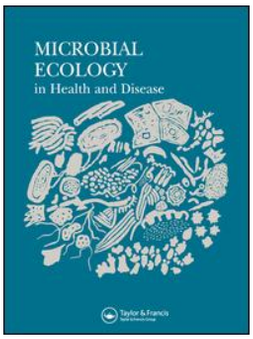

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ORIGINAL ARTICLE

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The Effect of Lactulose on the Composition of the Intestinal Microbiota and Short-chain Fatty Acid Production in Human Volunteers and a Computer-controlled Model of the Proximal Large Intestine

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The objective of this study was to compare the *in vivo* effect of lactulose on faecal parameters with the effect in a dynamic, computer-controlled *in vitro* model of the proximal large intestine (TIM-2). Faecal samples from 10 human volunteers collected before (non-adapted) and after 1 week of treatment (10 g/day) with lactulose (lactulose-adapted) were investigated. Parameters were compared immediately in the faecal samples, and after incubation in the *in vitro* model of the large intestine. After an adaptation period of the faecal microbiota in the *in vitro* model of the proximal colon, lactulose (10 g/day) was fed to the microbiota over a 48-h period. Samples taken from the model were investigated for microbiota composition and metabolite production (short-chain fatty acids (SCFAs) and lactate). No changes in the faecal parameters pH, dry weight or SCFA ratio were observed in the *in vivo* samples. However, the results show a major change in the ratio of SCFAs produced in the *in vitro* model, with a drastic reduction of butyrate production on lactulose. This was clear in the non-adapted microbiota by the observed arrest in butyrate production 24 h after the start of lactulose feeding. However, in the adapted microbiota butyrate production was already low from the start of the experiment. In fact, only the microbiota of one of the 10 individuals still produced significant amounts of butyrate after lactulose adaptation, the concentration in the other samples was extremely low. Similarly, in the *in vitro* model lactate production of the non-adapted microbiota started after approximately 24 h, whereas the adapted microbiota produced lactate from the start. In faecal (*in vivo*) samples no changes in microbiota composition were obvious, except for a significant increase in *Bifidobacterium* counts after lactulose feeding. With classic plating techniques, the *in vitro* samples showed an increase in *Lactobacillus* and *Enterococcus* species. With denaturing gradient gel electrophoresis, a clear change in banding pattern was observed, indicating a shift in microbiota composition. When the major bands that appeared after lactulose feeding in the *in vitro* model were excised and sequenced, the sequences showed homology to *Lactobacillus* and *Enterococcus* species. This is in agreement with the classic plating technique as well as with the observed increase in lactate production. Sampling *in vivo* at 'the site where it all happens' (the proximal colon) is difficult and inconvenient. We conclude that the *in vitro* model for the proximal colon reflects much better the fermentation of lactulose, in both metabolite production and changes in microbiota composition, than do faecal samples from an *in vivo* experiment. Therefore, the *in vitro* model is an excellent tool with which to study bioconversion of functional food components and/or drugs. *Key words:* Lactulose, *in vitro* large intestinal model, *in vivo* study, SCFA, microbiota, DGGE, FISH.

INTRODUCTION

Lactulose is a synthetic disaccharide (4-O-β-D-galactosyl-D-fructose, molecular weight 342.3) which does not exist in nature. It is produced in small amounts during heat treatment of milk. Lactulose can be made on a large scale from lactose by alkaline isomerization in which galactose is linked to fructose as a β-1-4-glycoside. Lactulose is thought to have many potential health applications, ranging from stimulation of beneficial bacteria in the intestinal microbiota to the treatment of severely ill, chronic hepatic encephalitic patients (1). Lactulose is neither digested in nor absorbed from the stomach and small intestine. Lactulose has been shown to increase Ca^{2+} absorption in postmenopausal women (2) and to affect the composition of the intestinal microbiota and short-chain fatty acid (SCFA) production *in vivo* (3, 4). In the caecum and proximal colon lactulose is anaerobically fermented by the indigenous microbiota. This is evidenced by the production of hydrogen and/or methane (5–7) and by the production of lactic acid (4, 8) and SCFAs (9), especially after adaptation to lactulose (4). *In vitro* studies with individual strains have

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shown that lactulose can act as a substrate for many lactic acid bacteria, including gastrointestinal genera and can support their growth. In healthy adults the intake of 3 g of lactulose per day for 2 weeks resulted in a significant increase in the number of *Bifidobacterium* in faecal samples. The numbers of (lecithinase-positive) clostridia and Bacteroidaceae decreased significantly (10, 11). In cirrhotic patients treatment with lactulose for 10 days resulted in a higher number of faecal lactobacilli (12). The production of lactic acid and SCFAs resulted in a lower colonic and faecal pH, the latter depending on the dose, ranging from pH 6 (20 g/day) (4) to pH 5 ($>$ 100 g/day) (3). The ultimate function of SCFAs in the large intestine, such as stimulation of mucosal proliferation and the suggested inhibitory effect of butyrate on cancer, is not completely understood. However, the possible health-promoting effects of butyrate in particular (13) have resulted in an increased interest in SCFA production in the large intestine and the effect of certain food or pharmaceutical compounds on the production of SCFAs.

However, one drawback of analysing faecal samples is the fact that they do not necessarily represent quantitatively what happens in the different parts of the colon. SCFAs are predominantly produced in the proximal part of the colon, where most of the carbohydrate fermentation takes place. But they will be absorbed for a considerable amount by the colonic epithelium during subsequent transport of the chyme to the distal colon. Thus, amount and ratio of SCFAs recovered in the faeces is not representative of those produced in the proximal colon. So, the human being is a 'model' with various drawbacks. The most important ones are ethical constraints, high costs and limitations in sampling from 'the site where it really happens'. Laboratory models which simulate the successive kinetic conditions in the gastrointestinal tract, including an active microbiota of human origin, can be an alternative. Several computer-controlled models have been developed for this purpose (14, 15). Also TNO has developed dynamic, computer-controlled *in vitro* gastrointestinal models, in which the successive conditions in the lumen of the gastric and small intestinal compartments (16) and in the large intestine (17) can be simulated in an accurate and reproducible manner. This offers the opportunity to compare different ingested products under identical and standardized conditions. These models are unique tools with which to study the stability, release, digestibility, absorption and bioconversion of nutrients, chemicals and pharmaceuticals in the gastrointestinal tract (18). The model simulating the (proximal) large intestine (TIM-2) has recently been shown to be a valuable tool in investigating the mechanistic effects behind the mode of action of prebiotics, such as inulin (19). This model has been developed and validated using data from sudden death individuals (17). In the model comprising the large intestine (TIM-2) the following standardized conditions are simulated: body temperature, pH in the lumen, delivery of a pre-digested substrate from the 'ileum', mixing and transport of the intestinal contents, absorption of water, and the presence of a complex, high density, metabolically active, anaerobic microbiota of human origin. To prevent growth inhibition of the microbiota by the microbially produced metabolites (primarily SCFAs and lactate), these metabolic products are absorbed from the system via a semi-permeable membrane inside the lumen of the model (17). In this way, a human microbiota could be maintained stably in TIM-2 for up to 3 weeks, without changes in composition or activity.

The objective of this study was to compare the composition of the microbiota and production of SCFAs in faecal samples derived from human volunteers with those in samples obtained from a dynamic, computer-controlled *in vitro* model of the proximal large intestine (TIM-2) inoculated with the faecal microbiota of the human volunteers, using lactulose as a test compound. We show here that the use of a validated model containing a complex microbiota of human origin allows a better exploration of the effect of lactulose 'at the site where it all happens' (the proximal colon) than analyses of faecal matter.

MATERIALS AND METHODS

Study substance

The study substance for the *in vivo* trial consisted of 10 g lactulose (Solvay Pharmaceuticals, Weesp, The Netherlands) per day, dissolved in 100 ml water with 100 mg benzoic acid. The pH of all solutions was between 3.0 and 3.2. The solutions were prepared by a pharmacy and packed in bottles in individual daily portions and stored in the refrigerator ($<8^{\circ}$C) until use. The study substance was consumed at breakfast for 7 days, starting at day 10.

For the experiments in the *in vitro* model of the large intestine (TIM-2; described below), 10 g lactulose per day were added to a pre-digested food (slightly modified from ref. 14) and introduced gradually into the model. The pre-digested food contained (per litre): 2.5 g $K_2HPO_4.3H_2O$, 4.5 g NaCl, 0.005 g $FeSO_4.7H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.45 g $CaCl_2.2H_2O$, 0.4 g ox bile, 0.4 g cysteine-HCl, 4.7 g pectin, 4.7 g xylan, 4.7 g arabinogalactan, 4.7 g amylopectin, 23.5 g casein, 39.2 g starch, 17 g Tween 80, 23.5 g bactopeptone, plus 1 ml of a vitamin mixture containing (per litre): 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B12, 10 mg pantothenate, 5 mg nicotinamide, 5 mg p-aminobenzoic acid and 4 mg thiamine.

Study subjects

Ten apparently healthy postmenopausal women aged between 55 and 64 years (average 60.6 ± 2.5) with body mass index between 22.4 and 30.0 kg/m^2 (average 25.2 ± 2.8) participated in this study. Exclusion criteria, apart from

general health parameters, included lactose intolerance, known hypersensitivity to lactulose or its components, known allergy to benzoic acid, smoking, alcohol consumption above three glasses per day, vegetarian, vegan or macrobiotic diet, and any medication that might influence the outcome of the study. All participants signed an informed consent before the start of the study. The study was approved by the Medical Ethics Committee of TNO under study-code 99/34.

Design of the human study.

The *in vivo* study period lasted 17 days during which the subjects were given a list of liquid, fermented dairy products and foods containing non-digestible carbohydrates and dietary fibre for exclusion from their diet during the total *in vivo* study period. For the rest, diet was not controlled. From day 10 to 17 lactulose was consumed during breakfast (Fig. 1). On day 8, 2 days before the start of the lactulose treatment, and on day 17, after 7 days of lactulose treatment, fresh faecal samples were collected and analysed for microbial composition and SCFA concentrations. In addition, these samples (hereafter called non-adapted microbiota (NAM) and adapted microbiota (AM) for the microbiota collected on day 8 and day 17, respectively) were used as inoculum for the *in vitro* experiments with lactulose in the TIM-2 *in vitro* system (described below). Body weight was measured before and after lactulose treatment.

On day 8 and on day 17 all subjects brought a faecal sample to the institute within 2 h after defecation. Handling of the fresh faecal samples took place in an anaerobic glove box. For bacterial enumeration 2–3 g of fresh faeces were taken from the centre of the bolus, transferred into transport media and homogenized with an ultraturrax. Three cryotubes containing glycerol (final concentration 20%) were filled with the faecal suspension and stored in liquid nitrogen (−196°C) until microbiological analysis. After measuring faecal pH, five samples were taken from the fresh faeces and stored at −20°C for analysis of dry matter content and SCFAs. The dry matter content was determined in all samples by drying about 1 g of a sample in a pre-weighed tube in an oven at 160°C until no loss of weight could be detected. Another 30-g portion of the fresh faecal sample was used to carry out the *in vitro* experiments in the TIM-2 system.

Dynamic in vitro model of the large intestine (TIM-2)

TNO's *in vitro* model of the proximal large intestine (TIM-2) simulates the average conditions in the lumen of the human proximal colon (17). The model consisted of a number of linked glass units with flexible walls inside (Fig. 2). Body temperature and peristaltic movements were achieved by pumping water at 37°C into the space between the glass jacket and the flexible wall at regular intervals. The computer controlled the sequential squeezing of the walls, causing the chyme to be mixed and transported through the system. The model was equipped with hollow-fibre semi-permeable membranes inside the lumen of the model to remove water and fermentation products, such as SCFAs. In addition, they maintained physiological concentrations of small molecules, such as electrolytes, and prevented product inhibition of enzymes due to accumulation of microbial metabolites. A constant volume of the luminal content was maintained by water absorption controlled by a level sensor. The environment in the model was kept strictly anaerobic by flushing with gaseous nitrogen, to allow for the growth of a dense, complex microbiota, comparable to that found in humans in the first part of the colon (caecum/proximal colon). The pH of 5.8 in the proximal colon was controlled via the computer by using a pH sensor in combination with NaOH secretion.

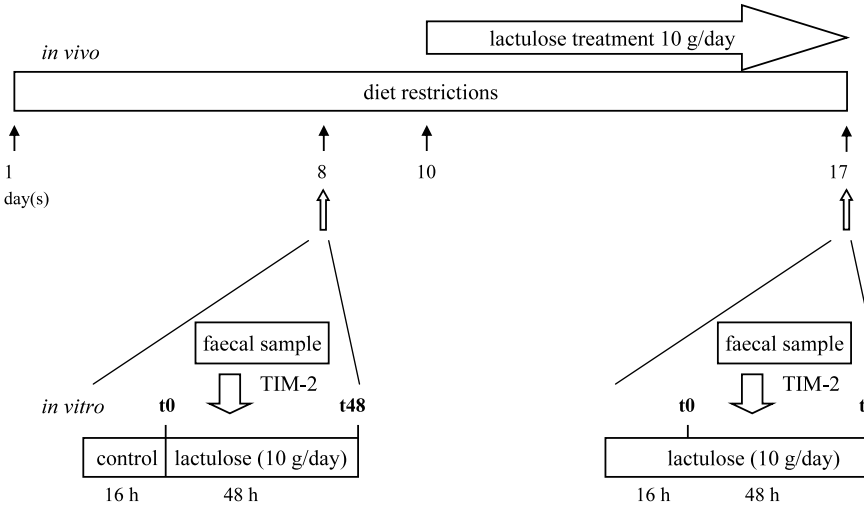

Fig. 1. Study design.

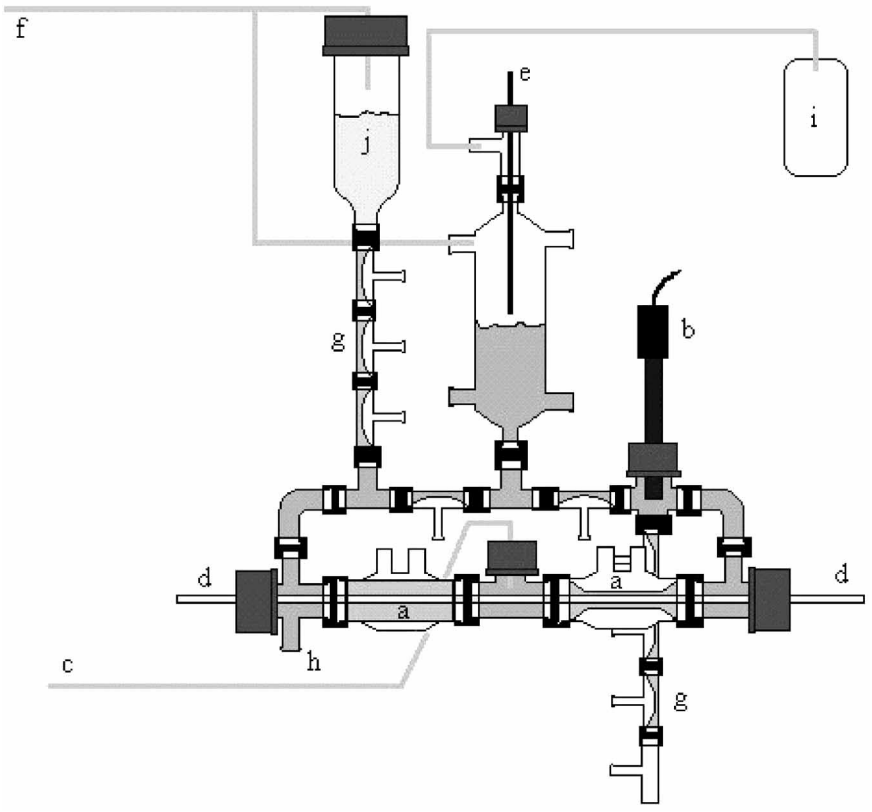

Fig. 2. Schematic representation of the *in vitro* model of the proximal colon (TIM-2) (17). a, peristaltic compartments; b, pH electrode; c, pH control by secretion of NaOH; d, hollow-fibre semi-permeable membranes; e, level sensor; f, N_2 gas inlet; g, inlet and outlet valves; h, sampling-port; i, gas collection bag; j, 'pre-digested food' container.

Study design of in vitro experiments

In four successive runs, two with NAM and two with AM, five TIM-2 systems were inoculated in parallel within 5–6 h after defecation by the human volunteers. The inoculum was a mixture of 30 g of faeces and 80 ml of pre-digested food. After stabilization of the microbiota for 16 h on the pre-digested food, 10 g of lactulose per 24 h were added to this medium and gradually introduced into the system. In the experiments with the microbiota adapted to lactulose (collected on day 17 of the *in vivo* study), the overnight stabilization was directly on the standard medium supplemented with 10 g of lactulose per 24 h (Fig. 1). Besides luminal addition of lactulose, the study substance was also added to the dialysis fluid (1 g/L) to prevent loss of lactulose during the dialysis process. During each experimental run, metabolites and microbiota composition were measured in all models after overnight stabilization (t0) and after 8, 24, 32 and 48 h of lactulose feeding.

SCFAs and lactate analyses

SCFAs (acetic, propionic, butyric, valeric, iso-valeric and iso-butyric acid) were analysed in fresh faeces collected from the human volunteers on days 8 and 17, and in both lumen and dialysis samples taken from the *in vitro* model. Analysis was performed on a gas chromatograph (GC; Chrompack CP9001, Varian, Bergen op Zoom, The Netherlands) according to the method described by Jouany (20). After centrifugation of the faecal and TIM-2 samples, the supernate was diluted (7% by volume) with a mixture of methanol, internal standard (2 mg/ml 2-ethyl butyric acid) and formic acid (20%). Of this mixture 0.5 µl was loaded onto a 'wide-bore' GC column (Stabilwax-DA; length 15 m; ID 0.53 mm; film thickness 0.1 µm; Restek, Bad Homburg, Germany) using a Chrompack CP9050 automatic sampler (Varian). L-Lactate and D-lactate were analysed enzymatically in the supernatant of the samples from the large intestinal model (not in the human faecal samples). The assay (Roche Biochemicals, Mannheim, Germany) was automated on a Cobas Mira Plus autoanalyser (Roche) and is based on the principle of conversion of NAD into NADH.

Microbiological methods

All samples were serially diluted 10-fold in peptone physiological salt solution (Oxoid, Haarlem, The Netherlands). Anaerobic bacterial groups were enumerated on pre-reduced media in a Bactron IV anaerobic glove box (IKS, Leerdam, The Netherlands). Total anaerobic bacteria, *Bifidobacterium* and Bacteroidaceae were counted on Reinforced Clostridium Blood-China Blue Agar (Oxoid), and (sulphite-reducing) *Clostridium* on Perfringens Agar Base

with a *B. cereus* selective supplement (Oxoid). These plates were incubated at 37°C under anaerobic conditions. The facultative anaerobic bacteria were plated and incubated under aerobic conditions, with the exception of lactobacilli which were incubated anaerobically after aerobic plating. *Lactobacillus* were counted on LAMVAB Agar (21), Enterobacteriaceae on Violet Red Bile Glucose Agar (Oxoid), and *Enterococcus* on Slanetz and Bartley Agar (Oxoid).

During the *in vitro* experiments samples were taken from the lumen of the TIM-2 system to determine the composition of the microbiota. These samples were immediately frozen in liquid nitrogen after addition of glycerol (20%) and stored at −196°C before analysis. LAMVAB selects for vancomycin-resistant lactobacilli. Although this resistance is common among lactobacilli isolated from the human microbiota, plating on LAMVAB may lead to an underestimation of their numbers.

Denaturing gradient gel electrophoresis

For denaturing gradient gel electrophoresis (DGGE) both RNA and DNA were isolated from the faecal and TIM-2 samples. One gram (faeces) or 1 ml (TIM-2 samples) were centrifuged and the cells were resuspended in 1 ml of TN150-buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl). Subsequently, 0.3 g zirconium beads and 150 µl acidified phenol were added and the cells were disrupted with a mini-Beadbeater 8™ (Merlin Diagnostic Systems, Rotterdam, The Netherlands). After mixing with 150 µl of a mixture of chloroform and iso-amylalcohol (24:1; v/v) it was centrifuged and the supernatant was used for isolation of RNA and DNA. For isolation of RNA the supernatant was further extracted with phenol/chloroform/iso-amylalcohol. The nucleic acids were precipitated with sodium acetate and ethanol. The precipitated nucleic acids were treated with 5 U DNAse in TNMC-buffer (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$) for 30 min at 37°C. After additional phenol/chloroform/isoamylalcohol extractions, the RNA was precipitated and resuspended in 100 µl of 10 mM Tris-HCl, pH 8.0. For DNA isolation, a similar protocol was used, except that RNAse (100 µg/ml final concentration) was used in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA was resuspended in 100 µl TE-buffer. RT-PCR and PCR were performed using established protocols. The enzymes used were rTth polymerase (Applied Biosystems, Foster City, CA, USA) and Taq polymerase (Roche), respectively. The primers used were U968-GC and L1401 (22). PCR amplification was carried out in the thermal cycler Hybaid Omnigene (Biozym, Landgraaf, The Netherlands). The reaction mixture (50 µl) contained approximately 25 pmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (Roche), 1 µl PCR buffer with $MgCl_2$ (Roche), 100 pmol of the target DNA and 2.5 U of Taq DNA Polymerase (Roche). DNA fragments were amplified as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 10 s, annealing at 56°C for 20 s, extension at 68°C for 40 s and a 7-min final extension step at 68°C. The products were stored at −20°C until analysis. The PCR products were separated on a Bio-Rad Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands), using a urea gradient from 50% to 60%. Electrophoresis was performed at 40 V for 24 h at 60°C. Silver staining of the separated PCR products was performed on a Hoefer Automated Gelstainer as described by the supplier (Amersham Biosciences, Roosendaal, The Netherlands).

Sequencing and sequence analysis

DNA from bands cut from the DGGE gel was isolated using the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. This DNA was used for re-amplification by PCR as described above, and the PCR product was sequenced directly with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using the supplier's protocol. The sequence products were analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequences (between 254 and 340 nucleotides long depending on the fragment) were compared to sequences deposited at the NCBI database by using the online BLAST service.

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed according to the protocol described by Langendijk et al. (22) with minor modifications. In short, after homogenization, 0.5 g of the samples was suspended in 4.5 ml of filtered PBS, vortex mixed and centrifuged at low speed (700 g) to remove large particles. Then 1 ml of the supernatant was mixed with 3 ml of fresh 4% paraformaldehyde solution and incubated overnight to fix the cells. Dilutions of the fixed cells in PBS were spread over the surface of a gelatin-coated slide. Hybridizations with fluorescein isothiocyanate-coupled probes were done overnight at 50°C in the presence of 0.9 M NaCl and 0.1% SDS. After hybridization, slides were washed at the same temperature for 30 min, rinsed in milli-Q water and air-dried. At this step DAPI (4′-6′-diamidino-2-phenylindole) was added at a final concentration of 1.25 ng/µl for 5 min at 20°C to stain all cells. The slides were washed again, dried and mounted with 6 µl of Vectashield® (Vector Laboratories, Peterborough, UK) on each well. Images were recorded on a Zeiss fluorescent microscope (Zeiss, Weesp, The Netherlands). Depending on the amount of fluorescent cells, 10–20 microscopic fields were counted. Fluorescein isothiocyanate probes (Isogen Bioscience, Maarssen, The Netherlands) used were Eub338, Bac303, Bif164, Chis150, Clit135, Ec1531, Ato291,

Rbro730, Rfla729 and Rec482 (22–29). FISH was done only with *in vivo* faecal samples.

Statistical analysis

Statistical analysis was carried out with an SAS statistical package, version 8.2. The data were compared using the paired Student's t-test. Differences between treatments with p values < 0.05 were considered to be significant. Data are expressed as mean (SD).

RESULTS

Body weight, faecal pH and dry matter content

The intake of 10 g of lactulose per day had no significant effect on body weight (72.6 ± 8.3 before lactulose treatment; 72.2 ± 8.3 after lactulose treatment; $p = 0.92$), faecal pH (6.9 ± 0.8 before and 7.1 ± 0.4 after lactulose treatment; $p = 0.60$) or dry matter content (25.9 ± 4.4 before and 24.6 ± 3.5 after lactulose treatment; $p = 0.45$). An effect on softening of the stool was not observed.

SCFAs and lactate

In vivo. Average molar ratios of SCFAs, as measured in fresh faecal samples, showed no significant differences between the faecal samples obtained from subjects before and after the intake of lactulose (Table I). Lactate was not analysed in fresh faecal samples.

In vitro. Results from the studies in the dynamic *in vitro* model of the large intestine were rather different from the above-described *in vivo* results. The cumulative total SCFA production *in vitro* in time was the same for both the NAM (Fig. 3A) and the AM (Fig. 3B). However, a statistically significant lower amount of propionate was found for the NAM (7.0 mmol ± 3.4 vs 12.0 mmol ± 6.6 for NAM and AM, respectively; $p = 0.049$), and a statistically significant lower amount of butyrate for AM (21.2 mmol ± 16.4 vs 3.1 mmol ± 5.1 for NAM and AM, respectively; $p = 0.005$). A clear effect on molar ratios of SCFAs produced by the different microbiotas was observed (Table I) with a decrease in the butyrate ratio for AM compared with NAM as the major difference. During the first 24 h the NAM produced almost linear amounts of all three SCFAs. Thereafter, acetate was still increasing, but the production of propionate and butyrate was arrested. During fermentation of lactulose by the NAM the production of lactate was observed only after 24 h (Fig. 3A). D-Lactate production was higher than L-lactate production (data not shown). For the AM butyrate production was low during the whole experiment (Fig. 3B). In fact, a significant amount of butyrate production could be detected in the microbiota of

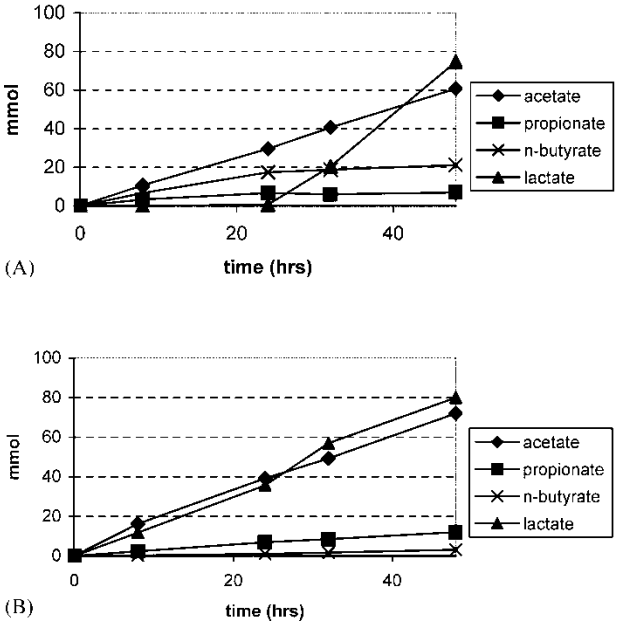

Fig. 3. Average SCFA (acetate, propionate and *n*-butyrate) and lactate production during 48 h of 10 *in vitro* experiments of the non-adapted microbiota (A) and lactulose adapted microbiota (B) in the large intestinal model.

Table I

SCFA ratios in fresh faecal samples from humans and in samples from the in vitro large intestinal model at t = 48 h (average ±SD; n = 10)

Sample		Acetate	Propionate	n-Butyrate	Lactate
Fresh faecal samples	NAM	62.4 (±6.5)	20.4 (±4.2)	17.2 (±3.5)	nd
	AM	63.8 (±5.5)	20.8 (±4.6)	15.4 (±2.8)	nd
In vitro samples*	NAM	68.7[a] (±7.6)	8.2[b] (±3.6)	23.0[c] (±8.3)	–
	AM	79.9[a] (±11.6)	16.8[b] (±9.3)	3.2[c] (±4.0)	–
In vitro samples†	NAM	47.0 (±17.5)	5.8 (±3.0)	17.4[d] (±11.5)	29.8 (±29.5)
	AM	41.9 (±20.1)	7.7 (±3.3)	1.5[d] (±2.1)	48.9 (±21.8)

NAM, non-adapted microbiota; AM, adapted microbiota; nd, not determined.
*Excluding lactate;
†including lactate.
[a]Difference between NAM and AM; $p = 0.02$.
[b]Difference between NAM and AM; $p = 0.01$.
[c]Difference between NAM and AM; $p < 0.00001$.
[d]Difference between NAM and AM; $p = 0.0008$.

only one of the 10 individuals. In all other nine microbiotas production was very low or below the limit of detection. As with the NAM, in the AM propionate production was arrested after 24 h. The AM was able to produce lactate from the start of the experimental period, immediately after lactulose feeding (Fig. 3B). L-Lactate was produced in higher quantities than D-lactate, which contrasted with the NAM (data not shown).

In vivo microbiological composition

Bacterial counts in fresh faecal samples reached between 10^{10} and 10^{11} cfu/g (wet weight) for both the samples taken at day 8 and day 17 (Table II). In comparing the average composition (Table II) of the NAM with the AM an increase in absolute numbers for *Bifidobacterium* ($p < 0.00001$ both for FISH and the classic plating technique) and (sulphite-reducing) *Clostridium* (NS) was found after lactulose treatment. In addition, the numbers of *Bifidobacterium* and *Bacteroides* were similar for the classic plating technique in comparison to the FISH technique. FISH was also used for some additional groups of microorganisms for three randomly selected faecal samples. Using the selective probes an increase in *Clostridium* ($p < 0.01$) and *Escherichia coli* ($p = 0.04$) was detected in these samples after lactulose treatment. The *Atopobia/Collinsella* and the *Eubacterium/Clostridium/Ruminococcus* groups stayed at similar levels. When evaluated separately, the *Ruminococcus* group decreased slightly in these samples after lactulose treatment ($p = 0.04$). It should be kept in mind that these analyses were performed on only 3 of the 10 samples, and therefore, at the moment it is not known how representative these findings are. With the probes used, only approximately 65% of the total microorganisms present were detected (Table II). Other probes will be needed to study changes in composition of the other bacterial groups.

In vitro microbiological composition

Table III shows the changes in microbiota composition (absolute and in percentages) during the experiments in the large intestinal model based on classic plating of the microorganisms on (s)elective plates. FISH was not performed on these samples. For the NAM, a relative increase of *Bifidobacterium* and decrease of *Bacteroides* occurred after lactulose treatment (Table III). The AM showed a relative decrease in *Bifidobacterium* (possibly caused by the increase in other groups, see below), whereas the *Bacteriodes* population stayed proportionally similar. Especially in the AM, but also detectable in the NAM, the *Lactobacillus* and *Enterococcus* ratio increased. Also, numerically an increase of lactobacilli and enterococci was observed after lactulose feeding (Fig. 4). This occurred, irrespective of previous lactulose feeding, for both the NAM and the AM ($p = 0.009$ and $p = 0.006$ for *Lactobacillus* for NAM and AM, respectively, and $p = 0.007$ and $p = 0.006$ for *Enterococcus* for NAM and AM, respectively). The effect was similar in both cases (Table III).

DGGE

Using DGGE of PCR fragments obtained from the 16S rRNA gene region 968–1401 (*E. coli* numbering; containing the variable regions V_6–V_8) the changes in dominant microbiota from one of the individuals in the TIM-2 samples were investigated (Fig. 5). Fig. 5 shows that after

Table II

Composition of the fresh faecal microbiota from humans determined by FISH and classic plating (average ±SD; n = 10)

Microorganism	Probe	NAM			AM		
		FISH		Plating	FISH		Plating
		$\log_{10}$ (cfu/g)	ratio (%)	($\log_{10}$ cfu/g)	$\log_{10}$ (cfu/g)	ratio (%)	($\log_{10}$ cfu/g)
Total bacteria	Eub338	10.6 (±0.2)	100	–	10.6 (±0.2)	100	–
Bifidobacterium	Bif164	8.6[a] (±0.4)	1.4	8.8[b] (±0.16)	9.5[a] (±0.2)	9.5	9.6[b] (±0.2)
Bacteroides	Bac303	9.4 (±0.7)	14.0	9.2 (±0.41)	9.6 (±0.5)	17.0	9.3 (±0.4)
*Clostridium**	Chis150/ Clit135	< 7.35†[c]	< 0.05	–	8.0[c] (±0.4)	0.3	–
*Atopobium/Collinsella**	Ato291	9.1 (±0.9)	6.5	–	9.3 (±0.4)	5.1	–
*E. coli**	Ec1531	< 7.35[d]	< 0.05	–	8.0[d] (±0.6)	1.3	–
*Ruminococcus**	Rfla729/ Rbro730	9.3[e] (±0.5)	6.2	–	9.1[e] (±0.1)	2.7	–
*Eubacterium/Clostridium/Ruminococcus**	Rec482	10.0 (±0.4)	31.0	–	10.1 (±0.2)	27.1	–
Sulphite-reducing *Clostridium*	–	–	–	5.0[f] (±1.3)	–	–	5.9[f] (±1.0)
Lactobacillus	–	–	–	4.7 (±1.6)	–	–	5.0 (±2.0)
Enterococcus	–	–	–	5.9 (±1.0)	–	–	5.81 (±1.3)
Enterobacteriaceae	–	–	–	6.4 (±1.4)	–	–	6.7 (±1.5)

*Average of three randomly selected faecal samples.

†Detection limit for FISH is $\log_{10}$ 7.35 cfu/g.

a, b, c, d, e, f: statistically significant difference between NAM and AM; $p < 0.05$.

Table III

Average (n = 10) bacterial composition in TIM-2 samples (absolute in cfu/ml and in percentages), inoculated with NAM and with AM, before (t0) and after 2 days of lactulose intake (t48)

Microorganism	NAM (log_{10} cfu/ml)		NAM (%)		AM (log_{10} cfu/ml)		AM (%)	
	t0	t48	t0	t48	t0	t48	t0	t48
Total bacteria	8.7 (±0.2)	8.9 (±0.2)	100	100	9.1 (±0.2)	9.2 (±0.2)	100	100
Bifidobacterium	8.4 (±0.5)	8.6 (±0.8)	48.3	54.3	8.9 (±0.1)	9.0 (±0.5)	62.9	37.5
Bacteroides	8.4 (±0.5)	8.5 (±0.5)	51.1	38.7	8.6 (±0.3)	8.8 (±1.2)	36.2	36.7
Lactobacillus	5.5 (±0.7)	7.1 (±1.0)	0.08	1.86	5.7 (±1.1)	7.1 (±0.9)	0.31	13.1
Enterobacteriaceae	5.8 (±1.1)	6.7 (±0.3)	0.13	0.06	7.0 (±0.8)	7.3 (±0.7)	0.07	3.53
Enterococcus	6.6 (±1.1)	7.9 (±0.5)	0.34	4.44	6.0 (±0.7)	7.5 (±1.1)	0.48	9.25

lactulose feeding a major shift occurred in the dominant microbiota. Several new bands appeared (in PCR reactions from DNA samples as well as from RNA samples) after lactulose feeding, whereas other bands disappeared. Similar shifts were seen in the microbiotas from the other individuals, although the exact positions of the bands that appeared were not always identical (data not shown). Several of the bands (circled in Fig. 5) were excised from the gel and the nucleotide sequences were determined and compared to sequences in the database (Table IV). From this comparison it is clear that the bands corresponded to *Lactobacillus* and *Enterococcus* species. It therefore appears that, as was determined with plating on (s)elective plates, numbers of lactobacilli and enterococci were higher after lactulose feeding. Since DGGE shows patterns for the dominant microbiota, it is also clear that no other unculturable microorganisms were stimulated by lactulose in this system in such a manner that they became part of the dominant microbiota. However, it cannot be ruled out that species present in very small amounts are also stimulated by lactulose, but still remain below the level of detection for DGGE.

DISCUSSION

We show here that addition of lactulose results in a profound reduction of butyrate production. In the microbiota not adapted to lactulose (NAM) butyrate production was arrested after 24 h; the microbiota adapted to lactulose (AM) produced low amounts of butyrate right from the start of the experiment. Propionate production was also affected, although to a lesser extent than butyrate and only markedly detectable in the NAM. It apparently takes between 8 and 24 h (no samples were taken in between) to change the metabolic activity of the NAM microbiota. This is also reflected in the onset of production of lactate by the NAM after 24 h. Lactate is immediately produced by the AM. This, and the immediate low butyrate production, shows that the AM was indeed adapted to lactulose. The only significant difference in composition of the faecal microbiota before and after lactulose treatment in humans was an increase in *Bifidobacterium*, with a trend of an increase in (sulphite-reducing) *Clostridium*. From the results in TIM-2 using both classic plating and DGGE, it is clear that *Lactobacillus* and *Enterococcus* are largely

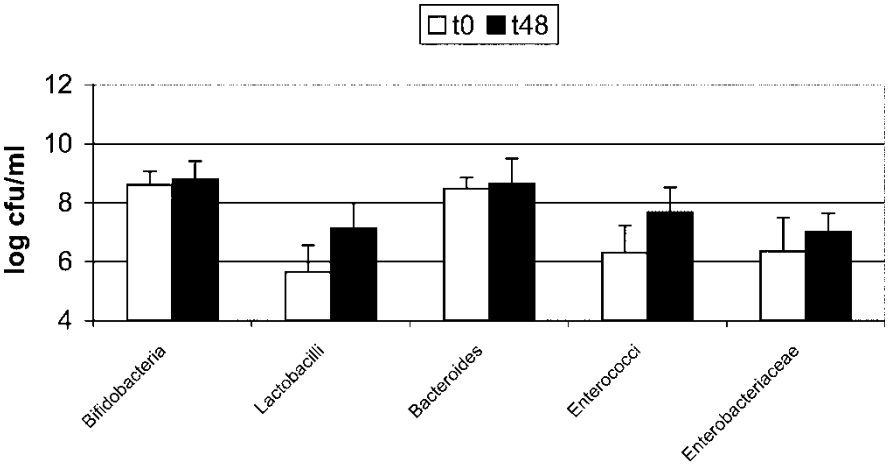

Fig. 4. Average (±SD) changes in microbiota composition in the large intestinal model. Data from all runs ($n = 20$) were taken. There were no significant differences between non-adapted and lactulose-adapted microbiotas.

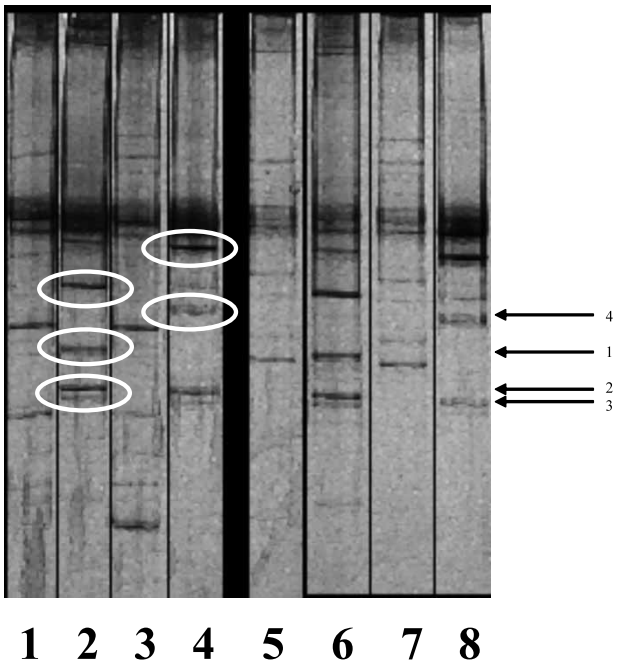

Fig. 5. DGGE analysis of samples from the large intestinal model from one of the individuals. DGGE analysis was done on both DNA samples (lanes 1–4) and RNA samples (lanes 5–8). Samples from the start of the experiment in the *in vitro* model of the large intestine (t0: lanes 1, 3, 5 and 7) were compared to samples taken at the end of the experiment (t48: lanes 2, 4, 6 and 8). Both NAM (lanes 1, 2, 5 and 6) and AM (lanes 3, 4, 7 and 8) samples were analysed. The numbered arrows correspond with the PCR products that were sequenced and of which the homology to database sequences is shown in Table IV.

Table IV

Comparison of the excised DNA bands from the DGGE gel with database sequences

	E-value	Homology (%)
PCR product 1		
Lb. sp. oral clone CX036	0	100
Lb. vaginalis ATCC49540	0	99.7
Lb. reuteri DSM 20016	e^{-128}	< 97
PCR product 2		
Uncultured bacterium S24-8	e^{-149}	98.6
Lb. reuteri DSM 20016	e^{-144}	97.6
PCR product 3		
Lb. sp. oral clone CX036	e^{-148}	97.9
Lb. vaginalis KC19	e^{-148}	97.9
Uncultured bacterium S24-8	e^{-148}	97.9
PCR product 4		
E. faecalis VRE no 1492	e^{-128}	99.2

For identical species, a sequence identity of = 99.7% is taken (30).

stimulated by addition of lactulose (over 1.5 log increase for both genera).

SCFAs (primarily acetate, propionate and butyrate) and D- and L-lactate are the major metabolic products due to microbial degradation of organic substrates (31, 32). Both total acid production and SCFA ratios are used in *in vivo* studies for the description of bacterial metabolic activities of a typical microbiota. A drawback of analysing faecal samples is the fact that they do not represent quantitatively what happens in the proximal part of the colon where most fermentation, including that of lactulose, takes place. SCFAs are predominantly produced in this part of the colon and will be absorbed by the body to a considerable extent during subsequent transit of the chyme to the distal colon and rectum. Consequently, the amount and ratio of SCFAs recovered in the faeces will not reflect those resulting from fermentation of lactulose in the proximal colon. Indeed, our results from the human study show no difference in faecal SCFA ratios between the control and lactulose period (Table I). Even if *in vivo* samples from the lumen of the proximal colon and the portal blood vein could be taken, not all the SCFAs produced would be measured (32). This is because butyrate in particular is used as a substrate by colonocytes and therefore only low amounts of this microbial metabolite are found in the bloodstream (32). In the *in vitro* model of the proximal colon used in the present study, these disadvantages are absent. All SCFAs are detected, either in the lumen of the model, or in the collected dialysis fluid. This makes it possible to study mechanistically the effects of lactulose or any other food compound or drug on microbial metabolite production. Similar to what we found, the data available in the literature show no effect of lactulose on butyrate in faecal samples at the same dosage as that used in the present study. At higher dosages (up to 160 g of lactulose per day), the ratio of acetate and lactate increased (13). This is consistent with the decrease in butyrate and propionate that we found in the TIM-2 experiments. In batch incubations, where metabolites accumulate, either a slight increase or a decrease in butyrate ratios is measured on lactulose. However, this is most likely caused by interconversion of lactate into butyrate, a process which is more likely to take place in a batch incubation in which the metabolites are not removed, than *in vivo*, where the metabolites produced are quickly taken up by the epithelium and do not accumulate to the same degree. Incubation times in batch cultures are usually long, allowing time for this interconversion to take place.

The TNO *in vitro* model has been developed and validated using data from sudden death individuals (17). Both with respect to composition and with respect to metabolic activity, a faecal inoculum in the model has been shown to simulate the data from these sudden death individuals very well. In addition, we have performed experiments in the model using different microbiotas from dogs. The microbiotas originated from the caecum and from faecal material (a microbiota from the terminal ileum was studied as well). The microbiotas maintained in TIM-2 that

originated from either the caecum or faeces were shown to have the same composition and microbial activity as those of the microbiota when freshly obtained from the caecum, supporting the hypothesis that the microbiota in TIM-2 does develop to resemble that of the caecum (unpublished data). We hypothesize that this is also the case for a human microbiota. A human microbiota could be maintained stably in TIM-2 for up to 3 weeks on the standard predigested food, without changes in microbial composition or activity (17).

The increase in bifidobacteria and clostridia in the human trial is in agreement with data reported in the literature (33, 34). However, as in the case for SCFA and lactate production, changes in the faecal microbiota do not reflect which microorganisms are responsible for fermentation of lactulose in the proximal colon. In fact, it is unlikely that the stimulation of *Clostridium* is a direct effect of lactulose, since *Clostridium* is known to produce butyrate, whereas we have found that butyrate production in particular was very low after lactulose addition. In the *in vitro* system, lactobacilli and enterococci were increased. This is in agreement with the observed reduction of production of butyrate and propionate, and the increase in lactate production. However, the observed effect *in vitro* seems to be in contrast to the effect found in the faecal samples. Since TIM-2 simulates the proximal colon, we postulate that the results in TIM-2 are a better reflection of the events taking place in this part of the colon than are the results from the faecal samples. In addition, the length of the experiments in TIM-2 (48 h) was different to those of the *in vivo* trial (7 days). Perhaps additional microbiological shift would have been found if the *in vitro* experiments had been carried out for a longer period.

The AM was different in metabolic activity immediately from the start of the experiment in TIM-2. Assuming that this difference is not caused by the minor increase in *Bifidobacterium*, which is corroborated by the fact that lactobacilli and enterococci are primarily stimulated, we concluded that the AM is metabolically adapted to lactulose, rather than compositionally adapted. This is a phenomenon we have seen before. Microbiotas from healthy individuals that do not seem to differ in composition (at least based on tools available to us at the moment), differ with respect to metabolic activity, and hence metabolite production (data not shown). If the composition was hardly or not changed, this must mean that the microbiotas were metabolically predisposed or adapted to lactulose. Alternatively, different strains of the various genera present in the complex faecal mixture are present with different enzymatic activities (e.g. saccharolytic versus proteolytic). The tools available are not sufficiently accurate and discriminative to detect these differences at the strain level.

The results from the classic plating and FISH, for those microorganisms for which the comparison can be made, are in perfect agreement (Table II). Another set of probes was used on a limited number of faecal samples. It would be worthwhile to increase the number of group-, genera- or species-specific probes to see if other changes in the composition of the microbiota can be identified. From the DGGE analyses it can be concluded that, apart from the bands that were excised and sequenced, some additional bands appear after lactulose addition. We are in the process of investigating which microorganisms these PCR fragments originate from. The results from classic plating and DGGE also seem to correlate well, since both show an increase in lactobacilli and enterococci; however, there is a discrepancy. The DGGE technique only investigates the dominant microbiota. Based on this, we would expect an even larger number of cfu/ml for lactobacilli and enterococci in the TIM-2 samples than is found with classic plating, even when taking into account the fact that DGGE is only semi-quantitative. We hypothesize that the (s)elective media used in classic plating may be too selective to allow for growth of all of the *Lactobacillus* and *Enterococcus* species in the complex large intestinal microbiota. The correlation between plating and DGGE is only accidental. If lactulose would have also stimulated an unculturable microorganism, there would be no correlation whatsoever between plating and DGGE. Therefore, the state-of-the-art molecular tools reveal changes in composition much better than classical plating.

CONCLUSIONS

In conclusion, we postulate that TIM-2 is a valuable tool for investigating, at a mechanistic level, the effect of food components on the composition and activity of the large intestinal microbiota and vice versa (results of present study and ref. 35). In contrast to analysis of faecal samples, in TIM-2 all metabolites that are actually produced can be measured at 'the site where it all happens'. In combination with molecular tools to investigate the composition of the microbiota it is even possible to determine which microorganisms are responsible for a certain effect. The present example shows that lactulose results in a reduction of butyrate production in the proximal colon in the *in vitro* experiments. Our planned experiments using stable isotopes in *in vivo* experiments should corroborate these findings in the *in vivo* situation. We hypothesize that lactulose is the preferred substrate, compared with the complex carbohydrates, for fermentation in the proximal part of the colon. Whether or not this means that the other carbohydrates present in the food are fermented to a lesser degree in the proximal colon and therefore may end up further in the colon to be fermented there is unknown at present. However, it is worthwhile investigating this further, since fermentation of carbohydrates in the transverse and distal colon is hypothesized to reduce proteolytic fermentation, with its concomitant production of various toxic metabo-

lites, such as ammonia, phenol and *p*-cresol. We are currently adapting TIM-2 to investigate this aspect.

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