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The Fermentation of Lactulose by Colonic Bacteria

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Sixty-four strains of intestinal bacteria were cultured under anaerobic conditions in lactulose-containing media to assess their ability to ferment lactulose. Some organisms were unable to metabolize the disaccharide, while others, e.g. clostridia and lactobacilli, metabolized lactulose extensively. Quantitative analyses of the fermentation products indicated that the major non-gaseous metabolites were acetic, lactic and butyric acids. Hydrogen and carbon dioxide were the only gases detected. Fermentation products were estimated for selected species throughout their growth cycles. The products of fermentation of lactulose by stool cultures varied with incubation conditions such as pH, but correlated well with those produced by pure cultures. These results are discussed in relation to the therapeutic uses of lactulose.

INTRODUCTION

The synthetic disaccharide lactulose (4-*O*-*β*-D-galactopyranosyl D-fructofuranose) cannot be hydrolysed by human intestinal *β*-galactosidases (EC 3.2.1.23; Dahlquist & Gryboski, 1965; Udupihille, 1974) and, except for slow permeation of the intact disaccharide, is not absorbed from the small intestine (Müller *et al.*, 1969; Carulli *et al.*, 1972). Consequently, following ingestion it passes to the colon, where it undergoes rapid fermentation by the colonic flora (Hoffmann *et al.*, 1964) to products which cause acidification of the stool contents and flatulence (Agostini *et al.*, 1972). Ingestion of sufficient amounts of lactulose produces a diarrhoea that is both osmotic and fermentative.

Because of this resistance to intestinal hydrolases, lactulose is used clinically for the treatment of acute constipation (Wesselius-De Casparis *et al.*, 1968) and portal systemic encephalopathy (e.g. Bircher *et al.*, 1966; Simmons *et al.*, 1970), and for the assessment of small bowel transit time (Bond & Levitt, 1975).

Although the therapeutic uses of lactulose involve, or may be modified by, bacterial degradation within the intestine, there is little detailed knowledge of this process. The few reported investigations indicate that some Gram-positive cocci and bacilli metabolize lactulose (Hoffmann *et al.*, 1964) as do several bacteroides (Ruttloff *et al.*, 1967). The former workers showed that lactic acid is the principal end-product, but reported no quantitative data.

The present investigation was undertaken to extend knowledge of the fermentation of lactulose by individual types of intestinal micro-organisms. Utilization of lactulose and formation of fermentation products have been estimated quantitatively for 64 strains of bacteria, cultured anaerobically, the behaviour of selected species being assessed throughout their growth cycles. These data have been compared with the results of anaerobic fermentations of lactulose by cultures of mixed organisms from faecal homogenates.

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METHODS

Organisms. Strains of lactobacillus were purchased from the N.C.I.B., Torry Research Station, Aberdeen. All other organisms were kindly provided by Dr A. Vince, The Rayne Institute, University College Hospital Medical School, London; Dr M. Hill, Bacterial Metabolism Research Laboratory, Central Public Health Laboratory, London, and Professor I. Phillips, Department of Microbiology, St Thomas's Hospital Medical School, London.

Media and growth conditions. All organisms were maintained on Oxoid 'Cooked Meat' medium (CM349) and stored at −70 °C, after the addition of glycerol to a final concentration of 15% (v/v). The purity of the strains was verified at regular intervals after culturing on blood agar. A standard sugar-free basal liquid medium (Holdeman *et al.*, 1977) was used, and when necessary, lactulose was added via a 0·22 μm bacteriological filter, to a final concentration of 40 mM.

Routine screening of the bacterial strains for their ability to utilize lactulose was carried out by inoculating (5%, v/v) liquid media (15 ml, with and without lactulose) with cultures which had been grown overnight in basal medium. They were then incubated at 37 °C for 48 h in an atmosphere of $N_2/H_2/CO_2$ (80:10:10, by vol.). Time-course experiments over a 24 h period were conducted similarly, with sampling every 4 h. Bacterial growth was measured turbidimetrically at 612 nm, and pH was measured by a microelectrode. Culture supernatants were obtained by centrifugation at 2000 ***g*** for 15 min.

Determination of carbohydrates. The carbohydrates in culture supernatants were separated by thin-layer chromatography on silica gel G and subsequently quantified by locating and scanning the appropriate bands (Menzies *et al.*, 1978). Alternatively, when lactulose was the only sugar present it was estimated by the method of Vachek (1971).

Determination of non-gaseous fermentation products. These were separated by gas chromatography (Pye-Unicam model 204, fitted with a flame ionization detector) on glass columns (2 m × 4·5 mm, internal diameter) packed with Diatomite C (acid washed, 60–80 mesh) coated with 10% polyethylene glycol 20M. The following conditions were employed: gas flow rates of 50, 750 and 45 ml min^{-1} for H_2, air and N_2, respectively; injection port temperature 120 °C, detector temperature 180 °C and a temperature programme of 8 °C min^{-1} from 60 °C to 160 °C, the final temperature being maintained for 15 min.

Quantification of non-gaseous products was achieved by comparing the integrated peak areas of standard compounds with those of experimental samples, using internal markers of butanol and malonic acid for volatile and non-volatile fatty acids, respectively.

In order to avoid losses due to evaporation, acetic and formic acids were determined directly on culture supernatants, using a standard test kit for acetic acid (Boehringer Corporation) and formate dehydrogenase (EC 1.2.1.2) for formic acid (Schutte *et al.*, 1976).

The other non-gaseous products were extracted from acidified culture supernatants (2·4 ml) containing the appropriate internal marker (0·1 ml). Volatile fatty acids were obtained directly by partition with diethyl ether (1·0 ml), while non-volatile fatty acids were methylated with 14% (v/v) boron trifluoride in methanol (2·5 ml) at room temperature, overnight, and then partitioned with chloroform (1·25 ml). This methylation procedure gave recoveries of 93 ± 2% for lactic acid.

Determination of gaseous fermentation products. These were initially detected and their total volumes estimated after collection in inverted sample tubes which had been placed in the media prior to inoculation. Qualitative analyses were then carried out by gas chromatography on glass columns (1·5 m × 4·5 mm i.d.) packed with silica gel (80–100 mesh; Phase Separations, Queensferry, Clywd). The following conditions were used: carrier gas (N_2) flow rate 50 ml min^{-1}; injection port temperature 50 °C; column temperature 50 °C; detector temperature 50 °C and a bridge current of the catharometer of 95 mA.

Selected bacteria were cultured, as described earlier, in gas-tight vials (Hypo-vials, Pierce and Warriner, Chester) under N_2. Samples (1 ml) were removed from the head space with a gas-tight syringe and injected on to the column. Retention times were compared with those of a standard gas mixture containing methane, ethane, propane and butane (Phase Separations) and also with hydrogen and carbon dioxide (British Oxygen, Wembley, Middlesex).

Incubation with mixed faecal organisms. These samples were kindly provided by Dr A. Vince. They were incubated anaerobically at 37 °C for 48 h in the presence of 90 mM-lactulose, as described elsewhere (Vince *et al.*, 1978). After centrifugation at 47500 ***g*** for 90 min at 4 °C the supernatants were removed and analysed for fermentation products as described above.

RESULTS

Utilization of lactulose

The amount of lactulose fermented over a 48 h period varied significantly, not only from genus to genus but also, in some cases, between different strains of the same species (Table 1).

Table 1. *Utilization of lactulose (40 mM) by colonic bacteria*

	Organism*	Percentage utilization after 48 h cultivation	Gas production†
Organisms utilizing >20% lactulose	*Bacteroides oralis*‡	21·0	–
	Bacteroides vulgatus‡	21·0	+
	Bifidobacterium bifidum	30·5	–
	Clostridium perfringens (8)	33·6–43·1	3+
	Lactobacillus casei sub. *casei*	33·5	–
	Lactobacillus spp. (4)	21·1–47·3	–
Organisms utilizing 5–20% lactulose	*Bacteroides distasonis*‡ (3)	11·5–19·5	–
	Bacteroides fragilis (6)	9·0–13·5	+
	Bacteroides ovatus (2)	5·0–8·1	+
	Bacteroides thetaiotaomicron (3)	5·0–8·0	+
	Bacteroides uniformis (3)	9·4–13·5	+
	Bacteroides vulgatus‡	9·8	+
	Clostridium butyricum (3)	7·5–19·0	2+
	Clostridium cadaveris (2)	5·0–17·5	2+
	Clostridium difficile (2)	5·0–13·8	2+
	Enterobacter aerogenes	12·0	+
	Escherichia coli	5·0	+
	Klebsiella aerogenes	9·6	–
	Lactobacillus acidophilus (2)	10·0–13·1	–
	Lactobacillus brevis	13·2	–
	Peptostreptococcus anaerobius	10·0	2+
	Proteus mirabilis	11·6	2+
	Proteus vulgaris	16·1	2+
	Staphylococcus aureus	7·0	–
	Streptococcus faecalis	6·0	+
	Streptococcus intermedius	6·6	+
	Streptococcus sp.	8·8	+
	Streptococcus viridans	6·0	+

The following organisms were found to utilize <5% of the added lactulose and no gaseous products were formed: *Bacteroides mel assacharolyticus, Clostridium paraputrificum, Eubacterium lentum, Eubacterium limosum, Fusobacterium nucleatum, Lactobacillus buchneri, Neisseria catarrhalis, Propionibacterium acnes, Veillonella parvula.*

* Number of strains shown in parentheses if more than one used.

† Scored on a scale + to 3+.

‡ Galactose and/or fructose found in the culture medium after growth.

Some 14% of the 64 strains examined showed negligible (<5%) utilization of lactulose, while the majority (61%) of the organisms fermented an 'intermediate' (5–20%) amount of the disaccharide. The latter group consisted of coliforms, bacteroides, streptococci and all but two species of clostridia. The culture supernatants of *Bacteroides distasonis, B. oralis* and *B. vulgatus* contained small quantities of galactose and fructose (Table 1).

Of the three organisms selected from those exhibiting over 20% utilization of lactulose, only *Clostridium perfringens* exhibited a typical growth pattern when cultured on 40 mM-lactulose (Fig. 1). Both *Bacteroides vulgatus* and the *Lactobacillus* sp. metabolized the majority of the disaccharide in the stationary phase, an example of deferred substrate utilization. In all three, the total quantities of lactulose utilized after 24 h were similar to those at the 48 h stage in the earlier screening experiment (Table 1). A fall in pH of 2·0–2·5 units was found over the 24 h fermentation (data not shown).

Fermentation products

In order to accurately quantify the products of lactulose fermentation, it was necessary to compare the types and amounts of the products after growth on basal medium and on lactulose-supplemented medium.

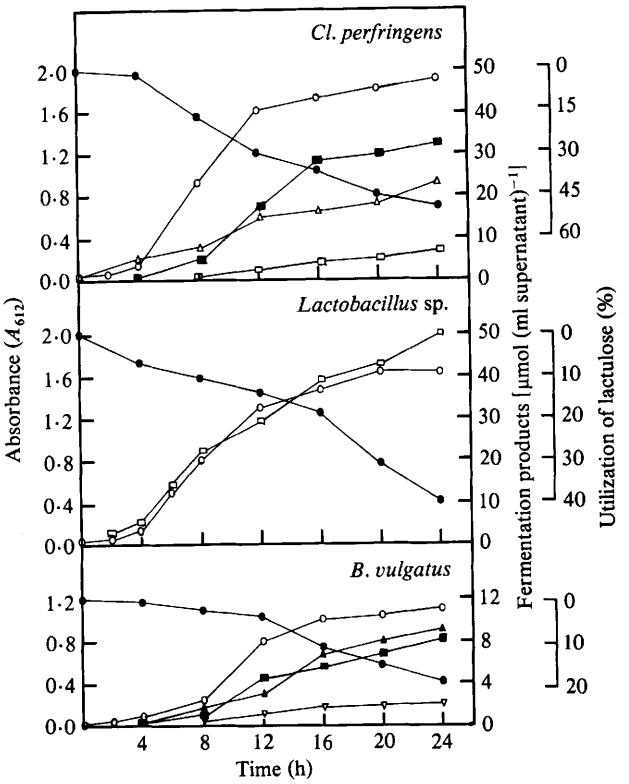

Fig. 1. Growth of selected organisms on 40 mM-lactulose. Absorbance (○), utilization of lactulose (●), concentrations of fermentation products – lactic acid (□), acetic acid (■), butyric acid (△), succinic acid (▲), propionic acid (▽).

Table 2. *Quantitative analysis of significant non-gaseous products of lactulose fermentation*

The values quoted show the increment in the fermentation product observed with the strains employed.

	Product [μmol (ml culture supernatant)$^{-1}$]				
Organism*	Acetic acid	Butyric acid	Isovaleric acid	Lactic acid	Succinic acid
B. fragilis (6)	3·2– 8·9	–	–	3·9– 7·25	2·1–4·1
Cl. perfringens (8)	7·3–11·3	14·05–25·4	–	3·2–15·4	–
E. coli	3·8	–	–	2·75	–
L. acidophilus	–	–	–	21·2	–
P. anaerobius	1·9	–	1·00	–	–
St. faecalis	4·5	–	0·7	3·0	–

* Number of strains shown in parentheses if more than one used.

Both qualitative and quantitative differences were found on addition of 40 mM-lactulose to the medium. Generally, large increases in acetic, lactic and butyric acids were found, with butyric acid being a characteristic product of clostridia. Bacteroides predominantly metabolized lactulose to acetic and succinic acids, but produced smaller quantities of higher fatty acids during lactulose fermentation than with basal medium alone. The lactobacilli exhibited a homolactic fermentation pattern. Formic acid was not detected by either enzymic or gas–liquid chromatographic analyses. Quantitative analyses of the non-gaseous fermentation products of representative species are shown in Table 2.

Table 3. *Non-gaseous products of in vitro incubation of 90 mM-lactulose with faecal homogenates*

Each value is the mean of duplicate incubations.

Incubation condition	Product* [μmol (ml culture supernatant)$^{-1}$]										
	Ac	Prop	iBut	But	iVal	Val	Cap	iCap	Lac	Fum	Succ
+ Lactulose, 0 h	19·9	2·4	0·3	2·8	0·4	0·4	0·4	0·4	0·0	0·0	0·0
+ Lactulose, 48 h	116·7	9·5	0·3	29·8	0·4	0·5	0·6	0·2	52·6	0·0	0·0
+ Lactulose, 48 h, titrated to pH $\geqslant$ 5·5	99·1	17·2	1·7	65·5	1·7	3·4	9·3	0·4	0·0	2·3	3·6

* Ac, acetic acid; Prop, propionic acid; iBut, isobutyric acid; But, butyric acid; iVal, isovaleric acid; Val, valeric acid; Cap, caproic acid; iCap, isocaproic acid; Lac, lactic acid; Fum, fumaric acid; Succ, succinic acid.

Of those organisms utilizing more than 5% of the available lactulose, some 63% produced gases during fermentation, with strains of *Cl. perfringens* evolving the largest quantities (Table 1). Carbon dioxide and hydrogen were the only gases detected in these cases, methane being absent.

Analyses of fermentation products throughout growth of the three selected bacteria showed that all the metabolites were excreted during each phase of growth (Fig. 1).

Incubations with faecal homogenates

The fermentation products of the mixed cultures from faecal homogenates, incubated with lactulose, differed depending on the condition of the incubations (Table 3). Untitrated incubations over a 48 h period showed large increases in acetic, butyric and lactic acids, but maintenance of pH $\geqslant$ 5·5 inhibited lactic acid formation while the production of butyric, succinic and longer-chain fatty acids was elevated under these conditions.

DISCUSSION

The amounts of lactulose fermented by the 64 strains of gut bacteria (Table 1) indicate that of the three quantitatively predominant gut genera (all approx. 10^{10} per g faeces; Drasar & Hill, 1974), eubacteria do not play a significant role in colonic lactulose metabolism, whereas strains of both bacteroides and bifidobacteria show high amounts of lactulose utilization *in vitro* (Table 1). Other quantitatively important colonic organisms such as streptococci and enterobacteria (both approx. 10^8 per g faeces) exhibit a rather low ability to use lactulose (5–10%, Table 1). Although both lactobacilli and clostridia do not occur in such high numbers in the lower intestine (10^5–10^6 per g faeces), the large amount of lactulose utilized by certain strains (Table 1), the extent of which was confirmed by their comparatively high rates of uptake during exponential and stationary phases of growth (Fig. 1), suggests that such organisms are important in the metabolism of lactulose in the colon.

The non-gaseous fermentation products of lactulose (Table 2) were qualitatively the same as those obtained when these organisms were cultured on glucose (Holdeman *et al.*, 1977), suggesting that similar glycolytic pathways are operative for both carbohydrates. This is the first report of butyric acid formation from lactulose and indicates that the mixture of fermentation products present in the colon during lactulose therapy is more complex than has been claimed previously (Conn & Lieberthal, 1979). These organisms are also able to utilize the aldose isomer lactose (Holdeman *et al.*, 1977), suggesting that prokaryotes are relatively non-specific with respect to β-D-galactoside metabolism, in contrast to human intestinal β-galactosidases (Dahlquist & Gryboski, 1965).

The formation of carbon dioxide and hydrogen during growth on lactulose (Table 1) correlates with the characteristic flatulence experienced after ingesting therapeutic doses of lactulose (Elkington, 1970). The evolution of hydrogen forms the rationale of tests for sugar absorption and intestinal transit time based on the appearance of this gas in expired air when a non-absorbed sugar, such as lactulose, enters the lower intestine (Bond & Levitt, 1975; Metz *et al.*, 1975, 1976).

Mixed populations of bacteria, derived from faecal homogenates and incubated with lactulose, generated increased amounts of acetic, lactic and butyric acids in the same way that occurred when individual organisms were cultured (Table 3) and is probably due to the activity of acid-tolerant lactobacilli, streptococci and clostridia. Viable cell counts of streptococci and clostridia do not alter significantly over 48 h in untitrated incubations, but bacteroides decrease markedly over the same period (Vince *et al.*, 1978). It is significant, therefore, that on maintenance of a pH $\geqslant$ 5·5 during incubation, the balance of fermentation products is altered in favour of succinic and higher fatty acids, presumably because bacteroides and other acid-sensitive organisms were metabolically more active at such pH values. Since high oral doses of lactulose, as used in the treatment of hepatic coma, produce acidification of colonic contents to below pH 5·5 (Conn & Lieberthal, 1979), acetic, butyric and lactic acids may become the predominant metabolic products in the colon.

The mode of action of lactulose in the alleviation of hepatic coma is still unclear (Conn & Lieberthal, 1979). However, blood ammonia levels, a major aetiological factor in this condition, do fall significantly during lactulose therapy (Conn *et al.*, 1977). Since significant quantitative changes in the gut flora of adults do not appear to be induced by oral lactulose administration (Vince *et al.*, 1974), a current hypothesis to explain this phenomenon is that lactulose fermentation in the colon enables certain unspecified organisms to increase the rate of incorporation of colonic ammonia into bacterial proteins (Vince *et al.*, 1978). Such investigations would be assisted by more detailed knowledge of bacterial interactions occurring in the colon, and also of the selective toxicity to bacterial species of fermentation products (Bergeim, 1940; Lee & Gemmell, 1971). Our present studies, which detail the products of lactulose metabolism and show which colonic organisms are capable of growth on lactulose, should facilitate future work on this complex subject.

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REFERENCES

AGOSTINI, L., DOWN, P. F., MURISON, J. & WRONG, O. M. (1972). Faecal ammonia and pH during lactulose administration in man: comparison with other cathartics. *Gut* **13**, 859–866.

BERGEIM, O. (1940). Toxicity of intestinal volatile fatty acids for yeast and *Escherichia coli*. *Journal of Infectious Diseases* **66**, 222–234.

BIRCHER, J., MÜLLER, J., GUGGENHEIM, P. & HAEMMERLI, U. P. (1966). Treatment of chronic portal-systemic encephalopathy with lactulose. *Lancet* **1**, 890–893.

BOND, J. H., JR & LEVITT, M. D. (1975). Investigation of small bowel transit time in man utilising pulmonary hydrogen (H_2) measurements. *Journal of Laboratory and Clinical Medicine* **85**, 546–555.

CARULLI, J., SALVIOLI, G. F. & MANENTI, F. (1972). Absorption of lactulose in man. *Digestion* **6**, 139–145.

CONN, H. O. & LIEBERTHAL, M. M. (1979). *The Hepatic Coma Syndromes and Lactulose*. Baltimore: Williams & Wilkins.

CONN, H. O., LEEVY, C. M., VLAHCEVIC, Z. R., RODGERS, J. B., MADDREY, W. C., SEEF, L. & LEVY, L. L. (1977). Comparison of lactulose and neomycin in the treatment of chronic portal-systemic encephalopathy. *Gastroenterology* **72**, 573–583.

DAHLQUIST, A. & GRYBOSKI, I. (1965). Inability of the human small intestine to hydrolyse lactulose. *Biochimica et biophysica acta* **110**, 635–636.

DRASAR, B. S. & HILL, M. J. (1974). *Human Intestinal Flora*. London: Academic Press.

ELKINGTON, S. G. (1970). Lactulose. *Gut* **11**, 1043–1048.

HOFFMANN, K., MOSSEL, D. A., KORUS, W. & VAN DE KAMER, J. H. (1964). Untersuchungen über die Wirkungsweise der Lactulose im Darm. *Klinische Wochenschrift* **42**, 126–130.

HOLDEMAN, L. V., CATO, E. P. & MOORE, W. E. C.

(editors) (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, Virginia, U.S.A.: V.P.I. Anaerobe Laboratory.

LEE, M. & GEMMELL, E. (1971). Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids. *Infection and Immunity* **5**, 1–7.

MENZIES, I. S., MOUNT, J. N. & WHEELER, M. J. (1978). Quantitative estimation of clinically important monosaccharides in plasma by rapid thin-layer chromatography. *Annals of Clinical Biochemistry* **15**, 65–76.

METZ, G., JENKINS, D. J. A., PETERS, T. J., NEWMAN, A. & BLENDIS, L. M. (1975). Breath hydrogen as a diagnostic method for hypolactasia. *Lancet* **1**, 1155–1157.

METZ, G., JENKINS, D. J. A., NEWMAN, A., BLENDIS, L. M. (1976). Breath hydrogen in hyposucrasia. *Lancet* **1**, 119–120.

MÜLLER, M., WALKER-SMITH, J., SMERLING, D. H., CURTIUS, H.-C. & PRADER, A. (1969). Lactulose: a gas–liquid chromatography method of determination and evaluation of its use to assess intestinal mucosal damage. *Clinica chimica acta* **24**, 45–49.

RUTTLOFF, H., TÄUFEL, A., HAENEL, H. & TÄUFEL, K. (1967). Die intestinal-enzymatische Spaltung von Galakto-oligosacchariden im Darm von Tier und Mensch mit besonderer Berücksichtigung von *Lactobacillus bifidus*. 3: Mitt. Carbohydratische Aktivität ausgewählter Mikroorganismen der Darmflora. *Nährung* **11**, 47–54.

SCHUTTE, H., FLOSSDORF, J., SAHM, H. & KULA, M.-R. (1976). Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *European Journal of Biochemistry* **62**, 151–160.

SIMMONS, F., GOLDSTEIN, H. & BOYLE, J. D. (1970). A controlled clinical trial of lactulose in hepatic encephalopathy. *Gastroenterology* **59**, 827–832.

UDUPIHILLE, M. (1974). *Factors affecting human intestinal permeability of non-metabolized oligosaccharides*. M.Phil., University of London.

VACHEK, J. (1971). Spectrophotometric determination of lactulose. *Československá farmacie* **20**, 96–98.

VINCE, A., ZEEGEN, R., DRINKWATER, J. E., O'GRADY, F. & DAWSON, A. M. (1974). The effect of lactulose on the faecal flora of patients with hepatic encephalopathy. *Journal of Medical Microbiology* **7**, 163–168.

VINCE, A., KILLINGLEY, M. & WRONG, O. M. (1978). Effect of lactulose on ammonia production in a faecal incubation system. *Gastroenterology* **74**, 544–549.

WESSELIUS-DE CASPARIS, A., BRAADBAART, S., VD BERGH-BOHLKEN, G. E. & MIMICA, M. (1968). Treatment of chronic constipation with lactulose syrup: results of a double-blind study. *Gut* **9**, 84–86.