

## Factors affecting fermentation reactions in the large bowel

BY G. T. MACFARLANE AND S. MACFARLANE

MRC Dunn Clinical Nutrition Centre, 100 Tennis Court Road, Cambridge CB2 1QL

Quantitatively, the main sources of C and energy for bacteria growing in the human large intestine are starches and plant cell wall polysaccharides that escape digestion in the small bowel (Macfarlane & Cummings, 1991). These substances are depolymerized by hydrolytic enzymes produced by colonic bacteria, and their component sugars are fermented to a range of products, including organic acids, alcohols and gases (Cummings *et al.* 1989; Macfarlane *et al.* 1992). The principal products are the short-chain fatty acids (SCFA) acetate, propionate and butyrate (Cummings, 1981). The physiological significance to the host of SCFA production in the colon is that the vast majority (about 90%) are absorbed from the gut and subsequently undergo a variety of metabolic fates within the body (for review, see Cummings & Macfarlane, 1991). Because of the interactions between bacterial fermentation products and host metabolism, it is important to understand the physical, chemical and biological factors that govern the degradation of carbohydrates by intestinal micro-organisms. The present paper describes *in vivo* and *in vitro* studies on carbohydrate fermentation using pure and mixed cultures of colonic bacteria to determine the role of substrate availability on endproduct formation.

### IN VIVO MEASUREMENTS OF FERMENTATION PRODUCTS

Due to the practical difficulties involved in routine sampling from the healthy large intestine, these studies were made using contents taken from different regions of the colon from persons who had died suddenly (Cummings *et al.* 1987). The results showed that the mean total concentration of SCFA in the proximal bowel (caecum and ascending colon) was 142 (SEM 14) mM ( $n$  8), and in the distal bowel (descending colon and sigmoid/rectum), 96 (SEM 18) mM ( $n$  6). These findings suggested that the difference in SCFA levels resulted from the greater availability of fermentable substrate in the proximal colon. This was confirmed in subsequent experiments where gut contents were incubated anaerobically *in vitro* for up to 48 h in the absence of any exogenously added substrate: SCFA generation was found to be approximately threefold higher in digesta from the proximal gut, compared with that from the distal colon (Macfarlane *et al.* 1992). Determinations of other fermentation acids in gut contents showed that concentrations of lactate and succinate, which are electron sink products formed by bacteria to dispose of excess reducing power during fermentation, were also greatest in the proximal colon, reflecting comparative substrate levels, and bacterial growth rates, as will be discussed in more detail later.

### GENERAL ECOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL FACTORS AFFECTING FERMENTATION PRODUCT FORMATION IN THE GUT

A number of studies have indicated that although the species composition of the gut microbiota in different people is broadly similar, there can be considerable inter- and

Table 1. *Cell population densities and fermentation products of saccharolytic anaerobes from the human large gut\**

Bacteria	Log <sub>10</sub> (/g dry wt faeces)		Products
	Mean	Range	
Bacteroides	11.3	9.2–13.5	A, P, S, L
Eubacteria	10.7	5.0–13.3	A, B, L
Bifidobacteria	10.2	4.9–13.4	A, f, l, e
Clostridia	9.8	3.3–13.1	A, P, B, L, e
Lactobacilli	9.6	3.6–12.5	L, A
Ruminococci	10.2	4.6–12.8	A

A, acetate; B, butyrate; e, ethanol; f, formate; L, lactate; P, propionate; S, succinate.

\* Cell count data is from Finegold *et al.* (1983), fermentation product information is from Holdeman *et al.* (1977).

Table 2. *Polysaccharides known to be degraded by certain human intestinal bacteria*

Bacteria	Polysaccharide
Bacteroides	Arabinogalactan, pectin, cellulose, xylan, guar gum, mucins, heparin, chondroitin sulphate, starch
Bifidobacteria	Arabinogalactan, pectin, starch, mucin, gum arabic, xylan
Ruminococci	Mucins, guar gum
Eubacteria	Starch, pectin
Clostridia	Starch, pectin

intra-individual variation in the relative cell population densities of the principal taxonomic groups (Finegold *et al.* 1983; Meijer-Severs & Van Santen, 1986). This is demonstrated in Table 1, which shows cell count data for the major anaerobic genera that occur in the large gut, together with their principal fermentation products. The significance of this information in the context of carbohydrate metabolism in the colon is that the fermentation strategies of bacteria degrading any particular substrate, as well as their absolute numbers, will affect the amounts and types of fermentation product that can be formed. Since these polymer-degrading bacteria participate in the breakdown of different polysaccharides (Table 2), it can be predicted that different substrates will give rise to different fermentation products.

The fermentation of carbohydrate by bacteria growing in the large bowel consists of a series of energy-yielding catabolic reactions that do not involve respiratory chains which use molecular O<sub>2</sub> or nitrate as terminal electron acceptors. ATP can be generated by substrate level phosphorylation reactions, or in some species, by electron-transport-linked phosphorylation. In fermentation, the terminal electron acceptor is a product of the original substrate, and the amount of energy that can be produced depends on the difference in redox potential between the substrate and endproducts. For example, acetate is a more oxidized product than butyrate, and in the butyrate-forming clostridia,

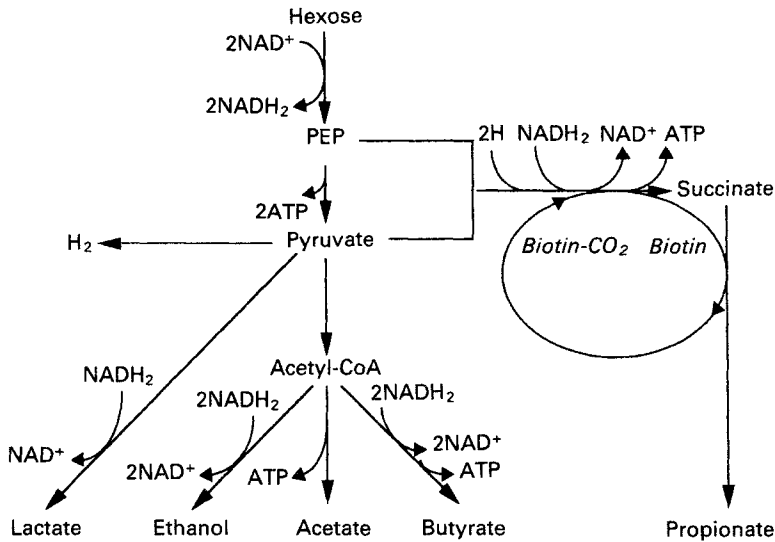


Fig. 1. Overview of pathways of carbohydrate fermentation in the human large intestine. PEP, phosphoenolpyruvate.

one mole of ATP is made for each mole of acetate produced, whereas two moles of butyrate must be formed to generate one mole of ATP. An important consideration in regulating fermentation reactions is the need to reoxidize reduced pyridine nucleotides and maintain redox balance. This is achieved by making reduced fermentation products (electron sinks) such as H<sub>2</sub>, ethanol, butyrate, succinate and lactate. A simplified overview of some of the major carbohydrate fermentation pathways occurring in the colon is shown in Fig. 1.

#### IN VITRO FERMENTATION EXPERIMENTS

They were carried out to investigate how different polysaccharides were degraded by mixed populations of gut bacteria. Four substrates were studied; starch, pectin, arabinogalactan and xylan. The fermentations were carried out in pH-controlled batch-culture fermenters, and periodic measurements were made of residual carbohydrate and endproduct formation. The results showed that the polysaccharides were broken down at different rates: starch was degraded most rapidly, followed by pectin, arabinogalactan and xylan. Since these substrates must be depolymerized before they can be fermented, the rate of substrate hydrolysis will strongly influence the rate at which carbohydrate becomes available to the bacteria. This, as will be seen later in the pure culture studies, is an important determinant of the amounts and types of fermentation products that can be formed. SCFA measurements from these experiments indicated that intestinal bacteria formed different fermentation products from different substrates. The molar ratios of acetate, propionate and butyrate were 50:22:29 with starch; 84:14:2 with pectin; 50:42:8 with arabinogalactan and 82:15:3 with xylan. As well as the rate of substrate depolymerization, the chemical composition and degree of reduction of the constituent sugars all influence fermentation product formation, with highly reduced

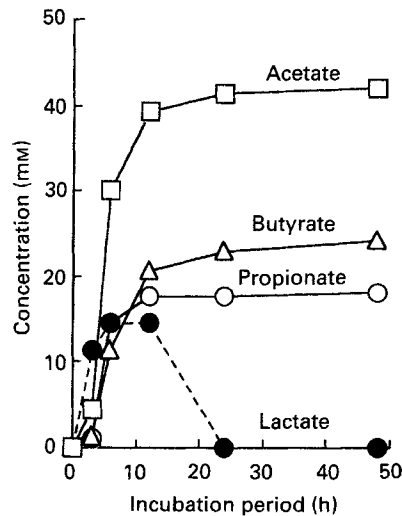


Fig. 2. Lactate and short-chain fatty acid production during fermentation of starch by mixed populations of human colonic bacteria. The bacteria were grown in pH-controlled batch-culture fermenters as described by Macfarlane & Englyst (1986).

substrates giving rise to more reduced fermentation products. The starch fermentations were particularly notable in that comparatively large amounts of lactate and butyrate were produced (Fig. 2). It can be seen that although lactate is the main fermentation acid in the initial stages of the incubation, its concentration decreases in the later stages, as it is converted to SCFA. Thus, measurements of this fermentation intermediate in either gut contents, or in *in vitro* fermentations with faecal bacteria do not necessarily reflect actual production values, but only the balance between that which is being produced and that which is being further metabolized. High levels of butyrate formation from starch have also been reported by Scheppach *et al.* (1988) to occur *in vivo*, in feeding studies with human volunteers. The relationship between starch fermentation and butyrate production is of special interest because this SCFA is an important fuel for colonic epithelial cells (Roediger, 1980), and in some circumstances, has been shown to inhibit the growth of certain neoplastic cell lines (Leder & Leder, 1975; Kim *et al.* 1982). Interestingly, most probable number (MPN) estimations of bacteria (tenfold dilutions; five replicates) that produce butyrate as a major fermentation product (>2.5 mM after 48 h incubation in peptone yeast-extract glucose broth) indicated that they occur in comparatively low numbers in faeces, comprising less than 1% of the total anaerobe count. Since butyrate accounts for approximately one-fifth of total SCFA in gut contents, these bacteria must be very active metabolically.

#### CONTINUOUS CULTURE FERMENTATIONS

It is necessary to study carbohydrate metabolism *in vitro* using mixed populations of gut bacteria, since this enables the observations to be related to events occurring in the colon. However, these experiments provide relatively limited information with respect to the physiological and biochemical factors that govern fermentation processes. Ideally,

they should be complemented, therefore, with studies using pure cultures of intestinal bacteria, to provide an insight into the underlying regulatory principles that are involved. For this reason, we studied the metabolism of carbohydrate in pure cultures of colonic bacteria grown in chemostats, to determine how nutritional availability can influence fermentation product formation. The four species studied were *Clostridium perfringens*, a bacterium that produces acetate and lactate as its major fermentation acids; *Bifidobacterium angulatum*, which forms a variety of products including acetate, lactate, formate and ethanol; *Bacteroides ovatus*, which produces acetate, succinate and propionate and *Clostridium butyricum*, an acetate- and butyrate-forming organism. In all these bacteria, C availability and growth rate strongly affected fermentation product formation.

Glucose fermentation by *C. perfringens* was studied at different dilution rates ( $D$  0.04–0.16/h) under C- and N-limited growth conditions. Acetate was the principal fermentation acid formed at all dilution rates during growth under C limitation, constituting approximately 80% of the total. Lactate production was negligible. However, when the cultures were changed to N limitation, the fermentation patterns were radically altered in that the percentage of acetate formed markedly decreased, ranging from 49% at  $D$  0.04/h to 18% at  $D$  0.16/h, whilst the respective lactate values were 35 and 80%. These results can be explained by the fact that pyruvate is the control point in carbohydrate metabolism in this bacterium, which under C limitation, is geared towards ATP production. Thus, more acetate is produced from acetyl phosphate and there is less need to divert C to lactate production to regenerate oxidized pyridine nucleotides. In contrast, when fermentable substrate is in excess, the decline in acetate formation reduces the efficiency of energy transduction, whilst a high throughput of C through the cells is maintained by using lactate as an electron sink (see Fig. 1).

Lactate and ethanol synthesis by *B. angulatum* was strongly influenced by dilution rate in C-limited cultures. At  $D$  0.03/h, their respective molar ratios were 9:25, whereas, at  $D$  0.51/h the values were 23:7. The inverse relationship between ethanol and lactate production in this species occurs because at low dilution rates, the bacteria were extremely energy-limited. Under these conditions, they maximized ATP formation by converting pyruvate to acetyl phosphate instead of lactate. Half the acetyl phosphate was used to make acetate and produce ATP and the other half was used to make ethanol, to facilitate oxidation of the pyridine nucleotides that are needed to make pyruvate from glyceraldehyde-3-phosphate (see Fig. 3). When the dilution rates were raised, this also increased the availability of C and energy in the chemostats, with the result that metabolism was diverted from extra ATP formation (acetate and ethanol synthesis) towards lactate production.

*B. ovatus* is one of the most nutritionally versatile members of the *B. fragilis* group and is able to ferment a wide range of carbohydrates (Macfarlane *et al.* 1990). During growth in C-limited continuous culture ( $D$  0.06–0.19/h) acetate, succinate and propionate were the main fermentation products. In this bacterium, succinate acts as an electron sink; however, ATP is also produced by the succinate pathway. Propionate is formed from succinate, and its synthesis is not an energy-generating mechanism, but instead serves to produce CO<sub>2</sub>, which is needed to convert C<sub>3</sub> acids to C<sub>4</sub> acids in the succinate pathway (see Fig. 1). Decarboxylation of succinate to propionate was inversely related to growth rate. Consequently, at low dilution rates, acetate and propionate predominated, whereas at high dilution rates, acetate and succinate were prevalent. It is likely that at high growth rates, as C flow through the bacteria was increased, sufficient CO<sub>2</sub> was generated

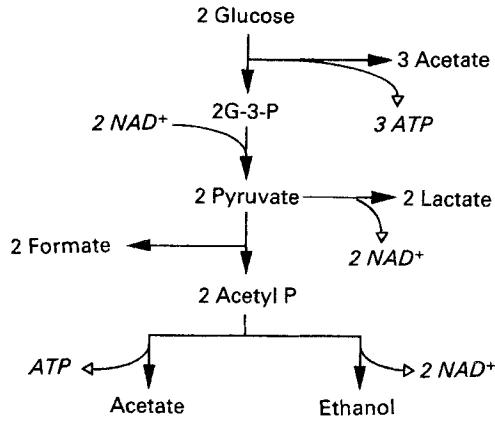


Fig. 3. Pathway of glucose fermentation in *Bifidobacterium angulatum*.  
G-3-P, glyceraldehyde-3-phosphate; acetyl P, acetyl phosphate.

by the conversion of pyruvate to acetyl-CoA, with the result that decarboxylation of succinate was not required. This hypothesis is supported by results from the N-limited chemostats, where acetate and succinate were the major fermentation products. The relative proportion of acetate produced decreased, whilst molar ratios of propionate and succinate were little affected by dilution rate, again reflecting preferred CO<sub>2</sub> formation from pyruvate. D-Lactate was also synthesized under these circumstances, probably as an additional electron sink.

The influence of C availability on butyrate production was studied with *C. butyricum*. In this species, acetate and butyrate synthesis is controlled at the level of acetyl-CoA and since two moles of this metabolite are needed to synthesize one mole of butyrate, and only one mole is required to form one mole of acetate, twice as much ATP is generated when acetate is produced (see Fig. 1). The bacteria were grown under C or N limitation at *D* 0.05/h and the respective molar ratios of acetate:butyrate in spent culture media were 32:68 and 20:80. The increase in butyrate formation in the N-limited cultures occurred because *C. butyricum* uses acetate synthesis to make ATP when substrate is limiting, and butyrate to dispose of excess reducing power and regenerate oxidized pyrimidine nucleotides when fermentable substrate is in excess, in a manner analogous to acetate and lactate production in *C. perfringens*.

#### EFFECT OF NITRATE ON FERMENTATION REACTIONS

Inorganic electron acceptors such as nitrate markedly affect the outcome of carbohydrate fermentation reactions by faecal bacteria by enabling them to produce more oxidized endproducts. This was demonstrated by the addition of 5 mM-KNO<sub>3</sub> to faecal slurries fermenting starch. Although nitrate had no significant effect on the total levels of SCFA formed, the relative amounts of individual fatty acids were significantly altered, as evidenced by the molar ratios of acetate, propionate and butyrate which were 76:12:10 and 59:16:25 respectively, in the presence and absence of nitrate. This occurred because dissimilatory nitrate reduction to ammonia acted as an electron sink, diverting electrons from H<sub>2</sub> and butyrate formation towards acetate synthesis, thereby enabling the bacteria

to increase ATP production and increase cell mass. This was also shown to occur in cultures of *C. butyricum*.

In summary, therefore, many different factors have been identified in the present study as being able to influence the fermentation reactions carried out by human intestinal bacteria. These include the amount and chemical composition of the substrate, the rate at which it is depolymerized, the relative numbers, substrate specificities, preferences and fermentation strategies of individual gut species, and the availability of inorganic terminal electron accepters.

#### REFERENCES

- Cummings, J. H. (1981). Short chain fatty acids in the human colon. *Gut* **22**, 763–779.
- Cummings, J. H., Gibson, G. R. & Macfarlane, G. T. (1989). Quantitative estimates of fermentation in the hind gut of man. *Acta Veterinaria Scandinavica* **86**, 76–82.
- Cummings, J. H. & Macfarlane, G. T. (1991). A review: the control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology* **70**, 443–459.
- Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P. E. & Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**, 1221–1227.
- Finegold, S. M., Sutter, V. L. & Mathisen, G. E. (1983). Normal indigenous flora. In *Human Intestinal Microflora in Health and Disease*, pp. 3–31 [D. J. Hentges, editor]. London: Academic Press.
- Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobic Laboratory Manual*, 4th edn. Blacksberg, Virginia: VPI Anaerobe Laboratory.
- Leder, A. & Leder, P. (1975). Butyric acid, a potent inducer of erythroid differentiation in cultural erythroleukemic cells. *Cell* **5**, 319–322.
- Kim, Y. S., Tsao, D., Morita, A. & Bella, A. (1982). Effect of sodium butyrate and three human colorectal adenocarcinoma cell lines in culture. *Falk Symposium* **31**, 317–323.
- Macfarlane, G. T. & Cummings, J. H. (1991). The colonic fermentation and large bowel digestive function. In *The Large Intestine: Physiology, Pathophysiology and Disease*, pp. 51–92 [S. F. Phillips, J. H. Pemberton and R. G. Shorter, editors]. New York: Raven Press Ltd.
- Macfarlane, G. T. & Englyst, H. N. (1986). Starch utilization by the human large intestinal microflora. *Journal of Applied Bacteriology* **60**, 195–201.
- Macfarlane, G. T., Gibson, G. R. & Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. *Journal of Applied Bacteriology* **72**, 57–64.
- Macfarlane, G. T., Hay, S., Macfarlane, S. & Gibson, G. R. (1990). Effect of different carbohydrates on growth, polysaccharidase and glycosidase production by *Bacteroides ovatus*, in batch and continuous culture. *Journal of Applied Bacteriology* **68**, 179–187.
- Meijer-Severs, G. J. & Van Santen, E. (1986). Variations in the anaerobic faecal flora of mice and on their resistance to infection. *Journal of Experimental Medicine* **115**, 1161–1171.
- Roediger, W. E. W. (1980). Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* **21**, 712–715.
- Scheppach, W., Fabian, C., Sachs, M. & Kasper, H. (1988). The effect of starch malabsorption on fecal short chain fatty acid excretion in man. *Scandinavian Journal of Gastroenterology* **23**, 755–759.