Influence of dietary protein supplements on the formation of bacterial metabolites in the colon

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Abstract

Background—To evaluate the influence of increased dietary protein intake on bacterial colonic metabolism in healthy volunteers.

Methods—Short chain fatty acids, ammonia, and volatile organic compounds in faecal samples, and phenols in the urine of five volunteers were measured after one week of basal nutrient intake and and after one week of a diet supplemented with a protein rich food (Fortimel; Nutricia, Zoetermeer, The Netherlands). Paired *t* tests and factor analysis were used for statistical analysis.

Results—Total energy and resistant carbohydrate intake remained unchanged in each study period. The percentage energy intake delivered as dietary protein, increased significantly (from 15·4% to 23·8%; p=0·007) during supplement intake. A significant increase in faecal ammonia (p=0·002), faecal valeric acid (p=0·02), and urinary *p*-cresol (p=0·04) was noted during supplementary protein intake. A total of 120 different volatile compounds were isolated from the faecal samples of which 10 increased significantly during dietary protein supplementation. The change in volatile pattern, especially for S containing metabolites, was clearly shown by a factor analysis model which made a distinction between the two dietary regimens for all volunteers.

Conclusion—An increase in dietary protein leads to altered product formation by colonic metabolism, mainly reflected by an increase in faecal ammonia, faecal volatile S substances, and urinary *p*-cresol.

(*Gut* 1997; 41: 70–76)

Optimal function of the colon, in particular its motor activity, epithelial cell metabolism, and absorptive capacity, is closely related to luminal bacterial digestive processes. Because of the difficulties in obtaining access to the human large intestine during normal digestion, most information about fermentation has been gained by analysis of breath (for example, hydrogen, methane, carbon dioxide), urine (volatile phenols), and mainly faeces. These techniques are indirect, but have proved their validity in many studies.[1] Metabolites formed by fermentation have an important role not only in energy supply, but also in the physiology and the pathophysiology of the colon.[2-6] The nature and extent of fermentation depend on the characteristics of the bacterial flora, colonic transit time, and the colonic availability of nutrients (carbohydrates and nitrogen).[7 8]

Carbohydrate fermentation has been intensively studied.[9] The end products such as hydrogen, methane, and especially short chain fatty acids (SCFAs) have been thoroughly investigated.[10-13] SCFAs are generally accepted to be beneficial to the host.[5] The lumen of the large bowel is also an intensely proteolytic environment.[14] Bacterial degradation of proteinaceous nutrients, however, has been less intensively studied. Metabolism of protein (dietary or endogenous) that escapes digestion and absorption in the small bowel will produce SCFAs and branched chain fatty acids (BCFAs; isobutyric acid and isovaleric acid),[15] but will also initiate less beneficial outcomes, such as the production of potentially toxic substances, for example, ammonia, amines, thiols, phenols, and indoles.[7 16 17]

The aim of this study was to evaluate the influence of an increase in dietary protein intake by volunteers on their colonic metabolism by determining the levels of SCFAs, ammonia, and volatile organic compounds (VOCs) in faeces and phenol derivatives in urine.

Methods

VOLUNTEERS, DIET, AND SAMPLE COLLECTION

Five healthy volunteers (two men and three women; mean age 30 (range 21–34) years), all members of the laboratory staff, were included in the study. (They have been indicated by the initial of their first names.) The volunteers had no gastrointestinal complaints and were free of antibiotics or any other medical treatment for at least three months before the study started. In the first week volunteers ate a diet of their own choosing. In the second week volunteers consumed a supplementary intake of a commercial protein rich food, based on whey, casein, and lactalbumin (table 1) (Fortimel; Nutricia, Zoetermeer, The Netherlands). Fortimel (200 ml) was taken at breakfast, lunch, and dinner additionally to normal food. The two consecutive study periods were not allocated in a randomised order, because no data are available on the nature (and duration)

TABLE 1 *Composition of 100 ml Fortimel*

Energy value	Mean analysis 420 kJ
Lipid	
From plants	2·10 g
From milk	—
Total	2·10 g
Protein	
Whey	1·94 g
Casein and lactalbumin	7·76 g
Total	9·70 g
Carbohydrate	
Lactose	4·40 g
Saccharose	4·20 g
Dextrin-maltose	1·80 g
Total	10·40 g

of changes in bacterial metabolism induced by an altered diet. Volunteers had to weigh and record all food and drink taken during each seven day period. Reliable dietary intake data are important for interpretation of the results. Numerous studies over the past 40 years have tried to establish the reliability or validity of the various methods for collecting dietary intake data. Of those capable of measuring individual dietary intake, the seven day record method has been used.[18][19] Each volunteer was additionally questioned about the recorded data. All data were analysed by computer to obtain energy intake results for each participant in the two periods (Nederlands voedings-stoffenbestand 1989–90, Voorlichtingsbureau voor de voeding, Den Haag, The Netherlands). The total daily protein supplementary load in the second period was 58·2 g.

Urine and faecal samples were collected on day seven of each period. Faecal samples were taken from bulk mass under a nitrogen atmosphere (anaerobic conditions) immediately after delivery, diluted in oxygen free phosphate buffered saline (pH 7) and homogenised under strict anaerobic conditions. Dilution depends on the nature of the compound, and is indicated further in the text. Immediately after voiding, urine samples were frozen at −20°C until further analysis.

The protocol was approved by the ethical committee of the Katholieke Universiteit Leuven.

SCFA (AND BCFA) ANALYSIS

Short chain carboxylic acids were determined following the method of Ghoos *et al.*[20] Freshly delivered faecal samples were diluted 50-fold by weight. One ml of the solution was acidified with 0·1 ml aqueous 20% (w/v) sulphosalicylic acid. A total of 0·25 ml aqueous 2-ethylbutyric acid solution (1·574 mmol/l) was added as internal standard. Vacuum distillation was performed as described by Vreman *et al.*[21] The distillate was adjusted to pH 9 and dried at 60°C. After adding 0·1 ml of silylating agents, the reaction was completed after 30 minutes. The solution was diluted and 1 μl was injected into the gas chromatograph (Carlo Erba, Milan, Italy). After separation on the analytical column (25 m×0·32 mm CP Sil 5 CB, film thickness 1·2 μm; Chrompack, Middelburg, The Netherlands), the acids were identified by ion trap technology (ITD 700; Finnigan, San José,

CA, USA). Quantitative results were obtained by the internal standard method. Results are expressed as concentrations. Total SCFA output is affected by wide variations in total daily stool excretion and therefore is less reproducible.

AMMONIA ANALYSIS

Ammonia analysis was performed by spectrophotometry with an enzymatic determination kit (Sigma Diagnostics, St Louis, MO, USA).[22] Freshly delivered faecal samples were diluted 200-fold by weight and centrifuged for 10 minutes at 1000 *g*. Quantitative enzymatic determination was performed on 0·2 ml of the supernatants at 340 nm and 37°C by an external standard method.

VOCs

Analysis of VOCs was performed by closed loop trapping combined with high resolution gas chromatography ion trap detection, following the method of Ghoos *et al.*[23]

Faecal samples were diluted 125-fold by weight. A 5 ml aliquot of the faecal suspension was transferred to a sample container, flushed with nitrogen, and firmly closed. The sample container was plugged into the closed loop trapping system after an incubation period of 24 hours at 37°C. Three internal standards – that is, bromochloromethane (IS 1), 1-chloro-2-bromopropane (IS 2), and 1,4-dichlorobutane (IS 3), were added, each in a concentration of 25 ng/5 ml faecal suspension. As each standard gave the same reproducibility, only IS 2 was used for further analysis. The aim of the closed loop trapping device is to release VOCs from the matrix – that is, the faecal suspension, and to trap these VOCs on an adsorption trap without interference of water. The injector was a thermodesorption cold trap injector (Chrompack). Separation of the VOCs was performed by gas chromatography (Vega 6000) (on a 25 m×0·32 mm CP-Sil 5 CB, film thickness 1·2 μm column; Chrompack). Identification of the VOCs was performed using ion trap technology (ITD 700; Finnigan).

The relative index (RI) technique was used for semiquantitative data. Each volatile compound is characterised by its mass spectrum with a specific m/z value. The peak area of this specific m/z value is divided by the peak area of the specific m/z value of IS 2 to give the RI for this volatile compound. The data obtained with this technique can be used to compare each volatile product between the two different periods of food intake.

An internal standard method was used for quantitative analysis (when possible).

ANALYSIS OF PHENOL AND *P*-CRESOL IN URINE SAMPLES

Phenol and *p*-cresol were analysed by gas chromatography ion trap technology. One ml urine was diluted with 3 ml distilled water. A total of 75 μl 2,6-dimethylphenol solution (20 mg/100 ml) was added as internal standard.

The pH was adjusted to 1 with concentrated H_2SO_4 and the solution was refluxed for 75 minutes to hydrolyse the conjugated phenols. After cooling to ambient temperature, phenols were extracted with 2 ml diethyl ether. An aliquot (1 μl) was injected into the gas chromatograph (Carlo Erba). After separation on the analytical column (25 m×0.25 mm CP-Sil 13 CB, film thickness 0·4 μm; Chrompack), the phenols were identified by ion trap technology (ITD 700, Finnigan). For quantitation specific m/z values were used: phenol (m/z 94); *p*-cresol (m/z 107); and 2,6-dimethylphenol (m/z 122). Quantitative results were obtained by the internal standard method. Results are calculated as concentrations and expressed relative to the creatinine content of the sample, which was measured by standard laboratory techniques.

STATISTICAL EVALUATION

The difference in food intake between the control and protein rich periods for each basic nutrient – that is, carbohydrates, dietary lipids, and dietary protein, and for the total caloric intake, was evaluated by a paired comparisons *t* test.[24] This test was also used to assess faecal ammonia and SCFA concentrations, as well as the urinary phenol concentration.

The change in volatile pattern between the two periods was evaluated by a factor analysis model.[24] This model made it possible to visualise changes in the volatile pattern by a biplot. Each volatile compound is projected on a bidimensional space, with the normalised *x* and *y* axes chosen as the two factors explaining most variance between the measured volatile

patterns. The length of each arrow represents the importance of that volatile compound in this two factor model. Each volunteer is given by the initial of his first name with an index of the test condition (1: normal diet and 2: with protein supplement). The relative amount of each volatile compound produced in each volunteer can be estimated after orthogonal projection of the subject on the vector in casu.

Results

FOOD INTAKE

Total energy (p=0·14), carbohydrate (p=0·17), and resistant carbohydrate (p=0·52) intake remained unaltered in each study period. The percentage energy intake delivered as dietary protein increased from a mean (SEM) of 15·4 (0·9)% during the control period to 23·8 (2·4)% during supplemental protein intake (p=0·007). The percentage energy intake delivered as dietary lipid intake decreased from 37·6 (0·7)% during the control period to 31·1 (1·5)% during supplemental protein intake (p=0·005) (table 2).

SCFA

Table 3 gives the results for the analysis of all SCFAs.

AMMONIA

The faecal ammonia concentration expressed in mmol/l increased significantly from a mean (SEM) of 0·237 (0·066) during the control period to 0·304 (0·076) during supplemental protein intake (p=0·002).

VOCs

A total of 120 different volatile organic compounds was isolated from faeces, of which 25 remained unidentified. In table 4 an attempt is made to classify these compounds according to their chemical nature. Additionally, specific m/z values used for computing RI values and numbers used for identification in the chromatograms are given. The difference

TABLE 2 *Mean (SEM) energy and resistant carbohydrate intake of the five volunteers during the control and protein rich periods*

Food intake	Control period	Protein rich period	p Value
Carbohydrates (%)	47·0 (0·7)	45·1 (1·4)	0·17
Dietary lipids (%)	37·6 (0·7)	31·1 (1·5)	0·005
Dietary protein (%)	15·4 (0·9)	23·8 (2·4)	0·007
Total energy intake (kJ)	7666 (543)	8933 (1129)	0·14
Resistant carbohydrate (g)	18·9 (1·5)	17·2 (3·5)	0·52

TABLE 3 *Mean (SEM) concentrations of the short chain fatty acids (SCFAs) (mmol/l) of the five volunteers after the control and protein rich periods*

Period	C2	C3	iC4	C4	iC5	C5	C6	Total SCFAs
Control								
T1	33·9	9·3	1·3	10·2	3	1·4	0·5	59·6
N1	37·8	13·3	0·9	14·2	2·5	1·4	0·7	70·8
E1	78·1	18·7	0·9	16·3	0·4	0·8	0·4	115·6
F1	47·3	18·3	1·3	2	2·5	0·9	0	72·3
H1	61·9	87	2·5	11·5	4·7	2	0·8	170·4
Mean (SEM)	51·8 (8·2)	29·3 (14·5)	1·4 (0·3)	10·8 (2·5)	2·6 (0·7)	1·3 (0·2)	0·5 (0·1)	97·7 (20·5)
Protein rich								
T2	56·5	15·6	3	10	3·8	1·8	0·4	91·1
N2	50·4	14	2·1	14	3·6	2·3	0·8	87·2
E2	42·9	15·4	1·2	4·6	3·3	1·4	0·8	69·6
F2	106·6	44·2	3·7	4·6	3·4	2·9	1·6	167
H2	86·7	53·4	2·5	10·5	3	3·5	2·4	162
Mean (SEM)	68·6 (12·1)	28·5 (8·4)	2·5 (0·4)	8·7 (1·8)	3·4 (0·1)	2·4 (0·4)	1·2 (0·4)	115·4 (20·4)
p Value (n=5)	0·33	0·94	0·06	0·44	0·34	0·02	0·12	0·49
p Value (n=4)*	0·5	0·34	0·05	0·51	0·06	0·07	0·28	0·46

Each volunteer is given by the initial of the first name with an index of the test condition. Index 1 is for the normal period; index 2 for the protein rich period.
*(n=4) is without volunteer H (see text).

TABLE 4 Volatile organic compounds, together with their m/z values used for computing relative indices, and their identification numbers used in the chromatograms, found in the faecal samples of the five volunteers after the control and protein rich periods

Compound	m/z	Identification no
Compounds		
Sulphur dioxide	64	1
Hydrogen sulphide	34	2
Methanethiol	47	4
Methylthiirane	45	9
Methyl sulphide	62	11
Carbondisulphide	76	13
Dimethysulphoxide	79	15
Methylthiocyanate	73	33
2-Methyl-2-propane thiol	41	34
Thioacetic acid	43	35
Dimethyl disulphide	94	44
Methanesulphonylchloride	79	50
3-Pentanethiol	43	60
2-Phenyl-4-(phenylmethylene)-5(4H)-thiazolone	77	68
Methylpropyl disulphide	80	74
2-Methyl-2-[(1-methylethyl)thio]propane	41	75
Dimethyl trisulphide	45	82
Methyl(methylthio)methyl disulphide	61	98
Dimethyl tetrasulphide	45	102
Acids and esters		
Acetic anhydride	43	24
Methylacetate	43	26
Ester	57	61
Ester	43	62
Ester	41	80
Ester	43	93
Ester	43	99
Ester	73	100
Ester	41	104
Ester	104	110
Ester	104	112
Ester	104	116
Ester	104	117
Alcohols		
4-Pentene-2-ol	45	5
3-Methyl-2-butanol	45	21
4-Methyl-2-pentanol	45	22
Phenyl compounds		
Benzene	78	31
Toluene	91	49
Phenyl compound	91	64
1,2-Dimethylbenzene	91	67
Phenyl compound	91	71
Benzaldehyde	105	78
Phenol	94	83
Ethylbenzene	105	86
3-Carene	93	88
Limonene	67	91
4-Methylphenol	107	94
4-Carene	93	95
Indole	117	105
Skatole	130	109
Phenol compound	205	119
Diphenylamine	169	120
Alkanes/alkenes		
1-Propene	41	6
2-Methylpropane	43	7
1,3-Pentadiene	67	10
Pentane	41	14
Methylpropene	41	25
1-Butene	41	30
3-Octene	41	54
Methyl-1,3-cyclopentadiene	79	81
Ketones/aldehydes		
4-Pentene-2-one	43	18
3-Methyl-2-butanone	43	29
3,3-Dimethyl-2-hexanone	43	32
2-Butenal	41	43
3-Methyl-2-pentanone	43	46
2-Hexanone	43	52
3-Methyl-3-pentene-2-one	55	58
4-Heptanone	43	66
2-Heptanone	43	70
6-Methyl-5-heptene-2-one	43	84
2-Nonanone	43	96
Undecanone	43	107
Halogenated compounds		
Trichloromethane	83	23
Fluoro compound	51	27
Fluoro compound	51	36
Bromohexane	43	42
Fluoro compound	51	47
Fluoro compound	51	55
2-Bromo-2-methylpentane	41	56
Tetrachloroethene	35	57
Fluoro compound	51	63
Fluoro compound	51	73
Miscellaneous/unknown		
Unknown	43	3
Diisopropyl ether	45	8
Unknown	59	12

TABLE 4 cont

Compound	m/z	Identification no
1,2-Dimethylhydrazine	42	16
Unknown	43	17
Unknown	51	19
Unknown	43	20
Unknown	41	28
2-Methyl-1-nitropropane	41	37
Hydroxylamino compound	41	38
Unknown	39	39
Furanone compound	41	40
Pyridine	52	41
1H-Pyrrole	67	45
Furanone compound	41	48
1,3-Epoxy-4-methylpentane	43	51
Unknown	41	53
Unknown	207	59
2,3-Dihydropyrane	41	65
Unknown	39	69
Unknown	41	72
Unknown	43	76
Unknown	57	77
β-Pinene	93	79
Unknown	41	85
Unknown	281	87
Unknown	57	89
Unknown	67	90
Unknown	41	92
Unknown	41	97
Tetradecyloxirane	43	108
Unknown	161	111
o-Decylhydroxylamine	41	113
Unknown	43	114
Caryophyllene	41	115
Unknown	43	118
Internal standards		
Bromochloromethane	49	IS 1
1-Chloro-2-bromopropane	41	IS 2
1,4-Dichlorobutane	55	IS 3

between the chromatograms of the faecal samples in the control (fig 1A) and protein rich periods (fig 1B) is obvious. Paired comparisons t test showed a significant increase of 10 products (table 5) after the protein rich regimen.

Figure 2 shows the results of the factor analysis performed on the data of each volunteer in the two test conditions – that is, before and after protein supplementation. Each numbered vector represents a volatile substance in a multidimensional (120) space (vector numbers are in concordance with identification numbers in table 4). All volunteers in basal test conditions are located to the left of line L and therefore are clearly separated from the condition after protein supplementation (all to the right of line L). This indicates that VOCs with an arrow left of this line are produced in excess in volunteers consuming a normal diet, while VOCs with an arrow right of this line are produced in excess after protein supplementation. For the sake of clarity Figure 3 shows only the S containing

TABLE 5 Volatile organic compounds that appeared or were increased (represented in terms of x-fold) in all volunteers after the protein rich period

| Compound	Appearance or increase	p Value*
Methanethiol	Appearance	0·019
1-Propene	3 to 16-fold increase	0·018
Diisopropyl ether	5 to 24-fold increase	0·024
1,3-Pentadiene	1 to 4-fold increase	0·004
3-Methyl-2-butanol	2 to 4-fold increase	0·005
Hydroxylamino compound	2 to 20-fold increase	0·032
Unknown	2 to 23-fold increase	0·022
Methylpropyl disulphide	2 to 7-fold increase	0·044
Dimethyl trisulphide	2 to 25-fold increase	0·040
Ethylbenzene	2 to 12-fold increase	0·015

*Significancy level of the change.
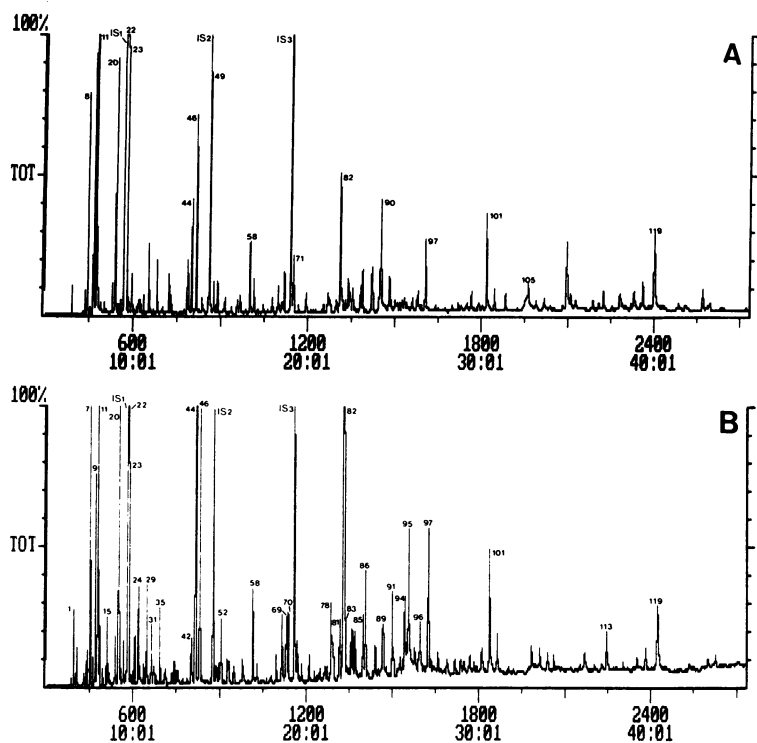
Figure 1: Total ion chromatogram of volatile organic compounds from a faecal sample after the (A) control and (B) protein rich periods. x axis: scan number and time (minutes, seconds). y axis: Peak height of compound. Peak numbers indicate the compound number listed in table 4.

Figure 3: S containing compounds of fig 2.

URINARY PHENOLS

The phenol concentration expressed in mg/g creatinine remained fairly stable during the two different regimens (p=0·35). The concentration of *p*-cresol expressed in mg/g creatinine increased in all volunteers after supplementation (p=0·04) (table 6).

compounds seen in Figure 2. This factor analysis model made a clear distinction between the two dietary regimens for all volunteers.

Figure 2: Biplot of the results of the factor analysis model performed on all data from each volunteer in the two dietary regimens. Each numbered vector represents a volatile substance (vector numbers are in concordance with the identification numbers in table 4). The length of each arrow represents the importance of that volatile in the model. Each volunteer is given by the initial of his first name with an index of the test condition (1: normal diet and 2: after protein supplementation). The relative amount of each volatile produced in each volunteer can be estimated after orthogonal projection of the subject on the vector in casu.
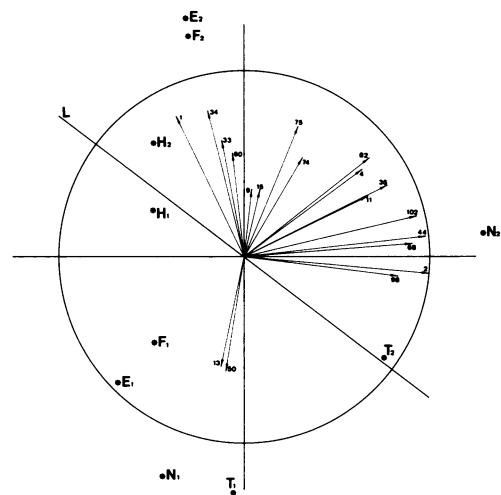
Discussion

In the past decade, there has been growing interest in the role of human colonic flora in health and disease.[3] [4] [6] [25] Through the process of fermentation, colonic bacteria are able to produce a wide range of compounds that have both positive and negative effects on gut physiology, as well as other systemic influences.[28] In general, fermentation of carbohydrates is considered to be beneficial, whereas fermentation of proteinaceous material (putrefaction) is considered to be detrimental to the host. For example, ammonia is involved in portal systemic encephalopathy and has been implicated in the pathogenesis of colon cancer.[4] The nature and extent of the fermentation process are influenced by composition of the flora, colonic transit time, and intraluminal pH. Nevertheless, the type of substrate (carbohydrate, nitrogen) as well as the amount

TABLE 6   *Mean (SEM) concentrations of urinary phenols (mg/g creatinine) of the five volunteers after the control and protein rich periods*

Period	Phenol	*p*-Cresol	Total
Control			
T1	1·6	17·24	18·84
N1	3·12	4·67	7·79
E1	31·68	7·69	39·37
F1	2·19	3·91	6·1
H1	2·48	5·02	7·5
Mean (SEM)	8·21 (5·87)	7·71 (2·47)	15·92 (6·29)
T2	2·82	32·26	35·08
N2	2·57	9·36	11·93
E2	57·51	11·48	68·99
F2	3·65	24·44	28·09
H2	1·57	10·41	11·98
Mean (SEM)	13·62 (10·98)	17·59 (4·58)	31·21 (10·47)
p Value (n=5)	0·35	0·04	0·04

Each volunteer is given by the initial of the first name with an index of the test condition. Index 1 is for the normal period; index 2 for the protein rich period.

of substrate (absolute and relative to each other) available in the large intestine are probably the most important factors affecting fermentation, as was suggested by several in vitro faecal incubation studies.[23] [26] [27] Few studies have been done on the influence of dietary changes on colonic fermentation.[7] Because of the inaccessibility of the colon, these studies have to rely on the analysis of urine and especially faecal samples. Although there is a difference between real colon content and its end product, – that is faeces, this indirect technique has produced reliable results on colonic fermentation.[1] [23] [26] [28] [29] Only five volunteers took part in this study. This number might be too small to provide exact data on inter- and intra-variability of the volatile pattern or on the reversibility of the induced changes. Nevertheless, the aim of this preliminary and descriptive study was essentially to evaluate the influence of increased dietary protein intake on the colonic formation of metabolites in healthy and well instructed volunteers.

The seven day food record of each volunteer was followed by an interview on dietary intake. Computing the percentage energy intake for carbohydrates, lipids, and protein showed clear differences between the two periods. Although total energy, carbohydrate, and resistant carbohydrate intake remained unchanged, protein intake increased significantly, mainly due to supplementary intake of a commercial protein rich food, based on milk proteins. Lipid intake decreased significantly.

These dietary changes led to a significant increase in the faecal ammonia concentration and an increase in the urinary excretion of volatile phenols. The increase in *p*-cresol, a unique fermentation metabolite of tyrosine, reached statistical significance.

All but one volunteer (represented by H) showed an increase in the faecal concentration of branched chain fatty acids (isobutyric acid: 1·1 mmol/l to 2·5 mmol/l; p=0·05, n=4, and isovaleric acid: 2·1 mmol/l to 3·5 mmol/l; p=0·06, n=4) (table 3).

A total of 120 volatile organic compounds were isolated from the faecal samples of the five volunteers by closed loop technique. The use of relative indices (very common in mass spectrometric analysis) made it possible to express the results of analysis semiquantitatively, and to compare the compounds in an intraindividual way. Statistical evaluation of these relative indices showed a significant increase in nine volatile products and the appearance of methanethiol after the protein rich regimen (table 5). Because of the lack of appropriate chemical references, only two compounds could be determined quantitatively. The mean concentration of 3-methyl-2-butanol increased from 2·01 mg/g faeces to 6·42 mg/g faeces, and the concentration of ethylbenzene showed a mean increase from 129·5 pg/g faeces to 946·8 pg/g faeces (mean) after the protein rich diet. The RI values of the 120 VOCs of each period for all volunteers resulted in a long data list. Factor analysis, however, offered (by projecting these compounds on a bidimensional plot) an elegant and powerful way of interpreting these numerous data (Fig 2). Ninety nine VOCs are situated to the right of line L, indicating that they are produced in excess in all volunteers after protein supplementation. Among them, phenol (no 83), *p*-cresol (no 94), indole (no 105), and skatole (no 109) are represented by long vectors, meaning that there is a clear increase in these compounds in the faeces after the protein rich diet. Seventeen of 19 S compounds (Fig 3) are found to the right of line L. As the length of each arrow represents the importance of that volatile compound, it is concluded that the S containing VOCs are among compounds the concentration of which changed most prominently in the faecal samples of the volunteers after protein supplementation. These S containing compounds are intermediary or end products of the breakdown of cysteine and methionine, two amino acids, present in casein.[30] As milk proteins are highly digestible,[31] the significant increase in S compounds and ammonia in the faecal samples and of *p*-cresol in urine samples is surprising. The supplementary load of proteins might saturate digestive capacity, resulting in increased protein putrefaction in the colon. Because the process of fermentation is complex, some caution is warranted in making conclusive statements. Increased availability of SO_4^{2-} (Fortimel contains SO_4^{2-} as $ZnSO_4$ at a concentration of 1·7 mg/100 ml) in the colon as a cause of increased S metabolite formation cannot be excluded. Neither can conclusions be drawn from our data concerning the origin of the surplus protein made available to the colon. Indeed, endogenous secretion of protein is also influenced by the dietary level.[32] To the best of our knowledge, no data are available on the influence of altered lipid intake on protein assimilation and fermentation.

The increase in S compounds is not only of interest in the field of flatology but may also be of importance in the pathogenesis of ulcerative colitis in which luminal reducing agents, mainly hydrogen sulphide and thioacetic acid, are involved.[33] Moreover, 96% of patients with ulcerative colitis carry sulphate reducing bacteria in their colon compared with about 50% in healthy individuals in the UK.[34] Therefore, high protein intake may be an environmental factor related to the aetiology of this disease. There is good experimental and epidemiological evidence to suggest that large bowel cancer is at least partially due to dietary factors. Fat and animal protein are implicated as the main factors in the diet, while dietary fibre is thought to exert a protective effect.[35] [36] The present study shows that an increase in dietary protein leads to an altered pattern of metabolites formed by the colonic microflora. Further research is necessary to investigate whether these metabolites have tumour inducing or promoting potential under physiological circumstances.

For all variables studied, one individual (represented by H) showed only minor response to the diet, as can be seen on the biplots of the factor analysis model where

points H1 and H2 are located close to each other (Figs 2 and 3).

It may be hypothesised that this individual had better protein absorption in the small intestine, increased absorption of metabolites in the colon, less fermentation activity, or perhaps a one week adaptation period was too short to evaluate any effect.

In conclusion, an increase in dietary protein intake leads to altered product formation by colonic metabolism. This is mainly reflected by a change in ammonia concentration, the pattern of S containing volatile substances in faeces, and a change in *p*-cresol concentration in urine.

1 Coates ME, Drason BS, Mallett AK, Rowland IR. Methodological consideration for the study of bacterial metabolism. In: Rowland IR, ed. *Role of the gut flora in toxicity and cancer.* London: Academic Press, 1988: 1–21.

2 Drason BS. The bacterial flora of the intestine. In: Rowland IR, ed. *Role of the gut flora in toxicity and cancer.* London: Academic Press, 1988: 24–38.

3 Simon GL, Gorbach SL. The human intestinal microflora. *Dig Dis Sci* 1986; **31:** 147–62S.

4 Royall D, Wolever TM, Jeejeebhoy KN. Clinical significance of colonic fermentation. *Am J Gastroenterol* 1990; **85:** 1307–12.

5 Scheppach W. Effects of short chain fatty acids on gut morphology and function. *Gut* 1994; **35:** S35–8.

6 Roberfroid MB, Bornet F, Bouley C, Cummings JH. Colonic microflora: nutrition and health. *Nutr Rev* 1995; **53:** 127–30.

7 Cummings JH, Hill MJ, Bone ES, Branch WJ, Jenkins DJ. The effect of meat protein and dietary fiber on colonic function and metabolism. II Bacterial metabolites in faeces and urine. *Am J Clin Nutr* 1979; **32:** 2094–101.

8 Stephen AM, Wiggins HS, Cummings JH. Effect of transit time on colonic microbial metabolism. *Gut* 1987; **28:** 601–9.

9 Macfarlane GT, Cummings JH. The colonic flora, fermentation, and large bowel digestive function. In: Phillips SF, Pemberton JH, Shorter RG, eds. *The large intestine: physiology, pathophysiology, and disease.* New York: Raven Press, 1991: 51–92.

10 Cummings J. Quantitating short chain fatty acid production in humans. In: Binder HJ, Cummings J, Soergel K, eds. *Short chain fatty acids.* London: Kluwer Academic Publishers, 1994: 11–9.

11 Christl SU, Murgatroyd PR, Gibson GR, Cummings JH. Production, metabolism, and excretion of hydrogen in the large intestine. *Gastroenterology* 1992; **102:** 1269–77.

12 Strocchi A, Levitt MD. Maintaining intestinal H$_2$ balance: credit the colonic bacteria. *Gastroenterology* 1992; **102:** 1424–6.

13 Mckay LF, Eastwood MA, Brydon WG. Methane excretion in man - a study of breath, flatus and faeces. *Gut* 1985; **26:** 69–74.

14 Cummings JH, Macfarlane GT. The control and consequences of bacterial fermentation in the human colon. *J Appl Bacteriol* 1991; **70:** 443–59.

15 Nordgaard I, Mortensen PB, Langkilde AM. Small intestinal malabsorption and colonic fermentation of resistant starch and resistant peptides to short-chain fatty acids. *Nutrition* 1995; **11:** 129–37.

16 Macfarlane GT, Allison C. Utilisation of protein by human gut bacteria. *FEMS Microbiology Ecology* 1986; **38:** 19–24.

17 Murray KE, Adams RF, Earl JW, Shaw KJ. Studies of the free faecal amines of infants with gastroenteritis and of healthy infants. *Gut* 1986; **27:** 1173–80.

18 Block G. A review of validations of dietary assessment methods. *Am J Epidemiol* 1982; **115:** 492–505.

19 Bingham SA. Limitations of the various methods for collecting dietary intake of data. *Ann Nutr Metab* 1991; **35:** 117–27.

20 Ghoos YF, Geypens BJ, Hiele MI, Rutgeerts PJ, Vantrappen GR. Analysis of short-chain carboxylic acids in faeces by gaschromatography with an ion-trap detector. *Analytica Chimica Acta* 1991; **247:** 223–7.

21 Vreman HJ, Dowling JA, Raubach RA, Wiener MW. Determination of acetate in biological material by vacuum microdistillation and gas chromatography. *Anal Chem* 1978; **50:** 1138–41.

22 da Fonseca-Wollheim F, Bergmeyer HU, Gutmann I. Ammoniak. In: Bergmeyer HU, ed. *Methoden der enzymatischen Analyse.* Weinheim: Verlag Chemie, 1974: 1850–3.

23 Ghoos YF, Claus DR, Geypens BJ, Hiele MI, Maes BD, Rutgeerts PJ. Screening method for the determination of volatiles in biomedical samples by means of an off-line closed-loop trapping system and high-resolution gas chromatography-ion trap detection. *J Chromatogr A* 1994; **665:** 333–45.

24 *SAS/STAT user's guide. Release 6.03. Edition 1.* Raleigh, NC: SAS Institute, 1988.

25 Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotic. *J Nutr* 1995; **125:** 1401–12.

26 Hiele MI, Ghoos YF, Rutgeerts PJ, Vantrappen GR, Schoorens D. Influence of nutritional substrates on the formation of volatiles by the faecal flora. *Gastroenterology* 1991; **100:** 1597–602.

27 Sabry ZI, Shadarevian SB, Cowan JW, Campbell JA. Relationship of dietary intake of sulphur amino-acids to urinary excretion of inorganic sulphate in man. *Nature* 1965; **4987:** 931–3.

28 Vince AJ, McNeil NI, Wager JD, Wrong OM. The effect of lactulose, pectin, arabinogalactan and cellulose on the production of organic acids and metabolism of ammonia by intestinal bacteria in a faecal incubation system. *Br J Nutr* 1990; **63:** 17–26.

29 Macfarlane GT, Hay S, Gibson GR. Influence of mucin on glycosidase, protease and arylamidase activities of human gut bacteria grown in a 3-stage continuous culture system. *J Appl Bacteriol* 1989; **66:** 407–17.

30 Kadota H, Ishida Y. Production of volatile sulfur compounds by microorganisms. *Annu Rev Microbiol* 1972; **26:** 127–38.

31 Mahé S, Huneau J, Marteau P, Thuillier F, Tomé D. Gastroileal nitrogen and electrolyte movements after bovine milk ingestion in humans. *Am J Clin Nutr* 1992; **56:** 410–6.

32 Corring T, Juste C, Jhoste EF. Nutritional regulation of pancreatic and biliary secretions. *Nutrition Research Reviews* 1989; **2:** 161–80.

33 Roediger WE, Duncan A, Kapaniris O, Millard S. Reducing sulphur compounds of the colon impair colonocyte nutrition: implication for ulcerative colitis. *Gastroenterology* 1993; **104:** 802–9.

34 Florin TH, Gibson GR, Neale G, Cummings JH. A role for sulphate reducing bacteria in ulcerative colitis [abstract]? *Gastroenterology* 1990; **98:** 170.

35 Armstrong B, Doll R. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* 1975; **15:** 617–31.

36 IARC Intestinal Microecology Group. Dietary fibre, transit time, faecal bacteria, steroids and colon cancer in two Scandinavian populations. *Lancet* 1977; ii: 207–11.