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Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids

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Abstract

Proteins and trichloroacetic acid-soluble peptides were present in high concentrations in human intestinal contents and faeces. Free amino acids were also detected in millimolar amounts in proximal and distal colon contents, with hydroxyproline, alanine, lysine and valine predominating, showing that a wide variety of organic N-containing compounds was available for fermentation by intestinal bacteria. Measurements of products of dissimilatory amino acid metabolism (ammonia, branched chain fatty acids) demonstrated that these substances occurred in all regions of the large bowel. Amino acid fermenting populations were enumerated in faeces obtained from five healthy donors by most probable number analysis. Counts ranged from 10^{10} to 10^{11} per gram dry weight faeces. Acetate, propionate and butyrate were the principal fermentation acids in the most probable number tubes. Bacteria forming branched chain fatty acids as major end products of metabolism ranged from 0.6% (isovalerate/2-methylbutyrate) to 40% (isobutyrate) of total peptide and amino fermenting populations. Plate counts also gave high values for peptide fermenting communities in the region of 10^{11} per gram dry weight faeces, though considerably lower numbers of organisms grew on plates containing either single amino acids or Stickland pairs. Clostridia and anaerobic Gram-positive cocci were the predominant isolates in these studies. Physiological investigations on the effects of pH and carbohydrate availability on peptide and amino acid fermentation by intestinal bacteria showed that two environmental characteristics of the proximal colon (low pH, high carbohydrate availability), reduced the rate and net ammonia production from peptides, while carbohydrate (starch) was more important in this respect in amino acid fermentation vessels. Starch reduced initial rates of production of branched chain fatty acids by approximately 35% in peptide fermentations, however, culture pH was a more significant determinant affecting formation of these metabolites. Comparisons of branched chain fatty acid formation by faecal bacteria at pH 6.8 and 5.5 showed that their production was reduced by over 60% in pH 5.5 cultures. These data demonstrate that by increasing bacterial requirements for organic N-containing compounds for use in biosynthetic reactions, and through fermentation acid production, carbohydrate availability plays a major role in regulating dissimilatory metabolism of peptides and amino acids in the human large intestine.

Keywords: Large intestine; Peptide; Amino acid; Fermentation

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1. Introduction

Approximately 13 grams of proteins and peptides are believed to enter the human large intestine every day in digestive materials, where they serve as N-sources for the gut microflora [1]. Mucus secretions and constant turnover of colonic epithelial cells also make a contribution to the pool of organic N-containing compounds in the large bowel [2]. These proteins must be degraded to peptides and amino acids before bacteria can assimilate them. We have previously shown that proteolytic activities in the large intestine comprise a complex mixture of bacterial proteases and peptidases, together with host derived pancreatic endopeptidases [3,4].

The colonic microbiota mainly consists of anaerobic bacterial populations, with some groups being present in very high numbers (ca. 10^{12} cells per gram dry weight of intestinal material). A significant proportion of these organisms are amino acid fermenting species [2,5]. Evidence suggests that intestinal anaerobes prefer to assimilate N in the form of peptides or ammonia, rather than as free amino acids [6–8]. Peptides have been shown to stimulate growth of many rumen organisms, for example *Prevotella ruminicola* utilises N from ammonia or peptides but not amino acids [8,9]. *Bacteroides fragilis* [10] and *Ruminobacter amylophilus* [11] also fail to use amino acids as the sole source of energy, however, amino acids are assimilated by these organisms via six transport systems and are rapidly incorporated into cellular material, provided a source of ammonia is available [12].

The acidic end products of bacterial protein fermentation are varied and include short chain fatty acids (SCFA), branched chain fatty acids (BCFA), which are exclusive products of branched chain amino acid breakdown [13,14], and a range of other non-volatile organic acids [4]. Other products of dissimilatory amino acid metabolism include CO_2, H_2, ammonia, phenols, indoles and amines [4,15,16]. Many of these substances are physiologically active in host tissues, with ammonia, phenols, indoles and amines being of toxicological significance [15,16]. For example, ammonia can alter the morphology and intermediary metabolism of intestinal epithelial cells, increase DNA synthesis and affect their lifespan [17], while high concentrations of this metabolite in the colonic lumen may select for neoplastic growth [18,19]. In patients with liver disease, ammonia formed by colonic bacteria contributes to the onset of portal systemic encephalopathy [20,21].

Despite the known toxicological characteristics of many of the end products of bacterial N metabolism, few data are available concerning the microbiological, physiological or environmental factors that regulate their formation in the large intestine. This study investigated the abilities of faecal bacteria to utilise peptides and amino acids. Samples from different areas of the colon were analysed for products of protein breakdown to assess the availability of substrate, and nature of fermentation products formed at each site. Since marked regional differences in carbohydrate availability and pH exist in the proximal and distal colons [22], their effects on dissimilatory amino acid metabolism were also investigated.

2. Materials and methods

2.1. Measurements of proteins, peptides, amino acids, ammonia and BCFA in intestinal contents and faeces

Human intestinal contents were obtained from six sudden death victims at autopsy. Samples from the small bowel (ileum), caecum and ascending colon (proximal bowel), as well as the descending colon and sigmoid rectum (distal colon) were taken between 2 and 4 h of death, to minimise post-mortem changes, as previously described [16]. Slurries (10% w/v) were made with intestinal material and fresh faeces obtained from 10 healthy donors, using anaerobic 100 mM sodium phosphate buffer (pH 7.0). The samples were homogenised and centrifuged ($30\,000 \times g$, 8 min) to obtain bacteria- and particle-free supernatants. These were frozen and stored at −20°C for subsequent analysis.

2.2. Enumeration of peptide utilising bacteria in faeces

Cell populations of bacteria producing SCFA from peptides and amino acids were determined in faeces from five healthy individuals using a most

probable number (MPN) procedure (10-fold dilutions, five replicates) as described by Alexander [23]. Aliquots (9 ml) of peptone yeast extract (PY) broth [5] were dispensed in 20-ml test tubes (MPN tubes) and autoclaved for 15 min at 121°C. The tubes were transferred while still boiling to an anaerobic cabinet containing an atmosphere consisting of H_2, CO_2 and N_2 (10:10:80). Portions (1 g) of fresh faeces were diluted in half-strength Peptone water to make 10% (w/v) slurries, and 10-fold dilution series were made by transferring 1-ml aliquots from the dilution tubes to replicate MPN tubes, which were incubated anaerobically at 37°C for 5 days. Uninoculated tubes were used as controls. After incubation, tubes showing turbidity were recorded as positive for growth, which gave the total anaerobe count. Samples (5 ml) were removed from the MPN tubes and centrifuged at $13\,000 \times g$ for 10 min. Cell pellets were used for dry weight measurements, while cell-free supernatants were frozen at −20°C for subsequent chemical analyses.

2.3. Enumeration and identification of amino acid fermenting anaerobes in faeces

Anaerobic amino acid fermenting bacteria were enumerated in three fresh faecal samples, using plate count methods as follows. Faeces (1 g wet wt.) were serially diluted in sterile anaerobic saline (0.9% w/v), and suitable dilutions were spread in duplicate onto plates containing a mineral salts based culture medium, solidified with 1.5% (w/v) purified agar, and enriched with individual or pairs of amino acids as energy and N sources. The basal medium consisted of (in g l^{-1} distilled water): NaCl, 4.5; KCl, 2.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.45; $CaCl_2 \cdot 2H_2O$, 0.15; cysteine, 0.8; $(NH_4)_2SO_4$, 2.0; yeast extract, 1.5; haemin, 0.05; Tween 80, 0.5. The medium also contained 2 ml l^{-1} each of vitamin, SCFA and trace element solutions [24]. The trace elements solution contained (in g l^{-1} distilled water): $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot H_2O$, 0.45; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4 \cdot 7H_2O$, 0.18; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2 \cdot 12H_2O$, 0.018; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; Na_2SeO_4, 0.19; $NiCl_2 \cdot 6H_2O$, 0.092. The vitamin solution consisted of (in mg l^{-1} distilled water): pyridoxine hydrochloride, 10.0; *p*-aminobenzoic acid, 5.0; nicotinic acid, 5.0; biotin, 2.0; folic acid, 2.0; vitamin B_{12}, 0.5; thiamine hydrochloride, 5.0; riboflavin 5.0. The SCFA solution comprised (g l^{-1}): sodium acetate, 17; sodium propionate, 6; sodium butyrate, 4; and in ml l^{-1}: *n*-valerate, 1; iso-valerate, 1; iso-butyrate, 1; 2-methylbutyrate,1. The vitamin and SCFA solutions were adjusted to pH 6.5 with 1 M NaOH, and sterilised through 0.2 μm pore size Sartorius filters, before being added to the autoclaved basal culture medium. The mineral salts base was supplemented with Peptone at a concentration of 5 g l^{-1}, or with single amino acids (glutamate, lysine, alanine, glycine, aspartate, proline, arginine, tyrosine, phenylalanine, tryptophan) at a concentration of 20 mM each, or with pairs of amino acids (phenylalanine and leucine, isoleucine and tryptophan, alanine and glycine) at 10 mM each. All manipulations were carried out in an anaerobic cabinet. All plates were preincubated under anaerobic conditions for 24 h at 37°C prior to inoculation, and were subsequently incubated for 5 days. Representative single colonies from the plates were purified by repeated passage on the same culture medium. The organisms were stored in Tryptone Soya broth, plus 10% glycerol at −50°C prior to being identified to genus level according to Gram reaction, morphology and fermentation products formed during growth in peptone yeast extract glucose broth [5]. Bacteria were further identified by use of API 20A anaerobe identification strips (Bio-Mérieux).

2.4. Effect of pH and carbohydrate availability on peptide and amino acid fermentation

Fresh faeces were used to prepare 20% (w/v) slurries in quarter-strength Peptone water, which had been autoclaved for 15 min at 121°C and cooled under O_2-free N_2. Particulate matter was removed from the slurries by centrifugation, as previously reported [25]. Aliquots of slurry (140 ml) were added to three glass fermentation vessels (280 ml working volume) containing sterile culture medium (140 ml) as described by Smith and Macfarlane [15,16]. 10 g l^{-1} Lintner's starch (BDH) was added to one vessel (pH 6.8). The other two vessels, with no added carbohydrate, were controlled at pH 6.8 and 5.5.

In other experiments, three fermenters were set up as above, except that Peptone water and Tryptone in

the culture media were replaced with a mixture of 19 amino acids as follows: aspartate, glycine, alanine, valine, histidine, methionine, leucine, isoleucine, proline, cysteine, glutamate, serine, lysine, ornithine, arginine and threonine to give individual concentrations of 10 mM. Tryptophan, phenylalanine and tyrosine were added at concentrations of 5 mM.

The pH of all fermentation vessels was regulated using Modular Fermenter pH Controllers (Gallenkamp). Growth temperature was 37°C, anaerobic conditions were maintained by sparging with O_2-free N_2 at a flow rate of 600 ml h^{-1}. Cultures were continuously stirred. Samples (5 ml) were taken periodically from all vessels for dry weight and chemical measurements.

2.5. Chemical analyses

Proteins (insoluble in 10% w/v trichloroacetic acid (TCA)) and peptides (soluble in 10% w/v TCA) in intestinal contents were determined by the Lowry method [26]. SCFA were measured by GC using a Pye Model 204 Gas Chromatograph fitted with a flame ionisation detector. SCFA were extracted using procedures outlined by Holdeman et al. [5], with addition of an internal standard (*tert*-butylacetate) at a concentration of 30 mM. SCFA were separated using Unicam 10% FFAP, 100/120 mesh Chromosorb WAW-DMCS in a 1.8 m×2 mm (i.d.) glass column. Injector, detector and column temperatures were 200, 300 and 155°C, respectively. Flow rates of the N_2 carrier gas, H_2 and air were set at 50, 30 and 370 ml min^{-1}. These column conditions did not separate the BCFA, 2-methylbutyrate and isovalerate. All samples were quantitated by comparison of sample peak heights, with those of authentic standards.

Ammonia was determined using the phenol-hypochlorite method of Solorzano [27]. Due to insufficient sample material, amino acid analyses were made with intestinal contents from only one person. They were undertaken using a standard LKB 4151 Alpha Plus Amino Acid Analyser, equipped with a potentiometric recorder and a Hewlett Packard 3392A integrator. The analyser was fitted with a 200 mm×4.6 mm stainless steel column filled with Ultropac 8 cation exchange resin; lithium form (LKB Biochrom). Detection with ninhydrin was at 570 and 440 nm.

For amino acid measurements, an internal standard of norleucine (10 mM) was added to samples before deproteinisation with 10% (w/v) TCA for 1 h at 4°C. The mixture was centrifuged at 13 000×*g* for 15 min, and the supernatant was filtered through a 0.22 μm membrane (Whatman). Samples (20 μl) were adjusted to pH 2.2 using 0.3 M LiOH. Amino acids were quantitated by automatic integration by comparison to an amino acid physiological standard solution.

2.6. Dry weight measurements

Dry weights of bacterial cultures were determined as described by Degnan and Macfarlane [28].

2.7. Chemicals

Bacteriological culture media were obtained from Oxoid. Unless stated otherwise, all other chemicals were purchased from Sigma.

3. Results

3.1. Protein, peptides, free amino acids, BCFA and ammonia in human intestinal contents

Although inter-individual variations were considerable, large amounts of soluble protein, TCA-soluble peptides and ammonia were found in the proximal and distal large intestines, as well as in faecal samples (Fig. 1). Ammonia and TCA-soluble peptide concentrations were markedly lower in faeces than in material removed from the bowel at autopsy, while total BCFA marginally increased in the distal colon and faeces. Due to insufficient sample material, free amino acids were only measured in intestinal contents taken from one person (Table 1). Aspartate, arginine, histidine, isoleucine, methionine, serine, threonine, cysteine, citrulline, γ-aminobutyric acid, hydroxylysine, proline and tryptophan were never detected in intestinal material. Hydroxyproline, taurine, glutamine, alanine and lysine were consistently present in the small and large intestines. In the large gut, availability of free amino acids increased distally, mainly due to glutamine, alanine, valine, leucine, tyrosine, phenylalanine, ornithine and lysine.

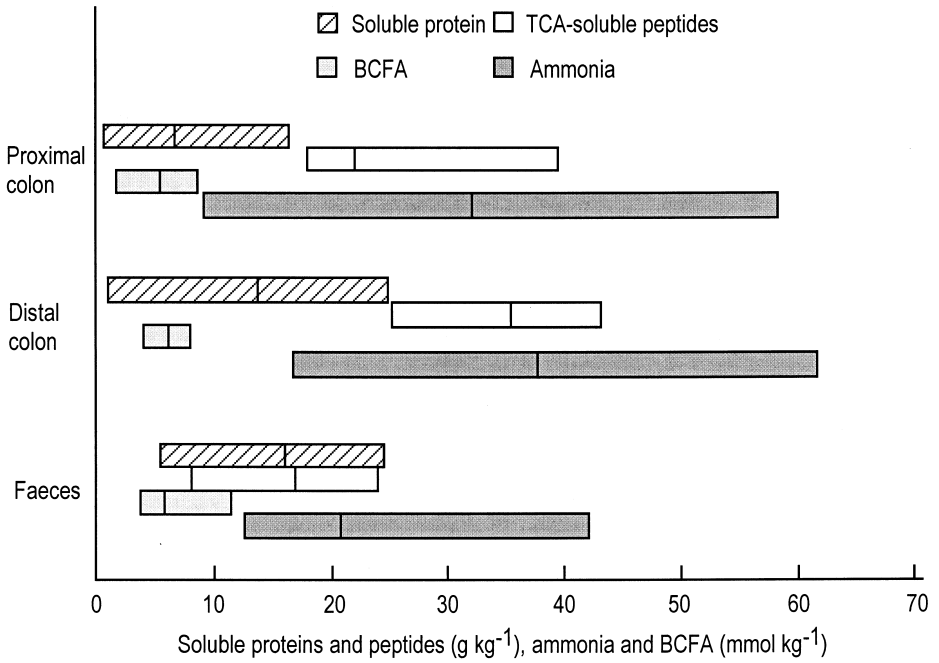

Fig. 1. Measurements of proteinaceous materials and bacterial fermentation products in proximal ($n=6$) and distal ($n=2$) colon contents and faeces ($n=10$).

3.2. Enumeration of intestinal bacteria producing SCFA from peptides

Large populations of amino acid fermenting bacteria were enumerated in the MPN studies, with counts of amino acid utilisers equating to total bacterial numbers in PY medium, in all five faecal samples (Table 2). SCFA production from peptides and amino acids was measured in MPN tubes. In all five faecal samples studied, acetate, propionate and butyrate producing bacteria were most prevalent in all dilution tubes, and they equated with total amino acid fermenter counts. On average, bacteria forming the BCFA isobutyrate constituted approximately 40% of the total anaerobe count, whereas greater inter-individual variation in carriage rates was seen with isovalerate/2-methylbutyrate producing species. They comprised about 0.6% of the total amino acid fermenting population. Valerate and isocaproate producing bacteria were minor components of the amino acid fermenting microbiota.

3.3. Enumeration, isolation and identification of amino acid fermenting microorganisms in faeces

A wide range of bacteria belonging to the genera *Clostridium*, *Fusobacterium*, *Bacteroides*, *Actinomyces*, *Propionibacterium* and a variety of Gram-positive cocci including micrococci, peptococci, peptostreptococci and ruminococci grew on the basal agar medium supplemented with single or pairs of amino acids. However, clostridia and peptostreptococci were the most prevalent isolates on the amino acid plates (Table 3). The highest numbers of bacteria were recovered on plates containing peptides as N and energy sources ($\log_{10}$ 11.3 (cells g dry wt. faeces)$^{-1}$). Addition of glutamate, arginine, glycine and serine stimulated growth of large numbers of bacteria in all samples. Counts on aromatic amino acid plates were variable: phenylalanine and tyrosine supported growth of comparatively high numbers of bacteria ($\log_{10}$ 8.5 and 8.1 cells (g dry wt. faeces)$^{-1}$) respectively, whereas considerably lower numbers of amino acid fermenting species ($\log_{10}$ 4.9 (cells g dry wt. faeces)$^{-1}$) utilised tryptophan as sole energy source. Aspartate also selected for a minor bacterial

Table 1
Concentrations of free amino acids in human intestinal contents[a]

Amino acid	Ileum	Proximal colon	Distal colon
Taurine	0.81	0.62	0.58
Aspartate	ND[b]	ND	ND
Hydroxyproline	1.90	1.59	1.50
Threonine	ND	ND	ND
Serine	ND	ND	ND
Glutamine	0.92	0.10	0.22
Proline	ND	ND	ND
Glycine	0.96	ND	ND
Alanine	0.60	0.36	0.91
Citrulline	ND	ND	ND
Valine	ND	0.05	0.57
Cysteine	ND	ND	ND
Methionine	ND	ND	ND
Isoleucine	ND	ND	ND
Leucine	ND	ND	0.45
Tyrosine	ND	ND	0.30
β-Alanine	ND	ND	0.09
Phenylalanine	ND	ND	0.35
γ-Aminobutyric acid	ND	ND	ND
Hydroxylysine	ND	ND	ND
Ornithine	ND	0.31	0.55
Lysine	0.23	0.24	0.68
Histidine	ND	ND	ND
δ-Aminovaleric acid	ND	0.04	0.08
Tryptophan	ND	ND	ND
Arginine	ND	ND	ND
Total	5.42	3.36	6.28

[a]Measurements were made on intestinal material obtained from one person.
[b]Not detected (<0.01 mmol kg^{-1}).

population identified as *Fusobacterium nucleatum*. Alanine supplemented plates supported growth of low numbers of bacteria in only one faecal sample. *Clostridium bifermentans* and *Clostridium indolis* were identified from plates containing Stickland pairs of amino acids.

3.4. Effect of pH and carbohydrate on peptide and amino acid fermentation

Because pH and carbohydrate availability are important variables in different regions of the colon, their effects on peptide and amino acid fermentation

Table 2
MPN counts of amino acid fermenting populations in faeces on the basis of SCFA measurements

SCFA produced	MPN (log_{10} (g dry wt. faeces)$^{-1}$)							
Faecal sample	1	2	3	4	5	Range	Mean	S.E.M.
Total amino acid fermenters	11.8	11.9	10.9	11.6	11.4	10.9–11.8	11.5	0.2
Acetate	11.8	11.9	10.9	11.6	11.4	10.9–11.8	11.5	0.2
Propionate	11.8	11.9	10.9	11.6	11.4	10.9–11.8	11.5	0.2
Butyrate	11.8	11.9	10.9	11.6	11.4	10.9–11.8	11.5	0.2
Valerate	9.6	6.9	8.9	9.5	7.8	7.8–9.6	8.5	0.5
Isocaproate	6.7	5.2	1.3	4.4	6.2	1.3–6.7	4.8	0.9
Isobutyrate	10.8	11.6	10.4	11.3	11.3	10.4–11.6	11.1	0.2
Isovalerate/2-methylbutyrate	10.3	11.3	10.1	11.4	3.4	3.4–11.1	9.3	1.5

Table 3
Enumeration of amino acid fermenting bacteria in faeces obtained from three healthy donors[a]

Substrate	Bacterial counts (log_{10} (g dry wt. faeces)$^{-1}$)			Predominant species
	Range	Mean	S.E.M.	
Glutamate	9.4–10.4	9.8	0.31	*Peptostreptococcus asaccharolyticus*
Lysine	7.2–9.3	8.4	0.62	*Clostridium bifermentans*
Alanine	0–1.2	0.4	0.40	*Clostridium propionicum*
Glycine	8.6–10.0	9.3	0.41	*Clostridium sporogenes*
Aspartate	0–5.9	3.2	1.72	*Fusobacterium nucleatum*
Proline	6.4–8.7	7.4	1.16	*Clostridium bifermentans*
				Clostridium sporogenes
				Clostridium clostridiiforme
Arginine	9.2–9.8	9.5	0.17	*Clostridium clostridiiforme*
				Clostridium sporogenes
				Actinomyces israelii
Tyrosine	7.1–9.0	8.1	0.55	*Bacteroides thetaiotaomicron*
Phenylalanine	8.2–8.9	8.5	0.20	*Clostridium sporogenes*
				Bacteroides thetaiotaomicron
Tryptophan	3.1–5.4	4.9	0.95	*Peptostreptococcus indolicus*
				Bacteroides putredinis
				Clostridium sporogenes
				Peptostreptococcus asaccharolyticus
Serine	8.8–9.7	9.2	0.25	*Megasphaera elsdenii*
				Clostridium leptum
				Propionibacterium acnes
Phenylalanine/leucine	8.6–9.1	8.9	0.19	*Clostridium bifermentans*
Isoleucine/tryptophan	7.4–8.9	8.3	0.47	*Clostridium indolis*
				Clostridium bifermentans
Alanine/glycine	7.0–9.2	8.1	0.64	*Clostridium bifermentans*
Peptone	10.4–11.3	11.0	0.29	

[a]Bacteria were enumerated by direct plate counting methods.

were investigated in batch culture fermentation studies, using faecal bacteria. Results are standardised to take account of variations in cell mass in the fermentation vessels.

In the peptide and amino acid fermenters, low pH and increased carbohydrate availability reduced initial rates of production and total accumulation of ammonia (Table 4, Fig. 2a,d). However, in amino

Table 4
Effect of pH and carbohydrate availability on production rates of bacterial metabolites from peptides and amino acids

Fermentation product	Culture conditions	mmol h^{-1} (g dry wt. bacteria)$^{-1}$	
		Amino acid substrates[a]	Peptide substrates[b]
Ammonia	pH 6.8	0.69 ± 0.24	1.08 ± 0.65
	pH 5.5	0.45 ± 0.20	0.43 ± 0.17
	pH 6.8+10 g l^{-1} starch	0.39 ± 0.08	0.68 ± 0.34
SCFA	pH 6.8	0.67 ± 0.12	0.97 ± 0.47
	pH 5.5	0.34 ± 0.03	0.47 ± 0.17
	pH 6.8+10 g l^{-1} starch	0.72 ± 0.23	1.35 ± 0.76
BCFA	pH 6.8	0.06 ± 0.03	0.33 ± 0.18
	pH 5.5	0.02 ± 0.02	0.06 ± 0.02
	pH 6.8+10 g l^{-1} starch	0.07 ± 0.02	0.22 ± 0.04

[a]Results are mean values from two experiments ± S.D.
[b]Results are mean values from three experiments ± S.D.

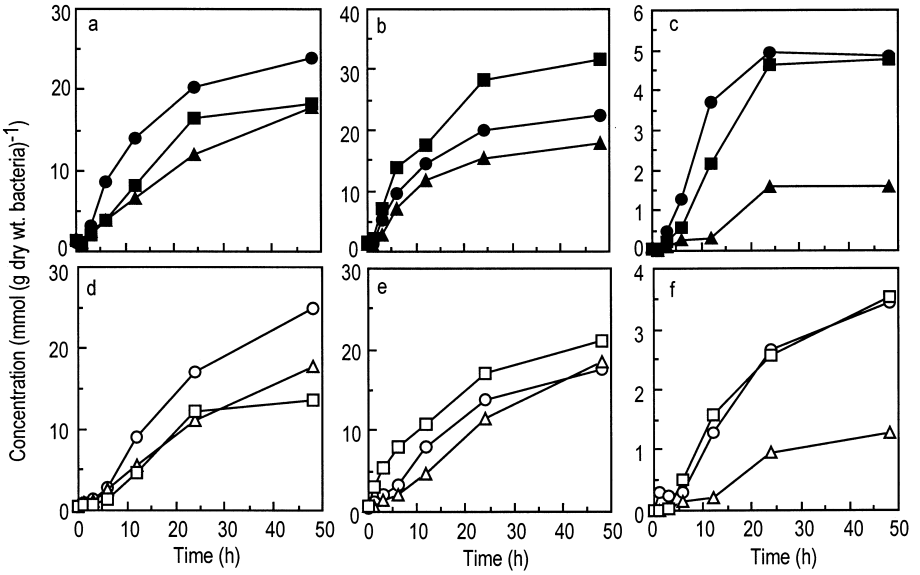

Fig. 2. Production of ammonia (a, d), SCFA (b, e), and BCFA (c, f) from peptides (closed symbols) and amino acid mixtures (open symbols) by human faecal bacteria grown under different cultural conditions: circles, pH 6.8; triangles, pH 5.5; squares, pH 6.8+10 g l^{-1} starch.

acid vessels, the reduction in ammonia formation was more evident (ca. 44%) in the presence of a fermentable source of carbohydrate (Fig. 2d).

Initial rates and total levels of production of SCFA were greatest in fermentation vessels containing starch, when both peptides and free amino acids served as N sources (Table 4, Fig. 2b,e). Acetate was the major SCFA formed in all vessels, while propionate, butyrate and valerate were relatively minor products (Table 5).

Starch reduced initial rates of production of BCFA from peptides by approximately 33%, although it had little effect on these fermentation products when free amino acids were used as N sources (Table 4). However, total BCFA production from free amino acids or peptides differed little after 48 h incubation (Fig. 2c,f). Qualitative differences in peptide fermentation were apparent in that although isovalerate/2-methylbutyrate were the principal BCFA produced, comparatively more isobutyrate

Table 5
Accumulation of SCFA in mixed cultures of intestinal bacteria grown on peptides or amino acids as sole sources of N after 48 h incubation

Fermentation product	Concentration (mmol (g dry wt. bacteria)$^{-1}$)					
	pH 6.8		pH 5.5		pH 6.8+10 g l^{-1} starch	
	Free amino acids[a]	Peptides[b]	Free amino acids[a]	Peptides[b]	Free amino acids[a]	Peptides[b]
Acetate	10.6 ± 0.4	15.0 ± 3.9	11.7 ± 0.4	10.5 ± 1.6	12.5 ± 0.9	20.6 ± 5.3
Propionate	2.8 ± 0.9	3.7 ± 1.9	3.2 ± 0.8	4.1 ± 1.9	2.8 ± 1.0	4.1 ± 2.7
Butyrate	1.8 ± 0.5	3.2 ± 1.2	3.2 ± 0.6	3.0 ± 1.0	3.1 ± 0.8	5.9 ± 1.1
Valerate	1.9 ± 0.9	3.9 ± 1.0	0.9 ± 0.5	2.3 ± 0.4	1.6 ± 0.3	4.4 ± 0.9
Isobutyrate	0.5 ± 0.1	1.5 ± 0.8	0.5 ± 0.4	0.4 ± 0.0	0.4 ± 0.2	1.4 ± 0.7
Isovalerate/2-methylbutyrate	2.6 ± 1.7	3.0 ± 1.7	0.9 ± 0.0	0.8 ± 0.3	2.8 ± 0.1	2.6 ± 1.7
Isocaproate	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0.0	0.4 ± 0.3	0.3 ± 0.1	0.8 ± 0.4
Total	20.5 ± 4.9	30.3 ± 8.5	20.4 ± 3.7	21.5 ± 3.1	23.5 ± 3.5	39.8 ± 11.8

Results are mean values from faecal samples obtained from [a]two or [b]three individuals ± S.D.

Table 6
Apparent rates of assimilation and production of amino acids by mixed populations of intestinal bacteria grown in batch culture[a]

Amino acid	mmol h^{-1} (g dry wt. bacteria)$^{-1}$		
	pH 6.8	pH 5.5	pH 6.8+10 g l^{-1} starch
Glycine	0.052 ± 0.011	0.017 ± 0.008	0.059 ± 0.015
Alanine	0.009 ± 0.002	0.005 ± 0.001	0.014 ± 0.007
Serine	0.157 ± 0.048	0.162 ± 0.060	0.109 ± 0.006
Threonine	0.068 ± 0.013	0.039 ± 0.011	0.045 ± 0.020
Valine	0.024 ± 0.004[b]	0.007 ± 0.002[b]	0.029 ± 0.002
Leucine	0.032 ± 0.003	0.008 ± 0.002	0.023 ± 0.002
Isoleucine	0.008 ± 0.003	0.005 ± 0.001	0.018 ± 0.005
Phenylalanine	0.003 ± 0.001[b]	0.004 ± 0.003	0.005 ± 0.001[b]
Tyrosine	0.007 ± 0.001	0.002 ± 0.001	0.004 ± 0.002
Tryptophan	0.009 ± 0.001	0.010 ± 0.009	0.012 ± 0.005[b]
Lysine	0.009 ± 0.001	0.018 ± 0.009	0.018 ± 0.004
Arginine	0.092 ± 0.021	0.121 ± 0.031	0.138 ± 0.051
Histidine	0.012 ± 0.006	0.006 ± 0.001	0.018 ± 0.002
Ornithine	0.020 ± 0.012	0.068 ± 0.012[b]	0.027 ± 0.017
Aspartate	0.141 ± 0.060	0.054 ± 0.005	0.075 ± 0.001
Glutamate	0.037 ± 0.001	0.029 ± 0.010	0.053 ± 0.004
Cysteine	0.024 ± 0.008	0.017 ± 0.013	0.034 ± 0.018
Methionine	0.013 ± 0.006[b]	0.007 ± 0.001[b]	0.019 ± 0.001[b]
Proline	0.022 ± 0.011	0.007 ± 0.002	0.057 ± 0.001
Hydroxyproline	0.004 ± 0.001	0.031 ± 0.008	0.025 ± 0.007

[a]Results are mean values from two experiments ± S.D.
[b]Net production of amino acid.

and isocaproate were formed in the presence of carbohydrate (Table 5). Culture pH was a more important physiological determinant of branched chain amino acid fermentation than carbohydrate availability, as evidenced by the fact that BCFA production from peptides declined by 67% at pH 5.5, and by 61% from free amino acids (Fig. 2c,f).

3.5. Amino acid utilisation

Mixed populations of intestinal bacteria assimilated free amino acids at varying rates (Table 6). There was an apparent sequential uptake, with serine, arginine and aspartate being metabolised most rapidly. Aromatic amino acid precursors of phenolic and indolic compounds were utilised very slowly by faecal microorganisms. Carbohydrate increased uptake rates of glycine, alanine, valine, isoleucine, phenylalanine, tryptophan, arginine, histidine, glutamate, cysteine, proline and hydroxyproline, whereas utilisation of serine, threonine, leucine, tyrosine, and aspartate was reduced. Acid pH reduced rates of uptake of most amino acids, but assimilation of lysine, serine, ornithine and hydroxyproline was markedly higher at pH 5.5.

4. Discussion

Analysis of digestive materials in different regions of the intestinal tract showed that large quantities of soluble protein and peptides were present throughout the gut (Fig. 1). The fact that TCA-soluble peptide concentrations were lower in faeces than in intestinal contents reflected utilisation of these substances by colonic bacteria, while the increase in protein in faecal material was indicative that a large component consisted of bacterial secretions and cell lysis products. Support for this comes from feeding studies which showed that the composition of normal faecal protein changed little when dietary protein intake was varied [29]. High levels of protein degradation products such as ammonia and BCFA were found in colonic contents demonstrating high levels of amino acid fermentation. Unlike the proximal large intestine, the distal colon is a carbohy-

drate and energy deficient environment, where bacterial growth is slow, and where protein provides an important source of fermentable carbon [22].

Studies have shown that SCFA [2] and ammonia [30] are present in human faeces, and it was initially thought that these metabolites were primarily products of polysaccharide fermentation [31] and bacterial ureolysis [32]. However, urea was not detected in intestinal material in this study (results not shown), while Chacko and Cummings [33] found very low levels in ileal effluent. Large quantities of ammonia and SCFA produced by gut bacteria growing on peptides and amino acids, in in vitro faecal incubation systems (Fig. 2) and in MPN experiments (Table 2), indicated that protein degradation accounts for the majority of ammonia occurring in the large intestine, while making a significant contribution to colonic SCFA. This supports work by Cummings et al. [34] who observed a doubling in faecal ammonia excretion when daily protein intake was increased from 63 to 136 g in volunteer feeding studies.

Previous investigations have suggested that only minor variations in the compositions of faecal amino acid pools exist among normal healthy adults living on uncontrolled Western diets [30,35]. Adibi and Mercer [36] and Padovan et al. [37] found low concentrations (<10 mM) of free amino acid residues resulting from protein digestion, which had escaped absorption in ileal fluids. This correlates with values obtained in our study (Table 1). Since the majority (80–85%) of N in ileal effluent is in the form of proteins and peptides [32], one of the most important aspects of their metabolism in the colon is peptidolytic digestion [3]. The composition of large intestinal amino acid pools is therefore largely determined by bacterial metabolism of substrate reaching the large bowel, this in turn will depend on diet and by release of intracellular contents from desquamated epithelial cells and dead bacteria, as well as pancreatic and colonic secretions and intestinal absorption.

Previous work in our laboratory [4] demonstrated that free amino acids did not accumulate in bacterial fermentations of casein, although TCA-soluble peptides did, indicating that peptide hydrolysis was the rate-limiting step in protein utilisation. In this study, broadly similar amino acid profiles were observed in the small intestine and colon (Table 1), although a greater variety was seen in the distal bowel. Free glycine was not detected in any region of the colon, which suggests that it is an important substrate for amino acid fermenting species. Interestingly, hydroxyproline, alanine and lysine were found in all regions of the gut, and in in vitro batch culture studies, these amino acids were slowly assimilated by intestinal bacteria, while aspartate was rapidly utilised (Table 6), and was not detected in colonic contents (Table 1). The higher levels of amino acids detected in the distal colon may be explained by reduced bacterial requirements for biosynthetic purposes, due to limiting carbohydrate, increased peptide hydrolysis and bacterial cell lysis, or possibly, bacterial excretion of unwanted peptide hydrolysis products.

Peptides and amino acids were the major C, N and energy sources in the MPN culture medium used to enumerate amino acid fermenting populations in faeces (Table 2). The high numbers of bacteria observed in these tubes demonstrate that dissimilatory amino acid metabolism is an important process supporting growth of significant cell mass in the intestine. Analysis of fermentation metabolites in MPN tubes showed that the end products of peptide breakdown are complex, with a range of SCFA being formed. Acetate, propionate and butyrate producing bacteria equated with total anaerobe counts. Estimates of isobutyrate producing populations in faeces showed that these bacteria formed a relatively large proportion (40%) of the total amino acid fermenting anaerobe count, whereas isovalerate/2-methylbutyrate producing species constituted a minor population. Nevertheless, isobutyrate was formed in relatively small amounts (results not shown), indicating that different branched chain amino acids are fermented by distinct bacterial sub-populations. For example, *Bacteroides ovatus* produces isovalerate from PYG broth but does not form isobutyrate [5].

Faecal samples from healthy donors were also screened for carriage of amino acid fermenting bacteria. Large numbers of organisms were able to grow on a relatively simple basal culture medium supplemented with growth factors and single amino acids (Table 3). Each individual amino acid selected for different bacterial species, reflecting generic variations in amino acid transport systems and fermentation pathways. The principal organisms isolated in these studies were clostridia. Putrefactive clostridia and anaerobic Gram-positive cocci have previously

been considered to be important amino acid fermenting bacteria [38–40]. All clostridia appeared to be able to attack single amino acids, with pairs of amino acids not being essential, although growth of *C. bifermentans* was enhanced when provided with Stickland amino acid pairs. Despite the fact that colonic bacteroides are saccharolytic, several species were isolated from the amino acid plates, suggesting that they contained trace amounts of carbohydrate.

Fermentation of organic N-containing compounds by faecal bacteria was profoundly influenced by environmental conditions. Culture pH and carbohydrate in the form of starch, affected the metabolism of amino acids and peptides in varying ways. Quantitatively, SCFA and ammonia were the major products of amino acid fermentation (Fig. 2). On the basis of their production rates, it was apparent that intestinal bacteria prefer to assimilate and ferment organic N in the form of peptides rather than as free amino acids (Table 4). This was also suggested by the observation that considerably higher numbers of bacteria grew on peptide agar plates (Table 3). Rates of production of SCFA from peptides in these batch cultures was approximately 30% faster than from amino acids, in the absence of carbohydrate. The amino acid content in peptone and tryptone would be marginally less than the amount present in amino acid containing fermenters [41]. Therefore, the amount of available N was not an important factor affecting fermentation, but the way in which it was presented to colonic bacteria influenced its metabolism. The ability of microorganisms to assimilate peptides instead of free amino acids is energetically advantageous, and would be physiologically important in an energy deficient environment such as the large intestine. Peptide stimulation of growth has been observed in *Prevotella ruminicola* [9], but is not only confined to single isolates, since mixed cultures of rumen microorganisms have been shown to utilise N from ammonia or peptides, but not from amino acids [42,43].

SCFA and BCFA production from peptides and free amino acids was markedly reduced at pH 5.5 (Table 4). Inhibition of amino acid fermentation was also reflected in lower net ammonia production (Fig. 2). Normal colonic pH is approximately 5.4 in the proximal large intestine, progressively rising to about 6.9 in the descending colon [22]. These studies therefore suggest pH may be a limiting factor affecting release of ammonia in vivo, especially from branched chain amino acids in the proximal bowel.

Addition of fermentable carbohydrate to batch cultures also reduced net ammonia production. This was independent of low pH, which inhibits the activity of deaminating enzymes in bacteria [44]. The ability of glucose to inhibit synthesis of alanine and glutamate deaminases and aspartase in *Escherichia coli* was first demonstrated by Epps and Gale [45], and its effects on synthesis of a variety of inducible and constitutive enzymes is now well documented [46]. However, glucose inhibition of amino acid fermentation does not occur in all intestinal bacteria. For example, peptide and amino acid fermentation in *Fusobacterium nucleatum* and *Prevotella melaninogenica* is not subject to catabolite repression by glucose [47,48]. Nevertheless, in human studies, faecal N excretion increases while ammonia levels fall when fermentation is stimulated by addition of fibre to the diet [34], with active carbohydrate fermentation routing N into bacterial protein. This principle has been exploited in the treatment of patients with liver disorders such as cirrhosis, where elevated levels of ammonia accumulate in body fluids, contributing to the onset of hepatic coma. Administration of the non-absorbable disaccharide lactulose reduces ammonia recycling by stimulating fermentation by the gut microflora [21,49]. Recent studies [50] have shown that lactulose decreases protein degradation in colonic contents through inhibition of amino acid metabolism, while Ito et al. [51] also observed suppression of amino acid fermentation in intestinal bacteria. They showed a positive correlation between the concentration of ammonia with isobutyrate and isovalerate concentrations in persons fed a diet supplemented with transgalactosylated disaccharides. Ammonia concentrations in the colon therefore represent a balance between deamination of amino acids by some organisms, subsequent uptake by bacterial cells as a N source for protein synthesis, and colonic absorption.

Acetate was the predominant SCFA formed from peptides and free amino acids in batch culture fermentation studies (Table 5), with smaller amounts of propionate and butyrate. Butyrate production was only greater than propionate upon addition of fermentable starch, this was previously demonstrated

by Englyst et al. [52], and if a similar change occurs in the large intestine after ingestion of fermentable starch, this might be of benefit to the host, because butyrate is an important metabolic fuel for colonic epithelial cells [53].

In this study, net BCFA formation after 48 h incubation was unaffected by carbohydrate, however, their initial rates of production from peptides in starch containing cultures was markedly reduced. It is therefore apparent that formation of these metabolites only became quantitatively significant when the carbohydrate source was depleted. Indeed it has been estimated that the potential contribution made by protein towards SCFA production in the large gut rises progressively from about 17% in the proximal colon, to approximately 38% in the distal bowel [13]. However, carbohydrate does not affect branched chain amino acid metabolism uniformly. For example, glucose represses BCFA formation by clostridia [54,55] and *Megasphaera elsdenii*, but stimulates the process in *Prevotella ruminicola* [56]. Therefore, whilst catabolite control of branched chain amino acid metabolism may not be important in individual species of colonic bacteria, an overall reduction in fermentation occurs in faecal material.

In peptide fermentations containing starch, isocaproate production was stimulated at pH 6.8, whereas isobutyrate and isovalerate/2-methylbutyrate were reduced. It has previously been noted that glucose suppresses production of isobutyrate and isovalerate (oxidation products) but not isocaproate in *Peptostreptococcus anaerobius* and *Clostridium bifermentans* [57]. Therefore, glucose appears to inhibit oxidative deamination of amino acids but not reductive deamination. This effect may be caused by glycolytic processes consuming co-factors such as NAD^+, which would otherwise be available for amino acid fermentation. Indeed, NAD^+ has been shown to be an important proton acceptor in oxidative deamination of amino acids [58].

Assimilation of free amino acids by intestinal bacteria was assessed under different conditions of pH and carbohydrate availability (Table 6). It is recognised that in these studies, the pool of amino acids potentially available for assimilation consisted not only of the amino acid additions, but also contained free amino acids formed as a result of proteolysis of endogenous polymers by intestinal microbes in the faecal slurry, and from lysed bacterial cells. Furthermore, the release of amino acids from peptides by anaerobic bacteria has been previously documented [59]. Overall, net production of valine, phenylalanine, ornithine and methionine was in fact observed in these cultures, concurrent with increases in citrulline and α-aminobutyrate (results not shown). Quantitatively, however, amino acids added to the fermenters were the main contributors to the pool of amino acids available for fermentation.

Tyrosine, tryptophan and phenylalanine were poorly assimilated by colonic bacteria in comparison to other amino acids, possibly reflecting the hydrophobic character of these substances. No other pattern was evident in uptake of other classes of amino acids (e.g. acid, basic, S-containing). Experiments with pure and mixed cultures of rumen bacteria, have previously indicated that the chemical composition of peptides affects their rate of breakdown [60,61]. Hydrophilic peptides were more rapidly degraded than those containing high levels of aliphatic, proline or bulky aromatic amino acid residues. Hydrophobicity was a more important factor than chain length in the regulation of peptide metabolism, and this can probably be extended to free amino acids. Alves et al. [62], showed that the outer membrane of Gram-negative bacteria can form a barrier to hydrophobic molecules. Studies with rumen organisms indicate that individual amino acids are fermented at different rates. For example, arginine and threonine are metabolised most rapidly, followed by lysine, phenylalanine, leucine and isoleucine, whereas valine and methionine are slowly degraded [63].

In general, addition of starch resulted in greater uptake of amino acids. However, the increase in assimilation of tryptophan detected in the presence of carbohydrate was not matched by a concomitant elevation in production of indolic compounds (results not shown). Likewise, increases in valine assimilation in the presence of starch was not reflected by a rise in the formation of isobutyrate, further implying that in starch vessels, bacteria were assimilating amino acids for incorporation into bacterial protein as opposed to dissimilatory metabolism.

In conclusion, proteins and peptides occur in large amounts throughout the human large intestine, while most free amino acids are assimilated by intestinal bacteria. However, dissimilatory metabolism of these

substances by colonic microorganisms is reduced in the presence of fermentable carbohydrates, such as starch, which increases bacterial requirements for peptides and amino acids for biosynthetic purposes, and in the proximal bowel will exert an indirect effect by reducing gut pH, thereby changing patterns of fermentation. This in large part explains in vivo observations, which suggest that dissimilatory amino acid metabolism is primarily a phenomenon associated with the distal colon.

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