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 & Magenat, 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 discoloration 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;
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 & Bittlingham, 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 melano- 

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°C in a shaking waterbath to give sample MP. Sample MP (20 g) was adjusted to pH 7 using 0.5 M NaHCO₃ and 15 ml was subjected to ultratulation 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 NaHCO₃). The pH of the mixture was adjusted to 7 over 30 min using 0.5 M NaHCO₃, before incubating at 40°C 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 octadecasila 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°C. The basal growth medium contained (per litre): 1 g yeast extract, 1 g peptone water, 0.05 g NaCl, 0.02 g KH₂PO₄, 0.02 g K₂HPO₄, 0.005 g MgSO₄.7H₂O, 0.005 g CaCl₂.2H₂O, 1 g NaHCO₃, 1 ml Tween 80, 0.025 g haemin, 5 μl phyloquinone, 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 (H₂–CO₂–N₂, 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°C with 40 ml paraformaldehyde. These cells were then washed with PBS (0.1 M, pH 7.0), resuspended in a mixture of PBS–10% ethanol (1 : 1, v/v) and stored at −20°C (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°C (Lab158 and Bac303) or 50°C (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 excitation filter was used to illuminate Cy3-stained cells. Any counts identified as DAPI-negative and Cy3-positive were scored as positive.

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

<table>
<thead>
<tr>
<th>Target bacteria</th>
<th>Probe name and hybridization temperature</th>
<th>Culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total anaerobes agar</td>
<td>DAPI, Bac303, 45°C</td>
<td>Wilkens Chalgren</td>
</tr>
<tr>
<td>Bacteroides</td>
<td></td>
<td>Brucella agar + (λ) 75 mg kanamycin; 5 mg haemin; 10 ml phyloquinone; 75 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>vancomycin; 50 ml laked horse blood</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>Bif164, 50°C</td>
<td>Beerens agar (Columbia agar + (λ) 5 g glucose; 0.5 g cysteine HCl; 0.5 g</td>
</tr>
<tr>
<td></td>
<td></td>
<td>propionic acid)</td>
</tr>
<tr>
<td>Clostridia</td>
<td>His150†, 50°C</td>
<td>Reinforced clostridial agar + (λ) 8 mg colistin; 8 mg novobiocin</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>Lab158, 45°C</td>
<td>Rogosa agar + 1.32 ml glacial acetic acid</td>
</tr>
</tbody>
</table>

† 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. These are mainly carbohydrate components such as short-chain fatty acids and free lysine, which could have resulted from bacterial action on 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

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**Table 2. Effects of the fermentation of melanoidins (sample M, Fig. 1) in batch culture fermenters on numbers of faecal bacteria (log₁₀ bacterial counts/g wet weight of faeces from triplicate dilution series) as evidenced by enumeration on agar plates†**

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Total anaerobes</th>
<th>Bacteroides</th>
<th>Clostridia</th>
<th>Bifidobacteria</th>
<th>Lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>8.71</td>
<td>0.1</td>
<td>7.16</td>
<td>2.12</td>
<td>7.4</td>
</tr>
<tr>
<td>6</td>
<td>10.1*</td>
<td>0.1</td>
<td>8.39*</td>
<td>0.25</td>
<td>8.63*</td>
</tr>
<tr>
<td>24</td>
<td>10.68*</td>
<td>0.1</td>
<td>10.25*</td>
<td>0.4</td>
<td>9.96*</td>
</tr>
</tbody>
</table>

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°C. 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 melanoids 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 auto-claving 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 melanoids and related materials as a substrate. Terasawa et al. (1996) demonstrated that Coriolus versicolor IFO 30340, Streptomyces werraensis TT14 and Paeilomyces 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 melanoids, are able to bind certain metal ions (Finot, 1990; O'Brien & Morrissey, 1997). O'Brien & Morrissey (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 melanoids 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 (6h, 24h), 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 Harmen, GR Gibson, P Elfferich, GC Raangs, ACM Wideboer-Veloo, A Argaz, 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 melanoids 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 melanoids. 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 melanoids in the large intestine may also play a role in the binding and/or release of other dietary components.

### Table 3. Effects of the fermentation of melanoids (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†

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Total anaerobes</th>
<th>Bacteroides</th>
<th>Clostridia</th>
<th>Bifidobacteria</th>
<th>Lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Mean</td>
<td>9.61</td>
<td>8.7</td>
<td>8.2</td>
<td>8.71</td>
<td>7.36</td>
</tr>
<tr>
<td>SD</td>
<td>0.2</td>
<td>0.25</td>
<td>0.1</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>6 Mean</td>
<td>9.62</td>
<td>9.03</td>
<td>7.97</td>
<td>8.7</td>
<td>7.61</td>
</tr>
<tr>
<td>SD</td>
<td>0.5</td>
<td>0.7</td>
<td>0.5</td>
<td>0.09</td>
<td>0.1</td>
</tr>
<tr>
<td>24 Mean</td>
<td>10.36</td>
<td>9.93*</td>
<td>9.12*</td>
<td>9.07</td>
<td>8.66*</td>
</tr>
<tr>
<td>SD</td>
<td>0.21</td>
<td>0.3</td>
<td>0.4</td>
<td>0.34</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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°C. The experiments were carried out in triplicate.
(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 melanoids and with the pepsin and pancreatin digestions.

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