Effects of *Lactobacillus casei* Shirota, *Bifidobacterium breve*, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans

Vicky De Preter, Tom Vanhoutte, Geert Huys, Jean Swings, Luc De Vuyst, Paul Rutgeerts, and Kristin Verbeke

1Department of Gastrointestinal Research, University Hospital Gasthuisberg, Leuven; 2Laboratory of Microbiology; 3Belgian Co-Ordinated Collections of Micro-Organisms/Laboratory of Microbiology Ghent Bacteria Collection, Ghent University, Ghent; and 4Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing, Department of Applied Biological Sciences, Vrije Universiteit, Brussels, Belgium

Submitted 1 February 2006; accepted in final form 17 September 2006

De Preter V, Vanhoutte T, Huys G, Swings J, De Vuyst L, Rutgeerts P, Verbeke K. Effects of *Lactobacillus casei* Shirota, *Bifidobacterium breve*, and oligofructose-enriched inulin on colonic nitrogen-protein metabolism in healthy humans. *Am J Physiol Gastrointest Liver Physiol* 292: G358–G368, 2007. First published September 21, 2006; doi:10.1152/ajpgi.00052.2006.—Pre- and/or probiotics can cause changes in the ecological balance of intestinal microflora and hence influence microbial metabolic activities. In the present study, the influence of oligofructose-enriched inulin (OF-IN), *Lactobacillus casei* Shirota, and *Bifidobacterium breve* Yakult on the colonic fate of NH3 and p-cresol was investigated. A randomized, placebo-controlled, crossover study was performed in 20 healthy volunteers to evaluate the influence of short- and long-term administration of OF-IN, *L. casei* Shirota, *B. breve* Yakult, and the symbiotic *L. casei* Shirota + OF-IN. The lactose[15N,15N]ureide biomarker was used to study the colonic fate of NH3. Urine and fecal samples were analyzed for 15N content by combustion-isotope ratio mass spectrometry and for p-cresol content by gas chromatography–mass spectrometry. RT-PCR was applied to determine the levels of total bifidobacteria. Both short- and long-term administration of OF-IN resulted in significantly decreased urinary p-cresol and 15N content. The reduction of urinary 15N excretion after short-term OF-IN intake was accompanied by a significant increase in the 15N content of the fecal bacterial fraction. However, this effect was not observed after long-term OF-IN intake. In addition, RT-PCR results indicated a significant increase in total fecal bifidobacteria after long-term OF-IN intake. Long-term *L. casei* Shirota and *B. breve* Yakult intake showed a tendency to decrease urinary 15N excretion, whereas a significant decrease was noted in p-cresol excretion. In conclusion, dietary addition of OF-IN, *L. casei* Shirota, and *B. breve* Yakult results in a favorable effect on colonic NH3 and p-cresol metabolism, which, in the case of OF-IN, was accompanied by an increase in total fecal bifidobacteria.

ammonia; p-cresol; bifidobacteria; biomarker

In recent years, several human studies (1, 10, 28, 29, 31) have evaluated the possible health effects related to pre- and/or probiotic administration. The underlying mechanisms responsible for most of the effects of pre- and/or probiotic intervention have been extensively investigated and can be roughly classified as modifications of host immune reactivity (inhibition of NF-κB, modulation of apoptosis, and activation of macrophages) (14, 30) on the one hand and interference with colonic bacterial metabolism, which might result in a modulation of the colonic microbial ecosystem, on the other hand (7, 32). In general, pre- and probiotic administration aims at increasing the saccharolytic activity of colonic microbiota because of the beneficial effects attributed to end products of carbohydrate fermentation (i.e., short-chain fatty acids). Concomitantly, proteolytic fermentation resulting in the formation of potentially toxic metabolites is expected to be reduced.

In previous studies, we developed two biomarkers, i.e., lactose[15N,15N]ureide and p-cresol, used to monitor, in vivo, the degree of proteolytic fermentation by quantification of the formation of potentially toxic metabolites in the colon. As a result, these markers have been proposed as efficient tools to measure the effect of different pre- and probiotic substrates (8, 12). Because of its stable isotope label, lactose[15N,15N]ureide is a safe biomarker that allows us to study the metabolic fate of ammonia in a noninvasive manner. Upon stimulation of bacterial growth and/or activity due to pre- or probiotic administration, it is generally assumed that more ammonia will be assimilated by bacteria, leading to reduced excretion in urine. The use of the p-cresol marker is based on the fact that it is a unique bacterial metabolite of tyrosine that is not formed by human enzymes. It is absorbed from the colon and excreted in urine after sulfate or glucuronide conjugation in the mucosa or liver. As a consequence, a decrease in urinary excretion reflects decreased proteolytic activity in the colon. Using these two biomarkers, we (9) have previously evaluated the efficacy of lactulose and the probiotic yeast *Saccharomyces boulardii* to reduce proteolytic colonic activity.

In the present study, the two biomarkers were used to study the effect of three commercially available pre- and probiotic substrates [i.e., bacterial strains *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult as well as oligofructose-enriched inulin (OF-IN)] on colonic protein metabolism in healthy volunteers. For this purpose, we distinguished the immediate metabolic effects of the substrates in the colon (short-term effects) from the effects induced by a long-term dietary intervention, which are presumably caused by a change in the protein profile (long-term effects).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
relative composition of microbiota. Hence, immediate effects were evaluated when pre- and probiotics were first administered, whereas long-term effects were evaluated after a 4-wk intervention period and in the absence of substrates. In addition, we investigated whether the changes in metabolic activity could be correlated to quantitative changes in the total fecal Bifidobacterium population.

MATERIALS AND METHODS

Subjects

Twenty healthy volunteers (10 women and 10 men, age: 21 ± 1 yr) participated in the study. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from an appendectomy). Subjects did not receive antibiotic treatment or had received any other medical treatment influencing gut transit or intestinal microbiota for at least 3 mo before the start of the study. The Ethics Committee of the University of Leuven (Leuven, Belgium) approved the study, and all subjects gave informed consent.

Experimental Design

The healthy volunteers were randomly assigned to two different treatment groups of a placebo-controlled crossover trial. The study was conducted over a 16-wk period consisting of three ingestion periods of 4 wk, each separated by a 2-wk washout period: a probiotic period (days 1–28), a prebiotic period (days 42–70), and a placebo period (days 84–112). In group 2, an additional symbiotic period (days 126–154) was added to the setup of the study. Each subject received two different substrates twice a day during the ingestion periods (Table 1). Throughout the study, the volunteers consumed their usual diet, taking care that the diet remained as stable as possible over the four periods. In addition, they were advised to avoid intake of fermented milk products and food components containing high quantities of fermentable carbohydrates.

Immediately before the start of the study, at the start of each treatment period (short-term influence) and at the end of each treatment (long-term influence) and washout periods, volunteers consumed a test meal containing lactose\(^{\text{15N}},\text{15N}\)ureide as a biomarker, as shown in Fig. 1. At the start of each dietary intervention period, the pre- and/or probiotic substrate was administered together with the labeled test meal as well as twice per day during the urine and fecal collection to evaluate the influence of the actual presence of the pre- and/or probiotic substrate in the colonic lumen. After each intervention period, no substrate was administered with the labeled test meal as an inert radiolabeled transit marker.

Pre- and Probiotic Substances

OF-IN (Synergy 1, Orafti, Tienen, Belgium) was chosen as the probiotic substrate (17, 26). IN is a mixture of β-(2-1) linear fructans obtained from chicory root with a degree of polymerization (DP) ranging between 2 and 60 (average DP: 12). OF (DP: 2–8, average DP: 4) is obtained by partial enzymatic hydrolysis of IN, whereas a long-chain IN known as HP (DP: 10–60, average DP: 25) can be obtained by combining IN HP and OF. In the present study, a 1:1 (wt/wt) mixture was used.

As probiotic substrates, L. casei Shirota (incorporated in a single-strain fermented milk product) and lyophilized B. breve Yakult (both provided by Yakult Honsha, Tokyo, Japan) were chosen (37). The placebo for the probiotic milk was an identical milk product without the L. casei Shirota strain (provided by Yakult Honsha); otherwise, maltodextrin (AVEBE Food, Foxhol, The Netherlands) was used as the placebo.

Test Meal

The test meal consisted of a pancake (8.4 g proteins, 11.2 g fat, and 26.7 g carbohydrates [243.5 kcal]) that contained 75 mg lactose\(^{\text{15N}},\text{15N}\)ureide. The latter substrate was synthesized according to the method of Schoorl (33) as modified by Hofmann (16) with \[^{15}\text{N},\text{15N}\]urea (obtained from Euroiso-top, St. Aubin Cédez, France). The absence of remaining \[^{15}\text{N},\text{15N}\]urea or lactose was confirmed using thin-layer chromatography (23). Whenever a correction for transit time was required, 185 kBq of \[^{3}\text{H}\]polyethylene glycol ([\(^{3}\text{H}\)]PEG, NEN Life Science Products, Boston, MA) was added to the test meal as an inert radiolabeled transit marker.

Sample Collection

Urine was always collected in dedicated receptacles to which neomycin was added for the prevention of bacterial growth. A basal urine sample was collected before volunteers consumed the test meal. After volunteers had consumed the test meal, a 48-h urine collection was performed in three fractions: 0–6, 6–24, and 24–48 h. After volumes were measured, samples were stored at –20°C until analysis. After volunteers had consumed the test meal, all stools were collected for 72 h by the volunteers. Upon delivery of the fecal samples to the laboratory, a 5-g (wet wt) aliquot was taken for DNA extraction and immediately frozen at –20°C. All stools collected on the same day were combined, weighed, and homogenized before further analysis. Samples of known weight were removed and freeze dried. Aliquots of the dried material were used for the subsequent analysis of total nitrogen, \[^{15}\text{N}\], and \[^{3}\text{H}\]PEG content or for separation into bacterial, fiber, and soluble fractions.

Analytical Procedures

Determination of urinary p-cresol content. The p-cresol content of all urine fractions was measured by gas chromatography (GC)-mass spectrometry (MS) technology. Urine samples with a volume of 950 μL were taken, and the pH of the samples was adjusted to pH 1 with concentrated H₂SO₄ (Merck, Darmstadt, Germany). This solution was heated for 30 min at 90°C to deproteinize and hydrolyze the conjugated phenols. After samples had been cooled down to room temperature, 50 μL of 2,6-dimethylphenol (20 mg/100 mL, Sigma-Aldrich Chemie, Steinheim, Germany) were added as an internal standard. p-Cresol was extracted with 1 mL of ethyl acetate (Merck). The ethyl acetate layer was dried, and 0.5 μL of this solution were analyzed by GC-MS (Trace GC-MS, ThermoFinnigan, San José, CA). The analytical column was a 30-m × 0.32-mm inner diameter, 1-μm AT5-MS (Alltech). Helium GC grade was used as a carrier gas with a constant flow of 1.3 ml/min. The oven was programmed from 75°C (isothermal

Table 1. Ingestion periods of the different groups

<table>
<thead>
<tr>
<th>Ingestion Period</th>
<th>Prebiotic Period</th>
<th>Probiotic Period</th>
<th>Symbiotic Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1</strong></td>
<td>2× 10⁹ B. breve Yakult + 2× 10 g placebo OF-IN</td>
<td>2× Placebo B. breve Yakult + 2× 10 g placebo OF-IN</td>
<td>2× Placebo B. breve Yakult + 2× 10 g placebo OF-IN</td>
</tr>
<tr>
<td><strong>Group 2</strong></td>
<td>2× 6.5 × 10⁹ L. casei Shirota + 2× 10 g placebo OF-IN</td>
<td>2× Placebo L. casei Shirota + 2× 10 g placebo OF-IN</td>
<td>2× Placebo L. casei Shirota + 2× 10 g placebo OF-IN</td>
</tr>
</tbody>
</table>

Ingestion periods were each for 4 wk; n = 10 volunteers/group. OF-IN, oligofructose-enriched inulin.
for 5 min) with 10°C/min to 160°C and to 280°C with 20°C/min. MS
detection (single quadrupole) was performed in EI full-scan mode
from m/z 59 to 590 at 2 scans/s. Results were expressed as total
p-cresol content (in mg) excreted in urine.

Determination of total nitrogen content and 15N enrichment in
urine, feces, and bacterial pellets. Total nitrogen content and 15N
enrichment were determined by a continuous-flow elemental analyzer
isotope ratio mass spectrometer (ANCA-2020, Europa Scientific,
Crewe, UK). Therefore, known amounts of urine (15 μL, adsorbed on
chromosorb (Elemental Microanalysis, Devon, UK)), feces (freeze
dried, 5–7 mg), or bacterial pellets (freeze dried, 5–7 mg) were
introduced in the oxidation-reduction module coupled to the isotope
ratio mass spectrometer. In this module, the samples were oxidized
using copper oxide, oxygen, and chromium oxide to nitrous oxides
[nitrite-nitrate (NOx)] at 1,000°C and subsequently reduced to nitro-
gen gas (N2) using copper at 600°C. This gas was lead to the ion
source of the mass spectrometer, where the total nitrogen content and
15N enrichment were measured. The 15N-to-14N isotope ratio of N2
was measured with reference to a calibrated laboratory standard (i.e.,
a standard ammonium sulphate solution). Results for urinary and fecal
samples were expressed as total nitrogen content and as percentages of
the administered dose of 15N (8). Total nitrogen and 15N excretion
in the bacterial fraction were expressed, respectively, as milligrams
per gram of bacteria and as nanograms per milligram of bacteria.

Determination of fecal [3H]PEG. The [3H]PEG content in fecal
samples was measured with liquid scintillation counting (model 3375,
Packard Tricarb Liquid Scintillation Spectrometer, Packard Instru-
m ents, Downers Grove, IL) after oxidation to [3H]H2O (model 306,
Packard Sample Oxidizer, Packard Instruments). 3H contents in fecal
samples were expressed as percentages of the administered dose
recovered over 72 h and were used to correct for gastrointestinal
transit by dividing the cumulative percentage of the administered dose
of 15N recovered over 72 h by the cumulative percentage of the
administered dose of 3H recovered over 72 h.

Separation of dried fecal samples into bacteria, fiber, and solu-
ble fractions. The separation of freeze-dried fecal samples was per-
formed according to the method described by Stephen and Cummings
(38). An aliquot of 500 mg freeze-dried fecal material was taken and
thoroughly mixed for 10 min with 30 ml formylsaline (1% formol in
water-soluble compounds, was discarded, whereas the sediment,
containing bacteria, was dissolved in 2 ml sterile pyrogen-free water.
After being weighed, the sediment was freeze dried, and the dried
material was weighed again.

DNA extraction from fecal specimens. Total bacterial DNA was
extracted from fecal samples using a modified version of the method
of Pitcher and co-workers (25) as previously described (40).

Real-time PCR analysis. Quantification of the fecal Bifidobacte-
rrium population was performed with the LightCycler system I (Roche,
Mannheim, Germany) using a FastStart DNA Master SYBR Green I
kit and a Bifidobacterium genus-specific PCR primer set (22). Data
were expressed as log10 bifidobacteria per gram wet weight.

Statistical Analysis

Results are expressed as medians plus interquartile ranges (IQR).
Statistical analysis was performed with SPSS software (SPSS 12.0 for
Windows, SPSS, Chicago, IL). Given the low numbers of subjects in
the treatment groups, nonparametric statistical analysis was used
regardless of the distribution of results (Friedman ANOVA, Kruskal-
Wallis test, Wilcoxon test, and Mann-Whitney test with the Bonferroni
correction). The level for statistical significance was set at P < 0.05.

RESULTS

One female subject of group 2 withdrew from the study
during the first ingestion period due to antibiotic intake. Data
from this subject were excluded from further analysis. All
other subjects completed the study.

Total Nitrogen and 15N excretion

The results of the urinary and fecal excretion of total
nitrogen and 15N as well as different fecal parameters in the
different test situations are shown in Figs. 2–9.

Urinary excretion. The cumulative nitrogen excretion was
similar throughout the study in each group and varied from 15 to
20 g/48 h. In group 1, short-term intake of B. breve Yakult did not
influence the cumulative percentages of 15N recovered in the 0–
48-h urine collection, whereas a tendency to decreased 15N re-
covery was noted upon long-term administration of B. breve
Yakult cells. On the other hand, the urinary 15N excretion was
significantly lower after both short-term (P = 0.005) and long-
term (P = 0.017) administration of OF-IN compared with base-
line values and compared with placebo (P = 0.005 for short-term
OF-IN intake and P = 0.022 for long-term intake). Also, in group
2, OF-IN administration significantly decreased urinary 15N con-
tent (P = 0.008 for short-term intake of OF-IN and P = 0.011 for
long-term administration of OF-IN) compared with the baseline
value and compared with placebo (P = 0.008 for both short and
long term). For L. casei Shirota cells, only short-term administra-
tion resulted in a significant effect compared with the baseline
condition (P = 0.038), although the urinary 15N excretion after
the administration of L. casei Shirota cells was not significantly
different from that after placebo intake. The symbiotic combina-
tion of L. casei Shirota with OF-IN resulted in a significant
reduction of the cumulative excretion of the label compared with
baseline (P = 0.008 for short- and long-term symbiotic adminis-
tration). Also, compared with placebo intake, the urinary 15N excretion
was significantly lower after symbiotic intake (P = 0.011 for short-term symbiotic administration and P = 0.008 for
long-term symbiotic administration, respectively). No changes in
urinary 15N content were observed after placebo treatment, and,
after each 2-wk washout period, urinary $^{15}$N values had returned to baseline values. No significant differences in effect between the synbiotic and prebiotic were found.

**Fecal excretion. Stool weight, fecal dry weight, pH, and total $^{15}$N excretion.** The results obtained in the different test situations are shown in Table 2. After probiotic or placebo treatment, no significant effect was found on the total (combined urinary and fecal) excretion of $^{15}$N. After the short-term administration of OF-IN, the decrease in urinary $^{15}$N excretion was compensated by an increase in the fecal excretion of the marker, resulting in a similar total excretion of the label. However, the long-term administration of OF-IN and short- and long-term administration of the synbiotic combination resulted in significantly decreased total $^{15}$N excretion, suggesting that a higher fraction of $^{15}$N was retained within the human body.

**Total nitrogen and $^{15}$N enrichment in bacterial pellets.** The results of total nitrogen content and labeled nitrogen of bacterial pellets after pre- and/or probiotic intake in both groups are shown in Fig. 10. No influence of the different substrates on total nitrogen in the bacterial fraction was found in either of the groups. The mean nitrogen content varied from 20 to 30 mg/g bacterial solids. In both groups, a significant increase in $^{15}$N content in the bacterial fraction was found after short-term OF-IN intake compared with baseline [from 2.58 (IQR: 1.33–7.44) to 6.10 ng/mg bacterial solids (IQR: 1.89–9.53), $P = 0.047$, in group 1 and from 2.79 (IQR: 1.32–6.32) at baseline...
to 8.90 ng/mg (IQR: 7.50–16.28), \( P = 0.008 \), in group 2].

Also, short-term intake of the synbiotic combination of OF-IN and \( L. \) casei Shirota cells resulted in a significant increase [2.79 (IQR: 1.32–6.32) vs. 12.14 ng/mg (IQR: 5.34–18.86), \( P = 0.008 \)]. On the contrary, no significant effect on \( ^{15}N \) content in the bacterial fraction was seen after long-term intake of OF-IN or synbiotic, correlating with the fact that no rise in the cumulative fecal excretion of \( ^{15}N \) was observed. Neither probiotic nor placebo intake resulted in a significant change of \( ^{15}N \) content in the bacterial fraction.

**Urinary p-Cresol Excretion**

The results of the effects of pre- and/or probiotic administration on urinary excretion of \( p \)-cresol are shown in Table 3. In group 1, short- and long-term intake of \( B. \) breve Yakult resulted in a significant decrease of \( p \)-cresol excretion compared with baseline (\( P = 0.028 \) and \( P = 0.005 \), respectively). In group 2, the long-term administration of \( L. \) casei Shirota decreased \( p \)-cresol excretion significantly (\( P = 0.038 \)), whereas a tendency toward a lower \( p \)-cresol excretion was observed after the short-term administration of the substrate. After the short-term OF-IN administration, the urinary excretion of \( p \)-cresol was significantly reduced compared with baseline in both groups (\( P = 0.013 \) and \( P = 0.025 \) for groups 1 and 2, respectively). The long-term OF-IN administration resulted in significantly reduced \( p \)-cresol excretion in group 2 (\( P = 0.025 \)), whereas in group 1 no significant effect was observed. However, the combination of the results of both groups after...
OF-IN administration resulted in a statistically significant reduction of p-cresol excretion compared with the combined baseline results ($P = 0.001$ and $P = 0.005$ for short- and long-term intake, respectively). The long-term synbiotic administration in group 2 resulted in a significant reduction of p-cresol excretion compared with baseline ($P = 0.021$). In both groups, placebo intake did not change p-cresol levels in urine.

Quantification of Fecal Bifidobacteria Levels Using Real-Time PCR.

Total fecal bifidobacteria were significantly increased after long-term OF-IN intake [from 7.54 (IQR: 6.70–8.03) log$_{10}$ bifidobacteria/g wet wt under baseline conditions to 8.19 (IQR: 7.97–9.08) log$_{10}$ bifidobacteria/g wet wt, $P = 0.006$]. In group 2, the long-term administration of the synbiotic resulted in a tendency to increased *Bifidobacterium* levels [7.88 (IQR: 6.81–9.10) log$_{10}$ bifidobacteria/g wet wt under baseline conditions vs. 8.76 (IQR: 7.92–10.04) log$_{10}$ bifidobacteria/g wet wt, $P$ = not significant].

**DISCUSSION**

The extent of urinary and/or fecal excretion of ammonia and p-cresol may give an indication of the degree of proteolytic colonic fermentation, which is considered to be relevant to colonic health (32). Both ammonia and p-cresol are considered important in the disease course of uremic syndrome (5) and
have been proposed as putative markers relevant to colon cancer risk (4, 24). Several studies have investigated the ability of different types of dietary intervention to decrease the proteolytic activity of colonic microbiota, thereby reducing the generation and accumulation of these potentially toxic metabolites and to simultaneously increase saccharolytic activity (26).

The results of the present study demonstrated that the extent of the production of the biomarkers of proteolytic activity in the colon was reduced after the administration of the selected prebiotic (i.e., OF-IN) and, to a lesser extent, after the administration of bacterial probiotic substrates (i.e., B. breve Yakult or L. casei Shirota), whereas placebo intake did not result in any significant effects. Similar effects have previously been observed after lactulose administration (9). Remarkably, the decrease in urinary $^{15}$N excretion observed after the long-term administration of OF-IN or the symbiotic combination of OF-IN and L. casei Shirota was not correlated with a corresponding increase in fecal $^{15}$N excretion, resulting in an increased body retention of $^{15}$N. Previously, it has been shown that after the administration of lactulose, the decrease in urinary $^{15}$N excretion was compensated by an increased fecal

![Fig. 8. Effect of ST and LT Pre and/or Pro administration on total fecal nitrogen excretion in groups 1 and 2. Values are medians + IQR.](image1)

![Fig. 9. Effect of ST and LT Pre and/or Pro administration on fecal $^{15}$N excretion corrected for transit in groups 1 and 2. Values are medians + IQR. *$P < 0.05$ compared with Bas. Corr, corrected values.](image2)
$^{15}$N excretion, resulting in a similar body retention of $^{15}$N (9). Possibly, the observed retention in the colon may be linked to the fact that OF-IN does not only have a stimulating effect on the microbiota in the lumen of the colon but also promotes mucosa-associated microbiota. Recently, Langlands et al. (20) demonstrated both in vivo and in vitro that supplementation of a mixture of OF and IN significantly increased mucosal bifidobacteria. Also, in the study by Kleessen et al. (19), a significant increase in mucosal bifidobacteria was observed after rats harboring human fecal flora had been fed an OF-IN-supplemented diet.

Evaluation of the rate of urinary and/or fecal excretion of $^{15}$N and $p$-cresol after a 2-wk washout period demonstrated that the modification in the metabolic activity of colonic microbiota induced by dietary intervention was only temporary and readily disappeared once administration of substrate had ceased. These observation are in line with previous studies (6, 13, 37) in which 1–2 wk after the end of the intervention period, the induced changes of the composition of microbiota (i.e., an increase of bifidobacteria), accompanied with higher saccharolytic activity, disappeared and baseline values were restored. As a consequence, it was suggested that a continuous stimulation of microbiota through pre- and/or probiotic administration might be necessary to maintain the beneficial effects.

Further evaluation of the results demonstrated that the effects of prebiotic intake on colonic ammonia metabolism were more pronounced than those caused by probiotic intake both in short and long term. The clear effects caused by the short-term administration of the prebiotic can be explained by the rapid fermentation of the carbohydrate in the colon, resulting in a lower colonic pH (33). The short-term administration of probiotics does not immediately influence the colonic environment and metabolic activity. In addition, a moderate but significant correlation was found between the effect of short-term OF-IN intake on urinary $p$-cresol and $^{15}$N content (Fig. 11; Pearson correlation coefficient = 0.574 and $P = 0.010$). Although the colonic processes measured with both markers are different (fate of ammonia and protein degradation, respectively) and not related to each other, these results suggest that the influence of short-term OF-IN administration is similar on both processes. After long-term administration, this correlation was less pronounced (Fig. 12; Pearson correlation coefficient = 0.432 and $P = 0.071$).

Conversely, the impact of both probiotics was clearly more pronounced on $p$-cresol excretion than on the fate of ammonia. Contrary to prebiotics, probiotic intervention does not provide energy to colonic microbiota. As a consequence, it was not surprising that less-pronounced effects on $^{15}$N excretion were observed since this biomarker mainly reflects the stimulation of bacterial activity and/or growth. It is assumed that the decreased excretion of $p$-cresol is caused by an increased uptake of the amino acid tyrosine or metabolic products of protein putrefaction by a higher colonic availability of probiotic cells or by reduced protein fermentation due to inhibition of proteolytic bacteria by probiotic cells.

It was anticipated that the effects caused by symbiotic administration would exceed those of the separate compounds.

### Table 2. Effect of pre- and/or probiotic administration on total $^{15}$N excretion

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>59.53 (53.71–72.53)</td>
<td>59.72 (46.02–74.85)</td>
</tr>
<tr>
<td>Probiotic ST</td>
<td>58.58 (49.36–64.32)</td>
<td>57.56 (45.98–61.70)</td>
</tr>
<tr>
<td></td>
<td>60.06 (56.18–61.01)</td>
<td>55.45 (38.99–70.16)</td>
</tr>
<tr>
<td>WO 1</td>
<td>59.83 (42.76–66.24)</td>
<td>56.80 (49.39–64.46)</td>
</tr>
<tr>
<td>Prebiotic ST</td>
<td>56.46 (30.52–62.93)</td>
<td>50.43 (44.93–59.74)</td>
</tr>
<tr>
<td></td>
<td>39.89 (24.57–55.22)*</td>
<td>42.34 (33.89–55.63)</td>
</tr>
<tr>
<td>WO 2</td>
<td>56.45 (47.45–59.14)</td>
<td>57.41 (50.11–66.44)</td>
</tr>
<tr>
<td>Placebo ST</td>
<td>58.03 (53.99–62.91)</td>
<td>53.79 (45.80–65.05)</td>
</tr>
<tr>
<td></td>
<td>53.60 (45.64–62.46)</td>
<td>58.88 (49.74–62.45)</td>
</tr>
<tr>
<td>WO 3</td>
<td>65.39 (52.26–69.77)</td>
<td></td>
</tr>
<tr>
<td>Symbiotic ST</td>
<td>37.86 (22.08–47.84)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.91 (23.52–48.47)*</td>
<td></td>
</tr>
</tbody>
</table>

Values are shown as medians (in %) with interquartile ranges in parentheses; $n = 10$ volunteers in group 1 and 9 volunteers in group 2. ST, short term; LT, long term; WO 1–3, washout periods 1–3. *Significantly decreased total $^{15}$N excretion (by Friedman ANOVA, $P < 0.05$).
However, in our study, we could not observe a significant additive value of the synbiotic compared with the prebiotic, although a clear tendency was observed.

In the present study, the effects of the substrates were evaluated under “normal” conditions (i.e., in healthy people without imposing standard diets) since pre- and probiotics are often recommended as food supplements for healthy individuals in normal circumstances. A relatively homogenous group of young volunteers was selected to minimize interindividual variability. Since it is known that the diversity of colonic microbiota decreases with aging, the impact of pre- and probiotics on colonic metabolism in middle-aged and elderly people might be different and should be investigated in future studies.

Furthermore, a reduction of proteolytic activity in the colon may not only benefit healthy individuals but could also play an important role in several pathological conditions. For instance, it has been demonstrated that as many as 50% of patients with irritable bowel syndrome (IBS) have abnormal colonic fermentation (18). The developed biomarkers could be used to determine whether \(^{15}\text{N}\) and \(\text{p-cresol}\) excretion in these patients differ from those of healthy volunteers and, second, to investigate whether pre- or probiotic administration could induce comparable effects as in healthy individuals.

There are indications that colonic microbiota can play a role in the pathogenesis of inflammatory bowel disease (IBD). Several studies have shown that inflammation is more abundant in those parts of the gastrointestinal tract where the highest numbers of bacteria are present (36). Furthermore, differences in the composition of colonic microbiota of patients with IBD compared with healthy volunteers have been demonstrated, which might lead to differences in the overall fermentation pattern (15, 21, 35). A higher degree of protein fermentation has the potential to cause damage to epithelial cells, resulting in higher inflammation levels. As a consequence, a modification of intestinal microbiota and changes in metabolic activity may have favorable effects on this syndrome (11).

Besides gastrointestinal conditions, a role for colonic microbiota was also described in the pathogenesis of chronic renal failure. Chronic renal failure is characterized by a progressive retention of a number of microbial metabolic end products (i.e., phenols, indoles, and polyamines) (39), which cannot be eliminated anymore due to kidney failure and can hardly be removed using dialysis techniques because of their high protein binding. In addition, Bammens et al. (2) demonstrated that the small intestinal assimilation (digestion and/or absorption) of...
proteins is impaired in the case of renal failure, resulting in an increased availability of proteins for fermentation in the colon. As a consequence, besides reduced renal excretion, renal failure is also characterized by increased production of proteolytic fermentation metabolites and, thus, an accumulation of p-cresol. Furthermore, it has recently been demonstrated that lower p-cresol levels are correlated with lower mortality in uremic syndrome (3). For this reason, strategies that contribute to a lower generation of protein fermentation metabolites might constitute a significant improvement in the management of those patients.

In the present study, the use of two biomarkers demonstrated that OF-IN, L. casei Shirota, and B. breve Yakult exert favorable effects on colonic protein and ammonia metabolism in healthy volunteers. In the case of OF-IN, this effect was accompanied by an increase in total bifidobacteria levels in feces. Further exploitation of 15N excretion and p-cresol as biomarkers would particularly be useful during clinical trials involving specific patient groups, e.g., patients diagnosed with IBS and IBD.

ACKNOWLEDGMENTS
The authors acknowledge T. Coopmans and E. De Brandt for excellent technical assistance and B. Pot for intellectual input.

GRANTS
This work was supported by Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen, Brussels, Belgium (Gene
risch BasisOnderzoek aan de Universiteiten Project No. 010054: “Development of a fast, non-invasive technological tool to investigate the functionality and effectiveness of pro- and prebiotics in normal healthy volunteers: the use of a labeled biomarker”), the Fund for Scientific Research-Flanders, the University Research Councils, and several companies. G. Huys is a postdoctoral fellow of the Fund for Scientific Research-Flanders.

REFERENCES
22. Morrison DJ, Dodson B, Preston T, Weaver LT. Rapid quality control analysis of 13C-enriched substrate synthesis by isotope ratio mass spec-
25. Roberfroid MB, Borven F, Bouley C, Cummings JH. Colonic micro-