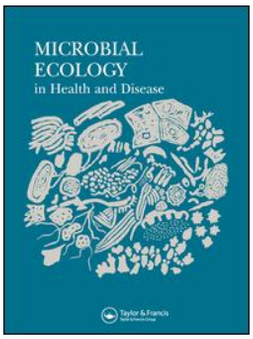

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Establishment of Methanogens in the Infant Intestine

M. J. HUDSON* and A. K. ROBERTS

Division of Pathology, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wilts., SP4 0JG, UK

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Fifty-five healthy, full-term babies were studied to determine the age at which methanogens establish as part of the developing intestinal microflora. The infants were stratified into diet groups according to whether they were fed breast milk, formula or formula supplemented with different amounts of an iron-binding protein. The faecal microflora, including methanogens, was enumerated at 1, 4, 8 and 12 wk of age. Methanogens colonised the infant intestine surprisingly rapidly. By 1 wk of age, 11 of 47 babies (23 per cent) had acquired methanogens and the proportion of infants colonised rose to 70 per cent, 86 per cent and 83 per cent at 4, 8 and 12 wk, respectively. Analysis of sequential samples from individual infants revealed that, once established, methanogens were rarely lost from the gut microflora. Diet did not appear to influence colonisation by methanogens directly. Acquisition of methanogens by 1 wk of age was associated with the equally early establishment of a relatively complex faecal microflora characterised by higher concentrations of bacteroides, clostridia and enterobacteria.

KEY WORDS—Methane; Methanogens; Microflora; Faeces; Intestine; Infant; Diet.

INTRODUCTION

Methanogens are found as part of the normal intestinal flora in man.[20,21,24] Methane produced in the intestine is excreted either directly in flatus or, following systemic absorption and pulmonary excretion, in end-expired breath.[4,17] The methods required for the isolation and characterisation of these exquisitely oxygen-sensitive anaerobes are technically demanding and therefore measurement of breath methane (BM) is commonly used for the study of intestinal methanogenesis. In adults BM status and the concentration of methane vary with the faecal methanogen concentrations.[21] The prevalence of BM excretion increases with age,[4,26] such that between 33 per cent and 49 per cent of healthy adult Europeans and North Americans are excretors.[4,12,26,28] In contrast, infants and children in these populations rarely excrete BM;[4] indeed, none of 72 infants or young children (aged 2 yr or less) in one series were BM excretors.[26] Children of BM-positive parents are themselves more likely to excrete BM,[4] but data from family and twin studies suggest that BM is determined more by environmental factors than by simple Mendelian inheritance.[4,9]

*Author to whom correspondence should be addressed.

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Interest in intestinal methanogenesis has been stimulated by observations that patients with colorectal cancer, a disease which is more common in the developed world, are more commonly BM excretors.[11,27] However, the implementation of BM measurement as a simple, non-invasive screening test for bowel cancer requires first a clear understanding of the microbiological basis of intestinal methanogenesis and the physiological factors which influence BM excretion. For example, several other 'western' diseases are associated with increased BM and these include diverticular disease[35] and aorto-iliac peripheral vascular disease.[19] BM is abolished or reduced after routine bowel preparation and enemas[16] and treatment with certain antimicrobials,[3] whereas diets rich in complex or malabsorbed carbohydrate enhance BM.[9,18,28] Interestingly, although ethnic origin has only a minor influence on BM prevalence in European and North American populations,[12,28] this is certainly not the case in developing countries where there is a far higher incidence of BM in local population groups, such that 72–84 per cent of adult black Africans are BM positive.[15,32] This marked difference suggests a strong environmental influence. It has been suggested that such differences in BM prevalence may simply be due to alterations in intestinal microflora, with

sulphate-reducing bacteria outcompeting with methanogens for hydrogen in the intestine of BM-negative subjects.[10]

Little is documented concerning when methanogens colonise the developing infant microflora, although BM studies suggest that this occurs after about 2 yr of age.[4,26] However, we noted that methanogens were present in the faecal microflora of five of six young Nigerian children (aged 2 yr or less)[8,15] and although BM was detected in three children, this did not correlate with presence or concentrations of faecal methanogens.

The ultimate composition of the infant faecal microflora, and the pattern of colonisation by different bacteria, is markedly influenced by diet.[2,6,29,30,33,34,36] The present study was designed to determine the pattern of colonisation of the infant faecal microflora by methanogens; it was part of a larger study of the influence of diet on the development of the faecal microflora during the first 3 months of life.[29-31]

PATIENTS AND METHODS

Infants and faecal samples

Fifty-five healthy, full-term, vaginally delivered babies born in a single maternity unit in Belgium were studied. They were fed either breast milk or one of three infant formulas: a standard adapted infant formula or the same formula with the iron-binding protein conalbumin (ovotransferrin) added at either 0·1 g/l or 1 g/l. Some mothers changed from wholly breast milk to wholly formula or mixed feeding of their infants at various times during the study period; two infants were switched to standard formula and six and one infants were switched to formula with the lower and higher levels of conalbumin, respectively. A total of 18 babies were breast fed for at least 2 wk. Babies who received antibiotic treatment during the study period were excluded from bacteriological analyses.

Faecal samples were collected from babies during routine follow-up visits at 1, 4, 8 and 12 wk of age; two babies were also examined at 16 wk. It was not possible to collect a complete series of samples for the 12 wk from every baby; a total of 156 samples from 55 babies were analysed and at least three sequential samples were obtained from 37 babies. Faeces collected on clean nappy-liners were sampled for various laboratory investigations with the minimum of delay, and usually within a few hours of defecation. Approximately 0·5 g of stool was added aspetically to 4·5 ml of glycerol broth,[7] which was frozen immediately and stored at below −35°C for microbiology. Exact weights of sample and diluent were determined by difference. Quantitative and qualitative changes to the faecal flora during frozen storage are minimised by dilution of faeces in peptone broth containing glycerol as a cryoprotectant.[7]

Microbiology

Faecal samples were processed in an anaerobic chamber.[5] A decimal dilution series was prepared in prereduced brain–heart infusion broth (Oxoid) and used to inoculate various non-selective, selective and differential media for the quantitative characterisation of the normal faecal microflora. Colony counts of each bacterial group were taken from the least selective medium and expressed as $\log_{10}$ c.f.u./g wet weight faeces. Isolates were identified where possible to genus level by Gram-reaction, morphology, phenotypic and biochemical tests and supported by fermentation end-product analysis by gas–liquid chromatography where appropriate.[5,14]

Methanogens were sought in BG-8, a non-selective methanogen enrichment medium, which was prepared by pre-reduced and anaerobically sterilised (PRAS) techniques.[13] BG-8 was a minor modification of BG-6 which had been used previously,[8,15] the peptone and liver digest content of BG-6 were halved in BG-8 to 1 g/l each. The complete PRAS medium was equilibrated with CO_2 (30 per cent) in hydrogen and dispensed into gassed-out autosampler vials (*c.* 23 ml) each containing 9 ml BG-8. The vials were crimp-sealed with butyl closures, sterilised by autoclaving (121°C for 15 min) and stored in the dark at room temperature for up to 4 mth before use.

Vials of BG-8 were inoculated by syringe with 1 ml aliquots for the decimal dilution series, routinely from the 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} dilutions. The limit of detection was therefore *c.* 10^2 methanogens/g faeces. Syringes were pre-reduced before use in order to minimise oxidation of the medium. Inoculated vials were removed from the anaerobic chamber and pressurised[1] aseptically with 30 per cent CO_2 in hydrogen to *c.* 2 bar, with appropriate safety precautions. The vials were incubated at 37°C for up to 21 d with occasional shaking; aseptic re-pressurisation[1] with gas mix after 7–10 d to replenish substrate CO_2 and H_2 for methanogenesis did not improve the specificity of the gas chromatographic (GC) assay and was not performed routinely.

Methane analysis

The gases and volatiles in each culture vial were analysed by autosampling head-space GC (F40 or F45, Perkin-Elmer) as described elsewhere.[15] Briefly, culture head-space gases were separated isothermally (50°C) on Porapak Q (100–120 mesh) with nitrogen as carrier gas. Methane was detected with a flame-ionisation detector (FID), identified by retention time and quantified using a computing integrator calibrated against a standard gas mix or the methane concentration in air (*c.* 2 p.p.m. or 0·09 μmol/l). The FID response was essentially linear over the range 1 to > 200 p.p.m. with a sensitivity of *c.* 0·2 p.p.m. The FID response to hydrogen was non-stoichiometric but was useful for confirming residual substrate for methanogenesis in the vials. The FID response to CO_2 was negligible.

Statistical comparisons

The influence of infant age and diet on the carriage and concentrations of the major phenotypic groups or genera present in the faecal microflora, including methanogens, and the interrelationships between the methanogens and the other genera were compared by the χ^2-test or Fisher's Exact test and by one-way Analysis of Variance (ANOVA) or ANOVA by Rank (Kruskall–Wallis test) as appropriate, and with due regard to the data distribution (Statgraphics, STSC Inc.); tests were two-tailed and, unless otherwise stated, a probability (*P*) value of $<0{\cdot}05$ was deemed significant. For statistical computations, bacteriological data with zero counts ('not detected') were assigned an arbitrary value of 2·0 $\log_{10}$ c.f.u./g; this was just less than the true limit of detection for most specimens.

RESULTS

Uninoculated or sham-inoculated BG-8 medium usually contained < 2 p.p.m. methane. Cultures with ≥ 10 p.p.m. head-space methane were considered positive for methanogens, as methanogenesis could be passaged reproducibly from these, but only inconsistently from cultures with < 10 p.p.m. methane. Subcultures heated to 75°C for 15 min did not produce methane. Cultures that produced large amounts of methane (> 100 p.p.m.) were noted to have consumed the pressurised gas overlay (30 per cent CO_2 in hydrogen), resulting in a negative pressure in the vials. Frozen storage of faeces for up to 6 mth in glycerol broth had no marked effect on the titre of methanogens.[15]

A most-probable number (MPN) assay was not performed and therefore methanogens were estimated from the titres of methane-positive tubes; an MPN assay would have necessitated multiple cultures from each of the 10 decimal dilutions and this was not practicable for this project. For simplicity, estimates of methanogens are given as $>10^2$/g wet weight faeces (i.e. from the lowest dilution tested), $>10^4$/g, $>10^6$/g, $>10^8$/g or $>10^{10}$/g. Viable counts of total culturable microorganisms (both obligate and facultative anaerobes) were usually in the range $10^{9\cdot0}$–$10^{10\cdot5}$/g wet weight. Quantitation of methanogens was conducted in parallel with the characterisation of the normal faecal microflora. All of the babies developed a normal flora that differed between infants and also according to diet (data not shown). Enteric pathogens were not isolated. *Clostridium difficile* was found commonly as part of the normal microflora in 28 of the 55 infants; the prevalence of *C. difficile* was similar at each sampling age and was 32 per cent (15 of 47 infants) at 1 wk, 28 per cent (15 of 53) at 4 wk, 31 per cent (13 of 42) at 8 wk and 17 per cent (2 of 12) at 12 wk of age.

Colonisation of the newborn infant intestine by methanogens (as evidenced by faecal carriage at 7 d) was surprisingly rapid and the prevalence increased with age (Table 1). The acquisition *de novo* of a methanogenic flora ($>10^2$/g) by 23 per cent of the babies aged 1 wk was highly significant ($\chi^2 = 14{\cdot}4$; $P<0{\cdot}0002$). Between 1 and 4 wk the colonisation rate increased markedly to 70 per cent ($\chi^2 = 21{\cdot}5$; $P<10^{-5}$) and there was also an increase between 4 and 8 wk to 86 per cent of infants colonised ($\chi^2 = 3{\cdot}33$; $P=0{\cdot}09$) and a similar prevalence (83 per cent) at 12 wk. There was a comparable increase in colonisation when higher concentrations of faecal methanogens were considered: for $>10^4$/g the rates were 21, 58, 67, and 75 per cent and for $>10^6$/g the rates were 11, 30, 31 and 58 per cent at 1, 4, 8 and 12 wk, respectively.

The faecal concentrations of methanogens for each infant during the period to 12 wk are shown in Figure 1; the marked trend for colonisation by increasing concentrations of methanogens with age was highly significant (Kruskall–Wallis statistic (w) = 116; $P<10^{-6}$). Only two infants were studied to 16 wk and both were colonised with methanogens at $>10^6$/g. They had acquired methanogens at different ages; one, a breast-fed infant, was already

Table 1. Age of acquisition of methanogens ($>10^2$/g) in the infant faecal microflora

	Age (wk)			
	1 ($n=47$)	4 ($n=53$)	8 ($n=42$)	12 ($n=12$)
Number with methanogens	11	37	36	10
Number positive (%)	23·4%*	69·8%†	85·7%†	83·3%†

*Significant increase *de novo* ($\chi^2 = 14{\cdot}4$; $P<0{\cdot}0002$).
†Significantly greater than prevalence at 1 wk ($\chi^2 > 14{\cdot}9$; $P<0{\cdot}0002$).

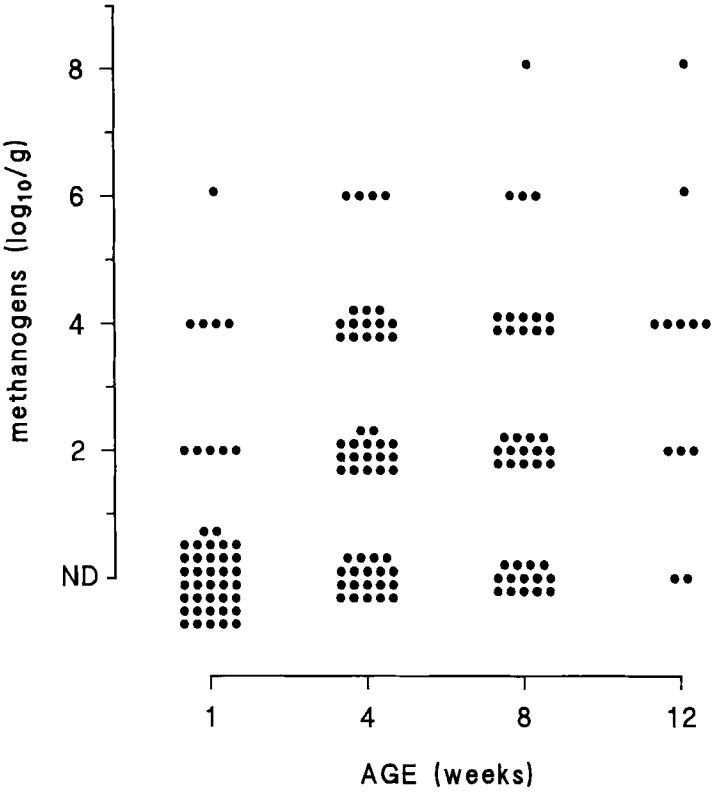

Figure 1. The effect of age (wk) on the estimated numbers of faecal methanogens ($\log_{10}$/g) for each infant. (ND = not detected.)

colonised by 4 wk whereas the other, a formula-fed infant, did not become colonised until 12 wk of age.

No relationship was discernible between the feeding regimen and the prevalence of methanogens (Table 2; χ^2-test). There was also no relationship between diet and either age of acquisition or the concentration of faecal methanogens found although the numbers of babies in each diet group were rather small (χ^2-test and Kruskall–Wallis). No sex differences were found for carriage rate or concentrations of methanogens.

At 1 wk of age, a direct relationship was found between the acquisition of methanogens and the early establishment of certain groups of bacteria as

Table 2. Acquisition of faecal methanogens ($> 10^2$/g) according to age and diet group

Diet	Age (wk) 1	4	8	12
Breast milk alone	4/15*	8/13	4/6	3/3
Breast milk changed to any formula	NA	3/5	7/7	2/2
Formula (no supplement)	0/9	7/11	6/8	0/1
Formula with:				
conalbumin, 0·1 g/l	3/12	10/12	9/10	4/5
conalbumin, 1·0 g/l	4/11	9/12	10/11	1/1
Any formula	7/32	29/40	32/36	7/9
All diets	11/47	37/53	36/42	10/12

NA = not applicable.
*Number positive for methanogens/number of infants in each diet group.

Table 3. Comparison of major phenotypic groups and genera in faeces of infants at 7 d based on the presence or absence of methanogenic bacteria

	Methanogens detected ($n = 11$)	Methanogens not detected ($n = 36$)
Total flora	9·38 ± 0·51 (100)*	8·94 ± 0·95 (100)
Obligate anaerobes	8·35 ± 1·41 (100)	7·37 ± 2·21 (94)
Bifidobacteria	6·49 ± 3·17 (73)	6·35 ± 3·07 (69)
Bacteroides	4·84 ± 2·54 (64)	2·93 ± 1·76 (28)
Clostridia	6·25 ± 1·15 (100)	3·77 ± 1·80 (64)
Facultative anaerobes	9·14 ± 0·63 (100)	8·23 ± 1·42 (100)
Enterobacteria	8·57 ± 0·73 (100)	6·34 ± 2·62 (78)
Enterococci	8·10 ± 2·16 (100)	7·84 ± 1·60 (97)
Lactobacilli	3·89 ± 2·80 (36)	2·64 ± 1·67 (17)

Results given are mean ± SD.
Figures in parentheses are percentage carriage rate.

the predominant flora. Infants with methanogens were more likely to have higher concentrations of total culturable microorganisms (Kruskall–Wallis statistic (w) = 2·3; P = 0·12) and higher levels of obligate anaerobes (w = 2·0; P = 0·16) and facultative organisms (w = 4·6; P = 0·03). Comparison of concentrations of each of the major genera revealed strong associations with bacteroides (w = 5·8; P = 0·016), clostridia (w = 13·7; P = 0·00021) and enterobacteria (w = 9·7; P = 0·0018), a weak association with lactobacilli (w = 2·39; P = 0·12) but no correlation with bifidobacteria (w = 0·41; P = 0·52) or enterococci (w = 0·93; P = 0·33). The differences in prevalence of bacteroides and clostridia in the methanogenic samples were also significantly greater ($P < 0·05$; Fisher's Exact test). The summary data are given in Table 3. Faecal samples in which bifidobacteria, lactobacilli and enterococci predominated tended to have a less complex microflora. In contrast to the 1 wk samples, no marked

microflora–methanogen correlations were evident in samples taken at 4, 8 and 12 wk, perhaps because the flora had become too complex or too varied between infants to reveal simple correlations (data not shown).

The stability of colonisation by methanogens was assessed in 37 of the infants who provided series of at least three faecal samples. Faecal concentrations changed between samples, but once established they were only rarely absent from subsequent samples. Inconsistent colonisation was seen in only four infants, all from different diet groups. In one infant the levels when positive never exceeded 10^2/g. Interestingly, an infant who had methanogens at $>10^8$/g at 1 wk became methanogen negative by 4 wk of age, coincident with a change of diet from breast milk to a formula, and by 8 wk the methanogens had recolonised to $>10^{10}$/g; there was, however, no obvious correlation between colonisation by methanogens in this infant and concomitant qualitative or quantitative changes of other bacterial groups.

DISCUSSION

The most striking result of this study was the surprising rapidity with which methanogens colonised the gut, such that nearly one-quarter of infants were colonised at only 1 wk of age and by 4 wk more than two-thirds were colonised. Once established, methanogens varied in concentration but were rarely lost from the intestinal microflora. The gut is usually sterile at birth and the establishment and development of the intestinal microflora is a complex process. It takes weeks or months to stabilise as a climax microflora, a process which is influenced by diet.[2,6,8,22,29,30,33,34,36] Enterococci and streptococci, lactobacilli, enterobacteria and bifidobacteria colonise the gut rapidly during the first few weeks, whereas other genera colonise more gradually; extremely oxygen sensitive (EOS) anaerobes are not commonly detected until after weaning.[22] It was generally assumed that this marked change in diet was concomitant with an increase in the density and complexity of the microflora sufficient to produce the highly anaerobic conditions that would enable EOS (such as the methanogens) to colonise. Although the babies in this 12 wk study were not weaned, the data presented here are consistent with this hypothesis. Early colonisation by methanogens correlated with the equally early establishment of a more complex intestinal (faecal) microflora, characterised by relatively high levels of putrefactive groups such as enterobacteria, bacteroides and clostridia. There was no correlation when more saccharolytic bacteria such as bifidobacteria, enterococci and lactobacilli predominated; these conflicting data for different anaerobes suggest that rapid colonisation of the neonate intestine by methanogens is not simply incidental to gut anaerobiosis *per se* but that other factors must play an important role; it is reasonable to assume that these may be, in part, nutritional since the methanogens derive their energy from the use of the simple carbon compounds and hydrogen that are themselves end-products of the complex bacterial fermentations in the intestine.

We observed no obvious differences in either the pattern or degree of colonisation by methanogens that could be attributed to diet (breast milk or formula) or to the inclusion of conalbumin supplements. This was in contrast to the marked effect of diet (breast milk vs. formula) on the pattern of establishment of the rest of the microflora, as has been observed in our own[29-31] and in other studies.[2,6,8,33,34,36] The influence of conalbumin on the growth of methanogens is undocumented, although iron is an essential mineral and stimulates methanogenesis *in vitro*.[25]

We did not use a selective medium[20] for the isolation of methanogens but passage of random methane-positive enrichment cultures confirmed that methanogens were present. A few faecal samples yielded low levels of methane which did not passage reproducibly. This effect had been noted previously[15] and was not investigated in detail; it was not a result of failure of anaerobiosis. Inasmuch as enrichment culture might be expected to encourage the growth of competing or inhibitory micro-organisms, the prevalence and numbers of methanogens determined in the faeces of these infants, calculated using a conservative cut-off value of 10 p.p.m. of methane, are likely to be underestimates.

The combined culture and detection method described here was similar to that used previously in studies on intestinal methanogens and prevalence of breath methane excretion by Nigerian subjects.[8,15] In that study, most (5/6) of the young children in whom faecal methanogens were sought by culture were 2 yr olds and thus not comparable to this present study; only one was an infant of 4 mth of age who was found to have $>10^2$/g faecal methanogens but was BM negative. In fact the majority of Nigerian children and adults carried faecal

methanogens but neither the presence nor concentrations were clearly related to BM excretion. Although five of six young children (2 yr old or less) had faecal methanogens, only one had $>10^4$/g; three of these six children were BM positive, but this did not correlate with methanogen concentrations. In a survey of BM excretion in rural Nigerians, only four (5·9 per cent) of 68 young children (< 2 yr) were BM positive; none of 25 breast-fed infants (4–12 wk) were BM positive.[15] These data are slightly higher, although generally in agreement with studies on European and North American children[4,26] in which, for example, none of 37 infants of between 1 and 12 wk of age were BM positive.[26] Clearly, the presence of methanogens in the gut does not reliably predict BM excretion in infants and children and vice versa; for both adults and for children, the many factors that influence the detection or quantitation of intestinal methanogens by BM measurement require elucidation.

In conclusion, methanogens colonise the infant intestine at a surprisingly early age. The source of methanogens, like the rest of the intestinal microflora, is not known precisely but is presumed to be the mother's faecal, perineal and skin microflora and the immediate environment. The observation that methanogens colonise at reasonable numbers in the gut argues against transient contamination from environmental sources and supports their establishment as part of the normal infant intestinal flora. It would be interesting to determine if the pattern of acquisition of methanogens is as strongly influenced by the infant's immediate environment, such as the maternity unit, as has been noted for bifidobacteria.[23,29] Such a study would, of course, necessitate the isolation and characterisation of the methanogens from infants and parents and the comparison of isolate by some typing scheme that could discriminate between strains. It would also be interesting to determine the age of acquisition of sulphate-reducing bacteria and determine at what age this microflora might begin to outcompete or exclude the methanogens.[10]

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REFERENCES

1. Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS. (1979). Methanogens: reevaluation of a unique biological group. *Microbiological Reviews* **43,** 265–296.
2. Benno Y, Sawada K, Mitsuoka T. (1984). The intestinal microflora of infants: composition of the faecal flora in breast-fed and bottle-fed infants. *Microbiology and Immunology* **28,** 975–986.
3. Bjorneklett A, Midvedt T. (1981). Influence of three antimicrobial agents—penicillin, metronidazole, and doxycyclin—on the intestinal microflora of healthy humans. *Scandinavian Journal of Gastroenterology* **16,** 473–480.
4. Bond JH Jr, Engel RR, Levitt MD (1971). Factors influencing pulmonary methane excretion in man: an indirect method of studying the *in situ* metabolism of methane-producing colonic bacteria. *Journal of Experimental Medicine* **133,** 572–588.
5. Borriello P, Hudson M, Hill M. (1978). Investigation of the gastrointestinal bacterial flora. *Clinics in Gastroenterology* **7,** 329–349.
6. Bullen CL, Tearle PV, Stewart MG. (1977). The effect of feeding 'humanised' milks and supplemented breast-feeding on the faecal flora of infants. *Journal of Medical Microbiology* **10,** 403–413.
7. Crowther JS. (1971). Transport and storage of faeces for bacteriological examination. *Journal of Applied Bacteriology* **34,** 477–483.
8. Drasar BS, Tomkins AM, Wiggins H, Hudson MJ. (1984). Breath methane levels and intestinal methanogenesis among rural Nigerians on a local diet. *Proceedings of the Nutrition Society* **43,** 86A.
9. Flatz G, Czeizel A, Métneki J, Flatz SD, Kühnau W, Jahn D. (1985). Pulmonary hydrogen and methane excretion following ingestion of an unabsorbable carbohydrate: a study of twins. *Journal of Pediatric Gastroenterology and Nutrition* **4,** 936–941.
10. Gibson GR, Macfarlane GT, Cummings JH. (1988). The occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. *Journal of Applied Bacteriology* **65,** 103–111.
11. Haines AP, Metz G, Dilawari J, Blendis L, Wiggins H. (1977). Breath methane in patients with cancer of the large bowel. *Lancet* **ii,** 481–483.
12. Haines AP, Imeson JD, Wiggins HS. (1984). Relation of breath methane with obesity and other factors. *International Journal of Obesity* **8,** 675–680.
13. Holdeman LV, Cato EP, Moore WEC (eds). (1977). *Anaerobe Laboratory Manual*, 4th edn. Virginia Polytechnic Institute and State University, Blacksburg, pp 141–148.
14. Hudson MJ, Hill MJ, Elliott PR, Berghouse LM, Burnham WR, Lennard-Jones JE. (1984). The microbial flora of the rectal mucosa and faeces of

patients with Crohn's disease before and during antimicrobial chemotherapy. *Journal of Medical Microbiology* **18,** 335–345.

15. Hudson MJ, Tomkins AM, Wiggins H, Drasar BS. (1993). Breath methane and intestinal methanogenesis in rural Nigerians. *Scandinavian Journal of Gastroenterology* **28,** 993–998.
16. Karlin DA, Jones RD, Stroehlein JR, Mastromarino AJ, Potter GD. (1982). Breath methane excretion in patients with unresected colorectal cancer. *Journal of the National Cancer Institute* **69,** 573–576.
17. Levitt MD, Inglefinger, FJ. (1968). Hydrogen and methane production in man. *Annals of the New York Academy of Sciences* **150,** 75–81.
18. McKay LF, Brydon WG, Eastwood MA, Smith JH. (1981). The influence of pentose on breath methane. *American Journal of Clinical Nutrition* **34,** 2728–2733.
19. McKay LF, Brydon WG, Eastwood MA, Housley E. (1983). The influence of peripheral vascular disease on methanogenesis in man. *Atherosclerosis* **47,** 77–81.
20. Miller TL, Wolin MJ. (1983). Enumeration of *Methanobrevibacter smithii* in human feces. *Archives of Microbiology* **131,** 14–18.
21. Miller TL, Wolin MJ. (1983). Stability of *Methanobrevibacter smithii* populations in the microbial flora excreted from the human large bowel. *Applied and Environmental Microbiology* **45,** 317–318.
22. Mitsuoka T. (1982). Recent trends in research on intestinal flora. *Bifidobacteria and Microflora* **1,** 3–24.
23. Mitsuoka T. (1984). Taxonomy and ecology of bifidobacteria. *Bifidobacteria and Microflora* **3,** 11–28.
24. Nottingham PM, Hungate RE. (1968). Isolation of methanogenic bacteria from feces of man. *Journal of Bacteriology* **96,** 2178–2179.
25. Patel GB, Khan AW, Roth LA. (1978). Optimal levels of sulphate and iron for the cultivation of pure cultures of methanogens in synthetic media. *Journal of Applied Bacteriology* **45,** 347–356.
26. Peled Y, Gilat T, Liberman E, Bujanover Y. (1985). The development of methane production in childhood and adolescence. *Journal of Pediatric Gastroenterology and Nutrition* **4,** 575–579.
27. Piqué JM, Pallarés M, Cusó E, Vilar-Bonet J, Gassull MA. (1984). Methane production and colon cancer. *Gastroenterology* **87,** 601–605.
28. Pitt P, de Bruijn KM, Beeching MF, Goldberg E, Blendis LM. (1980). Studies on breath methane: the effects of ethnic origins and lactulose. *Gut* **21,** 951–959.
29. Roberts AK. (1988). The Development of the Infant Faecal Flora. PhD Dissertation. Council for National Academic Awards.
30. Roberts AK, van Biervliet J-P, Harzer G. (1985). Factors of human milk influencing the bacterial flora of infant faeces. In: Schaub J. (ed.) *Composition and Physiological Properties of Human Milk.* Elsevier, Amsterdam, pp 259–267.
31. Roberts AK, Chierici R, Sawatzki G, Hill MJ, Volpato S, Vigi V. (1992). Supplementation of an adapted formula with bovine lactoferrin: 1. Effect on the infant faecal flora. *Acta Paediatrica Scandinavica* **81,** 119–124.
32. Segal I, Walker ARP, Lord S, Cummings JH. (1988). Breath methane and large bowel cancer risk in contrasting African populations. *Gut* **29,** 608–613.
33. Simhon A, Douglas JR, Drasar BS, Soothill JF. (1982). Effect of feeding on the infants' faecal flora. *Archives of Diseases in Childhood* **57,** 54–58.
34. Stark PL, Lee A. (1982). The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *Journal of Medical Microbiology* **15,** 189–203.
35. Weaver GA, Krause JA, Miller TL, Wolin MJ. (1986). Incidence of methanogenic bacteria in a sigmoidoscopy population: an association of methanogenic bacteria and diverticulosis. *Gut* **27,** 698–704.
36. Yoshioka H, Iseki K-I, Fujita F. (1983). Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics* **72,** 317–321.