EFFECTS OF LACTOBACILLUS CASEI SHIROTA, BIFIDOBACTERIUM BREVE, AND OLIGOFRUCTOSE-ENRICHED INULIN ON THE COLONIC NITROGEN-PROTEIN METABOLISM IN HEALTHY HUMANS

Pre- and probiotics on colonic metabolism

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ABSTRACT

Background: Pre- and/or probiotics can cause changes in the ecological balance of intestinal microbiota and hence influence the microbial metabolic activities. In the present study, the influence of oligofructose-enriched inulin (OF-IN), *Lactobacillus casei* Shirotta (LacS) and *Bifidobacterium breve* Yakult on the colonic fate of NH₃ and p-cresol was investigated.

Methods: A randomized, placebo-controlled, cross-over study was performed in 20 healthy volunteers to evaluate the influence of short- and long-term administration of OF-IN, LacS, *B. breve* Yakult and the synbiotic LacS + OF-IN. The lactose-[¹⁵N, ¹⁵N]-ureide biomarker was used to study the colonic fate of NH₃. Urine and faeces samples were analysed for ¹⁵N-content by combustion-IRMS and for p-cresol content by GC-MS. RT-PCR was applied to determine the levels of total bifidobacteria.

Results: Both short- and long-term administration of OF-IN resulted in a significantly decreased urinary p-cresol and ¹⁵N-content. The reduction of the urinary ¹⁵N-excretion after short-term OF-IN intake was accompanied by a significant increase in the ¹⁵N-content of the faecal 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 faecal bifidobacteria after long-term OF-IN intake. Long-term LacS and *B. breve* Yakult intake showed a tendency to decrease the urinary ¹⁵N-excretion, whereas a significant decrease was noted on p-cresol excretion.

Conclusion: Dietary addition of OF-IN, *L. casei* Shirotta and *B. breve* Yakult results in a favourable effect on the colonic NH₃ and p-cresol metabolism, which in the case of OF-IN, was accompanied by an increase in total faecal bifidobacteria.

Key words: ammonia, p-cresol, bifidobacteria, oligofructose-enriched inulin, probiotics, biomarker
INTRODUCTION

In recent years, several human studies have evaluated the possible health effects related to pre- and/or probiotic administration (1, 10, 28, 29, 31). 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 modification of the host immune reactivity (inhibition of NFκB, modulation of apoptosis, activation of macrophages) (14, 30) on the one hand and interference with the 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 the 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 have developed two biomarkers, i.e. lactose-[\textsuperscript{15}N, \textsuperscript{15}N]-ureide and p-cresol, used to monitor, \textit{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-[\textsuperscript{15}N, \textsuperscript{15}N]-ureide is a safe biomarker which allows to study the metabolic fate of ammonia in a non-invasive 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 a 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 which is not formed by human enzymes. It is absorbed from the colon and excreted in urine after sulphate- or glucuronide-conjugation in the mucosa or liver. As a consequence, a decrease in urinary excretion reflects a decreased proteolytic activity in the colon. Using
these two biomarkers, we have previously evaluated the efficacy of lactulose and the probiotic yeast *Saccharomyces boulardii* to reduce the proteolytic colonic activity (9).

In the present study, the two biomarkers were used to study the effect of three commercially available pre- and probiotic substrates (i.e. the bacterial strains *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult, and oligofructose-enriched inulin (OF-IN)) on the colonic protein metabolism in healthy volunteers. For this purpose, we have 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 relative composition of the microbiota. Hence, the immediate effects were evaluated when pre- and probiotics were first administered whereas the long-term effects were evaluated after a 4-weeks intervention period and in the absence of the substrates. In addition, it was investigated whether the changes in metabolic activity could be correlated to quantitative changes in the total faecal *Bifidobacterium* population.
MATERIALS AND METHODS

Subjects

Twenty healthy volunteers (10 women, 10 men; aged 21 ± 1 y) participated in the study. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from appendectomy). The subjects did not receive antibiotic treatment or any other medical treatment influencing gut transit or intestinal microbiota for at least three months before the start of the study. The Ethics Committee of the University of 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 cross-over trial. The study was conducted over a 16-wk period consisting of three ingestion periods of 4 weeks, each separated by a 2-weeks wash-out period: probiotic period (d1-d28), prebiotic period (d42-d70) and placebo period (d84-d112). In group 2, an additional synbiotic period (d126-154) was added to the set up 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 wash-out period, the volunteers consumed a test meal containing lactose-[\(^{15}\text{N}, \^{15}\text{N}\)]-ureide as biomarker, as represented in Figure 1. At the start of each dietary intervention period, the pre- and/or probiotic substrate was administered together with the labelled test meal, as well as twice per
day during the urine and faecal 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 labelled test meal nor during the urine and faecal
collection in order to exclude effects caused by the actual presence of the substrates.

Pre-and probiotic substrates

Oligofructose-enriched inulin (Synergy®, Orafti, Tienen, Belgium) was chosen as prebiotic
substrate (17, 26). Inulin is a mixture of β (2-1) linear fructans obtained from chicory root
with a degree of polymerisation (DP) ranging between 2 and 60 (average DP of 12).
Oligofructose (DP 2 to 8; DP_{av} = 4) is obtained by partial enzymatic hydrolysis of inulin
whereas a long-chain inulin known as inulin HP (DP 10 to 60; DP_{av} = 25) can be produced by
applying specific physical separation techniques. OF-IN is obtained by combining inulin HP
and oligofructose. In the present study, a 1:1 (w/w) mixture has been used.

As probiotic substrates, *Lactobacillus casei* Shirota, incorporated in a single strain fermented
milk product and lyophilized *Bifidobacterium breve* Yakult (both provided by Yakult®,
Yakult Honsha Co. Ltd, Tokyo, Japan) were chosen (37). The placebo for the probiotic milk
was an identical milk product without the *Lactobacillus casei* Shirota strain (provided by
Yakult®), otherwise maltodextrin (AVEBE B.A. Food, Foxhol, The Netherlands) was used as
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)), which contained 75 mg lactose-[\^{15}N, ^{15}N]-ureide. The latter substrate was
synthesized according to the method of Schoorl (33) as modified by Hofmann (16) with [\^{15}N,
^{15}N]-urea obtained from Euriso-top, St. Aubin, Cédex, France. Absence of remaining [\^{15}N,
\[^{15}\text{N}\]-urea or lactose was confirmed using thin layer chromatography (23). Whenever correction for transit time was required, 185 kBq of \([^{3}\text{H}]\)-polyethylene glycol (\([^{3}\text{H}]\)-PEG) (NEN Life Science Products Inc., Boston MA, USA) was added to the test meal as an inert radiolabelled transit marker.

**Sample collection**

Urine was always collected in dedicated receptacles to which neomycin was added for prevention of bacterial growth. A basal urine sample was collected before consumption of the test meal. After intake of the test meal, a 48-h urine collection was performed in 3 fractions: 0-6 h, 6-24 h and 24-48 h urine collections. After measurement of the volume, samples were stored at \(-20^\circ\text{C}\) until analysis.

After each test meal, all stools were collected for 72 h by the volunteers. Upon delivery of the faecal samples at the lab, a 5-g (wet weight) aliquot was taken for DNA extraction and immediately frozen at \(-20^\circ\text{C}\). 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 subsequent analysis of total nitrogen and \(^{15}\text{N}\), and \(^{3}\text{H}\)-PEG content or for separation into bacterial, fibre and soluble fractions.

**Analytical procedures**

*Determination of urinary p-cresol content*

The p-cresol content of all urine fractions was measured by gas chromatography–mass spectrometry technology. Urine samples with a volume of 950 \(\mu\text{l}\) were taken and the pH of the samples 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 deproteinize and hydrolyse the conjugated phenols. After cooling down to room temperature, 50 \(\mu\text{l}\) of 2,6 dimethylphenol (20
mg/100 ml) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added as internal standard. p-Cresol was extracted with 1 ml of ethyl acetate (Merck KgaA). The ethyl acetate layer was dried and 0.5 µl of this solution was analysed on a GC-MS (Trace GC-MS, ThermoFinnigan, San José, CA, USA). The analytical column was a 30 m x 0.32 mm i.d., 1 µm AT5-MS (Alltech Associates, Deerfield, USA). Helium GC grade was used as carrier gas with a constant flow of 1.3 ml/min. The oven was programmed from 75°C (isothermal for 5 min), with 10°C/min to 160°C and to 280°C with 20°C/min. Mass spectrometric detection (single quadrupole) was performed in E.I. full scan mode from m/z 59 to m/z 590 at 2 scans/sec. The results were expressed as total p-cresol content (mg) excreted in urine.

**Determination of total nitrogen content and ¹⁵N-enrichment in urine, faeces and bacterial pellets**

Total N content and ¹⁵N 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 µl, adsorbed on chromosorb (Elemental Microanalysis Limited, Devon, UK), faeces (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 oxidised using copper oxide, oxygen and chromium oxide to nitrous oxides (NO₃) at 1000 °C and subsequently reduced to nitrogen 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 the 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). The results for the urinary and faecal samples were expressed as total nitrogen content and as percentage of the administered dose of ¹⁵N (8). Total nitrogen and
$^{15}$N-excretion in the bacterial fraction were expressed respectively in mg/g bacteria and in ng/mg bacteria.

**Determination of faecal [$^3$H]-PEG**

The [$^3$H]-PEG content in faecal samples was measured with liquid-scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer, model 3375, Packard Instruments Inc., Downers Grove, IL, USA) after oxidation to [$^3$H]-H$_2$O (Packard Sample Oxidiser, model 306, Packard Instruments Inc., Downers Grove, IL, USA). The tritium contents in the faecal samples were expressed as percentage of administered dose recovered over 72 h and were used to correct for gastrointestinal transit by dividing the cumulative percentage of administered dose of $^{15}$N recovered over 72 h by the cumulative percentage of administered dose of $^3$H recovered over 72 h.

**Separation of the dried faecal samples into bacteria, fibre and soluble fractions**

The separation of the freeze-dried faecal samples was performed according to the method described by Stephen and Cummings (38). An aliquot of 500 mg freeze-dried faecal material was taken and thoroughly mixed for 10 min with 30 ml formylsaline (1% formol in 0.9% saline) and 0.30 ml of 10% sodium lauryl sulphate (SLS). The mixture was filtered through a 20-µm filter (Varian Inc., Palo Alto, CA, USA) under vacuum and the residue was washed with 30 ml of formylsaline, shaken and filtered again. This procedure was repeated three times. The fraction on top of the filter was the fibre fraction. The filtrate was ultracentrifuged at 25,000 g during 36 min. The supernatant, containing the water-soluble compounds, was discarded, while the sediment, containing the bacteria, was dissolved in 2 ml sterile pyrogen-free water. After weighing, the sediment was freeze-dried and the dried material was weighed again.
DNA extraction from faecal specimens

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

230 Real-time PCR analysis

Quantification of the faecal Bifidobacterium population was performed with the LightCycler system I (Roche, Mannheim, Germany) using the FastStart DNA Master SYBR Green I kit and a Bifidobacterium genus-specific PCR primer set (22). Data were expressed as log_{10} bifidobacteria/g wet weight.

235 Statistical analysis

Results are expressed as median plus interquartile range. The statistical analysis was performed with SPSS software (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL, USA). Given the low number of subjects in the treatment groups, non-parametric statistical analysis was used regardless of the distribution of results (Friedman analysis of variance (ANOVA), Kruskal-Wallis test, Wilcoxon test and Mann-Whitney test with 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.

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Total nitrogen and $^{15}$N-excretion

The results of the urinary and faecal excretion of total nitrogen and $^{15}$N, as well as different faecal parameters, in the different test situations are shown in Figures 2-9.

260 Urinary excretion

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

**Faecal excretion**

Stool weight, faecal dry weight, pH and total nitrogen excretion were not influenced by the diet intervention throughout the study in either of the groups. In both groups, a significantly higher 72 h-cumulative excretion of $^{15}$N corrected for transit was observed after short-term administration of OF-IN alone compared to baseline values ($p=0.004$ and $p=0.002$ for group 1 and 2, respectively) or in combination with the probiotic *L. casei* Shirota ($p=0.047$), whereas a tendency to higher $^{15}$N-excretion was observed after long-term intake of OF-IN. Intake of *L. casei* Shirota alone, *B. breve* Yakult or placebo did not change the 72 h cumulative excretion of $^{15}$N.

**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 faecal) excretion of $^{15}$N. After short-term administration of OF-IN, the decrease in urinary $^{15}$N-excretion was compensated by an increase in the faecal excretion of the marker resulting in a similar total excretion of the label. However, long-term administration of OF-IN and short-term and long-term administration of the synbiotic combination resulted in a 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 labelled nitrogen of bacterial pellets after pre- and/or probiotic intake in both groups are shown in Figure 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 to baseline: from 2.58 ng/mg (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 ng/mg (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 ng/mg (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 faecal excretion of $^{15}$N was observed. Neither probiotic or placebo intake resulted in a significant change of the $^{15}$N content in the bacterial fraction.

Urinary p-cresol excretion

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

Quantification of faecal bifidobacteria levels using real-time PCR

Total faecal bifidobacteria were significantly increased after long-term OF-IN intake: from 7.54 (IQR 6.70-8.03) log_{10} bifidobacteria/g wet weight at baseline conditions to 8.19 (IQR 7.97-9.08) (p=0.006). In group 2, long-term administration of the synbiotic resulted in a tendency to increased *Bifidobacterium* levels, 7.88 (IQR 6.81-9.10) at baseline conditions vs. 8.76 (IQR 7.92-10.04), p=NS).
DISCUSSION

The extent of urinary and/or faecal 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 the 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 the colonic microbiota, thereby reducing the generation and accumulation of these potentially toxic metabolites and to simultaneously increase the saccharolytic activity (26).

The results of the present study have 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. oligofructose-enriched inulin) and to a lesser extent after administration of bacterial probiotic substrates (i.e. Bifidobacterium breve Yakult or Lactobacillus casei Shirota), whereas placebo intake did not result in any significant effects. Similar effects had previously been observed after lactulose administration (9). Remarkably, the decrease in urinary $^{15}$N-excretion observed after long-term administration of OF-IN or the synbiotic combination of OF-IN and Lactobacillus casei Shirota was not correlated with a corresponding increase in faecal $^{15}$N-excretion, resulting in an increased body retention of $^{15}$N. Previously it has been shown that after administration of lactulose, the decrease in urinary $^{15}$N-excretion was compensated by an increased faecal $^{15}$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 the mucosa-associated microbiota. Recently, Langlands et al. demonstrated both in vivo and in vitro that supplementation of a mixture of oligofructose and inulin significantly increased the mucosal bifidobacteria (20). Also in the study of Kleessen et
a significant increase in the mucosal bifidobacteria was observed after feeding an OF-IN-supplemented diet to rats harbouring a human faecal flora (19).

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

Further evaluation of the results demonstrated that the effects of prebiotic intake on the colonic ammonia metabolism were more pronounced than those caused by probiotic intake both on short- and long-term. The clear effects caused by 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). 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 the urinary p-cresol and $^{15}$N content (Figure 11, Pearson correlation coefficient = 0.574 and p = 0.010). Although the colonic processes measured with both markers are different (fate of ammonia, respectively protein degradation) 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 (Figure 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 the colonic microbiota. As a consequence, it is not surprising that less pronounced effects on $^{15}$N-excretion were observed since this biomarker mainly reflects 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 a reduced protein fermentation due to an inhibition of proteolytic bacteria by the probiotic cells.

It was anticipated that the effects caused by synbiotic administration would exceed those of the separate compounds. However, in our study we could not observe a significant additive value of a synbiotic as compared to 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 has been selected in order to minimise interindividual variability. Since it is known that the diversity of the colonic microbiota decreases with ageing, the impact of pre- and probiotics on the colonic metabolism in middle-aged and elderly people might be different and should be investigated in future studies.

Furthermore, a reduction of the 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 described that as many as 50% of patients with irritable bowel syndrome have abnormal colonic fermentation (18). The developed biomarkers could be used to determine whether the $^{15}$N- and p-cresol excretion in these patients differs from that of healthy volunteers and secondly to investigate whether pre- or probiotic administration induced comparable effects as in healthy individuals.
There are indications that the 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 number of bacteria are present (36). Furthermore, differences in the composition of the colonic microbiota of patients with IBD compared to 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 the epithelial cells resulting in higher inflammation levels. As a consequence, a modification of the intestinal microbiota and changes in the metabolic activity may have a favourable effect on this syndrome (11).

Besides gastrointestinal conditions, also a role for the colonic microbiota has been described in the pathogenesis of chronic renal failure. Chronic renal failure is characterised by a progressive retention of a number of microbial metabolic end products (i.e. phenols, indols, 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., demonstrated that the small intestinal assimilation (digestion and/or absorption) of proteins is impaired in case of renal failure, resulting in an increased availability of proteins for fermentation in the colon (2). As a consequence, besides a reduced renal excretion, renal failure is also characterised by an 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 to a 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, *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult exert a favourable effect on the 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 the faeces. A further exploitation of $^{15}$N-excretion and p-cresol as biomarkers would particularly be useful during clinical trials involving specific patient groups e.g. diagnosed with IBS and IBD.
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References


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<td>+ 2x 10g placebo OF-IN</td>
<td>+ 2x 10g OF-IN</td>
<td>+ 2x 10g placebo OF-IN</td>
<td></td>
</tr>
<tr>
<td>Group 2 (n=10)</td>
<td>2x 6.5 $10^9$ <em>L. casei</em> Shirota</td>
<td>2x placebo <em>L. casei</em> Shirota</td>
<td>2x placebo <em>L. casei</em> Shirota</td>
<td>2x 6.5 $10^9$ <em>L. casei</em> Shirota</td>
</tr>
<tr>
<td></td>
<td>+ 2x 10g placebo OF-IN</td>
<td>+ 2x 10g OF-IN</td>
<td>+ 2x 10g placebo OF-IN</td>
<td>+ 2x 10g OF-IN</td>
</tr>
</tbody>
</table>
Table 2: Effect of pre- and/or probiotic administration on the total excretion of \(^{15}\text{N}\) in the 2 different treatment groups (median and IQR; Friedman ANOVA, \(\alpha=0.05\))

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=10)</th>
<th>Group 2 (n=9)</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>Probiotic (LT)</td>
<td>60.06 (56.18-61.01)</td>
<td>55.45 (38.99-70.16)</td>
</tr>
<tr>
<td>Wash-out 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>Prebiotic (LT)</td>
<td>39.89 (24.57-55.22)(^*)</td>
<td>42.34 (33.89-55.63)</td>
</tr>
<tr>
<td>Wash-out 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>Placebo (LT)</td>
<td>53.60 (45.64-62.46)</td>
<td>58.88 (49.74-62.45)</td>
</tr>
<tr>
<td>Wash-out 3</td>
<td></td>
<td>65.39 (52.26-69.77)</td>
</tr>
<tr>
<td>Synbiotic (ST)</td>
<td></td>
<td>37.86 (22.08-47.84)(^*)</td>
</tr>
<tr>
<td>Synbiotic (LT)</td>
<td></td>
<td>28.91 (23.52-48.47)(^*)</td>
</tr>
</tbody>
</table>

\(^*\) Significantly decreased total \(^{15}\text{N}\)-excretion (Friedman ANOVA; \(p<0.05\))
### Table 3: Effect of pre- and/or probiotic administration on the urinary excretion of p-cresol (mg/24h)

*(median and IQR; Friedman ANOVA, α=0.05)*

<table>
<thead>
<tr>
<th>Group 1 (n=10)</th>
<th></th>
<th>Group 2 (n=9)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>p-cresol</td>
<td>p-cresol</td>
<td>Test</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Baseline</td>
<td>21.17 (18.20-34.74)</td>
<td>24.43 (15.28-39.72)</td>
<td>Baseline</td>
</tr>
<tr>
<td>B. breve (ST)</td>
<td>17.03 (13.63-22.70)*</td>
<td>20.06 (13.09-28.26)</td>
<td>L. casei S (ST)</td>
</tr>
<tr>
<td>B. breve (LT)</td>
<td>16.66 (12.69-22.72)*</td>
<td>20.54 (12.40-35.35)*</td>
<td>L. casei S (LT)</td>
</tr>
<tr>
<td>Wash-out 1</td>
<td>22.08 (12.61-35.65)</td>
<td>28.60 (24.57-38.85)</td>
<td>Wash-out 1</td>
</tr>
<tr>
<td>OF-IN (ST)</td>
<td>15.71 (7.71-19.67)*</td>
<td>14.70 (7.07-31.34)*</td>
<td>OF-IN (ST)</td>
</tr>
<tr>
<td>OF-IN (LT)</td>
<td>21.32 (6.00-27.28)</td>
<td>13.41 (9.12-29.62)*</td>
<td>OF-IN (LT)</td>
</tr>
<tr>
<td>Placebo (ST)</td>
<td>23.24 (19.90-32.87)</td>
<td>23.59 (17.89-35.63)</td>
<td>Placebo (ST)</td>
</tr>
<tr>
<td>Placebo (LT)</td>
<td>21.97 (13.74-40.15)</td>
<td>25.84 (18.22-35.08)</td>
<td>Placebo (LT)</td>
</tr>
<tr>
<td></td>
<td>19.09 (14.34-42.40)</td>
<td></td>
<td>Wash-out 3</td>
</tr>
<tr>
<td></td>
<td>17.76 (12.00-29.95)</td>
<td>L. casei S + OF-IN (ST)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.79 (4.56-27.03)*</td>
<td>L. casei S + OF-IN (LT)</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly decreased p-cresol excretion (Friedman ANOVA; p<0.05)
Figure legends

**Figure 1:** Schematic representation of the study design: the study was conducted over a 16-wk period consisting of three ingestion periods of 4 weeks each separated by a 2-weeks wash-out period ( ). Immediately before the start of the study (BAS), at the start of each treatment period (short-term (ST) effect) and at the end of each treatment (long-term (LT) effect) and wash-out (WO) period a test was performed and each time urine (48-h) and faeces (72-h) were collected ( ). In group 2, an additional synbiotic period was added to the study.

**Figure 2:** Effect of short-term (ST) and long-term (LT) pre- and/or probiotic administration on total urinary N excretion in group 1 and 2 (values are medians + IQR)

**Figure 3:** Effect of short-term (ST) and long-term (LT) pre- and/or probiotic administration on the urinary $^{15}$N excretion in group 1 and 2 (values are medians + IQR).
* p<0.05 compared to baseline

**Figure 4:** Effect of pre- and/or probiotic administration on total faecal weight in group 1 and 2 (values are medians + IQR)

**Figure 5:** Effect of pre- and/or probiotic administration on the percentage dry weight in group 1 and 2 (values are medians + IQR)

**Figure 6:** Influence of pre- and/or probiotic intervention on the pH in group 1 and 2 (values are medians + IQR)
Figure 7: Influence of pre- and/or probiotic intervention on total faecal transit in group 1 and 2 (values are medians + IQR)

Figure 8: Effect of short-term (ST) and long-term (LT) pre- and/or probiotic administration on total faecal nitrogen excretion in group 1 and 2 (values are medians + IQR)

Figure 9: Effect of short-term (ST) and long-term (LT) pre- and/or probiotic administration on the faecal $^{15}$N excretion corrected for transit in group 1 and 2. (values are medians + IQR)
* p<0.05 compared to baseline

Figure 10: Effect of pre- and/or probiotic administration on the excretion of total nitrogen and $^{15}$N in the bacterial mass (BM) in group 1 (A) and 2 (B) (values are medians + IQR)

Figure 11: Difference in % dose $^{15}$N (48h) post – pre short-term OF-IN treatment vs. difference in mg p-cresol/24h post – pre short-term OF-IN treatment in urine ($r =$ Pearson correlation coefficient)

Figure 12: Difference in % dose $^{15}$N (48h) post – pre long-term OF-IN treatment vs. difference in mg p-cresol/24h post – pre long-term OF-IN treatment in urine ($r =$ Pearson correlation coefficient)
Figure 1

-3 0 28 42 70 84 112 126 154

Probiotic  Prebiotic  Placebo  Synbiotic

BAS ST LT WO ST LT WO ST LT WO ST LT
Figure 2

Total N excretion (g) in 0-48h urine collection

- Bas
- ST Pro
- LT Pro
- WO 1
- ST Pre
- LT Pre
- WO 2
- ST Pla
- LT Pla
- WO 3
- ST Syn
- LT Syn
Figure 8

Group 1 □ Group 2

Total faecal N excretion (g)
Figure 9

Corr faecal 15N excretion

- Group 1
- Group 2

* * *

*
Figure 10

A

\[ \Delta \text{15N in BM} \quad \bullet \text{Total N in BM} \]
B

15N in BM  Total N in BM

ng/mg $^{15}$N bacterial solids

mg/g N bacterial solids

Bas  ST Pro  LT Pro  ST Pre  LT Pre  ST Pla  LT Pla  ST Syn  LT Syn
Figure 11

$r=0.574$
$p=0.010$
Figure 12

$r=0.432$
$p=0.071$