOH, to lower or raise the pH of the medium to 0.2 pH unit above the desired pre-autoclaving pH. At this point in the procedure the flow of oxygen-free carbon dioxide was maintained until the pH of the medium had dropped to the desired pre-autoclaving pH. When the proper pre-autoclaving pH had been reached, the flow of oxygen-free carbon dioxide was discontinued and a flow of oxygen-free nitrogen, approximately 300 ml per min, was initiated to prevent a further drop in pH and to prevent atmospheric oxygen from gaining entry to the flask.

The medium was dispensed into tubes immediately after the flow of nitrogen was initiated. While the medium had been boiling, the

0.125 in amber latex tubing (#R5325-1, Scientific Porducts) had been removed from the cysteine-HCl solution and the dispensor assembly had been reassembled. The desired delivery volume was set by trial and error using a 10 ml graduated cylinder (#C9050-10, Scientific Products) or a 25 ml graduated cylinder (#C9050-25, Scientific Products). The syringe was set to deliver no more than 5 ml per stroke to minimize the jamming that tends to occur when a Cornwall syringe is used near its upper limit of delivery. Multiple strokes of the dispensing pump were used when the desired delivery was greater than 5 ml. The cysteine-HCl solution was pumped through the syringe when the volume was being set and the system was being purged of air bubbles, in order that reoxidation of the tubing would be avoided. If an agar-based medium was being prepared, agar-agar (#1400000, Gibco Diagnostics) was individually added to each tube, while the medium was boiled and cooled, after being weighed on the triple-beam balance.

When the medium was ready to be dispensed into tubes, the pinch clamp was removed from the dispensing cannula, the gas flow of oxygen-free nitrogen was raised to approximately 2300 ml per min, and a screw clamp (#C6080-Clamp, Scientific Products) was put on the gas supply line to the single cannula to throttle the gas flow. The gas flow was adjusted so that a trickle of oxygen-free nitrogen was bubbling through the medium to exclude air while most of the gas flow was directed to the dispensing cannula and was used to flush tubes with nitrogen.

Before the medium was dispensed into tubes, the dispensing pump was flushed with medium. After this was accomplished, the dispensing cannula was inserted to the bottom of an 18 mm anaerobic biochemical tube (#2046-18142, Bellco Glass Inc.) or a 25 mm anaerobic roll tube (#2046-25142, Bellco Glass Inc.). The required amount of medium was dispensed into the tube and the proper-sized stopper, number 1 (#1926-00001, Bellco Glass Inc.) for 18 mm tubes or number 4 (#1926-00004, Bellco Glass Inc.) for 25 mm tubes, was lightly held in the mouth of the tube as the cannula was partially withdrawn from the tube. The gas cannula was allowed to remain immediately under the bottom surface of the stopper so that any oxygen trapped under the stopper would be flushed out by the flow of oxygen-free nitrogen. The cannula was allowed to remain in this position for 5 sec after which the cannula was fully withdrawn and inserted to the bottom of the next tube in line while the stopper was lightly inserted into the neck of the filled tube. The filled tube was then inverted and the stopper was wiggled from side to side to admit a film of liquid medium to the space between the stopper and the tapered neck of the tube. This liquid seal is much more gas tight than a dry seal. The tube stopper was then tightly seated into the tapered neck of the tube with a pushing and twisting motion. During this process, the left hand, in the case of a right-handed person, was protected against cuts by a chain mail glove (#58722, Koch Supplies Inc.) covering the thumb and forefinger. The tube was held in the left

hand with the covered forefinger completely surrounding the neck of the tube and covering it so that no direct contact between the hand and the tube neck could occur. This dispensing procedure, beginning with the dispensing of medium into the tube, was repeated for subsequent tubes to be filled.

Since the tube stoppers are not securely held in the tubes, when the tubes are autoclaved the stoppers must be held in the tubes by means of a tube clamp (Holdeman and Moore, 1975). Stainless steel tube racks of the 18 mm size (#2027-18072, Bellco Glass Inc.) and the 25 mm size (#2027-25036, Bellco Glass Inc.), without feet, were used for autoclaving tubes of media. A 1-2 in thick piece of foam rubber was placed just above the bottom mesh of the rack to cushion the bottoms of the glass tubes and reduce tube breakage. The tubes to be autoclaved were placed in the rack, leaving the middle 2 or 3 rows vacant. These rows should be left vacant because flexion of the tube often causes tubes of media in the center rows of the rack to be ruined because the stoppers loosen during autoclaving and air is sucked into the tubes when they cool. After the tubes had been placed in the rack, the stoppers were covered with a layer of paper toweling (#237, Singlefold, Garland Sof-Knit Towels, Fort Howard Paper Company) to prevent stoppers from sticking to the top plate of the tube clamp. If tube stoppers should stick to the top plate of the tube clamp during autoclaving, when the rack of tubes is slid out of the tube clamp after cooling many of these stoppers will become loosened or

completely dislodged from the tubes. The paper toweling is easily peeled from the tops of the stoppers, after the rack is removed from the tube clamp, without dislodging the stoppers. After the rack of tubes had been placed in the tube clamp, the thumbscrews on the clamp were spun down until they rested against the top plate of the clamp with the top plate level. One's foot was then used to press down strongly on the top plate, while keeping it level, as the thumbscrews were tightened by hand as much as possible.

Clamped racks of media were autoclaved in the same manner as media in open tubes, with the exception that the autoclave was depressurized rapidly after the completion of autoclaving to prevent exposure of the medium to excessive heat. Racks of agar medium were inverted 6 times, immediately after removal from the autoclave, to counteract the stratification of broth and agar-agar that occurs during autoclaving. The thumbscrews on the tube clamp were retightened after removal of the medium from the autoclave and the racks of medium were allowed to cool to room temperature.

After the tubes of media had cooled to room temperature, the tube clamp was removed and the paper toweling was peeled from the tops of the tube stoppers. The medium was then ready for use and could be used immediately or stored at room temperature until used. Storage at 5 C is not recommended as accelerated oxidation of the tubes of media occurs at low temperatures.

After a long period of use, tube stoppers tend to become hard and brittle which results in the inability of the stopper to

properly seal the tube. Hard stoppers were immersed in a saturated solution of sodium hydroxide which was then steamed in the utensil sterilizer until the stoppers became soft again. The stoppers were then repeatedly washed in deionized water until all traces of the sodium hydroxide were removed. No soap was ever used to wash tube stoppers at any time because this tended to cause the stoppers to become slippery and they would tend to be forced out of the tubes by internal gas pressure of the tubes. In addition, slippery stoppers are nearly impossible to seat properly in the necks of culture tubes when a liquid seal is used between the tube neck and the stopper.

APPENDIX D - MEDIA DISPENSER

The media dispenser used in this study was based on the dispenser used at the VPI Anaerobe Laboratory. The present dispenser is more durable, more resistant to jamming, simpler in construction and operation, and more reproducible in the volume of media delivered than was the original VPI dispenser.

The entire dispenser unit was constructed on a hardwood baseboard 24 in long by 6 in wide by 1 in thick. A small board 6 in long by 2 in wide by 0.75 in thick was nailed across the board to provide a raised platform for the attachment of a hinge. This platform was attached so that the center axis was 5 in from one end of the baseboard.

The actuating lever arm consisted of a hardwood board 17 in long by 3 in wide by 1 in thick. A 2.5 in wide hinge was attached by wood screws to the end face of the lever arm. The other end of the hinge was attached to the upper face of the raised platform by wood screws, such that the hinge pin was 5 in from the nearest end of the baseboard. The lever arm was positioned so that its long axis was directly above the long axis of the baseboard and parallel to it.

A 10 ml Cornwall syringe (#2193 (1270 S), Becton, Dickinson and Company) was used as the pumping mechanism. The plunger spring (#3558 (1270 PS), Becton, Dickinson and Company) was used to return the plunger to the filled position after each stroke of the pumping

mechanism. The syringe body was passed through a hole in the lever arm, plunger end down, so the plunger would rest on the upper surface of the baseboard. The hole in the lever arm was drilled vertically through the upper surface of the lever arm with the center of the hole equidistant from the 2 long sides of the lever arm and 1.25 in from the end of the lever arm opposite the end where the hinge was attached. The hole was drilled just large enough so the Cornwall syringe barrel would pass through it but small enough that the retaining collar on the syringe barrel would not pass through and the syringe barrel would not wobble in the hole during operation of the dispenser.

The upper and lower limits of travel of the syringe plunger were determined by flat washers and nuts on a coarsely-threaded 0.25 in diameter threaded rod 7 in long. The threaded rod was passed through a hole in the baseboard which was equidistant from the 2 long sides of the baseboard and 6.125 in from the end of the baseboard opposite the end where the raised platform was located. An identical hole was drilled in the lever arm directly above the hole in the baseboard and the threaded rod was also passed through this hole. The rod was affixed to the baseboard by a nut under the baseboard, with a flat washer above the nut to protect the board, and a nut above the baseboard, with a flat washer under it to protect the board. The bottom of the threaded rod was set so as to be flush with the bottom of the lower nut. The stop for the lower limit of travel was constructed of 2 nuts locked together,

with a flat washer above them to protect the lever arm. The stop for the lower limit of travel was set so the syringe plunger would not quite reach its limit of travel when the lower stop was contacted by the lever arm. The stop for the upper limit of travel was made movable and allowed for the adjustment of the delivery volume of the dispenser. The upper stop consisted of a flat washer above the lever arm, a nut above the washer, and a wingnut above the nut. The upper limit of travel was set by trial and error, using a graduated cylinder, by changing the position of the nut until the correct delivery was achieved. When the nut was in the correct position, the wingnut was tightened against the nut so as to lock it in position.

The valving mechanism used in the media dispenser was the standard Cornwall syringe filling outfit (#3560 (1220 FO), Becton, Dickinson and Company) which was attached to the Luer-Lok fitting of the Cornwall syringe. The only change made to this valving mechanism was the substitution of 0.125 in bore, 0.031 in wall amber latex tubing (#R5325-1, Scientific Products) for the tubing originally supplied with the valving mechanism.

A screw eye was inserted into the free end of the lever arm and a 0.375 in diameter hole was drilled through the baseboard, directly below the screw eye, to allow the passage of the nylon cord used to operate the lever arm. The nylon cord passed through the baseboard and table top to a foot pedal on the floor. The pedal was constructed of a 7x21 in piece of 1 in thick pine from which

had been cut a foot pedal measuring 4.5x16.5 in. The far side and left side of the pedal corresponded to the far side and left side of the original board. The remaining piece of the original board was used to hold the pedal in place and the pedal was hinged to it using a 2.188x3 in hinge at the near end of the pedal. A screw eye was inserted into the end of the pedal to receive the nylon cord.

Stoppers from Vacutainer tubes (#6630-145-1137, Becton, Dickinson and Company) were inserted into holes drilled into the lower surface of the baseboard to prevent movement of the dispensing mechanism across the table top. One stopper was placed near each of the 4 corners of the baseboard.

The dispensing cannula assembly consisted of a 14 gauge, 4 in long blunt-tipped needle (#1789 (1250 NR), Becton, Dickinson and Company) silver-soldered parallel to an 18 gauge, 6 in long blunt-tipped needle (Popper and Sons, Inc.) so that the 18 gauge needle extended 10 mm below the tip of the 14 gauge needle. The 14 gauge needle was attached to a 36 in long piece of 0.125 in bore, 0.031 in wall amber latex tubing which was also connected to the outlet port of the valving mechanism of the Cornwall syringe. The 18 gauge needle was attached to the oxygen-free gas supply using 0.25 in inside diameter, 0.0625 in wall amber latex tubing (#R5325-3, Scientific Products).

Very little periodical maintenance was required with this dispensing mechanism. Between batches of medium, the Cornwall syringe was removed by raising the upper stop of the mechanism and

sliding the syringe out. The syringe was then disassembled and the plunger was lightly coated with Vaseline (Chesebrough-Ponds, Inc.). The syringe was then reassembled and reinstalled in the lever arm. The syringe was always flushed with distilled water between batches of medium and after the last batch of medium had been prepared each day. As much of the distilled water was pumped out of the syringe as was possible and the syringe was stored overnight in this condition. After the last batch of medium had been prepared each day, the valving mechanism was disassembled, the passages were brushed out, and the pieces were stored dry until the next use of the dispenser.

The amber latex tubing used for transporting the medium was chemically reduced by soaking it overnight in an aqueous solution of cysteine-HCl. The cysteine-HCl solution was produced by adding 4 ml of the resazurin solution (Holdeman and Moore, 1975) to 1 L of distilled water and steaming this in the utensil sterilizer for 10 min. A 250 ml Erlenmeyer flask (#F4257-250, Scientific Products) was then filled with the hot solution and the amber latex tubing was slowly fed into the flask, while avoiding the entrapment of air bubbles within the tubing. L-cysteine-HCl (#C-7880, Sigma Chemical Company) was then added to the flask in sufficient quantity to cause the resazurin to become colorless, after which the flask was stoppered with a number 6 stopper.

APPENDIX E - OXYGEN-FREE GAS SUPPLY FOR MEDIA DISPENSER

Oxygen-free gas for the media dispenser was produced by passing gas from gas cylinders through a heated bed of reduced copper turnings. This reduced gas was then distributed to the various gas cannulas used with the media dispenser.

Three tanks of compressed gas were used with the media dispenser: 1 carbon dioxide tank, 1 nitrogen tank, and 1 hydrogen tank. Single- or double-stage regulators were used to reduce the tank pressure to 10 psi which was used to supply the mixing manifold. The pressure regulators were connected to the mixing manifold of a salvaged anaesthesia machine (Heidbrink Kinet-o-meter, The Heidbrink Company). Unused ports on the manifold were plugged with rubber stoppers and a rubber stopper with a short section of 8 mm glass tubing (#G6050-8, Scientific Products) passing through it was used in the outlet port of the manifold to conduct the gas mixture out of the manifold. Amber latex tubing, 0.25 in inside diameter 0.0625 in wall, was used to connect the outlet of the mixing manifold to the inlet of the deoxygenation tube.

The deoxygenation tube consisted of a Vycor tube, 29 cm long and 2 cm in outside diameter, with the ends fused to short sections of Vycor tubing, 35 mm long and 9.5 mm in outside diameter. The large Vycor tube was filled with copper turnings and was wrapped with nichrome wire. The gauge and length of nichrome wire

was determined by trial and error and was such that the copper turnings in the tube were heated to the extent that the turnings would rapidly oxidize when a gas mixture containing oxygen was passed through the deoxygenation tube. This nichrome heating coil was energized by 110 VAC and was connected to a standard line cord. In order to achieve efficient heat transfer from the heating coil to the deoxygenation tube, the heating coil was held tightly against the tube by strips of asbestos tape (#A6820-9, Scientific Products) wrapped around the end of the tube, with wire wrapped around the outside of the asbestos strips to hold them in place. Amber latex tubing, 0.25 in inside diameter and 0.0625 in wall, was used to connect the deoxygenation tube to the gas-distributing manifold.

A homemade 6-port glass manifold was used to distribute the oxygen-free gas to the various cannulas used with the dispensing apparatus. The manifold was constructed of 8 mm outside diameter glass tubing, a long straight piece with 4 short pieces fused to the side. A Castaloy 3-pronged utility clamp (#5-768-10, Fisher Scientific Company) was used to hold the manifold in a horizontal position. Since only 4 of the ports were used, 2 of the ports were closed by attaching short pieces of 0.25 in inside diameter, 0.0625 in wall amber latex tubing to the unused ports and closing off the tubing with Castaloy pinchcocks (#5-849A, Fisher Scientific Company). One of the remaining ports was attached to the tubing from the deoxygenation tube and a second port was attached to a

section of amber latex tubing, 0.25 in inside diameter and 0.0625 in wall, leading to a fume hood which allowed the venting of excess hydrogen from the apparatus. This vent line was kept closed by the use of a pinchcock except when it was necessary to vent the system. Of the remaining 2 ports, one was used as the gas supply for the media dispenser while the other was connected to a section of amber latex tubing, 0.25 in inside diameter and 0.0625 in wall, leading to a 14 gauge, 4 in long blunt-tipped needle (#1789 (1250 NR), Becton, Dickinson and Company). This latter cannula was used to exclude air from the flask of medium being prepared and dispensed.

All new amber latex tubing was chemically reduced before its first use by soaking it in a reducing solution of cysteine-HCl. The same solution and procedure was used as was used for the media dispenser tubing. This procedure was necessary because oxidized tubing tends to oxidize reduced gas passing through it.

APPENDIX F - PH METER

The pH of solutions was measured using a pH meter (Corning Model 7, Corning Glass Works) equipped with a rugged combination pH electrode (#476051, Corning Glass Works). The meter was generally standardized in the range of pH 4 to pH 7 and was standardized in the range of pH 7 to pH 10 when necessary. The pH 4 buffers used were Sargent-Welch pH 4 buffer (#S-30141-10-A, Sargent-Welch Scientific Company) or Harleco potassium acid phthalate buffer (#2106, Harleco). The pH 7 buffers used were Sargent-Welch pH 7 concentrated buffer solution (#S-30143-10, Sargent-Welch Scientific Company) diluted as directed or Harleco dibasic sodium phosphate-monobasic potassium phosphate buffer (#21131, Harleco). The pH 10 buffers used were Sargent-Welch pH 10 buffer (#S-30141-10-C, Sargent-Welch Scientific Company) or Harleco boric acid-potassium hydroxide buffer (#2119, Harleco). The pH electrode was washed with distilled water between readings and the electrode was immersed in distilled water when not in use.

APPENDIX G - COLONY PICKING APPARATUS

The colony picking apparatus was built around an AO Spencer Series 58 Cycloptic Stereoscopic Dissecting Microscope with a Magni-Changer "M" feature, a set of 10X widefield eyepieces, and a substage mirror attachment (#58M-1, American Optical Company, Spencer Instrument Division). Added to the basic microscope were a 2X auxiliary lens (#265, American Optical Company, Spencer Instrument Division) and a single tube photographic adaptor (#638, American Optical Company, Spencer Instrument Division).

The base of the microscope was set into a hole cut into a 12x12x0.75 in piece of plywood such that the microscope was kept from sliding and at the same time was prevented from falling through the hole in the board. Four small metal strips held the microscope into the hole and prevented tipping of the microscope. The right side of the microscope was raised by attaching a piece of 0.5 in thick plywood to the right side of the piece of 0.75 in thick plywood, such that the right side of the latter board was held 5 in above the table top. The 2 boards were held together with wood screws.

Substage illumination was provided by a fluorescent illuminator (#31-33-36, Bausch and Lomb). A cutout was made in the 0.75 in thick plywood baseboard to allow the illuminator to be as close to the substage base of the microscope as possible and another cutout was made to clear the light path from the illuminator to the substage

mirror. In use, the illuminator was adjusted so it was parallel to and at the same level as the axis of rotation of the substage mirror.

The tube-holding stage was constructed of 0.25 in thick Plexiglas, with the base piece constructed of a single 4x5 in piece. The 25 mm roll tubes were held in place on the stage by 2 0.5x5 in pieces of Plexiglas. These pieces were glued atop the base, their long axes parallel to the long axis of the base piece, near the center of the base piece. These 2 strips were arranged so that a 25 mm roll tube would lie securely on them and the outside of the wall of the tube would not quite touch the base piece. This construction allowed a tube to be rotated without wobbling. A 1.5x1.75 in piece of Plexiglas was glued to the ends of the narrow strips and the end of the base piece to act as a stop to prevent the roll tube from sliding off of the end of the stage.

The 2 metal clips on the microscope stage were crossed and then the base piece of the movable stage was inserted under them. The movable stage was positioned so a roll tube would be held directly under the objective lens of the microscope, with the bottom of the roll tube pointing down at an angle toward the table top.

The gassing cannula consisted of an 18 gauge, 6 in long needle (Popper and Sons) cut to 2.75 in in length and bent to fit over the lip of a 25 mm roll tube. The bend in the needle was at approximately its midpoint after shortening. The last 0.5 in

of the needle was bent slightly in the opposite direction to reduce the tendency for the cannula to be accidentally plugged with agar. A 1.75 in long piece of glass tubing just large enough in diameter to fit inside of the Luer-Lok fitting of the cannula was cemented in place, while the other end was connected to a source of oxygen-free carbon dioxide by a length of amber latex tubing.

In use, the helical line on the roll tube was followed from the bottom of the tube to the top for counting and subculturing colonies. The movable stage was moved to follow the line. The microscope was set for 10X magnification, giving an actual magnification of 20X with the 2X auxiliary lens being used. Colonies were observed and picked with their bottoms toward the objective lens of the microscope, since the media used did not exhibit sufficient clarity to permit observation from both the top and the bottom. No above-stage illumination was used because this only caused glare problems and made it difficult to follow the forward progress of the picking needle down the tube when colonies were picked. The sterile picking needle was handled with no support and was inserted into the roll tube, being careful not to touch the agar or the gassing cannula, while observing the process with the naked eye. When the tip of the picking needle was near the colony, the process was observed through the dissecting microscope as the needle was used to sample from the center of the colony. The picking needle was then removed from the roll tube while being observed with the naked eye and was used to inoculate media.

APPENDIX H - DRYING OVEN

The oven used to dry fecal samples for moisture determinations was a Hotpack model 35738 drying oven (The Electric Hotpack Company, Inc.) operated at 70 C under a reduced pressure of 10 in Hg. In operation, filtered room air was allowed to enter the intake side of the manifold atop the oven. The valve on the intake side of the manifold was used to throttle the flow of air to allow adjustment of the pressure within the oven. The vacuum source was connected to the outlet side of the manifold and the valve on this side of the manifold was used to shut off the vacuum supply to the oven when the vacuum source was used for other purposes. The upper manifold port was closed by a solid number 2 rubber stopper which was sealed with vacuum grease.

The air intake filter consisted of a 25 mm outside diameter by 200 mm long test tube stuffed with absorbent cotton (#C8350, Scientific Products). This was fitted with a 2-holed rubber stopper which had 2 sections of 8 mm outside diameter glass tubing passing through it with approximately 10 mm of each section of tubing protruding from the outside surface of the stopper. One section of tubing had a total length of 50 mm while the other had a total length of 200 mm. After the components were assembled, the stopper with the 2 tubes was inserted into the 25x200 mm test tube so that there was an unbroken column of absorbent cotton between the outlet of the short tube and the outlet of the long tube.

Parafilm (American Can Company, Marathon Products) was used to seal the stopper to the test tube and 0.25 in inside diameter, 0.0625 in wall amber latex tubing was used to connect the long section of glass tubing to the intake port of the drying oven while red rubber vacuum tubing, 0.25 in inside diameter, 0.625 in outside diameter, and 0.1875 in wall thickness (#R5311-4, Scientific Products), was used to connect the outlet side of the drying oven to the vacuum source.

The source of vacuum was a type T-A refrigeration compressor salvaged from an old refrigeration unit. The compressor had an 8 in diameter pulley belt-driven by a 0.33 horsepower, model 5KH45KB62, 1725 rpm, General Electric AC motor with a 2.5 in diameter pulley. All unused ports on the compressor were plugged, leaving 1 port on the vacuum side and 1 port on the compression side unplugged. The port on the vacuum side was used as the vacuum source for the drying oven, while the port on the compression side was vented to a fume hood.

APPENDIX I - TRANSFER APPARATUS GAS SUPPLY

One tank of carbon dioxide and 1 tank of hydrogen were used as the gas supply for the transfer apparatus. Tygon tubing of 0.25 in inside diameter, 0.5 in outside diameter, and 0.125 in wall (#R5340 3X, Scientific Products) was used to connect the 1- or 2-stage regulator on each tank to a glass "Y" connector (#S-82765, Sargent-Welch Scientific Company).

A homemade flow reducer was inserted into the hydrogen supply line to reduce the amount of hydrogen escaping into the laboratory. The flow reducer consisted of a Pasteur pipette with a broken tip. The pipette was heated in a flame and was drawn out into a small capillary which was then broken off flush with the body of the pipette. This left a small orifice which reduced the hydrogen flow to the extent that the flow could be controlled using the gas regulator control.

The outlet from the "Y" connector was connected, using another section of Tygon tubing, to the deoxygenation tube (#S-36518, Sargent-Welch Scientific Company). The inlet to the tube was the short bottom tubing while the long upper tubing was the outlet. A furnace (#S-36517, Sargent-Welch Scientific Company) was used to heat the copper to a temperature sufficient to allow oxidation of the copper when oxygen was present in the gas stream.

The outlet of the deoxygenation tube was connected to a section of Tygon tubing which was connected to a glass "Y" tube.

The "Y" tube was used to divide the stream of oxygen-free gas from the deoxygenation tube between the main transfer apparatus and the colony picking apparatus. Pinchcocks were used to cut off flow to one apparatus or the other. Amber latex tubing, 0.25 in inside diameter and 0.0625 in wall, was used to connect the "Y" tube to the copper manifold on the transfer apparatus, while 0.125 in bore, 0.03125 in wall amber latex tubing 50 in long was used to connect the glass "Y" tube to the cannula of the picking apparatus.

APPENDIX J - TRANSFER APPARATUS

The design of this apparatus was based upon the design of the commercially available Bellco apparatus (#7790-11111, Bellco Glass, Inc.). It was hand made in the South Dakota State University Engineering Shop.

The base of the transfer apparatus was an aluminum radio chassis mounted on the table top, with the open end down. A piece of heavy sheet metal was bent at a 90 degree angle and attached to the left rear corner of the base. A "U" channel was mounted horizontally on the rear surface of the piece of sheet metal and was equipped with bushings to act as pivot points for the cannulas. Stainless steel truck brake tubing was used as the cannula supply tubing and a Luer-Lok fitting was affixed to the end of each of the 3 supply tubes used.

Each cannula consisted of an 18 gauge blunt-tipped needle 6 in long. The needle was locked into the Luer-Lok fitting, heated to a red color in a flame, bent in an arc so as to enter the center of a tube placed on the tube holder, and cut to length.

The syringe for the multiple inoculator was mounted in a pair of broom-holding clamps. A cutout was made in the base to accomodate the ratcheting mechanism which was salvaged from a typewriter. A metal rod was bent and attached to the "U" channel. The rod was equipped with a hand made clamp which held a Pasteur pipette over the left-hand tube holder. A 14 gauge needle was attached to the

syringe and was forced into a section of I.V. tubing which was attached to the Pasteur pipette.

Three foot pedals were constructed of small pieces of plywood hinged to a common plywood board taped to the floor with cloth tape. Eye screws at the tips of the pedals served as attachment points for nylon cords attached to the cannula supply tubes. Sections of amber latex tubing were connected to the pedals and the underside of the table to act as return springs.

FOOTNOTES

[1]It was desirable to fill the sample bag to minimize the surface area-to-volume ratio of the fecal mass and to obtain a large enough sample to minimize the effects of the heterogeneity of feces.

[2]Transportation temperatures which are too high or too low may be lethal to some bacteria of the fecal flora. In addition, an improper transportation temperature could allow the reproduction of some bacteria of the flora during transportation with the result being that the flora composition of the fecal sample, as determined in the laboratory, would not be a valid estimation of the flora composition of the feces of the particular animal sampled.

[3]The effect of oxygen exposure would be expected to be low in the sampling method employed because a large sample was used to reduce the surface area-to-volume ratio. Fecal material contains a large amount of organic material which keeps most of the sample in a reduced state. In addition, the total amount of oxygen the sample is exposed to is minimized by expressing most of the air from the bag before transport.

[4]The bag was not kneaded between subsamplings for moisture determination because the bag had previously been thoroughly kneaded at least twice before this step and nearly all of the sample was used in this step.

[5]In fact, this designation is correct only if exactly 1 g of feces is added to the first dilution tube. Other weights of fecal material require correction factors to be used in the calculation of colony counts and direct microscopic clump counts to correct them for the size of the fecal subsample actually used. In practice, it is desirable to keep the sample size at 1 g or less because larger samples contain too many clumps of solid material to be easily pipetted with a standard 1.1 ml pipette.

[6]This vigorous shaking of each dilution tube was performed to allow the glass beads to chop up the clumps of solid material in the fecal subsample in order to minimize pipette plugging, to suspend in solution any bacteria adhering to the clumps, and to homogenize the contents of the dilution tube.

[7]Vaspar was formulated following directions in the CDC Laboratory Manual (Dowell and Hawkins, 1974). A rubber "O" ring cemented to a rubber stopper was used as the tool to produce Vaspar rings of a reproducible size on a microscope slide. In use

the tool was first pressed on to the surface of solidified Vaspar and was then carefully pressed on the surface of the microscope slide. Care was taken to prevent sliding of the tool on the surface of the microscope slide. The Vaspar rings produced had an inside diameter of 12.1 mm, giving the smear an area of 0.0263 mm^2.

[8]The reagents used in the Kopeloff modification of the Gram stain were those described in the VPI Manual (Holdeman and Moore, 1975). The staining procedure used was that described in the Manual of Microbiological Methods (Society of American Bacteriologists, 1957.

[9]The microscopic factor of the microscope was calculated by a modification of the standard milk direct microscopic clump count procedure (Hausler, 1972).

diameter of smear=12.1 mm area of smear=115 mm^2
diameter of field=0.183 mm area of field=0.0263 mm^2

$$\frac{\text{area of smear}}{\text{area of field}} \times 100 = \text{microscopic factor}$$

$$\frac{115\ mm^2}{0.0263\ mm^2} \times 100 = 4.37 \times 10^5 = \text{microscopic factor}$$

The direct microscopic clump count (DMCC) was calculated using the standard milk DMCC procedure (Hausler, 1972).

$$\text{ave. field clump count} \times \text{microscopic factor} \times \text{reciprocal of dilution sampled} = \text{DMCC}$$

[10]The DMCC was corrected for the size of the fecal subsample placed in the 10^{-1} dilution tube and the fecal sample moisture content using the formulas on p. 107 of the VPI Manual (Holdeman and Moore, 1975).

[11]The colony count was corrected for the subsample size and sample moisture content using the formulas on p. 107 of the VPI Manual (Holdeman and Moore, 1975).

[12]The bent iron picking needle was constructed of Belgian stainless steel wire (#AD8B, Herters Inc.) held in a standard inoculating loop handle. The wire was 7.25 in in overall length and the free end was bent at a 90 degree angle 0.25 in from the end.

[13]The straight iron picking needle was constructed of a 7.25 in long piece of Belgian stainless steel wire held in a standard inoculating loop handle.

[14]The arithmetic mean was calculated using the formula:

$$\frac{\text{sum of values}}{\text{number of values}} = \text{arithmetic mean}$$

[15]The small sample standard deviation was calculated according to Mendenhall (1971).

$$\left[\frac{\sum_{a=1}^{n}(X_a - \text{mean of } X)^2}{\text{number of values}-1}\right]^{\frac{1}{2}} = \text{small-sample standard deviation}$$

[16]The 90% confidence interval was calculated using Student's t test according to Mendenhall (1971).

$$\text{mean of } X \pm \frac{t_{0.05} \times \text{small-sample standard deviation}}{(n)^{\frac{1}{2}}} = 90\% \text{ confidence interval}$$

[17]The percentage recovery was calculated using the following formula:

$$100 - \left[100 \times \frac{\text{DMCC} - \text{plate count}}{\text{DMCC}}\right] = \text{percentage recovery}$$

[18]The t test for the difference between 2 means was calculated according to Mendenhall (1971). The hypothesis was that the 2 means were equal.

$$\frac{\text{mean of X1} - \text{mean of X2}}{\left[\frac{1}{n1} + \frac{1}{n2}\right]^{\frac{1}{2}} \times \text{small-sample standard deviation}} = t$$

$n1 + n2 - 2 =$ degrees of freedom

[19]The cultural recovery was calculated using the following formula:

$$\frac{\text{number of isolates producing turbidity}}{\text{total number of isolates}} \times 100 = \text{cultural recovery}$$

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