

## The *in vivo* use of the stable isotope-labelled biomarkers lactose-[<sup>15</sup>N]ureide and [<sup>2</sup>H<sub>4</sub>]tyrosine to assess the effects of pro- and prebiotics on the intestinal flora of healthy human volunteers

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Amongst the various claimed beneficial effects of pro- and prebiotics for the human host, it has been hypothesised that functional foods are able to suppress the generation and accumulation of toxic fermentation metabolites (NH<sub>3</sub>, *p*-cresol). Direct evidence supporting this hypothesis is lacking mainly because of the unavailability of reliable biomarkers. Preliminary data indicate that lactose-[<sup>15</sup>N]ureide and [<sup>2</sup>H<sub>4</sub>]tyrosine may be potential biomarker candidates. The aim of the present study was to evaluate the effect of pro- and prebiotics on the colonic fate of these biomarkers in a randomised, placebo-controlled, cross-over study with nineteen healthy volunteers. At the start of the study and at the end of each 2-week study period, during which they were administered either a probiotic (*n* 10; 6.5 × 10<sup>9</sup> *Lactobacillus casei* Shirota cells twice daily) or a prebiotic (*n* 9; lactulose 10 g twice daily), the volunteers consumed a test meal containing the two biomarkers. Urine was collected during 48 h. Results were expressed as percentage of the administered dose. As compared with the placebo, the decrease in the percentage dose of *p*-[<sup>2</sup>H<sub>4</sub>]cresol in the 24–48 h urine fraction was significantly higher after probiotic intake (*P*=0.042). Similar changes were observed for the <sup>15</sup>N tracer (*P*=0.016). After prebiotic intake, a significantly higher decrease in the percentage dose of *p*-[<sup>2</sup>H<sub>4</sub>]cresol (*P*=0.005) and <sup>15</sup>N tracer (*P*=0.029) was found in the 0–24 h urine collection. The present results demonstrate that suppression of the generation and accumulation of potentially toxic fermentation metabolites by pro- and prebiotics can reliably be monitored *in vivo* by the use of stable isotope-labelled biomarkers.

### Probiotics: Prebiotics: Biomarkers: Stable isotopes

In recent years, there has been growing evidence in the literature indicating that functional foods, i.e. pro-, pre- and synbiotics, may play a substantial role in the maintenance of health or prevention of disease. A probiotic is a live micro-organism, which, when administered in adequate amounts, confers a health benefit to the host (Fuller, 1991). Prebiotics have been defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating growth, and/or activity, of one or a restricted number of bacteria in the colon (Gibson & Roberfroid, 1995). Some health advantages postulated to be associated with pre- and/or probiotic intake are the production of SCFA, the reduction of the symptoms of lactose intolerance, the stimulation of the immune system, a reduction in serum cholesterol levels and the prevention of cancer (Fuller, 1991; Salminen *et al.* 1998; Collins & Gibson, 1999; Saavedra & Tschernia, 1999).

Until now their pivotal role has not completely been established, but there is a general acceptance that they positively influence one or more functions in the organism. The main target site for functional foods is the colon, which is a highly complex organ that plays a major role in food assimilation processes that determine the physiological effects of the diet (Roberfroid *et al.* 1995). The nature of the bacterial metabolites formed in the large intestine depends on the characteristics of the bacterial flora, the transit time through the colon and the substrate availability (Gibson *et al.* 1995).

The main sources of C and energy for intestinal bacteria are complex carbohydrates and proteins. Carbohydrates are converted into SCFA, which are generally assumed to be beneficial for the colonocytes of the host (Cummings, 1981). Proteins, on the other hand, are degraded by bacterial fermentation into potentially toxic metabolites

**Abbreviation:** AP, atom percentage.

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such as amines, NH<sub>3</sub>, phenolic compounds and thiols (Smith & Macfarlane, 1996; Fooks *et al.* 1999). Protein fermentation products, especially NH<sub>3</sub> and phenols, are implicated in the pathogenesis of certain diseases. Phenols have been implicated in the pathogenesis of bladder and bowel cancers, whereas NH<sub>3</sub> has been shown to affect the intermediary metabolism and DNA synthesis of colonic epithelial cells and to reduce their lifespan (Bone *et al.* 1976; Visek, 1976).

There are indications that the administration of pro- and prebiotics suppresses the generation and accumulation of these toxic metabolites (Cummings & Bingham, 1987; Ziemer & Gibson, 1998; Cummings & Macfarlane, 2002) and through a suppression of toxic metabolites, the incidence of colon and bladder cancers may decrease. However, substantial evidence supporting these beneficial effects of pro- and prebiotics is currently lacking, mainly due to the inaccessibility of the colon and the unavailability of reliable tracers.

The aim of the present study was to investigate *in vivo* whether the administration of a selected probiotic (*Lactobacillus casei* Shirota cells), or prebiotic (lactulose), would result in a reduced concentration of one or more bacterial metabolites in the colon. For this purpose, a tracer technique using stable isotope-labelled biomarkers was used to study the colonic microenvironment *in vivo*. Importantly, this technique is easy to perform, non-invasive and completely safe.

The first biomarker used was egg protein in which <sup>2</sup>H (<sup>2</sup>H<sub>4</sub>tyrosine) is incorporated, according to the method of Evenepoel *et al.* (1997). After oral administration, the major part of the <sup>2</sup>H<sub>4</sub>tyrosine-labelled proteins is digested in the small intestine after which <sup>2</sup>H<sub>4</sub>tyrosine is absorbed and used by the human metabolism. However, the human metabolism is not able to convert <sup>2</sup>H<sub>4</sub>tyrosine into *p*-<sup>2</sup>H<sub>4</sub>cresol (*p*-cresol is a unique bacterial metabolite of tyrosine) and, as a consequence, the amount of the biomarker that is digested and absorbed does not interfere with the measurements. Based on literature data (Evenepoel *et al.* 1999), it is assumed that in physiological circumstances about 3 to 6% of the proteins ingested remains malabsorbed. When the non-digested proteins reach the colon, they are fermented by the colonic flora, resulting in the production of <sup>2</sup>H-labelled [ring-<sup>2</sup>H<sub>4</sub>]phenol and *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol. These compounds are largely absorbed from the colon, detoxified in the mucosa and in the liver (by glucuronide- and sulfate conjugation) and finally excreted in the urine. Since phenol and *p*-cresol originate exclusively from bacterial metabolism and not from human metabolism, the urinary output of phenol and *p*-cresol reflects the bacterial production of these compounds in the colon (Smith & Macfarlane, 1996). As a consequence, any influence of pre- and/or probiotics on the colonic protein fermentation should be reflected in the urinary concentration of [ring-<sup>2</sup>H<sub>4</sub>]phenol and *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol.

The second biomarker, lactose-[<sup>15</sup>N]ureide, was used to investigate the influence of a pro- and prebiotic on the fate of the colonic NH<sub>3</sub>. This substrate is an efficient vehicle to introduce a known amount of <sup>15</sup>N, in the form of NH<sub>3</sub>, into the colon. During the passage through the

intestinal tract, lactose-[<sup>15</sup>N]ureide is degraded by β-galactosidase, located in the brush border of the small bowel, resulting in the formation of galactose and glucose-[<sup>15</sup>N]ureide (Ruemmele *et al.* 1997). A recent study of Morrison *et al.* (2003) demonstrated that a fraction of glucose-[<sup>15</sup>N]ureide (approximately 10–15%) traverses the small intestine epithelium and appears in the urine. Therefore, the 0–6 h urine collection was not taken into account during the interpretation of the present results on the percentage recovery of <sup>15</sup>N, because this urine collection reflects the fraction of glucose-[<sup>15</sup>N]ureide absorption in the small intestine. When the non-absorbed part of glucose-[<sup>15</sup>N]ureide reaches the colon, the bond between glucose and [<sup>15</sup>N]urea is hydrolysed through *Clostridium innocuum* (Mohr *et al.* 1999) and the resulting [<sup>15</sup>N]urea is quantitatively hydrolysed to [<sup>15</sup>N]NH<sub>3</sub> by the microbial species. Part of the resulting [<sup>15</sup>N]NH<sub>3</sub> can be taken up by the micro-organisms whereas the remaining part is absorbed and, after conversion in the liver (hepatic urea synthesis; Weber, 1979; Weber *et al.* 1982), is renally excreted as [<sup>15</sup>N]urea. For this reason, the urinary excretion of <sup>15</sup>N is a measure for the fate of NH<sub>3</sub> in the colon and should reflect decreases in the colonic production of NH<sub>3</sub>, caused by the administration of a pro- or prebiotic.

## Materials and methods

### Subjects

Nineteen healthy volunteers (nine females and ten males; age range 22–45 years) participated in the study and were at random divided into two groups. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from appendectomy). The subjects were free of antibiotics or any other medical treatment influencing gut transit or intestinal flora for at least 3 months before the start of the study. The ethical committee of the University of Leuven approved the study and all subjects gave informed consent.

### Experimental design

A randomised, placebo-controlled, single-blind cross-over study with a 14 d washout between the intake of pro- or prebiotic and placebo was performed. The probiotic consisted of  $6.5 \times 10^9$  *L. casei* Shirota cells as a single-strain fermented milk product (Yakult<sup>®</sup>; Yakult Honsha Co. Ltd, Tokyo, Japan) and was evaluated in ten healthy volunteers (group 1; probiotic). Lactulose (Lactulose EG<sup>®</sup>; Eurogenerics, Brussels, Belgium) was selected as the prebiotic (Schumann, 2002; Bouhnik *et al.* 2004) and was evaluated in nine healthy volunteers (group 2; prebiotic). The placebo for the probiotic was an identical milk product without the *L. casei* Shirota strain (Yakult Honsha Co. Ltd, Tokyo, Japan); lactose was used as the placebo for lactulose. In each group, five individuals received for 2 weeks twice daily (once with breakfast, once with supper) respectively the prebiotic (10 g) or probiotic whereas the remaining five (or four) individuals received a placebo during the same period. After a washout

period of 2 weeks, the subjects crossed from the treatment to the placebo group and vice versa, enabling each volunteer to act as a control. At the start of the study and at the end of each test period (i.e. after 2, 4 and 6 weeks), the volunteers consumed a test meal containing both [ $^2\text{H}_4$ ]tyrosine incorporated in egg proteins and lactose- $^{15}\text{N}$ ureide as biomarkers.

#### Dietary intake

No standard diets were imposed on the volunteers. However, they were asked to maintain a regular eating pattern until the end of the study period and to avoid the intake of fermented milk products and food components containing high quantities of fermentable carbohydrates.

#### Test meal

The test meal consisted of a pancake (15.8 g proteins, 11.6 g fat and 21.1 g carbohydrates; 1066 kJ (255 kcal)), which contained the two different stable isotope-labelled substrates, i.e. [ $^2\text{H}_4$ ]tyrosine (14.75 mg), incorporated in egg proteins, and lactose- $^{15}\text{N}$ ureide (75 mg). The labelled egg proteins were prepared according to the method of Evenepoel *et al.* (1997). Briefly, laying hens were given free access to food containing 3 g L-[ring- $^2\text{H}_5$ ]phenylalanine/kg (98 mol %; Euriso-top, St Aubin Cédex, France). By the hen's metabolism, the dietary L-[ring- $^2\text{H}_5$ ]phenylalanine is converted to L-[ring- $^2\text{H}_4$ ]tyrosine, which is consequently incorporated in the egg protein. The L-[ring- $^2\text{H}_4$ ]tyrosine content of the egg protein was determined by GC-MS (Trace GC-MS; Thermofinnigan, San José, CA, USA) (Geypens *et al.* 1999). Lactose- $^{15}\text{N}$ ureide was synthesised according to the method of Schoorl (1903) as modified by Hofmann (1931) with [ $^{15}\text{N},^{15}\text{N}$ ]urea obtained from Euriso-top.

#### Urine collection

Urine was collected in receptacles to which neomycin was added for the prevention of bacterial growth. A basal urine sample was collected before the consumption of the test meal. After the intake of each test meal, a 48 h urine collection was performed in three fractions: 0–6 h; 6–24 h; 24–48 h. After measurement of the volume, samples were taken and stored at  $-20^\circ\text{C}$  until analysis.

#### Analytical procedures

**Determination of urinary phenolic compounds.** *p*-[ring- $^2\text{H}_4$ ]Cresol, *p*-[ring- $^2\text{H}_4$ ]phenol and total *p*-cresol content were measured by GC-MS technology. Therefore, the pH of 950  $\mu\text{l}$  urine was adjusted to pH 1 with concentrated  $\text{H}_2\text{SO}_4$  (Merck KgaA, Darmstadt, Germany). This solution was heated for 30 min at  $90^\circ\text{C}$  to deproteinise and hydrolyse the conjugated phenols. After cooling down to room temperature, 50  $\mu\text{l}$  2,6-dimethylphenol (20 mg/100 ml; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added as an internal standard. The phenols were extracted with 1 ml ethyl acetate (Merck KgaA). The ethyl acetate layer was dried and 0.5  $\mu\text{l}$  of this solution

was analysed on a GC-MS (Trace GC-MS). The analytical column was a 30 m  $\times$  0.32 mm internal diameter, 1  $\mu\text{m}$  AT5-MS (Alltech Associates, Deerfield, IL, USA). He gas, GC grade, was used as a carrier at a constant flow rate of 1.3 ml/min. The oven temperature was programmed from  $75^\circ\text{C}$  (isothermal for 5 min), and increased by  $10^\circ\text{C}/\text{min}$  to  $160^\circ\text{C}$  and by  $20^\circ\text{C}/\text{min}$  to  $280^\circ\text{C}$ . Mass spectrometric detection was performed in electron impact full scan mode from  $m/z$  59 to  $m/z$  590 at two scans/s. Results for *p*-[ring- $^2\text{H}_4$ ]cresol and *p*-[ring- $^2\text{H}_4$ ]phenol were expressed as percentage of the administered dose of L-[ring- $^2\text{H}_4$ ]tyrosine recovered in 0–24 h and 24–48 h collections, as described by Evenepoel *et al.* (1999).

**Determination of urinary total nitrogen content and  $^{15}\text{N}$ .** Total N content and  $^{15}\text{N}$  enrichment were determined by a continuous flow elemental analyser isotope ratio mass spectrometer (ANCA-2020; Europa Scientific, Crewe, UK). Therefore, a known amount of urine (15  $\mu\text{l}$ ) was absorbed on chromosorb (Elemental Microanalysis Limited, Okehampton, Devon, UK) in a tin capsule, which was introduced in the oxidation–reduction module coupled to the isotope ratio mass spectrometer. In this module the samples were oxidised using copper oxide,  $\text{O}_2$  and chromium oxide to nitrous oxides at  $1000^\circ\text{C}$  and then reduced to  $\text{N}_2$  using Cu at  $600^\circ\text{C}$ . This gas was led to the ion source of the mass spectrometer where the total N content and the  $^{15}\text{N}$  enrichment were measured. The  $^{15}\text{N}:$  $^{14}\text{N}$  isotope ratio of  $\text{N}_2$  was measured with reference to a calibrated laboratory standard (i.e. a standard ammonium sulfate solution). Results for  $^{15}\text{N}$  were expressed as percentage of the administered dose of  $^{15}\text{N}$  recovered in the 6–24 h and 24–48 h urine collections (Evenepoel *et al.* 1999). The percentage of the administered dose of  $^{15}\text{N}$  recovered was calculated as follows:

$$\text{Percentage dose } ^{15}\text{N} = 100 \times \frac{\text{mg excess } ^{15}\text{N}}{\text{mg } ^{15}\text{N administered}},$$

where

$$\text{mg excess } ^{15}\text{N} = \frac{\text{AP}_t - \text{AP}_{\text{bas}}}{100} \times \text{N}_{\text{tot}},$$

and where  $\text{AP}_t$  is the measured  $^{15}\text{N}$  enrichment of a specified urine sample, expressed in atom percentage (AP),  $\text{AP}_{\text{bas}}$  is the  $^{15}\text{N}$  enrichment of a basal urine sample (expressed in AP) and  $\text{N}_{\text{tot}}$  is the total N content in a specified urine sample.

#### Statistical analysis

Results are expressed as mean values and standard deviations. The statistical analysis was performed with Statistica software (Statistica 6.0; Statsoft Inc. 1984–2001, Tulsa, OK, USA). Statistical evaluation of the data was performed by applying a Student's *t* test on the post- and pre-treatment values. To compare the effects of the pro- and prebiotic with the placebo, a Student's *t* test was performed on the differences between post- and pre-administration values. The level of statistical significance was set at  $P < 0.05$ .

## Results

### *Effects of probiotic Lactobacillus casei Shirota cells on urinary p-[ring-<sup>2</sup>H<sub>4</sub>]cresol and <sup>15</sup>N excretion*

After intake of the probiotic *L. casei* Shirota cells the percentage dose of p-[ring-<sup>2</sup>H<sub>4</sub>]cresol significantly decreased from 1.88 (SD 0.56) to 1.18 (SD 0.75) ( $P=0.032$ ) whereas the percentage dose of <sup>15</sup>N significantly decreased from 11.57 (SD 2.85) to 8.41 (SD 3.39) ( $P=0.047$ ), both in the 24–48 h urine collection. No influence of the probiotic on the <sup>15</sup>N (6–24 h urine collection) or <sup>2</sup>H marker (0–24 h urine collection) was seen (Table 1). Significant changes were not observed during the placebo intake period. The concentration of [<sup>2</sup>H<sub>4</sub>]phenol was too low for further calculations.

### *Effects of the prebiotic lactulose on urinary p-[ring-<sup>2</sup>H<sub>4</sub>]cresol and <sup>15</sup>N excretion*

After intake of the prebiotic, a significant decrease in the percentage dose of p-[ring-<sup>2</sup>H<sub>4</sub>]cresol was found in the 0–24 h urine collection, from 1.77 (SD 1.43) to 0.52 (SD 0.49) ( $P=0.035$ ). Also the decrease in the percentage dose of <sup>15</sup>N in the 6–24 h urine collection was statistically significant; from 36.95 (SD 10.64) to 26.16 (SD 9.74) ( $P=0.046$ ). No significant differences were found in the placebo intake period (Table 2).

### *Differences between pro- or prebiotic and placebo intake (percentage dose of p-[ring-<sup>2</sup>H<sub>4</sub>]cresol and percentage dose of <sup>15</sup>N)*

The decrease in the percentage dose of <sup>15</sup>N excreted in the 24–48 h urine collection after probiotic intake was significantly higher as compared with the placebo ( $P=0.016$ ; Table 3). Similar changes were found for the p-[ring-<sup>2</sup>H<sub>4</sub>]cresol tracer in the 24–48 h urine collection ( $P=0.042$ ; Table 3). The decrease in the percentage dose of p-[ring-<sup>2</sup>H<sub>4</sub>]cresol excreted in the 0–24 h urine collection after prebiotic intake was significantly higher as compared with placebo ( $P=0.005$ ; Table 4). Also a significantly more pronounced decrease in the percentage dose of <sup>15</sup>N excreted in the 6–24 h urine collection after prebiotic intake was found in comparison with the placebo intake period ( $P=0.029$ ; Table 4).

**Table 1.** Influence of the probiotic (*Lactobacillus casei* Shirota cells) v. placebo administration on the percentage dose of p-[ring-<sup>2</sup>H<sub>4</sub>]cresol and percentage dose of <sup>15</sup>N in the urine of ten volunteers\* (Mean values and standard deviations)

	Urine collection (h)	Probiotic					<i>P</i> value†	Placebo				
		Pre		Post		Pre		Post				
		Mean	SD	Mean	SD	Mean		SD	Mean	SD	<i>P</i> value†	
Percentage dose [ <sup>2</sup> H <sub>4</sub> ] cresol	0–24	0.56	0.35	0.65	0.38	NS	0.65	0.31	0.82	0.45	NS	
	24–48	1.88	0.56	1.18	0.75	0.032	1.78	1.18	1.93	1.77	NS	
Percentage dose <sup>15</sup> N	6–24	29.94	10.39	31.50	10.61	NS	31.29	9.57	32.12	10.74	NS	
	24–48	11.57	2.85	8.41	3.39	0.047	9.45	1.38	9.95	2.78	NS	

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's *t* test.

### *Effects of probiotic Lactobacillus casei Shirota cells and prebiotic lactulose on the total urinary p-cresol and nitrogen excretion*

Evaluation of the total urinary p-cresol after probiotic and prebiotic intake showed similar results as found with the labelled biomarkers. Urinary N excretion only demonstrated a similar result after probiotic intake in comparison with the labelled biomarkers (Tables 5 and 6). The total p-cresol and N content decreased significantly in the 24–48 h urine collection after intake of the *L. casei* Shirota cells (p-cresol,  $P=0.007$ ; N,  $P=0.044$ ). After lactulose intake, a significant difference could be demonstrated for the total p-cresol content in the 0–24 h urine collection ( $P=0.024$ ). No decrease could be demonstrated for the total N content. The placebos did not cause significant changes in the urinary excretion of either p-cresol or N.

### *Differences between pro- or prebiotic and placebo intake (total p-cresol and nitrogen)*

No significant differences were found for the total N content when the differences between pro- or prebiotic and placebo intake were analysed statistically (Tables 7 and 8). The decrease in total p-cresol excreted in the 24–48 h urine collection after probiotic intake was found to be significantly higher than the placebo effect ( $P=0.009$ ; Table 7). Similarly, the decrease of total p-cresol excreted in the 0–24 h urine collection after prebiotic intake was significantly higher than the placebo effect ( $P=0.018$ ; Table 8).

## Discussion

The aim of the present study was to evaluate whether the administration of a pro- and/or prebiotic reduces the production of potentially toxic bacterial metabolites in the colon. In order to demonstrate the potential effects of the selected pro- and prebiotic, two biomarkers, i.e. a <sup>15</sup>N and <sup>2</sup>H marker, were used in an adult control group without medical history. The availability of substrates labelled with stable isotopes allowed us to study the effect of a probiotic (*L. casei* Shirota cells) and prebiotic (lactulose) on the fermentation processes by means of a non-invasive tracer technique. The use of [<sup>2</sup>H<sub>4</sub>]tyrosine in proteins as a biomarker mainly reflects protein degradation whereas lactose-[<sup>15</sup>N]ureide reflects the bacterial incorporation of N.

**Table 2.** Influence of the prebiotic (lactulose) v. placebo administration on the percentage dose of  $p$ -[ring- $^2\text{H}_4$ ]cresol and percentage dose of  $^{15}\text{N}$  in the urine of nine volunteers\*

(Mean values and standard deviations)

	Urine collection (h)	Lactulose				$P$ value†	Placebo				$P$ value†
		Pre		Post			Pre		Post		
		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Percentage dose [ $^2\text{H}_4$ ] cresol	0–24	1.77	1.43	0.52	0.49	0.035	0.72	0.41	0.77	0.33	NS
	24–48	2.51	1.99	2.11	1.46	NS	1.89	1.26	1.79	1.29	NS
Percentage dose $^{15}\text{N}$	6–24	36.95	10.64	26.16	9.74	0.046	35.42	14.51	33.75	2.64	NS
	24–48	7.65	2.60	11.29	4.67	NS	11.34	3.10	11.45	4.88	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's  $t$  test.**Table 3.** Differences in the percentage dose of  $p$ -[ $^2\text{H}_4$ ]cresol and percentage dose of  $^{15}\text{N}$  between probiotic (*Lactobacillus casei* Shirota cells) and placebo intake for ten volunteers\*

(Mean values and standard deviations)

	Urine collection (h)	Difference (post – pre probiotic)		Difference (post – pre placebo)		$P$ value†
		Mean	SD	Mean	SD	
		Percentage dose [ $^2\text{H}_4$ ] cresol	0–24	0.10	0.37	
	24–48	–0.70	0.81	0.46	1.34	0.042
Percentage dose $^{15}\text{N}$	6–24	1.56	9.36	0.83	9.73	NS
	24–48	–4.76	6.06	1.13	2.47	0.016

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's  $t$  test.**Table 4.** Differences in the percentage dose of  $p$ -[ $^2\text{H}_4$ ]cresol and percentage dose of  $^{15}\text{N}$  between prebiotic (lactulose) and placebo intake for nine volunteers\*

(Mean values and standard deviations)

	Urine collection (h)	Difference (post – pre probiotic)		Difference (post – pre placebo)		$P$ value†
		Mean	SD	Mean	SD	
		Percentage dose [ $^2\text{H}_4$ ] cresol	0–24	–0.87	0.68	
	24–48	–0.72	0.86	–0.1	0.89	NS
Percentage dose $^{15}\text{N}$	6–24	–13.88	8.81	3.04	9.14	0.029
	24–48	1.56	3.37	0.42	4.22	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's  $t$  test.**Table 5.** Comparison of the total  $p$ -cresol and nitrogen content with probiotic (*Lactobacillus casei* Shirota cells) v. placebo administration for ten volunteers\*

(Mean values and standard deviations)

	Urine collection (h)	Probiotic				$P$ value†	Placebo				$P$ value†
		Pre		Post			Pre		Post		
		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
$p$ -Cresol (mg)	0–24	26.78	10.75	33.43	16.37	NS	21.34	13.43	29.78	11.59	NS
	24–48	47.64	24.67	31.48	21.52	0.007	37.00	19.45	39.29	25.11	NS
N (g)	6–24	6.69	3.38	7.41	3.67	NS	6.98	3.00	7.21	3.76	NS
	24–48	11.09	3.44	8.08	2.46	0.044	10.41	2.99	9.45	3.22	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's  $t$  test.

**Table 6.** Comparison of the total *p*-cresol and nitrogen content with prebiotic (lactulose) *v.* placebo administration for nine volunteers\* (Mean values and standard deviations)

	Urine collection (h)	Prebiotic				<i>P</i> value†	Placebo				<i>P</i> value†
		Pre		Post			Pre		Post		
		Mean	SD	Mean	SD		Mean	SD	Mean	SD	
<i>p</i> -Cresol (mg)	0–24	33.98	22.21	22.76	15.86	0.024	31.12	17.80	39.41	15.53	NS
	24–48	30.84	21.95	39.77	22.09	NS	46.44	30.50	53.06	35.21	NS
N (g)	6–24	9.35	3.59	7.14	2.66	NS	8.69	4.29	6.76	2.82	NS
	24–48	10.21	4.24	11.24	3.52	NS	11.57	5.36	11.59	4.61	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's *t* test.**Table 7.** Differences in total *p*-cresol and nitrogen content between probiotic (*Lactobacillus casei* Shirota cells) and placebo intake for ten volunteers\* (Mean values and standard deviations)

	Urine collection (h)	Difference (post – pre probiotic)		Difference (post – pre placebo)		<i>P</i> value†
		Mean	SD	Mean	SD	
		<i>p</i> -Cresol (mg)	0–24	6.64	13.82	
	24–48	–16.16	13.54	8.28	20.98	0.009
N (g)	6–24	0.71	2.08	0.22	2.23	NS
	24–48	–2.18	3.29	–0.96	2.16	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's *t* test.

The results of the present placebo-controlled cross-over study demonstrated that administration of the prebiotic lactulose resulted in a significant decrease of the urinary concentrations of *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol, total *p*-cresol and <sup>15</sup>N.

Lactulose (β-galactosido-fructose) is not digested in the small intestine and passes unchanged into the large bowel, where it is fermented by the colonic microflora to SCFA, hydrogen and lactate. The production of SCFA results in a decrease of the colonic pH, which reduces the protease activity in the colon, since most colonic proteases have a neutral pH optimum (Schepach, *et al.* 2001). Both acidification and a process of so-called catabolite repression result in an inhibition of the deamination of the amino acids in the colon. In addition, carbohydrate fermentation provides energy to the colonic microflora, stimulating an increased uptake of N into the bacteria for bacterial growth and/or metabolism (Weber 1996, 1997).

The significant reduction in *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol excretion provides direct evidence that also *in vivo*, colonic protein degradation is reduced by the administration of lactulose as a fermentable carbohydrate, resulting in a lower concentration of potentially toxic metabolites. The fact that carbohydrate fermentation mainly occurs in the proximal part of the colon is concordant with the finding that its effect on *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol excretion was observed only in the 0–24 h urine collection. It can be speculated that carbohydrate fermentation in the proximal colon may result in only a delay of normal protein fermentation resulting in an increased protein metabolism upon exhaustion of the carbohydrates in more

distal parts of the colon. However, in the 24–48 h urine collection, there was still a decrease in *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol excretion upon administration of lactulose, although not significantly different from the placebo effect. Exactly the same effect was demonstrated for the total *p*-cresol content.

Based on literature data, it was assumed that the observed effect after administration of lactulose on the urinary <sup>15</sup>N excretion is probably caused by an increased bacterial incorporation of N, resulting in a lower colonic concentration of NH<sub>3</sub> (Vince & Burrige, 1980; Vince *et al.* 1990; Mortensen, 1992; Weber, 1997). Analogously to *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol, the effect of lactulose was observed in the 0–24 h urine collection and not in the 24–48 h urine collection. On the other hand, the urinary excretion of total N was not influenced by the administration of lactulose. This might be explained by the fact that the urinary output of N is determined in the first place by the endogenous N body pool of the host and only in the second place by the fraction of N absorbed from the colon. As a consequence, a decrease in colonic N absorption is not necessarily reflected in the total urinary N output. Earlier studies in patients with low N body pools or low protein intake did show a reduced urinary N excretion after administration of lactulose (Lupton & Marchant, 1989; Weber, 1997). It has been assumed that the increased colonic bacterial metabolism, secondary to the administration of lactulose, resulted in a flux of urea from the blood to the colon and hence a lower urinary excretion of urea. However, the present study was performed in healthy volunteers, who had a normal protein intake and, hence, had a normal N reserve.

**Table 8.** Differences in total *p*-cresol and nitrogen content between prebiotic (lactulose) and placebo intake for nine volunteers\*

(Mean values and standard deviations)

	Urine collection (h)	Difference (post – pre probiotic)		Difference (post – pre placebo)		P value†
		Mean	SD	Mean	SD	
<i>p</i> -Cresol (mg)	0–24	– 11.22	12.14	8.29	19.07	0.018
	24–48	8.93	29.41	6.61	18.93	NS
N (g)	6–24	– 2.21	3.96	– 1.93	5.01	NS
	24–48	1.03	3.23	0.02	3.40	NS

\* For details of procedures, see p. 441.

† Statistical significances were assessed by Student's *t* test.

Upon administration of the probiotic, *L. casei* Shirota cells, a significant decrease in the urinary excretion of *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol, total *p*-cresol and <sup>15</sup>N was also observed. Contrary to the effect of lactulose, the effects of the probiotic were found in the 24–48 h urine collection and not in the 0–24 h collection, suggesting that the effects evolved in the more distal parts of the colon. It is assumed that the decreased excretion of *p*-[ring-<sup>2</sup>H<sub>4</sub>]cresol and total *p*-cresol is caused by an increased uptake of the amino acids tyrosine or metabolic products of protein putrefaction by the increased bacterial activity of the gut. On the other hand, the degradation of the proteins can also be inhibited (inhibition of deamination) when the concentration of SCFA increases in the large intestine (Cummings & Bingham, 1987). Analogously to the effect of lactulose, the influence of *L. casei* Shirota cells on <sup>15</sup>N excretion was assumed to be caused by an increased uptake of N into the bacterial fraction. Also, even though a significant reduction in total N was observed, this effect was not significantly different from the placebo effect.

In the present study, the effects of administration of pro- and prebiotics were evaluated under 'normal' conditions since pro- and prebiotics are often recommended as food supplements for 'normal' individuals in 'normal' circumstances. Therefore, no standard diets were imposed and the volunteers were asked only to keep a regular eating pattern. As a consequence, no data are available on the components reaching the colon during the period of urine collection. However, since the volunteers were not able to discriminate effective treatment from placebo, it was considered unlikely that the observed significant effects were due to casual changes in the diet and, as a consequence, in the components reaching the colon.

The present study describes the influence of pro- and prebiotics on the urinary excretion of labelled substrate metabolites. For further characterisation of the impact of pro- and prebiotics, it might be helpful to study also the kinetics of faecal excretion of the marker and to include also physiological characteristics such as oro-caecal transit time in future evaluations.

In conclusion, the results obtained in the present study have clearly shown that the administration of a pro- or prebiotic can significantly reduce the concentration of potentially toxic metabolites in the colon. Moreover, the biomarkers lactulose-[<sup>15</sup>N]ureide and [<sup>2</sup>H<sub>4</sub>]tyrosine are excellent tools to measure these effects on a quantitative and direct basis.

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