FACTORS INFLUENCING THE HUMAN INTESTINAL FLORA

A thesis submitted by

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for

The degree of Doctor of Philosophy

in the

University of London

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1971

ABSTRACT

The numbers of viable bacteria in faeces from healthy persons living in Uganda, India, Japan, America and Britain have been compared. Faeces from persons in Britain and America (where the diets included animal and vegetable products) contained more bacteroides and bifidobacteria and fewer streptococci, enterobacteria, bacilli, veillonellae and fungi than did those from persons in Uganda, India and Japan (where the diets were principally vegetarian).

The numbers of bacteria appeared to be unrelated to the age, sex or racial origins of the host, and their relationships to diet and environment were not always clear.

Sarcina ventriculi, an organism not previously reported in faeces from healthy adults, was found in numbers up to 10^8 per g in faeces from 80 out of 123 persons in India, Uganda, Japan and strict vegetarians in England, but it occurred in faeces of only one out of 122 persons living on mixed diets in Britain and America. It is suggested that diet influences the colonisation of the intestine by sarcinae.

Little evidence was found for direct interaction between two different organisms, but the counts of streptococci, enterobacteria, lactobacilli, veillonellae and bacilli were correlated negatively with the faecal concentrations of

deoxycholic, lithocholic and cholanic acids (which are formed from primary bile acids by microbial activity) and it is suggested that degraded bile acids may be amongst the factors which control these organisms in the intestine.

Faeces from persons in England, Scotland and America, where the incidence of colon cancer is high, contained more bacteroides and bifidobacteria (which actively degraded bile-steroids) than faeces from persons in Uganda, India and Japan, where the cancer incidence is low, and it is postulated that bacteria may be involved in the aetiology of the disease.

The methods for transporting faeces were validated.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Professor R.E.O. Williams for his kind encouragement and guidance throughout this study and to Dr. B.S. Drasar and Dr. M.J. Hill for stimulating my interest in gut bacteriology and for offering much technical advice.

I am indebted to Dr. R. Blowers (at the time Professor of Bacteriology at Makerere University College, Kampala, Uganda), Professor S.J. Baker (Wellcome Research Unit, Christian Medical College Hospital, Vellore, India), Dr. T. Shigematsu (Tottori University School of Medicine, Yonago City, Japan), Dr. P.S. Brachman (National Communicable Disease Center, Atlanta, Georgia), Dr. J.G. Collee (University Medical School, Edinburgh), Dr. F.R. Ellis (Kingston and Long Grove Hospital, Kingston-upon-Thames, Surrey), Professor I. Macdonald (Guy's Hospital, London) and Dr. I. McLean Baird (West Middlesex Hospital, Middlesex) for collecting the faecal specimens. Professor E.A. Dawes (Department of Biochemistry, University of Hull) kindly gave me the culture of *Sarcina ventriculi*.

I am grateful to Mrs. D. Irving for her statistical advice and to Miss R. Wood (of the University of London Intercollegiate Unit in Medical Statistics) for writing the

computer programmes.

This study was part of a joint project involving Dr. M.J. Hill, Dr. B.S. Drasar and Dr. V.C. Aries and I should like to thank all for their patient co-operation. I also thank Dr. Hill for permission to use his chemical analyses in section 3.4 and Dr. Drasar for permission to use unpublished collaborative results in Tables 15 and 16. Dr. R.R. Davies kindly identified the fungi listed in Table 18.

Miss R. Scutt, Miss R. Steward, Mr. D. Antebi, Miss F. Fernandez and Miss K. Johnson all gave valuable technical assistance at various times throughout the study and their help is greatly appreciated.

I also acknowledge the Cancer Research Campaign for their generous financial support.

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INTRODUCTION AND AIMS OF THE PRESENT STUDY

Each region of the human gastrointestinal tract has its own distinctive anatomy and physiology, and these together encourage or discourage the development and maintenance of microbial populations. Within broad limits the same species of micro-organisms are found at all sites but their activities and relative numbers change from site to site. Each site will influence each other: body secretions and bacteria and their metabolic products swept by peristalsis from proximal sites, will contribute to the ecology of distal parts. As food components are digested by gut enzymes and absorbed, the contents of the gut lumen will change qualitatively and quantitatively, and this will bring about changes in the microbial ecology.

The large intestine is the most densely populated region in the gastrointestinal tract, but we know little of the factors which control the flora in health or in disease. Nor do we fully understand what beneficial or detrimental effects these organisms may have on the human host. Recent studies on the 'blind-loop syndrome' (see reviews by Donaldson, 1968; Tabaqchali, 1970; Gorbach, 1971) show that changes in the distribution and activities of the bacteria within the small bowel, can seriously impair the health of the host. Similarly, abnormal distributions or activities of bacteria in the large

bowel could also produce nefarious results - for example, bacterial metabolism of nitrogenous substrates, such as amino acids or urea, may be important in the pathogenesis of hepatic coma (Sherlock, 1958; Donaldson, 1968).

The large intestine of man is difficult to sample, and for preliminary studies it seems reasonable to use faecal specimens to assess the microbial populations of the large intestine. The faecal flora of persons in North America and Western Europe has been studied by many workers, but that of persons living in other parts of the world has received little attention.

This thesis describes the microbial populations of faeces from persons living in Uganda, India, Japan, Britain and America, and attempts to define some factors which control them. The work is part of a wider study involving Drs. M.J. Hill, V.C. Aries and B.S. Drasar, in which we have attempted to find the ways in which human intestinal bacteria degrade bile salts, and in particular, we have emphasised the possible formation of carcinogens. These biochemical studies have not been my direct concern and have been published elsewhere (Aries and Hill, 1970a and b; Hill, 1971a and b; Hill and Aries, 1971). We have also attempted to relate the numbers and activities of the intestinal bacteria to the worldwide distribution of cancer of the large bowel in man.

S E C T I O N 1

REVIEW OF PREVIOUS WORK

1.1 THE INTESTINAL BACTERIA

The microbial populations within the intestines of man and some animals have been considered by Dubos (1965) and Mata, Carrillo and Villatorro (1969) to form two components: the first is a resident (or autochthanous) component containing organisms which are always present in large numbers, whilst the second is a transient component containing organisms which are present in very variable numbers. According to Dubos, organisms in the second group include enterobacteria, enterococci, spore-forming bacilli and clostridia. Organisms in the first group are principally non-sporing anaerobes and are now considered to be the most important components of the flora. Many isolates are difficult to grow, identify and preserve, and these problems have prevented ready exchange of strains between laboratories. Several diverse taxonomic schemes have therefore evolved - for example, Breed, Murray and Smith (1957), Prevot (1966) and Beerens and Tahon-Castel (1965). These have been compared in a review by Smith and Holdeman (1968) and the same workers are currently attempting to unify the taxonomic criteria and identification methods and so bring order to chaos (see Cato et al., 1970). The different species isolated from human and animal intestines have been listed by Rosebury (1962) and Donaldson (1968).

The Gram-positive, non-sporing anaerobes (bifidobacteria)

The Gram-positive, non-sporing anaerobic bacteria of man are thought to belong to four families: Lactobacillaceae, Corynebacteriaceae, Propionibacteriaceae and Actinomycetaceae. Although the family status and the speciation of many organisms are still in dispute - compare Smith and Holdeman (1968) with Cato et al. (1970) - several genera can be defined by their acid end-products of glucose fermentation. Fermentation patterns of those genera commonly found in faeces are shown in Table 1.

In our survey it was not possible to identify all Gram-positive anaerobes to the genus level and in this thesis the term 'bifidobacteria' embraces all Gram-positive, strictly anaerobic non-sporeforming rods.

The Gram-negative, non-sporing anaerobes (bacteroides)

The Gram-negative anaerobes are no better understood than the bifidobacteria, and their taxonomy is also in flux. The family Bacteroidaceae was divided into three genera, Bacteroides, Sphaerophorus and Fusobacterium by Smith and Holdeman in 1968, but in 1970 the same authors (Cato et al.) combined Sphaerophorus and Fusobacterium into one genus Fusobacterium. In a study using numerical taxonomy, Barnes and Goldberg (1968) verified that Sphaerophorus and

Table 1. Genera of obligately anaerobic non-sporing bacteria (from Cato et al., 1970)

Gram stain	Major volatile acids produced from glucose	Genus
Gram-positive cells present	Propionic and acetic	Propionibacterium and Arachnia
	Acetic and lactic	Bifidobacterium
	Lactic sole major product	Lactobacillus
	Succinic and lactic; some acetic and formic	Actinomyces
	Butyric and others; acetic and formic; or no major acids	Eubacterium
Only Gram-negative cells present	Butyric (without much isobutyric and isovaleric)	Fusobacterium
	Lactic only	Leptotrichia
	Not as Fusobacterium or Leptotrichia	Bacteroides

Fusobacterium formed one phenon, which was distinct from the genus Bacteroides.

Different workers have tried to separate the genera using differential sensitivity to antibiotics (Finegold, Miller and Posnick, 1965; Barnes and Goldberg, 1968), and stimulation of growth by bile (Beerens et al., 1963; Barnes and Goldberg, 1968; Shimada, Bricknell and Finegold, 1970). The most useful differential test is the detection of the acid products of glucose fermentation (Guillaume, Beerens and Osteux, 1956; Barnes and Goldberg, 1968; Cato et al., 1970). Bacteroides spp. produce acetic, lactic and succinic acids (and sometimes propionic and iso-valeric acids), whilst Fusobacterium spp. produce predominantly butyric acid (see Table 1).

A few species of Bacteroides and Fusobacterium are relatively well defined, but many isolates cannot be identified using the present criteria, and some cannot even be placed in the appropriate genus. In this study specific identification has not been attempted, and the term 'bacteroides' includes all strictly anaerobic, Gram-negative non-sporing rods.

Sarcina ventriculi

In the work described in this thesis, Sarcina ventriculi was found regularly in faeces from persons in Uganda and India.

As this organism has never been reported previously in the intestines of healthy persons, it requires some introduction.

Sarcina ventriculi was first observed by Goodsir (1842) in the stomach contents of a patient with "gastric fermentation". It was subsequently reported in similar cases and in patients with pyloric ulcers or gastric carcinomas (Smit, 1933; Professor E.A. Dawes, personal communication, 1970). In these patients food is withheld for long periods and the sarcinae multiply, even in the presence of high gastric acidity. Sarcina ventriculi was first isolated from the human stomach by Beijerinck (1911) who showed that it was a strict anaerobe; Beijerinck (1905) had previously shown that the same organism occurred widely in the soil.

There has been considerable confusion in the literature about the taxonomic relationships between the aerobic and anaerobic packet-forming cocci. However, the genus Sarcina is now restricted to the obligately anaerobic, Gram-positive cocci which form cubical packets (Kocur and Martinec, 1965; Canale-Parola, Mandel and Kupfer, 1967). The sarcinae have been reviewed recently by Canale-Parola (1970). The only species identified in this study was Sarcina ventriculi.

1.2 DISTRIBUTION OF BACTERIA IN THE GASTROINTESTINAL TRACT

The mouth

The human mouth contains very diverse microbial populations, which include obligate anaerobes as well as facultative organisms. Although most oral organisms can usually be isolated from anywhere in the mouth, particular sites, such as tooth surfaces, gingival crevices and the tongue, favour particular genera (Hoffman, 1966; Bowden and Hardie, 1971). Saliva also supports a varied flora: 'viridans' streptococci are usually the most numerous members (approximately 10^6 per g saliva), but anaerobic organisms, including bacteroides, veillonellae, bifidobacteria and fusobacteria are present in counts of approximately 10^4 per g; enterobacteria and enterococci, which are commonly considered as faecal organisms, are sometimes found (Richardson and Jones, 1958; Rosebury, 1962; Drasar, Shiner and McLeod, 1969).

The stomach and small intestine

The microbial populations of the stomach and upper small intestine in fasting healthy persons are relatively sparse compared with those of the mouth and consist predominantly of yeasts and Gram-positive, facultative bacteria in concentrations up to approximately 10^3 per ml. fluid

(Dellipiani and Girdwood, 1964; Donaldson, McConnell and Deffner, 1967; Gorbach, Plaut, Nahas and Weinstein, 1967). Gram-negative organisms are usually absent, but enterobacteria and bacteroides have occasionally been found (French, 1961; Kalser et al., 1966; Drasar et al., 1969; Hamilton et al., 1970). The numbers of bacteria generally increase progressively down the gut so that in the terminal ileum the total may be 10^8 per g; Gram-negative organisms, including enterobacteria and bacteroides, become numerous components of the flora, and the relative numbers of the different groups approach those found in faeces (Gorbach et al., 1967; Drasar et al., 1969; Hamilton et al., 1970). The literature describing the microbial populations of the small intestine has been reviewed by Donaldson (1968) and Gorbach (1971).

The large intestine

The bacterial flora of the proximal large intestine of healthy adults has been studied little, because of the considerable difficulties in sampling. Gorbach et al. (1967) found that caecal contents of healthy persons sampled by tubes passed orally, contained between $10^{6.4}$ and $10^{9.1}$ bacteria per g, and Seeliger and Werner (1963) found 10^7 - 10^9 bacteria per g in healthy appendices taken at operation. Unfortunately, simultaneous faecal specimens were not examined in either of

these studies. The bacterial flora of caecal contents and faeces sampled post-mortem were found to be the same by Haenel and Müller-Beuthow (1958). William-Smith (1965) also found that caecal contents and faeces were the same for 15 different species of laboratory animals examined immediately post-mortem, and this was confirmed in our laboratory for four animal species (Hawksworth, Drasar and Hill, 1971). Therefore, it is generally assumed in the literature that samples of faeces provide a reasonable assessment of the microbial populations of the large intestines of man (see Haenel, 1970).

The bacterial flora of faeces

The bacterial flora of faeces of persons from America and Western Europe has been studied by many workers, but with a few exceptions the faecal bacteria of persons living in other parts of the world is unknown. The work has been reviewed by Rosebury (1962), Donaldson (1964, 1968) and Drasar (1967a) and that published since 1967 is summarised in Table 2.

It is well established that the predominant organisms in faeces from Western persons are non-sporing anaerobes. Some authors claim that the most numerous organisms are Bacteroides (Mata et al., 1969; Levinson and Kaye, 1969), others bifidobacteria (Bendig, Haenel and Braun, 1968), whilst others have found equal numbers of bifidobacteria and bacteroides

Table 2. Bacterial flora of faeces from healthy persons

	Gorbach et al., 1967	Bendig et al., 1968	Floch et al., 1968	Drasar et al., 1969	Levinson and Kaye 1969	Mata et al., 1969	Miller, 1969	Cordaro et al., 1970	Conn and Floch, 1970
No. persons examined	5	265	8	25	5	12	8	4	-
Total anaerobes	9.7	9.8	9.4	-	-	10.5	10-11	8-11	9.3
Bifidobacteria	7.1	9.4	-	10.5	9.5	9.4	8-11	-	-
Bacteroides	-	9.1	9.4	10.5	9.8	10.3	9-11	6-10	8.9
Enterobacteria	5.9	7.3	7.1	6.0	8.0	8.7	4-8	5-9	6.8
Streptococci	6.6	6.7	6.4	4.0	-	-	-	-	5.7
enterococci	-	-	-	3.2	6.0	7.9	7-9	2-8	-
Streptococcus salivarius	-	-	-	2.2	-	-	-	2-8	-
Lactobacilli	6.1	6.7	8.2	4.0	4.4	8.6	5-8	3-9	7.9
Clostridia	-	3.7	-	2.6	5.5	9.3	-	-	-
Veillonellae	-	-	-	3.0	-	9.2	-	-	-
Bacilli	-	3.8	-	-	-	-	-	-	-
Staphylococci	5.6	4.1	-	1.1	1.7	5.0	-	-	-
Fungi	3.2	3.9	-	1.0	2.3	4.0	-	-	-

(Drasar et al., 1969; Miller, 1969; Moore, Cato and Holdeman, 1969). It is noteworthy that although the various authors used different bacteriological techniques, they generally agree that approximately 10^{10} viable anaerobes can be recovered from one gram of faeces.

In contrast, facultative organisms such as coliforms, lactobacilli and streptococci are found in much lower numbers and the counts differ widely between surveys. This variation can be attributed to the small number of persons examined in some surveys, and to the real variation in counts of these organisms from person to person.

1.3 FACTORS INFLUENCING MICROBIAL GROWTH IN THE GASTROINTESTINAL TRACT

The stomach and small intestine

The microbial populations of the stomach and small intestine of healthy persons remain sparse, even though there are ample nutrients for growth. The reasons for this are not fully understood, but gastric acidity, intestinal motility, components of bile and secretory immunoglobulins may all be involved.

In normal persons the pH of gastric juice is below 3.5 and inhibits bacterial growth in the stomach. Free hydrochloric acid is one of the toxic agents (Bartle and Harkins, 1925). The numbers of viable organisms in gastric juice decrease with increasing gastric acidity (Gorbach *et al.*, 1967; Drasar *et al.*, 1969) and in persons with gastric achlorhydia more organisms are found than in normal persons.

Gastric acidity may also be one factor limiting the microbial populations in the upper small intestine. Persons with gastric achlorhydria usually contain more bacteria in the upper small intestine than normal persons. Bacteria passing into the small intestine immediately after a meal are usually removed rapidly in normal persons, but persist much longer in achlorhydrics (Drasar *et al.*, 1969).

Bile may be important in controlling the bacterial

populations in the upper small intestine but evidence in man is lacking. It is conflicting that whilst bile tends to inhibit Gram-positive but not Gram-negative bacteria *in vitro* (Stacey and Webb, 1947), the few bacteria in the upper small intestine of normal persons are predominantly Gram-positive aerobes; bile tolerant organisms, enterobacteria, enterococci and bacteroides, begin to increase in numbers only in the terminal ileum (Drasar *et al.*, 1969; Hamilton *et al.*, 1970), and by then most of the conjugated and some of the free bile acids will have been reabsorbed from the ileum and returned to the enterohepatic bile salt pool (Hislop, Hofmann and Schoenfield, 1967). The effects of bile and bile salts on the growth of intestinal bacteria *in vitro* are reviewed below.

Intestinal peristalsis appears to be important in the mechanical removal of bacteria from the small intestines of monkeys and dogs (Dack and Petran, 1934) and rats (Dixon, 1960) and a similar mechanism may operate in man. Where regions of stasis develop in the human small intestine, due to impaired motility or altered anatomy, a richer microbial flora may evolve: enterobacteria, bacteroides, clostridia and enterococci increase to numbers approaching those found in the terminal ileum. Such bacterial overgrowth may occur in patients with diverticulosis (Drasar and Shiner, 1969), intestinal obstructions (Bishop and Allcock, 1960), Crohn's

disease (O'Grady et al., 1970), or result from surgically created stagnant loops as in Polya partial gastrectomy (Drasar and Shiner, 1969).

Immunological mechanisms may also be important host defence mechanisms in the human gastrointestinal tract. Immunoglobulins of the class IgA are the predominant immunoglobulin species in the nasal, gastric and intestinal secretions and tissues of man (Tomasi and Bienenstock, 1968; Plaut and Keonil, 1969; Johnson, 1970). They are chemically, physically and antigenically distinct from the IgA antibodies of serum, and have been called secretory antibodies. In the gut secretory antibodies are synthesised locally in the lamina propia of the intestinal mucosa; they also occur in bile and gastric and pancreatic secretions.

Studies on cholera (Freter, 1962) and polio (Ogra et al., 1968) suggest that oral administration of antigen may stimulate better local antibody formation than the parenteral routes, and in patients with cholera, IgA antibodies are excreted in the faeces (Waldman et al., 1971). It is therefore presumed that secretory antibodies are formed against any micro-organism entering the gastrointestinal tract, and they may protect the host tissues and their indigenous microbial populations against invasion by exogeneous species. The mechanism by which secretory antibodies destroy bacteria in the gut is unknown, but

both lysozyme and complement may be involved (Adinolphi, Glynn, Lindsay and Milne, 1966).

The large intestine and faeces

Mechanisms controlling the microbial populations in the human large intestine are even less well understood than those of the stomach and small intestine. This is due, in part, to the greater difficulties in sampling from the large intestine and to the greater number of variables likely to influence the richer microbial populations. Further, whilst most attention has focussed on the association of bacterial overgrowth in the small intestine with malabsorption disorders, such as the blind loop syndrome (see reviews by Donaldson, 1968; Gorbach and Levitan, 1970; Tabacqhali, 1970), there is no chronic disease which is known to be caused by an atypical distribution of bacteria in the large intestine.

The large intestine possesses some antibacterial mechanisms in common with those in the small gut: lysozyme (Florey, 1930) and IgA antibodies (Crabbé and Heremans, 1966; Tomasi and Bienenstock, 1968) are found in colonic and rectal mucosa. Monteiro et al. (1971) have shown that IgG antibodies are the predominant class of antibodies in colonic and rectal mucosa, but this has to be confirmed. These mechanisms may protect the host from invasion by bacteria at the tissue

surfaces, but it is not known whether they play any part in controlling the numbers and activities of the indigenous bacteria in the gut lumen.

It is reasonable to suppose that the factors governing the distribution of bacteria in faeces may also govern their distribution in the large intestine. This section describes previous surveys designed to find some factors which control the faecal flora of man.

Effects of age

At birth the human infant is colonised by bacteria from the environment. During the first three days of life the intestinal flora consists almost entirely of facultative organisms such as lactobacilli, streptococci and Escherichia coli in counts up to 10^{10} per g; bacteroides and clostridia may be present in some infants in counts up to 10^{10} and 10^{8} per g respectively. On the third day bifidobacteria become the predominant components of the flora (Smith and Crabb, 1961; Haenel, 1961, 1970). Infants fed on breast-milk may contain fewer bacteroides, veillonella, clostridia (Haenel, 1970), enterobacteria and enterococci (Gyllenberg and Roine, 1957) than those fed on cow's milk. Other workers have found little difference between breast-fed and bottle-fed infants (Smith and Crabb, 1961; Mata et al., 1969). Haenel suggests that breast-

feeding alone is not the determining factor. The effects of breast-feeding are only pronounced in relatively clean environments; with poor hygiene, the faecal flora of breast-fed infants approaches that of bottle-fed infants.

The numbers of lactobacilli, enterococci and enterobacteria may fall gradually during the first year so that when the infant is one year old the counts may be less than a tenth of their level during the first few days of life; counts of enterobacteria and streptococci in faeces from calves, piglets and rabbits are high during the first few days of life but may fall during subsequent weeks (Smith and Crabb, 1961).

The faecal flora of children aged 5 months to 6 years in Alaska (Miller, 1969) and children aged 6-16 years in Germany (Bendig, Haenel and Braun, 1968) was the same as that of young adults (Table 3). Bendig and co-workers could find no correlation between the counts of bacteria and the age or sex of the children.

During adult life the relative numbers of each of the different micro-organisms may change. Older men and women carried fewer bifidobacteria and more enterobacteria than did young adults in Germany (Haenel, 1963) and in America (Gorbach, Nahas, Lerner and Weinstein, 1967). Older persons also carried more clostridia, streptococci and lactobacilli (Haenel, 1963), but these results were not confirmed by Gorbach et al. (1967).

Table 3. Faecal flora of persons of different ages living on mixed diets (mean $\log_{10}$ viable count per g faeces)

Reference	Bendig et al. 1968	Gorbach et al., 1967			Haenel, 1963	
Age	6-16	20-39	40-69	70-100	22 (mean)	66-88
No. persons examined	265	60 in all	-	-	59	19
Country	Germany	U.S.A.	U.S.A.	U.S.A.	Germany	Germany
Total anaerobes	9.8	9.3	9.8	9.1	9.6	9.6
Bifidobacteria	9.1	7.2	7.0	5.9[a]	9.4	9.3[b]
Bacteroides sp	9.4	9.3	9.8	9.1	-	-
Enterobacteria	7.3	6.4	7.6	7.5[a]	6.4	7.9[a]
Streptococci	6.7	5.7	5.6	5.9	6.7	8.2[a]
enterococci	-	-	-	-	5.9	7.8[a]
Lactobacillus spp	-[c]	5.3	4.3	4.9	-[c]	-[c]
Clostridia: Lecithinase-positive	3.7	-	-	-	4.6	6.0[a]
Bacillus spp	3.8	-	-	-	-	-
Staphylococci	4.1	5.2	4.1	5.0	4.1	4.3
Yeasts	3.9	1.8	1.1	3.3[a]	4.4	4.3
Total aerobes	7.6	7.3	8.1	7.9	7.0	8.7[a]

a Significant difference between young and old age-groups ($P < 0.01$)

b only 76% of persons carried bifidobacteria

c lactobacilli and streptococci counted as one group

- count not reported

It is difficult to isolate the effects due to a person's age from those due to dietary, environmental and racial factors. Nevertheless, it is possible to recognise general trends and it seems reasonable to conclude from the literature that after an infant has been weaned, an intestinal flora is established which, within broad limits, remains stable from childhood to adult life; as old age approaches, numbers of bifidobacteria decrease and those of enterobacteria and clostridia increase.

Effects of diet

Interest in the ways in which man's dietary habits influence his intestinal bacteria began at the end of last century, when it was generally believed that many diseases were caused by toxins produced in the intestine by bacteria. Metchnikoff (1903, 1908) suggested that toxins could not be formed if lactobacilli were the predominant bacteria in the gut. Bacteriologists therefore sought diets to encourage *in vivo* growth of lactobacilli. Some claimed success, others failure. Their work has been reviewed by Rettger and Cheplin (1921). The most conclusive studies were those of Rettger *et al.* (1935) who showed that the numbers of *Lactobacillus acidophilus* excreted in the faeces could be increased only by consuming viable lactobacilli together with large amounts of lactose;

after withdrawal of lactose therapy the numbers of lactobacilli returned to their former level. Failure of the lactobacilli to colonise the gut even after such radical measures, provided the first evidence that the human intestinal flora is very stable. More recent work has confirmed that feeding lactose does not increase the numbers of lactobacilli in faeces (Haenel, 1961).

In the 1950's Haenel and his co-workers were the first ecologists to recognise the claims of Eggerth and Gagnon (1933) that the most numerous bacteria in faeces were non-sporing anaerobes, and they investigated the effects of extreme diets on the faecal flora of man.

Vegetarian diets

Haenel, Müller-Beuthow and Scheunert (1957) found that faeces from eight persons in Germany who had lived for 14 days on a strict vegetarian diet contained the same bacterial flora as during control periods on mixed diets; bifidobacteria were the most numerous organisms in all specimens. This was confirmed in part by Moore, Cato and Holdeman (1969) who studied 12 teenage American girls living on a vegetarian diet for 10 weeks. Three kinds of predominant faecal flora were found - one primarily bifidobacteria, another primarily bacteroides and the third a mixture of bifidobacteria and bacteroides; after the girls had lived on a mixed diet for a year, they had the

same predominant flora as they had previously on the vegetarian diet.

Diets rich in animal protein

Haenel et al. (1957) and Hoffman (1964) found that the faecal flora of healthy persons living from 2-11 weeks on diets containing high levels of animal protein was no different from that during control periods on normal mixed diets; Haenel et al. (1957) found that in some persons the numbers of coliforms, enterococci and lactobacilli increased slightly, but not significantly.

High fat diet

All previous workers except Hoffman (1964) have neglected to study the ways in which the dietary fat may influence the faecal flora. Hoffman showed that when a healthy volunteer was fed a high fat diet containing 252 g fat per day for 81 days, the numbers of bifidobacteria and enterococci decreased whilst those of bacteroides increased. The pH of the faeces varied from 6.2 to 7.0, and was the same as during the control period on a mixed diet.

Chemically defined liquid diets

A new approach to the study of the microbial ecology of the adult intestine was found by Winitz, Adams, Seedman and Graff (1966) and Winitz et al. (1970) who described a chemically defined liquid diet containing water-soluble carbohydrates, vitamins, amino acids, fats and minerals, designed to be used in the place of conventional foods. The concentration of each component can be varied at will. The diet is claimed to be absorbed almost completely from the gut and therefore leaves a very low residue. Winitz et al. (1970) found that a liquid diet containing glucose as the sole carbohydrate lead to a faecal flora in which the total counts, predominantly bacteroides, were only 10^5 per g faeces compared with levels of 10^9 per g whilst on mixed diets; coliforms and enterococci were present in counts of 10^3 to 10^5 per g whilst bacilli, lactobacilli, clostridia and yeasts were rarely found. If sucrose was used in place of glucose, the counts of bacteroides were the same as the counts on a mixed diet, but those of clostridia, lactobacilli, bacilli and enterococci usually decreased.

Effects of chemical nature of faeces

The chemical nature of faeces is very complex (Table 4) and some components may affect the microbial populations. The chemical nature is directly and indirectly influenced by diet.

Table 4. Composition of faeces from healthy persons living on mixed diets

Water: 70-80%

Solids: 20-30%

Bacteria: approximately 30% of the solids.

Organic components:

Fatty acids (approx. 18% of the solids)

Long chain: mainly stearic, oleic and palmitic in the form of soaps, glycerides or free acids.

Short chain: mainly formic, acetic, iso-butyric, iso-valeric and caproic.

Bases (approx. 50 mg/g solids): ammonia, methylamine, trimethylamine, pyrrolidine, piperidine, cadaverine, putrescine, indole, skatole.

Steroids (approx. 16 mg/g solids)

Neutral steroids (approx. 10 mg/g solids): cholesterol,

Direct effects would be due to dietary components, or their degraded products, which have escaped absorption from the intestine. Indirect effects would be due to those changes in gut physiology, including those which result from changes in diet. An important physiological change is the fall in concentration of bile acids in the faeces in response to a low fat diet (Hill, 1971b). Hill showed that when healthy persons changed from a mixed diet containing approximately 120 g fat per day, to a mixed diet containing only 30 g fat per day, the faecal concentration of bile acids fell from 5.5-7.5 mg/g to 1.5-2.0 mg/g faeces, and maintained this low level until a normal diet was resumed; the cholesterol and coprostanol levels in the faeces also fell during a low fat diet. Since bile and bile acids affect bacterial growth *in vitro*, it seems reasonable to suggest that they may do so *in vivo*.

Bile and bile steroids

Bile is a complex mixture of bile salts (conjugates of bile acids with taurine or glycine), bile pigments (urobilins), free cholesterol, fatty acids and inorganic ions (Haslewood, 1967). Only two bile acids, cholic and chenodeoxycholic acids are synthesised by the liver. These are secreted into the bile as salts with taurine or glycine. It is now well established that *in vivo* bile salts can be deconjugated by the intestinal

bacteria to give free bile acids and these may be further degraded (see reviews by Gray, Nicholson and Quincey, 1968; Shimada, Bricknell and Finegold, 1969). Most of the undegraded and degraded bile acids are reabsorbed from the ileum and returned to the liver to be again excreted in the bile. Approximately 10-15% so circulating escapes into the large intestine and may be further degraded before reaching the faeces (Gray et al., 1968). In a literature survey Haslewood (1967) found that 31 molecular species of bile acids have been reported in the faeces of healthy human adults; two of these are the primary acids (cholic and chenodeoxycholic acids) whilst the remainder are formed from them by bacterial activity. Similarly, biliary cholesterol is degraded by gut bacteria and the resultant products, principally coprostanol and coprostanone, are excreted in the faeces (Coleman and Bauman, 1957; Hill and Aries, 1971). The ways in which intestinal bacteria can degrade bile steroids have been reviewed by Bergström, Danielsson and Samuelsson (1960), Midtvedt and Norman (1967) and Shimada, Bricknell and Finegold (1969). Recent work by my colleagues Drs. Hill and Aries has been reported in detail elsewhere (Aries and Hill, 1970a and b).

Effects of bile on bacteria

(a) Inhibitory effects

The toxic effects of bile towards some bacteria have been known since the beginning of the century. Neufeld (1900) showed that *Streptococcus pneumoniae* is soluble in bile, whereas other streptococci are not. Weissenbach (1918) showed that incorporation of 10% ox-bile into broth medium inhibited growth of all streptococci except enterococci, and Bragger (1926) used this as the basis of a medium for distinguishing enterococci from other streptococci. Other early workers considered only aerobic pathogens and showed that in general bile was much more toxic to Gram-positive than to Gram-negative bacteria (Leifson, 1935; Stacey and Webb, 1947). The antibacterial properties are due to the detergent action of the bile salts, which may act either by stimulating activity of autolytic enzymes within the cells (Dubos, 1937; Wilson and Miles, 1964) or by dissolving some components of the Gram-positive cell wall, such as teichoic acids and polysaccharides (Hill, 1967).

There is little information which of the many undegraded and degraded bile steroids or other compounds present in whole bile are the inhibitory factors. Leifson (1935) found that deoxycholic acid was more toxic than whole bile to staphylococci, streptococci (other than enterococci) and

unspecified Gram-positive bacilli, but was without effect on Gram-negative intestinal bacilli. Deoxycholic acid (3α-, 12α-dihydroxycholanic acid) is much more toxic towards Staphylococcus aureus than is cholic acid (3α-, 7α-, 12α-trihydroxycholanic acid) which suggests that the antibacterial properties of these bile acids may depend on the number and position of the hydroxyl groups (Stacey and Webb, 1947). Cholic acid, at concentrations commonly found in human faeces, inhibits lactobacilli but not enterobacteria or enterococci (Floch, Gershengoren, Diamond and Hersh, 1970).

The toxicity of deoxycholic acid to enterobacteria is enhanced by some inorganic compounds, including sodium phosphate and sodium chloride (Allen, Pierce and Pashley, 1952), and by sodium salts of some organic acids, especially acetic, propionic, butyric and citric acids (Liefson, 1935). Culture medium containing sodium deoxycholate and sodium citrate inhibits Escherichia coli but not species of Salmonella or Shigella and Liefson used this technique to isolate these pathogens from faeces.

(b) Stimulatory effects

The effects of whole bile on anaerobic Gram-negative intestinal bacteria have been studied more recently, primarily as a method of classifying these organisms. Barnes and

Goldberg (1968) found that growth of most strains of Bacteroides spp. (except those of B. melaninogenicum) was stimulated by 10% bile, whereas growth of Fusobacterium spp. and Sphaerophorus spp. was not. Beerens et al. (1963) found that some strains of Bacteroides fragilis had an obligate requirement for an unidentified growth factor present in whole ox-bile and these authors suggested that such organisms should be assigned to a new genus, Eggerthella.

There appears to be no information showing how bile affects bacterial growth in vivo. Furthermore, only the sparse populations of the upper small intestine will be subject to the influences of whole bile. Bacteria in the colon will be subject to only the portion of bile which has escaped reabsorption in the ileum and any bile salts in this may be deconjugated, dehydroxylated or further degraded.

Part of this thesis attempts to find relationships between the concentrations of bile acids and the microbial populations in faeces.

Microbial factors

In any natural environment a given micro-organism may inhibit the growth of its neighbours (by the production of toxins) or may stimulate the growth of its neighbours (by the production of essential nutrients), or each organism may simply

grow independently of all others. In the human intestine, where there are very many different species of bacteria, the inter-relationships are necessarily complex, and although it is believed in the literature that the organisms themselves are important in controlling their numbers, the evidence for this is very scant. When intestinal strains of bacteroides, bifidobacteria, enterobacteria, enterococci and staphylococci are grown together in continuous culture, their relative numbers approximate to those found in faeces (Collard and Gosling, 1969). This provides some evidence that many factors responsible for the balance of the intestinal flora are properties of the organisms themselves rather than of the human host.

Antagonistic interactions

Competitive activities between the intestinal flora were sought to substantiate Metchnikoff's hypothesis (1908) that the lactobacilli in the human intestine were capable of inhibiting 'putrefactive' bacteria. Torrey and Kahn (1923) showed that *in vitro* *Lactobacillus acidophilus* inhibited proteolysis by, but not the growth of *Clostridium sporogenes*, *Cl. histolyticum* and *Cl. botulinum*; the high acidity of the culture medium was shown to be the inhibitory factor. Other workers have since shown that *in vitro* *L. acidophilus* also inhibits *E. coli* (Sabine, 1963; Tramer, 1966), *Staphylococcus aureus* (Wheater,

Hirsch and Mattrick, 1952) and Candida albicans (Young, Krasner and Yudkofsky, 1956). The inhibitory agent was probably lactic acid, although inhibition of S. aureus might also involve unidentified proteins of low molecular weight (Troller and Frazier, 1963). Bifidobacterium bifidus inhibits growth of E. coli and Streptococcus faecalis in vitro by the production of acetic and formic acids; lactic acid is also produced but it is much less inhibitory (Upton, 1929).

The normal bacterial flora in the mammalian intestine is thought to protect the host against invasion by enteric pathogens. Several studies have verified this and the early work has been reviewed by Rosebury (1962) and Meynell (1963). More recently, Hentges (1970) has shown that Bacteroides fragilis inhibits growth of Shigella flexneri in vitro by the production of acetic and propionic acids. Other species of Bacteroides, Propionibacterium and Eubacterium, all numerous components in the intestines of Western persons, also produce these acids and it seems likely that they too will prevent colonization of the gut by shigellae. Enterobacteria also produce acetic and lactic acids and are known to inhibit growth of shigellae in vitro (Halbert, 1948) and in vivo in the mouse caecum (Freter, 1962b). Production of these acids by the intestinal flora may be responsible for the failure by species of Salmonella, Proteus, Pseudomonas, and Staphylococcus to colonise mice (Meynell, 1963; Miller, 1969).

Many bacteria produce bacteriocins. These are toxins which inhibit growth of different strains of the same species but are non-toxic to those of other species. The subject has been reviewed extensively by Reeves (1965) and Nomura (1967). The colicines of Escherichia coli have received most attention. Serotypes of E. coli which actively produce colicines appear to prevent colonisation of the human intestine by other serotypes; weak colicine producers permit other serotypes to flourish (Branche et al., 1963).

Bacteriocins are also produced by enterococci. Those produced by Streptococcus faecium are true bacteriocins and inhibit only other strains of Str. faecium, but those produced by Str. faecalis may not be bacteriocins in the strict sense of the word, since, besides inhibiting other strains of S. faecalis, they also inhibit lactobacilli, bacilli, staphylococci and clostridia (Brock, Peacher and Pierson, 1963).

There appear to be no reports of bacteriocins produced by non-sporing anaerobes.

Co-operative interactions

An organism may promote growth of another by synthesising nutrients, or by creating an acidic or anaerobic environment favourable for growth. The co-operative effects of organisms within the human gut are not known; those of some aerobic

pathogens have been studied *in vitro* and have been reviewed by Rosebury (1962) and by Donaldson (1964, 1968).

It must be emphasised that the mutual interactions of two micro-organisms *in vitro* may not be the same as their interactions *in vivo*. For example, a strain of *Streptococcus faecalis* which inhibited *Salmonella typhimurium* on agar culture plates, would not do so when both organisms were bicontaminants in gnotobiotic mice (Wagner and Starr, 1967). Therefore one must extrapolate cautiously from two-component systems *in vitro* to the complex ecology of the gut.

Part of this thesis describes attempts to show microbial interactions within the healthy human intestine.

1.4 BACTERIA AND THE AETIOLOGY OF CHRONIC INTESTINAL DISEASES

Whilst many acute diseases of the intestine are caused by bacteria, several chronic diseases are still of unknown aetiology. However, evidence is now accumulating that bacteria may be involved in some of them.

Whipple's disease, Crohn's disease and ulcerative colitis

Whipple's disease is a rare condition in which the jejunal mucosa becomes invaded by large numbers of rod-shaped bacteria. Attempts to cultivate the organism have failed (Sherris, Evans and Porus, 1965). Nevertheless, most authors agree that a bacterial infection may be important in the pathogenesis of Whipple's disease since, during antibiotic therapy, the organisms disappear from the gut wall and the patient recovers (Roberts, Thiemann, Knust, Preston and Donaldson, 1970).

In Crohn's disease any part of the gastro-intestinal tract, but especially the ileum, may become inflamed, ulcerated and eventually obstructed. All attempts have failed to isolate a causative micro-organism (Schapiro, Ravdin and Johnson, 1939; Law, 1969). However, Mitchell and Rees (1970) showed that when homogenates of ileal or lymph-node tissue from a patient with the disease, were injected into the footpads of mice, granulomatous lesions developed which were similar to those

observed in Crohn's disease. The nature of this transmissible antigen is still unknown, but the possibility of a bacterial agent cannot be excluded.

In a search for the cause of ulcerative colitis, evidence has been presented in favour of hypersensitivity reactions to dietary proteins (Rider and Moeller, 1962) and autoimmune phenomena in which antibody is produced against the intestinal mucosa (Broberger and Perlmann, 1963). There is also evidence that some bacteria in the gut, especially 014 serotypes of Escherichia coli (Perlmann, Hammarstrom, Lagercrantz and Gustafsson, 1965) and strains of Clostridium difficile (Hammarstrom, Perlmann, Gustafsson and Lagercrantz, 1969) possess antigens in common with those on gut mucosa and it seems possible that such organisms could enhance, or initiate, the production of anticolon antibodies. Some strains of E. coli isolated from faeces of patients with ulcerative colitis, but not those from healthy controls, could also produce necrotic lesions when inoculated into the ileum of rabbits (Cooke, 1968). However, the role of these organisms in the pathogenesis of ulcerative colitis in man is still obscure.

Cancer of the large bowel

Cancer of the large bowel in man is of unknown aetiology. Epidemiological studies show that it is much more common in

North-West Europe and North America than it is in East Africa, India, Japan and South America (Doll, Payne and Waterhouse, 1966; Doll, 1967, 1969). The areas with a low incidence of the disease, with the exception of Japan, have a low standard of living, whilst the high incidence areas have a high standard. These geographical differences cannot be attributed to racial differences since Japanese who migrate to California retain their low incidence experience, provided they retain their original cultural habits; adoption of Western habits either in California (Buell and Dunn, 1965), or in Japan (Wynder et al., 1969), seems to be associated with a trend towards a higher incidence of colon cancer. The three racial groups in Hawaii differ in their incidence of colon cancer as do the different racial groups in South Africa (Doll, 1969).

Several studies have indicated a possible relation of colon cancer to diet - either fat (Wynder and Shigematsu, 1967), or protein (Gregor, Toman and Prusova, 1969), and the possibility that these foods contain carcinogens must be considered. Dietary differences might also explain the observed differences of the disease if the diet determined the composition or the metabolic activities of the bacterial flora of the gut, especially if the bacteria were able to produce carcinogens from food components or from the intestinal secretions.

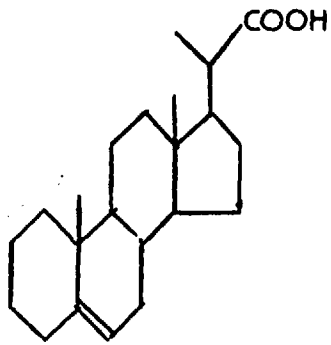
There are few reports of the conversion of food components

into carcinogens by micro-organisms; peanut kernels are commonly contaminated with the mould Aspergillus flavus, which produces aflatoxin, a liver carcinogen (Wright, 1968); cycasin, a glycoside found in the cycad nut, can be hydrolysed by the intestinal flora of the rat to give the carcinogenic aglycone, methoxyazomethanol (Laqueur and Spatz, 1968). However, there seem to be no reports of the production of carcinogens by gut bacteria from other foodstuffs or from natural secretions.

Some bile acids are known to be carcinogenic, particularly deoxycholic acid (Badger et al., 1940; Cook, Kennaway and Kennaway, 1940) which is produced by the intestinal bacteria from cholic acid in the bile. Apocholic acid and bis nor $\Delta 5$ cholanic acid (Fig. 1) are also claimed to be carcinogenic (Lacassagne, Buu-Hoi and Zadjdela, 1961, 1966) and Dr. Hill is investigating the production of these by gut bacteria in this laboratory. Inhoffen (1953) has reviewed the structural similarity between steroids and the polycyclic aromatic carcinogens. Cook and Haslewood (1933) discussed ways in which deoxycholic acid can be converted chemically, via dehydronorcholene, into 20 methylcholanthrene, a very potent polycyclic aromatic carcinogen. An unidentified enterobacterium isolated from human faeces can convert dehydronorcholene, which is not carcinogenic, into a carcinogenic

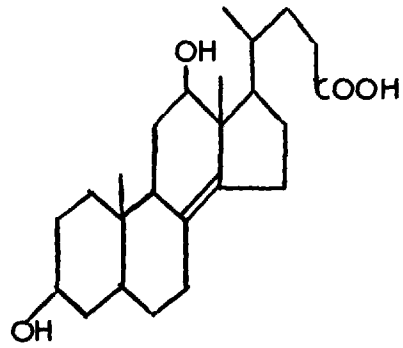
Fig. 1. Bile acids known to be carcinogenic.

BILE ACIDS WHICH HAVE BEEN SHOWN TO BE CARCINOGENS



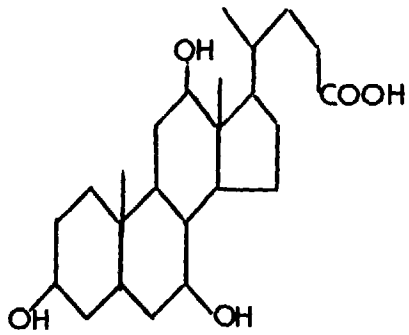
bis nor Δ^5 cholenic acid

Lacassagne et al, Nature 1966 209 1026



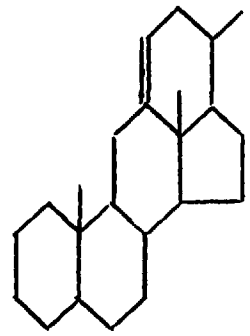
apocholic acid

Lacassagne et al, Nature 1961 190 1007



deoxycholic acid

Cook et al, Nature 1940 145 627
V. Ghiron Proc. 3rd Int.Canc.Con. 1939.116

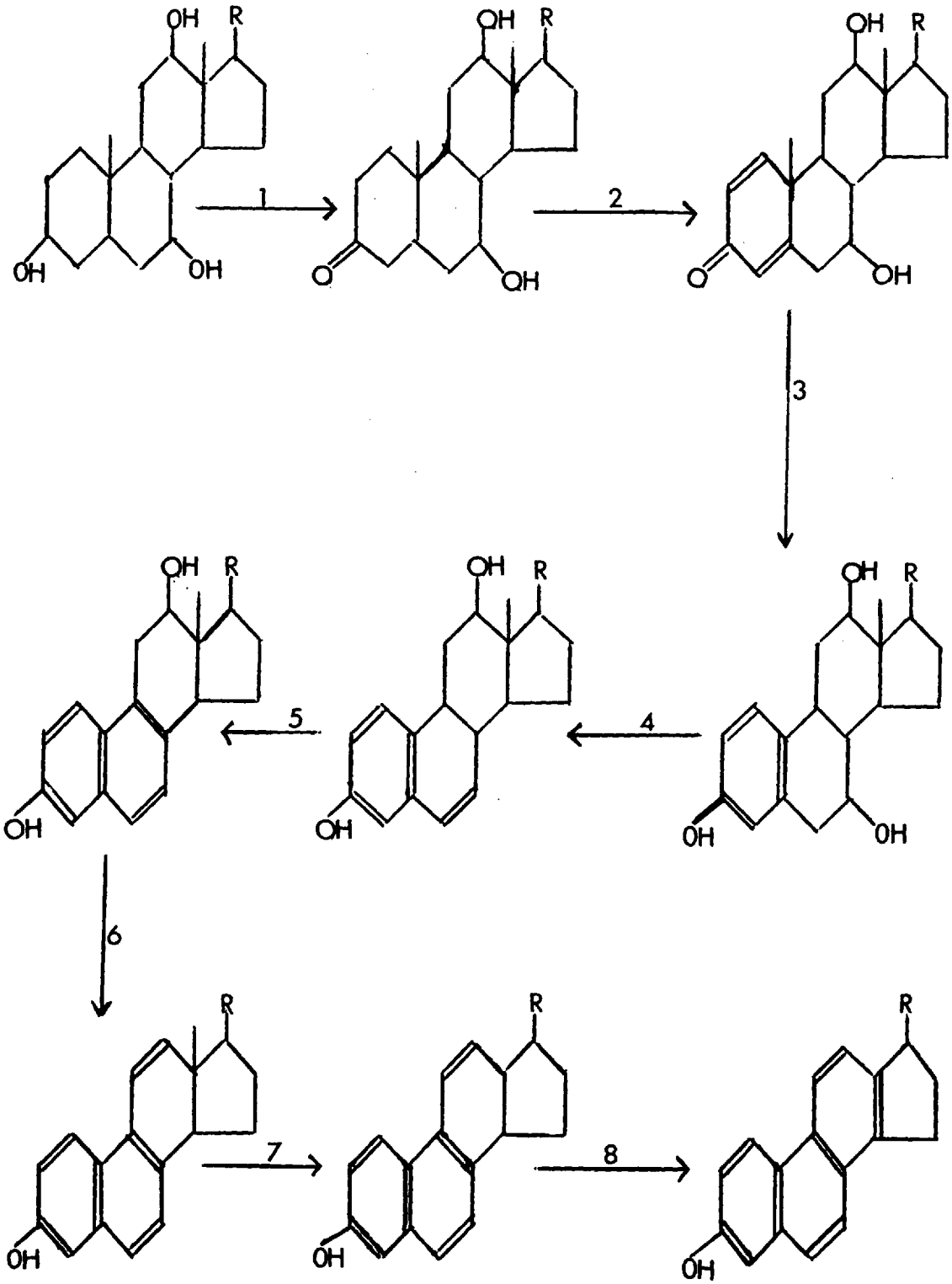


dehydronorcholene

Druckrey et al, Naturwissenschaften 1941 29 63

metabolite (Druckrey, Richter and Vierthaler, 1941). It seems possible therefore, that some of the many different species of bacteria present in the human intestine, could alone or in sequence, convert bile acids or steroids from the diet, into potent carcinogens. Hill (1971a) has suggested some possible ways by which the bacteria could achieve this (Fig. 2).

Fig. 2. Possible reaction sequence for the conversion of cholic acid to polycyclic aromatic carcinogens by bacteria (From: Hill, 1971a).


OH
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SECTION 2

MATERIALS AND METHODS

2.1 POPULATIONS STUDIED

Faeces were collected from healthy persons living on traditional diets in Uganda, South India and Japan, where the incidences of large-bowel cancer are low, and England, Scotland and the United States of America, where the incidences are high.

For additional studies, faeces were collected from healthy volunteers living on special diets.

Persons living on normal diets

Most of the subjects were between 20 and 50 years of age and there were approximately equal numbers of men and women. Precise dietary details of each individual person were not generally known, but all persons ate their normal day to day diet.

The persons were chosen in each of the countries as follows:

Uganda: These were villagers from Bantu tribes living in the Kyadondo district. Many were mothers accompanying children to the M.R.C. Malnutrition Clinic in Kampala; others were hospital staff. Their diet was principally matoke (boiled mashed bananas), supplemented with cassava, maize, beans, other vegetables and tea. The diet was characteristically

high in carbohydrate and low in protein and fat and contained little animal matter (H.J.L. Burgess, 1962; A.P. Burgess, 1962; Burgess, Morton and Burgess, 1962).

India: The Indians lived in villages near Vellore, South India, and were healthy controls in a study of tropical sprue. They lived on a traditional rice and vegetable diet, containing almost no animal protein or animal fat. However, small amounts of vegetable oils, principally from ground nuts, were consumed (Rao and Rao, 1958).

Japan: The Japanese were villagers living in Sakaiminato, near Yonago. Most were farmers and housewives. All lived on the traditional diet containing rice, fruit and vegetables, but little meat or fat.

England: The English persons were nurses, students and staff at St. Mary's Hospital Medical School, London, mostly aged 20-40. Some older hospital patients recently admitted to orthopaedic wards were also studied; none had any known gastro-intestinal disorder nor was taking antibiotics. All persons lived on normal, Western mixed diets.

A small group of persons were studied who lived on

strict vegetarian (vegan) diets consisting of fruit, vegetables and nuts but no animal matter. They consumed as much protein and more than half the amount of fat as those people living on mixed diets; some fortified their diet with the B group of vitamins (Ellis and Mumford, 1967).

Scotland: These people were all students and staff at Edinburgh University Medical School. All lived on normal random Western mixed diets.

United States: The Americans were staff at the Center for Disease Control, Atlanta, Georgia. White and black groups were studied; all lived on normal Western mixed diets.

Volunteers consuming special diets

Chemically defined diets

Three healthy male medical students consumed a chemically defined liquid diet for 10 days. The diet was supplied by The Vivonex Corporation, Mountain View, California, as a powder containing essential and non-essential amino acids, vitamins, minerals, carbohydrates and flavourings as shown in Appendix A1; the fat content of the diet was only 0.75% (w/w). The diet is claimed to be almost totally absorbed (Winitz *et al.*, 1970). It was diluted with water and consumed *ad libitum*. No other

foods were permitted during the period on the diet.

Faeces were collected from each person during the week immediately before starting the diet, during 10 days on the diet and afterwards when a normal diet was resumed.

Low fat diet

The normal Western mixed diet contains 100-120 g fat per day. To find the effects of the fat on the gut bacteria, a healthy male volunteer lived for one month on a diet containing only 30 g fat per day. The diet was otherwise a typically normal Western mixed diet and was designed by the Dietetic Department of St. Mary's Hospital. It excluded all fats used for cooking, fat from meat, eggs, butter, margarine, nuts, milk and cheese. Skimmed milk and cheese made from skimmed milk were permitted. Fruit and vegetables were allowed ad libitum.

Faeces were collected before starting the diet, during one month on the diet and after resumption of a normal diet.

Incidence rates for cancer of the large bowel

The reported incidence rates for cancer of the large bowel throughout the world are not always directly comparable. This is mainly due to the different facilities for diagnosing and recording the disease. In developing countries with inadequate medical services, only mortality rates, obtained from autopsy

series, are known. Doll (1967, 1969) therefore derived factors for converting mortality rates into incidence rates and produced figures for different countries which were much more comparable. He found that an age range of 35-64 gave the most comparable data. This range includes ages where colon cancer is frequent and excludes ages where the figures are likely to be unreliable. Doll's estimated incidence rates for the disease in Britain, the United States of America, Uganda, India and Japan are shown in Table 5. The zero figure recorded for Ugandan men aged 35-64 is obviously unreliable, especially as the disease is recorded for Ugandan men of younger or older age-groups (Doll, Payne and Waterhouse, 1966).

Comparable figures have not been published for the incidence rates of colonic cancer amongst European immigrants in Uganda. However, Davis, Knowelden and Wilson (1965) claimed that the cancer experience of Europeans in Kampala was similar to that of Europeans living in Europe.

The most reliable cancer rates published for India are those for Bombay (Doll, 1969). Although this area is remote from Vellore in South India where the subjects of this survey lived, there is no reason to believe that there would be a substantial difference between the two areas.

Cancer rates have not been published for persons living on strict vegan diets.

Table 5. Populations studied

Country	Incidence of colon cancer[1]: Annual rates per 100,000 persons Aged 35-64			Number and occupations of persons studied			Diet	Method of transporting faeces to London[4]
	Men	Women	Mean for men and women	Men	Women			
England (South Metropolitan)	15.8	20.5	18.2	29	39	Students and staff at St. Mary's Hospital Medical School; some hospital patients.	Normal Western mixed	none; freshly voided faeces emulsified and frozen at -78° or -55°C
"	-	-	-	7	11	Housewives and office workers	'Vegan' diet containing no animal matter	sent by post; specimens emulsified and frozen at -55°C
Scotland (Edinburgh)	28.2	34.1	31.2	20	3	Students and staff at Edinburgh University Medical School.	Normal Western mixed	frozen at -78°C
U.S.A. (Atlanta) (white)	26.6	29.6	28.1	16	6	Caucasian staff at Center for Disease Control, Atlanta	" " "	" " -196°C
U.S.A. (Atlanta) (black)	25.6	31.9	28.8	1	11	Negro staff at Center for Disease Control, Atlanta	" " "	" " "
India (Bombay)	6.6	4.8	5.7	-	-	Villagers living near Vellore, South India: controls in a study of tropical sprue	Rice and vegetable diet; no meat and little fat	" " "
Uganda (Kyadondo)	0.0	1.2	0.6	9	39	Villagers living in Kyadondo region: mothers accompanying children to mal-nutrition clinic in Kampala; hospital staff	Matoke (boiled mashed bananas), vegetables; little fat	" " -78°C[5]
	-	-	-	11[2]	6[3]	English immigrants; hospital staff in Kampala	Normal Western mixed	" " "
Japan (Miyagi)	5.0	4.8	4.9	7	10	Villagers near Yonago; mostly farmers and housewives	Fruit, rice and other vegetables. Little meat and fat	" " -196°C

1. Standardized for age (from Doll, 1969).
2. Quantitative bacteriology available for only 5.
3. " " " " " 3.
4. All samples emulsified 1 in 10 in 10% glycerol broth before freezing. Suspensions were kept frozen at -55°C until cultivation, unless stated otherwise.
5. Suspensions stored at -78°C until cultivation.

2.2 BACTERIOLOGICAL METHODS

Preservation and transport of faeces

Since most of the specimens had to be carried considerable distances to the Wright-Fleming Institute, a preservation system had to be used that would permit neither the death nor the multiplication of any bacteria present. The method described by Drasar, Shiner and McLeod (1969) was therefore adopted.

Samples of freshly voided faeces were diluted 1 in 10 in meat infusion broth (Lab-Lemco, Oxoid) containing 10% glycerol, emulsified carefully using a sterile swab stick and frozen in dry-ice (-78°C) or liquid nitrogen (-196°C). Preliminary experiments showed that bacteria survived without significant losses when specimens of faeces were stored under these conditions for one month (the evidence is presented in Appendix B4). For field studies the suspensions were placed either in screw-capped plastic bottles (Henley Medical Supplies) packed in dry-ice inside vacuum flasks, or in polypropylene centrifuge tubes (Measuring and Scientific Equipment) immersed in liquid nitrogen in a portable liquid nitrogen refrigerator (Union Carbide), and flown to London. Specimens were stored in dry-ice or in a refrigerator at -55°C and examined within one month.

When shorter distances were involved, specimens of untreated

faeces were sent to the laboratory by post and diluted and frozen in glycerol broth immediately on arrival. With the exception of the enterobacteria, none of the counts of any group of bacteria changed significantly, provided the transit time in the post did not exceed 24 hr (see Appendix B4).

Cultivation of specimens

It is well established that the most numerous bacteria present in the faeces of healthy human adults are non-sporing anaerobes, and many of them are sensitive to prolonged exposure to oxygen (Smith and Holdeman, 1968; Moore, Cato and Holdeman, 1969; Cato et al., 1970). When these organisms are cultivated by conventional techniques they are necessarily exposed to oxygen dissolved in the diluents and culture media. In quantitative work, the spreading of specimens on the surface of agar plates to isolate single bacteria, considerably enhances the lethal effects. Therefore, to recover the maximum possible number of viable cells it is advisable to use techniques which minimize their exposure to air.

Hungate (1950) described a method for cultivating highly oxygen-sensitive bacteria from the bovine rumen; media were prepared, dispensed into tubes and inoculated under a stream of oxygen-free nitrogen in such a way that the media and the organisms were never exposed to the air. Although this approach

has many advantages over conventional techniques, it has considerable manipulatory difficulties, and these make the technique cumbersome for routine use.

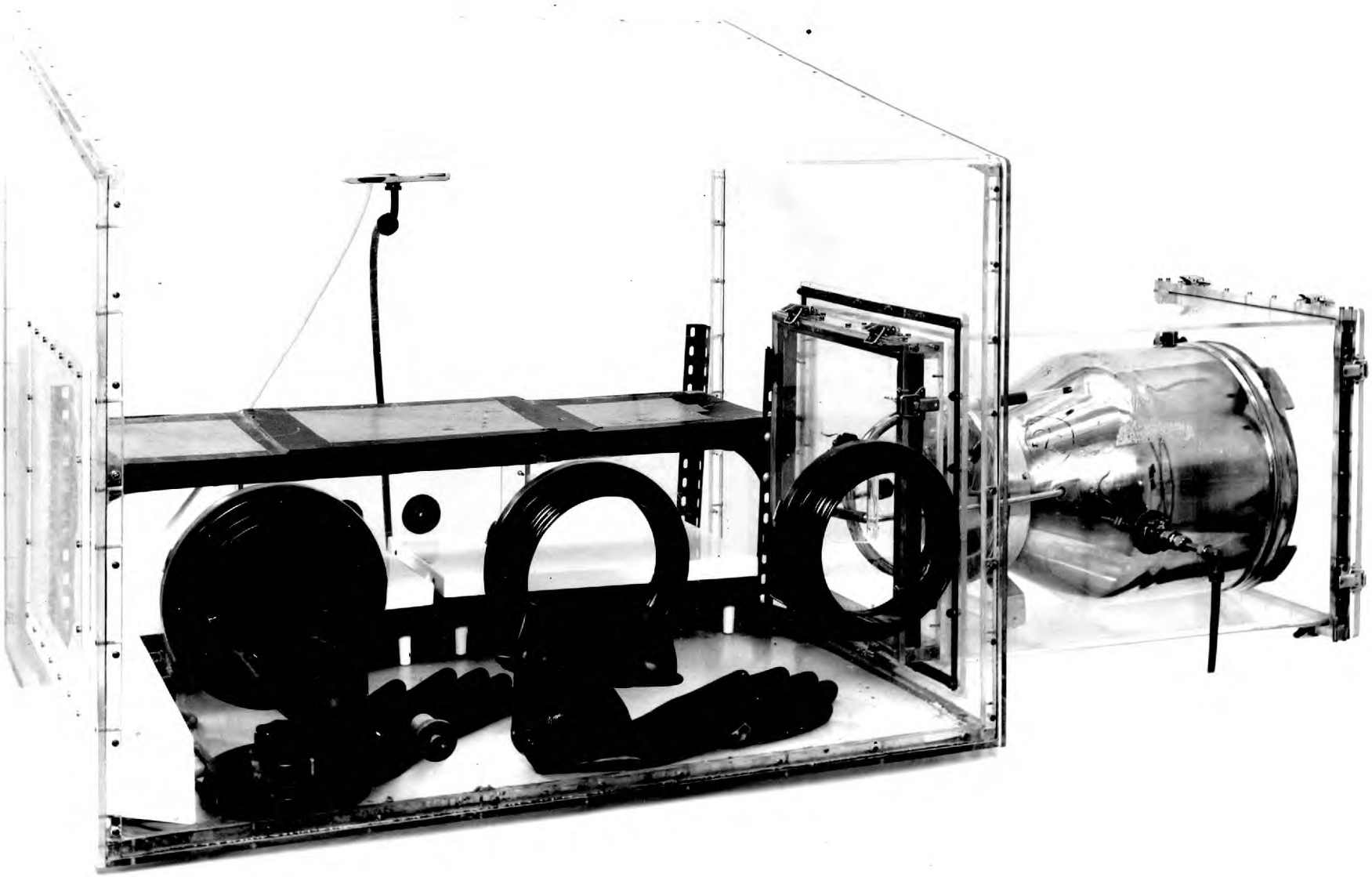
Socransky, MacDonald and Sawyer (1959) overcame these difficulties by constructing an anaerobic cabinet for the surface cultivation of Treponema microdentium. Various kinds of oxygen-free cabinets have since been described for isolating anaerobic bacteria from human faeces (Drasar, 1967b), from the mouse caecum (Lee, Gordon and Dubos, 1968; Aranki, Syed, Kenney and Freter, 1969), and from rumen fluid (Hobson and Mann, 1970). Drasar's technique, with minor modifications, was used for the present study.

Isolation of non-sporing anaerobes

(a) The anaerobic cabinet

The anaerobic cabinet was built by Drasar (1967b) and consisted of a gas-tight, perspex, glove-box filled with oxygen-free nitrogen containing 5% carbon dioxide (Fig. 3). A gas-tight airlock enabled material to be passed to and from the cabinet without disturbing the anaerobic atmosphere within. To prepare the cabinet for use the oxygen was removed by burning a spirit lamp within it and at the same time flushing it with 20 litres per min. oxygen-free nitrogen containing 10% carbon dioxide. The residual traces of oxygen in the cabinet and any

Fig. 3. The anaerobic cabinet. Within the body of the cabinet can be seen two photographic warming plates for drying plates. The airlock contains a converted milking machine pail, which is used in place of a conventional anaerobic jar.
(From: Drasar and Crowther, 1971)



further traces diffusing into the system from the external environment, were removed by continuously recycling the cabinet's atmosphere through a deoxygenating column (Fig. 4).

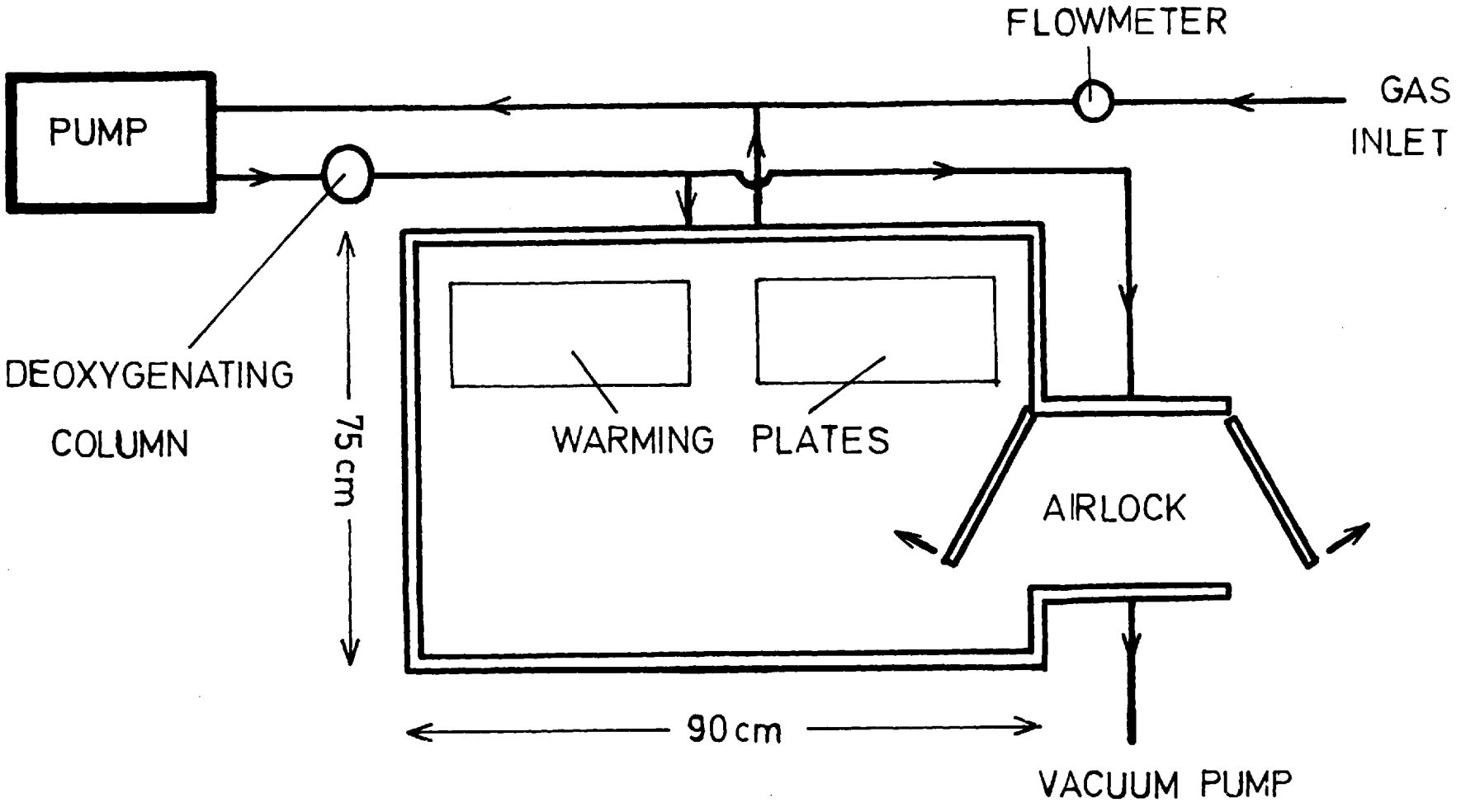
The column was constructed by Drasar (1967) along lines described by Moore (1966) and consisted of an inner Pyrex glass tube filled with copper wire and heated to 400°C by a 210 watt electrical heating tape wound round it; the whole was insulated by an outer Pyrex glass tube. When gas from the cabinet was pumped through the inner tube, any oxygen present readily combined with the hot copper wire; the effluent gas was therefore completely free from oxygen and was returned to the cabinet. After approximately 3 months of use the column was reactivated by passing hydrogen through it.

(b) Preparation of anaerobic media and diluent

The principle of Drasar's method was to prepare the culture medium and diluent in an oxygen-free state before they were autoclaved. Medium was transferred to the cabinet, and culture plates were poured, dried and inoculated in a completely anaerobic environment.

The constituents of the medium were placed in a serum bottle closed with a well fitting cap and put in a boiling water-bath. The dissolved oxygen in the medium was removed by a stream of oxygen-free nitrogen passed into the bottle by means of

Fig. 4. The gas circuit. The gas mixture (90% nitrogen and 10% carbon dioxide) is deoxygenated before being passed into the cabinet. The atmosphere within the cabinet is continuously recirculated through a deoxygenating column.


FLOWMETER
PUMP
GAS
INLET
DEOXYGENATING
COLUMN
75 cm
WARMING
PLATES
AIRLOCK
90 cm
VACUUM PUMP

hypodermic needles inserted through the cap. When all the solids had dissolved the needles were removed, the bottles autoclaved, and transferred to the cabinet for use. For the diluent, Drasar used the same medium without the agar, prepared and deoxygenated in the same way.

Although this method of preparation ensures that the oxygen is completely removed from the media, it has the disadvantage of being very slow; fresh materials have to be prepared immediately before use and the time taken to do this necessarily limits the number of specimens which can be examined on any one day. Therefore, to increase the efficiency of Drasar's cultivation procedure without any loss of reliability, the method was simplified by using reheated media in conjunction with the anaerobic cabinet.

Reinforced clostridial agar (RCA) (Oxoid) was fortified with 1% liver digest (Oxoid), the pH adjusted to 7.5 and filled into bottles to give a minimum air-gap; after autoclaving at 115° for 20 min. the final pH was 7.3. The caps were screwed down and the agar stored. Brain-heart infusion broth (Oxoid) containing 0.05% cysteine hydrochloride was used as the diluent: the broth was adjusted to pH 7.3 and autoclaved as above. When required for use bottles of agar or diluent were steamed for one hour with the caps loose, the caps being tightened immediately on removal from the steamer, and transferred to the

cabinet for use. Agar plates were poured, allowed to set and dried by inverting them over two 150 watt photographic warming dishes (Photax) placed inside the cabinet.

Culture plates prepared from reheated agar were equally effective in recovering fastidious anaerobic bacteria from faeces as those prepared using Drasar's original method (see Appendix B1 However, the survival of the fastidious anaerobes on either medium was often very poor; by the time colonies of the slower growing cells had developed, those of the faster growers were already dying. When 10% horse-blood was added to the agar (within anaerobic cabinet) more colonies could be subcultured. The blood was not deoxygenated before use. Reinforced clostridial agar fortified with 1% liver digest and 10% horse-blood was therefore used throughout the present survey.

(c) Inoculation and incubation of plates

The diluent was dispensed into tubes in the cabinet and 10-fold serial dilutions of faeces were prepared using weight-calibrated pipettes. 0.10 ml of suspension was spread onto the surface of each plate and the plates were transferred to a stainless-steel milking-machine churn (Fullwood Bland & Co.), placed in the airlock. The churn was used in place of a conventional anaerobe jar as suggested by Schaedler, Dubos and Costello (1965) and was fitted with a vacuum-tight lid,

vacuum tap, a platinum cold catalyst ('D' catalyst, Engelhard Industries) and a methylene blue anaerobic indicator. The churn was evacuated before being placed in the airlock. On removal from the airlock, the atmosphere within the churn was twice replaced by a gas mixture containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide, and incubated at 37°C for four days.

Isolation of Clostridium, Veillonella and Sarcina spp.

Whilst the non-sporing anaerobic bacteria require demanding techniques for their quantitative recovery, species of Clostridium, Veillonella and Sarcina, also obligate anaerobes, are more oxygen tolerant and can be counted using conventional anaerobic techniques.

Species of Clostridium and Sarcina form heat-resistant spores and can be isolated from faeces using simple heating techniques. Besides destroying all vegetative cells present, heat-treatment has the additional benefit of shocking the spores and so allows better germination of some species (Freame and Fitzpatrick, 1971; Hobbs, Williams and Willis, 1971). Serial dilutions of faeces were heated for 10 min. in a water-bath at 70°C. 0.10 ml of each dilution was spread onto freshly prepared infusion agar containing lactose, neutral red and egg-yolk (Willis and Hobbs' medium without antibiotics) (Willis and Hobbs, 1959) and incubated anaerobically. Colonies of

sarcinae could readily be distinguished from those of clostridia and aerobic spore-bearing bacilli. Sarcinae formed pale yellow colonies which were 2-4 mm in diameter and were usually surrounded by a yellow halo in the medium. All colonies were examined microscopically and subcultured aerobically and anaerobically.

Veillonella spp. do not form spores, and some of the Clostridium spp. which show lecthinase activity (such as Cl. perfringens) do so only occasionally. They were counted using conventional media and techniques (Table 6). Plates were freshly poured, or stored anaerobically until needed, and incubated anaerobically immediately after inoculation. Colonies were Gram-stained and subcultured aerobically and anaerobically.

Isolation of enterobacteria, fungi and species of Lactobacillus, Streptococcus and Bacillus

A series of eight conventional, selective and non-selective media were used to count eight broad groups of micro-aerophilic and facultatively anaerobic bacteria, yeasts and moulds known to be present in human faeces. These media are listed in Table 6 together with details of their use.

Control of quantitative bacteriological methods

As it was impossible to find a suitable method to control

Table 6. Media for the isolation of intestinal bacteria

Medium[1]	Incubation of plates		Organism	Notes, sources and references
	Atmosphere	Time (days)		
Reinforced clostridial agar (RCA)	An	4	Bacteroides spp. Bifidobacteria	Oxoid with 1% liver digest and 10% horse blood. pH adjusted to 7.5±0.1 before autoclaving. Plates poured, dried and inoculated in cabinet.
Rogosa's agar[2] (V)	An	4	Veillonella spp.	Rogosa et al. (1958)
Tomato juice agar[2]	An	1	Sarcina spp.	Oxoid. pH adjusted to 7.0
Willis & Hobbs' agar[2]	An	4	Clostridia (lecithinase-positive)	Willis and Hobbs (1959) Neomycin 50 µg/ml.
Willis & Hobbs' agar[2]	An	1	Clostridia (lecithinase-positive and -negative) Sarcina spp.	Inoculum heated for 10 min. at 70°C. No antibiotics.
Azide agar	0	1	Enterococci	Schaedler et al. (1965)
Blood agar	0	1	Total aerobes; 'viridans' streptococci	
MacConkey's Agar	0	1	Enterobacteria, enterococci	Oxoid
Mannitol Salt Agar	0	3	Staphylococci and micrococci	Oxoid. Incubate 30°C.
Rogosa's Agar (L)	90% CO_2	3	Lactobacillus spp.	Rogosa et al. (1951)
Sabouraud's Agar	0	5	Yeasts, filamentous fungi	Chloramphenicol 40 µg/ml. duplicates incubated 37°C and 22°C.
S_1 Agar	0	2	Strep. salivarius	Williams and Hirsch (1950)
Nutrient agar	0	1	Bacillus spp.	Inoculum heated for 10 min. at 70°C.

0 - aerobic
An - anaerobic
1 - formulae and details of preparation are given in Appendix A1.
2 - plates used freshly poured or stored anaerobically before use.

the quantitative bacteriology during the survey, the following precautions were taken. The anaerobic cabinet was always used to help recover the delicate anaerobes, and all manipulations, including the preparation of serial dilutions and inoculation of plates, were carried out by the same person using the same techniques throughout. Samples of faeces from English people were included when each batch of specimens from other countries was cultivated.

Further supplies of each medium had to be prepared from time to time, sometimes by different persons, and minor fluctuations in formulation, or those caused by autoclaving or storing, might produce errors in counting. However, preliminary tests found no significant variations in the viable counts of organisms recovered from faeces on different batches of any of the conventional media tested (see Appendix B2); on the other hand, the strictly anaerobic and nutritionally exacting Bacteroides group (but not the Bifidobacterium group) did show significant variation in counts ($P < 0.05$) on different batches of Oxoid reinforced clostridial agar (fortified with liver digest and horse-blood) inoculated in the anaerobic cabinet.

The quantitative bacteriological methods have been validated in Appendix B.

Identification of organisms

After incubation, colonies of each recognisable type were counted at a suitable dilution on each medium. Two colonies of every type were subcultured from most specimens for identification and storage; in the case of the Bacteroides and Bifidobacterium groups, five colonies were picked. The isolates were placed in broad groups based on the following criteria:

Bifidobacterium group: Gram-positive anaerobic, non-sporing rods or cocco-bacillary forms; catalase-negative or -positive; some strains are highly pleomorphic; cells showing true branching (Y and V forms) are common.

Bacteroides group: Gram-negative, anaerobic, non-sporing rods; some strains show bipolar staining; catalase-negative; pleomorphism is common but cells with true branching (Y forms) are rare.

Some colonies of the Bifidobacterium and Bacteroides groups are difficult to distinguish on non-selective media. Their numbers were therefore estimated by multiplying the total plate count by the proportion of Gram-positive (or Gram-negative) organisms found in smears of 20 colonies picked strictly at random.

Enterobacteria: Gram-negative, catalase-positive rods, producing typical colonies on MacConkey's agar. Two strains were isolated from most specimens and identified as described below.

Streptococci:

'viridans' streptococci: Gram-positive, catalase-negative cocci growing aerobically on blood agar.

Streptococcus salivarius: Gram-positive, catalase-negative cocci producing mucoid colonies on sucrose agar but not growing on MacConkey's agar.

enterococci: Gram-positive, catalase-negative cocci producing white or pale blue colonies on methylene blue agar containing sodium azide (Schaedler, Dubos and Costello, 1965) and red colonies on MacConkey's agar; isolates were further identified as shown below.

Lactobacillus spp.: Gram-positive, catalase-negative rods growing at pH 5.4 in an atmosphere of 90% carbon dioxide and 10% air; this group was not subdivided.

Clostridium spp.: Gram-positive, catalase-negative anaerobic rods, often forming spores.

Lecithinase-positive group: those clostridia producing

diffusible opalescence when grown on a medium containing egg-yolk (Willis and Hobbs' medium). The group includes only *Clostridium perfringens*, *Cl. bifermetans*, *Cl. sordellii* and *Cl. novyi* type C.

Lecithinase-negative group: those clostridia producing no or only non-diffusible opalescence when grown on Willis and Hobbs' agar; the group contains all the remaining species of *Clostridium*.

Veillonella spp.: anaerobic cocci, usually Gram-negative, but sometimes showing slight Gram-positivity; catalase-positive or -negative.

Bacillus spp.: Gram-positive, catalase-positive, aerobic rods forming spores resistant to heating at 70° for 10 mins.

Sarcina ventriculi: obligately anaerobic, Gram-positive, catalase-negative cocci, forming cubical packets of eight; forms spores resistant to heating at 70° for 10 min. Strains were identified as described in Appendix A4.

Staphylococci and micrococci: Gram-positive, catalase-positive cocci growing on mannitol-salt-agar; no attempt was made to subdivide this group.

Fungi: Organisms growing aerobically in three days on

Sabouraud's agar containing 40 µg/ml chloramphenicol.

Yeasts: Unicellular Gram-positive organisms dividing by budding or fission.

Filamentous fungi: Organisms showing true mycelial growth. Duplicate plates were incubated at 22° and 37°C.

Identification of non-sporing anaerobes

Some of the obligately anaerobic, non-sporing rods were identified using a modification of the scheme devised by Cato et al. (1970). These workers suggested that within either the Gram-positive or Gram-negative groups, the genera could be separated by the nature of the volatile fatty acids produced from glucose (Table 1). Genera of the Gram-negative anaerobes were divided into species on the basis of conventional tests such as indole production, gelatin hydrolysis and the production of acid from carbohydrates (Table 7). The analytical methods to detect the volatile fatty acids produced from glucose are described in Section 2.3. Details of the conventional tests are given in Appendix A3.

Identification of the enterobacteria

The enterobacteria were divided into the Escherichia,

Table 7. Differentiation of Bacteroides spp.

Test	Bacteroides fragilis	B. oralis	B. ruminicola	B. hypermegas	B. trichoides
Gelatinase	-	-	v	-	w
Indole production	v	-	-	-	-
Acid from:					
glucose	+	+	+	+	+
lactose	+	v	+	+	+
sucrose	+	+	v	+	+
maltose	+	+	v	+	+
fructose	+	+	+	+	+
mannose	+	+	+	+	+
mannitol	-	-	-	+	-
glycerol	-	-	-	-	-
arabinose	v	-	v	+	-
xylose	+	-	+	+	w
Aesculin hydrolysis	+	+	+	+	+
Nitrate reduction	-	-	-	-	-

Key: + 90% or more positive
- 90% or more negative
w weak reaction
v different biochemical types

Arizona, Citrobacter, Klebsiella, Aerobacter, Hafnia and Proteus groups using a simplification of the scheme of Edwards and Ewing (1962) (Table 8). The media and tests used are described in Appendix A3.

Identification of the enterococci

The enterococci were identified as Streptococcus faecium, Str. faecalis or as belonging to the Str. bovis/Str. equinus group using the criteria shown in Table 9; the media and methods shown in Appendix A3.

Identification of Sarcina spp.

Most strains of sarcinae isolated from faeces could not be preserved for detailed examination. Some remained viable and were compared with a known strain of Sarcina ventriculi as described in Appendix A4. All Gram-positive, anaerobic cocci showing the same cultural appearances as those of S. ventriculi, were presumptively identified as S. ventriculi.

Table 8. Differentiation of the Enterobacteriaceae [1]

	Escherichia coli	Arizona group	Citrobacter group	Klebsiella group	Aerobacter group A	Aerobacter group C	Hafnia group	Proteus mirabilis
Indole	+	-	-	-	-	-	-	-
Methyl red	+	+	+	-	-	d	+	+
Voges-Proskauer	-	-	-	+	+	d	d	-
Citrate	-	+	+	+	+	d	d	d
Urease	-	-	-	(+)	- or (+)	- or (+)	-	+
Growth in KCN	-	-	+	+	+	+	+	+
Gelatin liquefaction	-	(+)	-	-	(+)			+
Gas from glucose	+	+	+	+	+	+	d	+
lactose	+	+ or x	+ or x	+	+	- or (+)	x	-
sucrose	d	-	d	+	+	+	x	+
mannitol	+	+	+	+	+	+	+	-
dulcitol	d	-	d	d	-	-	-	-
salicin	d	-	d	+	+ or (+)	+	d	(+) or -
adonitol	-	-	-	+	d	-	-	-
inositol	-	-	- or x	+	-	+	-	-

1 from Edwards and Ewing (1962)
+ 90% or more positive
- 90% or more negative
(+) delayed positive reaction
x late and irregularly positive
d different biochemical types

Table 9. Differentiation of the enterococci [1]

Test [2]	Streptococcus			
	faecalis	faecium	bovis	equinus
Growth on MacConkey's agar	+	+	+	+
Growth on potassium tellurite agar	+	-	-	-
Hydrolysis of arginine	+	+	-	-
Reduction of tetrazolium	+	-	v	-

1 Adapted from Barnes (1956) and Deibel (1964)

2 The media and methods are given in Appendix A3

\+ 90% or more positive

\- 90% or more negative

v different biochemical types

2.3 BIOCHEMICAL METHODS

Analysis of volatile acids by gas-liquid chromatography

The volatile products of glucose metabolism (ethanol and the low molecular weight fatty acids) were detected using gas-liquid chromatography along lines described by Cato *et al.* (1970).

The gas-liquid chromatograph

This was an isothermal '104' Series Chromatograph, fitted with a flame-ionisation detector (Pye Instruments, Cambridge). It was fitted with a 5 ft. glass column packed with 'Diatomite C' which had been treated with phosphoric acid and impregnated with 10% polyethylene glycol. The operating temperature was 125°C and the carrier gas was oxygen-free nitrogen passed through at 30 ml per min. Responses were recorded on a 'Servoscribe' pen recorder (Smiths Industries).

Analysis of ethanol and volatile fatty acids

4 ml of a 4 day culture in basal medium containing 1% glucose (prepared as described in Appendix A3) were acidified with 0.5 ml 50% aqueous sulphuric acid and extracted with 4 ml diethyl ether. The mixture was centrifuged to break the emulsion and the ether layer pipetted off and dried over anhydrous magnesium sulphate. 1.0 µl was injected onto the column. The remainder was kept for analysis of pyruvic, lactic

and succinic acids. The peaks in the chromatogram were identified by comparison with those of a reference solution containing ethanol and volatile fatty acids prepared as described by Cato et al. (1970).

Samples of uninoculated glucose broth were similarly analysed to verify that all the acids had been produced by the bacteria.

Analysis of pyruvic, lactic and succinic acids

Pyruvic, lactic and succinic acids are unstable at 125°C and therefore cannot be detected using the above procedure, but after methylation their methyl esters are stable and readily separate on the column.

The remainder of the ether extract of the culture was methylated by adding 2-3 drops of diazomethane freshly prepared by the method described by De Boer and Backer (1954). 1.0 µl was injected onto the column which was used under the same conditions as described above. The peaks in the chromatogram were identified by comparison with those of a reference solution containing pyruvic, lactic and succinic acids.

Chemical analysis of faeces

The chemical components of faeces shown in Table 10 were estimated quantitatively by Drs. M.J. Hill and V.C. Aries by

Table 10. Chemical components of faeces estimated quantitatively during the present survey by Drs. M.J. Hill and V.C. Aries.

Steroids

Bile acids

cholic acid

deoxycholic and chenodeoxycholic acids

lithocholic acid

cholanic acid

Neutral steroids

cholesterol

coprostanol

Bile pigments (total urobilins)

Faecal pH

Moisture content

methods described elsewhere (Hill and Aries, 1971).

To estimate the pH of faeces a 1 in 10 suspension in distilled water was spotted onto pH indicator paper (British Drug Houses) and the colour compared against a standard colour chart.

The moisture content of faeces was estimated by finding the loss in weight after drying overnight in a vacuum freeze-drier (Edwards (High Vacuum) Limited).

2.4 STATISTICAL METHODS

Since bacteria occur in faeces in very large numbers, all counts were expressed as: $\log_{10}$ (viable counts per g wet faeces). All calculations were done by computer using logarithmic counts.

The computers

Olivetti '101' computer

All calculations, except those for constructing correlation matrices, were made on an Olivetti '101' desk-top computer (British Olivetti Ltd.) supplied with magnetic programme cards. Before starting any calculation, the appropriate card was checked for accuracy using test calculations recommended by the manufacturer.

The CDC computer

The counts of bacteria in each specimen of faeces, together with the corresponding chemical analyses (kindly supplied by Dr. M.J. Hill) were transferred to punched cards. Programmes now in the computer library in the Department of Medical Statistics and Epidemiology at the London School of Hygiene, were used on the CDC computer at the University of London Computing Centre, to construct and interpret correlation

matrices as described below. The CDC computer was also used to check calculations done on the Olivetti computer.

Presentation of counts of bacteria in each population

The mean counts of bacteria in a population of N people was expressed, with its standard deviation as:

$$\frac{1}{N}.\sum \log_{10} x \pm \left[\frac{\sum}{N}(\log_{10} x - \frac{\sum \log_{10} x}{N})^2\right]^{\frac{1}{2}}$$

where x is the viable count of bacteria per g faeces for each person.

Comparison of counts of bacteria in different populations

Student's t-test and Mann-Whitney test

The bimodal distribution of counts of some groups of organisms within any population indicated that the Mann-Whitney rank test (Snedecor and Cochran, 1967) was more appropriate than the student t-test for comparing distributions within two populations. However, Miss R. Wood, of the Intercollegiate Unit in Medical Statistics, kindly tried the Mann-Whitney test on extreme examples of my data and found levels of significance which agreed with those obtained by Student's t-test. Owing to its greater ease of calculation the Student t-test was

therefore used in preference to the Mann-Whitney test to compare counts of all groups of bacteria in different populations. Values of t were calculated on the Olivetti '101' computer and levels of significance obtained from statistical tables.

Analysis of variance

When more than two populations were being compared simultaneously, analysis of variance was used. Mean-square ratios were calculated on the Olivetti computer and significance levels found from tables (Snedecor and Cochran, 1967).

Linear correlations

Correlation between two variables

To test whether two variables were correlated, the Olivetti computer was used to calculate the correlation coefficient and the constants of the regression line: $(y = bx + a)$. Confidence limits about the line were calculated as described by Moore and Edwards (1965). Levels of significance for the correlation coefficients were found from statistical tables.

Construction of large correlation matrices

When a large number of variables were being studied, coefficients of linear correlation were calculated on the CDC computer. The programme used was based on the method of

Hills (1969) and was written expressly for my data by Miss R. Wood of the Intercollegiate Unit in Medical Statistics. The linear correlation coefficient, r, was calculated for every possible pair selected from the different variables.

Half normal plotting was used to assess which of the correlations were statistically significant. A slight modification of the technique described by Hills (1969) was made to allow for missing data. Each correlation coefficient was converted to a normal deviate by means of the z-transformation. The normal deviates thus obtained were standardised and used to construct a half normal plot. Significant correlations were identified by visual assessment of this plot alongside others made with high values of the standard normal deviate omitted.

Partial correlations

The interactions between the micro-organisms themselves were assessed using partial correlations to eliminate the effects of the bile acids and neutral steroids. Only Bacteroides spp. bifidobacteria, 'viridans' streptococci, enterococci, enterobacteria, Lactobacillus spp. and Veillonella spp. (those organisms thought to be the most important) were considered, and to avoid the mathematical difficulties of compensating for missing data, only those specimens having a complete set of steroid analyses and bacterial counts

were included in the calculations. 106 specimens satisfied these criteria and came from Scotland (14), U.S.A. (34), England (31, of which 13 were from vegans), Japan (17), India (8) and Uganda (2).

A matrix of the linear correlation coefficients between each of the seven groups of organisms and the concentrations of the total bile acids and total neutral steroids was first computed as part of the programme described above. The partial correlation coefficient between organisms 1 and 2 in the presence of constant concentrations of bile acids (B) and neutral steroids (N), was obtained from the linear correlations using the formula:

$$r_{12.BN} = \frac{r_{12.N} - r_{1B.N}\, r_{2B.N}}{\sqrt{(1 - r^2_{1B.N})(1 - r^2_{2B.N})}}$$

as described by Snedecor and Cochran (1967). The computer programme to calculate the partial correlations was also kindly written by Miss R. Wood. The significant correlations were identified by the use of the table given by Snedecor and Cochran (1967).

SECTION 3

RESULTS

Introduction

The counting methods used here could not detect less than 10^2 viable organisms per g faeces. Thus, a sterile culture plate could indicate either that the organism was absent, or that it was present in counts ranging from 1 to 99 per g. Where an organism was present at less than 10^2 per g it was never considered worthwhile to find the precise count, since organisms occurring in such small numbers must be negligible components of the flora.

The results of the population studies have therefore been presented in two ways. Firstly, those persons with counts of 10^2 or more per g of any organism have been considered to be carriers of that organism and those with less than 10^2 as non-carriers; the proportions of carriers of each organism in the different populations have been presented in Table 14.

Secondly, to compare the population means, all persons have been considered as carriers and those carrying less than 10^2 per g have been considered to carry 10^1 organisms per g. Counts of organisms have been expressed as the $\log_{10}$ viable count per g faeces, and the population means as the arithmetic means of the logarithmic counts; the standard deviations are also in logarithms. The distribution of counts of organisms in the different populations have been shown by spot diagrams.

3.1 EFFECT OF AGE ON THE FAECAL FLORA

The effect of age on the distributions of 15 groups of faecal organisms was examined in 29 men and 39 women aged from 20 to 85 years; all lived in London and ate typical Western mixed diets.

Bifidobacteria and *Bacteroides* spp. were the most numerous organisms in all persons. Analysis of variance showed no significant differences between the numbers of bifidobacteria carried by the young (less than 30 yr.), middle-aged (30-60 yr.) or elderly (over 60 yr.) age groups for men or for women and regression lines showed no significant trends in the counts (Fig. 5). Similarly, the counts of *Bacteroides* spp., enterobacteria, 'viridans' streptococci, enterococci, *Streptococcus salivarius*, *Veillonella* spp., *Bacillus* spp., staphylococci and yeasts, showed no significant differences with age in either men or women. Typical distributions are shown in Figs. 6-8. Counts of lecithinase-positive clostridia (Fig. 9) and filamentous fungi decreased with age in men and women, but neither trend was significant. Older women, but not older men, tended to carry more *Lactobacillus* spp. (Fig. 10) and lecithinase-negative clostridia than the younger (Fig. 11); these trends were on the borderline of significance ($P < 0.05$).

The numbers of persons examined from other countries were

Fig. 5. Relationships between age and the counts of bifidobacteria in faeces. Each spot represents the count from one person (black spots for men, circles for women). The calculated regression line is shown as a solid line for men and a broken line for women. 95% confidence limits about the regression line for women are shown as broken lines; those for men are similar and have been omitted. Neither trend is significant.

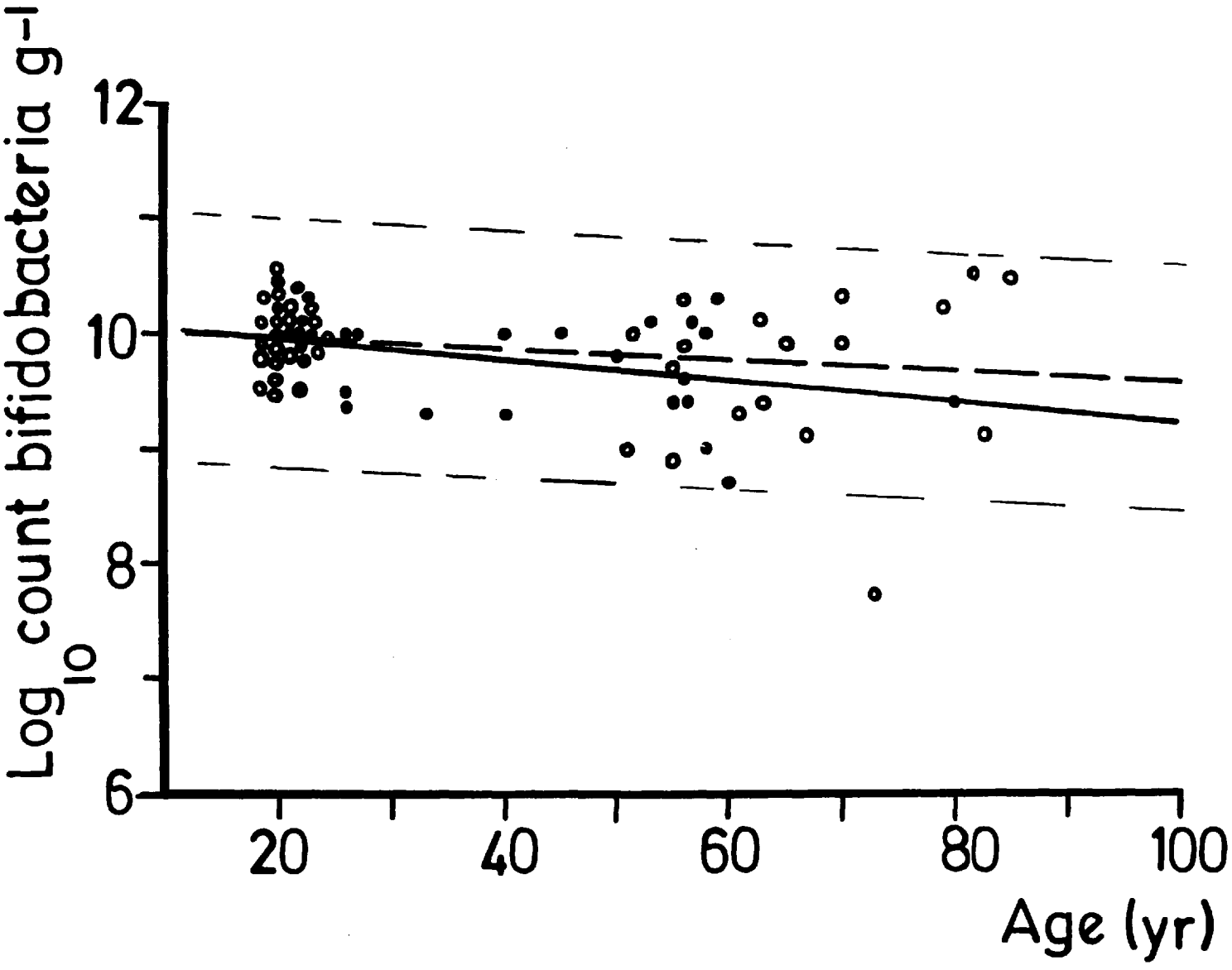

Log10 count bifidobacteria g-1
12
10
8
6
20
40
60
80
100
Age (yr)

Fig. 6. Relationships between age and the numbers of *Bacteroides* spp. in faeces.
Key: as Fig. 5. The 95% confidence limits about the regression line for men are shown as dotted lines. Neither trend is significant.

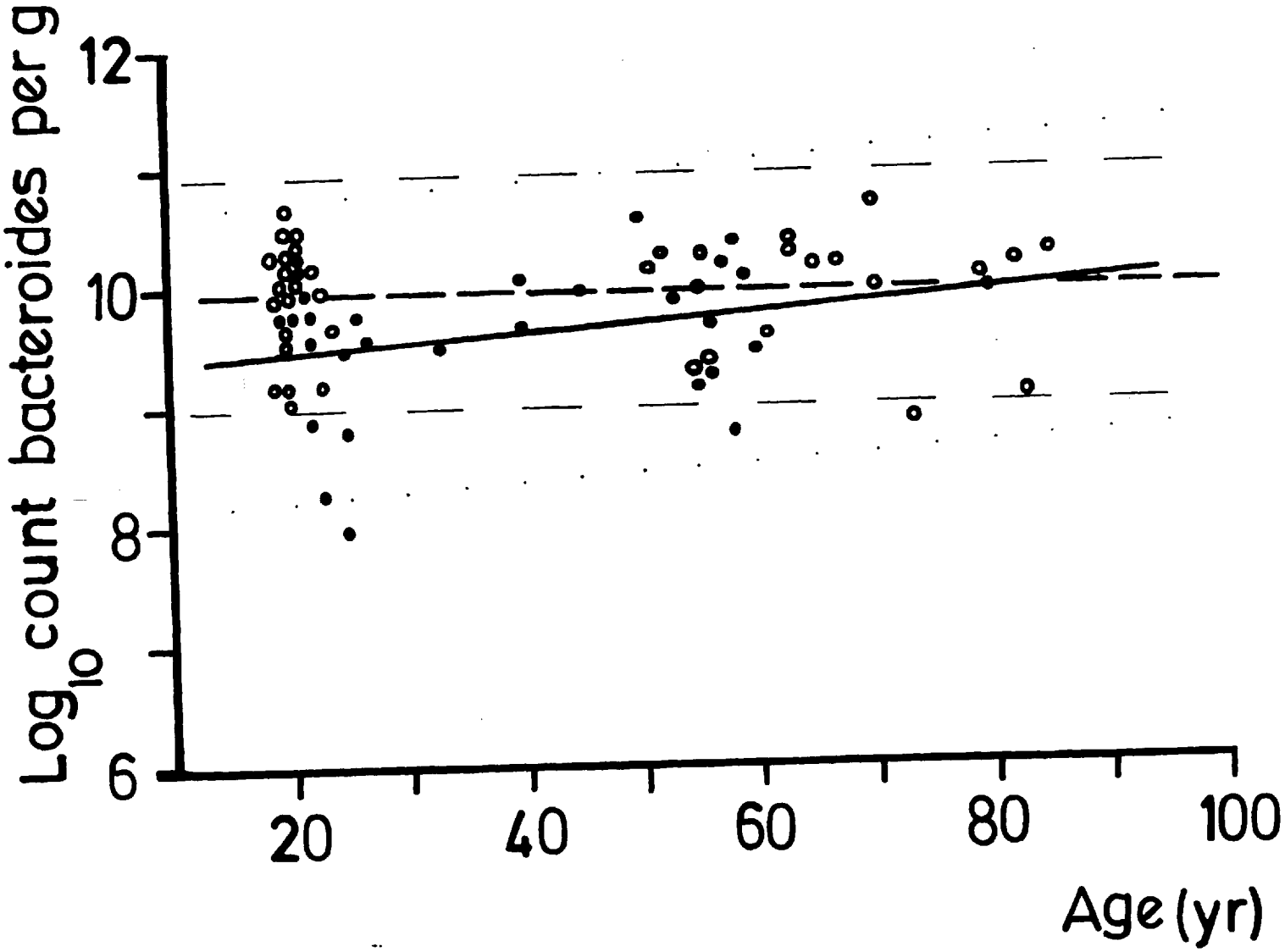

Log10 count bacteroides per g
12
10
8
6
20
40
60
80
100
Age (yr)

Fig. 7. Relationships between age and the numbers of enterobacteria in faeces. Key: as Fig. 6. Neither trend is significant.

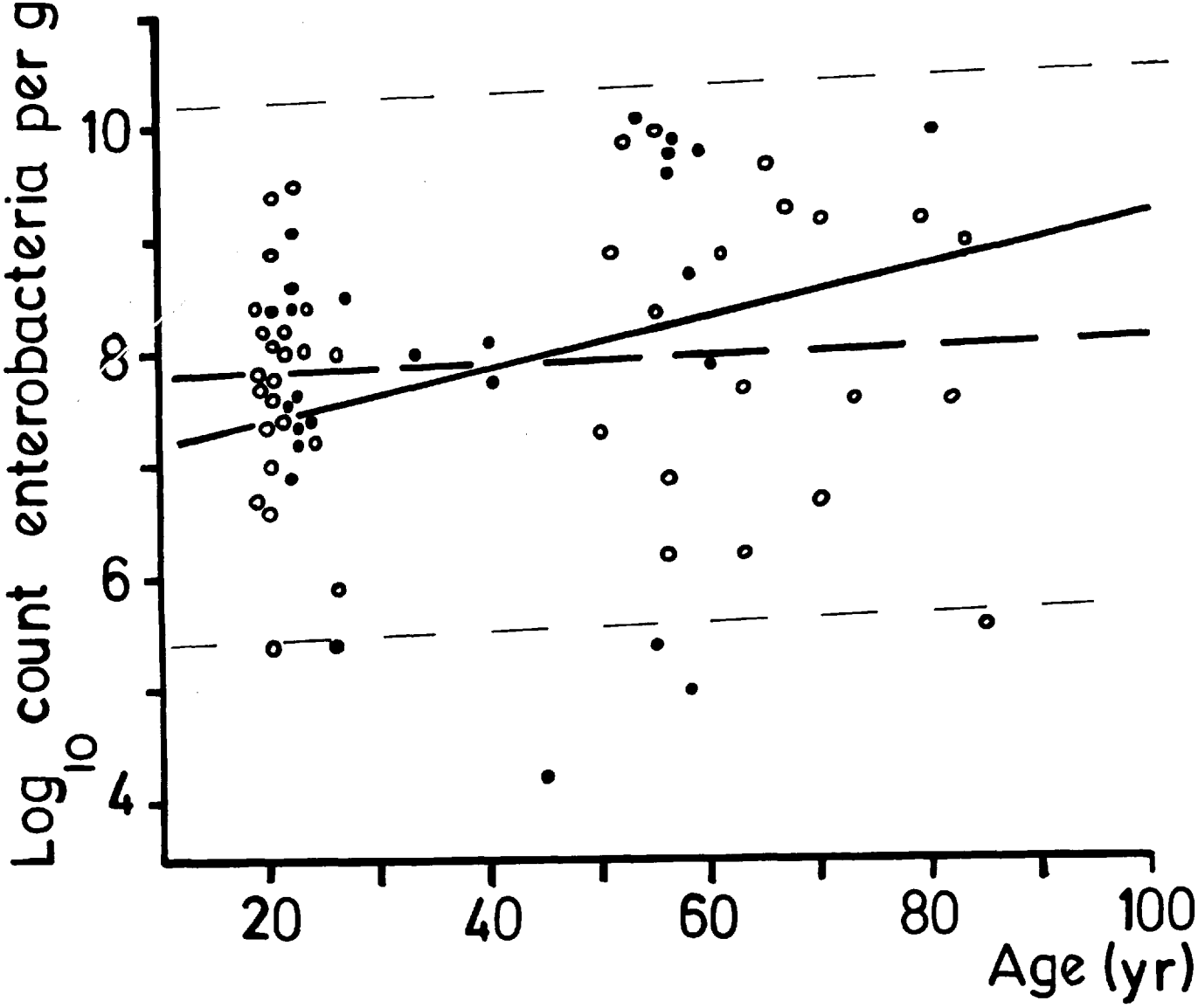

Log10 count enterobacteria per g
10
8
6
4
20
40
60
80
100
Age (yr)

Fig. 8. Relationships between age and the numbers of 'viridans' streptococci in faeces.

Key: as in Fig. 6. The trends are not significant.

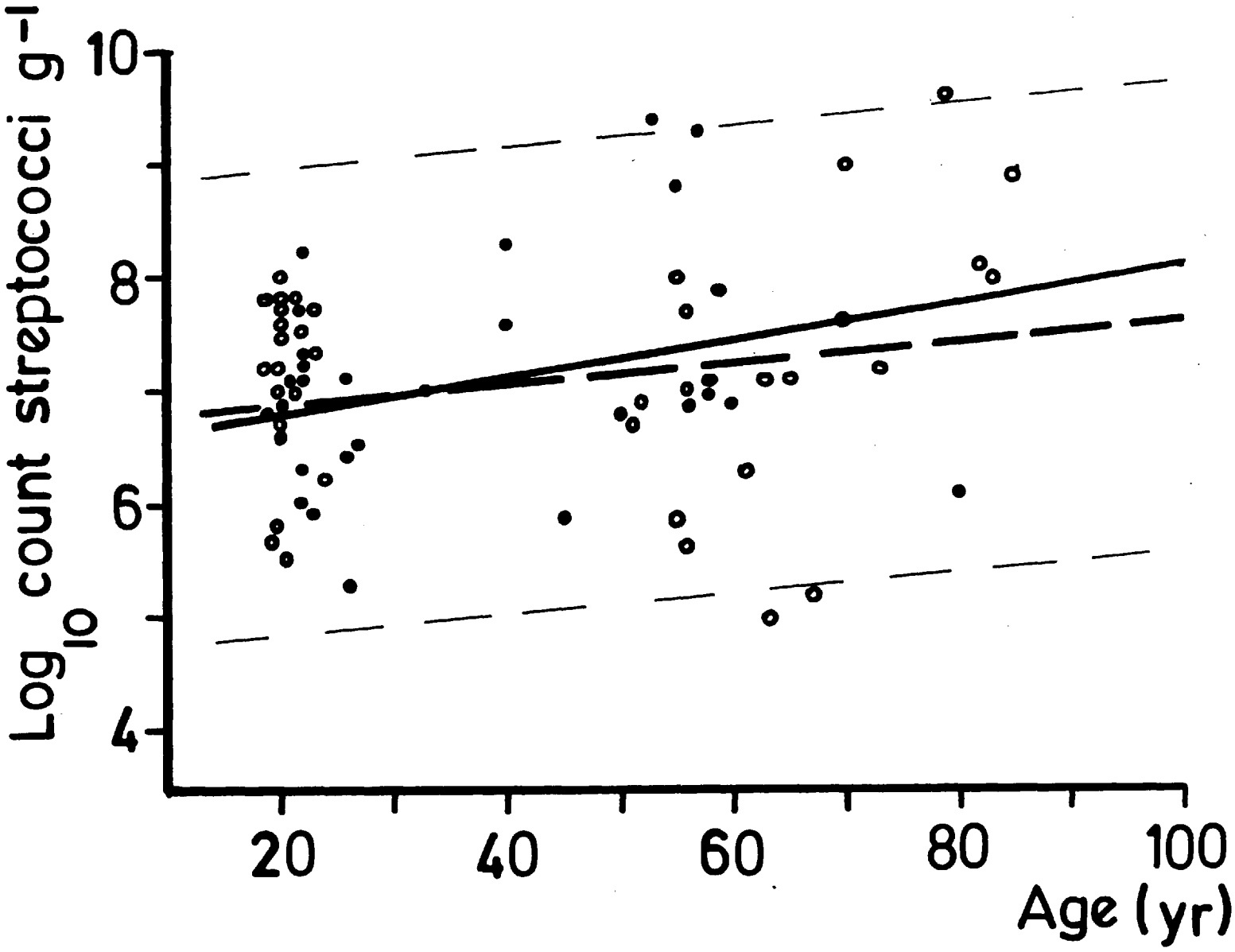

Log10 count streptococci g-1
10
8
6
4
20
40
60
80
100
Age (yr)

Fig. 9. Relationships between age and the counts of lecithinase-positive clostridia in faeces.

Key: as in Fig. 6.

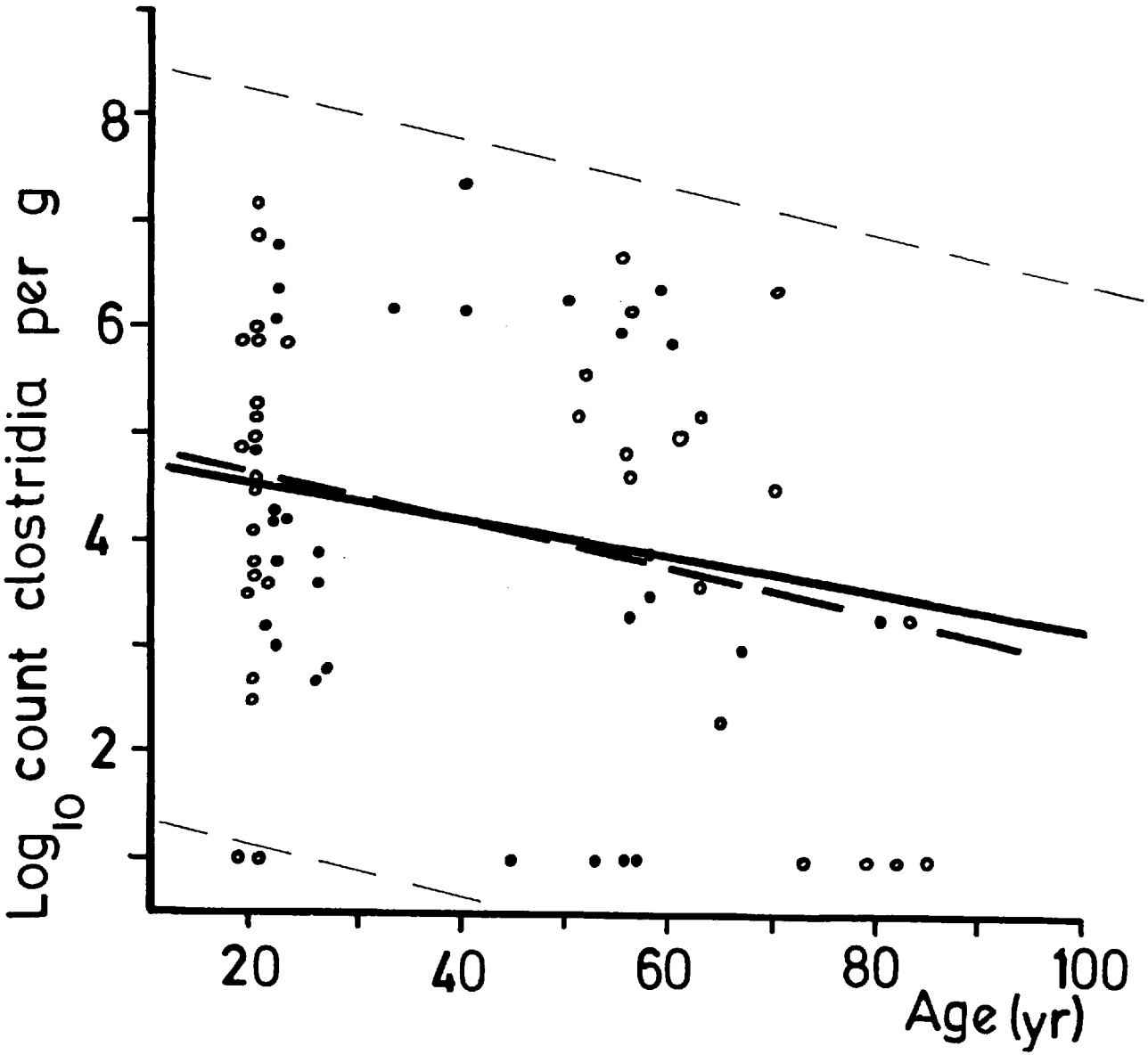

Log10 count clostridia per g
8
6
4
2
20
40
60
80
100
Age (yr)

Fig. 10. Relationships between age and the counts of *Lactobacillus* spp. The increase with age may be significant for women ($P = 0.05$), but not for men. Key: as in Fig. 6.

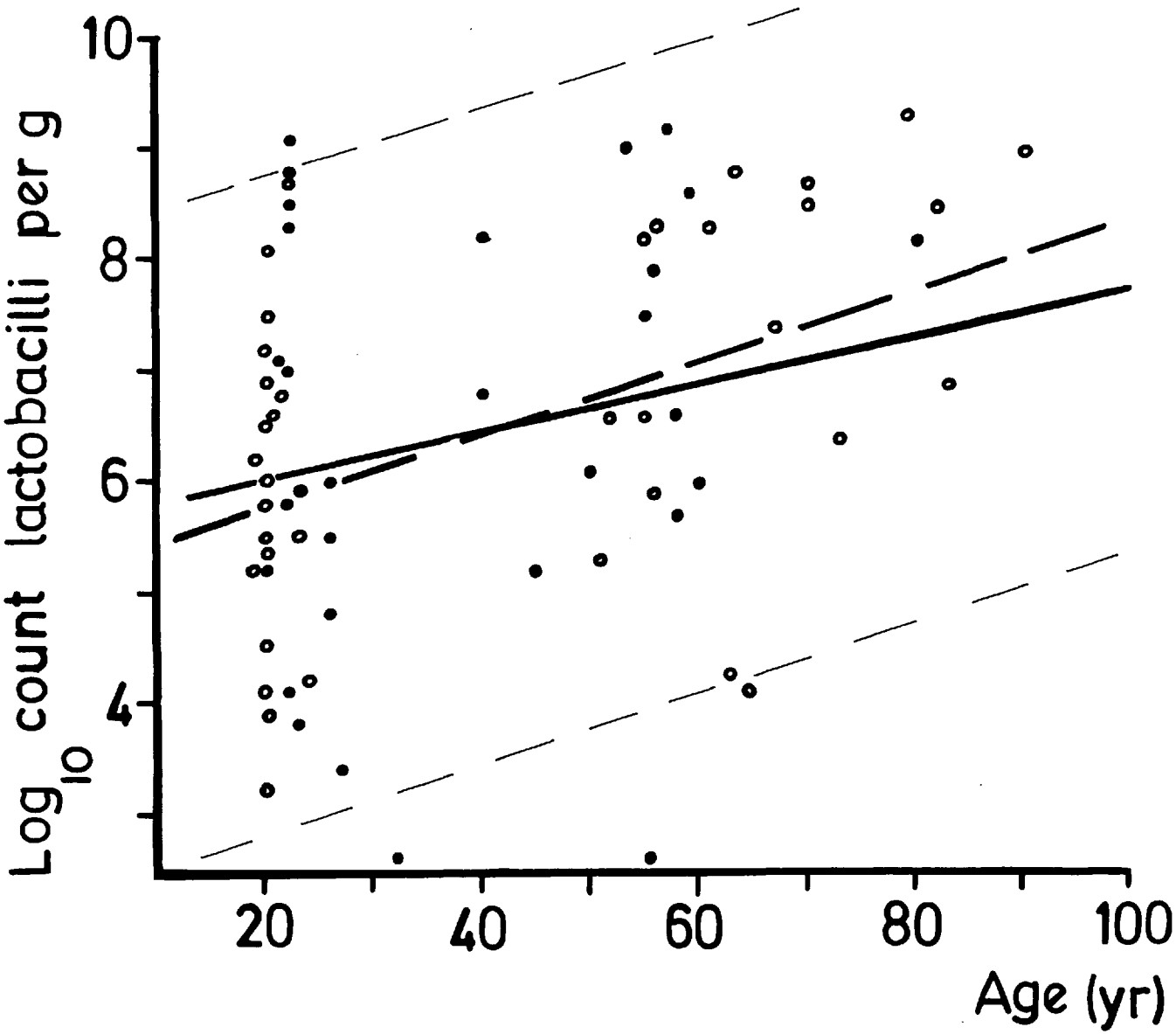
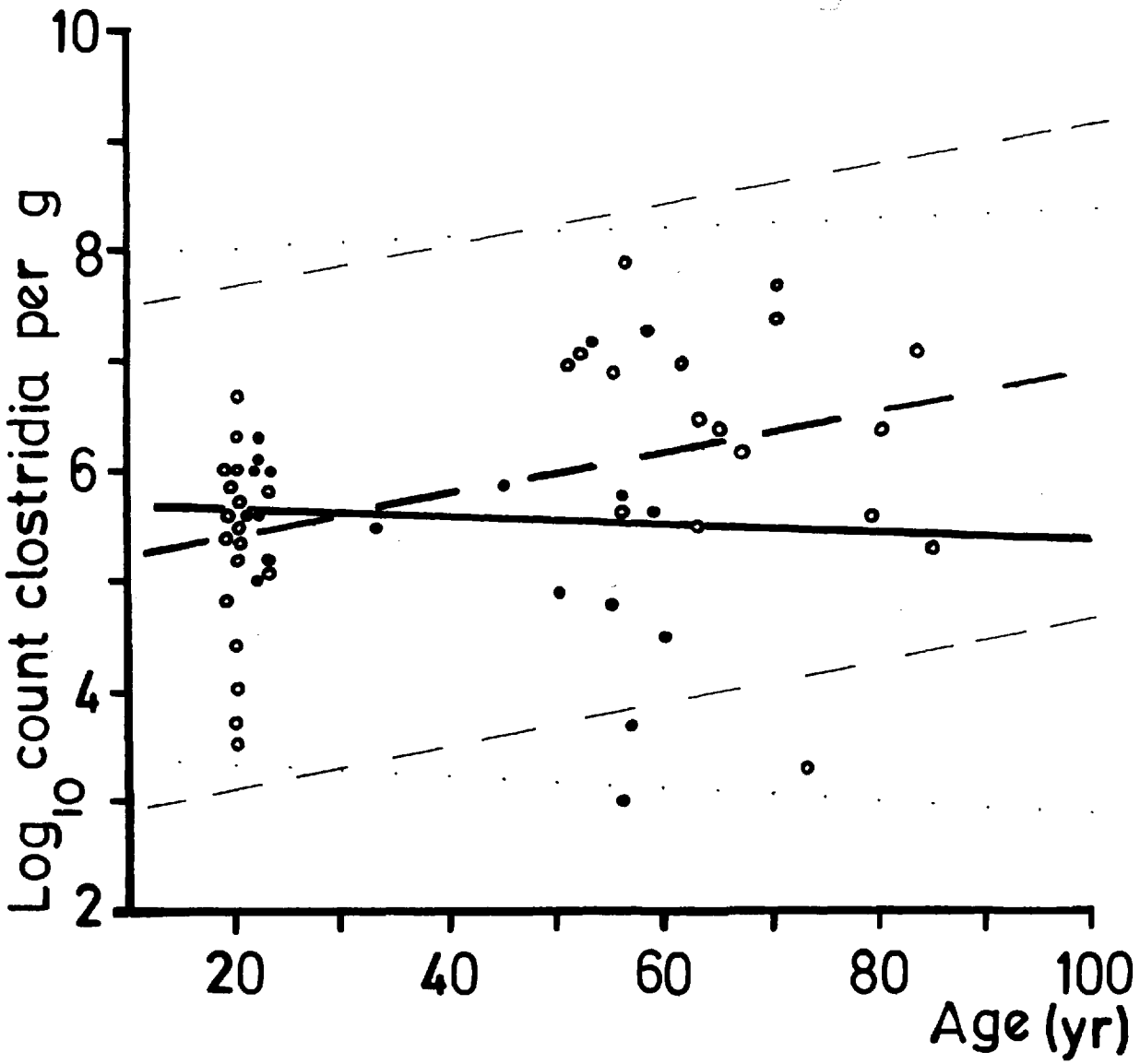

Log10 count lactobacilli per g
10
8
6
4
20
40
60
80
100
Age (yr)

Fig. 11. Relationships between age and the counts of lecithinase-negative clostridia in faeces. The counts show an increase with age for women ($P < 0.05$) but not for men.

Key: as in Fig. 6.


10
8
6
4
2
Log10 count clostridia per g
20
40
60
80
100
Age (yr)

not sufficient to justify similar analyses. However, there is no reason to believe that the results would be different from those reported here for English people.

3.2 DIFFERENCES IN FAECAL FLORA BETWEEN MEN AND WOMEN

Since age had little effect on the distribution of faecal micro-organisms in English men or women, the two sexes were considered as two separate homogeneous populations and the numbers of faecal bacteria in each were compared. Similar comparisons were made between men and women living in the U.S.A., Uganda and Japan and those in England who lived on strict vegan diets.

Men and women showed no significant differences in counts of bifidobacteria in any of the countries (Table 11) but women carried significantly more Bacteroides spp. than did men in England ($P < 0.01$) and the U.S.A. ($P < 0.05$), but not in Uganda or Japan. Men carried more ($P < 0.05$) enterococci, Lactobacillus spp. and lecithinase-negative clostridia than did women in the U.S.A. but not in England, Uganda or Japan. Men carried more 'viridans' streptococci ($P < 0.05$), Streptococcus salivarius, Bacillus spp. and filamentous fungi ($P < 0.01$), than did women in Uganda, but not in England, the U.S.A. or Japan.

Men and women who lived on strictly vegan diets in England carried similar numbers of each group of organisms.

Table 11. Differences in faecal flora between men and women (Mean $\log_{10}$ viable count per g wet faeces)[a]

	England		U.S.A. (White)		Uganda		Japan		England (Vegans)	
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
No. persons examined:	29	39	16	6	9	39	7	10	7	11
Bifidobacteria	9.8	9.8	10.1	10.0	9.2	9.3	9.8	9.7	9.7	9.6
Bacteroides spp.	9.7	10.0[2]	9.6	10.1[1]	8.3	8.2	9.7	9.3	9.6	9.7
Enterobacteria	7.9	8.0	7.5	7.2	8.6	7.8	9.6	9.4	7.1	7.0
'Viridans' streptococci	7.1	7.1	7.2	6.3	8.3	7.7[1]	8.2	8.7	6.6	6.6
Enterococci	5.7	5.8	6.5	4.3[2]	7.1	6.9	8.2	8.3	4.5	5.0
Streptococcus salivarius	4.4	3.4	4.2	2.9	6.9	4.5[2]	2.4	2.4	4.2	4.8
Lactobacillus spp.	6.4	6.5	6.9	5.1[1]	7.3	7.2	7.5	7.4	6.8	7.7
Clostridia (lecithinase-positive)	4.2	4.2	3.6	2.6	4.6	4.0	5.0	4.4	2.6	2.7
Clostridia (lecithinase-negative)	5.6	5.8	5.8	4.5[1]	4.3	5.4	5.5	5.6	5.1	5.6
Veillonella spp.	4.1	4.3	3.8	2.8	5.9	5.2	4.8	4.5	5.3	4.4
Bacillus spp.	3.6	3.8	3.6	3.8	5.4	4.3[2]	4.1	4.5	3.1	3.5
Sarcina ventriculi	1.0	1.0	1.2	1.0	5.7	5.1	1.8	1.4	3.1	2.6
Staphylococci	1.7	1.7	2.4	2.1	3.8	1.7[2]	1.8	1.7	2.7	2.2
Yeasts	2.3	1.9	1.3	1.7	4.2	3.2	1.6	2.9[1]	2.8	3.2
Moulds growing at 37°	1.7	1.3	1.1	1.0	2.2	1.3[2]	1.0	1.4	1.0	1.6
Moulds growing at 22°	1.6	1.6	1.3	1.2	2.8	2.3	1.8	2.1	1.7	1.7

a The standard deviations of the means are not shown here. However, they were similar for men and women; the combined values are shown in Table 13.

1 Significantly different counts between men and women: $P < 0.05$

2 " " " " " " " $P < 0.01$

3.3 DIFFERENCES IN FAECAL FLORA BETWEEN WHITE AND BLACK AMERICANS

Faeces from 12 black and 22 white Americans, all working at the Center for Disease Control in Atlanta and living on mixed diets, are compared in Table 12. The two groups carried the same numbers of all organisms except *Lactobacillus* spp. and *Veillonella* spp. which were more numerous in the black, and staphylococci, which were more numerous in the white persons ($P < 0.05$).

Table 12. Differences in faecal flora between white and black Americans
(Mean $\log_{10}$ viable count per g wet faeces ± SD)

Organism	White	Black
Bifidobacteria	10.04 ± 0.56	9.97 ± 0.57
Bacteroides spp.	9.70 ± 0.58	9.77 ± 0.24
Enterobacteria	7.40 ± 1.25	7.33 ± 1.21
'viridans' streptococci	6.92 ± 1.32	7.15 ± 1.04
Enterococci	5.90 ± 1.89	4.98 ± 1.96
Str. salivarius	3.86 ± 2.42	5.43 ± 1.76
Lactobacillus spp.	6.45 ± 1.58	7.96 ± 1.53[1]
Clostridia (lecithinase-positive)	3.34 ± 1.45	3.66 ± 1.75
Clostridia (lecithinase-negative spore-formers)	5.42 ± 1.44	5.12 ± 1.29
Veillonella spp.	3.51 ± 1.77	4.81 ± 1.72[1]
Bacillus spp.	3.63 ± 0.70	3.86 ± 0.43
Sarcina ventriculi	1.11	-
Staphylococci	2.31 ± 1.67	1.28 ± 0.66[1]
Yeasts	1.44 ± 0.86	1.44 ± 0.88
Filamentous fungi growing at 37°	1.09 ± 0.29	-
Filamentous fungi growing at 22°	1.22 ± 0.53	1.65 ± 0.90

The differences are not significant unless stated otherwise.

1 Differences probably significant $P < 0.05$

\- Organisms not found

Discussion

The results described here show that the dominant microbial flora of human faeces is very stable throughout adult life. The increases in enterobacteria with age found by Haenel (1963) and Gorbach, Nahas, Lerner and Weinstein (1967) were not confirmed here. However, the increase in Lactobacillus spp. with age reported by Haenel, was confirmed here for women but not for men.

The tacit assumption in the literature that men and women have the same faecal flora is confirmed by these results, except that in England and in America women carried more Bacteroides spp. than did men. Some differences were found in the number of the minor components of the flora, but none was consistent in direction from population to population and none attained a high level of statistical significance. It seems likely that these differences may have arisen by chance, and that there are no real differences in the faecal flora between men and women. Similarly, the differences in the faecal flora between white and black Americans living on mixed diets in the same environment, were not found to be highly significant and it seems likely that they also may have arisen by chance.

Since age and sex produce little effect on the faecal flora, it seems reasonable to conclude that the distribution of faecal bacteria within a small sample of healthy men and

women of different ages, would provide a reliable estimate of the distribution of bacteria within a larger population.

3.4 EFFECTS OF DIET AND ENVIRONMENT ON THE FAECAL FLORA

3.4.1 Faecal flora of persons living on normal diets

Faeces were examined from healthy people living in parts of the world where the traditional diets were very different. The populations chosen have been described in Table 5 together with details of their diets. Sixteen broad groups of micro-organisms were counted as described in Section 2.2. The counts from men and women were combined to obtain a population distribution for each country. These are shown in Figs. 12-26, and the population means are summarised in Table 13. The mean counts from each population were compared with those from the 68 Londoners living on mixed diets.

Six groups of organisms, Bifidobacteria, Bacteroides spp., enterobacteria, 'viridans' streptococci, Lactobacillus spp., and Bacillus spp. were found in all the specimens of faeces, whereas the remaining ten groups, Sarcina ventriculi, Veillonella spp., lecithinase-positive and lecithinase-negative clostridia, enterococci, Streptococcus salivarius, staphylococci, yeasts and filamentous fungi, were not found in some. Table 14 shows the percentage of persons from each country found to carry the latter groups of organisms.

Table 13. Faecal flora of healthy persons living in different countries
(mean $\log_{10}$ viable count per g wet faeces ± SD)

Diet	Western mixed			Vegan	Banana and vegetable	Western mixed	Vegetable and fruit	Vegetable and fruit
Country of origin	U.S.A.[d]	Scotland	England	England	Uganda	Uganda	India	Japan
No. persons examined	22	23	68	18	48	8	51	17
Bifidobacteria	10.0 ± 0.6	9.9 ± 0.5	9.8 ± 0.5	9.6 ± 0.4	9.3 ± 0.6[c]	9.5 ± 0.2	9.6 ± 0.5[a]	9.7 ± 0.5
Bacteroides spp.	9.7 ± 0.6	9.8 ± 0.6	9.8 ± 0.6	9.7 ± 0.3	8.2 ± 1.1[c]	9.8 ± 0.4	9.2 ± 0.6[c]	9.4 ± 0.5[b]
Enterobacteria	7.4 ± 1.3	7.6 ± 1.1	7.9 ± 1.3	7.0 ± 1.8[a]	8.0 ± 0.9	7.4 ± 0.4	9.9 ± 1.2	9.4 ± 0.6[c]
Streptococci:								
'viridans' streps.	6.9 ± 1.3	6.8 ± 1.0	7.1 ± 1.0	6.6 ± 1.2	7.8 ± 0.9[c]	6.4 ± 0.9[c]	7.9 ± 1.1[c]	8.5 ± 1.1[c]
enterococci	5.9 ± 1.9	5.3 ± 1.8	5.8 ± 1.5	4.8 ± 1.3[a]	7.0 ± 1.3[c]	5.3 ± 1.9	7.3 ± 1.1[c]	8.1 ± 0.9[c]
Str. salivarius	3.9 ± 2.4	4.1 ± 2.5	3.8 ± 2.7	4.6 ± 2.1	5.0 ± 2.3[a]	3.6 ± 2.1	5.7 ± 1.9[c]	2.4 ± 2.0[a]
Lactobacillus spp.	6.5 ± 1.6	7.7 ± 1.5[b]	6.5 ± 1.8	7.4 ± 1.4	7.2 ± 1.1[a]	5.3 ± 1.3	7.6 ± 1.2[c]	7.4 ± 0.9[a]
Clostridia:								
lecithinase-positive	3.3 ± 1.5	4.2 ± 1.8	4.2 ± 1.8	2.7 ± 1.9[b]	4.0 ± 1.9	2.3 ± 1.5[b]	4.2 ± 2.2	4.7 ± 1.5
lecithinase-negative	5.4 ± 1.4	5.6 ± 1.0	5.7 ± 1.1	5.42 ± 0.8	5.1 ± 1.4	4.7 ± 0.5[a]	5.7 ± 1.2	5.6 ± 0.8
Veillonella spp.	3.5 ± 1.8	3.8 ± 1.9	4.2 ± 2.1	4.8 ± 2.0	5.3 ± 1.5[b]	2.2 ± 1.1[a]	5.8 ± 1.9[c]	4.6 ± 1.4
Bacillus spp.	3.6 ± 0.7	3.3 ± 0.7[a]	3.7 ± 0.8	3.4 ± 0.8	4.5 ± 0.9[c]	2.9 ± 0.5[b]	4.9 ± 1.0[c]	4.4 ± 1.0[b]
Sarcina ventriculi	-[e]	-	-	2.5 ± 2.2	5.2 ± 2.2	-	4.6 ± 2.4	1.6 ± 1.0
Staphylococci and micrococci	2.3 ± 1.7	2.4 ± 1.5[a]	1.7 ± 1.2	2.4 ± 1.4	2.1 ± 2.1	2.2 ± 1.8	2.8 ± 1.5[c]	1.7 ± 1.1
Fungi: yeasts	1.4 ± 0.9	2.8 ± 1.4	2.1 ± 1.5	3.0 ± 1.7[a]	3.4 ± 1.7[c]	-	2.9 ± 1.6	2.3 ± 1.1
moulds (growing at 37°)	1.1 ± 0.3	1.1 ± 0.3	1.2 ± 0.5	1.4 ± 0.7[b]	1.5 ± 0.8	-	2.1 ± 0.9[c]	1.3 ± 0.5
moulds (growing at 22°)	1.2 ± 0.5	1.8 ± 1.1	1.6 ± 0.7	1.7 ± 0.8	2.4 ± 0.9[c]	-	2.2 ± 0.8[c]	2.0 ± 0.7[a]

a counts (probably) significantly different from the English $P < 0.05$
b " " " " " " $P < 0.01$
c " " " " " " $P < 0.001$
d counts of white persons only; taken from Table 12
e organism isolated from one person only
- organism not isolated

Table 14. Percentages of persons in each country found to carry 10 groups of micro-organisms

Organism	Country and number of persons studied							
	U.S.A.		Scotland	England		Uganda	India	Japan
	white 22	black 12	23	normal 68	vegan 18	48	51	17
Enterococci	100	100	96	100	100	100	100	100
Str. salivarius	68	92	61	59	83	85	94	44
Clostridia:								
lecithinase-positive	86	92	91	85	56	90	80	100
lecithinase-negative	100	100	100	100	100	96	98	100
Veillonella spp.	82	92	87	84	89	100	94	100
Sarcina ventriculi	5	0	0	0	33	84	75	28
Staphylococci	45	17	30	22	56	29	67	39
Fungi: yeasts	23	25	61	46	66	77	67	72
moulds (growing at 37°C)	9	0	4	20[b]	22	31[a]	69	28
moulds (growing at 22°C)	45	42	39	50[b]	50	80[a]	76	72

a only 30 persons studied

b " 56 " "

Bifidobacteria. The Gram-positive, non-sporing anaerobes, the bifidobacteria, were the most numerous organisms in faeces from all populations (Table 13). The counts were always distributed closely about the means. The widest scatter occurred in the counts from the Ugandans, where the standard deviation was 0.62 log unit (Fig. 12). Faeces from persons living on typical Western mixed diets in England, Scotland and the U.S.A. contained more bifidobacteria than those from persons living on principally vegetarian diets in Uganda, India or Japan. The mean count from the Ugandans was significantly lower ($P < 0.001$) and that from the Indians was probably significantly lower ($P < 0.05$) than that from English persons living on mixed diets. English persons living on strict vegan diets carried similar numbers of bifidobacteria to those living on mixed diets.

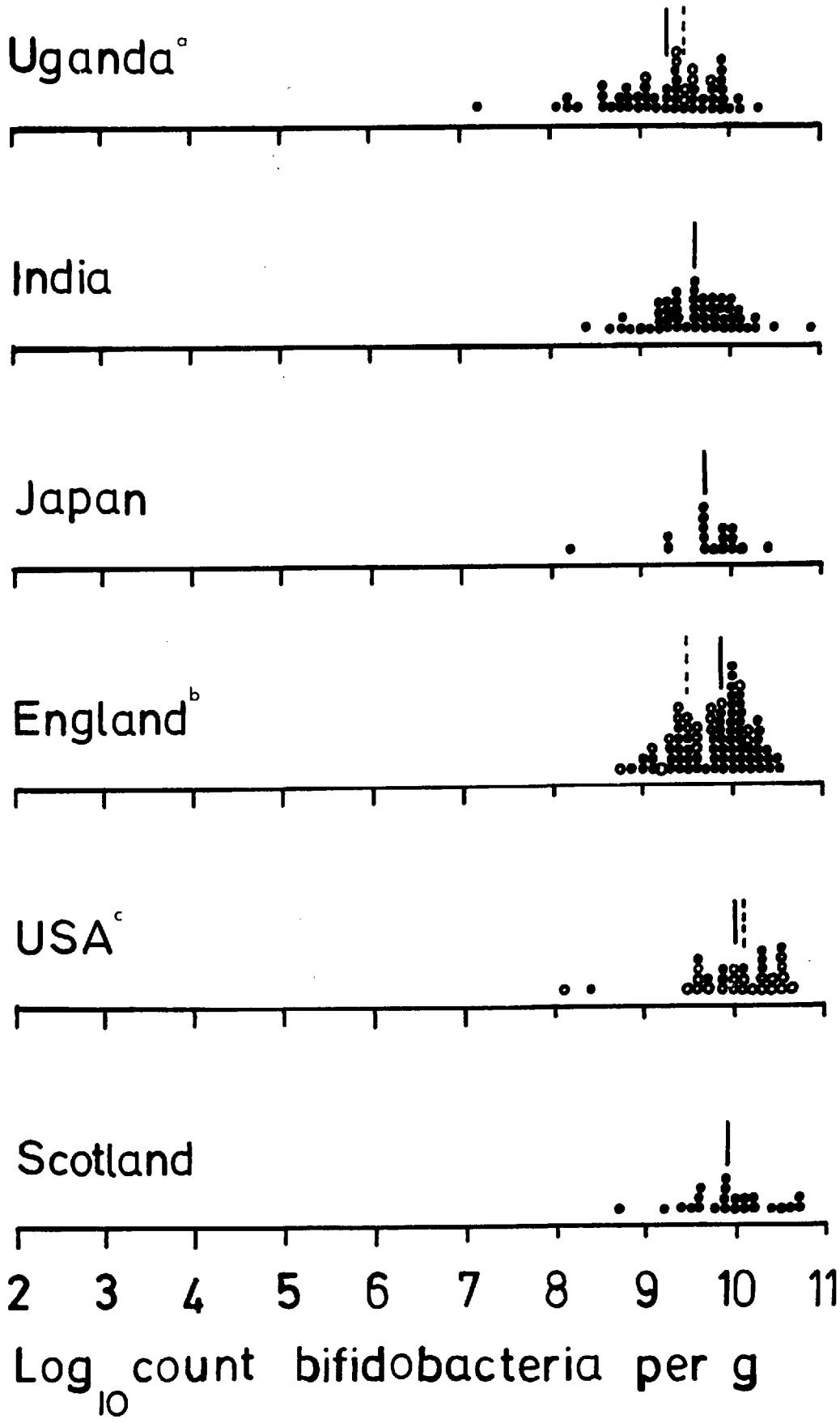
During the early stages of the work pure strains of bifidobacteria were found difficult to keep alive and so their identification has been far from satisfactory. Methods for freeze-drying Bifidobacterium bifidus described by Obayashi, Ota and Arai (1961) were not suitable for our isolates, nor were those employing horse-serum and glucose broth described by Cruikshank (1965). However, we have recently found that cultures of bifidobacteria (and bacteroides) survive well if they are frozen at -55°C in Robertson's cooked meat broth

Fig. 12. Distribution of bifidobacteria in faeces from persons living in different countries. One spot represents the count for one person. A vertical line represents the mean count (from Table 13). Where two distinct populations from one country have been compared, two different spots are used.

Legend a. Counts for Ugandans are shown as black spots and those for English immigrants as circles. The mean for Ugandans is given by a bold line and that for immigrants as a broken line.

b. Counts for persons living on mixed diets are shown as black spots and those for persons living on vegan diets as circles. Their means are represented by bold and broken lines respectively.

c. Counts are shown as black spots for black, and circles for white, Americans. Means are given by bold and unbroken lines respectively.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
2 3 4 5 6 7 8 9 10 11
Log10 count bifidobacteria per g

containing 10% (v/v) glycerol (Drasar and Crowther, 1971). Therefore, we have begun to re-isolate strains from all specimens of faeces and to identify them as Bifidobacterium, Propionbacterium and Eubacterium spp. with the help of gas-liquid chromatography as described in section 2.2.

Our preliminary results are very promising and show that whilst Bifidobacterium spp. were isolated more frequently than Propionibacterium from Japanese and Indians, the converse was found for Americans and Scots: Bifidobacterium spp. formed 44 out of 72 (61%) of isolates from Japanese, and 19 out of 40 (48.5%) of isolates from Indians, but only 12 out of 41 (29%) of isolates from Americans and 4 out of 21 (19%) from Scots; conversely, Propionibacterium species composed 22 out of 41 (54%) of isolates from Americans and 11 out of 21 (52%) from Scots, and only 11 out of 72 (15%) from Japanese and 11 out of 40 (27.5%) from Indians. Eubacterium spp. formed a similar proportion of isolates from all populations. Although we isolated over 137 Ugandan and 57 English strains of bifidobacteria - which were tested for their abilities to degrade bile salts, see Aries, Crowther, Drasar and Hill (1969) - only 10 of them (4 English and 6 Ugandan) survived for further identification. We are now re-isolating English and Ugandan strains, together with more from India, Scotland and America, for detailed examination.

Bacteroides spp. Gram-negative, non-sporing anaerobes, the Bacteroides group, were the second most dominant organisms in all populations (Table 13). The counts were distributed closely about the means, except for those for Uganda, where the standard deviation was 1.1 $\log_{10}$ unit (Fig. 13).

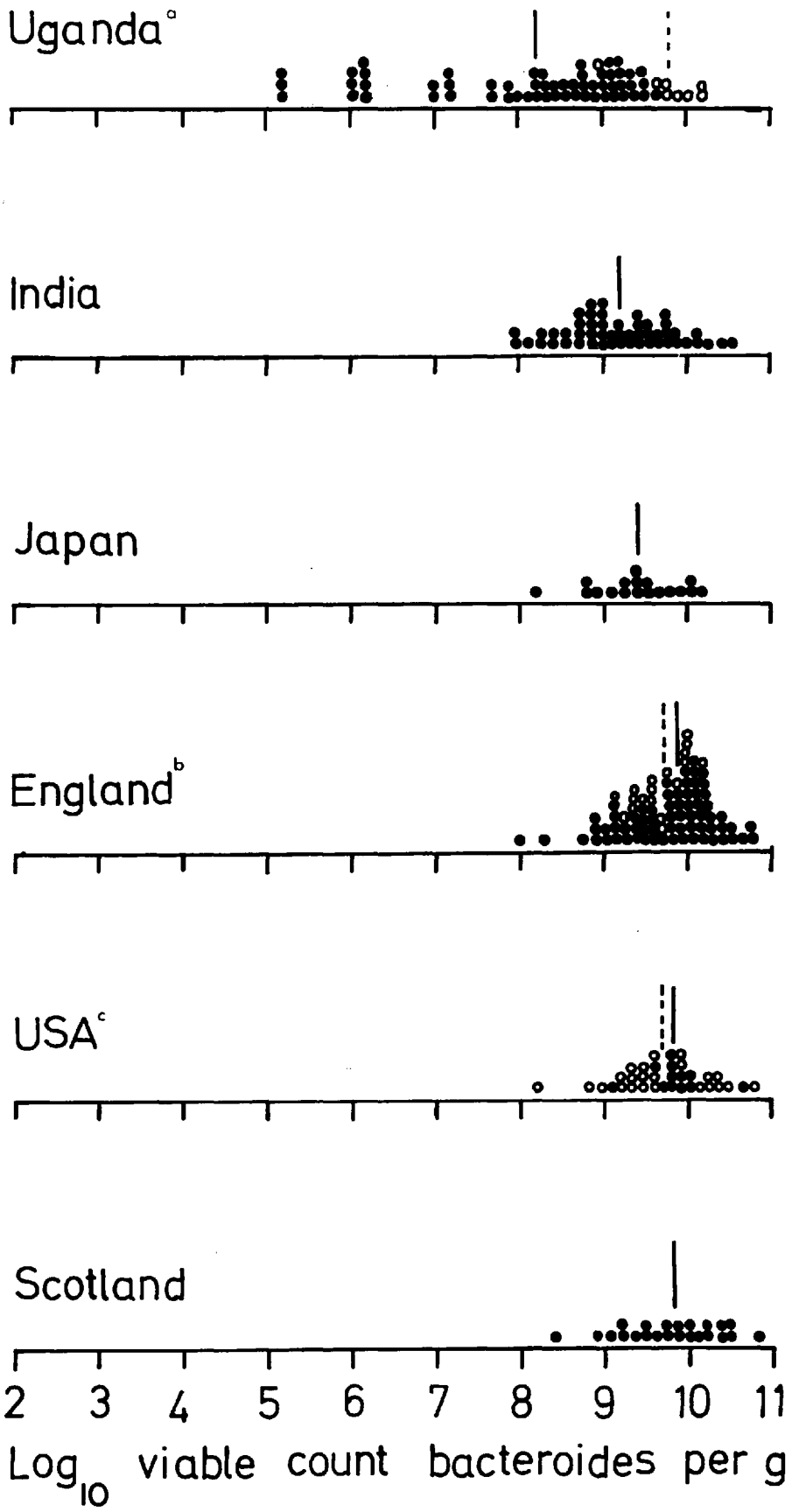
Faeces from persons living on mixed diets in England contained significantly more Bacteroides spp. than those from Ugandans, Indians, or Japanese living on vegetarian diets ($P < 0.001$). However, specimens of faeces from persons in England who lived on strict vegan diets contained the same number as those from people living on mixed diets. The number of Bacteroides spp. in faeces from people living on mixed diets in England, Scotland and America were not significantly different.

Strains of bacteroides were also difficult to keep alive. Freeze-drying was unsatisfactory for our isolates, although it is satisfactory for species of Bacteroides isolated from the caeca of chickens (Barnes, Impey and Goldberg, 1966). Latterly, we have preserved strains frozen at -55^{o} in Robertson's cooked meat broth containing 10% (v/v) glycerol. Species of Bacteroides isolated from the bovine rumen are known to survive better frozen than when freeze-dried (Latham and Sharpe, 1971).

Eighty-one of 85 strains of Gram-negative, non-sporing anaerobes were identified as Bacteroides spp., and four as

Fig. 13. Distribution of *Bacteroides* spp. in different populations.

Key: as Fig. 12.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
2 3 4 5 6 7 8 9 10 11
Log10 viable count bacteroides per g

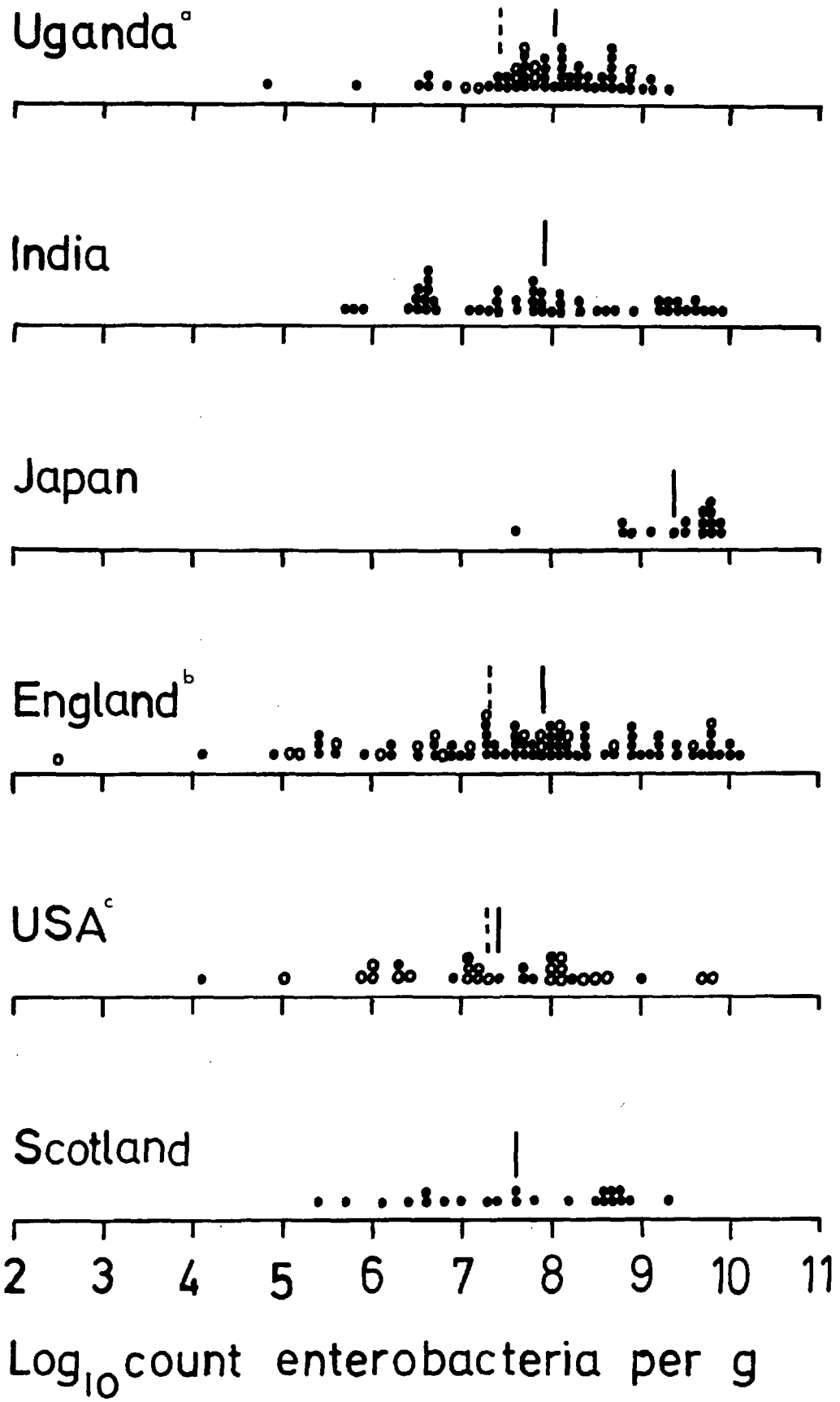
Fusobacterium spp. Of the 81 strains of Bacteroides 52 (64%) were further identified as *Bacteroides fragilis* and four as other species; the remaining 25 strains (31%) could not be identified as species described by Cato *et al.* (1970). There was no apparent distribution of the known species between the different countries. Further strains are now being isolated and identified.

Enterobacteria. Enterobacteria were found in all specimens of faeces but usually in much lower numbers than those of the non-sporing anaerobes; also the distributions about the means were much wider than those of the non-sporing anaerobes (Fig. 14). The numbers in faeces from Uganda, India, Scotland or America were not very different from those from England, but those from Japan were significantly higher ($P < 0.001$). The numbers of enterobacteria in faeces from English persons living on strict vegetarian diets were lower than those from persons living on mixed diets, but the difference did not attain statistical significance.

Enterobacteria isolated from the countable plate of each specimen were identified as described in section 2.2. *Escherichia coli* comprised more than 90% of isolates from persons in Scotland, America, Uganda and England (including those living on vegan diets), but only 69% and 78% of

Fig. 14. Distribution of enterobacteria in different populations.

Key: as Fig. 12.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
2 3 4 5 6 7 8 9 10 11
Log10 count enterobacteria per g

isolates from persons in India and Japan respectively (Table 15). Klebsiella and Aerobacter spp. were occasionally present in large numbers and the former were found more frequently in specimens from Japan than in those from elsewhere. Organisms tentatively identified as belonging to the Hafnia group were occasionally isolated from persons living on vegetarian diets in Uganda, India and England, but never from persons living on mixed diets. Arizona spp., Citrobacter spp. and Proteus mirabilis were sometimes the dominant aerobic organisms.

Aerobic streptococci

Viridans streptococci. The second most numerous group of facultative organisms in faeces were usually 'viridans' streptococci. In all populations the counts were distributed about the means with approximately unit standard deviation (Fig. 15). Faeces from persons living on vegetarian diets in Uganda, India and Japan contained significantly more than those living on mixed diets in England, Scotland or America ($P < 0.001$). Persons living on vegan diets in England contained fewer than those from persons living on mixed diets, but the difference was not significant.

Enterococci. The enterococci were present in almost all specimens of faeces. Within any population the counts

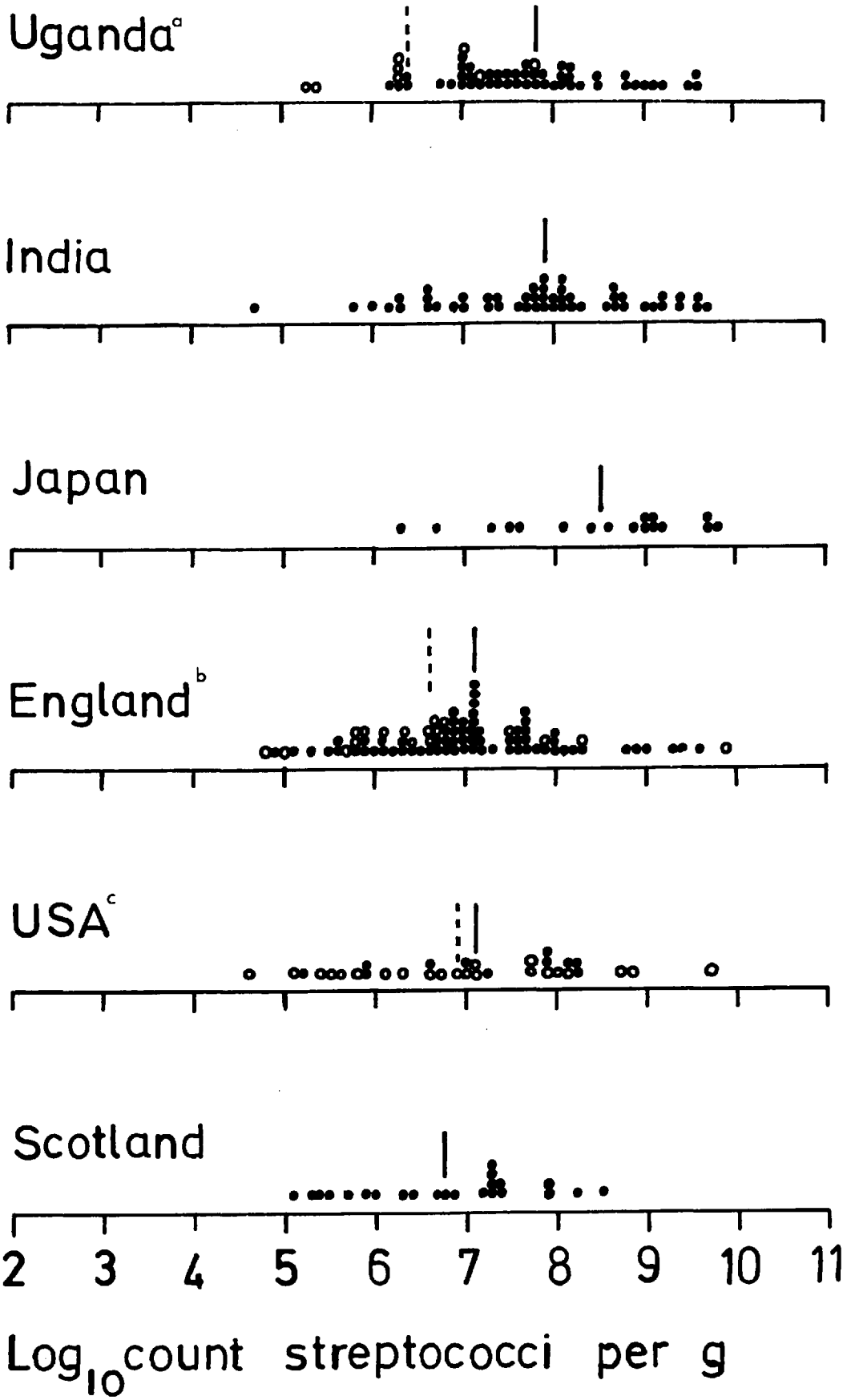
Table 15. Distributions of the dominant enterobacteria in faeces from persons living in different countries

Country of origin	No. strains tested	No. strains identified as:								
		Escherichia coli	Arizona group	Citrobacter group	Klebsiella group	Aerobacter group A	Aerobacter group C	Hafnia group	Proteus mirabilis	unidentifiable
U.S.A.										
white	32	29	1	-	1	1	-	-	-	-
black	26	23	-	-	2	-	-	-	-	-
Scotland	44	40	-	-	1	1	-	-	-	2
England	50	48	-	-	2	-	-	-	-	-
England	33	30	1	-	-	1	-	1	-	-
Uganda	50	47	-	-	2	-	-	1	-	-
India	51	40	-	1	3	3	1	1	1	1
Japan	36	25	-	-	10	1	-	-	-	-

- indicates that the organism was never found

Fig. 15. Distribution of 'viridans' streptococci in different populations.

Key: as Fig. 12.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
2 3 4 5 6 7 8 9 10 11
Log10 count streptococci per g

differed widely, and for the Americans and Ugandans the standard deviation was as high as 1.9 log units. Persons living in Uganda, India and Japan contained significantly more ($P < 0.001$) than those living in Scotland, America and England (including those living on vegan diets) (Fig. 16).

Enterococci isolated from the countable plate of each specimens were identified as Streptococcus faecalis, Str. faecium, or as belonging to the Str. bovis/Str. equinus group, as described in section 2.2. The proportions belonging to the three groups differed between countries (Table 16). Streptococcus faecalis was found more frequently than other enterococci in faeces from persons who lived on mixed diets in America and England and from persons in Uganda. Streptococcus faecium was found more frequently than other enterococci in faeces from persons in India and Japan. Streptococcus faecalis and Str. faecium were isolated with approximately equal frequency from persons in Scotland, and from vegan persons in England. Enterococci tentatively identified as Str. bovis or Str. equinus were isolated more frequently from Ugandan and Indian persons than from others; this group was not differentiated further.

Streptococcus salivarius. This organism was not found in all persons and the percentages of carriers ranged from 44

Fig. 16. Distribution of enterococci in different populations.

Key: as Fig. 12.

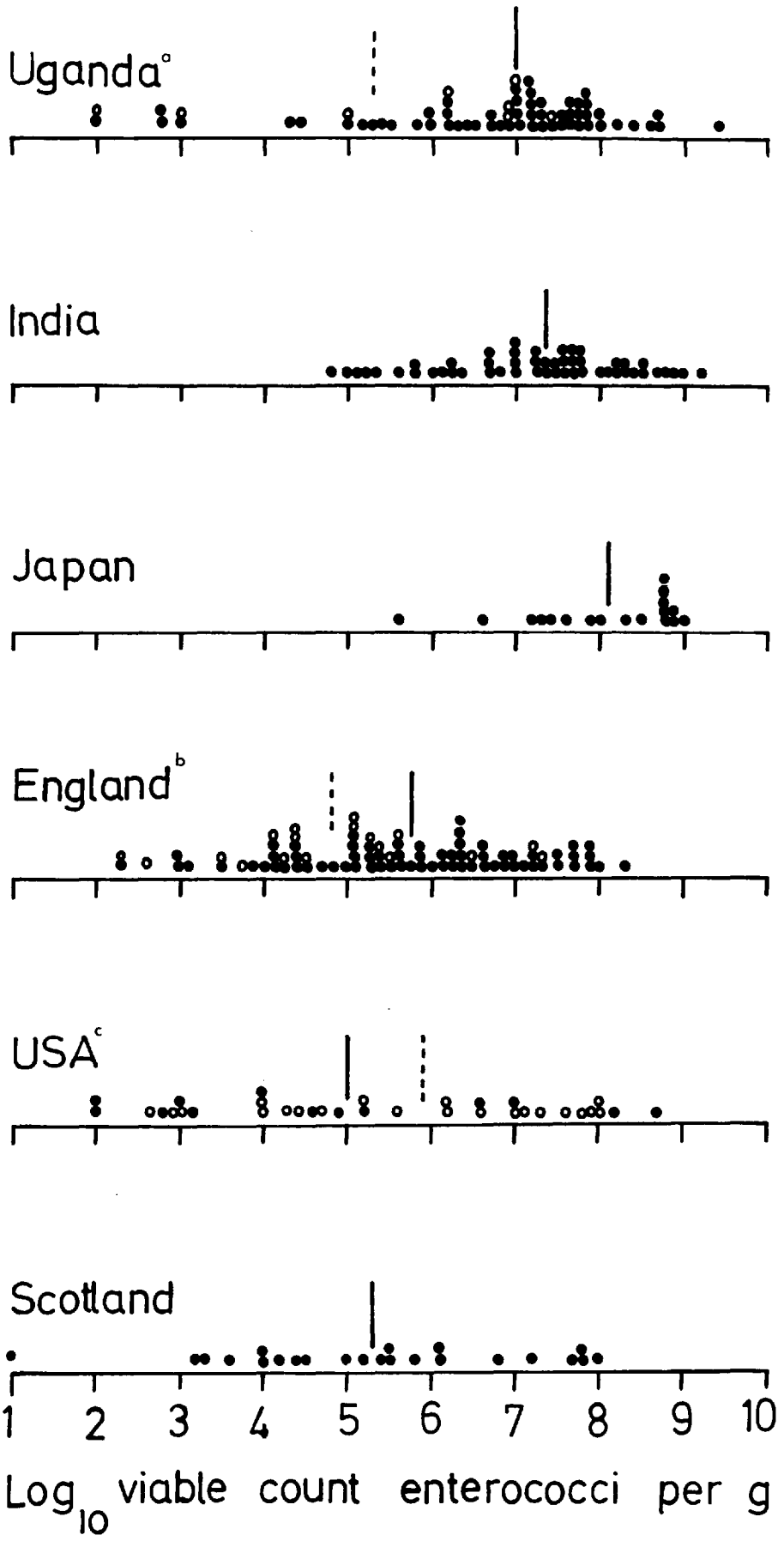

Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
1 2 3 4 5 6 7 8 9 10
Log10 viable count enterococci per g

Table 16. Distribution of the dominant enterococci in faeces from persons living in different countries

Country of origin	No.persons studied	No.strains tested	No.strains identified as:		
			Str. faecalis	Str. faecium	Str. bovis/equinus
U.S.A. (white)	17	17	15	2	-
(black)	12	20	18	2	-
Scotland	23	44	22	18	4
England (normals)	68	77	49	25	3
(vegans)	18	27	12	14	1
Uganda[1]	45	116	57	32	27
India	50	82	18	53	11
Japan	17	32	13	19	-

1 Native Ugandans only

- Organism not found

for Japanese, to 94 for Indians (Table 14). Within each population the counts were widely distributed, and the standard deviations often exceeded two log. units (Fig. 17 and Table 13). The Indians contained significantly more ($P < 0.001$), and the Ugandans probably significantly more ($P < 0.05$) than did English persons living on mixed diets. No consistent relationship was shown between the diet and the numbers of Str. salivarius in the faeces.

Lactobacillus spp. These were found in all persons, although the counts varied widely (Fig. 18). More were found in Ugandans, Indians, Japanese and Scots than in English or Americans. English persons on vegan diets contained more than those on mixed diets.

Clostridium spp. Clostridia were divided into the lecithinase-positive and lecithinase-negative groups as described in section 2.2.

Lecithinase-negative clostridia. These organisms produced heat-resistant spores and were found in almost all specimens of faeces, although in very much fewer numbers than non-sporing anaerobes. The mean logarithmic count per g faeces was approximately 5.5 with unit standard deviation for all

Fig. 17. Distribution of Streptococcus salivarius in different populations.

Key: as in Fig. 12. The numbers in parenthesis to the left of any distribution diagram indicate the proportion of persons not found to carry the organism.

d. The proportion of immigrants not carrying the organism.

e. The proportion of vegans not carrying the organism.

f. The proportion of white Americans not carrying the organism.

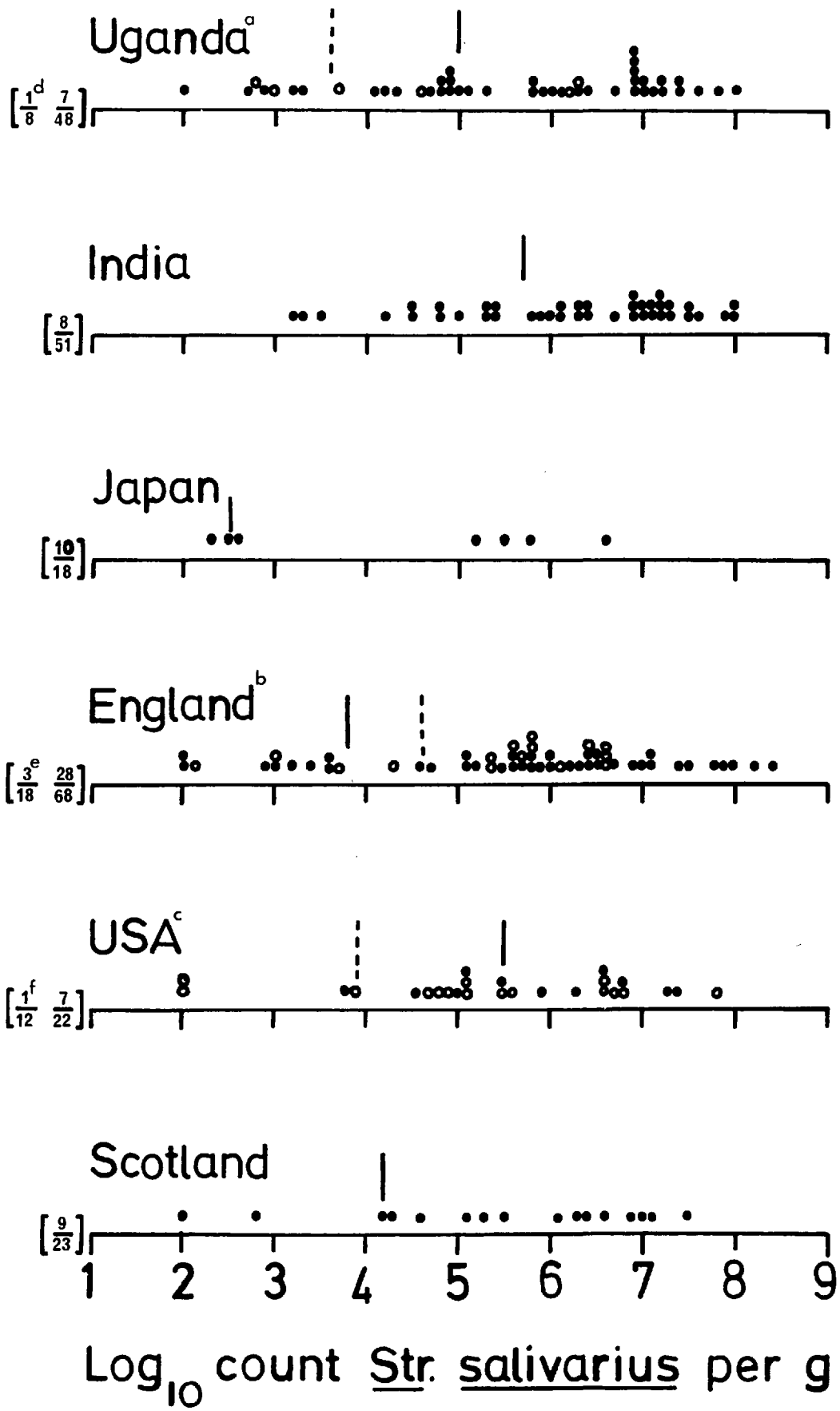
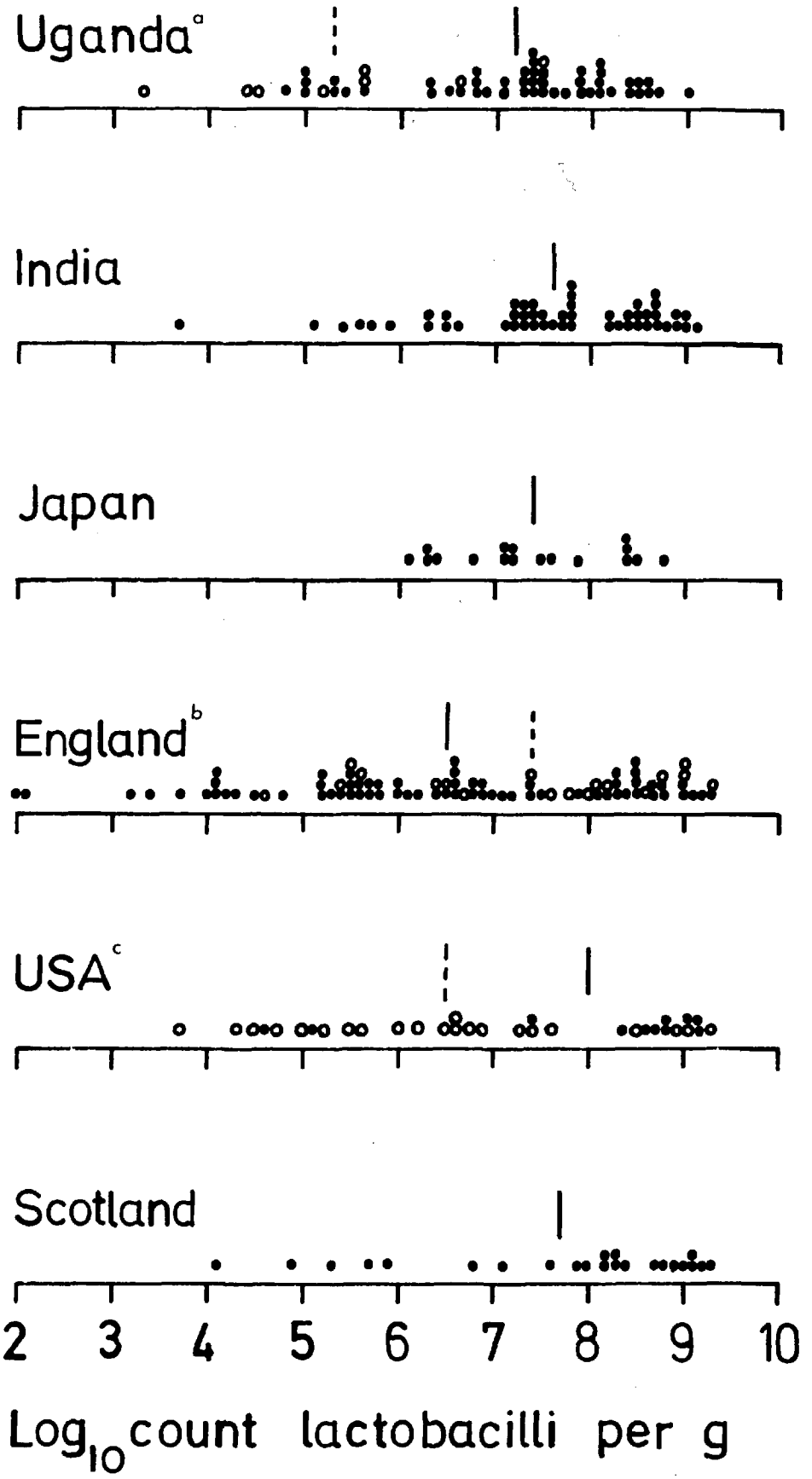

Uganda[a]
[1/8[d] 7/48]
India
[8/51]
Japan
[10/18]
England[b]
[3/18[e] 28/68]
USA[c]
[1/12[f] 7/22]
Scotland
[9/23]
1 2 3 4 5 6 7 8 9
Log10 count Str. salivarius per g

Fig. 18. Distribution of Lactobacillus spp. in different populations.

Key: as Fig. 12.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
2 3 4 5 6 7 8 9 10
Log10 count lactobacilli per g

populations, and therefore seemed to be unrelated to the nature of the diet (Fig. 19).

Lecithinase-positive clostridia were found in over 80% of faecal specimens from Britain, America, Uganda, India and Japan. The mean counts were lower than those for the lecithinase-negative clostridia and were similar for all populations (Fig. 20). However, only 56% of specimens from the English vegans contained these organisms and the mean count, $10^{2.3}$ per g, was lower than that ($10^{4.2}$ per g) for persons living on mixed diets ($P < 0.01$).

Veillonella spp. were found in faeces from most persons. Within any population the distribution of counts was very wide and had a standard deviation of approximately two log units (Fig. 21). The Ugandans and Indians contained more veillonellae than did persons in England living on mixed diets ($P < 0.01$). The English vegans and the Japanese also contained more, but the differences were not significant.

Bacillus spp. All persons were found to carry Bacillus spp., but those in Uganda, India and Japan carried more than those in Britain and America (Fig. 22). English vegans carried the same number as did persons living on mixed diets.

Fig. 19. Distribution of lecithinase-negative clostridia.

Key: as Fig. 17.

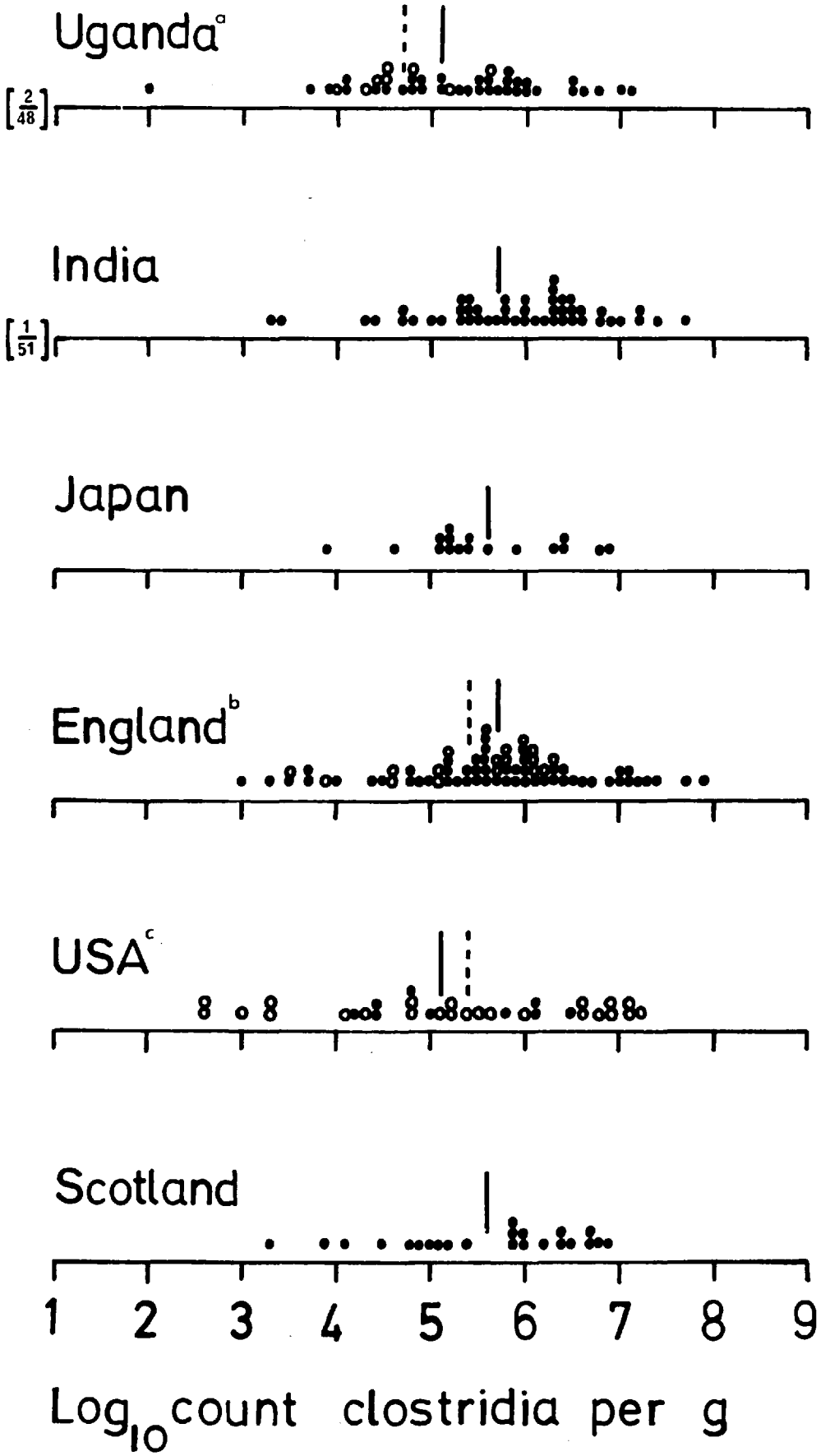

Uganda[a]
[2/48]
India
[1/51]
Japan
England[b]
USA[c]
Scotland
1 2 3 4 5 6 7 8 9
Log10 count clostridia per g

Fig. 20. Distribution of lecithinase-positive clostridia.

Key: as Fig. 17.

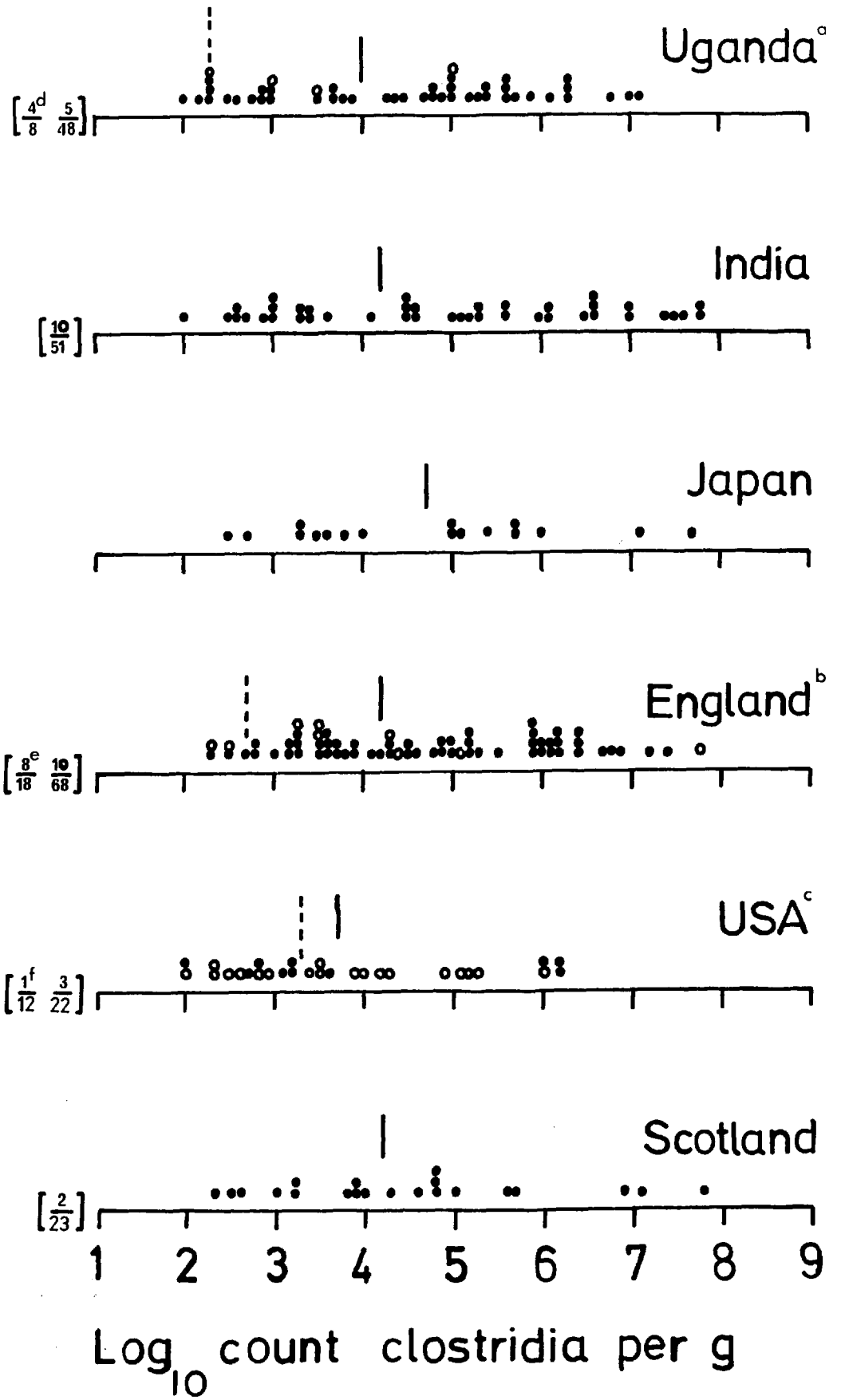

Uganda[a]
[4[d]/8 5/48]
India
[10/51]
Japan
England[b]
[8[e]/18 10/68]
USA[c]
[1[f]/12 3/22]
Scotland
[2/23]
1 2 3 4 5 6 7 8 9
Log10 count clostridia per g

Fig. 21. Distribution of Veillonella spp. in different populations.

Key: as Fig. 17.

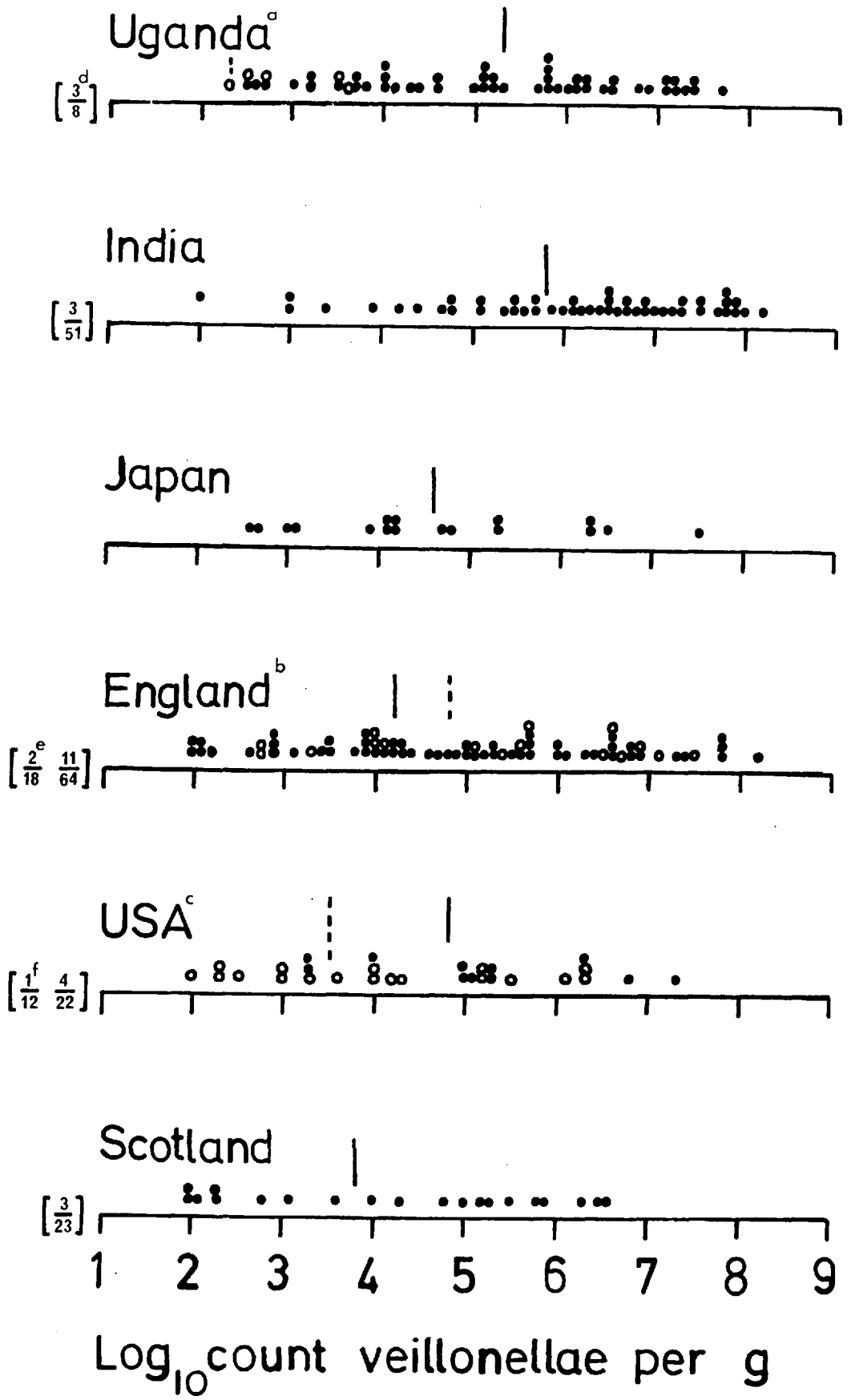
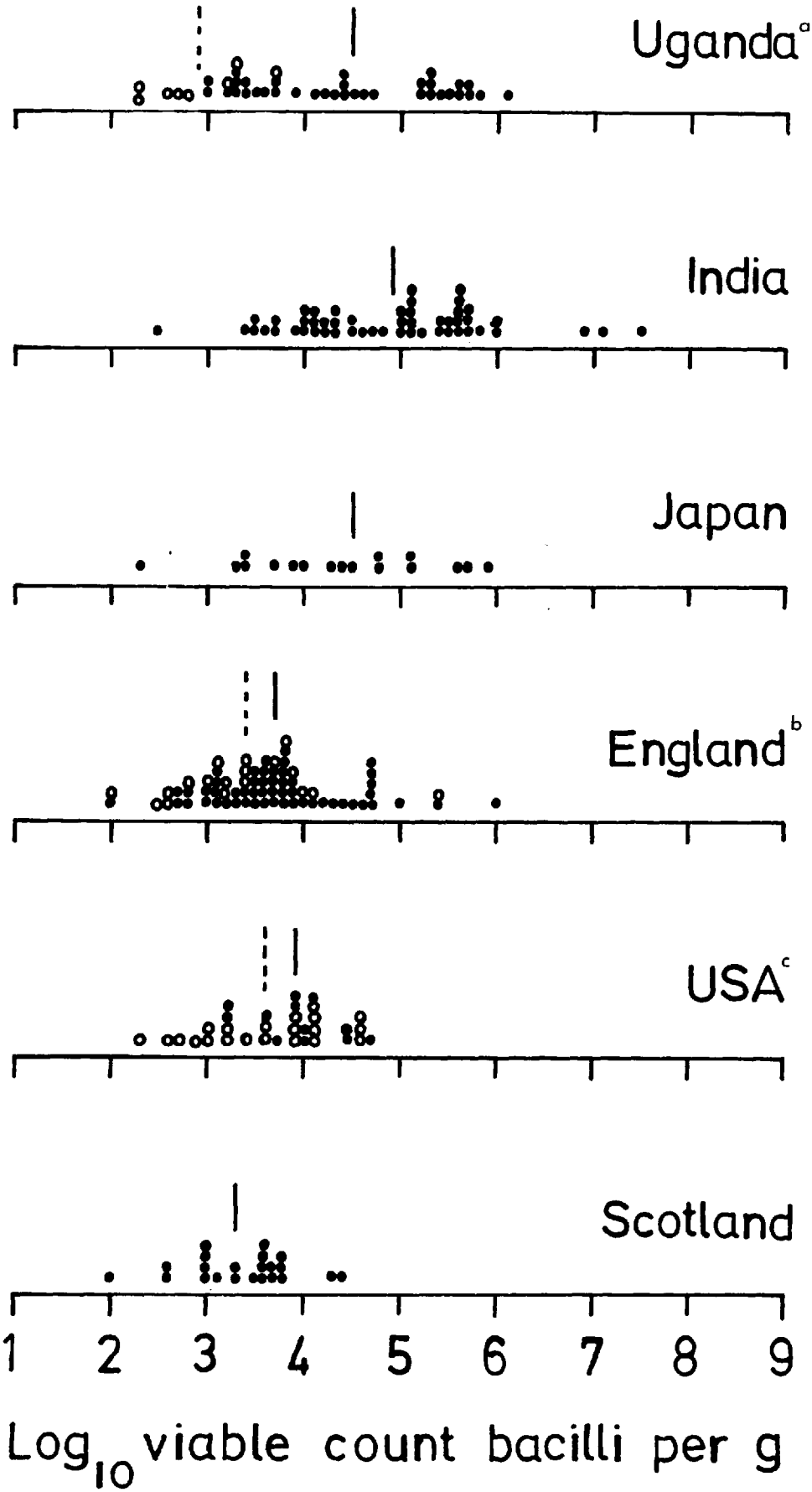

Uganda[a]
[3/8][d]
India
[3/51]
Japan
England[b]
[2/18 11/64][e]
USA[c]
[1/12[f] 4/22]
Scotland
[3/23]
1 2 3 4 5 6 7 8 9
Log10 count veillonellae per g

Fig. 22. Distribution of Bacillus spp. in different populations.

Key: as in Fig. 17.


Uganda[a]
India
Japan
England[b]
USA[c]
Scotland
1 2 3 4 5 6 7 8 9
Log10 viable count bacilli per g

Sarcina ventriculi. The obligate anaerobe Sarcina ventriculi showed very striking differences in distribution from population to population (Table 17). It was carried by 84% of Ugandans, 75% of Indians, 33% of English vegans and 28% of Japanese (all of whom lived on vegetarian diets), but it was never carried by persons living on mixed diets in England or Scotland. It was found in faeces from one white American living on a mixed diet. The counts of sarcinae varied widely within each population; counts of 10^6 organisms per g faeces were common and the highest count recorded was 10^8 per g (Fig. 23). It was not found possible to increase the yield of sarcinae from faeces by the use of acid enrichment media described by Canale-Parola and Wolfe (1960) for the isolation of S. ventriculi from the soil.

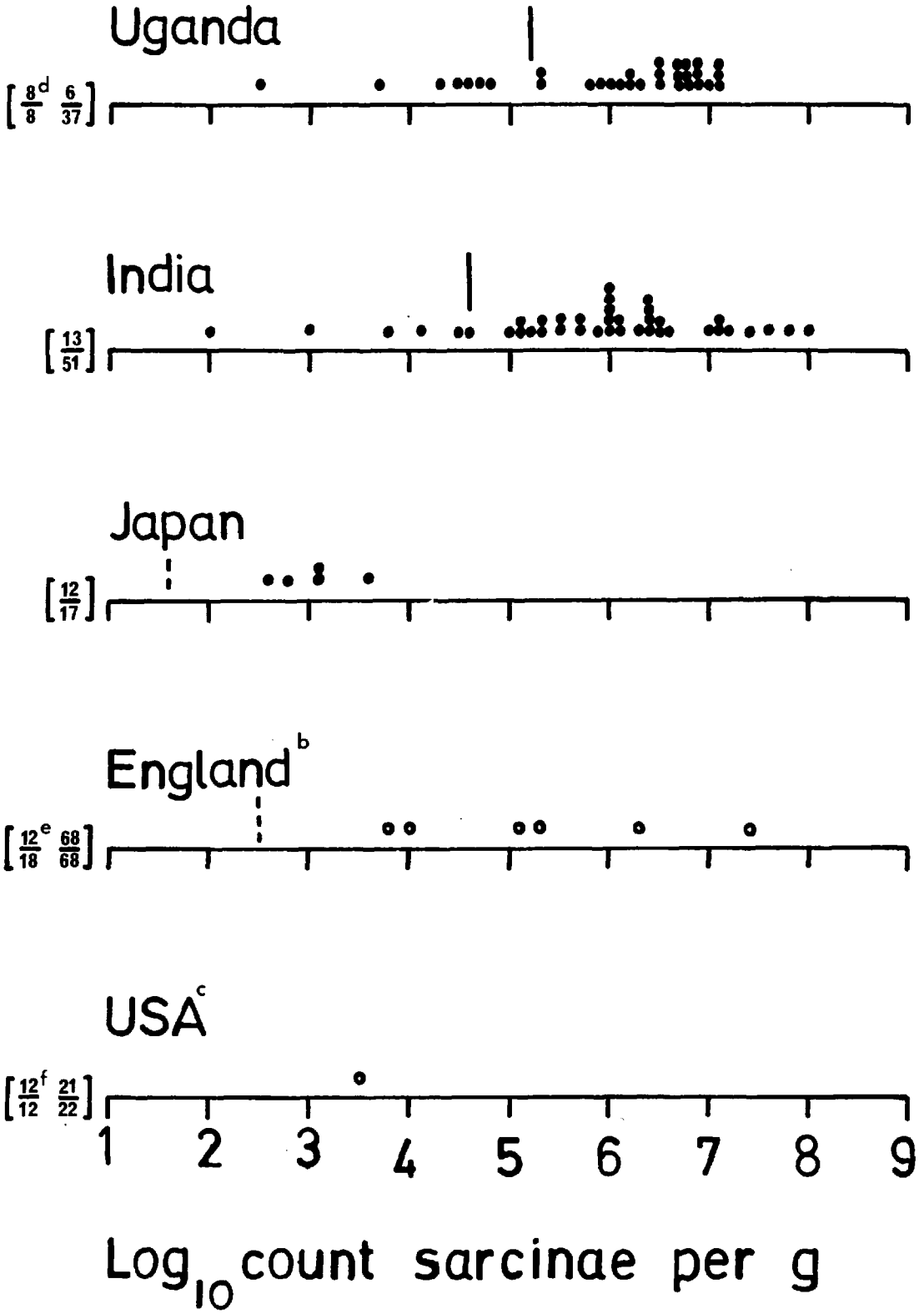
Sarcinae were found difficult to keep alive so that it was impossible to examine large numbers of strains in detail. Six strains (three from Ugandan, two from Indian and one from English vegan persons) were found to be almost identical to a known culture of S. ventriculi. The tests and results are given in Appendix A4. Since sarcinae from all carriers had the same cultural and morphological characteristics during primary isolation as those of Sarcina ventriculi, it was decided to record them all as S. ventriculi.

Table 17. Distribution of Sarcina ventriculi in faeces of persons living in different countries

Country of origin	Diet of population	No. persons studied	No. found to carry sarcinae
Japan	Vegetarian	17	5
Uganda	"	37	31
India	"	51	38
England	Vegan	18	6
England	Western mixed	55	0
Scotland	"	23	0
Uganda (English immigrants)	"	16	0
U.S.A. (white)	"	16	1
(black)	"	12	0

Fig. 23. Distribution of *Sarcina ventriculi* in different populations.

Key: as Fig. 17.


Uganda
[8/8[d] 6/37]
India
[13/51]
Japan
[12/17]
England[b]
[12/18[e] 68/68]
USA[c]
[12/12[f] 21/22]
1 2 3 4 5 6 7 8 9
Log10 count sarcinae per g

Staphylococci and micrococci. Staphylococci and micrococci were considered together as a single group. They were always very minor components of the flora. The mean logarithmic count per g faeces in all populations was approximately two, with unit standard deviation. Occasional specimens from Uganda and India contained more than 10^6 organisms per g (Fig. 24).

The fungal flora of faeces

The three very broad groups of fungi (described in section 2.2) were all very minor components of the flora.

Yeasts. Yeasts were found in higher numbers in persons living on vegan diets in England and banana diets in Uganda, than in persons living on mixed diets in Britain and America. Persons in India and Japan contained similar numbers to those in persons in Britain (Fig. 25).

Filamentous fungi were found more frequently and in higher numbers in persons from Uganda, India and Japan than in those from Britain and America. Organisms growing at 22° were isolated more frequently than those capable of growth at $37^{\circ}C$ (Fig. 26).

Dr. R.R. Davies kindly identified the isolates from

Fig. 24. Distribution of staphylococci in different populations.

Key: as Fig. 17.

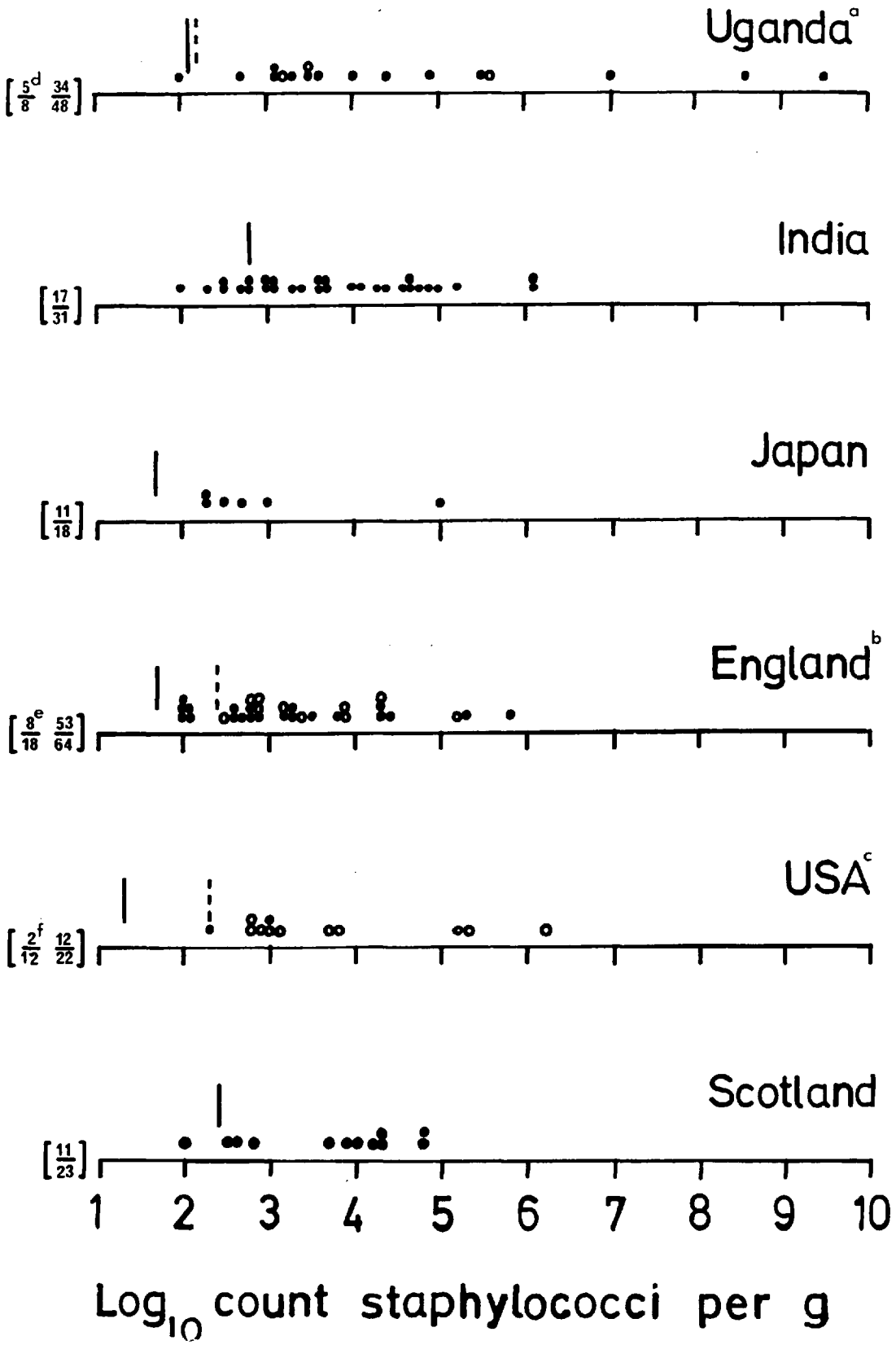

Uganda[a]
[5[d]/8 34/48]
India
[17/31]
Japan
[11/18]
England[b]
[8[e]/18 53/64]
USA[c]
[2[f]/12 12/22]
Scotland
[11/23]
1 2 3 4 5 6 7 8 9 10
Log10 count staphylococci per g

Fig. 25. Distribution of yeasts in different populations.

Key: as in Fig. 17.

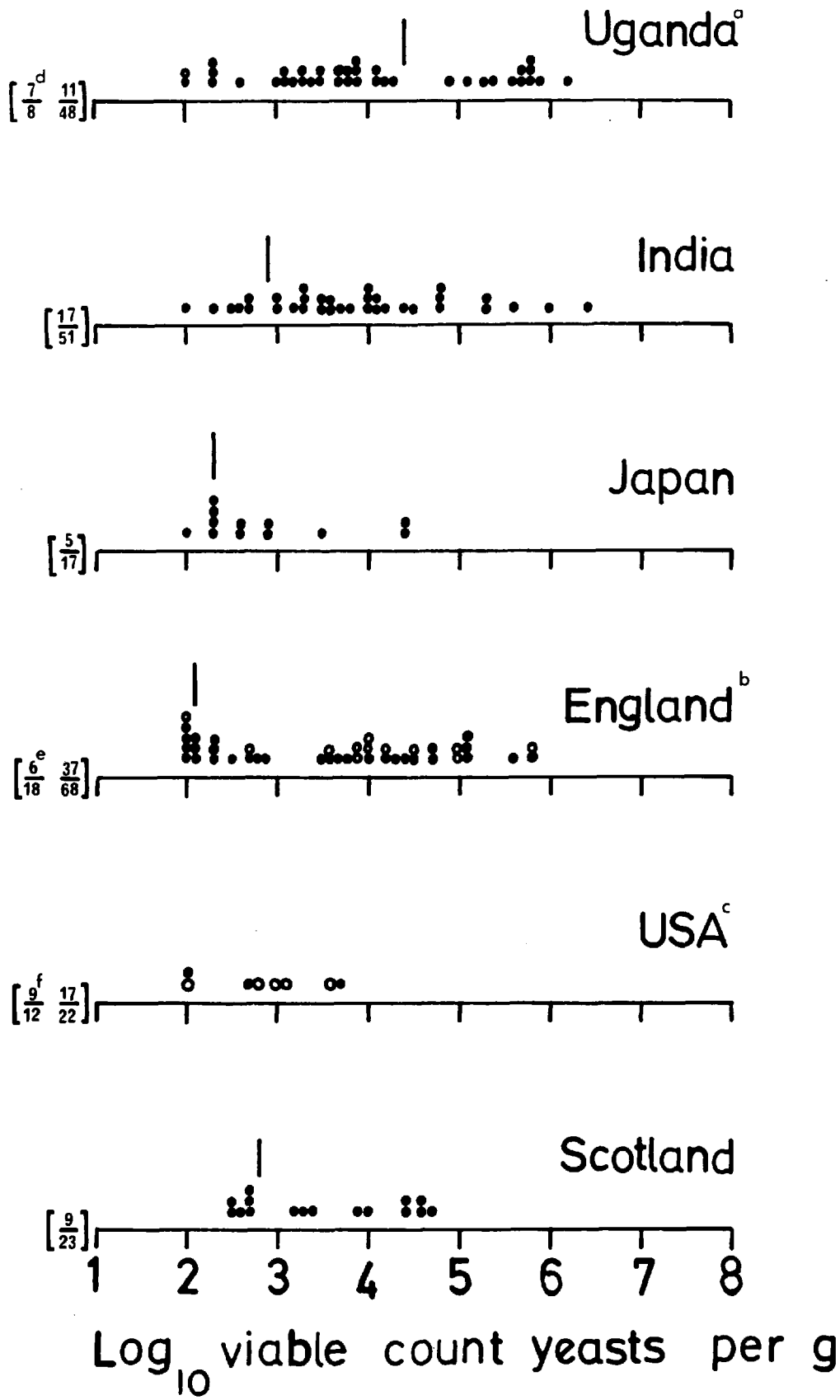
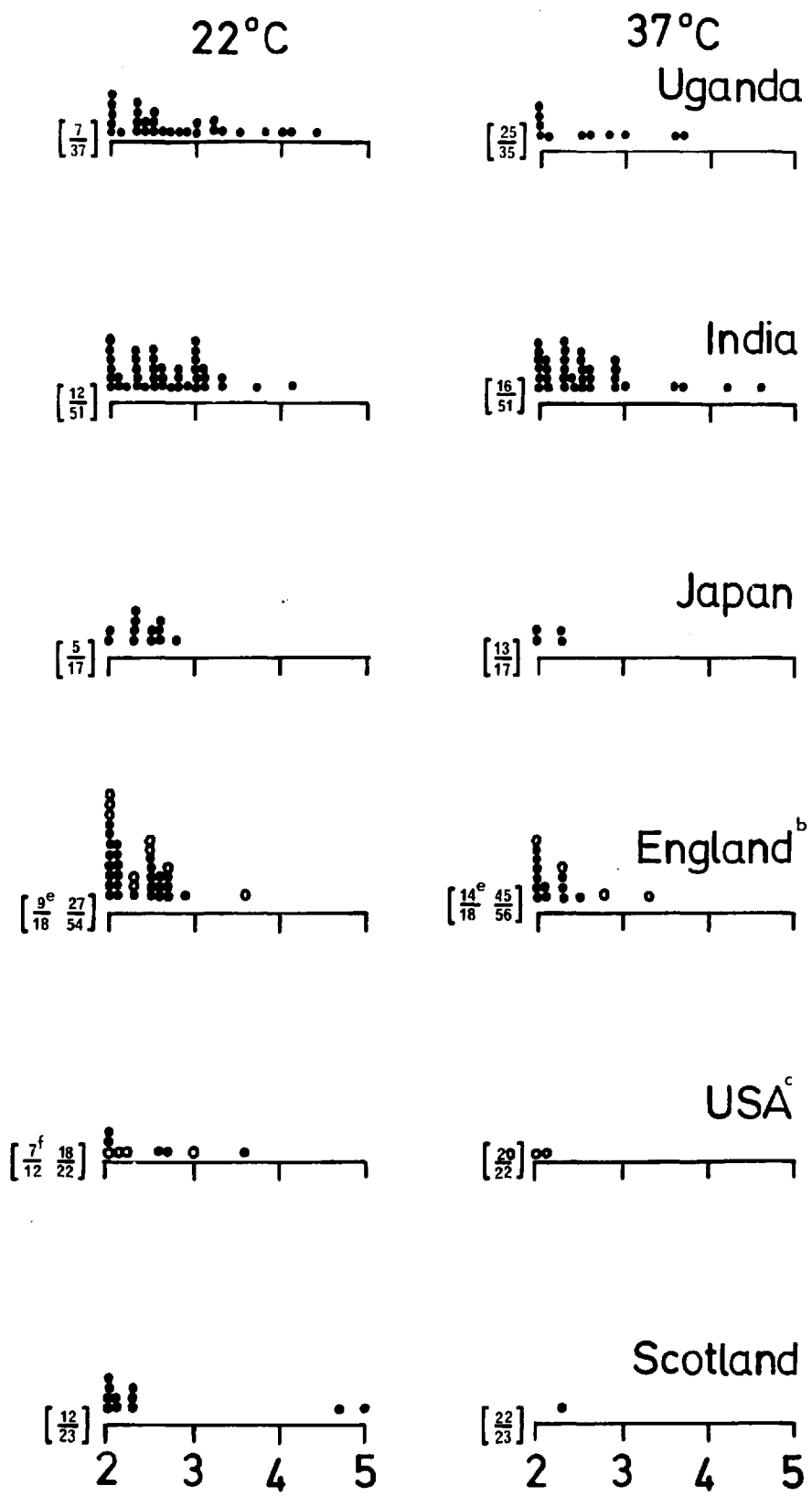

Uganda[a]
[7/8[d] 11/48]
India
[17/51]
Japan
[5/17]
England[b]
[6/18[e] 37/68]
USA[c]
[9/12[f] 17/22]
Scotland
[9/23]
1 2 3 4 5 6 7 8
Log10 viable count yeasts per g

Fig. 26. Distribution of filamentous fungi in different populations. Organisms capable of growth at 37°C are recorded separately.
Key: as Fig. 17.


22°C
37°C
Uganda
[7/37]
[25/35]
India
[12/51]
[16/51]
Japan
[5/17]
[13/17]
England[b]
[9[e]/18 27/54]
[14[e]/18 45/56]
USA[c]
[7[f]/12 18/22]
[20/22]
Scotland
[12/23]
[22/23]
2 3 4 5
2 3 4 5
Log10 viable count fungi per g

Ugandans and Indians and compared the results with his previous study of the filamentous fungi carried by English persons (Davies and Leese, 1968). Table 18 shows that the fungal flora differs qualitatively in all three populations.

Aspergillus species

Aspergillus spp. formed 60% of the isolates from Indians, 29.1% from Ugandans and only 15.6% of those from English persons. The species Aspergillus terreus and A. flavus were isolated from Indians and Ugandans but never from English persons. The species A. repens, A. chevaleri, A. clavatus, A. flavipes, A. candidus and A. ochraceus were found in only Ugandans and Aspergillus nidulans in only Indians.

Penicillium species

Penicillium spp. comprised 54% of the filamentous fungi isolated from English persons, 27.3% from Ugandans and only 10% of those from Indians. The species P. viridicatum, P. claviforme, P. diversum, P. expansum, P. granulatum, P. luteum, P. steckii, P. spinulosum and P. simplicissimum, which Davies and Leese isolated from English persons, were never found in Ugandans and Indians. On the other hand, P. purpurogenum, P. fumiculosum and P. digitatum were found in only Ugandans.

Table 18. Frequency with which filamentous fungi were isolated from faeces of persons living in different countries

Organism	Country of origin and no. of persons studied		
	England 456[a]	Uganda 30	India 14
Aspergillus niger	3	-	8
A. terreus	-	1	2
A. nidulans	-	-	3
A. flavus	-	3	1
A. sydowi	1	-	1
A. versicolor	3	1	1
A. repens-ruber series	-	4	-
A. chevalieri	-	1	-
A. clavatus	-	1	-
A. flavipes	-	1	-
A. candidus	1	1	-
A. ochraceus	-	1	-
A. repens	-	1	-
Aspergillus spp. not identified	-	-	2
Aspergillus spp. total carriage	32	16	18
Penicillium purpurogenum	-	3	-
P. cyclopium	21	2	-
P. chrysogenum	6	2	-
P. brevicompactum	5	4	-
P. fumiculosum	-	2	1
P. digitatum	-	1	-
P. roqueforti	27	1	-
Penicillium spp. not identified	28	-	2
Penicillium total carriage	111	15	3
Paecilomyces varioti	2	1	2
Epicoccum spp.	-	3	-
Helminthosporium spp.	-	-	1
Absidia corybifera	-	-	1
A. lichtheium	-	2	-
Cephalosporium spp.	6	-	1
Rhizopus stolonifer	-	1	1
Mucor racemosus	3	3	-
M. pusilus	1	5	-
M. plumbeus	1	1	-
Mucor spp. not identified	-	-	2
Cladosporium cladosporiodes	-	1	1
C. herbarum	-	1	-
Cladosporium spp. not identified	3	2	-
Curvularia lunata	-	1	-
Trichoderma viriale	-	1	-
Scopulariopsis brevicatus	-	1	-
Oidiodendron fuscum	-	1	-
Botrytis cinerea	1	2	-
Geotrichum candidum	22	1	-
Phoma spp. not identified	-	2	-
Sterile mycelium	27	8	-

- organism not isolated
a from Davies and Leese (1968)

Other genera

Geotrichum candidum which comprised 22 out of 205 isolates in Davies and Leese's study of English persons, was found only once in Ugandan and never in Indian persons. Phoma spp., Epicoccum spp., Absidia lichtheium, Cladosporium cladosporiodes, Cladosporium herbarum, Curvularia lunata, Trichoderma virale, Scopulariopsis brevicatus and Oidiodendron fuscum were found in Ugandans only. Absidia corybifera and Helminthosporium spp. were found in Indians only.

3.4.2 Persons living on special diets

Low fat diet

A healthy volunteer lived on a diet containing less than 30 g fat per day, but which was otherwise normal (see section 2.1). The faecal flora changed as shown in Fig. 27. Counts of bacteria in three consecutive specimens collected immediately before starting the diet were compared with those in the last three obtained after one month on the diet.

There were no apparent changes in the counts of bifidobacteria or *Bacteroides* spp. Counts of 'viridans' streptococci increased significantly ($P < 0.01$); those of *Streptococcus salivarius* and enterococci also increased but neither change was significant. There were no consistent changes in counts of enterobacteria, *Lactobacillus* or *Veillonella* spp.

Chemically defined, liquid diets

Three volunteers lived for 10 days on a chemically defined, liquid diet, as described in section 2.2. (Two specimens of faeces collected from each person immediately before starting the diet, were accidentally frozen before being diluted in glycerol broth. This probably caused a drop in the viable counts and must be remembered when interpreting the results.)

Fig. 27. Changes in bacteria and steroids in faeces of a person living on a low fat diet.

Key: Bacteria (black lines)

A bifidobacteria

B *Bacteroides* spp.

C 'viridans' streptococci

D *Streptococcus salivarius*

E enterobacteria

F enterococci

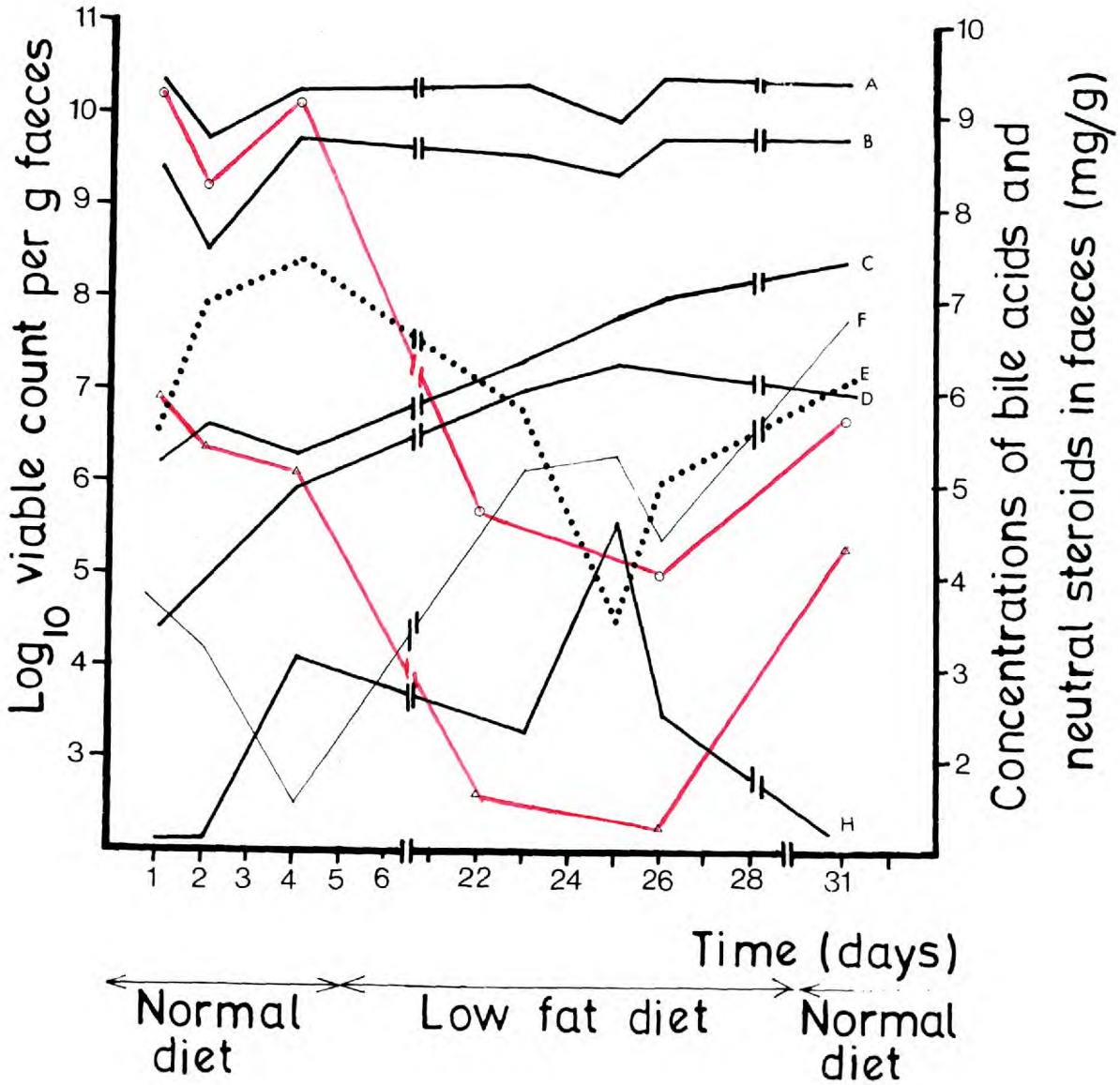
G *Lactobacillus* spp.

H *Veillonella* spp.

Steroids (red lines)

triangles, bile acids

circles, neutral steroids.


Log10 viable count per g faeces
Concentrations of bile acids and neutral steroids in faeces (mg/g)
A
B
C
D
E
F
H
1 2 3 4 5 6 22 24 26 28 31
Time (days)
Normal diet
Low fat diet
Normal diet

Counts of bifidobacteria and Bacteroides spp. in faeces from all three persons after 8-10 days on the diet were the same as those in control specimens taken when on normal diets. One person produced very fluid faeces as a result of starting the diet (a common occurrence), and this caused a drop in the total count; by the sixth day the faeces were again solid and the count returned to 10^{10} per g (Fig. 29).

In all three persons the counts of enterococci fell considerably during the period on the diet, and in one of them the counts fell below 10^2 per g, which is the lowest level detectable with these techniques; when a normal diet was resumed, the counts of enterococci returned to their former levels (Fig. 28). Similarly, Streptococcus salivarius disappeared from the faeces of one person during the liquid diet and reappeared later (Fig. 29). There were no consistent changes in counts of the other organisms.

In all three subjects the change from a mixed diet to a liquid diet was accompanied by a marked reduction in the mean weight of faeces excreted per day. The mean daily output fell from 116 to 54 g in one person; from 107 to 57 g in the second and from 278 to 85 g in the third.

Fig. 28. Changes in bacteria and steroids in a healthy person living on a chemically defined liquid diet.

Key: as Fig. 27.

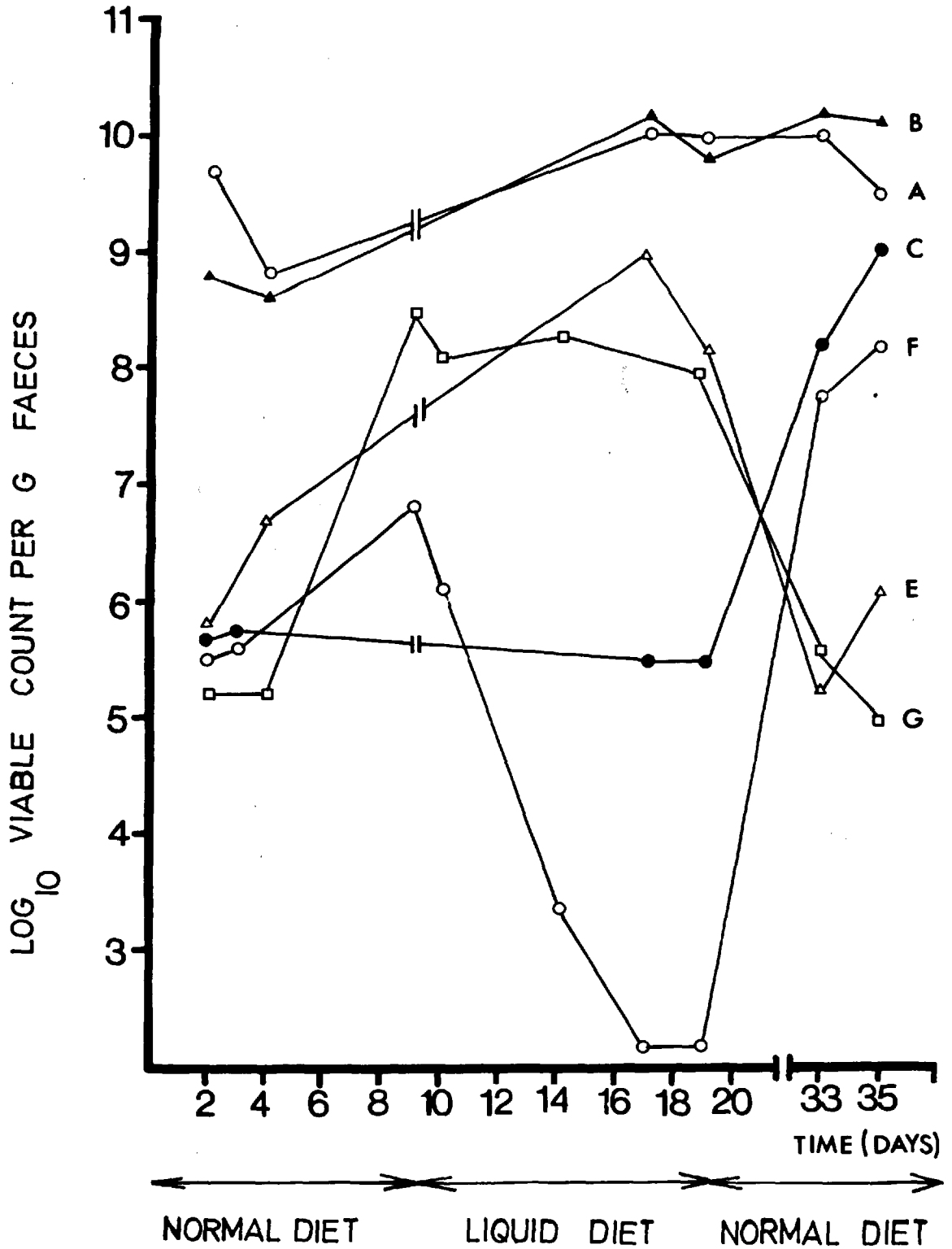
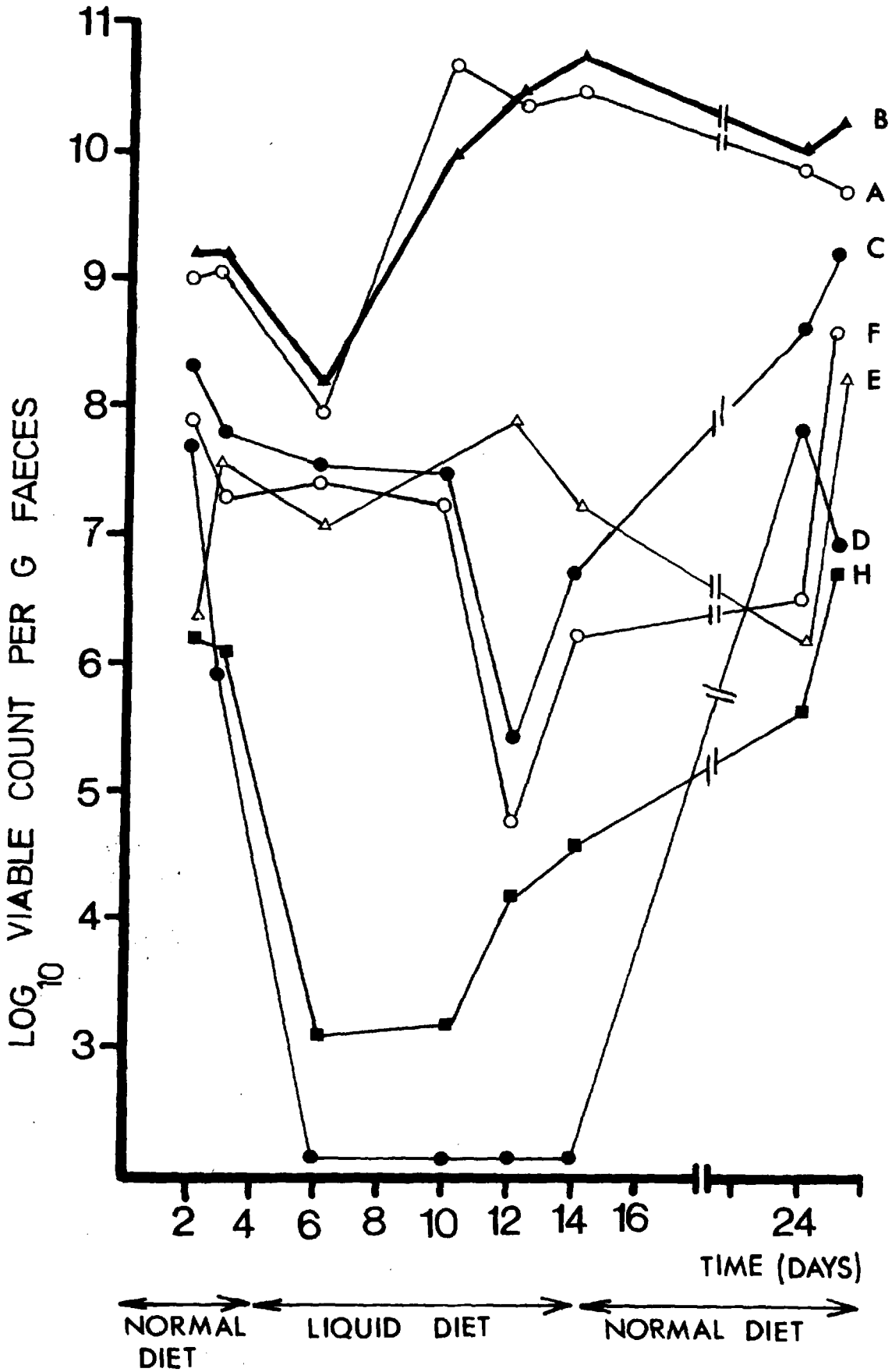

LOG10 VIABLE COUNT PER G FAECES
11
10
9
8
7
6
5
4
3
2 4 6 8 10 12 14 16 18 20 33 35
TIME (DAYS)
B
A
C
F
E
G
NORMAL DIET
LIQUID DIET
NORMAL DIET

Fig. 29. Changes in bacteria and steroids in another healthy person living on a chemically defined liquid diet.
Key: as Fig. 27.


11
10
9
8
7
6
5
4
3
LOG10 VIABLE COUNT PER G FAECES
B
A
C
F
E
D
H
2
4
6
8
10
12
14
16
24
TIME (DAYS)
NORMAL DIET
LIQUID DIET
NORMAL DIET

Starvation followed by a liquid diet

Figs. 30 and 31 show the changes in faecal bacteria when two female patients, suffering from extreme obesity, lived on a starvation diet for two weeks, followed by chemically defined, liquid diets containing increasing amounts of amino acids and carbohydrates.

In one patient a change from a ward diet to a liquid diet, led to a fall in the numbers of non-sporing anaerobes and enterococci; the numbers of enterobacteria, Clostridium and Lactobacillus species remained the same. During starvation for two weeks the counts of enterobacteria increased, whilst those of all other groups remained the same. Introduction of a liquid diet containing only amino acids and minerals led to a slight increase in the counts of all groups of organisms, except enterococci, which were not found. When increasing amounts of soluble carbohydrates were added to the diet, the total count remained steady at $10^{8.5}$ per g; enterococci were again found, but lactobacilli were not; counts of enterobacteria and clostridia fluctuated widely. It was not possible to obtain further control specimens after the patient had resumed a normal diet.

The changes observed in the second patient were similar (Fig. 31). The liquid diet led to the disappearance of the

Fig. 30. Changes in faecal bacteria in an obese patient living on liquid diets of various formulae following a period of starvation.

Key:

A total anaerobes

E enterobacteria

F enterococci

G *Lactobacillus* spp.

J *Clostridium* spp.

The broken line shows the mean weight of faeces excreted per day during the fortnight on each diet.

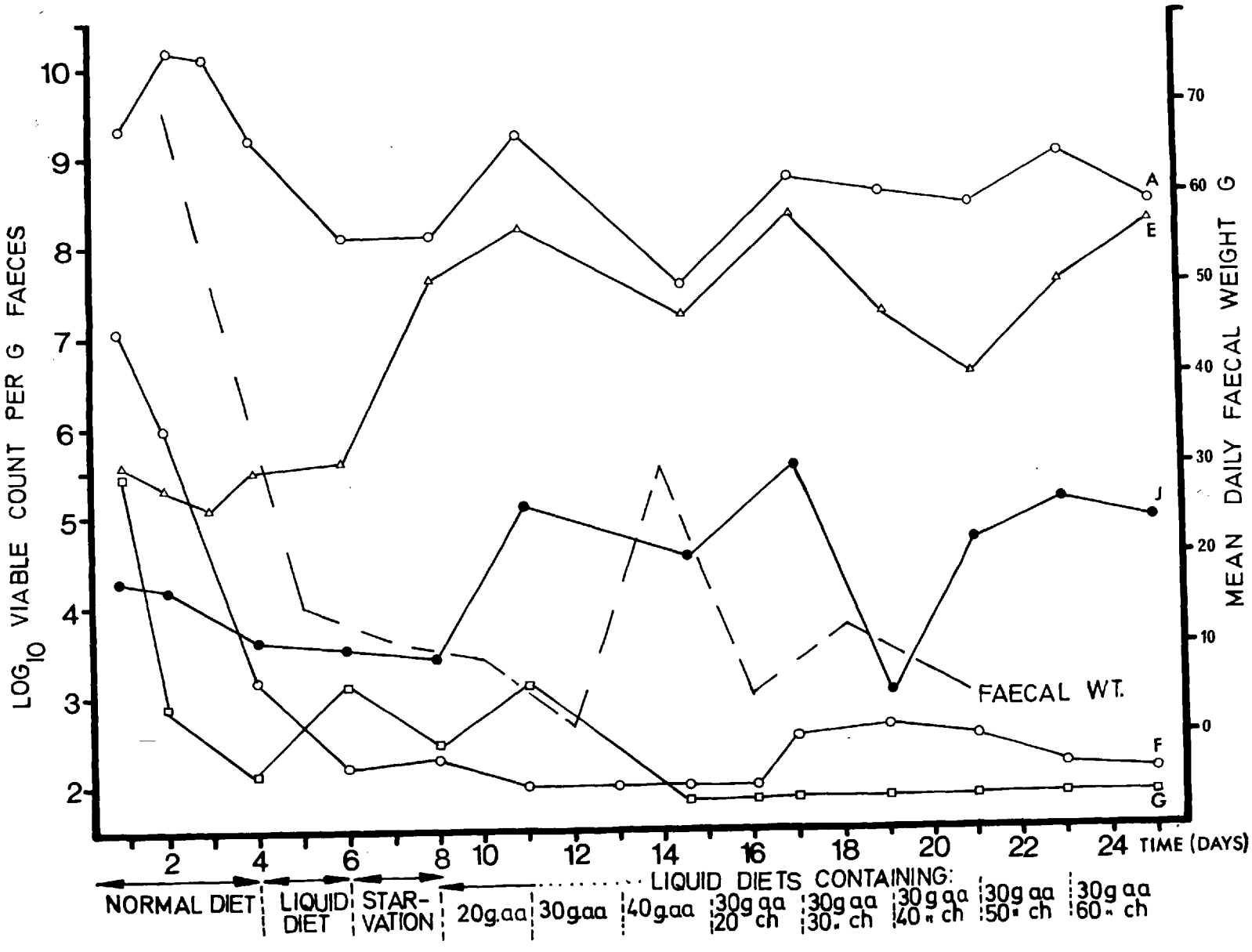
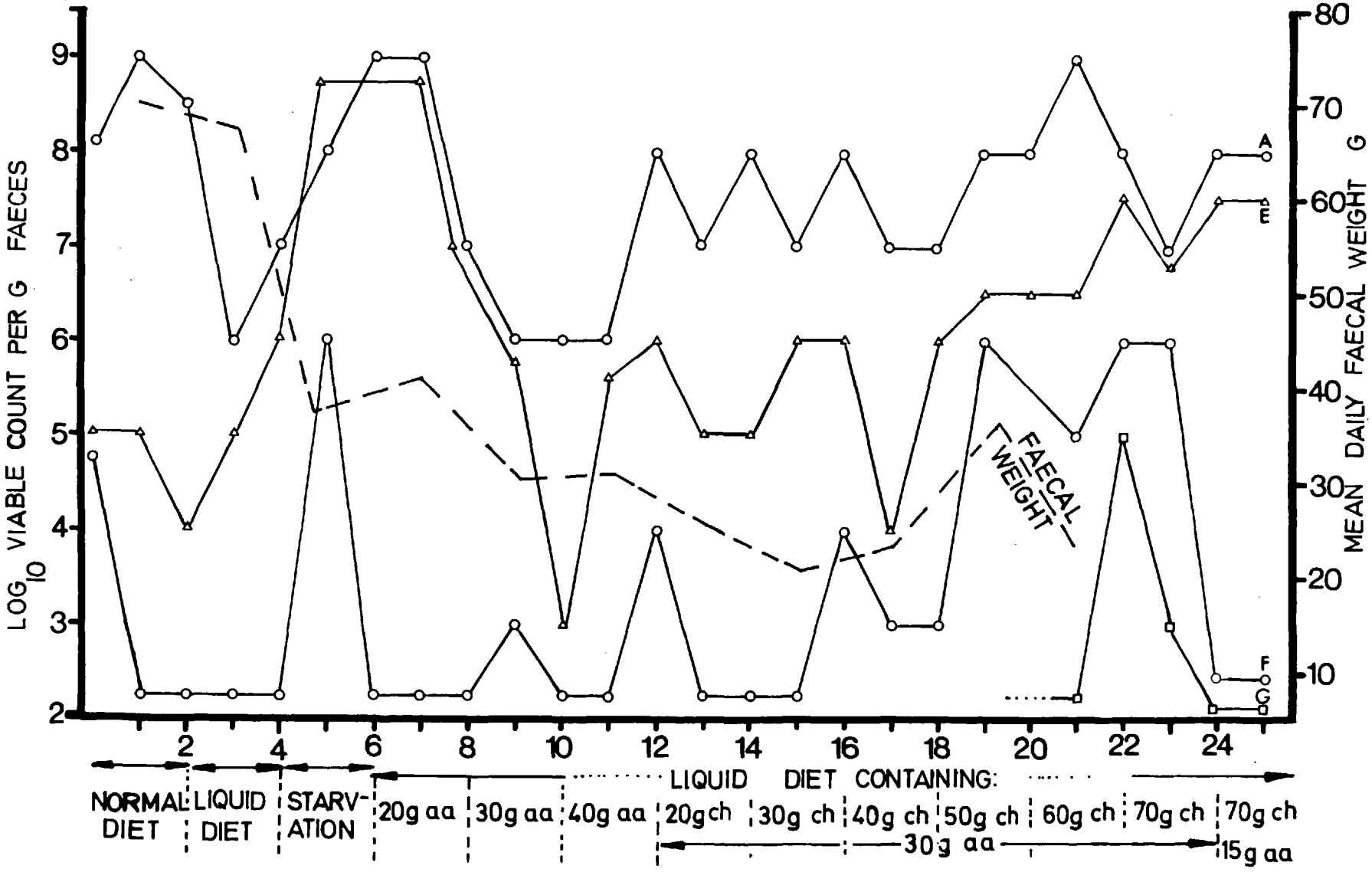

LOG10 VIABLE COUNT PER G FAECES
MEAN DAILY FAECAL WEIGHT G
TIME (DAYS)
A
E
J
F
G
FAECAL WT.
NORMAL DIET
LIQUID DIET
STAR-VATION
LIQUID DIETS CONTAINING:
20g.aa
30g.aa
40g.aa
30g aa 20 ch
30g aa 30 ch
30g aa 40 ch
30g aa 50 ch
30g aa 60 ch

Fig. 31. Changes in faecal bacteria in an obese patient living on liquid diets of various formulae following a period of starvation.

Key: as Fig. 30.


LOG10 VIABLE COUNT PER G FAECES
MEAN DAILY FAECAL WEIGHT G
FAECAL WEIGHT
A
E
F
G
NORMAL DIET
LIQUID DIET
STARV-ATION
20g aa
30g aa
40g aa
LIQUID DIET CONTAINING:
20g ch
30g ch
40g ch
50g ch
60g ch
70g ch
70g ch
30g aa
15g aa

enterococci and a slight drop in the total count. During starvation the counts of enterobacteria rose to 10^9 per g but fell again to a steady level of approximately 10^6 per g when the liquid diet was resumed. The counts of the anaerobes remained steady at $10^{7.5}$ per g; counts of clostridia, lactobacilli and enterococci fluctuated greatly and could not be correlated with the changing proportions of amino acids and carbohydrates in the diet.

In both patients a change from a ward diet to a liquid diet was accompanied by a fall in the mean weight of faeces excreted per day (Fig. 30 and 31).

3.4.3 The effect of the chemical components of faeces on the faecal microflora

(The chemical analyses in this part of the work were done by Drs. M.J. Hill and V.C. Aries and the computer analyses by Mrs. D. Irving)

Since persons living on very different diets were found to carry the same qualitative faecal microflora (except that *Sarcina ventriculi* was carried only by some persons living on vegetarian diets), it seemed reasonable to consider all persons from the six countries as belonging to one population, and to study the relationships between the faecal bacteria and faecal steroids within it. The bacteriological results used for the calculations are those given in Figures 12-26; Dr. Hill's steroid analyses of the same specimens of faeces are those which contributed to the mean values given in Table 22 and have been reported in detail elsewhere (Hill and Aries, 1971). A computer was used to calculate linear correlation coefficients between the viable counts of each of the 16 groups of organisms and the concentrations of each of the 10 chemical constituents in faeces from 267 persons. The significant correlations were identified as described in section 2.4. Only those correlations which were significant at a

probability level of 1% (or less) are discussed here (Table 19).

Bacteroides spp. and bifidobacteria

Counts of Bacteroides spp. and bifidobacteria were each correlated positively with the faecal concentrations of total bile acids, total neutral steroids and with the urobilins. Fig. 32 shows the relationship between the counts of Bacteroides spp. and the concentrations of bile acids in faeces. Bifidobacteria, but not Bacteroides spp., were correlated with cholanic acid. Bacteroides spp., but not bifidobacteria, were correlated with lithocholic and the dihydroxycholanic acids (deoxycholic and chenodeoxycholic acids).

Streptococci

Counts of enterococci and 'viridans' streptococci were each correlated negatively with the faecal concentrations of lithocholic, cholanic and dihydroxycholanic acids, cholesterol, coprostanol and urobilins. The relationships between the total concentrations of bile acids and the counts of 'viridans' streptococci and the counts of enterococci, are shown in Figures 33 and 34 respectively. Streptococcus salivarius showed no significant correlations.

Table 19. Linear correlation coefficients between counts of viable bacteria and concentrations of some chemical compounds in faeces[1]

Organism[3]	Compound[2] in faeces							
	Bile acid				Neutral steroid			
	Deoxycholic and chenodeoxycholic acids	Lithocholic acid	Cholanic acid	Total bile acids	Cholesterol	Coprostanol	Total neutral steroids	Urobilins
Bacteroides spp.	+0.34	+0.30		+0.51	+0.44	+0.37	+0.41	+0.32
Bifidobacteria			+0.31	+0.40	+0.36	+0.29	+0.35	+0.36
'Viridans' streptococci	-0.44	-0.52	-0.43	-0.37		-0.30	-0.30	-0.28
Enterococci	-0.44	-0.47	-0.48	-0.43		-0.30	-0.28	-0.29
Bacillus spp.	-0.42			-0.52	-0.41	-0.33	-0.37	-0.36
Veillonella spp.				-0.37	-0.37	-0.30	-0.33	
Sarcina ventriculi		-0.35		-0.56	-0.42	-0.26	-0.31	-0.33
Enterobacteria	-0.40	-0.36	-0.37	-0.34				
Lactobacillus spp.			-0.45		-0.33		-0.23	
Yeasts				-0.38	-0.35	-0.25	-0.29	-0.40
Moulds (growing at 37°)					-0.26			
Moulds (" " 22°)				-0.34	-0.25		-0.22	

1 Only those correlations significant at $P < 0.01$ have been included.

2 The pH, moisture content and concentrations of cholic acid in faeces showed no significant correlations with any of the organisms tested and have been omitted from the table.

3 Streptococcus salivarius, Clostridium spp. and Staphylococcus spp. showed no significant correlation with any of the compounds tested and have been omitted from the table.

Fig. 32. Relationship between counts of *Bacteroides* spp. and the total concentrations of bile acids in faeces. Counts from seven populations are included. The calculated line shows a significant regression ($r = 0.41$; $P < 0.001$). The broken lines show the 95% confidence limits.

Key:

- ● England
- ○ Scotland
- ■ U.S.A. (white and black persons)
- □ Japan
- ▲ India
- △ Uganda
- ▼ England (vegan persons)

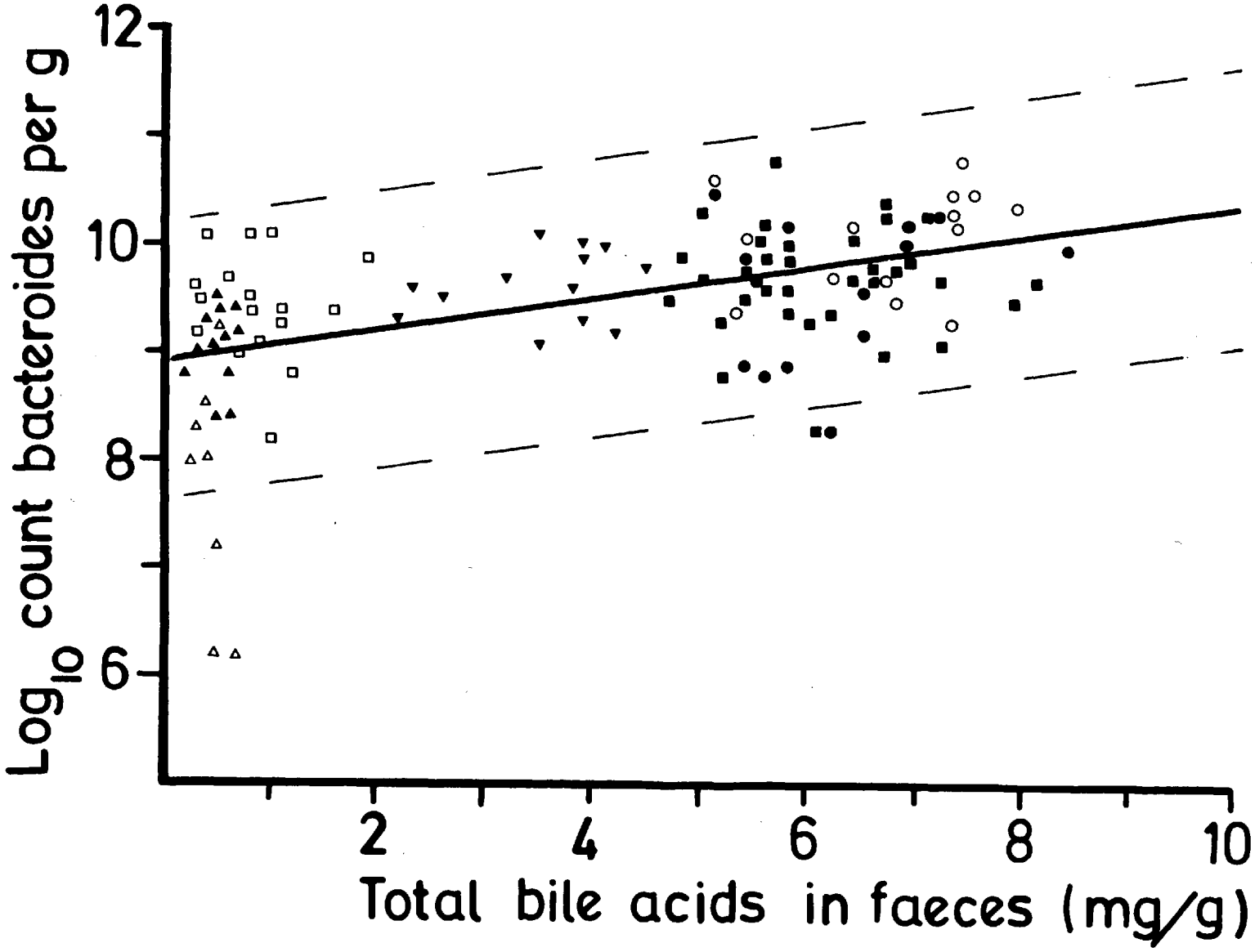

12
10
8
6
Log10 count bacteroides per g
2
4
6
8
10
Total bile acids in faeces (mg/g)

Fig. 33. Relationship between the counts of 'viridans' streptococci and the total concentrations of bile acids in faeces.
Key: as Fig. 32.

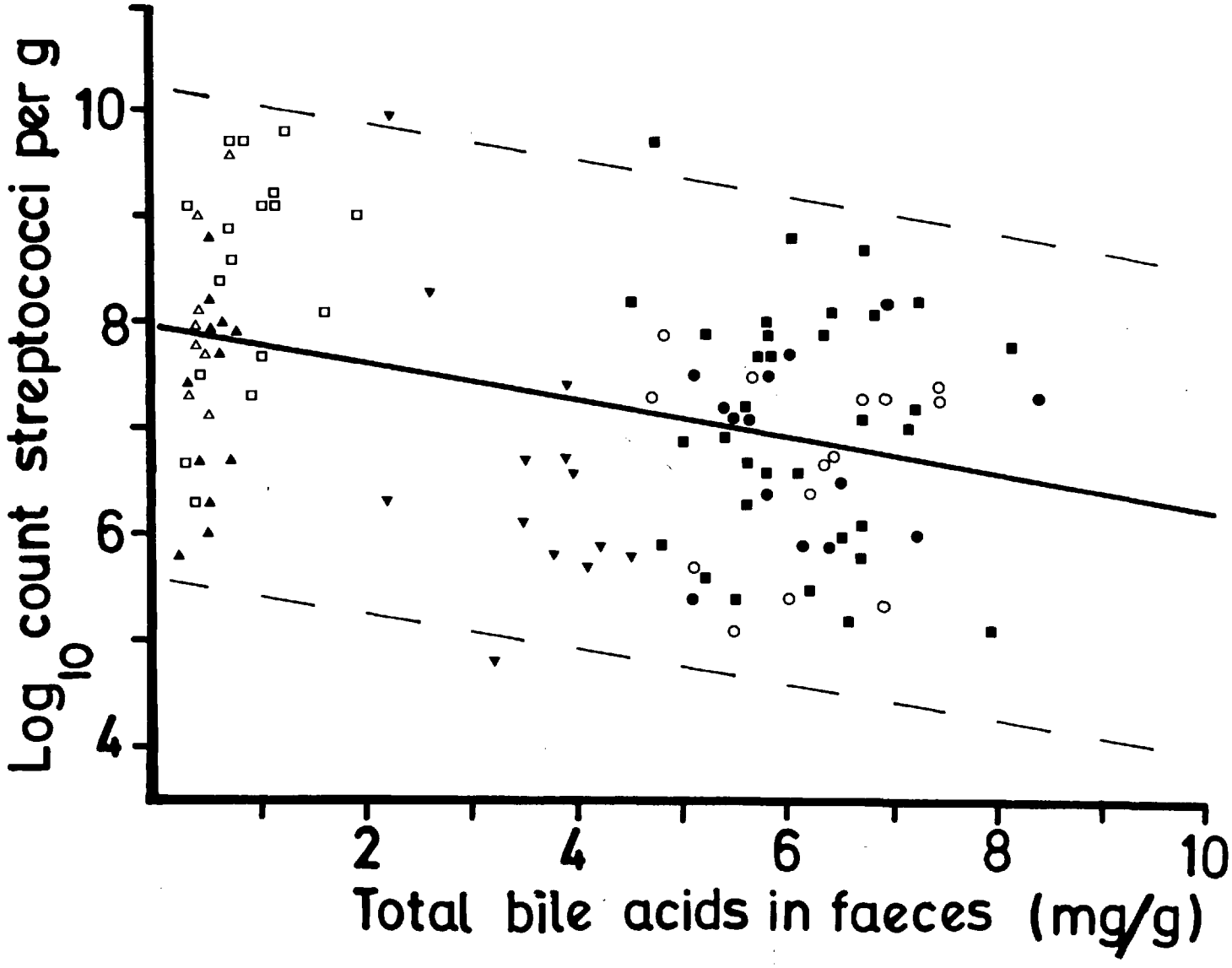
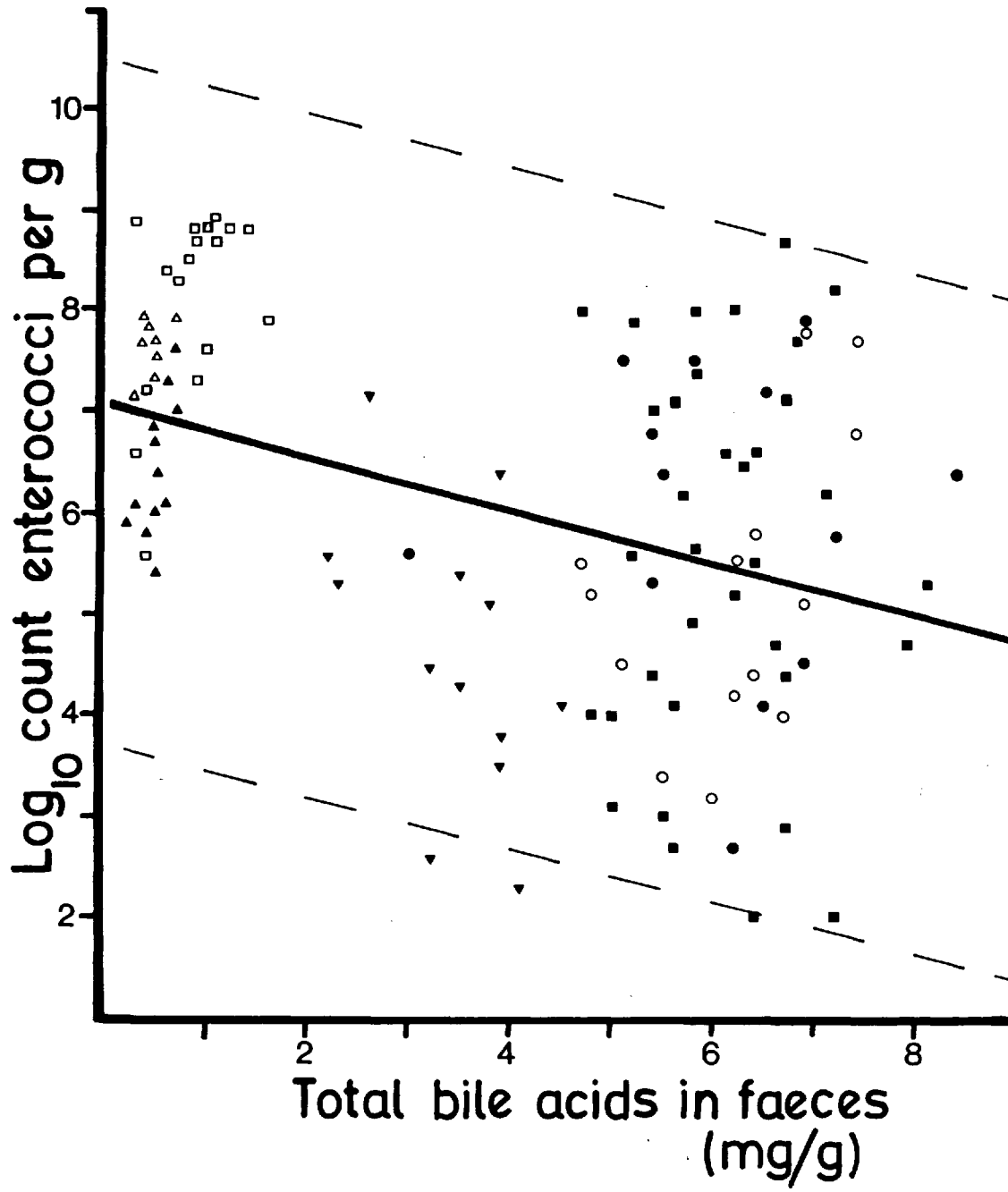

Log10 count streptococci per g
10
8
6
4
2
4
6
8
10
Total bile acids in faeces (mg/g)

Fig. 34. Relationships between the counts of enterococci and the total concentrations of bile acids in faeces.
Key: as Fig. 32.


Log10 count enterococci per g
10
8
6
4
2
2
4
6
8
Total bile acids in faeces (mg/g)

Enterobacteria

Enterobacteria were correlated negatively with each of the bile acids (lithocholic, cholanic and dihydroxycholanic acids), but not with cholesterol, coprostanol or the urobilins.

Veillonella, *Bacillus*, *Sarcina* spp. and yeasts

These organisms were all correlated negatively with the total concentration of bile acids and with cholesterol and coprostanol. Only *Bacillus* spp. was correlated (negatively) with dihydroxycholanic acids, and only *Sarcina* spp. was correlated (negatively) with lithocholic acid. *Bacillus* spp., *Sarcina* spp. and yeasts, but not *Veillonella* spp., were correlated (negatively) with the urobilins.

Lactobacillus spp.

These were correlated (negatively) with cholanic acid, cholesterol and with the total neutral steroids.

Filamentous fungi

Those filamentous fungi growing at 22°C were correlated negatively with cholesterol, with the total neutral steroids and with the total bile acids. Those fungi growing at 37°C were correlated with only cholesterol.

Clostridia and staphylococci

These showed no significant correlations with any of the components tested.

None of the 16 groups of micro-organisms showed a significant correlation with the concentration of cholic acid, or with the water content or pH of the faeces.

Discussion

The study described here appears to be the first survey in which the faecal flora of healthy persons living on very different traditional diets in different parts of the world have been examined under controlled conditions. The validity of the results depends very much on the reliability of our method for transporting specimens to the laboratory. All previous workers have studied freshly voided faeces (Floch *et al.*, 1968; Drasar *et al.*, 1969; Mata *et al.*, 1969; Levinson and Kaye, 1969; Winitz *et al.*, 1970) or faeces stored for a few hours in refrigerators at about 3°C (Zubrzycki and Spaulding, 1962; Gorbach, Nahas, Lerner and Weinstein, 1967). Clearly these methods could not be used for the present survey where there were large distances between the laboratory and the persons studied and so a preservation technique had to be devised. Extensive tests (described in Appendix B4) showed that all groups of bacteria, including the delicate non-sporing anaerobes, survived without significant losses when faeces were suspended in broth containing 10% v/v glycerol and frozen at -78°C or below. Other delicate organisms such as *Neisseria gonorrhoeae* (Ward and Watt, 1971) and *Str. pneumoniae* (Hollander and Nell, 1954; Smith, 1961) also survive without significant losses when frozen in the same way.

In a survey of this kind it is difficult to isolate the effects due to diet from those due to environmental, racial or other factors, since each factor may influence each other. It has already been shown that neither race, sex nor age may themselves greatly influence the faecal bacteria, although accumulative effects of these and other factors are possible, so that it seems likely that the greatest influences will be dietary and environmental.

The results of the study agree with the small-scale studies of Haenel, Müller-Beuthow and Scheunert (1957), Hoffmann (1964), Gorbach, Nahas, Lerner and Weinstein (1967) and Moore, Cato and Holdeman (1969) and show that even with extreme diets the predominant components are the same. However, there were differences in the numbers of several of the bacterial groups. Faeces from all populations living on traditional mixed diets containing large amounts of protein and fat, had more of the strictly anaerobic Bacteroides spp. than those from persons living on vegetable diets containing low amounts of protein and fat. Conversely, faeces from all the vegetarian populations, other than the English vegans, contained more aerobic streptococci (especially enterococci), Lactobacillus spp., Bacillus spp., yeasts and filamentous fungi, than did those from populations living on mixed diets. These results agree in part with those of Hoffmann (1964) who found

that when one person lived on a diet rich in fat, the numbers of Bacteroides spp. in the faeces increased, whilst the numbers of enterococci and bifidobacteria decreased. Torrey and Montu (1931) had also shown that persons living on all meat diets had lower counts of enterococci and other streptococci and Lactobacillus spp. than during control periods on mixed diets.

Possible reasons for the differences in counts of the aerobic components of the flora are discussed below, but the reasons for the lower counts of the total anaerobic bacteria in faeces from vegetarian populations, in particular the Ugandans, are not known. The diet of the Ugandan Bantu is mainly cooked bananas and vegetables, and contains large amounts of indigestible fibre (Burgess, Morton and Burgess, 1962). The diet of the South African Bantu is similar, except that other fruits, such as guavas, are used in place of bananas, and its fibre content varies considerably depending on the variety of fruit in season (Walker, Walker and Richardson, 1970). The mean daily output of faeces by Ugandans in 452 g per day compared with approximately 150 g for English persons (Dr. D.P. Burkitt, personal communication, 1971, and Burkitt, in press) and approximately 300 g for Indians in Vellore (Professor S.V. Baker, personal communication). Hence, the total numbers of bacteria excreted each day in the faeces of Ugandan, Indian and English persons is calculated (from Table 13) to be 9.5×10^{11},

1.7×10^{12} and 2.0×10^{12} respectively. Although these figures are very approximate (because the excretion rates and bacterial counts were not measured on the same persons), they are similar, and suggest that the lower numbers of viable bacteria in faeces of Ugandans and Indians may, in effect, be due to dilution of the intestinal contents, probably by unabsorbed components of the diet.

Identification of all the anaerobic bacteria in faeces would be a very complex procedure. Moore, Cato and Holdeman (1969) have shown that over 100 colonies have to be picked from the countable anaerobic plate of each specimen and identified, in order to define the predominant bacterial flora. During the present survey the non-sporing anaerobes were found very difficult to preserve in pure culture and so it has been impossible to identify the most numerous organisms in detail. Accordingly, many subtle differences between the different countries may have been lost.

Of the surviving Gram-negative anaerobes Bacteroides fragilis was the most frequent isolate. This could mean either that B. fragilis is indeed the most common species of Bacteroides in human faeces, as claimed (for American persons) by Smith and Holdeman (1968) and Moore et al. (1969), or that B. fragilis is more robust and survives better than other species.

Nor has it been possible to divide the obviously heterogeneous group, bifidobacteria, into its several components, Bifidobacterium, Propionibacterium and Eubacterium, and with confidence present comparable counts for each of the dietary groups. Most of the surviving strains from Japan and India were identified as Bifidobacterium spp., those from America and Scotland were identified as Propionibacterium spp. Again this could indicate either a true difference in the predominant flora of the different populations, or a difference in the ability of different isolates to survive under our culture conditions. Strains of Bifidobacterium bifidus are known to vary greatly in their ability to survive in pure culture (de Vries and Southamer, 1968). Whether Bifidobacterium spp. or Propionibacterium spp. is the predominant organism in the gut is important, since the two organisms are metabolically distinct: in pure culture the former produces acetic and lactic acids from glucose, and the latter propionic acid (Cato et al., 1970). These three acids inhibit different bacteria to different extents (Upton, 1929; Bergheim, 1940; Bergheim et al. 1941). If all species have the same metabolic pathways in the gut as they do in vitro, then which of the two genera were numerically dominant might be expected to have a considerable impact on the physiology of the gut, and in particular, on the relative numbers of the less numerous microbes.

The faecal flora of English persons living on strict vegetarian (vegan) diets containing no animal matter at all was (with the exception of Sarcina ventriculi) almost identical to that of persons living on mixed diets, and this supports the work of Haenel et al. (1957) in Germany and Moore et al. (1969) in the U.S.A. At first these observations appear to be at variance with the studies on vegetarian persons in Uganda, India and Japan. However, the vegan diet contains the same amounts of protein and carbohydrate and more than half the amount of fat consumed by people on a mixed diet (Ellis and Mumford, 1967). On the other hand, the vegetarian persons we studied in Uganda and India consumed diets which contained very low amounts of protein and fat and high amounts of carbohydrate (Burgess, 1962; Burgess, Morton and Burgess, 1962); such diets produce kwashiorkorr, which is very common amongst children in Kampala.

Thus, it seems possible that the differences in the faecal flora of persons in Uganda and Asia on the one hand, and America and Britain (including those living on strict vegan diets) on the other, are not due to the vegetarian nature of the diets as such, but may be due to differences in the relative proportions of protein, carbohydrate and fat, regardless of their animal or vegetable origins. The general cleanliness of the environment may also be important and will be discussed later.

A diet low in fat, but otherwise typically 'Western' led to an increase in the numbers of streptococci and a fall in the concentrations of bile acids and neutral steroids in the faeces, whilst the numbers of other organisms, including Bacteroides spp., remained the same (Fig. 27). These observations suggest that changes in the concentration of steroids (or other associated factors) in the faeces, brought about by corresponding changes in the amounts of fat in the diet (Hill, 1971b), might be one factor controlling the numbers of streptococci, but not the numbers of bacteroides, in the faeces.

To test this hypothesis the relationships between the different faecal organisms and the faecal concentrations of degraded and undegraded bile acids and neutral steroids have been assessed. The total bile acids and the total neutral steroids were each correlated positively with the counts of Bacteroides spp. and bifidobacteria and negatively with the streptococci, Bacillus spp., Veillonella spp. and yeasts. Further, Lactobacillus spp. were correlated negatively with cholanic acid but not with deoxycholic or lithocholic acids; Bacillus spp. were correlated negatively with deoxycholic acid but not with lithocholic and cholanic acid, and Sarcina ventriculi was correlated with lithocholic acid but not with deoxycholic or cholanic acids.

Now, a positive correlation between the concentration of a degraded steroid and the counts of a chosen organism could mean that the degraded steroid was produced by the organism, or conversely, that the steroid stimulated growth of the organism, or, that both organism and steroid were correlated merely because of their common association with other factors, which remain unidentified. On the other hand, a negative correlation could mean that the organism was inhibited by the steroid, or that both were again associated with unknown parameters.

The bile acids have long been known to inhibit growth of some aerobic bacteria, especially 'viridans' streptococci, bacilli and lactobacilli (Stacey and Webb, 1947; Floch et al., 1970) and to stimulate growth of some species of Bacteroides (Beerens et al., 1963; Barnes and Goldberg, 1968; Shimada et al., 1970) in vitro, and it seems likely that similar growth patterns could also occur in vivo. In short-term experiments in which healthy English volunteers changed from normal diets to diets low in fat (whether all liquid diets or low fat mixed diets), the numbers of Bacteroides spp. remained unchanged even though the concentrations of acid and neutral steroids fell sharply. This suggests that high levels of bile acids are not essential for the maintenance of a typically 'Western' anaerobic flora.

Therefore, it seems reasonable to suggest that the high

concentrations of bile steroids in the intestines of Western persons, brought about by high levels of dietary fat, may suppress growth of aerobic bacteria - the different bile acids selectively inhibiting different organisms. Conversely, the low concentrations of relatively undegraded bile acids in the intestines of Ugandans and Asians, brought about by low intakes of dietary fat, would allow a more luxurient aerobic flora to develop. It has been shown that when deoxycholic acid is added to mixed populations of faecal bacteria growing in continuous-flow culture, the numbers of streptococci and staphylococci decrease, whilst the numbers of bacteroides and bifibacteria remain unchanged (Collard and Gosling, 1969).

In general the same groups of organisms were correlated with neutral steroids (mainly cholesterol and coprostanol) and urobilins as with bile acids. Diets rich in fat (which stimulate secretion of bile acids) also contain high levels of cholesterol, and so lead to high levels in the gut (Hill, 1971b). The influence of cholesterol and its degradation product coprostanol, on the growth of organisms <u>in vivo</u> or <u>in vitro</u> are unknown. However, the negative correlation between coprostanol and the streptococci, and in particular the enterococci, suggests that coprostanol (or again, other compounds, such as urobilins, associated with it but which we have not sought in this study) could inhibit these organisms.

However, not all the evidence is compatible with the thesis that the numbers of some groups of bacteria are related to the concentrations of bile acids in the faeces. English persons living on vegan diets contained on average only 3.50 mg/g faeces bile acids per gram of faeces compared with 6.13 mg/g in persons living on mixed diets (Aries et al., 1971) and so might have been expected to contain more enterobacteria, enterococci, 'viridans' streptococci, Bacillus spp. yeasts and filamentous fungi than persons living on mixed diets. In fact, the numbers of these bacteria carried by the two groups were not significantly different. The reasons why the vegan flora is the same as that of persons living on mixed diets and yet shows some differences between persons living on vegetarian diets in Uganda and Asia are not clear, but it is possible that environmental factors may be involved.

In the present survey it was not possible to study systematically how geography, climate, season or the general hygienic conditions of man's daily life effect his intestinal flora, but some points are striking.

We were able to compare the bacteriology of faeces from eight healthy English immigrants living and working in Kampala, with that of the native Ugandans and with that of English persons living in London. The Ugandans were found to carry

fewer Bacteroides spp. but more streptococci, lactobacilli, veillonellae, bacilli and fungi than English persons in London or in Kampala; only the Ugandans were found to contain Sarcina ventriculi. These results show that the differences in the flora between Ugandan and English persons cannot be explained by climate or geography alone and suggest that other factors, such as diet or hygiene, may also be important. Diet may explain why Ugandans and English vegans carried Sarcina ventriculi, but it may not be the only explanation why Ugandans (or Indians or Japanese) carried more facultative organisms than did vegans or persons living on Western diets.

The persons in India, Japan and Uganda (except the immigrants) lived in poorly developed communities with inadequate hygiene in comparison with those in Britain and America. This might be expected to increase the numbers of bacteria from the environment likely to colonise the gut and so lead to a flora which is typical of the community. Even in Western countries with relatively high standards of living, "community specific" types of flora have been claimed. Bendig, Haenel and Braun (1968) compared the faecal flora of children in 13 different classes in one school and found evidence for "class-specific" types of flora: the pupils in one class all had high counts of the Klebsiella-Enterobacter group of organisms compared with those in other classes; pupils in a

second class had an anaerobic flora in which Bacteroides hypermegas was the predominant organism, whilst in all other classes the sphaerophorus group was dominant. It is known that in healthy persons (Sears, Brownlee and Uchiyama, 1950; Branche et al., 1963) and in hospital patients (Cooke, Ewins and Shooter, 1969) the dominant serotypes of E. coli in the faeces change from time to time. The new invading strains may be ingested as contaminants on food (Cooke et al., 1970). Patients may also acquire strains of Ps. aeruginosa from the hospital food and environment and excrete them in the faeces (Shooter et al., 1966, 1969).

Thus it is possible that the different distributions of bacteria in persons living in different countries may be influenced by environmental and hygienic factors in addition to any brought about by diet. The frequent carriage of Klebsiella spp. by Japanese but not by other persons may be analagous to the "community specific" Klebsiella-flora found amongst German school-children by Bendig, Haenel and Braun (1968). Similarly, Streptococcus faecalis was found more frequently than Str. faecium in faeces from Americans and Britons, whilst the reverse was shown in those from Indians. It is known that Streptococcus faecalis is more common than other enterococci in faeces from persons in Britain (Cooper and Ramadan, 1955) and the U.S.A. (Bartley and Slanetz, 1960), whilst Str. faecium

is more common in persons from continental Europe (Deibel, 1964). Different species of enterococci are found in faeces from different wild and domestic animals (Mundt, 1963; Cooper and Ramadan, 1955), on some plants (Mundt, Coggin and Johnson, 1962) and in the soil (Deibel, 1964). Hence, it seems possible that the relative frequencies of Str. faecalis and Str. faecium in faeces from persons in Uganda, India, Japan, Britain and the U.S.A., may reflect their different frequencies in the respective environments.

Environment might be expected to change the nature of those transient components of the flora such as spores of bacilli, clostridia and fungi, which are inevitably ingested as contaminants on food and may, or may not colonise the gut. In England persons excrete more filamentous fungi in the faeces in summer than in winter and this may be attributed to the more widespread use of uncooked salads and fruits in summer (Davies and Leese, 1968). In the present study different species of fungi were found in Ugandans and in Indians and these were different again from those found in English persons by Davies and Leese. It therefore seems reasonable to attribute the different distributions of the fungi in faeces from Ugandan, Indian and English persons, to the different distributions of fungi in the respective environments.

The reasons for the general overall similarity in the faecal flora of persons living on very different diets may be attributed to highly efficient absorption of nutrients from the small intestine, which to some extent buffers the bacterial flora of the large intestine from dietary influence. Only if some component of the diet saturates this absorptive capacity, or like the synthetic carbohydrate lactulose, cannot be absorbed at all, will diet directly influence the flora. In healthy man, the principal nutrients remaining for the microbial flora of the large gut, will be intestinal secretions and insoluble dietary residues, such as vegetable fibres. Cellulose is the main substrate for the anaerobic bacteria in the bovine rumen (Hungate, 1950) and in the horse caecum (Davies, 1971) and can be degraded by strains of Aerobacter aerogenes isolated from the soil (Clarke and Tracey, 1956), but I have seen no reports that it can be utilised by human intestinal bacteria. When radioactively labelled cellulose is fed to volunteers, a small quantity of labelled carbon dioxide is exhaled, showing that the cellulose has been degraded (Dr. G. Milton-Thompson, 1970, personal communication). Thus, it appears likely that human intestinal bacteria can degrade cellulose in vivo, but the energy provided may be only a small contribution to the total energy requirement.

If, in healthy man, only small amounts of unabsorbed nutrients escape into the caecum, other nutrients must be available to support the luxurient microbial population. Our study showed that if the human body was deprived food (except water, containing essential minerals and vitamins) for two weeks and then given a liquid diet containing only 20 g soluble amino acids per day (which is claimed to be absorbed completely), faeces were still produced in which the bacterial counts never fell below 10^7 viable organisms per g, although the daily output of faeces was low. Adding up to 70 g per day of soluble carbohydrate to the diet increased neither the bacterial counts, nor the faecal mass. This suggests that there exists a truly indigenous bacterial population which thrives on endogenous nutrients. The origins of these nutrients are not known; saliva, mucus and epithelial cells of the gut mucosa which are shed into the gut lumen, are likely to be major sources. However, when an all liquid diet was fed ad libitum to healthy volunteers, the numbers of bacteria in the faeces remained at the normal level of approximately 10^{10} per g and the faecal mass increased from a starvation level to approximately half the normal level. It is possible that an ad libitum diet might exceed the absorptive capacity of the small gut. Nutrients passing into the caecum would supplement endogenous nutrients and so enable the numbers of bacteria

to increase above indigenous levels.

Other workers have also found evidence for microbial populations in the human gut thriving on endogenous substrates. Winitz et al. (1970) found that healthy persons living on chemically defined liquid diets produced approximately 50 g faeces per day containing up to 10^{10} viable bacteria per gram. These authors claimed that the diet was completely absorbed and that the faecal bacteria represented an intestinal flora which survived on endogenous substrates. This appears to be analagous to the situation in human babies and suckling young animals, where the milk diets may also be almost completely absorbed, and the developing intestinal flora (as described by Smith and Crabb, 1961) thrives on endogenous nutrients. Rubinstein (1968) found that the concentrations of volatile fatty acids in faeces of patients living on starvation diets containing only methylcellulose, were about 25% of the levels for healthy adults, and Rubinstein concluded that these acids were derived either from cellulolytic bacteria or from bacterial metabolism of endogenous substrates.

Thus, it is possible to visualise the intestinal flora as a basal population living on endogenous substrates and which, in the limit when absorption is complete, is independent of the nature of the diet. When excess nutrients reach the bowel there will be an increase in the total numbers of bacteria and

the total amount of faeces. The relative proportions of the different microbes may also change depending on the nature of the nutrient. A principally vegetarian diet may increase the numbers of the facultative anaerobes, but whether bile acids or other factors are responsible for this, is not yet proven. Superimposed on this indigenous flora is a transient flora of oral organisms and contaminants from the external environment, which under favourable conditions may be able to colonise the gut.

Particular attention was paid to Sarcina ventriculi, a strictly anaerobic coccus which previously has never been found in the faeces of healthy adults. Our survey shows that Sarcina ventriculi occurs frequently in the faeces of healthy human adults living in the tropics but only rarely in faeces of people in temperate countries (Table 17). However, in Kampala none of the English immigrants, who ate diets containing animal foods, carried sarcinae, whereas they were carried by many of the Ugandans, whose principal diet was boiled bananas; furthermore, in London, sarcinae were found only in those persons eating strictly vegetarian diets. This suggests, therefore, that diet and not environment is a major factor controlling the distribution of S. ventriculi in man.

Sarcina ventriculi is widespread in the soil (Smit, 1933)

and it seems inevitable that it is ingested as a contaminant on food. Since in the Ugandans and Indians it occurs in faeces in numbers equal to those reported for coliforms, it seems likely that it multiplies, presumably in the large intestine, since the stomach and small intestine contain only a very sparse bacterial flora (Drasar, Shiner and McCleod, 1969; Gorbach, Plaut, Nahas and Weinstein, 1967). The precise factors which control its growth are not clear. Lithocholic acid may be especially important, since the faecal concentrations were negatively correlated with the numbers of sarcinae. The nature and concentrations of the volatile fatty acids produced by the intestinal bacteria may also be important; acetic and lactic acids are known to inhibit Sarcina ventriculi in vitro (Smit, 1933).

It is interesting to note that Dr. Prema Bhat (personal communication, 1971) working in southern India with the bacteriological techniques developed during the present survey, has confirmed that sarcinae occur frequently in counts up to 10^7 per g in faeces from healthy Indians and Indians with gastro-intestinal disorders.

I have proposed (Crowther, 1971a) that Sarcina ventriculi should now be considered as part of the normal intestinal flora of man. However, its significance in the intestine is not yet known.

Our survey suggests that the nature and concentrations of the intestinal steroids, brought about by the dietary fat, may be important factors controlling the intestinal microflora. A search for other factors failed - probably because it was not possible to measure more compounds in the faeces. It might have been rewarding, for example, to measure the concentrations of long-chain unsaturated fatty acids which are present in faeces (Gompertz and Sammons, 1963; Rubinstein, 1968) and in bile (Kozlowski, 1925; Haslewood, 1967) and are known to inhibit bacteria on the skin and *in vitro* (Ricketts, Squire and Topley, 1951). In our study the urobilin concentration, moisture content and pH of the faeces were not found to be important. Gorbach, Nahas, Weinstein, Levitan and Patterson (1967) could find no relationships between the number of viable organisms and the water content of ileostomy fluid, even though both varied considerably. The pH is important in determining the numbers of organisms in the stomach (Gorbach, Plaut, Nahas and Weinstein, 1967; Drasar *et al.*, 1969) but it does not appear to be so in the faeces. Neither Haenel, Müller-Beuthow and Scheunert (1957) nor Hoffmann (1964) could find relationships between the pH and the numbers of faecal bacteria when healthy persons changed from normal to extreme diets, and Gyllenberg and Roine (1957) found that counts of bifidobacteria, enterobacteria

and enterococci were independent of the pH in faeces from breast-fed or bottle-fed babies.

Reasons for failure to demonstrate relationships between the bacteria and the intestinal acidity may be twofold: firstly, had it been possible in this study, or in those of Haenel et al. and Hoffmann, to examine the numerically dominant non-sporing anaerobes at the species level, rather than by very broad groups, then some differences might have emerged. Secondly, analysis of the short-chain fatty acids, which contribute to the acidity of the faeces, might also have been rewarding.

3.5 MICROBIAL INTERACTIONS IN THE GUT - CORRELATIONS BETWEEN THE VIABLE COUNTS OF DIFFERENT ORGANISMS

The computer analysis also provided linear correlation coefficients between the viable counts of every possible pair of the 16 groups of micro-organisms. The significant correlations are shown in Table 20. Bacteroides spp. were correlated positively with bifidobacteria and negatively with 'viridans' streptococci, enterococci, Bacillus spp., Sarcina ventriculi and filamentous fungi. Lecithinase-positive and lecithinase-negative clostridia were correlated with each other but with no other organisms. Staphylococci were correlated with only Sarcina ventriculi. Yeasts, filamentous fungi, Bacillus spp., Veillonella spp. and Sarcina ventriculi were all positively correlated with each other. The only significant negative correlation was between Streptococcus salivarius and enterobacteria.

Since counts of two organisms may have been correlated because of their common association with the intestinal steroids (or some related factor), the calculations were repeated with only the seven most numerous groups of organisms and keeping the concentrations of neutral and acid steroids constant as described in section 2.4. Bifidobacteria were then correlated with Bacteroides spp. and 'viridans' streptococci, but not with

Table 20. Matrix of linear correlation coefficient between counts of viable organisms in faeces[1]

Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *Bacteroides* spp.		+0.56		-0.17	-0.21				-0.24		-0.28				-0.22	-0.46
2 Bifidobacteria									-0.21							-0.26
3 Enterobacteria				+0.35	+0.38	-0.20										
4 'Viridans' streps					+0.70		+0.36		+0.19	+0.19				+0.29	+0.17	+0.29
5 Enterococci							+0.33		+0.18	+0.26	+0.32			+0.23	+0.29	+0.32
6 *Str. salivarius*							+0.19			+0.23				+0.43		+0.23
7 *Lactobacillus* spp.														+0.28		
8 Staphylococci																+0.21
9 Yeasts														+0.16	+0.25	+0.20
10 Filamentous fungi (37°)											+0.49			+0.27	+0.34	+0.21
11 " " (32°)														+0.19	+0.24	+0.24
12 *Clostridium* spp.[2]													+0.24		+0.23	
13 *Clostridium* spp.[3]																
14 *Veillonella* spp.															+0.35	+0.24
15 *Bacillus* spp.																+0.22
16 *Sarcina ventriculi*																

1. Only those correlations significant at $P < 0.01$ have been included.

2. Lecithinase-positive.

3. Lecithinase-negative.

any of the other organisms. Lactobacillus spp., 'viridans' streptococci and enterococci were all positively correlated with one another. Neither enterobacteria nor Veillonella spp. showed a significant correlation with any other organism (Table 21).

Table 21. Matrix of partial correlation coefficients[1] between the most numerous organisms in faeces, after eliminating the effects due to bile acids and neutral steroids

Organism	1	2	3	4	5	6	7
1. *Bacteroides* spp.	·	+0.33					
2. Bifidobacteria		·		+0.36			
3. Enterobacteria			·	+0.33	+0.38	+0.23	
4. *Viridans* streps				·	+0.68	+0.26	
5. Enterococci					·		
6. *Lactobacillus* spp.						·	
7. *Veillonella* spp.							·

1 Only those correlations significant at $P < 0.01$ have been included.

Discussion

Many workers have attempted to show interference or co-operation between two or more species of intestinal bacteria on agar plates (Rosebury, Gale and Taylor, 1954; Sabine, 1963), in fluid media (Upton, 1929; Freter, 1962b; Collard and Gosling, 1969; Hentges and Maier, 1970) or in specifically contaminated gnotobiotic animals (Miller, 1959; Tanami, 1966; Wagner and Starr, 1969). The work presented here attempts to show relationships *in vivo*.

Counts of the two most numerous organisms in faeces, *Bacteroides* spp. and bifidobacteria were correlated with each other and it is suggested that the same, or related factors, control them both. These factors are not known. Low counts of both organisms could be attributed to dilution of the faeces by dietary residues, or they could be influenced by some of the many compounds present in faeces which were not studied in this survey.

Counts of *Lactobacillus* spp., 'viridans' streptococci and enterococci were correlated with each other and it seems likely that the same factors control them all. Krieg and Pelzcar (1961) have shown that *Streptococcus faecalis* and *Lactobacillus plantarum* grow together, but not alone, in a medium lacking tryptophane and folic acid; the lactobacillus synthesises folic acid, which is required by the streptococcus, whilst the

latter synthesis phenylalanine required by the lactobacillus. It seems possible that a similar mechanism, albeit much more complex, may exist amongst the streptococci and lactobacilli in gut. It also seems possible that bile acids could be controlling factors. The negative correlations between the bile acids and the lactobacilli or the 'viridans' streptococci seem reasonable since both organisms are inhibited by bile acids in vitro (Stacey and Webb, 1947; Floch et al., 1970). The negative correlation found between bile acids and enterococci (which have long been known to be bile tolerant) can be explained if the enterococci are dependent on growth factors supplied by bile-sensitive organisms. However, the negative correlation between the counts of enterococci and the faecal concentrations of coprostanol suggests that the latter may also be a factor controlling the enterococci.

Filamentous fungi which develop at 22° but not at 37°C are presumably unable to colonise the bowel, and may be regarded as transient components of the flora. It is therefore interesting that these organisms were correlated with those filamentous fungi growing at 37°C and with two genera of spore-formers, Bacillus and Sarcina spp., and it seems probable that these groups are also transient components and reach the gut as contaminants on food. Under favourable conditions some may colonise the bowel - such as the possible colonisation of

vegetarian persons by *Sarcina ventriculi*. Correlated with these 'transient' organisms were yeasts and *Veillonella* spp., suggesting that these are transient too. Both groups are commensals in the mouth and are carried into the intestine with food (Drasar *et al.*, 1969). Both were correlated negatively with coprostanol and with the bile acids, suggesting that these (or factors related to them but not evaluated in this study) may be involved.

3.6 FAECAL BACTERIA AND THE INCIDENCE OF LARGE BOWEL CANCER

The populations in the six countries were chosen because they are reported to have very different incidence rates of cancer of the large bowel. The bacteriological results presented in section 3 were therefore related to the published incidences of the cancer. The persons living on strict vegan diets in London and the English immigrants in Uganda were excluded from the study because their cancer incidences are not known. The mean count of each group of faecal microorganisms found in each population was plotted against the respective cancer incidence. Linear regression lines and coefficients of linear correlation were calculated by computer.

All the results could be expressed as linear relationships, except those for Bacteroides spp. and Sarcina ventriculi, which appeared to be hyperbolic (Figs. 35 and 36). Bacteroides spp. were present in significantly higher counts in faeces from Britain and America, than in those from Uganda, India or Japan. Conversely, Sarcina ventriculi was found only once in faeces from Britain and America but frequently in those from Uganda and India. Counts of bifidobacteria increased significantly with increasing cancer incidence (correlation coefficient $r = 0.89$; $P < 0.01$), but counts of all other organisms

Fig. 35. Relationships between the counts of faecal bacteria and the incidences of cancer of the large bowel.
In this Figure and in the next, the counts of bacteria are taken from Table 13 and the cancer incidences from Table 5.
Key: black spots, *Bacteroides* spp.
circles, *Sarcina ventriculi*.

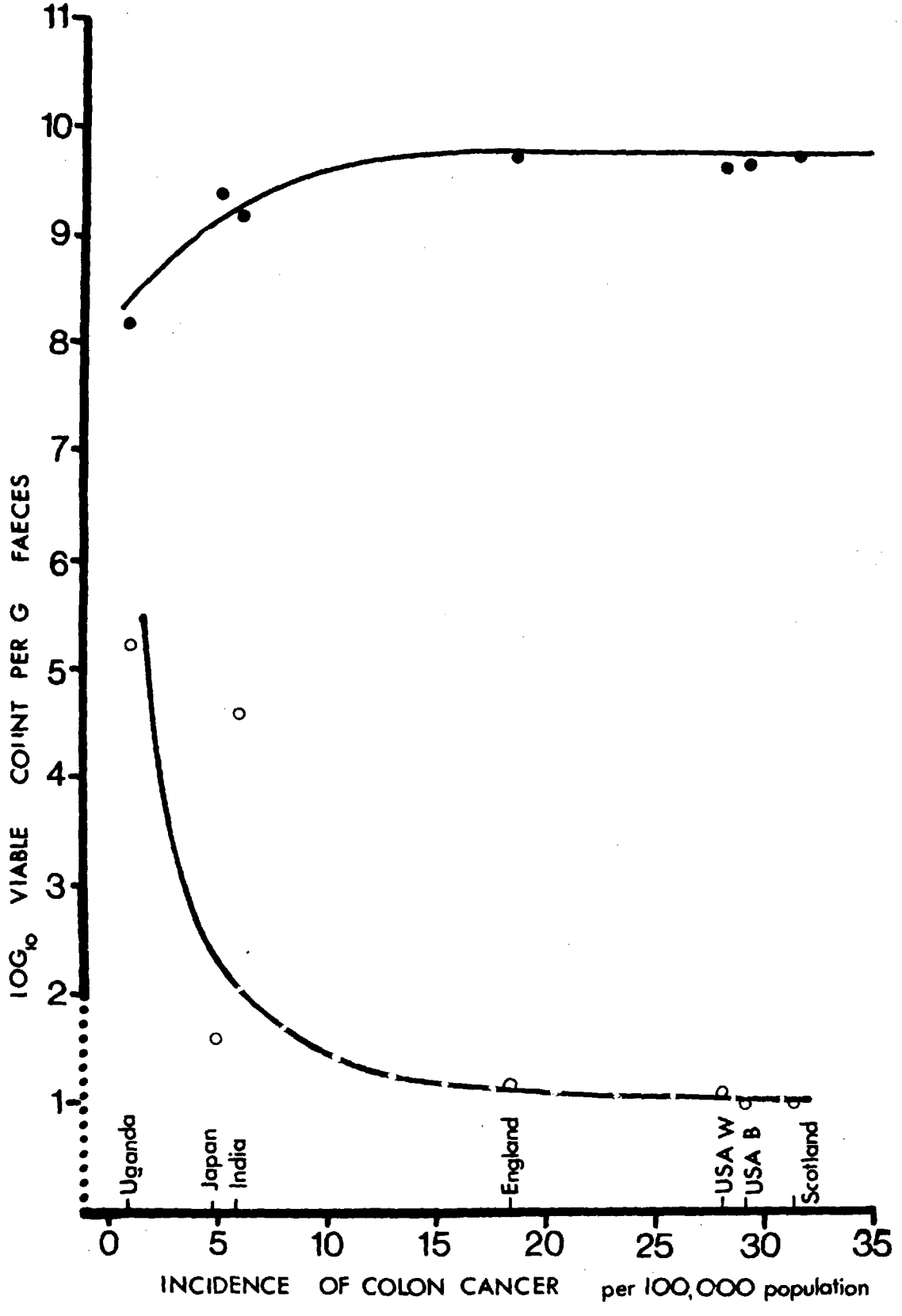

11
10
9
8
7
6
5
4
3
2
1
LOG_{10} VIABLE COUNT PER G FAECES
Uganda
Japan
India
England
USA W
USA B
Scotland
0
5
10
15
20
25
30
35
INCIDENCE OF COLON CANCER per 100,000 population

Fig. 36. Relationships between the counts of faecal bacteria and the incidences of cancer of the large bowel.

Key:

A = bifidobacteria

B = 'viridans' streptococci

C = enterococci

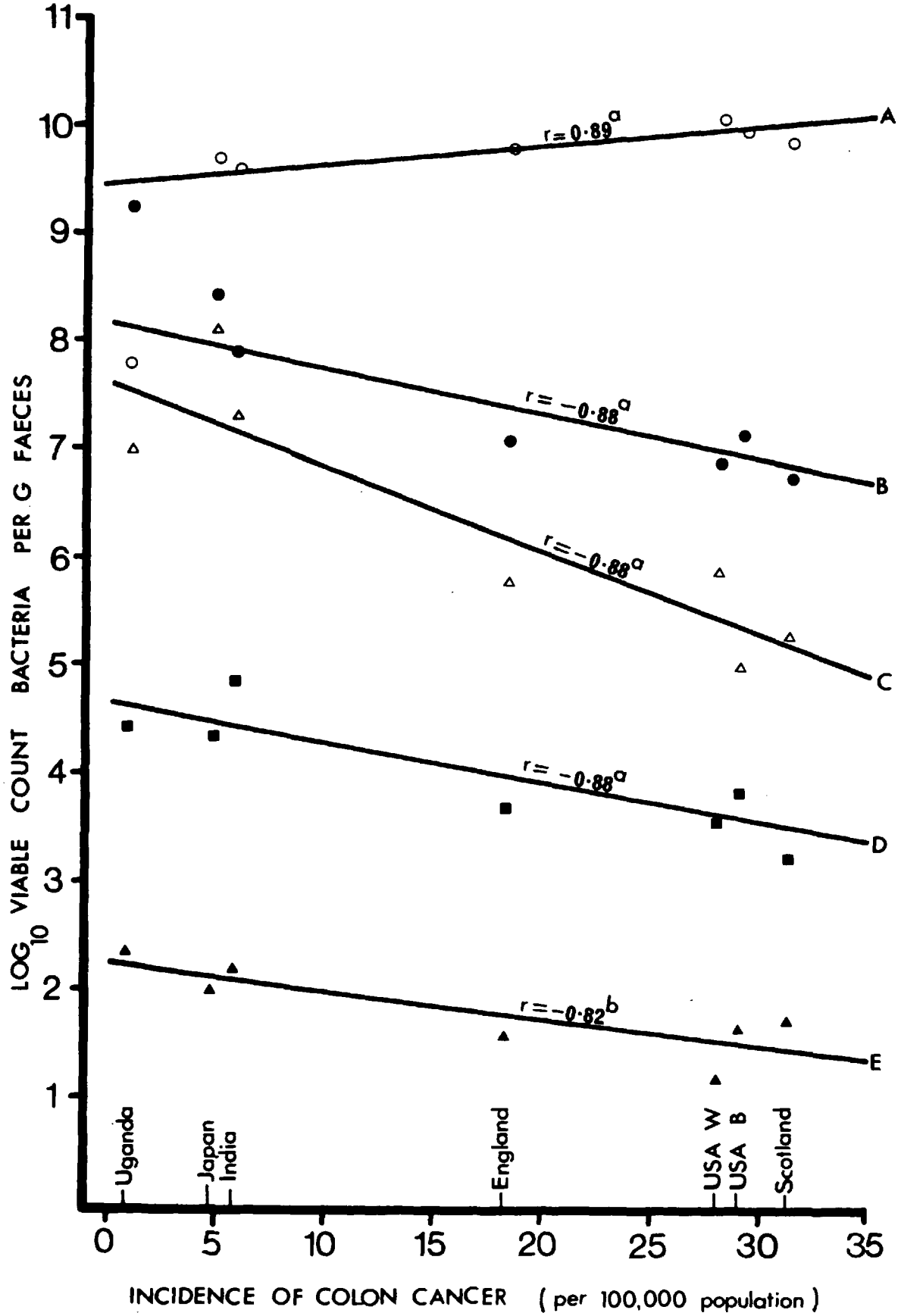
D = *Bacillus* spp.

E = filamentous fungi

r = coefficient of linear correlation

a = significant correlation ($P < 0.01$)

b = " " ($P < 0.05$).


LOG10 VIABLE COUNT BACTERIA PER G FAECES
INCIDENCE OF COLON CANCER (per 100,000 population)
r = 0·89a
r = −0·88a
r = −0·88a
r = −0·88a
r = −0·82b
A
B
C
D
E
Uganda
Japan
India
England
USA W
USA B
Scotland
11
10
9
8
7
6
5
4
3
2
1
0
5
10
15
20
25
30
35

decreased. The counts of 'viridans' streptococci, enterococci and Bacillus spp. decreased significantly ($P < 0.01$); the decreasing count of filamentous fungi was on the borderline of significance ($P < 0.05$). Counts of enterobacteria, Lactobacillus spp., Veillonella spp., Clostridium spp., staphylococci and yeasts also decreased with increasing cancer incidence, but the correlations were not significant.

Discussion

The work described here was part of a wider survey designed to test the hypothesis that the geographical variations in the incidence of large-bowel cancer might be due to differences in composition and activity of the intestinal flora, brought about by differences in the diets. Before any conclusions can be drawn, the bacteriological results must be discussed in the light of the work of my colleagues Drs. Hill and Aries who estimated the concentrations of undegraded and degraded steroids in the same specimens of faeces studied bacteriologically here.

Drs. Hill and Aries found that faeces from Britain and America contained significantly higher concentrations of bile acids and neutral steroids than those from Uganda, India or Japan (Table 22). The steroids in faeces from the Western

Table 22. Steroid concentrations in faeces[1]

	England	Scotland	United States		Uganda	Japan	India
			White	Black			
No. samples examined	26	18	24	12	11	18	18
Total steroids	16.86	16.28	16.73	16.64	2.37	6.40	2.02
Neutral steroids	10.81	10.10	10.73	10.37	1.82	4.52	1.51
Cholesterol	3.32	2.89	3.83	2.93	0.81	2.59	0.57
Coprostanol	6.77	6.68	6.19	6.79	0.90	1.89	0.73
Coprostanone	0.72	0.53	0.71	0.65	0.11	0.04	0.21
Acid steroids	6.15	6.18	6.00	6.27	0.45	0.88	0.51
Cholic acid (tri-substituted)	0.48	0.31	0.60	-	0.10	0.22	0.05
Deoxycholic and chenodeoxycholic (Di-substituted)	1.47	2.47	2.22	-	0.13	0.50	0.29
Disubstituted keto acids	1.02	0.37	0.42	-	0.07	0.05	0.06
Lithocholic (mono-substituted) and cholanic (unsubstituted)	3.08	3.03	2.74	-	0.15	0.11	0.11

[1] Modified from Hill et al. (1971)

countries were also considerably more degraded than those in faeces from Uganda and Asia. When pure strains of the different groups of bacteria were tested for their ability to degrade bile steroids *in vitro*, those from Britain and the U.S.A. showed much more steroidase activity than those from Uganda or Asia. *Bacteroides* spp. and bifidobacteria, which were present in significantly higher numbers in faeces from Britain and America, were particularly active in degrading bile steroids, and a higher proportion of strains from Britain and America showed degrading activity than did those from India or Uganda. Table 23 shows the proportion of strains from each country able to convert cholic acid to deoxycholic acid. These results have been presented in detail elsewhere (Aries *et al.*, 1969; Hill *et al.*, 1971).

Persons living in areas with a high incidence of large-bowel cancer tend to live on diets rich in fat and animal protein, whilst those who live in areas with a low incidence are principally vegetarians and eat little animal fat or protein. It is possible that a high fat diet may increase the anaerobic and decrease the aerobic intestinal bacteria in the manner described previously (section 3.4). In general the anaerobic bacteria were able to degrade bile steroids much more actively than the aerobes. Consequently, higher

Table 23. Conversion of cholic acid to deoxycholic acid by intestinal bacteria[1]

	Origin of strain									
	England		Scotland		United States		Uganda		India	
	A	B	A	B	A	B	A	B	A	B
Bacteroides spp.	50	44	24	56	10	50	42	33	20	5
Bifidobacterium	57	40	24	56	10	50	137	4	20	5
Clostridium spp.	70	34	38	45	10	60	60	6	14	0
Enterococci	90	11	30	40	0	-	162	3	24	0
Enterobacteria	87	0	30	0	0	-	180	0	32	0

A = no. of strains tested

B = % of strains performing this conversion.

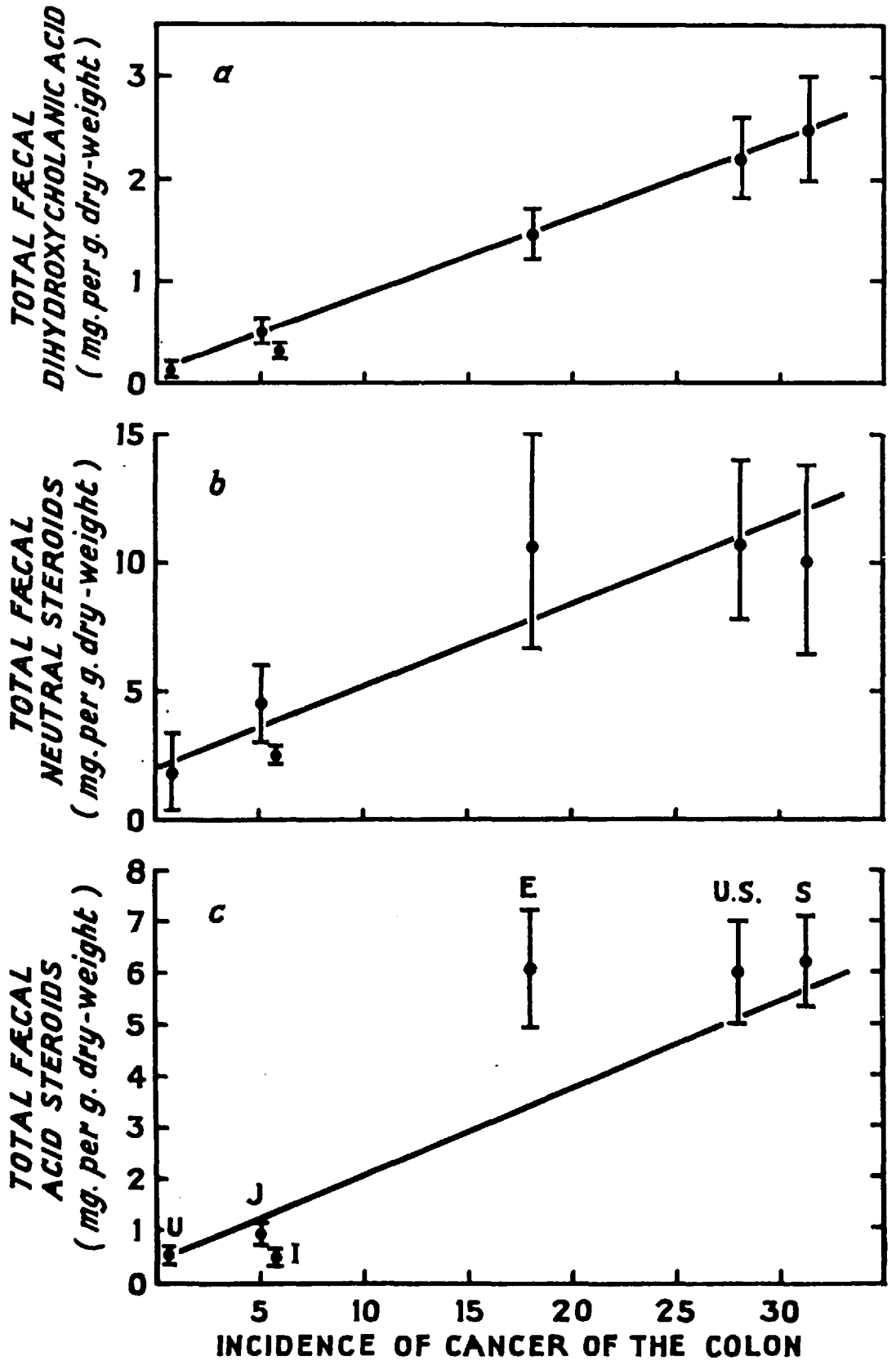
1. From Hill *et al.*, 1971.

concentrations of degraded steroids were excreted in faeces from persons living in high risk areas.

We still do not know whether intestinal bacteria could convert bile salts into carcinogens. However, deoxycholic acid has been considered to be carcinogenic (Cook *et al.*, 1940; Badger *et al.*, 1940) and in our studies it was produced from cholic acid by pure strains of *Bacteroides* spp., bifidobacteria and *Clostridium* spp. *Bacteroides* and bifidobacteria isolated from faeces of persons in Britain and America, not only were better producers of deoxycholic acid than those from Uganda, India or Japan, but were also present in much higher numbers; clostridia were equally prevalent in all populations, but those strains from Western persons were much more active producers of deoxycholic acid (Table 23). As a result, dihydroxycholanic acids (of which the main component was deoxycholic acid) were found in much higher concentrations in faeces from Western populations than in those from Uganda or Asia. Furthermore, the concentrations of dihydroxycholanic acids appear to correlate highly with the incidence of large bowel cancer in the six countries and we suspect that deoxycholic acid might itself be a carcinogen, or a precarcinogen (Fig. 37). Therefore, we have postulated that the intestinal bacteria, by degrading steroid secretions of the body, may be aetiologically related to cancer of the large bowel (Hill *et al.*, 1971). Work is

Fig. 37. Relationship between faecal concentration of (a) dihydroxycholanic acid (most of which is deoxycholic acid); (b) total neutral steroids; (c) total bile acids and incidence of cancer of large bowel in different countries. U = Uganda; J = Japan; I = India; E = England; U.S. = United States; S = Scotland. The steroid concentrations are shown as mean ± standard deviation.

From Hill et al. (1971).


TOTAL FÆCAL DIHYDROXYCHOLANIC ACID (mg. per g. dry-weight)
a
0
1
2
3
TOTAL FÆCAL NEUTRAL STEROIDS (mg. per g. dry-weight)
b
0
5
10
15
TOTAL FÆCAL ACID STEROIDS (mg. per g. dry-weight)
c
E
U.S.
S
J
U
I
0
1
2
3
4
5
6
7
8
5
10
15
20
25
30
INCIDENCE OF CANCER OF THE COLON

continuing to find ways in which intestinal bacteria can unsaturate the steroid nucleus to produce polycyclic aromatic hydrocarbon carcinogens.

The work so far in this laboratory has been directed towards finding ways in which bacteria might produce carcinogens within the human intestine. However, it seems worthwhile considering the possibility that some bacteria could also prevent carcinogenesis. There are several ways in which the intestinal bacteria could do this.

Firstly, the bacteria could excrete compounds which would inhibit the growth of tumour cells. Some streptococci, especially strains of Streptococcus pyogenes and Str. faecalis are known to produce such agents - albeit under very different conditions to those inside the human gut (Havas, Donnelly and Porreca, 1963). Although Str. pyogenes is never found in faeces of healthy persons, large numbers of other streptococci are, and it is possible that some of them could also show antineoplastic activity. It is noteworthy that faeces from the three areas with a low-risk of colon cancer contained on average about eight times more streptococci and about 43 times more enterococci than those from the three high-risk areas.

Secondly, it is not unreasonable to consider the possibility that an incipient carcinoma cell in the human intestine could be

invaded and lysed by one of the many bacteria in intimate contact with it, in the same way that tumour cells in mice are selectively invaded by Clostridium sporogenes, Cl. butyricum and Cl. tetani (Möse and Möse, 1964). All three species are found in human faeces, but in the work described here no attempt was made to identify them. Moreover, faeces from persons in high- and low-risk areas, were found to contain the same number of clostridia.

Finally, if some components of the intestinal flora, singly or in concert, can convert naturally occurring or dietary steroids into potent carcinogens, there exists the possibility that other components could further degrade the resultant carcinogens, or the carcinogen precursers, to produce less toxic molecules. Many species of soil bacteria and fungi can degrade the steroid nucleus (Capek, Hanc and Tadra, 1966) and it seems possible that some intestinal bacteria could do so too. Therefore, the quantities of any carcinogen, pre-carcinogen or co-carcinogen produced by the intestinal bacteria, would then depend on the intricate balance of these two opposing forces.

> "..... cancer might be regarded, in a final analysis, as a symptom of sterol poisoning."
>
> Aldous Huxley (1939),
> "After Many a Summer"

A P P E N D I X A

THE CULTURE MEDIA AND METHODS FOR IDENTIFYING AND PRESERVING ORGANISMS

1. Formulae of the culture media, the glycerol transport broth and the liquid diet

Reinforced clostridial agar (RCA) (for strict anaerobes) was prepared as described in section 2.2.

Willis and Hobbs' agar (for Clostridium and Sarcina spp.) was prepared as described by Cruikshank (1965). 50 µg/ml neomycin sulphate was added to isolate Clostridium spp. The medium was also used without antibiotics to isolate spores of clostridia and sarcinae from suspensions of faeces heated at 70° for 10 min.

Tomato juice agar (for Sarcina ventriculi) was obtained from Oxoid and constituted as directed; the pH was adjusted to 7.0 before autoclaving.

Rogosa's agar (for Veillonella spp.) (Rogosa et al., 1958). This medium contained: tryptone (Oxoid), 5.0 g; yeast extract powder (Oxoid), 3.0 g; sodium thioglycollate, 0.75 g; Tween '80', 1.0 g; sodium lactate (70% w/v aqueous solution), 18.0 ml; basic fuchsin (1% w/v aqueous solution) 0.2 ml; agar, 20 g and distilled water 1 litre. The pH was adjusted to 7.5 and the medium autoclaved at 115°C for

20 min. 7.5 µg/ml vancomycin were added before the plates were poured.

Rogosa's agar (for Lactobacillus spp.) (Rogosa et al., 1951). This medium contained: tryptone (Oxoid, 10 g; yeast extract powder (Oxoid), 5 g; potassium dihydrogen phosphate, 6 g; ammonium citrate, 2 g; glucose, 20 g; tween '80', 1.0 g; sodium acetate (anhydrous), 15.1 g; acetic acid (glacial), 1.32 ml; $MgSO_4.7H_2O$, 0.575 g; $MnSO_4.2H_2O$, 0.12 g; $FeSO_4.7H_2O$, 0.034 g; agar, 20 g and distilled water 1 litre. The pH was adjusted to 5.4 and the medium autoclaved at 115°C for 20 min.

Blood agar (for aerobic organisms) was prepared by adding 10% (v/v) horse-blood to molten blood agar base (Oxoid) constituted as directed.

Azide agar (for enterococci) (Schaedler et al., 1965). The medium contained: proteose peptone (Oxoid), 10 g; tryptose (Oxoid), 10 g; glucose, 5 g; sucrose, 10 g; K_2HPO_4, 4 g; KH_2PO_4, 1.5 g; NaCl, 5 g; sodium azide (1% w/v aqueous solution), 5 ml; glycerol, 2.5 ml, methylene blue solution, 4 ml; agar, 20 g and distilled water, 1 litre. The pH was adjusted to 6.9 and the medium autoclaved at 115°C for

20 min. The methylene blue solution was prepared by dissolving 1 g methylene blue in 100 ml acetone and 100 ml 95% alcohol; it was kept at +3°C.

Sucrose agar (for Streptococcus spp.) (Williams and Hirsch, 1950). 50 g sucrose were added to one litre of nutrient agar (Oxoid, Number 2) and constituted as directed. Before pouring plates 5% (w/v) horse-serum, 0.25 mg % crystal violet and 1.0 mg % potassium tellurite were added.

Sabouraud's agar (for fungi). This medium contained: mycological peptone (Oxoid), 10 g; glucose, 40 g; agar, 18 g and distilled water, one litre. The pH was adjusted to 6.5 and the agar autoclaved at 115° for 20 min. Before pouring the plates 40 µg/ml chloramphenicol was added.

MacConkey's agar (for enterobacteria and enterococci), mannitol salt agar (for staphylococci and micrococci) and nutrient agar (Number 2) (for Bacillus spp.) were all obtained from Oxoid Ltd., and reconstituted as directed.

Glycerol transport broth (Drasar, Shiner and McCleod, 1969). This was prepared by dissolving 10 g Lab-lemco powder (Oxoid) and 100 ml glycerol in 900 ml distilled water;

the broth was adjusted to pH 7.3, dispensed in 4.5 ml amounts and autoclaved at 115° for 20 min. For some experiments described in Appendix B4, the glycerol was replaced with distilled water.

Enrichment cultures for isolating Sarcina ventriculi

Enrichment medium, designed to isolate Sarcina ventriculi from the soil (Canale-Parola and Wolfe, 1960), was prepared by dissolving 10 g malt extract and 2 g maltose in 100 ml water; the medium was adjusted to several pH values from 2.3 to 6.0 and autoclaved at 115°C for 20 min.

Formula of the chemically defined diet

A chemically defined diet 'Vivasorb' was supplied as a powder by the Vivonex Corporation, Mountain View, California. It was identical to that described by Winitz et al. (1970) except that 64.5% of the glucose was replaced by maltose and some higher molecular weight oligosaccharides (Dr. S. Orford, personal communication, 1970). When dissolved in water as directed, 1,800 ml diet contained: 38 g L-amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, valine, alanine, arginine, aspartic acid, glutamine, glycine, histidine, proline, serine and tyrosine), vitamins (A, B_{12}, C, D_2 and E, p -aminobenzoic acid, biotin, pantothenate, choline, folic acid,

inositol, menadione, niacin, pyridoxine, riboflavine and thiamine) and minerals (sodium, potassium, calcium, magnesium, manganese, iron, copper, zinc, cobalt and molybdenum as phosphate, acetate, sulphate, iodide, gluconate, sorbate or benzoate). The only fat was ethyl linoleate (1.36 g). The carbohydrates were glucose, 134 g; maltose, 15 g; other oligosaccharides, 224 g.

2. Preservation of organisms

During the study strains of intestinal bacteria were preserved for further identification and for investigations by Drs. Hill and Aries into the bacterial metabolism of bile salts and other steroids. The methods of preservation are shown in Table 24 and were chosen to give ready access to all strains. Since satisfactory methods for preserving fastidious anaerobes were lacking, the following methods were devised.

Preservation of non-sporing anaerobes

Two ml sterile glycerol were thoroughly mixed into a two-day culture in Robertson's cooked meat broth (RCMB) (Southern Group Laboratories) and frozen at -55°C in a refrigerator. When required for use the cultures were thawed at room temperature. Over 80% of our cultures remained viable for at least nine

Table 24. Methods of preserving organisms

Organism	Medium and growth conditions before storage	Storage temperature (°C)
Bacteroides spp. Bifidobacteria	2 day culture in Robertson's cooked meat broth (RCM)[1]. Two ml sterile glycerol mixed into culture before freezing.	-55
Enterobacteria and enterococci	Overnight culture on Dorset's egg slopes[1]	Room temperature
Lactobacillus spp.	3 day culture on slopes of tomato juice agar (Oxoid)	3
Veillonella spp.	3 day culture on slopes of tryptone yeast extract agar (Oxoid)	3
Bacillus spp.	Overnight cultures on nutrient agar slopes	Room temperature
Clostridium spp.	2 day culture in RCM[1]	Room temperature
Sarcina spp.	Overnight culture in RCM[1] containing 1% glucose	3

1. Supplied by Southern Group Laboratories

months when frozen in this way (Drasar and Crowther, 1971).

Preservation of Sarcina ventriculi

It is well established that pure cultures of S. ventriculi readily die (Smit, 1933; Canale-Parola, 1970). Techniques claimed to preserve soil isolates of the organism (Canale-Parola and Wolfe, 1960) were found to be unsatisfactory for faecal isolates. A simpler technique was therefore used (Crowther, (1971a).

1% glucose was added to Robertson's cooked meat broth (RCMB) and autoclaved at 115°C for 20 min. The medium did not require deoxygenating before use. Cultures were grown overnight at 37°C and stored at +3°C. With this technique strains have remained viable for over a year without subculture.

3. Tests and media for the identification of organisms

The methods used to identify non-sporing anaerobes were those of Cato et al. (1970), unless stated otherwise. Whilst the anaerobic cabinet was necessary for the quantitative isolation of these organisms, conventional procedures on the open bench were usually adequate for their identification.

Culture media were not deoxygenated before use, but immediately after inoculation, cultures were incubated anaerobically in milk churns fitted with freshly activated catalysts (see Section 2.2). The inoculum used for all tests was a two-day culture in Robertson's cooked meat broth (RCMB). Methods for detecting the acid products of glucose metabolism are described in section 2.3.

Other organisms were identified using tests described by Cowan and Steel (1965), unless stated otherwise. All cultures were incubated at 37°C.

Aesculin hydrolysis

For strict anaerobes

1.0 g aesculin was dissolved in 100 ml basal medium (see 'fermentation tests'), adjusted to pH 7.3 and autoclaved at 115°C for 20 min. The medium was inoculated and incubated anaerobically for four days. Two drops 1% ferric chloride solution were added: a black colour was recorded as positive.

Arginine hydrolysis (Niven, Smiley and Sherman, 1942)

The medium contained: yeast extract powder (Oxoid) 5.0 g; tryptone (Oxoid) 5.0 g; K_2HPO_4, 2.0 g; glucose 0.05 g; L-arginine monochloride, 3.0 g and distilled water, one litre. The medium was adjusted to pH 7.0 and autoclaved at 115°C for 20 min. After inoculation, cultures were incubated for 7 days.

0.5 ml culture was added to 4.5 ml distilled water and 0.5 ml Nessler's reagent added. If the organism had split arginine to form free ammonia, the solution turned orange. Uninoculated broth was used as a control.

Catalase

For anaerobes

A 2-day culture on either RCA (Oxoid) or Willis and Hobbs' agar, was exposed to the air for at least 30 min. When 3% hydrogen peroxide was dropped onto the growth, bubbling was recorded as positive and no bubbles as negative.

For aerobes

Organisms were grown aerobically on nutrient agar (except for Lactobacillus spp. which were grown microaerophilically on Rogosa's agar); hydrogen peroxide was added and reactions recorded as above.

Cellulose production

Sarcina ventriculi is one of the few bacteria known to produce an adherent layer of extracellular cellulose (Canale-Parola, 1970). Two methods were used to detect this.

Sarcinae from an overnight culture in basal medium containing 2% glucose (see 'fermentation tests') were boiled in

1% NaOH, washed with water and suspended in Schultze's stain for cellulose (Canale-Parola, Borasky and Wolfe, 1961). The cellulose stained reddish purple. Staphylococcus aureus contains no cellulose and was used as a negative control; the cells remained colourless.

Cells grown overnight as above were suspended in buffer at pH 4.0 containing cellulase (British Drug Houses) and incubated overnight. The cultures were examined microscopically for disruption of the packets into smaller clusters and single cells. Suspensions without cellulase were used as controls.

Citrate utilisation

Organisms were grown in Koser's citrate (Southern Group Laboratories) and examined daily for up to seven days for growth. Turbidity was recorded as citrate utilized, and no turbidity as citrate not utilized.

Controls: positive Klebsiella aerogenes; negative Escherichia coli WF 96.

Fermentation tests

For non-sporing anaerobes (Cato et al., 1970)

1.0 g of each carbohydrate (but only 0.5 g arabinose or 0.8 ml glycerol) was added to 100 ml basal medium containing: 2.0 g peptone; 1.0 g yeast extract; 0.05 g cysteine

hydrochloride; 0.001 g $CaCl_2$; 0.001 g $MgSO_4$; 0.005 g K_2HPO_4; 0.005 g KH_2PO_4; 0.05 g $NaHCO_3$ and 0.01 g NaCl. The media were adjusted to pH 7.3 and autoclaved at 115°C for 20 min. The media were inoculated and incubated anaerobically for 4 days. Acid production was tested using 0.04% aqueous bromocresol purple; uninoculated medium was used as a control. The glucose cultures were retained for analysis of volatile acids (see section 2.3).

For Sarcina spp.

2.0% of each carbohydrate were added to the above basal medium, the pH adjusted to 7.0 and autoclaved as above. Two drops of an overnight culture in RCMB (containing 1% glucose) were used as the inoculum. Cultures were incubated anaerobically at 37°C and examined for acid production as described above.

For enterobacteria

Organisms were grown in peptone water sugars containing phenol red as indicator of acid production and Durham tubes for gas production (Southern Group Laboratories). Negative tests were examined up to 30 days.

Gelatin liquefaction

Charcoal gelatin discs (Oxoid) were added to Robertson's cooked meat broth (for anaerobes) or peptone water (for other

organisms) and incubated for up to 14 days. Liquefaction was indicated by the appearance of free charcoal particles in the medium.

Controls: positive, Bacillus cereus; negative, E. coli WF 96.

Gram stain

The method used was Preston and Morrell's modification of Lille's method, as described by Cowan and Steel (1965).

Some stains of Gram-positive anaerobes readily lose their Gram-positivity. Hence, if any Gram-positive cells were present, the organism was recorded as Gram-positive. If only Gram-negative cells were present the organism was recorded as Gram-negative (Cato et al., 1970).

Indole production

For anaerobes

2 ml xylene were added to a 2-day culture in RCMB and shaken well. 0.5 ml Ehrlich's reagent was added down the side of the bottle. After 15 min. a pink ring in the xylene was recorded as positive, a yellow ring negative.

For other organisms

1 ml ether was added to an overnight culture in peptone

water and proceeded as above.

Controls: positive, Escherichia coli WF 96; negative, Klebsiella aerogenes.

KCN resistance

Organisms were inoculated into broth containing 0.0075% KCN and control broth without KCN (both supplied by Southern Group Laboratories), the caps screwed down well and incubated for two days. Growth in both broths was recorded as positive; growth in the control only was recorded as negative.

Controls: positive, Klebsiella aerogenes; negative Escherichia coli WF 96.

Litmus milk test

Litmus milk (Southern Group Laboratories) was inoculated and incubated anaerobically. Cultures were examined daily for clotting and for acid production (a pink colour in the medium).

Control: Clostridium perfringens.

MR (methyl red) and V-P (Voges-Proskauer) reactions

MR and V-P medium (Southern Group Laboratories) was inoculated and incubated for two days. Two drops methyl red solution were added and the culture shaken. A red colour was recorded as MR positive, a yellow colour MR negative. After completing

the methyl red test 0.6 ml 5% ethanolic α-naphthol solution and 0.2 ml 40% aqueous KOH solution were added, shaken and examined after 15 min. and 1 hr. A red colour was recorded as V-P positive. Escherichia coli WF 96 and Klebsiella aerogenes were used as controls.

Nitrate reduction

For non-sporing anaerobes

0.5 ml 0.8% sulphanilic acid (in 5M acetic acid) and 0.5 ml 0.6% α-naphthylamine (in 5M acetic acid) were added to a 4-day culture in Indole-Nitrate medium (Baltimore Biological Laboratories). A red colour was recorded as positive. After 5 min. a pinch of zinc dust was added to each negative culture; a red colour indicated no reduction of nitrate; no colour reaction indicated complete reduction of nitrate.

For Sarcina spp.

2% glucose was added to nitrate broth (Difco) and autoclaved at 115°C for 20 min. Cultures were incubated anaerobically overnight. Nitrate and nitrite were detected as described above.

Escherichia coli WF 96 was used as a positive control.

Tellurite resistance

The organisms were streaked onto Hoyle's medium, prepared as described by Cruikshank (1965), and incubated for 2 days. The plates were viewed with a hand-lens and if any colonies were seen the organism was recorded as tellurite resistant; no growth was recorded as tellurite sensitive. Each culture plate was always streaked with Streptococcus faecalis CEI2 and Str. faecium NCTC 7379 as controls.

Tetrazolium reduction

Reduction of tetrazolium (2:3:5 triphenyltetrazolium chloride) was tested by the method described by Barnes (1956). RCA (Oxoid) was constituted as directed, adjusted to pH 6.0 and autoclaved at 115°C for 20 min. A solution of 2:3:5 triphenyltetrazolium chloride (sterilized by filtration) was added to the agar to give a final concentration of 0.02%. Freshly poured plates were dried, inoculated and incubated for 2 days. Reduction of tetrazolium was indicated by the formation of red or pink colonies; colonies of non-reducing strains remained white. As controls, each culture plate was also inoculated with Streptococcus faecalis CE12 and Str. faecium NCTC 7379.

Urease activity

A slope of Christensen's urea medium (Southern Group Laboratories) was inoculated and examined daily for 7 days. A red colour indicated hydrolysis of urea.

Controls: positive, Proteus vulgaris; negative, Escherichia coli WF 96.

4. Identification of Sarcina ventriculi

Sarcinae are now defined as obligately anaerobic, Gram-positive cocci, forming cubical packets of eight (Kocur and Martinec, 1965; Canale-Parola, Mandel and Kupfer, 1967). Only two species capable of utilising sugars are recorded, Sarcina maxima and Sarcina ventriculi (Breed, Murray and Smith, 1957). The main differences between them are that S. maxima has no extracellular cellulose and produces butyric acid from glucose, whilst S. ventriculi has extracellular cellulose and produces ethanol and not butyric acid from glucose.

A known strain of Sarcina ventriculi (HU1) originally isolated from soil (Stephenson and Dawes, 1970) was obtained from Professor E.A. Dawes, Department of Biochemistry, University of Hull, and maintained as described above. No type culture of S. maxima was available.

Six strains of sarcinae, three from Ugandan, two from Indian and one from English faeces, were compared with a known strain of Sarcina ventriculi using media and tests described in Appendix A3. No type culture of S. maxima was available. All the strains were obligate anaerobes, contained cellulose and required a carbohydrate for growth; all produced ethanol but not butyric acid from glucose (Table 25). All strains produced acid and gas from glucose, fructose, sucrose, maltose, lactose, galactose and raffinose; none could utilise arabinose, dulcitol, glycerol or starch; salicin, mannitol, inulin and xylose were utilised by some strains although growth was usually late. All strains, including the known culture of S. ventriculi, reduced nitrate to nitrite, which was then further reduced.

Although some strain to strain variations in biochemical reactions were found, the results show that the sarcinae isolated from human faeces were Sarcina ventriculi.

Most strains of sarcinae isolated from faeces could not be preserved for detailed investigation. All Gram-positive, anaerobic cocci, showing the same cultural appearances as those of Sarcina ventriculi, were therefore presumptively identified as S. ventriculi.

Table 25. Comparison of six faecal strains of Sarcina ventriculi with S. ventriculi HU1 isolated from soil

Test	Results of test with strains of sarcinae derived from				Results with strain HU1 derived from soil
	Uganda (1) India (1)	Uganda (1) India (1)	Uganda (1)	England (1)	
Aerobic growth	-	-	-	-	-
Growth without carbohydrate	-	-	-	-	-
Fermentations at 30 days:					
glucose	+	+	+	+	+
fructose	+	+	+	+	+
sucrose	+	+	+	+	+
maltose	+	+	+	+	+
lactose	+	+	+	+	+
galactose	+	+	+	+	+
raffinose	+	+	+	+	+
arabinose	-	-	-	-	-
dulcitol	-	-	-	-	-
glycerol	-	-	-	-	-
starch	-	-	-	-	-
salicin	-	+	(+)	(+)	-
mannitol	-	-	-	(+)	-
inulin	-	-	+	-	(+)
xylose	-	-	-	(+)	-
Production of:					
ethanol from glucose	+	+	+	+	+
butyric acid from glucose	-	-	-	-	-
extracellular cellulose	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Litmus milk	(AC)	(AC)	(AC)	(AC)	AC
Gelatin liquefaction	-	-	-	-	-

+ = positive
- = negative
(+) = positive after 7 days
AC = acid clot
(AC) = acid clot after 7 da s

A P P E N D I X B

VALIDATION OF THE QUANTITATIVE BACTERIOLOGICAL METHODS

1. Simplification of Drasar's anaerobic medium

Culture plates and diluent freshly prepared using Drasar's original method (described in section 2.2) were compared with those prepared more simply from reheated materials. Freshly prepared and reheated Oxoid RCA (containing 1% liver digest) were similarly compared; Oxoid brain heart infusion was used as the diluent. One specimen of faeces (already diluted in glycerol broth) was serially diluted in each of the diluents and plated in triplicate on agar prepared in the same way as the appropriate diluent. All manipulations were carried out in the anaerobic cabinet.

Table 26 shows that the counts of anaerobes recovered from faeces using reheated diluent and agar were the same as those using freshly prepared materials. Further, the anaerobes grew as well on commercially prepared RCA as on Drasar's original rich agar. The former is known to support good growth of Bacteroides spp. from the caeca of chickens (Barnes, Impey and Goldberg, 1966).

Fastidious anaerobes were therefore counted in the anaerobic cabinet on Oxoid RCA containing 1% liver digest; 10% (v/v) horse-blood was also added to increase the survival of the bacteria.

Table 26. Recovery of anaerobes from faeces using different media poured and inoculated in the anaerobic cabinet
(log_{10} total anaerobes per g faeces (mean of triplicate counts))

Agar	Diluent	Method of deoxygenation	
		Drasar's method: agar and diluent freshly prepared; deoxygenated before autoclaving	Conventional method: sterile agar and diluent from stock; steamed before use
Drasar's Rich Agar, with 1% liver digest	Rich Agar Medium without the agar	9.929	10.064
Reinforced Clostridium Agar (RCA) with 1% liver digest	Brain Heart Infusion with 0.05% cysteine hydrochloride	10.037	10.097

None of the differences was statistically significant

2. Reproducibility of the media

Fresh batches of each medium had to be prepared from time to time throughout the survey. As minor changes from batch to batch could readily lead to differences in the recovery of viable bacteria, the reproducibility of the media was tested.

Samples from three different batches of each medium were collected, and culture plates poured in the usual way. Triplicate plates of each batch of medium were inoculated with the same suspension of faeces, incubated and counted. All manipulations were carried out by the same person.

With one exception, none of the media tested produced significant batch to batch differences in the counts of viable bacteria recovered from faeces (Table 27). Whilst the three batches of RCA gave consistent counts of Bifidobacteria, the counts of Bacteroides spp. showed small, but significant differences.

The reasons for these differences is not known. However, fastidious anaerobic bacteria are known to be very susceptible to traces of inhibitors, such as organic peroxides, produced in the medium from proteins, reducing agents or even agar, during steaming or autoclaving (Smith and Holdeman, 1968). It is probable that occasional batches of medium received marginally more heat than others; this could result in the inhibition

Table 27. Reproducibility of viable counts of bacteria on different batches of media ($\log_{10}$ viable count per g faeces mean of triplicate plates)

Medium	Organism counted	Batch		
		1	2	3
RCA with 1% liver digest and 10% horse-blood	Bifidobacteria	10.04	10.09	10.09
"	Bacteroides spp.	10.11	10.10	9.89[1]
MacConkey's agar	Enterobacteria	7.85	7.86	7.79
Blood agar	'Viridans' streptococci	6.36	6.41	6.62
Dubos azide agar	Enterococci	5.31	5.17	5.41
Sucrose agar	Streptococcus salivarius	4.95	4.96	4.87
Rogosa's (lactobacillus) agar	Lactobacillus spp.	5.08	5.01	4.99
Willis and Hobbs' agar	Clostridia (lecithinase-positive)	6.53	6.54	6.50
Rogosa's (veillonella) agar	Veillonella spp.	3.64	3.67	3.61

1 Significant difference between batches, $P < 0.01$ (obtained by one-way analysis of variance on the 3 sets of triplicate counts).

of sensitive strains of Bacteroides spp.

3. Reproducibility of the diluting and plating technique

Suspension of faeces are often difficult to homogenise and this could readily contribute to counting errors. Since all the counts shown in Fig. 38 (a and b) were obtained from the same original suspension of faeces, and since none changed significantly on keeping, it seemed reasonable to use them to assess the errors. The eight sets of three counts (in logarithms) were submitted to a one-way analysis of variance and the residual terms used to calculate errors (Table 28). The counts of Bacteroides spp. showed the greatest variability and could be estimated within only 0.46 log. unit of the mean, with 95% confidence. Counts of enterobacteria and enterococci were the most reproducible.

of bacteria in 24 replicate dilutions
he data in Fig. 38 a and b)

	Dilution counted	Mean colony count ± S.D.	95% Confidence Limits of viable counts ($\log_{10}$ mean) ± (2 S.D.)[b]
	10^{-7}	66 ± 34	9.82 ± 0.46
	10^{-7}	103 ± 39	10.01 ± 0.32
	10^{-5}	136 ± 70	8.13 ± 0.36
	10^{-3}	160 ± 29	6.20 ± 0.12
	10^{-3}	186 ± 64	6.27 ± 0.12
	10^{-4}	385 ±170	7.59 ± 0.18
	10^{-3}	169 ± 76	6.23 ± 0.28
	10^{-1}	13 ± 5	3.10 ± 0.32
	10^{-2}	75 ± 45	4.88 ± 0.30

Crowther (1971)

sidual term in variance analysis

4. Validation of the methods for transporting faeces

When faeces have to be transported considerable distances for quantitative bacteriology, it is necessary to have a method that permits neither growth nor death of any bacteria present. Stuart (1959) designed a low-nutrient, soft-agar medium for transporting delicate pathogenic bacteria at ambient temperatures; Cary and Blair (1964) modified this medium to improve the survival of enteric pathogens in faecal specimens without the growth of nonpathogens. Although the Cary-Blair medium and further modifications of Stuart's medium give good recoveries of Escherichia coli, Clostridium perfringens, Proteus vulgaris, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella spp. (Gästrin, Kallings and Marcetic, 1968), a transport medium has never been designed to recover all groups of bacteria quantitatively from faeces. For transport by post moreover, fluid media are unsatisfactory, since it is often difficult to avoid contamination.

Drasar, Shiner and McLeod (1969) reported no apparent reduction in viable counts of Bacteroides melaninogenicus and Escherichia coli when pure cultures were suspended in broth containing 10% (v/v) glycerol and frozen on dry ice, and they

used this technique to transport specimens of intestinal juice for quantitative bacteriological examination. Their technique was therefore tested as a method for preserving faeces.

Methods

The survival of faecal bacteria was tested both in 10% (w/v) suspensions of faeces in glycerol broth (prepared as described in Appendix A) and in undiluted faeces. The suspensions were stored at -196° (in sealed ampoules immersed in liquid nitrogen), -78° (in screw-capped bottles in solid carbon dioxide) and at -25° and -12°C in refrigerators. Whole faeces were stored at -196°, -78°, +3°, +15°, +32° and +37°C. Further samples of faeces were examined after 24 or 48 hr in the post. Several replicate samples of each specimen were kept at each temperature, one being examined on each day of testing. All samples of faeces were from one individual.

The specimens were cultivated as described in section 2.2, using the cabinet to recover fastidious anaerobes. To minimise minor fluctuations in media, each medium was poured on each day of testing from stocks prepared on one single occasion. All dilutions and inoculations were carried out by the same person.

Results

Storage of faeces at sub-zero temperatures

The total viable count for undiluted faeces stored overnight at -78° was only 94% of the initial count (Table 29); the percentage survival for faeces suspended in glycerol broth was 99%, which is not significantly different from 100%. The glycerol and not the dilution in the broth appeared to be the protective factor.

Further tests showed that many different bacterial species survived well when the faeces were suspended in glycerol broth and held at a wide range of sub-zero temperatures (Figs. 38 and 39). With two exceptions, all groups of bacteria survived at -196°, -78° and -25° with no significant losses after one month. Lactobacilli showed a reduction in count immediately on freezing at 196°, but once frozen, showed no further losses. Veillonellae, although stable at -196° and -78° showed slight but significant losses when stored at higher temperatures.

The survival of bacteria in faecal suspensions held at -12° was not as good as at the lower temperatures; enterobacteria and veillonellae showed significant losses, but all other groups survived well.

Table 29. Survival of anaerobic bacteria in faeces frozen at - 78°C (mean $\log_{10}$ viable bacteria per g. wet faeces)

	Before freezing	After freezing		
		Faeces diluted (1:10) in broth containing:		
		Undiluted faeces	No addition	10% (v/v) glycerol
Viable count	9.8	9.2	9.1	9.7
% viability	100	94*	93*	99

* Significant loss: P 0.01 (obtained from Student t-test)

Fig. 38. Survival of bacteria in faeces suspended in glycerol broth.

Key: (a) suspensions frozen at -196°

(b) " " " -78°

A = bifidobacteria

B = *Bacteroides* spp.

C = 'viridans' streptococci

D = *Streptococcus salivarius*

E = enterobacteria

F = enterococci

G = *Lactobacillus* spp.

H = *Veillonella* spp.

J = *Clostridium* spp.

The vertical bars show the range of triplicate counts.

1 = significant reduction in viable count ($P < 0.05$)

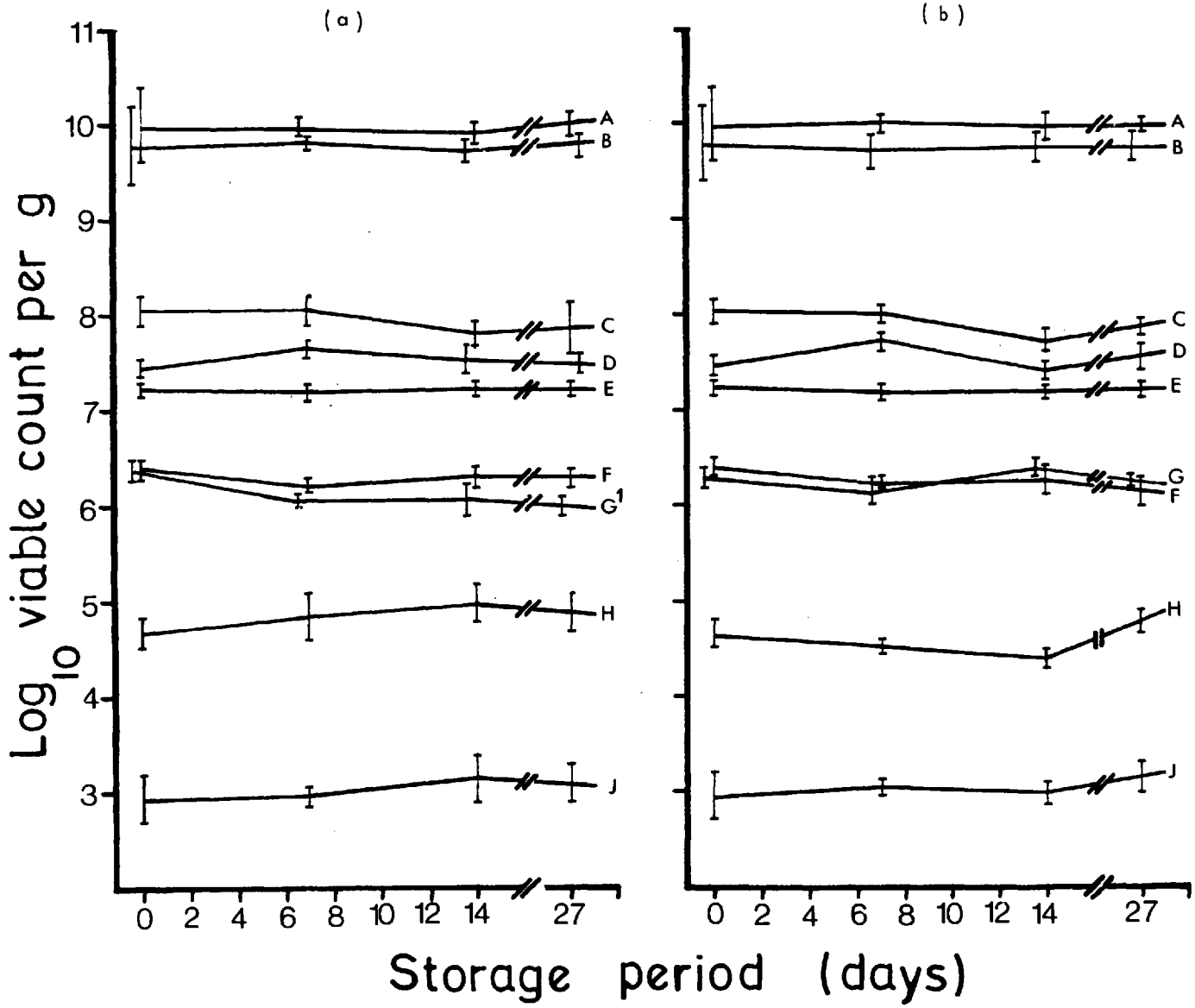

(a)
(b)
11
10
9
8
7
6
5
4
3
A
B
C
D
E
F
G
H
J
0 2 4 6 8 10 12 14 27
Log10 viable count per g
Storage period (days)

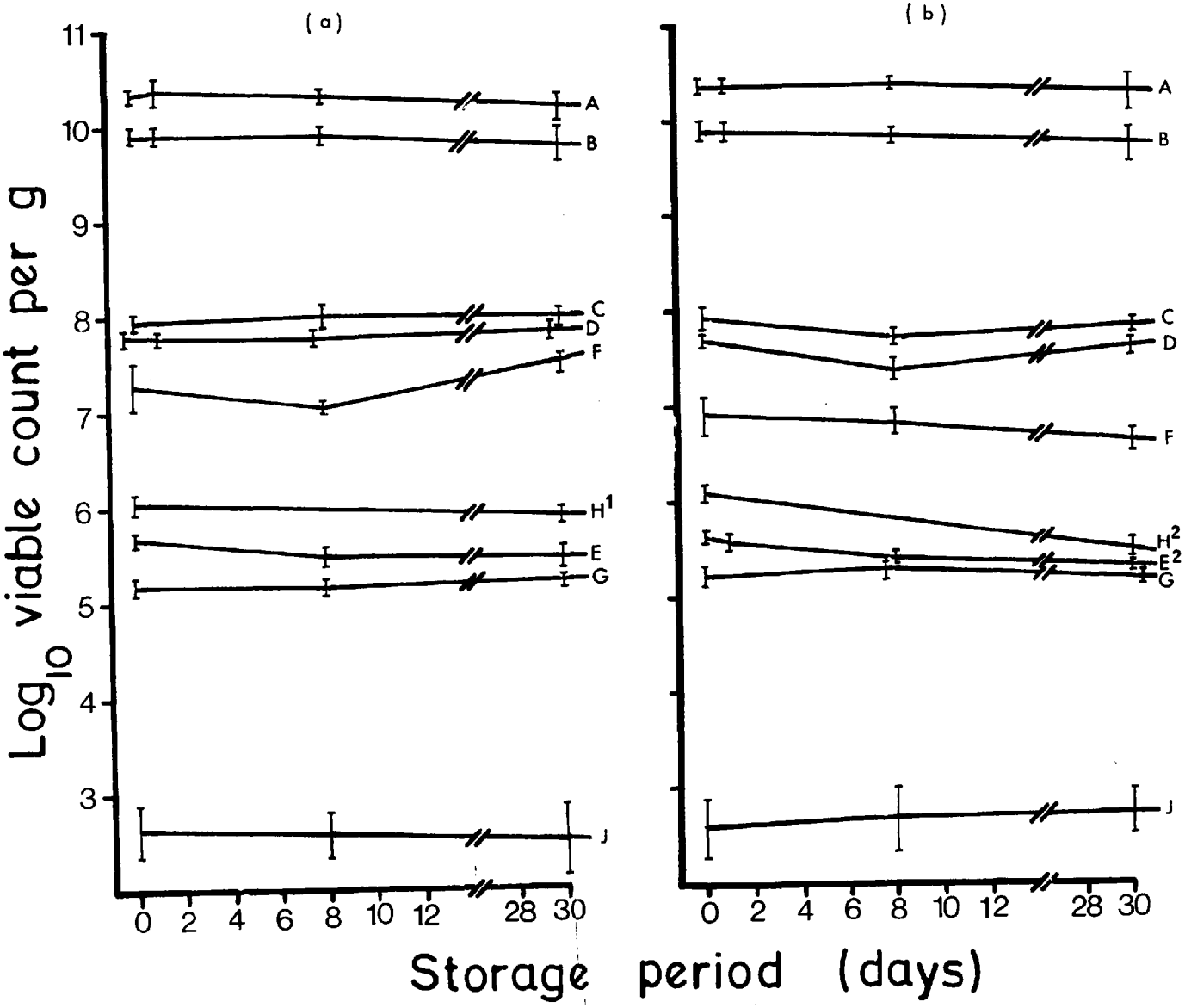
Fig. 39. Survival of bacteria in faeces suspended in glycerol broth.

Key: (a) suspensions frozen at -25°

(b) suspensions frozen at -12°

1. significant reduction in viable count ($P < 0.05$)
2. significant reduction in viable count ($P < 0.01$)

For description of other signs see Fig. 38.


(a)
(b)
Log10 viable count per g
11
10
9
8
7
6
5
4
3
A
B
C
D
F
H1
E
G
J
H2
E2
0 2 4 6 8 10 12 28 30
Storage period (days)

Storage of faeces at temperatures above zero

Survival curves for bacteria in undiluted faeces stored in air at temperatures above zero are shown in Fig. 40. No organism showed a significant change in viable count after storage at +3° for 24 hr, and even after 8 days the counts of bifidobacteria, enterobacteria, lactobacilli and clostridia had not changed significantly.

At 15° counts of bifidobacteria, bacteroides, 'viridans', streptococci, veillonellae and clostridia remained unchanged after 48 hr. At 32° there was an increase in the counts of enterobacteria and lactobacilli, and a decrease in the counts of veillonellae and Str. salivarius. These changes were even more pronounced when the specimens were stored at 37°C. The viable counts of the dominant anaerobes, bacteroides and bifidobacteria, were reduced significantly only after storage at 37° for 48 hr.

When faeces were examined after spending 24 hr in the post, the counts of only enterobacteria and lactobacilli, both minor components of the bacterial flora, had changed significantly; after 48 hr, however, counts of only bacteroides, bifidobacteria, veillonellae and clostridia remained unchanged.

Discussion

These results support the work of Hollander and Nell (1954)

Fig. 40. Survival of bacteria in undiluted faeces stored in air.

Key: (a) stored at 3°

(b) stored at 15°

(c) stored at 32°

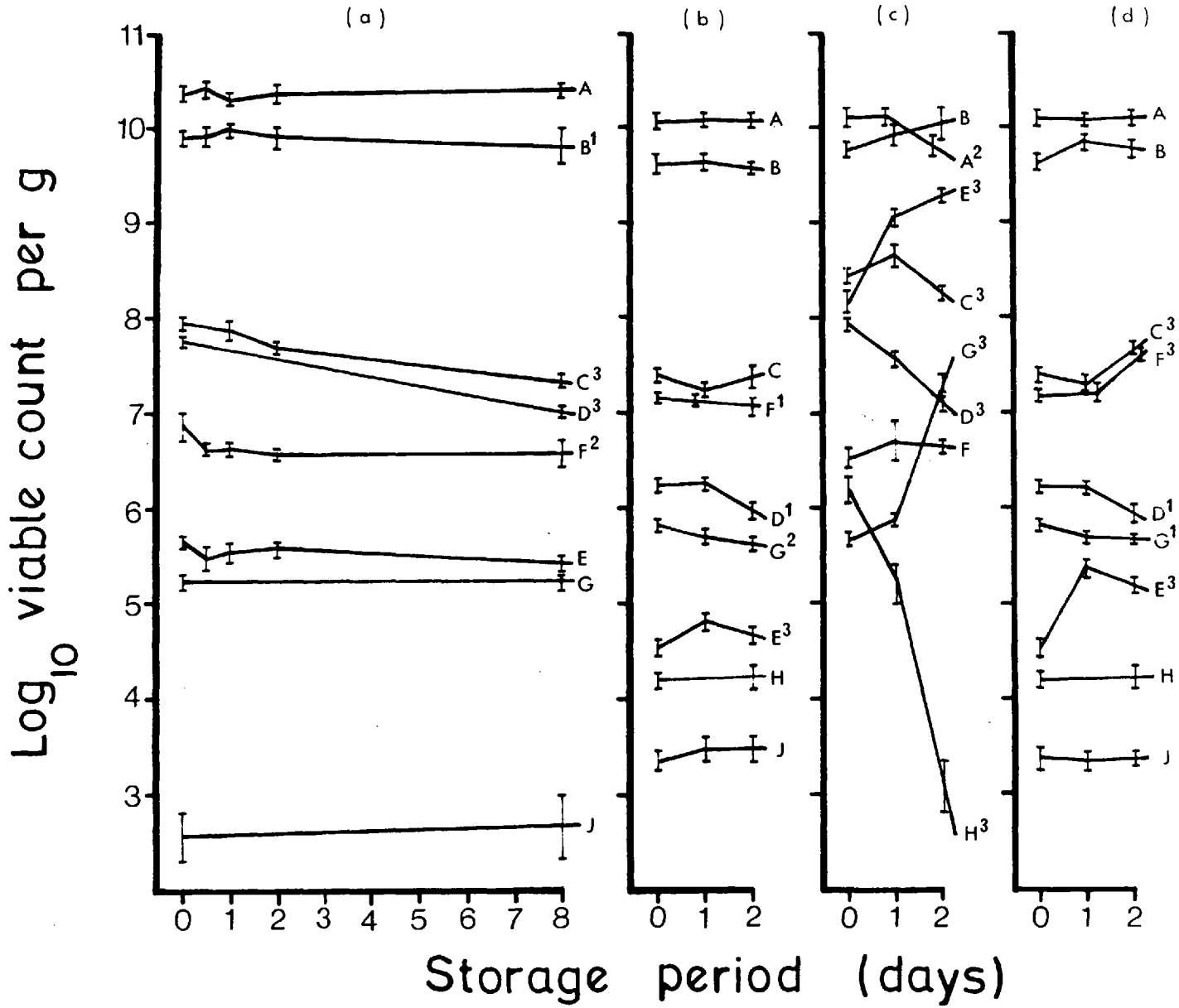
(d) specimens sent by post

1 significant change in viable count ($P < 0.05$)

2 significant change in viable count ($P < 0.01$)

3 significant change in viable count ($P < 0.001$)

For description of other signs see Fig. 38.


(a)
(b)
(c)
(d)
Log10 viable count per g
11
10
9
8
7
6
5
4
3
A
B1
C3
D3
F2
E
G
J
A
B
C
F1
D1
G2
E3
H
J
B
A2
E3
C3
G3
D3
F
H3
A
B
C3
F3
D1
G1
E3
H
J
0 1 2 3 4 5 6 7 8
0 1 2
0 1 2
0 1 2
Storage period (days)

and Drasar, Shiner and McLeod (1969) and show that even delicate anaerobic bacteria can be recovered from suspensions of faeces frozen in broth without significant losses, if glycerol is used as a protective agent. In this work no toxic effects of glycerol on the bacteria were found, even after contact for one month.

These experiments were carried out on different specimens of faeces from the same person, but it seems unlikely that there would be more differences from person to person.

From these results it is apparent that it will be satisfactory to transport faeces undiluted, if the transit time is less than 24 hr; for transport requiring longer periods of time, a 10% suspension of faeces in 10% (v/v) glycerol frozen at -25° or below, has proved satisfactory.

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Aries, Vivienne, Crowther, J.S., Drasar, B.S., Hill, M.J. and Williams, R.E.O. (1969). Bacteria and the aetiology of cancer of the large bowel. Gut, 10, 334.

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Crowther, J.S. (1971). Distribution of Sarcina ventriculi in human faeces. J. med. Microbiol.

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Bacteria and the aetiology of cancer of the large bowel

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Reprinted from The Journal of The British Society of Gastroenterology—GUT
Volume 10, page 334, 1969

BRITISH MEDICAL ASSOCIATION · TAVISTOCK SQUARE · LONDON · W.C.1

Gut, 1969, **10**, 334-335

Bacteria and the aetiology of cancer of the large bowel

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Cancer of the large bowel shows marked variations in geographical distribution (Doll, 1967; Doll, Payne, and Waterhouse, 1966; Davis, Knowelden, and Wilson, 1965) and, with the exception of Japan, the disease is more prevalent in developed than in underdeveloped countries. The reason for this variation is not known but epidemiological evidence suggests that environmental factors may be involved. It is claimed that immigrants from areas with a low incidence of cancer of the large bowel tend to show the same high incidence of this cancer as the local population (Haenszel and Dawson, 1965; Buell and Dunn, 1965). Changes in dietary habit may be especially important (Wynder and Shigematsu, 1967; Buell and Dunn, 1965) and diet is known to affect the nature and distribution of bacteria in the faeces (Hoffmann, 1964; Dubos, 1965).

Among the important metabolic activities of intestinal bacteria is the degradation of bile salts (Hill and Drasar, 1968). It seems possible that some of the bacteria in the bowel could convert bile salts, or steroids in the diet, into carcinogens; Haddow (1958) has reviewed the ways in which it is possible, in the laboratory, to convert deoxycholate into 20-methylcholanthrene, a potent carcinogen.

We have, therefore, compared the bacterial flora of the faeces from people in England, an area with a high incidence of cancer of the large bowel, with that from people in Uganda, where the incidence is low. We have also compared the abilities of English and Ugandan strains of faecal bacteria to degrade bile salts and have examined the products of bile degradation in English and Ugandan faeces.

MATERIALS AND METHODS

Samples of freshly voided faeces from 48 healthy Ugandan adults living in and around Kampala and from 40 healthy English adults living in London were examined. Specimens were preserved for transport and storage as a 10% suspension in meat infusion broth containing 10% glycerol frozen on solid carbon dioxide (Drasar, Shiner, and McLeod, 1969); the bacteria have been found to survive well under these conditions. Specimens were cultivated by the methods described previously (Drasar, 1967) with minor modifications. Approximately equal numbers of English and Ugandan specimens were examined on each day of testing in order to compensate for minor fluctuations in culture media, incubation temperatures, and operational techniques.

Methods for investigating the degradation of bile salts are described elsewhere (Hill and Drasar, 1968; Aries, Crowther, Drasar, and Hill, 1969).

RESULTS AND DISCUSSION

Our findings are summarized in the Table. The same groups of bacteria occurred in both populations but there were significant quantitative differences. Although the dominant bacteria in both populations were non-sporing anaerobes (bacteroides and bifidobacteria), the English specimens contained 30 times more bacteroides than did the Ugandan. Streptococci, enterococci, lactobacilli, and yeasts occurred in significantly greater numbers in the Ugandan specimens. No significant differences were

TABLE

BACTERIAL COUNTS OF FAECES FROM 40 ENGLISH AND 48 UGANDAN ADULTS[1]

Organism	*English*	*Ugandan*	P[2]
Bacteroides	9·7 ± 0·6	8·2 ± 1·1	<0·001
Bifidobacteria	9·9 ± 0·3	9·3 ± 0·6	<0·001
Aerobic streptococci	7·0 ± 0·8	7·8 ± 0·9	0·01
Enterococci	5·7 ± 1·3	7·0 ± 1·2	0·01
Lactobacilli	6·0 ± 1·6	7·2 ± 1·1	0·01
Yeasts	1·3 ± 1·8	3·1 ± 2·0	0·01
Enterobacteria	7·5 ± 1·2	8·0 ± 0·8	>0·05
Clostridia	4·4 ± 1·8	4·0 ± 1·9	>0·05
Veillonellae	4·4 ± 2·1	5·3 ± 1·4	>0·05
Filamentous fungi	1·4 ± 1·2	2·2 ± 1·2	>0·05[3]

[1]Arithmetic mean of $\log_{10}$ organisms per g wet weight ± standard error.
[2]Agreed values obtained from both the student *t* test and the χ^2 test.
[3]Agreed value obtained from both a rank test and the χ^2 test.

found in the numbers of clostridia, enterobacteria, veillonellae, or filamentous fungi.

The observed differences in the faecal flora of the two populations may be attributable to the radical differences in the diets. The English people we studied lived on diets containing mixed animal and vegetable foods. The Ugandans lived on a high carbohydrate vegetarian diet containing very little fat and almost no animal protein.

As reported elsewhere (Aries *et al*, 1969), English and Ugandan strains of the same group of bacteria were equally active in degrading bile salts, the bacteroides and clostridia being by far the most active in both deconjugation and further degradation of bile salts. Preliminary results, however, indicate that the bile salts present in English faeces are much more degraded than those in Ugandan and this presumably reflects the much higher numbers of bacteroides; clostridia, though equally active and equally prevalent in both populations, are present in much smaller numbers. The more degraded bile salts are presumably more likely to be converted into carcinogens.

The results presented here are compatible with the hypothesis that differences in the bacterial flora of the bowel, by leading to different degrees of degradation of bile salts, might be one cause of the varying geographical incidence of carcinoma of the large bowel. We hope to extend this survey to study the faeces of other populations with a high or low incidence of large bowel cancer.

SUMMARY

In a study designed to discover whether the frequency of large bowel cancer could be related to the bacterial flora of the bowel, the faeces of healthy English and Ugandan adults were compared bacteriologically. The faeces from the English subjects contained significantly more bacteroides and bifidobacteria but fewer streptococci and lactobacilli than those from the Ugandans. Bacteroides are very active in the degradation of bile salts and it is suggested that they might produce carcinogens.

We thank Professor R. Blowers at Makerere University College, Kampala, Uganda, and the director and nursing staff of the Medical Research Council Child Nutrition Unit, Kampala, for their invaluable work in obtaining the Ugandan faeces for us. Dr W. R. S. Doll of the Statistical Research Unit, University College Hospital Medical School, London, and Dr F. Avery Jones and Dr E. N. Rowlands of the Medical Research Council Gastroenterology Research Unit, Central Middlesex Hospital, London, all gave valuable help and advice.

We also acknowledge the very capable technical assistance of Miss R. Scutt and Miss R. Steward. The project was supported by grants from the British Empire Cancer Campaign (V.C.A., J.S.C., M.J.H.) and the Medical Research Council (B.S.D.).

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Gut, 1969, 10, 575-576

Degradation of bile salts by human intestinal bacteria

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We have previously reported that many strains of enterococci and the strictly anaerobic bacteria of the intestine (Bacteroides, Bifidobacteria, Clostridia, and Veillonella) hydrolyse bile acid conjugates (Drasar, Hill, and Shiner, 1966; Hill and Drasar, 1968). More detailed studies on the deconjugating enzyme, cholanylglycine hydrolase, obtained from Bacteroides spp, Bifidobacterium spp, Clostridium spp, and *Strep. faecalis* (two strains of each) are reported elsewhere (Aries and Hill, 1969). Here we report our studies on the metabolism of cholate and deoxycholate, and further studies on deconjugation.

METHODS

Samples of faeces from normal persons living on a mixed diet in England, and from normal persons living on a vegetarian diet in Uganda were examined bacteriologically using the methods described previously (Drasar, 1967), modified to facilitate the isolation and identification of Bacillus spp, Pseudomonas spp and anaerobic Sarcina. The differences between the faecal flora of Ugandans and English people are discussed elsewhere (Aries, Crowther, Drasar, Hill and Williams, 1969).

The bacteria were grown at 37°C under the conditions listed in Table I. Bacteria from 50 ml of broth culture were harvested by centrifugation and resuspended in 0·5 ml of supernatant. This suspension was mixed with 0·5 ml of a 0·1% solution of substrate (taurocholate, cholic acid, or deoxycholic acid) in 0·02 M phosphate buffer solution *p*H 7 and incubated at 37°C for 48 hours. Degradation products were separated by thin layer chromatography on silica gel G (Merck) using the solvents described by Eneroth (1963). Standard solutions were included on all plates; these contained sodium taurocholate (Maybridge Research Chemicals Ltd, Launceston, Cornwall), cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid (all from Koch-Light Laboratories), cholanic acid (Steraloid Ltd), 7 oxodeoxycholic acid, 12 keto lithocholic acid, and 7,12 diketo lithocholic acid, all prepared by oxidation methods from the parent hydroxyl compound (Fieser and Rajagopalan, 1949; Bergstrom and Haslewood, 1939).

TABLE I

GROWTH MEDIA AND CONDITIONS OF THE VARIOUS ORGANISMS STUDIED

Bacteria	*Medium*	*Period of Growth (hr)*	*Atmosphere*	*Other Conditions*
Enterobacteria	Glucose broth	24	Air	
Enterococci	Glucose broth	24	Air	
Str. salivarius	Reinforced clostridial medium	48	Air	
Lactobacilli	Reinforced clostridial medium	48	90% CO_2 + 10% air	
Clostridia	Reinforced clostridial medium	48	10% CO_2 in H_2	
Veillonella	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bacteroides	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bifidobacteria	Reinforced clostridial medium	72	10% CO_2 in H_2	
Bacillus spp	Glucose broth	24	Air	Shaking
Anaerobic Sarcina	Reinforced clostridial medium	24	10% CO_2 in H_2	

RESULTS

Groups of organisms unable to deconjugate sodium taurocholate in the more concentrated solutions used in our previous studies were unable to do so in the dilute solutions used here.

Many strains of Bacteroides spp, Bifidobacterium spp, Clostridium spp, Veillonella spp and enterococci were able to degrade cholate and deoxycholate yielding one or more products (Table II). Strains of these genera also deconjugated bile salts. In addition, many enterobacteria, none of which were able to deconjugate bile salts, were able to metabolize the bile acids. The reaction was subject to substrate inhibition and very little reaction with 0·5% cholate was detected (Hill and Drasar, 1968).

TABLE II

ORGANISMS ABLE TO DEGRADE TAUROCHOLATE, CHOLATE, AND DEOXYCHOLATE

Organism	*Source of Faeces*	*No. Tested*	*Percentage Able to Degrade Bile*		
			Taurocholate	*Cholate*	*Deoxycholate*
Enterobacteria	Uganda	180	0	53	32
	England	87	0	78	39
Enterococci	Uganda	162	90	68	18
	England	90	93	81	29
Str. salivarius	Uganda	37	0	0	0
	England	45	0	0	0
Lactobacilli	Uganda and England	48	0	0	0
Clostridia	Uganda	60	96	91	43
	England	70	94	87	47
Bifidobacteria	Uganda	137	86	47	18
	England	57	74	56	21
Bacteroides	Uganda	17	71	48	18
	England	37	82	79	30
Bacilli	Uganda	36	11	25	0
	England	45	49	9	0
Veillonella	Uganda	72	36	19	1
	England	20	95	90	30
Anaerobic Sarcina	Uganda	12	67	58	0

(It is possible that a higher proportion of strains would prove to be active at still lower substrate concentrations.)

A number of strains of each of the active genera are under more extensive investigation. In general the enterobacteria produce only a single degradation product from cholic acid, usually 7 ketodeoxycholate. The Bacteroides and Clostridia are much more active, producing a number of products, some of which have been identified as deoxycholic acid, lithocholic acid, and possibly cholanic acid together with 7 ketodeoxycholate. In addition, a number of products in which the α hydroxyl groups have been inverted to the β form (presumably by way of keto intermediates) have been tentatively identified.

DISCUSSION

The conversion of cholate to deoxycholate (Midtvedt, 1967; Coccucci, and Ferrari, 1963) and other products by bacteria is now well documented. Midtvedt and Norman (1967) have screened a number of anaerobic bacteria and identified the products of cholate metabolism as 3α 12α dihydroxy 7 keto cholanate, 3α hydroxy 7,12 diketo cholanate, and 3, 7, 12 triketo cholanate. We have not detected the two latter metabolites but this may be a result of the reaction conditions; Midtvedt used a much lower substrate concentration and incorporated it in the growth medium. The concentration used in our work (0·05%) is the lowest that can conveniently be used with our detection methods.

In conclusion, our results demonstrate that, in the metabolism of bile salts and acids by intestinal bacteria, the strictly anaerobic bacteria are of major importance. Our investigations on human faecal steroids indicate that this is probably true *in vivo* as well as in the situation *in vitro* described here.

We would like to acknowledge the financial support of the Medical Research Council (M.J.H. and B.S.D.) and the British Empire Cancer Campaign (V.C.A. and J.S.C.). We would also like to thank Miss Rosalind Steward and Miss Rosalind Scutt for their technical assistance and Professor R. Blowers for obtaining the Ugandan faecal samples for us.

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Reprinted from THE JOURNAL OF PATHOLOGY
Vol. 103, No. 1, pp. 54–56, 1971

The Effect of a Strict Vegetarian Diet on the Faecal Flora and Faecal Steroid Concentration

BY

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THE EFFECT OF A STRICT VEGETARIAN DIET ON THE FAECAL FLORA AND FAECAL STEROID CONCENTRATION

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CANCER of the colon is primarily a disease of developed countries; the incidence is highest in Europe and North America and low among the indigenous natives of Asia, Africa and South America (Doll, Payne and Waterhouse, 1966). This variation in incidence, supported as it is by other observations (e.g., Wynder *et al.*, 1969), suggests that diet may be among the most important factors involved. The effect of diet might be mediated by the action of intestinal bacteria if, for example, some strains have the capacity to produce carcinogens from dietary components or from intestinal secretions (Aries *et al.*, 1969*b*).

As part of our study of this hypothesis we have investigated the faecal flora and faecal steroids in a group of strict vegetarians who do not eat animal products of any kind, and compared the findings with those in English people living on a normal mixed diet. Though there was no gross difference in the faecal bacterial flora in the two groups, there were interesting differences in the concentration and composition of the faecal acid steroids and in the ability of the gut bacteria to degrade bile acids.

MATERIALS AND METHODS

Faeces were collected according to the method of Aries *et al.* (1969*b*); bacterial specimens were cultivated by the methods described by Drasar (1967) and Drasar and Crowther (1970); faecal steroid extractions and analyses were performed by the technique of Hill and Aries (unpublished), and the activity of bacteria in degrading bile salts was investigated by the method of Aries *et al.* (1969*a*). The results are presented in tables I and II.

DISCUSSION

The one striking difference between the faecal bacterial flora in the two groups was the occurrence of anaerobic sarcinae in faeces from the strict vegetarians (table I); these organisms have not previously been reported as being present in faeces from healthy adults, though we have isolated them in large numbers from faeces of a high proportion of people living on substantially vegetarian diets in Uganda and South India. Apart from this finding, there was no highly significant difference in the numbers of the major groups of bacteria in faeces from the two groups of people.

Although the faecal flora of the strict vegetarians was similar to that of people on a mixed diet, the strains of bacteria isolated from them were less active in degrading bile salts and acids *in vitro*. Only 20 per cent. of the cultures of *Bacteroides* spp. isolated from faeces of strict vegetarians had the enzyme 7-dehydroxylase (Aries and Hill, 1970) compared with 44 per cent. of those isolated from people on a mixed diet. The dehydroxylase enzyme produces mono- and un-substituted bile acids from the di- and tri-substituted cholanic acids secreted in the bile. Only 30 per cent. by weight of the faecal bile acids of the strict vegetarians are mono- or un-substituted compared with 49 per cent. of the faecal bile acids of people on a mixed diet (table II). Further, the total concentration of acid steroids is much lower in the faeces of strict vegetarians than in the faeces of people on a mixed diet. It has been shown that the faecal bile acid concentration depends on the amount of dietary fat (Hill,

Received 17 Apr. 1970; *accepted* 15 July 1970.

in press), and that strict vegetarians consume a smaller amount of fat than do people on a mixed diet (Ellis and Mumford, 1967).

TABLE I

Viable counts of bacteria in faeces from 40 adults living on mixed diets and 18 adults living on strict vegetarian diets

Organism	Mean $\log_{10}$ organism count (±standard error) per g wet weight faeces in adults on		P for difference between A and B
	a mixed diet* (A)	a strict vegetarian diet (B)	
Bacteroides	9·7±0·6	9·7±0·3	>0·05†
Bifidobacteria	9·9±0·3	9·6±0·4	>0·05†
Enterobacteria	7·5±1·2	7·0±1·8	>0·05†
Aerobic streptococci	7·0±0·8	6·6±1·2	>0·05†
Enterococci	5·7±1·3	4·8±1·3	>0·05‡
Lactobacilli	6·0±1·6	7·4±1·4	>0·05‡
Clostridia	4·5±1·7	2·7±1·9	>0·05‡
Veillonellae	4·5±1·9	4·8±2·0	>0·05‡
Yeasts	1·9±1·3	3·0±1·7	>0·05‡
Filamentous fungi	1·8±0·7	1·7±0·8	>0·05‡
Sarcinae	Not found	2·4±2·2§	...

* = Data from Aries *et al.* (1969*a*).
† = Values obtained from Student's *t* test and the χ^2 test.
‡ = Values obtained from the rank test and the χ^2 test.
§ = Organisms isolated from only six of 18 specimens.

TABLE II

The faecal acid and neutral steroids of strict vegetarians and of people on a normal mixed diet

Diet	No. of people tested	Neutral steroids (mg per g dry weight faeces)			Percentage composition of the acid steroids				Total acid steroids, (mg per g dry weight faeces)
		chol-esterol	copro-stanol	total	tri-substituted	di-substituted	mono-substituted	un-substituted	
Mixed	20	3·32	6·77	10·81	8	43	44	5	6·13
Strict vegetarian	15	1·91	5·88	8·91	18	52	28	2	3·50

A number of bile acids have been shown to be carcinogenic (Druckrey, Richter and Vierthaler, 1941; Lacassagne, Buu-Hoi and Zajdela, 1961, 1966). The bile acid, deoxycholic acid, can be chemically converted into the potent carcinogen, 20-methylcholanthrene (Haddow, 1958), and it is possible that bacteria can perform the same conversion. Such reactions would be more likely to occur in the intestine of people on a mixed diet since their gut bacteria are more active against bile acids, and the reaction could be more important in these people since the substrate concentration in their intestine is higher.

SUMMARY

As part of a study of the aetiology of cancer of the large bowel the faecal bacterial and fungal flora and the faecal steroids of English people living on a strict vegetarian diet have been compared with those of English people living on a mixed diet. There was no gross difference in the number or the species of organisms present in the two groups, but fewer of the species of *Bacteroides* in the faeces of strict vegetarians could dehydroxylate di- and tri-substituted cholanic acids, and the faeces of strict vegetarians contained relatively smaller quantities of mono- and un-substituted acid steroids, and lower concentrations of total bile acids, than those of persons on a mixed diet.

We would like to thank the strict vegetarians for their helpful co-operation in this study and to acknowledge the excellent technical assistance of Miss Deirdre Blann and Miss Rosalind Steward. This work was supported by the British Empire Cancer Campaign for Research.

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The Cultivation of Human Intestinal Bacteria

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The bacterial flora of the intestine is very complex. The aerobic bacteria, such as enterobacteria and enterococci, are easy to grow and attention has in the past focused upon them. However, non-sporing anaerobes were first isolated from faeces many years ago (Disato, 1914) and their numerical dominance was demonstrated over thirty years ago (Sanborn, 1931; Eggerth and Gagnon, 1933). Since anaerobic bacteria appear to be of great importance we have tried to develop methods for their quantitative recovery.

Although the aerobic bacteria in faeces probably protect the anaerobic bacteria by the production of an anaerobic micro-environment, preservation of specimens is essential if the relative numbers are to be maintained for any time.

The cultivation of oxygen-sensitive bacteria presents difficulties: using conventional techniques anaerobic bacteria are exposed to oxygen dissolved in diluents and culture media. The spreading of specimens on plates isolates single bacterial cells in the presence of oxygen and thus maximizes its lethal effects. Even in an anaerobic jar diffusion of dissolved gas from agar plates is slow and the exposure of anaerobic bacteria to oxygen is prolonged.

The preparation, dispensing and inoculation of media under a stream of oxygen-free gas, as suggested by Hungate (1950), has considerable advantages, but the manipulatory difficulties of the technique are considerable and, furthermore, plates cannot be used. We have used an anaerobic cabinet to overcome the manipulatory difficulties of the Hungate technique while preventing access of oxygen to the culture media during the preparation of culture plates, inoculation and incubation.

In addition to the preservation and cultivation of specimens the maintenance of pure cultures of non-sporing anaerobes is important.

The Preservation and Transport of Specimens

We have routinely preserved specimens of faeces and intestinal juice frozen as a 1:10 dilution in a glycerol broth containing 1% Lab Lemco (Oxoid) and 10% glycerol (Drasar, Shiner and McLeod, 1969). Detailed bacteriological analysis of replicate samples of faeces taken before freezing and at periods of up to one month after freezing showed no significant change in the viable counts of the various organisms present in the sample (Crowther, 1971, in preparation for *J. appl. Bact.*)

The Anaerobic Cabinet and its use

The cabinet (Fig. 1) consisted of an airtight Perspex glove box fitted with

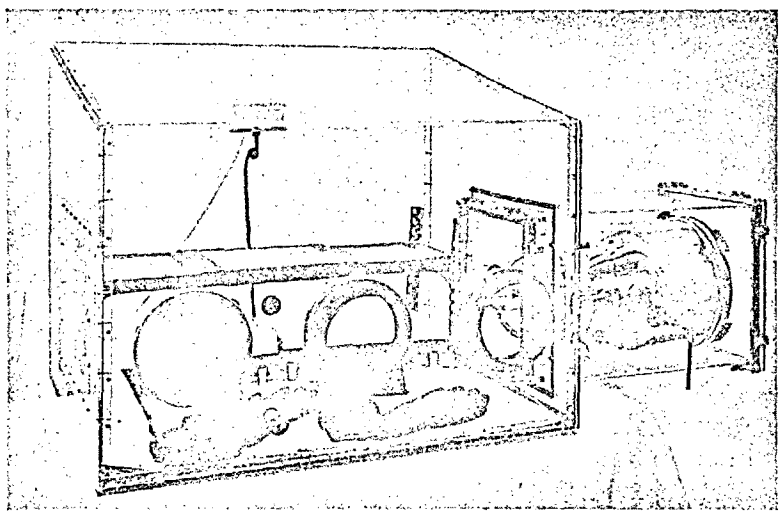

FIG. 1. The anaerobic cabinet. Within the body of the cabinet can be seen 2 photographic warming plates for drying plates. A converted milking machine pail is in the air-lock. The body of the cabinet (excluding the air-lock) is 3 ft long.

gas-tight doors. The bulk of the oxygen was removed from the cabinet by burning a spirit lamp within it; at the same time the cabinet was flushed at approximately 20 l/min with nitrogen containing 10% carbon dioxide; the final traces of oxygen were removed by recirculating the cabinet's atmosphere through a hot, copper-packed deoxygenating column* (Moore, 1966) at 27 l/min. During culture of bacteria a slight positive pressure was maintained in the cabinet; the gas flow was reduced to 5 l/min.

Stainless steel milking-machine pails with vacuum tight lids (Fullwood Bland and Co.) fitted with vacuum taps and cold catalysts ("D" catalyst, Engelhard Industries) were used in place of conventional anaerobic jars

* See also p. 134.

as suggested by Schaedler, Dubos and Costello (1965). When used in conjunction with the cabinet the pails were evacuated before being placed in the airlock.

Preparation of media for use in the cabinet

Reinforced Clostridial Agar (Oxoid) fortified with 1% liver digest (Oxoid) was used for the culture of the strict anaerobes. The pH was adjusted to 7·5 giving a final pH of 7·3 after autoclaving at 121°/15 min. The medium was prepared in bulk and stored until required. The agar was steamed with the cap loose, the cap being retightened on removal from the steamer. The molten agar was passed into the cabinet, the blood added, and the plates poured; they were dried on a warming plate.

TABLE 1. Media for the isolation of intestinal bacteria

Medium	Incubation Atmosphere	Incubation Days	Organism	Notes, sources and references
Reinforced Clostridial Agar	An	4	Total anaerobes	Oxoid with 1% liver digest and 10% horse blood. pH adjusted to 7·5±0·1 before autoclaving. Plates poured, dried and inoculated in cabinet.
Rogosa's Agar (V)	An	4	*Veillonella*	Rogosa (1956), Rogosa *et al.* (1958)
Tomato Juice Agar	An	1	Anaerobic sarcinae	Oxoid; pH adjusted to 7·0
Willis & Hobbs' Agar	An	4	Clostridia	Willis and Hobbs(1959) Neomycin 40 μg/ml
	An	1	Clostridia, Anaerobic sarcinae	Inoculum heated for 10 min at 70°. No antibiotics
Azide agar	0	1	Enterococci	Schaedler *et al.* (1965)
Blood agar	1	1	Total aerobes	
MacConkey's Agar	0	1	Enterobacteria, Enterococci	Oxoid
Mannitol Salt Agar	0	3	*Staphyloccus aureus*, *Bacillus* spp	Oxoid, incubate 30°
Rogosa's Agar (L)	90%CO_2	3	Lactobacilli	Rogosa *et al.* (1951)
Sabouraud's Agar	0	5	Yeasts, filamentous fungi	Chloramphenicol 40 μg/ml duplicates incubated 37° and 22°
S_1 Agar	0	2	*Streptococcus salivarius*	Williams and Hirsch (1950)
Nutrient agar	0	1	*Bacillus* spp	Inoculum heated for 10 min at 70°

O, aerobic; An, anaerobic (90% (v/v) H_2 and 10% (v/v) CO_2). Incubation, 37°.

Brain-heart infusion broth (Oxoid) with L-cysteine HCl (0·05%) was used as a diluent. The broth was steamed before use.

Inoculation of media

Table 1 gives details of the media routinely employed. Specimens were inoculated on to Reinforced Clostridial Agar into the cabinet; the other media were inoculated on the open bench.

Tenfold dilutions of the specimens were prepared in the cabinet. An 0·1 ml sample of each dilution was spread on to the prepared plates; these were then packed into an anaerobic jar in the airlock. The jar was removed and its atmosphere twice replaced by a mixture of 90% hydrogen and 10% carbon dioxide before incubation at 37°.

To count anaerobic sarcinae and spore formers serial dilutions of the specimen were heated for 10 min at 70° before plating out. After anaerobic incubation sarcinae could be readily distinguished (Crowther, 1971).

The Preservation of Strains of Strictly Anaerobic Bacteria

Strains of strictly anaerobic bacteria were preserved by the addition of sterile glycerol to a final concentration of 10% to a 4-day culture of the organism in Robertson's meat broth (Southern Group Labs.). The cultures were stored frozen at —20° or below. Over 80% of the cultures remain viable for at least 9 months. Cooked meat broth prepared from tablets (Oxoid) does not support the growth of non-sporing anaerobes and therefore should not be used.

Discussion

The media and methods described here enable us to count the major broad groups of bacteria present in the intestine. The methods are similar to those described previously (Drasar, 1967) but the use of reheated media in the cabinet simplifies the procedure for the cultivation of oxygen-sensitive bacteria.

Oxygen cannot dissolve in the media or diluents within the cabinet and this reduces the exposure of oxygen sensitive bacteria to its influence. The use of an anaerobic cabinet enables one to isolate numerous oxygen-sensitive bacteria not readily cultivated by conventional techniques (Drasar 1967, Lee, Gordon and Dubos, 1968; Aranki *et al.*, 1969).

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Reprinted from THE LANCET, *January* 16, 1971, *pp.* 95-100

BACTERIA AND ÆTIOLOGY OF CANCER OF LARGE BOWEL

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Summary The geographical variations in the incidence of carcinoma of the colon seem to be correlated with variation in the fat content of the diet. It was postulated that intestinal bacteria might be able to produce carcinogens from dietary fats or from bile steroids, and that the variations in the incidence of colon cancer might depend partly on differences in the composition of the intestinal bacterial flora brought about by the differences in diet. Samples of fæces from six areas were examined for their content of bacteria and steroids. Fæces from people in Britain and the U.S.A., where the incidence of colon cancer is high, had higher counts of *Bacteroides* and lower counts of enterococci and other aerobic bacteria than fæces from people in Uganda, South India, or Japan, where the incidence of the disease is low. Fæces from people in the " Western " countries contained higher concentrations of steroids than those from the African and Eastern countries, and the steroids were also more degraded. The results are consonant with the thesis that the intestinal bacteria may be ætiologically related to cancer of the colon.

Introduction

CANCER of the large bowel is much commoner in North-West Europe and in North America than it is in East Africa, Asia, and South America.[1-3] The areas with a low incidence of the disease, with the exception of Japan, have a low standard of living and the high-incidence areas have a high standard. These geographical differences do not seem to be explicable on a racial basis, since Japanese who migrate to California retain their low-incidence experience provided they retain their original cultural habits; the process of " westerni-

sation", either in California [4] or in Japan,[5] seems to be associated with a trend towards a higher incidence of colon cancer. Nor can geography alone explain the difference: the three racial groups in Hawaii differ in their incidence of colon cancer as do the different racial groups in South Africa.[3]

On the other hand, several studies have indicated a possible relation of colon cancer to diet—either fat [6] or protein.[7] Although the existence of carcinogenic compounds in cooked food has been postulated (e.g., benzpyrenes derived from cooking-oils, aflatoxins formed by moulds), none of these could explain the geographical distribution of colon cancer.

Dietary differences might, however, explain the observed differences in the disease if the diet determined the composition of the bacterial flora of the gut and if bacteria were able to produce carcinogenic (or cocarcinogenic) substances from food or from the intestinal secretions. Such a hypothesis has the added attraction that diet might have its effect both by altering the supply of the substrate for carcinogen production and by altering the numbers and nature of the bacteria available to act on it.

Although there are a few reports of the production of carcinogens by microorganisms, such as aflatoxin by *Aspergillus* and the release of the aglycone methoxyazomethanol from cycasin, there do not seem to be any reported studies of the possible production of carcinogens by gut bacteria from foodstuffs or secretions. However, some bile-acids have been shown to be carcinogenic—e.g., deoxycholic acid,[8–10] bis nor-Δ5-cholenic acid,[11] and apocholic acid.[12] Inhoffen [13] reviewed the structural similarity between steroids and the polycyclic aromatic carcinogens, and deoxycholic acid can be converted chemically into the very potent carcinogen 20-methyl-cholanthrene [14] via dehydronorcholene (fig. 1). Possibly bacteria can perform a similar conversion. Dehydronorcholene, which is not carcinogenic, can be converted into a carcinogenic

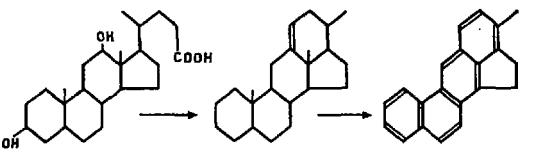

Fig. 1—Chemical conversion of deoxycholic acid to 20-methylcholanthrene via dehydronorcholene.

TABLE I—POPULATIONS STUDIED

Country of origin	*Incidence of colon cancer	Population studied	Diet
England	18·2	People working at St. Mary's Hospital Medical School	Normal Western mixed
Scotland	31·2	People working at Edinburgh University Medical School	" " "
U.S.A.	28·1	People working at Center for Disease Control, Atlanta	" " "
India	5·7	Villagers living close to Vellore, South India: controls in a study of tropical sprue	Rice diet, no meat and little fat
Uganda	0·6	Villagers living in Kyandondo region: parents accompanying children to kwashiorker clinic	Matoke (boiled mashed bananas)
Japan	4·9	Villagers living near Yonaga: mostly farmers and housewives	Fruits and vegetables. Little meat and fat

* Mean values for men and women, age-adjusted (R. Doll, personal communication).

metabolite by the action of intestinal bacteria.[15]

Evidence that diet can alter the composition of the intestinal bacterial flora was presented by Hoffmann,[16] but large-scale studies are lacking.

We have demonstrated that the change from a normal mixed diet to one low in fat is followed by a fall in the fæcal concentration of acid steroids,[17] presumably by reducing the rate of secretion of bile.

We report here a study in which we have examined the fæcal bacterial flora in groups of normal people living in six areas—three with a high incidence and three with a low incidence of colon cancer. We have also determined the composition and concentration of the acid steroids in the fæces of the same subjects and have investigated the ability of bacteria isolated from their fæces to degrade steroids or dietary fats. The results are consonant with the hypothesis that diet might influence the incidence of colon cancer and that the gut bacteria might play an important part in the ætiology of the disease.

Methods

Populations Studied

The characteristics of the groups of people included in this study are set out in table I. They were healthy individuals of each sex, mostly aged 20–50. The English were all staff, students, or nurses at St. Mary's Hospital and Medical School, London, and were mostly aged 20–30. The Scots were staff and students at Edinburgh University Medical School. The Americans were staff members at the Center for Disease Control, Atlanta, Georgia. All the people in these three groups lived on diets containing animal and vegetable matter and rich in animal fat and protein. The Ugandans were parents of children attending a malnutrition clinic in Kampala and all lived on a diet consisting largely of matoke (boiled mashed bananas) and containing very little animal matter and very little fat. The Indians were from a village close to Vellore in South India and were " healthy controls " in a study of tropical sprue. They lived on a rice-based diet containing little, if any, animal matter and very little fat. The Japanese were from a small village near Yonaga and were mostly from farming families. Their diet consisted largely of fruit and vegetables with very little fat or animal matter.

Completely reliable statistics for the incidence of carcinoma of the colon, or its mortality, in various countries are not available; in particular mortality statistics are likely to be very misleading in developing countries. However, Doll [3] has collected figures for the incidence of this disease from a number of special surveys (table I). The figures for the

United States refer to white individuals, whereas our results are from black and white; and the Indian figures are for Bombay, which is remote from the Southern Indian area in which our subjects lived, but there is no reason to think that there would be a substantial difference between the two areas.

Bacteriological Methods

Fresh samples of fæces were diluted 1/10 in 10% glycerol broth and immediately frozen in solid carbon dioxide or in liquid nitrogen for transport to London. We have not been able to detect any significant loss of living bacteria from samples of fæces stored in this way for over 1 month.[18]

The methods used for isolating and counting the bacteria have been described in detail by Drasar [19] and by Drasar and Crowther [20]; the definition of the bacterial groups are as described by Drasar,[19] except that, in accordance with current usage, the term " anaerobic lactobacillus " has been replaced by " *Bifidobacterium* ". The standard error of the means of groups of 24 replicate samples has been found to be of the order of 0·05 $\log_{10}$ counts. In order to provide some control on the bacteriological methods, samples of fæces from English subjects were included among each batch of specimens from other countries.

Preliminary investigations showed that, among adults, age and sex had no effect on the fæcal bacterial flora.

Biochemical Methods

Fresh samples of whole fæces were frozen in solid carbon dioxide or liquid nitrogen for transport and storage. The methods used for measuring and characterising the fæcal steroids are described in detail by Hill and Aries.[21]

Results

Fæcal Bacteria

The same broad groups of bacteria were found in fæces from all the populations studied, except that *Sarcina ventriculi* was present only in the fæces of the vegetarian people.[22] There were, however, substantial differences in relative numbers of several of the bacterial groups. The British and American subjects yielded many more of the gram-negative non-sporing anaerobes (*Bacteroides* spp.) than did the Ugandans, Indians, or Japanese. Conversely the Indians and Japanese had many more aerobic bacteria (streptococci and enterobacteria), so that the ratio anaerobes/aerobes was much higher in the people living on a Western diet than in those on the largely vegetarian diets (table II).

More detailed bacteriological studies revealed further differences. For example, the numbers of enterococci

TABLE II—BACTERIAL FLORA OF FÆCES

—	Mean $\log_{10}$ colonies per g. wet weight						
	England	Scotland	United States		Uganda	Japan	India
			White	Black			
No. of samples examined	68	23	22	12	48	17	51
Bacteroides spp.	9·8	9·8	9·7	9·8	8·2	9·4	9·2
Bifidobacterium spp.	9·8	9·9	10·1	9·9	9·3	9·7	9·6
Clostridium spp. lecithinase + ...	4·2	4·2	3·4	3·6	4·0	4·6	4·2
Clostridium spp. lecithinase −	5·7	5·6	5·5	4·9	5·1	5·5	5·7
Veillonella spp.	4·2	3·8	3·4	5·2	5·3	4·7	5·8
Sarcina ventriculi	−	−	−	−	5·2	+	4·6
Lactobacillus spp.	6·5	7·7	6·5	8·0	7·2	7·4	7·6
Bacillus spp.	3·7	3·3	3·6	3·9	4·5	4·4	4·9
Escherichia spp. and other enterobacteria ..	7·9	7·6	7·4	7·3	8·0	9·3	7·9
Streptococcus spp.	7·1	6·8	7·0	7·1	7·8	8·5	7·9
Enterococci	5·8	5·3	5·9	5·0	7·0	8·1	7·3
Total anaerobes	10·1	10·2	10·2	10·2	9·3	9·9	9·7
Total aerobes	8·0	7·7	7·5	7·5	8·2	9·4	8·2
$\text{Log}_{10}\frac{\text{total anaerobes}}{\text{total aerobes}}$	2·1	2·5	2·7	2·7	1·1	0·5	1·5

+ =Colonies found but not quantitated. − =None found in 0·1 g. fæces.

per gramme were $10^{5 \cdot 8}$ in fæces from English subjects and $10^{7 \cdot 3}$ in fæces from the Indians, but preliminary investigations indicate that, whereas 64% of the English enterococci were *Streptococcus fæcalis* and 33% *Str. fæcium*, only 22% of the enterococci from the Indians were *Str. fæcalis* and 65% were *Str. fæcium*. Thus, although the difference in the total count of enterococci was 30-fold, the difference in the count of *Str. fæcium* was 60-fold.

In an attempt to elucidate the factors leading to these differences we examined fæces from a small group of English people living in Uganda on a normal Western diet. These had a fæcal flora virtually the same as that of English people living in England on a mixed diet. Further, black Americans living in Atlanta had a fæcal bacterial flora qualitatively and quantitatively the same as white Americans in Atlanta. These results suggest that neither race nor climate is a major determinant of the fæcal bacterial flora. A small group of strict vegans living in London had a fæcal bacterial flora indistinguishable, except for the presence of *Sarcina ventriculi*, from that of the London people living on a mixed diet. The vegans, although living on a strictly vegetarian diet containing no animal matter whatever, nevertheless consumed as much protein and more than half the amount of fat consumed by people on a mixed diet.[23] The striking difference, therefore, between the diet of the British (including the vegans) and Americans and that of the remaining groups is the low fat intake of the latter.

Fæcal Steroids

Steroids were present in much lower concentration in fæces from the Ugandan, Indian, and Japanese than in the fæces of the British and Americans (table III); possibly the low cholesterol content of the diet of the former group resulted in a reduced rate of loss of steroids in fæces.[24]

The neutral-steroid concentration in fæces was very low in Ugandans and Indians, intermediate in Japanese, and high in British and Americans. Coprostanol and coprostanone, which are the bacterial metabolites of cholesterol and are formed in the large bowel,[25] constituted a much smaller proportion of the total neutral steroids in the Ugandans (55%), Indians (62%), and Japanese (43%) than in the Western group (English 69%, Scottish 71%, white Americans 64%, black Americans 72%). Except for the Indians, these differences are statistically significant and suggest that

TABLE III—STEROID CONCENTRATIONS IN FÆCES

	England	Scotland	United States		Uganda	Japan	India
			White	Black			
No. of samples examined	26	18	24	12	11	18	18
(*a*) *Total steroids*	16·86	16·28	16·73	16·64	2·37	6·40	2·02
(*b*) *Neutral steroids:*	10·81	10·10	10·73	10·37	1·82	4·52	1·51
(*c*) Cholesterol	3·32	2·89	3·83	2·93	0·81	2·59	0·57
(*d*) Coprostanol	6·77	6·68	6·19	6·79	0·90	1·89	0·73
(*e*) Coprostanone	0·72	0·53	0·71	0·65	0·11	0·04	0·21
% $\frac{d+e}{b}$	69	71	64	72	55	43	62
(*f*) *Acid steroids:*	6·15	6·18	6·00	6·27	0·45	0·88	0·51
(*g*) Trisubstituted	0·48	0·31	0·60	—	0·10	0·22	0·05
(*h*) Disubstituted Total	2·49	2·84	2·64	—	0·20	0·55	0·35
Dihydroxy	1·47	2·47	2·22	—	0·13	0·50	0·29
(*i*) Monosubstituted and unsubstituted ..	3·08	3·03	2·74	—	0·15	0·11	0·11
% $\frac{i}{f}$	51	49	46		33	13	21
Ratio *i*/*g*	6·4	9·8	4·6	—	1·5	0·5	2·2

the people living in Britain and North America, with a high incidence of colon carcinoma, have an intestinal bacterial flora that is much more active at degrading cholesterol than that in those from Uganda and Japan.

There are even larger differences in the fæcal concentration of acid steroids derived from bile-salts; the concentration of these was 11 times greater in the British and Americans than in the Ugandans and Indians and almost 7 times greater than in the Japanese. Moreover, there were corresponding differences in the degree of degradation. Dehydroxylation is one of the principal ways in which bile-acids are degraded by intestinal bacteria, and the ratio of monosubstituted and unsubstituted to trisubstituted cholanate gives a crude measure of the amount of dehydroxylation occurring during a single transit of the gut. This ratio was 9·8 for the Scots, 6·4 for the English, and 4·6 for the Americans, compared with only 1·5 for the Ugandans, 2·2 for the Indians, and 0·5 for the Japanese.

Ability of Pure Cultures of Bacteria to Degrade Bile-salts

The ability of pure cultures of various intestinal bacteria from the different countries studied were tested for their ability to degrade bile-salts. The bile degradative enzymes studied (fig. 2) were:

(*a*) Cholanoyl hydrolase, which deconjugates bile-salts, releasing the free bile-acid.

(*b*) Hydroxycholanoyl dehydrogenases, active on the 3α, 7α, and 12α hydroxyl groups.

(*c*) Hydroxycholanoyl dehydroxylase, which removes the 7α hydroxyl group from cholic acid (giving deoxycholate) and from chenodeoxycholate (releasing lithocholate).

Tests for these enzymes were performed on strains of a wide range of bacterial species and genera isolated

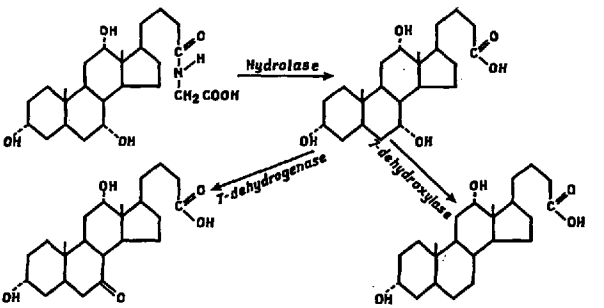

Fig. 2—Action of bile degradative enzymes studied.

from fæces of English and Ugandan people.[26] Lactobacilli, streptococci other than those of Lancefield's group D, *Bacillus* spp., yeasts, and fungi were virtually inactive, whilst the *Sarcina ventriculi* strains and the veillonellæ had enzymes of only low activity.

Many of the strains of *Bacteroides* spp., *Bifidobacterium* spp., group-D streptococci, and *Clostridium* spp., but no strain of *Escherichia coli*, possessed the cholanoyl hydrolase, whilst a large proportion of all the species just mentioned (including *Esch. coli*) were able to dehydrogenate the 7α hydroxyl group; a smaller number were able to dehydrogenate the 3α and 12α groups. There were no great differences in the proportion of English and Ugandan strains producing hydrolases and dehydrogenases. However, whereas very few Ugandan strains had 7α dehydroxylase, about 40% of the English *Bacteroides* spp., *Clostridium* spp., and *Bifidobacterium* spp. were able to perform this reaction, as were 11% of the enterococci (table IV).

In the examination of strains of bacteria from other countries only the *Escherichia coli*, group-D streptococci, *Bacteroides* spp., *Bifidobacterium* spp., and *Clostridium* spp. were tested (since English and Ugandan strains of the other genera had little or no steroidase activity) and only the 7α-dehydroxylase was examined (since high proportions of strains from both England and Uganda were able to perform the other reactions). Again, although very few Indian strains were able to 7α-dehydroxylate, 40–50% of the anaerobic bacteria of American and Scottish people were able to perform this reaction. Thus not only are there many more of the non-sporing anaerobic bacteria in the fæces of people from the areas in which the incidence of colon carcinoma is high, but a much higher proportion of their strains are able to perform this very important bile-degradation reaction.

Discussion

The conclusion from these investigations can be no more than tentative because of the small numbers of subjects whom we have been able to study and the considerable element of doubt surrounding the real incidence of colon carcinoma in various countries. Nevertheless the results strongly support the postulate that the geographical differences in the incidence of colon carcinoma may be related to dietary habits, and that these could operate through their influence on the nature and numbers of the intestinal bacteria.

TABLE IV—7α-DEHYDROXYLASE PRODUCTION BY INTESTINAL BACTERIA

	England		Scotland		United States		Uganda		India	
	A	B	A	B	A	B	A	B	A	B
Bacteroides spp.	50	44	24	56	10	50	42	33	20	5
Bifidobacterium spp. . .	57	40	24	56	10	50	137	4	20	5
Clostridium spp.	70	34	38	45	10	60	60	6	14	0
Enterococci	90	11	30	40	0	—	162	3	24	0
Enterobacteria	87	0	30	0	0	—	180	0	32	0

A = no. of strains tested. B = % of strains with this activity.

People living in the areas with a high recorded incidence of carcinoma of the colon tend to live on diets containing large amounts of fat and animal protein, whereas those who live in areas with a low incidence live on largely vegetarian diets with little fat or animal matter. The latter group have a fæcal flora containing relatively larger numbers of streptococci (especially of group D) and enterobacteria than the former and somewhat smaller numbers of bacteroides; the relation between the counts of these groups, and also between the ratio, total anaerobes/total aerobes, and

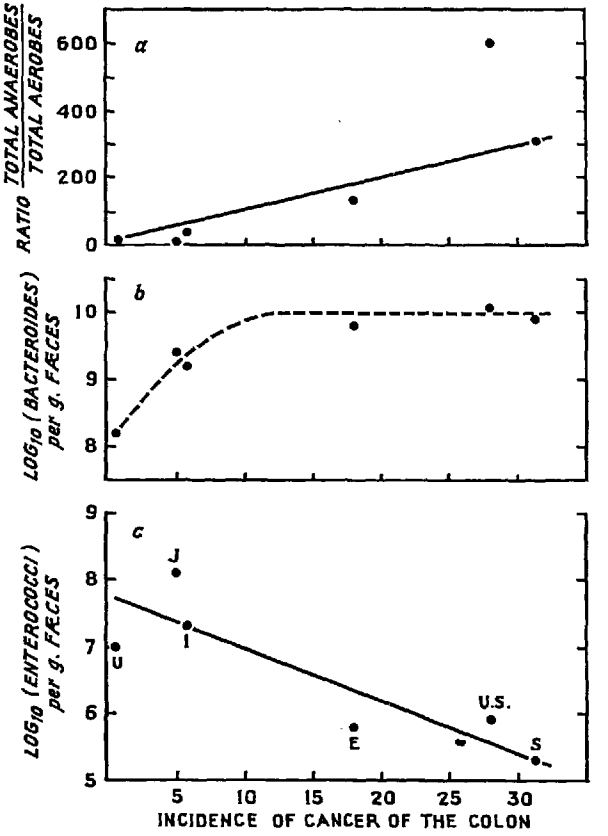

Fig. 3—Relationship between (a) ratio of anaerobes/aerobes in fæces; (b) log count of bacteroides per gramme wet weight of fæces; (c) log count of enterococci per gramme wet weight of fæces, and incidence of cancer of colon in countries studied.

U=Uganda, J=Japan, I=India, E=England, U.S.=United States, S=Scotland.

the incidence of cancer of the colon are shown in fig. 3.

In general, the anaerobic bacteria metabolise steroids more actively than the aerobes. The high-fat diet which is associated with the outgrowth of the steroid-degrading organisms also results in a high fæcal concentration of acid steroids. On average the people living in high-risk areas have about 5×10^9 bacteria per g. of fæces able to dehydroxylate cholic acid at the 7α position, compared with only about 5×10^7 per g. of fæces in people from the low-risk areas. It is therefore hardly surprising that the fæcal steroids are much more extensively dehydroxylated in people from high-risk areas than in those from low-risk areas.

It has been suggested that deoxycholic acid, which is recognised to be produced by bacterial degradation of bile-salts, is in certain circumstances a carcinogen; in the light of this it is striking how closely the incidence of colon carcinoma in the six countries appears to correlate with the fæcal concentration of dihydroxycholanic acids (most of which is almost certainly deoxycholic acid) in our subjects (fig. 4a). This correlation might be fortuitous, but in view of the previous work it makes one suspect that deoxycholate may indeed be a carcinogen, or a pre-carcinogen or co-carcinogen. One can calculate that the total cumulative dose of deoxycholate passing through the colon in fifty years might be some 1300 g., so that it need not be a very active agent to account for the incidence of the disease in Londoners of about 18 per 100,000 (in the age-group 45–64). The correlation of the cancer incidence with the fæcal concentration of dihydroxy acid is closer than that with total acid, or total neutral steroids, but the magnitude of the differences is actually greater for the total steroids than for the dihydroxy acids.

We have started an investigation of the possibility that bacteria might modify the steroid nucleus of the bile-salts to produce recognisable polycyclic aromatic hydrocarbon carcinogens. This requires the insertion into the nucleus of double bonds. We have already shown that two double bonds can be inserted in conjugation with a keto group.[27] There are several alternative routes by which bacteria can unsaturate the steroid nucleus,[28] some of which have been demonstrated with soil bacteria, and it is now necessary to determine the extent to which intestinal bacteria can aromatise the nucleus in vitro so that we can seek the

partially or fully aromatised molecules in fæces.

We have also demonstrated that intestinal bacteria can produce nitrosamines from nitrate and secondary amines.[29] Nitrosamines are well known to be potent carcinogens, and both nitrate and secondary amines are present in the gut. However, it is likely that most of the dietary nitrate is absorbed from the upper small

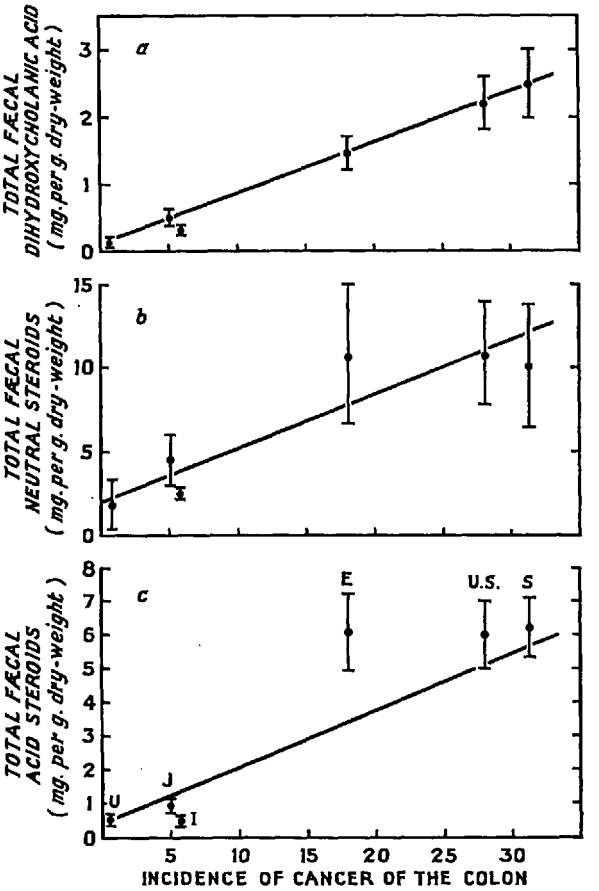

Fig. 4—Relationship between fæcal concentration of (a) dihydroxycholanic acid (mg./g. dry weight fæces); (b) total neutral steroids (mg./g. dry weight fæces); (c) total acid steroids (mg./dry weight fæces) and incidence of cancer of colon in countries investigated.

U=Uganda, J=Japan, I=India, E=England, U.S.=United States, S=Scotland. The steroid concentrations are shown as mean ± standard deviation.

intestine undegraded, so that opportunities for nitrosamine formation in the area of the gut with a high bacterial population may be small; indeed, the production of nitrosamines is more likely to take place in the urine of people with bladder infections. Present evidence suggests that nitrosamines rarely produce tumours at the site of application.[80]

There is clearly much work to be done before we can pretend to understand the ætiology of carcinoma of the colon, but we believe that the results of our present survey indicate that further exploration of the part played by bacteria may well be very rewarding.

This work was supported financially by the Cancer Research Campaign (M. J. H., V. C. A., and J. S. C.), the Wellcome Foundation (B. S. D.), and the British Nutrition Foundation (G. M. H.). We are greatly indebted to colleagues who arranged for the collection and dispatch of the fæcal samples: Dr. R. Blowers (Uganda), Dr. P. Brachman (Atlanta), Dr. T. Shigematsu (Yonaga), Prof. S. Baker (Vellore), and Dr. G. Collee (Edinburgh); and to Prof. Richard Doll for much helpful advice.

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22-—MICROBIOLOGY

SARCINA VENTRICULI IN HUMAN FAECES

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Received 29 July 1970; *accepted* 10 Sept. 1970.

SARCINA VENTRICULI was first observed by Goodsir (1842) in the stomach contents of a patient with gastric fermentation. The organism was subsequently reported in similar cases, but it was not isolated in pure culture from the human stomach until 1911, when Beijerinck showed that *S. ventriculi* could be grown only by using strictly anaerobic techniques; he had previously shown that the same organism occurred widely in the soil (Beijerinck, 1905).

Although *S. ventriculi* has been found frequently in the gastric contents and faeces of patients with gastro-intestinal disorders (Smit, 1933), it has never been reported as present in the faeces of healthy human adults. However, during a survey in which faeces from healthy adults in Britain, the United States, Uganda and South India were examined (Aries *et al.*, 1969), *S. ventriculi* has been found in the faeces of some persons.

This paper describes the techniques used to isolate sarcinae and reports the distribution of the organism in human faeces.

MATERIALS AND METHODS

Source of faeces. Specimens of freshly voided faeces were collected from 106 people living on principally vegetarian diets and from 123 people living on mixed diets containing animal and vegetable foods. Each group contained men and women and all except one 8-yr-old Sudanese girl were adults.

Transport of faeces. Specimens of freshly voided faeces were transported to the Wright-Fleming Institute frozen in glycerol broth (Drasar, Shiner and McLeod, 1969) from all sources except the London vegetarians in which case the faeces were sent by post and frozen in glycerol broth immediately on arrival.

Isolation of sarcinae. Sarcini ventriculi is difficult to maintain alive in pure culture. However, it is known to form heat-resistant spores (Knöll, 1965; Canale-Parola, 1970) and on primary isolation from soil and mud it is relatively resistant to acid and to heat at 60°C (Smit); these properties provided the basis for selective isolation methods.

The faecal suspensions were thawed, serially diluted in Brain Heart Infusion (Oxoid) and heated for 10 min. in a waterbath at 70°C; 0·1 ml of each dilution was spread on freshly poured meat infusion agar containing lactose, neutral red and egg-yolk (Willis and Hobbs' Medium without neomycin), as described by Willis and Hobbs (1958), and incubated overnight at 37°C in an atmosphere of 90 per cent. hydrogen and 10 per cent. carbon dioxide. Stainless steel milking-machine pails with vacuum-tight lids (Fullwood Bland and Co.) fitted with vacuum taps and cold catalysts (" D " catalyst, Engelhard Industries) as described by Schaedler, Dubos and Costello (1965) were used in place of conventional anaerobic jars. The heating destroyed all non-sporing organisms. Colonies of sarcinae could readily be distinguished from those of clostridia and aerobic spore-bearing bacilli. Sarcinae formed pale yellow colonies 2–4 mm in diameter, and were usually surrounded by a yellow halo

in the medium. Colonies were examined microscopically and subcultured aerobically and anaerobically.

Sarcinae were also isolated by plating either unheated or heated dilutions on freshly poured Tomato Juice Agar (Oxoid) adjusted to *p*H 7·0 and incubated anaerobically (Crecelius and Rettger, 1943).

Malt extract enrichment cultures designed to isolate *S. ventriculi* from the soil (Canale, Parola and Wolfe, 1960) were adjusted to several *p*H values over the range 2·3 to 6·0, inoculated with faeces and incubated anaerobically. Cultures were examined daily for sarcinae by plating on Willis and Hobbs' medium and incubating anaerobically.

Maintenance of strains for biochemical tests. Cultures of sarcinae isolated from faeces were maintained at +3°C in Robertson's cooked meat broth (Southern Group Laboratories) to which 1 per cent. of glucose has been added before they were autoclaved at 115°C for 20 min. The medium did not require deoxygenation before inoculation. Cultures were grown overnight at 37°C and stored at +3°C. With this technique, strains have remained viable for 6 mth without subculture.

A culture of *S. ventriculi* (HU1) originally isolated from soil (Stephenson and Dawes, 1970) was obtained from Professor E. A. Dawes, Department of Biochemistry, University of Hull, and maintained as described above.

Biochemical tests

Detection of cellulose. Two methods were used to detect cellulose in overnight cultures in basal medium containing 2 per cent. glucose. The bacterial cells were boiled in 1 per cent. sodium hydroxide, washed with water and suspended in Schultze's stain for cellulose (Canale-Parola, Borasky and Wolfe, 1961). The cellulose stained reddish-purple. Cells from each overnight culture were suspended in buffer at *p*H 4·0 containing cellulose (British Drug Houses) and incubated overnight at 37°C. The cultures were examined microscopically for disruption of the double tetrad packets into smaller clusters and single cells. Suspensions without cellulase were used as controls.

Fermentation tests. The fermentation tests were modifications of those described by Cato *et al.* (1969).

2·0 g of the test carbohydrate was added to a 100-ml volume of basal medium containing: 2·0 g peptone, 1·0 g yeast extract, 0·05 g cysteine hydrochloride, 0·001 g $CaCl_2$, 0·001 g $MgSO_4$, 0·005 g K_2HPO_4, 0·005 g KH_2PO_4, 0·05 g $NaHCO_3$ and 0·01 g NaCl. The media were adjusted to *p*H 7·1±0·1 with 8N-NaOH or 5N-HCl and autoclaved at 115°C for 20 min. They did not require deoxygenation before inoculation. Two drops of an overnight culture in Robertson's cooked meat broth containing 1 per cent. glucose were used as the inoculum.

Gas-liquid chromatography. Volatile products of glucose metabolism (ethanol and acetic and butyric acids) were detected by modifications of the methods described by Cato *et al.*

4 ml of an overnight culture in basal medium containing 2·0 per cent. glucose were acidified with 0·5 ml 50 per cent. aqueous sulphuric acid and extracted with 4 ml diethyl ether. The mixture was centrifuged to break the emulsion and the ether layer was pipetted off and dried over magnesium sulphate. 1·0 µl was injected on to a column containing 10 per cent. polyethylene glycol on phosphoric acid-treated diatomite contained in a " 104 " Series chromatograph (Pye Instruments Ltd, Cambridge). A flame-ionisation detector was used. Reference solutions of ethanol and volatile fatty acids were prepared as described by Cato *et al.*

Nitrate reduction. 2·0 per cent. glucose was added to Nitrate Broth (Difco) and autoclaved at 115°C for 20 min. Cultures were incubated anaerobically at 37°C. Nitrate and nitrite were detected by conventional procedures (Cowan and Steel, 1965).

Gelatin liquefaction. Charcoal gelatin disks (Oxoid) were added to Robertson's cooked meat broth containing 1 per cent. glucose; the test organism was inoculated and the culture

was incubated at 37°C for 14 days. Liquefaction was indicated by the appearance of free charcoal particles in the medium.

Fermentation tests. All test cultures were incubated anaerobically.

RESULTS

Sarcinae were readily isolated on either tomato juice agar or Willis and Hobbs' medium from faeces of people living on vegetarian diets. Tomato

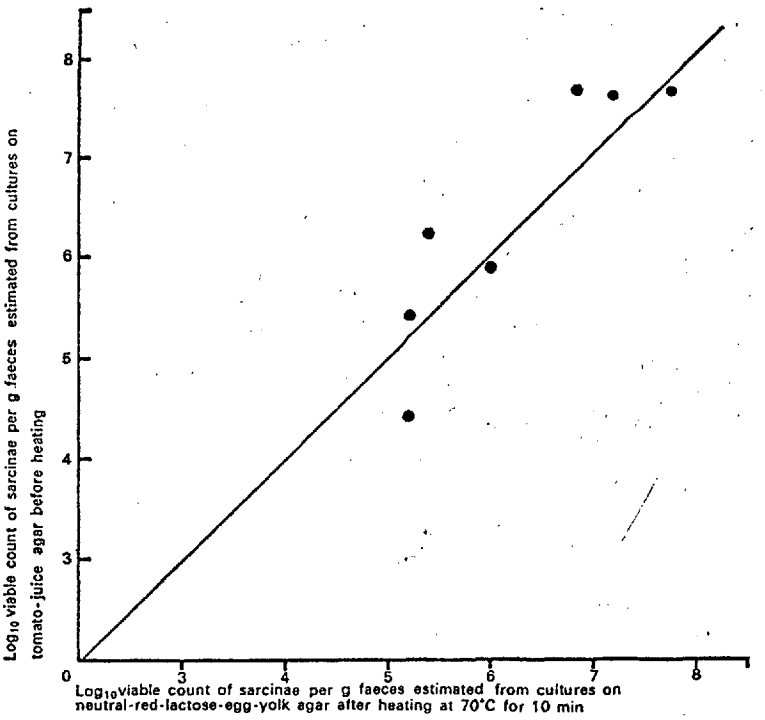

FIG. 1.—Comparison of methods of enumeration of viable *Sarcina ventriculi* in seven specimens of human faeces.

juice agar was the more selective for unheated specimens, but the heating of suspensions before they were plated on Willis and Hobbs' medium avoided overgrowth of the sarcinae by non-sporing organisms without consistently decreasing the viable counts of sarcinae (fig. 1).

It was not found possible to increase the yield of sarcinae from faeces by the use of the acid enrichment media described by Canale-Parola and Wolfe for the isolation of *S. ventriculi* from soil. Although cultivation at *p*H 4·0 was highly selective, many strains of sarcinae were inhibited. At higher *p*H values the sarcinae were often outgrown by other organisms.

Distribution of sarcinae in human faeces

The simple heating method with subculture on Willis and Hobbs' medium was used to count sarcinae in faeces from healthy adults living on different

TABLE I

The occurrence of anaerobic sarcinae in faeces from healthy adults on vegetarian and on mixed diets

Population sampled	Diet of population sampled	Number of specimens	
		cultivated	found to contain viable sarcinae
Ugandans living in and around Kampala	Vegetarian	37	31
Indians living in and around Vellore	Vegetarian	51	38
Vegans living in London	Vegan	18	6
London students and laboratory staff	Mixed	55	0
Edinburgh students and laboratory staff	Mixed	23	0
English immigrants in Kampala	Mixed	16	0
Caucasian laboratory staff in Atlanta	Mixed	16	1
Hospital staff in Khartoum	Mixed	13	1*

* Eight-year-old girl.

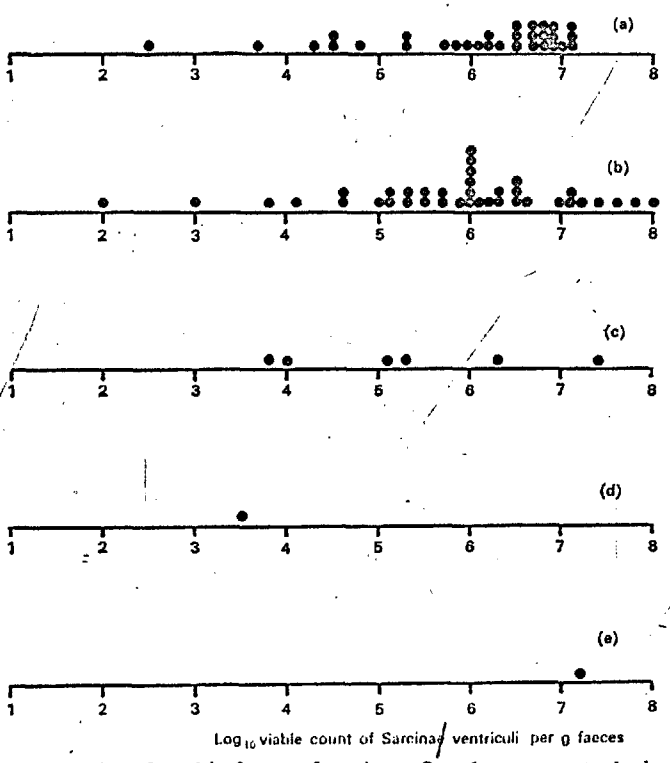

FIG. 2.—Numbers of sarcinae found in faeces of carriers. One dot represents the $\log_{10}$ viable count of sarcinae in the faeces of one person. The people lived in: (a) Kampala; (b) Vellore, South India; (c) London (these people ate strictly vegetarian diets); (d) Atlanta; (e) Khartoum. The sole carrier from Khartoum was an 8-yr-old girl.

diets in various parts of the world. Sarcinae were isolated from 75 out of 106 people living on principally vegetarian or strict vegetarian diets, but from only 2 out of 123 people eating mixed diets containing animal and vegetable foods (table I). In the carriers, the counts of sarcinae ranged from 10^2 to 10^8 per g faeces (fig. 2). Counts of 10^6 and more were frequently found and this indicates

TABLE II

Comparison of six faecal strains of Sarcina ventriculi with S. ventriculi strain HU1 isolated from soil

Test	Result of test with strains of *S. ventriculi* derived from				Results with strain HU1 derived from s oil
	Uganda (1) India (1)	Uganda (1) India (1)	Uganda (1)	England (1)	
Aerobic growth	−	−	−	−	−
Growth without carbohydrate	−	−	−	−	−
Fermentations at 30 days:					
glucose	+	+	+	+	+
fructose	+	+	+	+	+
sucrose	+	+	+	+	+
maltose	+	+	+	+	+
lactose	+	+	+	+	+
galactose	+	+	+	+	+
raffinose	+	+	+	+	+
arabinose	−	−	−	−	−
dulcitol	−	−	−	−	−
glycerol	−	−	−	−	−
starch	−	−	−	−	−
salicin	−	+	(+)	(+)	−
mannitol	−	−	−	(+)	−
inulin	−	−	+	−	(+)
xylose	−	−	−	(+)	−
Production of:					
ethanol from glucose	+	+	+	+	+
butyric acid from glucose	−	−	−	−	−
extracellular cellulose	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Litmus milk	(AC)	(AC)	(AC)	(AC)	AC
Gelatin liquefaction	−	−	−	−	−

* + = Positive; − = negative; (+) = positive after 7 days; AC = acid clot; (AC) = acid clot after 7 days.

that sarcinae were often as numerous as coliform organisms, streptococci and lactobacilli in faeces.

Identification of sarcinae

Sarcinae are now defined as obligately anaerobic, Gram-positive cocci forming cubical packets of eight (Kocur and Martinec, 1965; Canale-Parola, Mandel and Kupfer, 1967). Only two species capable of utilising sugars are recorded, *Sarcina maxima* and *S. ventriculi* (Breed and Smit, 1957). The main differences between them are that *S. maxima* has no extracellular cellulose and

produces butyric acid from glucose, whilst *S. ventriculi* has extracellular cellulose and produces ethanol and not butyric acid from glucose.

Six strains of sarcinae, three from Ugandan, two from Indian and one from English faeces, were compared with a known strain of *S. ventriculi*. No type culture of *S. maxima* was available. All the strains were obligate anaerobes, formed cellulose and required a carbohydrate for growth; all produced ethanol but not butyric acid from glucose (table II). All strains produced acid and gas from glucose, fructose, sucrose, maltose, lactose, galactose and raffinose; none could utilise arabinose, dulcitol, glycerol or starch; salicin, mannitol, inulin and xylose were utilised by some strains, but growth was usually late. All strains, including the known culture of *S. ventriculi*, reduced nitrate to nitrite which was then further reduced.

Although some strain-to-strain variations in biochemical reactions were found, the results show that the sarcinae isolated from human faeces were *S. ventriculi*.

Discussion

The results of the present study indicate that *Sarcini ventriculi* occurs frequently in the faeces of healthy human adults living in the tropics, but only rarely in faeces of people in temperate countries. However, none of the English immigrants in the Kampala series carried sarcinae whereas these organisms were carried by many of the Ugandans. The diet of the English immigrants was mixed and included animal meat whereas the principal item of the Ugandan diet was boiled bananas; furthermore, in London, sarcinae were found only in those English persons eating strictly vegetarian diets. This suggests, therefore, that diet and not environment is a major factor controlling the distribution of *S. ventriculi* in man.

S. ventriculi is widespread in the soil (Smit, 1933) and it seems inevitable that it is ingested as a contaminant on food. Since it occurs in facees in numbers equal to those reported for coliforms, it seems likely that it multiplies, presumably in the larger intestine, as the stomach and small intestine are rarely colonised by bacteria (Drasar *et al.*, 1969). It is not known whether vegetarian foods stimulate the growth or whether animal foods inhibit the growth of the sarcinae. However, it seems likely that the addition of animal foods to the diet leads to an increase in the numbers and possibly a change in the types of the non-sporing anaerobes such as *Bacteroides* species and bifidobacteria (Aries *et al.*, 1969). It is possible that some metabolites of these anaerobes, particularly organic acids, could inhibit the sarcinae. The short-chain fatty acids are known to inhibit *S. ventriculi* (Smit).

It is interesting that the known culture of *S. ventriculi* and the six strains isolated from faeces all reduced nitrate to nitrite which was then further reduced. Previous workers have not reported this reduction of nitrate, perhaps because, having shown that nitrite was absent, they omitted to test for residual nitrate.

Although *S. ventriculi* was often observed during the last century in the stomach contents of patients with gastric disorders, similar cases have been reported only rarely since. It is therefore interesting that Dawes (personal

communication), during the period 1959–60, found *S. ventriculi* in the stomach contents of patients in Glasgow with similar symptoms to those of the patient reported by Goodsir (1842).

It is suggested that *S. ventriculi* should now be considered to be part of the intestinal bacterial flora of man, but its significance in the intestine is not known.

SUMMARY

Anaerobic sarcinae were quantitatively isolated from suspensions of faeces heated at 70°C for 10 min. by plating on meat infusion agar containing lactose, neutral red and egg-yolk and incubating anaerobically at 37°C. *Sarcina ventriculi* was found in numbers up to 10^8 per g in faeces from 75 out of 106 healthy human adults living on vegetarian diets, but the organism occurred in the faeces of only 2 out of 123 people living on diets containing animal products. The identification and distribution of the organism are discussed and it is concluded that diet influences the colonisation of sarcinae in the human intestine.

I am indebted to Dr R. Blowers (at the time Professor of Bacteriology at Makerere University College, Kampala, Uganda), Professor S. J. Baker (Wellcome Research Unit, Christian Medical College Hospital, Vellore, India), Dr P. S. Brachman (National Communicable Disease Center, Atlanta, Georgia), Dr J. G. Collee (University Medical School, Edinburgh) and Dr F. R. Ellis (Kingston and Long Grove Hospitals, Kingston-upon-Thames, Surrey) for collecting the faecal specimens. Professor E. A. Dawes (Department of Biochemistry, University of Hull) kindly gave me the culture of *S. ventriculi* and allowed me to use some of his unpublished results. I thank Miss R. Steward for her technical help. The work was supported by the British Empire Cancer Campaign.

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Transport and Storage of Faeces for Bacteriological Examination

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(*Received* 12 *October* 1970)

SUMMARY. The survival of bacteria in specimens of human faeces transported and stored at different temperatures was examined quantitatively. No significant losses of bacteroides, bifidobacteria, *viridans* streptococci, *Streptococcus salivarius*, enterococci, enterobacteria, and clostridia were observed when 10% (w/v) suspensions of faeces in broth containing 10% (v/v) of glycerol were frozen for one month at −25, −78 or −196°; lactobacilli showed significant losses at −196°; veillonellae showed significant losses at −25°. At −12° veillonellae and enterobacteria showed significant losses. Most bacteria survived well for 24 h in undiluted faeces sent by post.

WHEN FAECES have to be transported considerable distances for quantitative bacteriology, it is necessary to have a method that permits neither growth nor death of any bacteria present. Stuart (1959) designed a low nutrient, soft agar medium for transporting delicate pathogenic bacteria at ambient temperatures and Cary & Blair (1964) modified it to improve the survival of enteric pathogens in faecal specimens without the growth of nonpathogens. Although the Cary-Blair medium and further modifications of Stuart's medium give good recoveries of *Escherichia coli*, *Clostridium perfringens*, *Proteus vulgaris*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella* spp. (Gästrin, Kallings & Marcetic, 1968), a transport medium has never been designed to recover all groups of bacteria quantitatively from faeces. For transport by post, moreover, fluid media are unsatisfactory, since it is often difficult to avoid contamination.

Drasar, Shiner & McLeod (1969) reported no apparent reduction in viable counts of *Bacteroides melaninogenicus* and *E. coli* when pure cultures were suspended in broth containing 10% v/v of glycerol and frozen on dry ice, and they used this technique to transport specimens of intestinal juice for quantitative bacteriological examination.

This paper reports the effects of storage at different temperatures on the recovery of bacteria from faeces.

Materials and Methods

The technique used by Drasar, Shiner & McLeod was to dilute intestinal juice in 10% (v/v) glycerol broth before freezing. This technique was therefore tested as a method for preserving the oxygen sensitive, strictly anaerobic bacteria that comprise >99% of the viable bacteria in faeces.

Glycerol broth

This was prepared by dissolving 10 g Lab-Lemco (Oxoid) and 10 ml of glycerol in 90 ml of water, adjusting the pH to 7·3 and autoclaving at 110° for 20 min. For control experiments the glycerol was replaced with distilled water.

Methods of storing faeces

The survival of faecal bacteria was tested both in 10% (w/v) suspensions of faeces in glycerol broth, and in undiluted faeces. The suspensions were stored at −196° (in sealed ampoules immersed in liquid nitrogen), −78° (in screwcapped bottles in solid CO_2) and at −25° and −12° in refrigerators. Whole faeces were stored at −196°, −78°, 3°, 15°, 32° and 37°. Further samples of undiluted faeces were examined after up to 48 h in the post.

Several replicate samples of each specimen of faeces were kept at each temperature, one being examined on each day of testing. All samples were from one individual.

Bacteriological technique

Specimens were examined bacteriologically immediately before storage (or posting) and at intervals for up to 1 month.

The dominant bacteria in faeces are nonsporing anaerobes, bacteroides and bifidobacteria. To minimize the exposure of these delicate organisms to air, all manipulations of the specimens were carried out in a gas-tight glove box filled with oxygen-free nitrogen (Drasar, 1967). Suspensions were diluted in triplicate in Brain-Heart Infusion (BHI) broth (Oxoid), containing 0·05% (w/v) of cysteine hydrochloride as a reducing agent, and inoculated on to a series of selective and nonselective media to enumerate bacteroides, bifidobacteria, enterobacteria, *viridans* streptococci, enterococci, lactobacilli, *Streptococcus salivarius*, veillonellae and clostridia as described by Drasar & Crowther (1971) (see Table 2). To minimize minor fluctuations each medium was poured on each day of testing from stocks prepared on one single occasion. All dilutions and inoculations were carried out by the same person.

Analysis of variance was used to assess the statistical significance of any changes in the viable counts.

Results

Storage of faeces at subzero temperatures

The total viable count for undiluted faeces stored overnight at −78° was only 94% of the initial count (Table 1); the percentage survival for faeces suspended in glycerol broth was 99, which is not significantly different from 100%. The glycerol and not the dilution in the broth appeared to be the protective factor.

Further tests showed that many different bacterial species survived well when the faeces were suspended in glycerol broth and held at a wide range of subzero temperatures (Figs 1, 2). With 2 exceptions, all groups of bacteria survived at −196, −78 and −25° with no significant losses after 1 month. Lactobacilli showed a significant reduction in count immediately on freezing at −196°, but once frozen showed no

TABLE 1

Survival of anaerobic bacteria in faeces frozen at −78°

	Mean log viable count/g wet faeces			
	Before freezing	After freezing: Faeces diluted (1 : 10) in broth containing		
		Undiluted faeces	No addition	10% (v/v) glycerol
Viable count	9·8	9·2	9·1	9·7
% viability	100	94*	93*	99

* Significant loss: $P = < 0{\cdot}01$ (obtained from Student *t*-test).

further losses. Veillonellae, although stable at −196 and −78°, showed slight but significant losses when stored at higher temperatures.

The survival of bacteria in faecal suspensions held at −12° was not as good as at the lower temperatures; enterobacteria and veillonellae showed significant losses, but all other groups survived well.

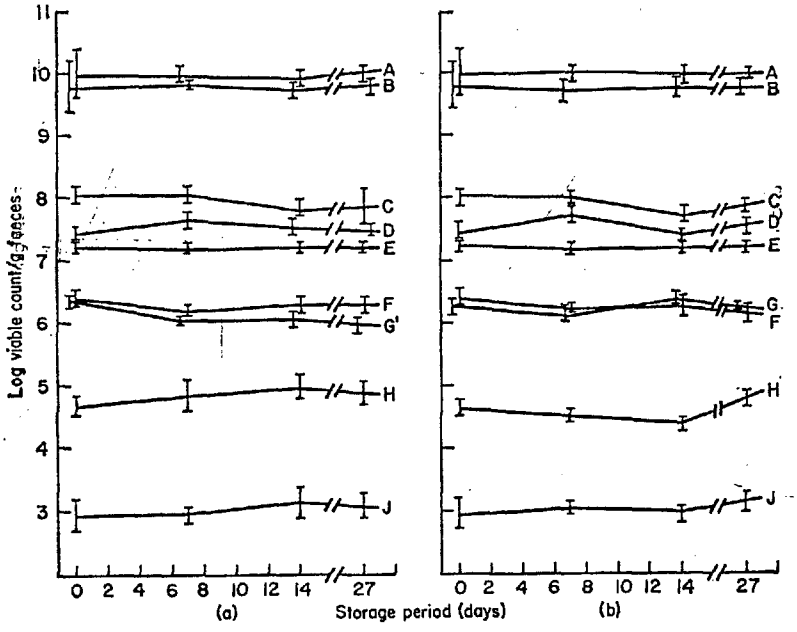

Fig. 1. Survival of bacteria in faeces suspended in glycerol broth. (*a*) Suspensions frozen at −196°; (*b*) suspensions frozen at −78°. A, bifidobacteria; B, bacteroides; C, viridans streptococci; D, *Streptococcus salivarius*; E, enterobacteria; F, enterococci; G, lactobacilli; H, veillonellae; J, clostridia. The vertical bars show the range of triplicate counts. [1], Significant reduction in viable count ($P = 0{\cdot}05$).

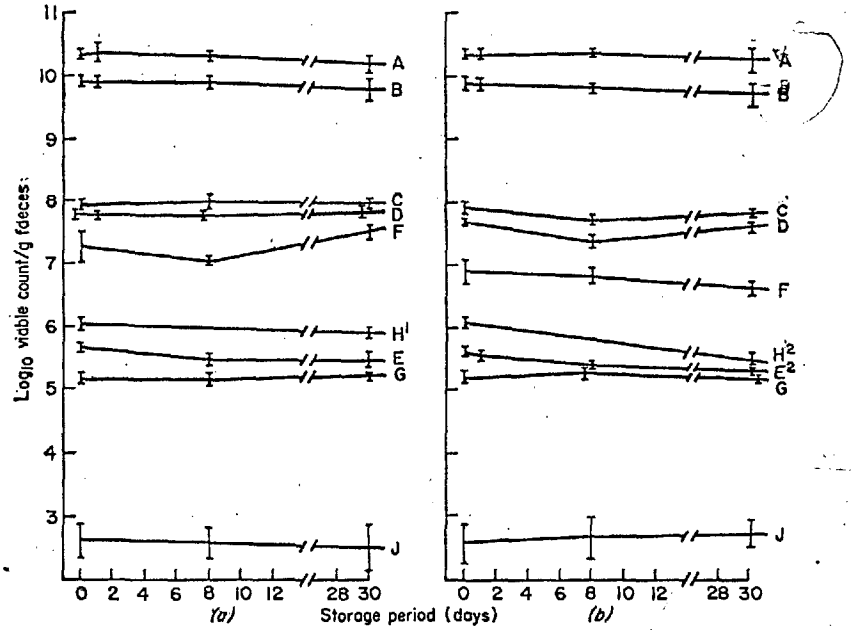

Fig. 2. Survival of bacteria in faeces suspended in glycerol broth. (*a*) Suspensions frozen at −25°; (*b*) suspensions frozen at −12°. For description of signs, see Fig. 1. [1], Significant reduction in viable count ($P = 0{\cdot}05$); [2], Significant reduction in viable count ($P = 0{\cdot}01$).

Storage of faeces at temperatures above zero

Survival curves for bacteria in undiluted faeces stored in air at temperatures above zero are shown in Fig. 3. No organism showed a significant change in viable count after storage at 3° for 24 h, and even after 8 days the counts of bifidobacteria, enterobacteria, lactobacilli and clostridia had not changed significantly.

At 15° counts of bifidobacteria, bacteroides, viridans streptococci, veillonellae and clostridia remained unchanged after 48 h. At 32° there was an increase in the counts of enterobacteria and lactobacilli, and a substantial decrease in the counts of veillonellae and *Strep. salivarius*. These changes were more pronounced when the specimens were stored at 37°. The viable counts of the dominant anaerobes, bacteroides and bifidobacteria, were reduced significantly only after storage at 37° for 48 h.

When faeces were examined after 24 h in the post, only the counts of enterobacteria and lactobacilli, both minor components of the bacterial flora, had changed significantly; after 48 h, however, only counts of bacteroides, bifidobacteria, veillonellae and clostridia remained unchanged.

Errors in counting bacteria in faeces

Suspension of faeces are often difficult to homogenize and this could readily contribute to errors in the counting technique. Because all the counts shown in Fig. 1(*a*) and (*b*) were obtained from the same original suspension of faeces and because none changed

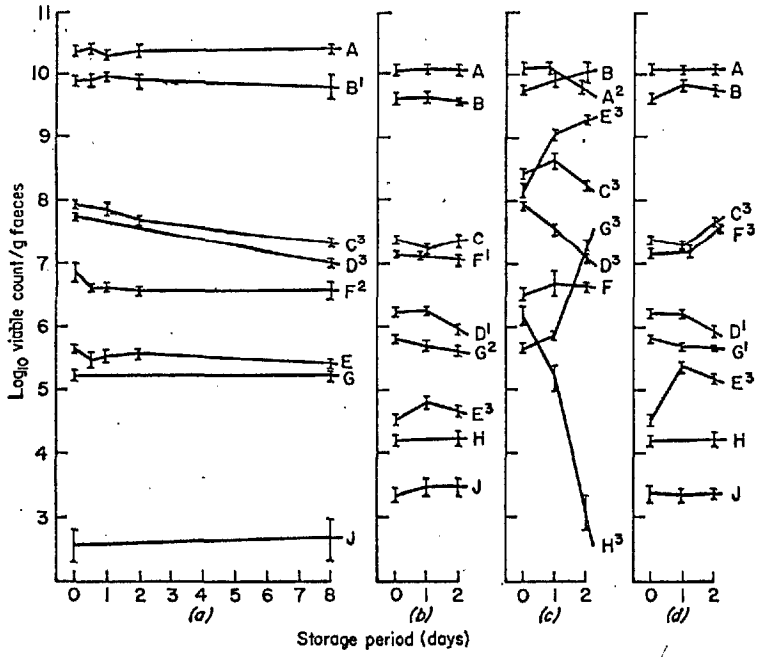

Fig. 3. Survival of bacteria in undiluted faeces stored in air. (*a*) stored at 3°; (*b*) stored at 15°; (*c*) stored at 32°; (*d*) specimens sent by post. For description of signs, see Fig. 1. [1], Significant change in viable count ($P=0{\cdot}05$); [2], Significant change in viable count ($P=0{\cdot}01$); [3], Significant change in viable count ($P=0{\cdot}001$).

TABLE 2

Errors in counting bacteria in faeces

Organism counted	Medium*	Dilution counted	Mean colony count ± S.D.	95% Confidence limits of viable counts [log mean ± 2 S.D.†]
Bacteroides	RCA with 1% (w/v) liver digest and 10% (v/v) horse blood	10^{-7}	66 ± 34	9·82 ± 0·46
Bifidobacteria		10^{-7}	103 ± 39	10·01 ± 0·32
Viridans streptococci	Blood agar	10^{-5}	136 ± 70	8·13 ± 0·36
Enterobacteria	MacConkey's agar	10^{-3}	160 ± 29	6·20 ± 0·12
Enterococci	Azide agar	10^{-3}	186 ± 64	6·27 ± 0·12
Streptococcus salivarius	Sucrose agar	10^{-4}	385 ± 170	7·59 ± 0·18
Lactobacilli	Rogosa's agar (L)	10^{-3}	169 ± 76	6·23 ± 0·28
Clostridia	Willis & Hobb's agar	10^{-1}	13 ± 5	3·10 ± 0·32
Veillonellae	Rogosa's agar (V)	10^{-2}	75 ± 45	4·88 ± 0·30

Means and standard deviations of counts of bacteria in 24 replicate dilutions of the same suspension of faeces (from the data in Fig. 1).

*, Drasar & Crowther (1971); †, obtained from residual term in variance analysis.

significantly on keeping, it seemed reasonable to use them to assess the errors. The 8 sets of 3 counts (converted to logs) were submitted to a one-way analysis of variance and the residual terms used to calculate errors (Table 2). The counts of the highly oxygen-sensitive bacteroides group showed the greatest variability and could only be estimated within 0·46 log unit of the mean, at $P=95\%$. Counts of enterobacteria and enterococci were the most reproducible.

Discussion

The results of the work support the findings of Hollander & Nell (1954) and Drasar, Shiner & McLeod (1969) and show that even delicate anaerobic bacteria can be recovered almost quantitatively from suspensions of faeces frozen in broth if glycerol is used as a protective agent. In this work no toxic effects of glycerol on the bacteria were found, even after contact for 1 month.

These experiments were made on different specimens of faeces from the same person, but it seems unlikely that there would be more differences between persons. The tests reported were not designed to enumerate bacteria present in small numbers, and for this reason no satisfactory results are available for bacilli, staphylococci, pseudomonads, sarcinae, yeasts or moulds.

The calculated confidence limits in the methods used here to count viable bacteria in faeces are similar to those reported by Gorbach, Nahas, Lerner & Weinstein (1967) using different bacteriological techniques.

From our results it is apparent that it will be satisfactory to transport faeces undiluted, if the transit time is <24 h; for transport requiring longer periods of time a 10% suspension of faeces in 10% (v/v) glycerol frozen at $-25°$ or below has proved satisfactory. We have used the latter during surveys in which samples were transported from Uganda to London (Aries, Crowther, Drasar, Hill & Williams, 1969).

I am indebted to Professor R. E. O. Williams for help and discussion and to Mrs D. Irving for statistical advice. I thank Miss R. Scutt for valuable technical assistance. The work was supported by the Cancer Research Campaign.

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