The effect of a model melanoidin mixture on faecal bacterial populations *in vitro*

Jennifer M. Ames*, Anthony Wynne, Andrea Hofmann, Saskia Plos and Glenn R. Gibson

Department of Food Science and Technology, The University of Reading, Whiteknights, PO Box 226, Reading RG6 6AP, UK

(Received 6 January 1999 – Revised 17 May 1999 – Accepted 11 June 1999)

The Maillard reaction produces coloured, macromolecular materials (melanoidins) in a variety of foods, on heating. Significant quantities may enter the human gut on a daily basis, but there is little information on their metabolism in the human colon. As the large bowel contains a diverse population of bacteria involved in normal bowel function, it is possible that melanoidins are metabolized therein. Depending on the bacteria involved, there may be disease or health implications. The aim of the present study was to use *in vitro* models to determine the digestibility of melanoidins and the effect of melanoidins on colonic bacteria in the gastrointestinal tract. Melanoidins were prepared and the effects of simulated upper-gut secretions on their stability determined in a model system. The effects of faecal bacteria were also determined, in batch culture, with a combination of phenotypic and genotypic (probes) criteria being used to identify the microbial diversity involved. Simulation of peptic and pancreatic digestion showed that the melanoidins did not produce detectable amounts of low-molecular-mass degradation products. However, melanoidins affected the growth of gut bacteria during mixed culture growth. The effect was to cause a non-specific increase in the anaerobic bacteria enumerated. This *in vitro* study indicates that melanoidins can affect the growth of human large-bowel bacteria and serves to demonstrate possible effects that may occur *in vivo*. Given the large and varied number of food items that contain Maillard reaction products, this may have relevance for lower-gut health.

Maillard reaction: Melanoidins: Gut fermentation: Gene probes

The Maillard reaction is a complex network of reactions that occurs in both food and the human body (O'Brien *et al.* 1998). It occurs when reducing sugars, like glucose and lactose, react with components possessing a free amino group, such as amino acids and peptides (Ledl & Schleicher, 1990; Ames, 1992). The final products of the reaction are coloured, macromolecular materials known as melanoidins, which are ubiquitous components in the typical Western diet (Ames, 1998). Structures of melanoidins and related materials are poorly defined (Ames & Nursten, 1989; Rizzi, 1997). No single melanoidin has been isolated and characterized.

The degradation of melanoidins under physiological conditions and the effects of micro-organisms on Maillard reaction products have been reviewed (O'Brien & Morrissey, 1989). Studies using rats indicated that only a small proportion of melanoidins prepared from glucose and glycine (or lysine) were absorbed through the gut wall, most being excreted in faeces (Finot & Magnenat, 1981; Homma & Fujimaki, 1981; Nair *et al.* 1981; Finot, 1990; Lee *et al.* 1992). However, 90 d toxicity studies of Class I and Class IV caramels using rats resulted in discolouration of the mesenteric lymph nodes that was attributed to the intestinal absorption of high-molecular-mass caramel components (Noltes & Chappel, 1985).

Investigations involving melanoidins and micro-organisms have hitherto focused on (a) the potential of melanoidins to affect the growth of bacteria, including the possible extension of food shelf-life (e.g. Viswanathan & Sarma, 1957; De Lara & Gilliland, 1985), (b) the ability of micro-organisms to degrade or decolorize melanoidins (Murata *et al.* 1992; Terasawa *et al.* 1996) and, (c) the effect of melanoidins on gut microflora composition (Jemmali, 1969; Horikoshi *et al.* 1981). There is very little information on microbial interactions between human colonic bacteria and Maillard reaction products.

The colon is the most metabolically active site in the human body. This is because of the resident microbiota, which comprises about 10^{14} prokaryote cells in total (Salminen *et al.* 1998). The nature of the gut fermentation may impact heavily on host health and welfare (Freter, 1983) and the gut flora is thought to play a central role in homeostasis, digestion and the prevention of diseases, such as acute gastroenteritis and bowel cancer (Roberfroid *et al.* 1995;

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization.

* **Corresponding author:** Dr Jennifer Ames, fax +44 (0)118 931 0080, email j.m.ames@afnovell.reading.ac.uk

Gibson *et al.* 1996). Probiotics and prebiotics have been developed to help maintain, or even improve, the gut microbiota composition, i.e. sustain benign or beneficial species residing in the gut (Fuller, 1992, 1997; Gibson & Roberfroid, 1995).

Bacteriological metabolism can be monitored by determining the formation of typical fermentative end-products like short-chain fatty acids and gases. However, this approach is limited in that it is important to identify the bacteria involved in metabolism, as it is recognized that the gut flora contains potentially pathogenic as well as harmless groups (Simon & Gorbach, 1984; Gilliland, 1990; Fuller & Gibson, 1997).

Microbiological changes in response to the fermentation of growth substrates are usually carried out by plating onto selective agars. However, the approach does have serious limitations, e.g. the media are never wholly selective and the technique is subject to operator subjectivity.

Molecular procedures have recently been applied to gastrointestinal microbiology (Kok *et al.* 1996; McCartney *et al.* 1996; Wilson & Blitchington, 1996; Salminen *et al.* 1998; Collins & Gibson, 1999). One such approach, that offers increased reliability over plating procedures, is the application of 16S rRNA-targetted probes (Langendijk *et al.* 1995). Here, a faecal specimen may be treated with a battery of oligonucleotide probes designed to hybridize with major components of the gut flora. To facilitate quantification, the probes are labelled with a fluorescent marker and applied in an *in situ* hybridization technique (fluorescent *in situ* hybridization, FISH).

In the present paper, we report the metabolism of melanoidins by human gut bacteria. Melanoidins were prepared from an aqueous glucose–lysine model system. We used *in vitro* systems to determine the degree of melanoidin degradation in the upper gastrointestinal tract and to assess the fermentation of melanoidins by human gut bacteria by a combination of phenotypic and FISH techniques.

Methods

Materials

Chemical reagents for the fermentation studies were obtained from Sigma (Gillingham, Dorset, UK). The bacterial culture reagents were from Oxoid (Basingstoke, Hants., UK), whilst oligonucleotide probes were synthesized and labelled by Eurogentec UK Ltd (Abingdon, Oxon., UK). D-(+)-Glucose (ACS grade) and L-lysine monohydrochloride (99+% grade, which was subsequently recrystallized from 100 ml/l aqueous ethanol), for preparing the melanoidins, were obtained from Aldrich (Gillingham, Dorset, UK). Pepsin (porcine, *EC* 3.4.23.1, catalogue no. P7000), pancreatin, containing many enzymes including amylase, trypsin, lipase, ribonuclease and protease, no *EC* number stated (porcine, catalogue no. P1750), and bile (porcine, catalogue no. B8631) were obtained from Sigma. Methanol for HPLC was obtained from Rathburn Chemicals Ltd (Walkerburn, Borders, UK) and water for HPLC was prepared in the laboratory using a Purite Labwater RO50 unit (Purite Ltd, High Wycombe, Bucks., UK).

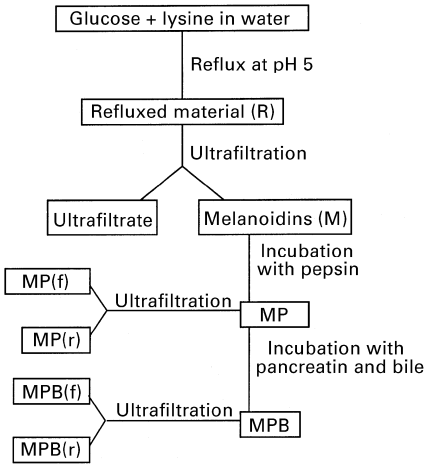

Fig. 1. Preparation of melanoidins and their *in vitro* digestion.

Preparation of melanoidins

Fig. 1 summarizes the preparation of melanoidins and their subsequent incubation and digestion. Preparation of the melanoidins was as described by Bailey *et al.* (1996) and Royle *et al.* (1998). Briefly, a solution of lysine (0·1 mol) and glucose (0·1 mol) in 100 ml water was refluxed for 2 h with the pH maintained at 5 throughout heating by the intermittent addition of 3 M-NaOH solution and pH monitoring using an autoclavable electrode. The resulting material, R (15 ml), was subjected to ultrafiltration using an Amicon ultrafiltration cell model 8050 (Amicon, Beverly, MA, USA), equipped with a 3000 Da nominal molecular mass cut-off membrane, to give a retentate volume of about 2 ml. The retentate was washed by diluting with water (5 ml) and reduced to about 0·5 ml using the 3000 Da membrane. This washing procedure was repeated twice more, in order to remove low-molecular-mass material. The final retentate was diluted to 3 ml with distilled water to give the melanoidin sample, M. This sample was subjected to elemental analysis in duplicate.

In vitro *digestion*

The fate of melanoidins under conditions that attempted to simulate those in the stomach and small intestine was examined using *in vitro* model systems, based on the procedure described by Minihane *et al.* (1993). Sample M (1 ml) was diluted with 30 ml distilled water. After adding freshly prepared pepsin (3·2 g/20 ml 0·1 M-HCl), the pH was adjusted to 2·0 using 6 M-HCl. The weight of the sample was adjusted to 50 g with distilled water and the mixture was incubated at 37° in a shaking waterbath to give sample MP. Sample MP (20 g) was adjusted to pH 7 using 0·5 M-$NaHCO_3$ and 15 ml was subjected to ultrafiltration using the 3000 Da membrane. The resulting filtrate and retentate were called MP(f) and MP(r) respectively.

Sample MP (25 g) was mixed with 5 ml freshly prepared pancreatin–bile mixture (0·4 g pancreatin and 2·5 g bile in 100 ml 0·1 M-$NaHCO_3$). The pH of the mixture was adjusted to 7 over 30 min using 0·5 M-$NaHCO_3$, before incubating at 40° for 2 h in a shaking waterbath to give sample MPB.

Sample MPB (15 ml) was subjected to ultrafiltration using the 3000 Da membrane and the filtrate, MPB(f), was collected.

Incubations were also performed without melanoidins and without enzymes. All incubations were performed in triplicate.

Melanoidins and samples derived from them were analysed by HPLC using a reverse-phase octadecasilica column and a water–methanol gradient with diode array detection and monitoring at 280 nm (Bailey *et al.* 1996).

Bacteriological fermentation

Anaerobic batch culture fermenters were operated at 37°. The basal growth medium contained (per litre): 1 g yeast extract, 1 g peptone water, 0·05 g NaCl, 0·02 g K_2HPO_4, 0·02 g KH_2PO_4, 0·005 g $MgSO_4.7H_2O$, 0·005 g $CaCl_2.6H_2O$, 1 g $NaHCO_3$, 1 ml Tween 80, 0·025 g haemin, 5 μl phylloquinone, 0·25 g cysteine HCl and 0·25 g bile salts. Initially, three fermenters were set up containing melanoidins (M), digested melanoidins (MPB), each at a final concentration of 10 g/l, and no melanoidins (control) respectively. This experiment was monitored using plate counts only. The second fermentation experiment was carried out in triplicate with melanoidin (M) at 10 g/l and the microflora were enumerated using both phenotypic and genotypic characterization methods. The operating volume of the fermenters was 50 ml and contained a final concentration of 10 g faeces/l. Samples were donated by three healthy volunteers (one female, two males, aged 25–37 years) who had not been taking antibiotics for at least 3 months before the study and had no history of gastrointestinal disorder. Fermenters were inoculated immediately after the stools had been passed. Samples for bacterial analysis and ultrafiltration were removed after 0, 6 and 24 h. Ultrafiltrates were analysed by HPLC (Bailey *et al.* 1996). The experiments were carried out in triplicate.

Cultural identification of gut bacteria

Three 1 ml samples were removed from each fermenter and were serially diluted (up to 10^{-12}) in an anaerobic cabinet (H_2–CO_2–N_2, 10 : 10 : 80, by vol.), using 500 g/l peptone water with 0·5 g/l cysteine HCl (pH 7), and plated out, in triplicate, onto agar plates designed to select for the predominant groups of gut anaerobes. These were bacteroides, bifidobacteria, lactobacilli, clostridia and total anaerobes (Table 1). After incubation, colonies were enumerated and identified. Details of the cultivation technique and phenotypic identification of isolates were as described by Wang & Gibson (1993) and Gibson *et al*. (1995).

Detection of gut bacteria by 16S rRNA probes

The probes used in the study were Bif164 (Langendijk *et al.* 1995), Bac303 (Manz *et al.* 1996), His150 (Franks *et al.* 1998) and Lab158 5′GGTATTAGCA(T/C)CTGTTTCCA, specific for bifidobacteria, bacteroides, clostridia (*Clostridium perfringens/histolyticum* subgroup) and lactobacilli–enterococci respectively. The probes were commercially synthesized and 5′-labelled with the fluorescent dye Cy3 (Eurogentec UK Ltd). The nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI) was used for total bacterial counts (Porter & Feig, 1980). Samples were diluted (1 : 3, v/v) and fixed overnight at 4° with 40 ml/l paraformaldehyde. These cells were then washed with PBS (0·1 M, pH 7·0), resuspended in a mixture of PBS–100 % ethanol (1 : 1, v/v) and stored at −20° (Langendijk *et al*. 1995). The cell suspension (32 μl) was then added to 200 μl prewarmed hybridization buffer (40 mM-Tris-HCl, 1·8 M-NaCl, 2 g/l SDS, pH 7·2) and 48 μl HPLC-grade water (Fisher Scientific, Middleton, Manchester, UK). The hybridization mixture (45 μl) was added to 5 μl each probe (50 ng/μl) and hybridized for 24 h at either 45° (Lab158 and Bac303) or 50° (Bif164 and His150). The cells were then re-suspended at their respective hybridization temperatures for 30 min in 5 ml wash buffer (20 mM-Tris-HCl, 0·9 M-NaCl, pH 7·2) and 20 μl DAPI (500 ng/μl). Subsequently, cells were vacuum filtered onto a 0·2 μm GTBP Isopore black membrane filter (Millipore Corporation, Watford, Herts., UK). The filter was mounted onto a microscope slide with SlowFade (Molecular Probes, Leiden, The Netherlands) and examined using a Nikon Microphot EPI fluorescent microscope (Nikon, Kingston upon Thames, Surrey, UK). The DM400 excitation filter was used to illuminate DAPI-stained cells and the DM510

Table 1. Oligonucleotide probes and selective growth media used for the enumeration of gut bacteria*

Target bacteria	Probe name and hybridization temperature	Culture media
Total anaerobes agar	DAPI	Wilkens Chalgren
Bacteroides	Bac303, 45°	Brucella agar + (/l) 75 mg kanamycin; 5 mg haemin; 10 ml phylloquinone; 75 mg vancomycin; 50 ml laked horse blood
Bifidobacteria	Bif164, 50°	Beerens agar (Columbia agar + (/l) 5 g glucose; 0·5 g cysteine HCl; 0·5 g propionic acid)
Clostridia	His150†, 50°	Reinforced clostridial agar + (/l) 8 mg colistin; 8 mg novobiocin
Lactobacilli	Lab158, 45°	Rogosa agar + 1·32 ml glacial acetic acid

DAPI, 4′,6-diamidino-2-phenylindole.

* Bacterial characterization, after growth on the agar plates, was as described by Wang & Gibson (1993) and Gibson *et al.* (1995).

† Probe His150 selects for the *Clostridium perfringens/histolyticum* subgroup, whilst Lab158 recovers both lactobacilli and enterococci. Probe descriptions are given by Porter & Feig (1980), Langendijk *et al.* (1995), Manz *et al.* (1996) and Franks *et al.* (1998).

filter was used to count the hybridized cells. A minimum of fifteen fields of view were counted on each slide, with more fields being counted if an abnormal distribution was observed. Each probe was counted in triplicate using this approach.

Statistical analysis

For both the cultural enumerations and FISH counts, statistically significant changes in bacterial groups at the various incubation times were determined by means of a paired Student's *t* test.

Results and discussion

The role of lower-gut function in nutrition and health is currently being researched by a number of groups. One critical aspect of this is a determination of the potential substrates for microbial growth in the colon and the nature of species involved in the metabolism of food products. Bacteria in the large bowel mainly rely on dietary residues for their growth substrates. These are mainly carbohydrate and proteinaceous in nature (Macfarlane *et al.* 1996; Macfarlane & Gibson, 1996). The present study used synthesized melanoidins to determine their degradation under *in vitro* conditions resembling those in the human gastrointestinal tract. Incubation of sample M with pepsin, and sample MP with pancreatin and bile, resulted in no melanoidin degradation products being observed by HPLC on analysis of MP(f) and MPB(f). These results indicate that no melanoidin degradation products with nominal molecular masses below 3000 Da were formed using these incubation conditions. In the batch culture fermenters inoculated with gut bacteria, there was no convincing evidence for the presence of melanoidin degradation products in the ultrafiltrates. This may be because they were metabolized by the bacteria as soon as they were formed or because the chosen HPLC conditions were not suitable for the analysis of components such as short-chain fatty acids and free lysine, which could have resulted from bacterial action on the melanoidins. Such materials may contribute towards energy gain from the fermentation process and butyrate is a preferred fuel for colonocyte function (Roediger, 1980; Cummings, 1981). The use of radiolabelled melanoidins and monitoring of labelled melanoidin degradation products would help to clarify their fate.

The results in Tables 2 and 3 show bacterial population differences in response to the fermentation of melanoidins (M). The data indicate that in a substrate-limited environment, such as that imposed on the faecal inoculum here, bacterial increases occur in response to melanoidin fermentation. That is, sample M, or products of its bacterial metabolism, acted as an electron donor in the anaerobic environment of the fermenter.

Table 2 used plate culture techniques to identify the community dynamics. For the total anaerobes, bacteroides and clostridia, a statistically significant increase was evident after both 6 h and 24 h incubations. For the bifidobacteria, this occurred after 24 h, whilst there was no marked increase in lactobacilli. In contrast, the use of a molecular probing procedure indicated that statistically significant changes occurred after 24 h incubation, and that this was the case for bacteroides, clostridia and lactobacilli, but not total anaerobes or bifidobacteria (Table 3). No significant increases were detected, using probes, after 6 h. Counts of lactobacilli were always higher through the genotypic approach. This was, at least partially, due to the fact that the probe used (Lab158) was also designed to hybridize with enterococci.

The fermentation data clearly indicate that the melanoidins affected the gut bacteria, although HPLC evidence for the presence of melanoidin degradation products was inconclusive. The observed increases in lactobacilli agree with both pure culture (Jemmali, 1969) and rat (Horikoshi *et al.* 1981) fermentation studies with Maillard reaction products. The influence of Maillard reaction products, prepared from glucose and glycine, on some intestinal bacteria (*Lactobacillus arabinosus*, *L. casei*, *L. acidophilus* and *Escherichia coli*) *in vitro* has been reported (Jemmali, 1969). The total Maillard reaction products were shown to reduce the lag phase of the lactobacilli by 10–30 %, but to increase that of *E. coli*. The amount and rate of growth increased for lactobacilli only. However, such pure culture studies do not give an adequate reflection of *in situ* events, where competitive interactions between the resident microbiota are intense. In a study using rats, it was demonstrated that the feeding of total Maillard products (high and low molecular mass) caused increases in the growth of

Table 2. Effects of the fermentation of melanoidins (sample M, Fig. 1) in batch culture fermenters on numbers of faecal bacteria ($\log_{10}$ bacterial counts/g wet weight of faeces from triplicate dilution series) as evidenced by enumeration on agar plates†

(Mean values and standard deviations)

Incubation time (h)		Total anaerobes	Bacteroides	Clostridia	Bifidobacteria	Lactobacilli
0	Mean	8·71	7·16	7·4	8·37	5·32
	SD	0·1	2·12	0·1	0·08	2·14
6	Mean	10·1*	8·39*	8·63*	8·93	6·15
	SD	0·1	0·25	1·2	0·09	1·74
24	Mean	10·68*	10·25*	9·96*	10·1*	6·06
	SD	0·1	0·4	0·06	0·1	2·37

Mean values were significantly different from those for 0 h: *$P < 0{\cdot}05$.

† Fermenters were inoculated with human faecal bacteria (10 g/l final concentration), operated under anaerobic conditions and at 37°. The experiments were carried out in triplicate.

Table 3. Effects of the fermentation of melanoidins (sample M, Fig. 1) in batch culture fermenters on numbers of faecal bacteria (log_{10} bacterial counts/g wet weight faeces from triplicate dilution series) as evidenced by fluorescent *in situ* hybridization (FISH) probes†

(Mean values and standard deviations)

Incubation time (h)		Total anaerobes	Bacteroides	Clostridia	Bifidobacteria	Lactobacilli
0	Mean	9·61	8·7	8·2	8·71	7·36
	SD	0·2	0·25	0·1	0·1	0·3
6	Mean	9·62	9·03	7·97	8·7	7·61
	SD	0·5	0·7	0·5	0·09	0·1
24	Mean	10·36	9·93*	9·12*	9·07	8·66*
	SD	0·21	0·3	0·4	0·34	0·2

Mean values were significantly different from those for 0 h: *$P < 0·05$.
† Fermenters were inoculated with human faecal bacteria (10 g/l final concentration), operated under anaerobic conditions and at 37°. The experiments were carried out in triplicate.

lactobacilli, but had no effect on numbers of enterococci, coliforms or clostridia (Horikoshi *et al.* 1981).

There are three possible mechanisms by which melanoidins may influence the growth of the gut bacteria, i.e. toxic effects, provision of substrates, and limiting the availability of metal ions due to binding. The nature of these effects will differ according to the composition of the melanoidin material. Elemental analysis of the sample used for the present study gave values of: C 44·34 %, H 7·31 %, N 6·95 %, giving O (by difference) 41·40 %, corresponding to an empirical formula of $C_{7·44}H_{14·62}NO_{5·22}$. This suggests a mixture of components which differ in empirical formulas and also little unsaturation or aromaticity (since the C : H ratio is about 1 : 2).

Viswanathan & Sarma (1957) demonstrated that autoclaving lactose with various proteins or amino acids resulted in the formation of a substance which inhibited the growth of a *Lactobacillus*. Selected bacteria appear to be able to use model melanoidins and related materials as a substrate. Terasawa *et al.* (1996) demonstrated that *Coriolus versicolor* IFO 30340, *Streptomyces werraensis* TT14 and *Paecilomyces canadensis* NC-1 were all able to decolorize model melanoidin prepared from xylose and glycine, but only the former two organisms could decolorize melanoidin made from glucose and lysine. The ability of gut bacteria to degrade these melanoidins was not investigated by the authors. It is well known that Maillard reaction products, including melanoidins, are able to bind certain metal ions (Finot, 1990; O'Brien & Morrisey, 1997). O'Brien & Morrisey (1997) reported binding of Mg, Cu, Ca and Zn to Maillard reaction products prepared from glucose and monosodium glutamate, while Homma *et al.* (1986) demonstrated that coffee pigments are able to bind Cu, Fe and Zn. In the gut, the prevailing pH will affect complexation of metal ions by melanoidins and thus influence metal ion availability.

The data in Tables 2 and 3 show that probe-based counts were always higher than the equivalent agar plate at the start of the incubation period. This probably reflected the presence of non-culturable diversity in the samples, which would not be recovered through the plating procedure. However, during later stages of the fermentation (6 h, 24 h), counts using the plate procedure were similar, or higher, than those recovered from probes. This probably indicated some adaptation of the microflora towards typical plate cultural nutrients also included in the fermenter basal media.

Traditionally, gut microbiology procedures have relied on the use of viable counting procedures to assess microbiota changes, either in human volunteer trials (Gibson *et al.* 1995) or by using *in vitro* fermentation studies (Wang & Gibson, 1993). However, there is now a shift towards the use of molecular principles for detecting gut bacterial responses to fermentable substrates (Collins & Gibson, 1999). These include gene-sequencing procedures that can more effectively identify bacterial colonies on plates (Ward *et al.* 1992; Snel *et al.* 1995), as well as direct community analyses that obviate the need for culture procedures (Amann *et al.* 1995; Zoetendal *et al.* 1998). One attractive approach is the use of 16S rRNA-based probes that can identify the microbiota of mixed samples such as gut contents. On a quantifiable basis, FISH has been used for this purpose (Langendijk *et al.* 1995; HJM Harmsen, GR Gibson, P Elfferich, GC Raangs, ACM Wideboer-Veloo, A Argaiz, MB Roberfroid and GW Welling, unpublished results).

In the present study we applied both plate culture and the FISH technology to determine how predominant gut bacteria react to the fermentation of Maillard reaction products. The rationale is that such products are widespread in the typical Western diet and may have an impact on gut bacterial metabolism. Use of both microbiological procedures showed that melanoidins are metabolized by faecal micro-organisms. However, 16S-rRNA probing (Table 3) indicated a slower fermentation than did the use of viable plate counting (Table 2). Using a phenotypic approach, a significant increase in bifidobacteria occurred (Table 2). However, this was not the case with probes (Table 3). As the probes used here have been validated for their use in gut contents, it likely that this approach will give the more accurate reflection of fermentation events.

The conclusions of this study are two-fold. (1) Human gut microflora are affected by melanoidins. O'Brien & Morrissey (1989) suggested that the effects of Maillard reaction products on gut microflora may be similar to those of lactose and other poorly digestible carbohydrates. The degradation of melanoidins in the large intestine may also play a role in the binding and/or release of other dietary components.

(2) A genotypic approach to the fermentation shows discrepancies with the more frequently used plate culture technique. Imminent developments should encompass both approaches to the bacteriology. However, the adaptation of molecular principles to gut fermentation opens up the possibility of volunteer trials that exploit a high fidelity approach and allow multiple-centre trials to be carried out (as the samples can be stored before detection of the bacteria).

Acknowledgements

Dr R. G. Bailey and Dr A. M. Minihane are thanked respectively for help and advice with the preparation and HPLC of the melanoidins and with the pepsin and pancreatin digestions.

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