THE EFFECTS OF pH ON COLONIC BACTERIA GROWN IN CONTINUOUS CULTURE

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SUMMARY. A model of the promixal colon was used to investigate the effects of pH on fermentation by colonic bacteria *in vitro*. Twelve continuous anaerobic cultures of human faecal bacteria were maintained at constant pH in a medium simulating ileostomy effluent. Five cultures were maintained at pH 7, five at pH 6, and two at pH 5. The pH of each of three further cultures was altered after they had reached steady state, either from 7 to 6 and then to 5, or from 5 to 6 to 7.

Both experimental designs showed that the pH exerted an important effect on bacterial metabolism without causing major changes in bacterial populations. Osmolality was lower in cultures run at a low pH. Total volatile fatty acid concentration was decreased at pH 5, and the production of propionic acid rather than acetic acid was favoured at pH 6. Changing the pH had no significant influence on the production of ammonia in these systems.

INTRODUCTION

Recent evidence suggests that the colonic fermentation of unabsorbed carbohydrate to volatile fatty acids (vfa) and their consequent absorption may make an important contribution to the energy intake of the host (Cummings, 1981), may provide nutrients necessary for the survival of colonic enterocytes (Roediger, 1980) and may prevent the osmotic diarrhoea caused by the entry of unabsorbed carbohydrate into the colon (McNeil, Cummings and James, 1978; Ruppin *et al.*, 1980; Saunders and Wiggins, 1981).

Fermentation of carbohydrate to vfa in the colon causes the pH to fall. Bown *et al.* (1974) reported a caecal pH of 4.8 after ingestion of the unabsorbable disaccharide, lactulose. Perman, Modler and Olson (1981) concluded that the reduction in hydrogen response to a lactulose drink that occurred following intake of a lactulose diet for 7 days was related to the fall in pH, because the amount of hydrogen produced by fresh human faeces from glucose was pH-dependent. This may have a marked influence on colonic metabolism. A reduction in pH also inhibits ammonia production in batch cultures (Vince, Killingley and Wrong, 1978), and this may explain why lactulose reduces blood ammonia concentrations in patients with hepatic encephalopathy. The

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colonic pH has an important influence on the growth of colonic bacteria; some species, such as lactobacilli, exhibit optimal growth at acidic pH (5.4–6.4) whereas others, such as *Clostridium* spp., prefer a higher pH of 6.5–7.5. The inhibition of bacterial growth by vfa and bile acids is greater at acid pH (Percy-Robb and Collee, 1972; Binder, Filburn and Floch, 1975; Russell, Sharp and Baldwin, 1979).

The metabolic function of the colonic bacteria is difficult to study *in situ* because of the inaccessibility of the proximal colon. Batch cultures, in which bacterial suspensions are exposed to finite amounts of substrate *in vitro*, may provide limited information because they are essentially static or dying cultures. We have developed an in-vitro model in which colonic bacteria are maintained viable and metabolically active in steady-state conditions simulating those found in the proximal colon. The model consists of faecal bacteria cultured anaerobically for up to 25 days in a continuous flow of medium resembling ileostomy effluent. We have now used this model of the proximal colon to investigate the effects of varying the pH on the colonic bacteria and on the ability of these populations to ferment carbohydrate and produce ammonia.

METHODS

The proximal colon model

The inoculum from normal human volunteers was 20–40g of faeces, homogenised in 250 ml of pre-reduced sterile medium, based on the composition of ileostomy effluent. This contained 60mM NaCl, 40mM NaHCO₃, 10mM KCl, tryptone (Oxoid) 10g/L, porcine bile extract (Sigma) 5g/L, haemin 5×10^{-5} g/L, starch 1.2g/L, glucose 5g/L and maltose 6g/L. The apparatus consisted of a modified Gallenkamp modular fermenter and is diagrammatically represented in fig. 1. The culture was contained within a 1-L fermentation vessel (A) and stirred at 100 rpm with a magnetic stirrer. A 13-ml volume of fresh medium was introduced over a 2-min period every hour by a peristaltic pump (B), which simultaneously removed excess culture to maintain the culture volume at 300 ml. Anaerobic conditions were maintained by gassing both the medium and fermentation vessels with 95% N₂/5% CO₂ and by using tubing which was impermeable to gas (Butyl XX tubing; Esco (Rubber) Ltd, Middlesex). The pH of the culture was maintained constant by a pH-stat unit which infused 1.5M NaHCO₃ when necessary (C). The system was housed in a constant temperature room at 37°C.

Protocol

The effect of pH was studied in two ways. (1) Continuous cultures were set up and run at constant pH for at least 21 days; two cultures were run at pH 5, five at pH 6 and five at pH 7. (2) After the cultures had attained steady state the pH was varied; in two cultures the pH was held initially at pH 7 for the first 7 days, then reduced to 6 for 5 days, and reduced again to 5 for a further 5 days. A third culture was run at pH 5 for 7 days, then the pH was raised to 6 for 5 days and then to 7 for a further 5 days. These three cultures were paired with control cultures, which were seeded with the same inoculum, run at the same time, but maintained at constant pH. Two cultures were run at pH 5, the other at pH 7.

The steady-state cultures allowed bacterial metabolism to be studied for long periods at a stable pH. The experiment in which the pH was varied provided a means of overcoming some of the variability between cultures by having each culture serving as its own control.

Measurements

Every 24 h the medium flasks were renewed, the outflow flasks emptied, and the overflow volume recorded. The Eh and pH of the culture were measured and a 20-ml sample was taken

FIG. 1.—The apparatus used for the continuous culture of faecal bacteria. A is fermentation vessel; B is peristaltic pump and timer; C is pH stat unit; D is pH meter and pump control unit; E is electrometer.

from the culture vessel for analysis of *vfa*, ammonia, osmolality and bacterial identification. The amount of base used during the previous 24 h was noted.

Effect of pH on osmolality and redox potential. To determine whether changing the pH of the sterile medium had any direct effect on osmolality and redox potential, measurements were made after the addition of HCl had reduced the pH of sterile medium in stages of half a pH unit to pH 5.

Chemical assays

Osmolality was measured by depression of the freezing point (Osmometer Model 3WII, Advanced Instruments Ltd, MA, USA). **pH** and **redox potential** (Eh) of the culture were monitored daily with a pH glass combination electrode (FBL-730-010E-Gallenkamp, London) and an Eh platinum combination electrode (CMR pt; Russell pH Ltd, Fife, Scotland) incorporated into the system in conjunction with a pH meter (FBL 720-Gallenkamp, or model 7020 Electronic Instruments Ltd, Surrey) and a high impedance electrometer (Model 602; Keighley, Cleveland, OH, USA), respectively.

Ammonia was assayed by an adapted Berthelot reaction (Varley, Gowenlock and Bell, 1980).

Vfa were assayed by gas liquid chromatography (GLC) after centrifugation of the sample to remove the sediment, dilution with an internal standard (1 ml of sample to 1 ml of 20 mM valeric acid) and acidification with 20% H_2SO_4 (0.2 ml to 1 ml of dilute sample). The gas-liquid chromatograph (Varian model 3700, Palo Alto, CA, USA) had a 2-m column of 4 mm internal diameter packed with AT200 10% and H_3PO_4 1% on Chromosorb WAW (80–100 mesh). Column temperature was 121°C and gas flow rates were H_2 30 ml/min, air 300 ml/min and N_2 30 ml/min. The output of the flame ionisation detector was fed into an integrator (model 3390A, Hewlett Packard, Waltham, MA, USA) which integrated the areas under the peaks. External standards of acetic, propionic, *n*-butyric and valeric acids were used to calibrate the responses.

Bacterial identification

Samples of culture were plated out on primary selective plates (table I) with a standard loop and plating procedure. A semiquantitative assay of bacterial population was obtained by a

TABLE I
The bacteriological media used for identification and quantitation of bacteria growing in continuous culture

Medium	Method of incubation	Principal bacteria detected
Blood agar	Aerobically 37°C	General (aerobes)
MacConkey agar (Oxoid)	Aerobically 37°C	Enterobacteria, Enterococci
Sabouraud agar (Oxoid)		Yeasts
Rogosa agar (Oxoid)	Anaerobically 37°C	Lactobacilli
Reinforced clostridial medium containing 0.1% cotton blue (Willis <i>et al.</i> , 1973)	Anaerobically 37°C	Bifidobacteria
BM Kanamycin agar (Holbrook, Ogston and Ross, 1978)	Anaerobically 37°C	Clostridia
BM Kanamycin/vancomycin agar	Anaerobically 37°C	Bacteroides
Blood agar	Anaerobically 37°C	General (anaerobes and facultative species)

system of scoring on a scale of 1–5 (Rotimi and Duerden, 1981). If growth occurred in the pool, a score of 1 was recorded (*c.* 10^4 cfu/ml); if in the first streak, a score of 2 and so on up to a maximum of 5 ($> 10^8$ cfu/ml).

The anaerobic plates were incubated in a BTL (Baird and Tatlock Ltd, London) or Whitley (Don Whitley Scientific, Shipley, W. Yorkshire) jar with an atmosphere of H₂ 90% and CO₂ 10% obtained by an evacuation-replacement technique (Collee *et al.*, 1972).

After 48 h, representative colonies of each type were subcultured. These were then identified according to colony morphology, cell morphology and results obtained in a range of conventional tests (Cowan, 1974; Willis, 1977; Rotimi, Faulkner and Duerden, 1980).

Statistical methods

The results of cultures maintained at constant pH were analysed by Student's 't' test; results of cultures in which the pH was varied were compared by a paired 't' test (Hill, 1971).

RESULTS

Cultures maintained at constant pH

Mixed cultures (12) were successfully grown from human faecal inocula. All cultures continued to ferment carbohydrate to vfa and maintain viable anaerobic bacterial populations for periods up to 25 days.

Redox potential (Eh). A highly negative redox potential (Eh), suitable for the growth of anaerobic bacteria, was maintained in all cultures. The Eh was not significantly lower in cultures run at a lower pH (fig. 2; table II), although the Eh of sterile medium became more positive by 54 mV for each drop of one pH unit.

FIG. 2.—The redox potential and osmolality of individual cultures maintained at a constant pH 7, or 6 or 5 (mean $\pm$ SEM).

Presumably the chemical effect of the acid was balanced in part by the effect of a change in bacterial metabolism.

Osmolality. The osmolality of cultures run at pH 7 (532–588 mosmol/kg) was significantly greater than the osmolality of the sterile medium (285 mosmol/kg) or the osmolalities of cultures run at more acid pH (fig. 2; $p < 0.001$). The differences in osmolalities between cultures run at pH 6 and pH 5 were also significant ($p < 0.05$). A heavy sediment was occasionally seen when cultures were run at pH 5. There was no variation in osmolality when the pH of the sterile medium was changed by the addition of acid.

Volatile fatty acid production. Volatile fatty acids were produced in all cultures. Acetic acid and propionic acids always amounted to 90% of the total. Some but not all cultures produced *n*-butyric acid (two cultures at pH 7, four at pH 6 and one at pH 5).

There was no significant difference between the total vfa outputs of cultures run at pH 6 and at pH 7, but vfa output was significantly lower in cultures run at pH 5 ($p < 0.01$) (fig 3). Acetic acid production was highest at pH 7 (21.3 ± 2.4 mmol/day; mean $\pm$ SEM) and lower in cultures at pH 6 and 5 ($p < 0.01$) (table II). Propionic acid production was higher at pH 6 (15.3 ± 1.2 mol/day) than at pH 5 or pH 7 ($p < 0.01$). There was no obvious relationship between the production of *n*-butyric acid and pH.

Ammonia production. Ammonia output did not decrease significantly when the cultures were run at a lower pH (table II).

TABLE II
The Eh, osmolality, volatile fatty acid (vfa) and ammonia production in cultures maintained at constant pH

pH	Redox potential (mV)	Osmolality (mosmol/kg)	Ammonia conc. (mmol/L)	Ammonia output (mmol/day)	Vfa concentration mmol/L			Vfa output mmol/day		
					Total	Acetic	Propionic	Total	Acetic	Propionic
7 (n=5)	-385 ± 29	589 ± 22	23.1 ± 4.4*	9.6 ± 2.1*	85.3 ± 8.2	57.0 ± 6.8	25.2 ± 4.5†	31.8 ± 2.0	21.3 ± 2.4	8.8 ± 1.6†
6 (n=5)	-351 ± 8	432 ± 8†	21.2 ± 3.2	7.4 ± 1.2	88.4 ± 5.4	39.4 ± 2.5†	41.2 ± 4.1	30.4 ± 1.6	13.7 ± 0.7	15.3 ± 1.2
5 (n=2)	-344 ± 18	351 ± 33‡	14.5 ± 5.9	4.6 ± 1.6	36.0 ± 9.3†‡	18.3 ± 4.0†	12.7 ± 3.4‡	11.9 ± 4.0†‡	5.7 ± 1.6	4.1 ± 0.9‡

The results are expressed as mean ± SEM.

*n=2

† Significantly less than pH 7.

‡ Significantly less than pH 6.

FIG. 3.—The vfa outputs of individual cultures maintained at a constant pH of 7, or 6 or 5 (mean $\pm$ SD).

Bacteria. The mean bacterial scores at each pH are shown in fig. 4. Populations of *Bacteroides* spp. and *Escherichia coli* were well maintained in all cultures. *Klebsiella* spp. and *Streptococcus faecalis* were present in most cultures, and diphtheroids and clostridia were present in a few. Lactobacilli and bifidobacteria were only recovered from cultures at pH 5.

The effect of changing the pH of an ongoing culture

Redox potential. Unlike our findings with cultures maintained at steady pH, the Eh in these cultures decreased as the pH fell (table III). The difference between values at pH 7 and at pH 6 was not statistically significant but the difference in Eh between pH 6 and pH 5 was ($p < 0.02$). However, these differences were not significant when the results were corrected for the reduction that occurred by adding acid to sterile medium.

Osmolality. As with cultures maintained at constant pH, the osmolality declined in proportion with the pH (table III).

Volatile fatty acid production. The vfa output profile of a typical culture is shown in fig. 5, along with that of its paired control culture which was maintained at a constant

FIG. 4.—The mean scores gained by each bacterial species in individual cultures; five were maintained at pH 7, five at pH 6 and two at pH 5.

TABLE III

The pH, osmolality, vfa and ammonia production in cultures in which pH was varied

pH	Redox potential (mV)	Corrected redox potential (mV)	Osmolality (mosmol/kg)	Ammonia production (mmol/day)	Total vfa production (mmol/day)	Acetic acid production (mmol/day)	Propionic acid production (mmol/day)	n-Butyric acid production (mmol/day)
7	-451 ± 9.2	-451 ± 9.2	$713 \pm 31^*$	5.2 ± 2.4	$19.8 \pm 6.0^*$	13.9 ± 4.2	$4.8 \pm 2.7^*$	1.4 ± 0.8
6	-375 ± 24	-429	$460 \pm 7^\dagger$	7.3 ± 2.7	$23.6 \pm 6.9^\dagger$	18.8 ± 3.9	$14.4 \pm 2.6^\dagger$	0.6 ± 0.2
5	-303 ± 33	-411	$371 \pm 11^{*\dagger}$	6.0 ± 3.8	19.0 ± 3.2	9.7 ± 2.6	7.4 ± 0.6	0.3 ± 0.3

Results are expressed as mean $\pm$ SEM.

* Statistically significant difference from pH 6.

† Statistically significant difference from pH 7.

pH 7. There was a significantly greater total production of vfa at pH 6 than at pH 7 ($p < 0.02$ for paired values from individual cultures). This total vfa production at pH 6 was also greater than at pH 5, but these values were not significantly different. Total vfa production in paired control cultures remained stable and was lower than that measured in the test cultures run at pH 6 ($p < 0.05$), but not at pH 5 or 7.

The increase at pH 6 was caused by an increase in propionic acid in all three cultures ($p < 0.005$), and an increase in acetic acid production in two of the three cultures. In contrast with cultures run at constant pH, there was no significant difference in the total and individual vfa output between pH 7 and pH 5.

FIG. 5.—The total vfa output profiles of a culture in which the pH was changed from 7 to 6 and then to 5 (----) and a control culture the pH of which was maintained at a constant pH 7.

FIG. 6.—The changes in bacterial scores that occurred in two cultures (——) when the pH was altered from pH 7 to 6 and then to 5, and in one culture (----) when the pH was changed from 5 to 6 and then to 7.

Ammonia production. Changing the pH did not significantly affect ammonia production (table III).

Bacteria. Changing the pH had little effect on the populations of most of the bacteria in these cultures (fig. 6). Numbers of *Bacteroides* spp. were well maintained at all pH values. Lactobacilli and bifidobacteria survived in the culture run initially at pH 5, but disappeared when the pH rose to 6 and then to 7. *C. perfringens* survived in cultures run at pH 7, but disappeared when the pH fell to 6 and then to 5.

DISCUSSION

The results of this study show that a mixed culture of faecal bacteria can be maintained *in vitro* under steady-state conditions for long periods, and can continue to produce volatile fatty acids (vfa). Moreover, the culture responded to changes in pH in the range that would be encountered in the colon. Two different experimental designs were used: in one the pH was maintained at a steady level throughout the experiment, and in the other the pH was changed after values had achieved a steady state for a few days.

In general, the different designs provided similar results. They both showed that the osmolality fell as the pH decreased, that the production of propionic acid was favoured at pH 6, that ammonia production was not altered by changes in pH, and that populations of bacteroides, enterococci and *E. coli* were well maintained at each pH; clostridia survived better at pH 7, whereas lactobacilli and bifidobacteria were favoured at pH 5. However, there were some differences in the results obtained with the two experimental designs. Acetic acid production was greater at pH 6 than at pH 7 in cultures where the pH was changed, but was greater at pH 7 in cultures run at one pH. A reduction in Eh was observed as the pH was decreased in those cultures where the pH was changed and could be explained in part by the physical effect of pH on Eh. This effect was only seen in cultures of this type. The variation in metabolic activity between cultures run at constant pH may have masked the effect of pH on Eh. Changing the pH of the same culture avoided this variability and may have provided a more sensitive index of metabolic change.

In both types of culture, more propionic acid was produced at pH 6. This result differs from that obtained in studies with continuous rumen cultures (Slyter, Bryant and Wolin, 1966) where the output of propionic acid was reduced at pH 6 but to a lesser extent than the output of acetic acid. The increase in production of propionic acid in our cultures at lower pH may be related to a change favouring the bacteria that produce propionic acid. On the other hand, it could be explained by an increased level of NADH in the bacterial cells. This would promote the metabolic pathways leading to propionic acid rather than those leading to acetic acid. This contrasts with the effect seen in the rumen in the presence of methanogenic bacteria which lower NADH levels in the cells by removing hydrogen ions from the rumen fluid; in turn this promotes acetic acid production at the expense of propionic acid production (Wolin, 1981).

The fall in osmolality is difficult to explain, especially as there was more vfa production at pH 6 than at pH 7. It may, however, be related to an increased association of the vfa at low pH or to the effect of pH on the production or dissociation of other constituents in the culture.

The observation that reduction in pH had no significant effect on production of ammonia contradicts the observations on batch cultures, in which a pH of 5 or less caused a significant reduction in ammonia production (Vince *et al.*, 1978). If we can assume that our continuous cultures bear a closer resemblance to conditions in the proximal colon *in vivo*, the beneficial effect of lactulose in decreasing blood ammonia levels in patients with hepatic encephalopathy may not be due to the effect of a reduced caecal pH on ammonia production (Vince *et al.*, 1978). Instead it is probably explained by ionisation of ammonia to ammonium which is poorly absorbed by the colon (Down *et al.*, 1972).

Changing the pH had little effect on bacterial numbers. Most of the bacteria maintained steady populations in all cultures regardless of pH. Lactobacilli and bifidobacteria, however, survived only at pH 5. This was also noted by Veilleux and Rowland (1981) who cultured rat faecal material in continuous culture.

Although our model attempts to simulate the fermentation of carbohydrate in the proximal colon, this is a very complex environment and contains important growth factors not supplied in our artificial system. Moreover, to allow adequate sampling and mixing, our culture was liquid and hence markedly different from the viscous semisolid contents of the proximal colon, which must contain much larger concentrations of bacteria and provide numerous attachment sites. The energy sources for the bacteria in our cultures were simple sugars and starch, whereas *in vivo* the fermentation of mucin and complex polysaccharides may be important. The porcine bile extract provided both primary and secondary bile salts. Long-chain fatty acids, magnesium and calcium ions were omitted from the medium because of precipitation, though these substrates may have important influences on bacterial growth.

Our study has shown that pH affects the metabolism of faecal bacteria without affecting bacterial numbers. The effect of pH may be of great importance in the proximal colon where the majority of bacterial fermentation takes place. The pH of the proximal colon varies considerably (Bown *et al.*, 1974), falling dramatically when unabsorbed carbohydrate enters from the small intestine. The resultant effect on bacterial metabolism changes the pattern and amount of vfa produced. Thus, changes in pH may have a major influence upon the health of colonic enterocytes and the efficiency of the colon to salvage unabsorbed carbohydrate by conversion into vfa, which are rapidly absorbed and utilised by the host.

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