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ORIGINAL CONTRIBUTION

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Does the biomarker ^{15}N-lactose ureide allow to estimate the site of fermentation of resistant starch?

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Abstract We evaluated the effect of resistant starch (RS) and resistant starch with wheat bran (RS+WB) on the colonic ammonia metabolism in healthy volunteers using the biomarker ^{15}N-lactose ureide (^{15}N-LU). Particularly, it was investigated whether this biomarker allowed to estimate differences in the site of fermentation. Ten volunteers were included in a placebo-controlled crossover study. They consumed in random order 2 × 15 g RS/day for 2 weeks and placebo for 2 weeks separated by 2 weeks washout. At baseline, on the first day of each intake period and after each intake period, they consumed a ^{15}N-labelled test meal and collected all urine in different fractions for 48 h. In ten other volunteers, the effect of 2 × 15 g RS/day and of 2 × 15 g RS + 2 × 6 g WB was compared. These volunteers collected urine and feces for 72 h. ^{15}N-content of urine and feces was measured using combustion-isotope ratio mass spectrometry. RS exerted a significant decrease in cumulative urinary ^{15}N-excretion which was different from placebo. The effect was most pronounced in the 6–24 h urine fraction which suggest fermentation in the proximal colon. The effect of RS+WB on cumulative urinary ^{15}N-excretion was not significantly different from the effect of RS. A less pronounced decrease in the 6–24 h fraction was observed suggesting less fermentation in the proximal colon whereas no indications for more distal fermentation were observed. Since about 80% of the cumulative urinary ^{15}N was recovered within 24 h, it was concluded that the biomarker ^{15}N-LU was useful to monitor processes in the proximal colon rather than in the distal colon.

Key words stable isotope – prebiotic – wheat bran – resistant starch – site of fermentation

Introduction

Nitrogen-15 labelled lactose ureide (^{15}N-LU) has been introduced by Jackson et al. to evaluate the colonic handling of ammonia [15]. Recently, this molecule has been further investigated as a non-invasive biomarker to evaluate the efficacy of pre- and probiotics in modulating the colonic bacterial metabolism [5, 11].

The molecule ^{15}N-LU can be considered as a vehicle to bring a known amount of ^{15}N-ammonia in the colon. The ureide bond in lactose ureide resists enzymatic cleavage by intestinal enzymes during

passage through the small bowel but is hydrolysed upon arrival in the colon by colonic bacteria [21]. Consequently, the resulting [^{15}N, ^{15}N]-urea is rapidly hydrolysed by bacterial urease activity yielding ^{15}N-ammonia. ^{15}N-ammonia can be taken up by the bacterial microbiota for their own metabolism and growth after which the ^{15}N-label is faecally excreted. Alternatively, it can be absorbed through the colonic epithelium and, after transportation to the liver where it is converted to ^{15}N-labelled urea, urinary excreted. Stimulation of colonic bacterial metabolism is expected to result in higher uptake of ^{15}N-ammonia by the bacteria and a lower absorption through the colonic mucosa and hence, to cause a shift from urinary to faecal excretion of ^{15}N [11].

An important factor that stimulates bacterial activity is the availability of fermentable carbohydrates. Fermentation of carbohydrates results in the production of short chain fatty acids and in this way, in a reduction of the colonic pH. A lower pH results in a lower proteolytic activity since the pH optimum of most proteases is neutral to alkaline. The availability of carbohydrates is most prominent in the proximal colon and decreases upon progression through the large intestine, resulting in a higher pH of the distal colonic environment. As a consequence, the processes of protein degradation and amino acid fermentation are mainly situated in the distal colon. Whereas carbohydrate fermentation is generally accepted to be beneficial to the host, protein fermentation is not because of the generation of potentially toxic compounds such as ammonia. These marked regional differences in metabolic processes occurring in the colon might be related to some degree with the observed regional differences in disease activity. For instance, 60% of the large bowel cancers lie within the rectum, sigmoid and descending colon [3] and might be related to the fermentation of proteins in that region.

Therefore, it might be desirable to shift the fermentation of carbohydrates to more distal parts of the colon. Govers et al. have achieved a more distal fermentation of resistant starch (RS) in pigs by addition of wheat bran (WB) to the diet [12]. It was postulated that wheat bran increased the transit of the digesta through the colon by bulking and increased water binding capacity [12].

In the present study, we evaluated in vivo the influence of RS on the colonic ammonia metabolism in healthy volunteers using ^{15}N-LU and compared it to the influence of RS combined with WB. Particular attention was paid as to whether ^{15}N-LU could detect a shift in the site of fermentation between RS and RS with WB. It was hypothesised that the kinetics of urinary ^{15}N-excretion would reflect the site of fermentation and that more distal fermentation would affect ^{15}N-excretion in later urine fractions as compared to proximal fermentation.

We have distinguished between short and long term effects of RS administration. The short term effect comprises the effect of the actual fermentation of RS alone or in combination with WB on the ^{15}N-excretion. Evaluation of the long term effect was meant to investigate whether expected changes in composition of the microbiota were accompanied with changes in metabolic activity. These tests were performed after a 2-weeks intake period of RS (+WB) but in the absence of the carbohydrate in order to exclude the effect of its actual fermentation.

Materials and methods

Subjects

Two series of ten healthy volunteers participated in the study. In series 1, four male and six female volunteers with a mean age of 24 ± 1 years were included whereas series 2 consisted of four males and six females with a mean age of 26 ± 8 years. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (except 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 Ethics Committee of the University of Leuven approved the study and all subjects gave informed consent.

Substrates

Lactose-[^{15}N,^{15}N]-ureide was synthesized according to the method of Schoorl [22] as modified by Hofmann [14] with [^{15}N, ^{15}N]-urea obtained from Euriso-top (Saint-Aubain, Cédex, France). Absence of remaining [^{15}N, ^{15}N]-urea or lactose was confirmed using thin layer chromatography [18].

Resistant starch was a type-3 resistant starch (Actistar) obtained from Cerestar (Vilvoorde, Belgium). It has been prepared from Tapioca starch and has a low water binding capacity and low viscosity. The product has been shown to contain 54% of type 3 RS that reaches the colon for fermentation [2]. Previous results have shown that this type of RS does not influence proximal gastrointestinal parameters such as gastric emptying and protein digestion [8].

Coarse wheat bran was obtained from AVEVE (Leuven, Belgium).

Maltodextrin (AVEBE B.A. Food, Foxhol, The Netherlands) was used as placebo.

Study design

Study 1

The study was a single blind placebo controlled cross-over study of 6 weeks consisting of 3 consecutive periods of 2 weeks: intake of RS (2 × 15 g/day), wash-out and intake of placebo (2 × 15 g maltodextrin/day) or vice versa. The substrates were dissolved in a glass of water and consumed together with the breakfast and evening meals. The volunteers of series 1 were randomly assigned to one of the treatment orders. Four days before the start of the study, on the first day of each intake period and on the day after the end of each intake period, the volunteers perfomed a test. The study design is schematically presented in Fig. 1. On the day of the test, the volunteers came to the laboratory after an overnight fast and received a standard pancake test meal [8.4 g proteins, 11.2 g fat and 26.7 g carbohydrates (243.5 kcal)] containing 75 mg of ^{15}N-LU. Before consumption of the test meal, a basal urine sample was collected and from that time on, all urine was collected for 48 h in different fractions (0–6, 6–24, 24–48 h). Dedicated plastic receptacles to which neomycine was added for prevention of bacterial growth were used for all urine collections. When performing test T2 and T4, the volunteers consumed the substrate (RS or placebo) together with the test meal whereas no substrate was administered with the test meal in test T1, T3 and T5.

Study 2

The study was an open label cross-over intervention study of 6 weeks consisting of 3 consecutive periods of 2 weeks: intake of RS (2 × 15 g/day), wash-out and intake of RS (2 × 15 g/day) with WB (2 × 6 g/day) or vice versa. The study design was similar to that of Study 1. Substrates were always consumed together with breakfast and evening meals. The volunteers of series 2 were randomly assigned to one of the treatment orders. Four days before the start of the study, on the first day of each 2-weeks period and on the day after the end of each intake period, the volunteers came to the laboratory after an overnight fast and received a standard pancake test meal containing 75 mg of ^{15}N-LU and 185 kBq of ^{3}H-labelled PEG. Before consumption of the test meal, a basal urine sample was collected and from that time on, all urine was collected for 72 h in different fractions (0–6, 6–24, 24–48, 48–72 h). In addition, all stools were collected for 72 h.

Dietary intake

The volunteers were asked to avoid consumption of pre- and probiotics during the study period and to maintain a regular eating pattern. However, no standard diets were imposed.

Analytical procedures

Determination of total nitrogen and ^{15}N-enrichment in urine and faeces

Total nitrogen content and ^{15}N enrichment were determined by a continuous flow elemental analyser isotope ratio mass spectrometer (ANCA-2020; Europa Scientific, Crewe, UK) as previously described [5]. Results were expressed in percentage of administered dose (^{15}N).

Determination of ^{3}H-PEG recovery

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 $^{3}H–H_2O$ (Packard Sample Oxidiser, model 307, Packard Instruments Inc.). The cumulative

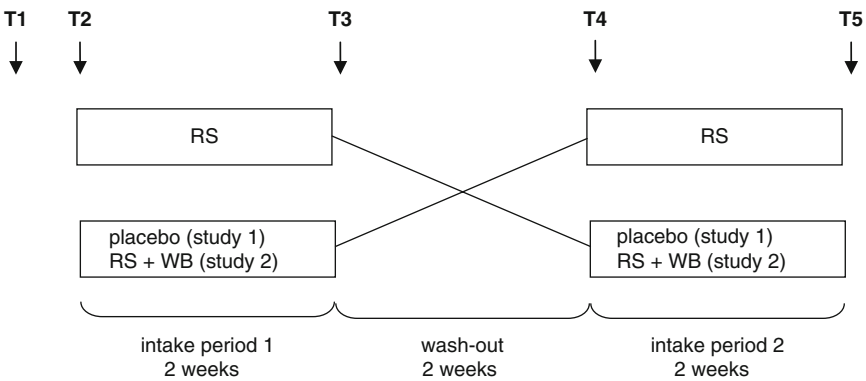

Fig. 1 Schematic presentation of the study design of study 1 (RS versus placebo) and study 2 (RS versus RS+WB). The *arrows* indicating T1–T5 represent the tests consisting of the consumption of a test meal and the collection of urine for 48 h (study 1) or the collection of urine and stools for 72 h (study 2)

Table 1 Influence of short term and long term administration of RS on urinary ^{15}N-excretion

	Urinary ^{15}N-excretion (percentage of administered dose)		
	Baseline	Short term RS intake	Long term RS intake
0–6 h	8.36 ± 3.54	10.14 ± 5.90	8.53 ± 5.30
6–24 h	33.41 ± 12.49	20.37 ± 10.52*	22.24 ± 10.32*
24–48 h	9.99 ± 4.87	7.85 ± 3.69	8.31 ± 3.23
0–48 h	50.93 ± 14.41	38.36 ± 10.92*	38.65 ± 12.04*

Values are means ± SD, $n = 20$
* Significantly different from baseline ($P < 0.05$)

Table 2 Influence of short and long term administration of RS and placebo on cumulative urinary ^{15}N-excretion (0–48 h) in subgroup 1

	Cumulative urinary ^{15}N-excretion (0–48 h) (percentage of administered dose)
Baseline	47.64 ± 18.66
Short term RS intake	40.30 ± 12.93
Long term RS intake	38.32 ± 13.84
Short term placebo intake	48.49 ± 10.72
Long term placebo intake	43.69 ± 12.52

Values are means ± SD, $n = 10$.

tritium recovery in stool over 72 h was expressed as percentage of administered dose and was used to correct the data of ^{15}N recovery in stools for gastrointestinal transit by dividing the cumulative percentage of administered dose of ^{15}N recovered over 72 h by the cumulative percentage of the administered dose of ^{3}H recovered over 72 h.

Statistical analysis

Results are expressed as mean values and standard deviations. Non-parametric statistical evaluation [Friedman analysis of variance (ANOVA), Wilcoxon test and Mann–Withney test] was performed using SPSS software (SPSS 14.0 for Windows; SPSS Inc., Chicago, IL, USA). The level for statistical significance was set at $P < 0.05$.

Results

Effect of resistant starch on urinary ^{15}N-excretion

To evaluate the influence (short term and long term) of RS on the colonic ammonia metabolism, the data of both groups were pooled and statistical analysis was performed on 20 volunteers.

From the results presented in Table 1, it is clear that administration of RS to healthy volunteers results in a significant decrease of the cumulative urinary ^{15}N-excretion over 48 h. This effect was observed upon the first administration of resistant starch ($P = 0.012$) as well as after a 2-weeks administration period ($P = 0.003$). The effect on the cumulative ^{15}N-excretion was found to be mainly due to a significant decrease in the 6–24 h fraction ($P = 0.006$ and $P = 0.009$ for short term and long term administration of RS, respectively).

In a subgroup of subjects, the effect of RS was compared to the effect of placebo. In Table 2, the mean cumulative urinary ^{15}N-excretion (0–48 h) for each of the five tests is shown whereas Table 3 presents the differences in %dose ^{15}N-excretion between the test at the start of RS or placebo intake and baseline (short-term effects) and between the test at the end of RS or placebo intake and baseline (long-term effects). A positive figure indicates that the ^{15}N-excretion was increased by the intervention whereas a negative figure points at a decrease in urinary ^{15}N-excretion. In this subgroup, the cumulative ^{15}N-excretion at the start of the RS intake period and after the RS intake period was not statistically significantly different from that at baseline. However, the decrease in the cumulative urinary ^{15}N-excretion after the first administration of resistant starch intake was significantly higher than the placebo effect ($P = 0.032$), whereas the effect observed after a 2-weeks intervention period was not statistically significantly different from the placebo effect ($P = 0.255$).

In the other subgroup of ten subjects, the effect of resistant starch as such was compared to the effect of

Table 3 Change in urinary ^{15}N-excretion (percentage of administered dose) induced by short term and long term administration of RS and placebo

	Short term effect (test at start of intake period—baseline)		Long term effect (test at end of intake period—baseline)	
	RS (2 × 15 g/day)	Placebo	RS (2 × 15 g/day)	Placebo
0–6 h	+1.42 ± 3.67	−0.37 ± 7.42	+0.74 ± 5.62	+1.72 ± 9.06
6–24 h	−6.05 ± 14.72	+2.42 ± 14.42	−8.47 ± 16.68	−2.84 ± 16.12
24–48 h	−2.71 ± 5.12	−1.20 ± 4.92	−1.58 ± 4.94	−2.82 ± 7.17
0–48 h	−7.34 ± 17.18	+0.86 ± 14.56*	−9.31 ± 15.21	−3.94 ± 14.50

Values are means ± SD, $n = 10$. Positive figures indicate increased ^{15}N-excretion whereas negative figures point at a decreased urinary ^{15}N-excretion by the dietary intervention
* Short term placebo effect is significantly different from RS effect ($P < 0.05$)

Table 4 Influence of short and long term administration of RS and RS+WB on cumulative urinary ^{15}N-excretion (0–72 h) in subgroup 2

	Cumulative urinary ^{15}N-excretion (0–72 h) (percentage of administered dose)
Baseline	57.02 ± 8.93[a]
Short term RS intake	38.35 ± 9.39[b]
Long term RS intake	42.00 ± 10.78[b]
Short term RS+WB intake	40.14 ± 10.75[b]
Long term RS+WB intake	50.44 ± 9.08[a]

Values are means ± SD, $n = 10$
[a, b]values with a different superscript letter are significantly different (Anova, $P < 0.05$)

RS combined with WB. At the start of both interventions and after the intervention with RS, the cumulative (0–72 h) ^{15}N-excretion was significantly lower as compared to baseline (Table 4). In Table 5, the effect of RS is compared to the effect of RS combined with WB. Although no statistically significant difference was obtained in effect caused by both treatments on the cumulative ^{15}N-excretion (0–72 h) ($P = 0.065$ and 0.070 for short and long term effects, respectively), the effect of RS was statistically significantly higher than that of RS+WB ($P = 0.017$) in the 6–24 h urine fraction. However, no clear time difference between RS and RS+WB could be observed, i.e. both treatments evoked the most pronounced effect in the 6–24 h urine fraction.

Effect of resistant starch and resistant starch + wheat bran on faecal parameters

The volunteers participating in the second study collected all stools for 72 h after consumption of the test meal. Short or long term administration of RS or RS+WB did not affect total faecal weight, faecal dry weight or transit as can be seen from Table 6.

The ^{15}N-excretion in faeces (corrected for transit) increased after short or long term administration of RS and to a lesser extent after administration of RS+WB. However, these effects were not statistically significant.

Discussion

The physiological effects of RS in the colon have mainly been attributed to the generation of short-chain fatty acids. Especially butyric acid, which acts as an energy source for colonocytes, is considered as an important factor in the maintenance of colonic health [23]. It has been shown that fermentation of RS produces more butyrate than does fermentation of non starch polysaccharides such as arabinogalactan, xylan and pectin [9].

The fermentation rate for resistant starches as well as for other fermentable carbohydrates such as pectin, guar gum and oat bran is relatively rapid and

Table 5 Change in urinary ^{15}N-excretion (percentage of administered dose) induced by short term and long term administration of RS and RS+WB

	Short term effect (test at start of intake period—baseline)		Long term effect (test at end of intake period—baseline)	
	RS (2 × 15 g/day)	RS (2 × 15 g/day) + WB (2 × 6 g/day)	RS (2 × 15 g/day)	RS (2 × 15g/day) + WB (2 × 6g/day)
0–6 h	3.82 ± 6.01	+0.05 ± 5.97	+0.43 ± 6.82	+3.49 ± 7.56
6–24 h	−20.02 ± 10.15	−13.45 ± 12.00*	−13.88 ± 9.62	−11.72 ± 7.77
24–48 h	−1.59 ± 6.23	−2.27 ± 7.97	−1.79 ± 5.68	+0.73 ± 8.76
48–72 h	−0.88 ± 1.80	−1.22 ± 2.34	+0.22 ± 1.68	+0.92 ± 1.73
0–72 h	−18.67 ± 11.82	−16.88 ± 12.84	−15.02 ± 8.88	−6.58 ± 13.44

Values are means ± SD, $n = 10$. Positive figures indicate increased ^{15}N-excretion whereas negative figures point at a decreased urinary ^{15}N-excretion by the dietary intervention
* Effect of RS+WB is significantly different from effect of RS ($P < 0.05$)

Table 6 Influence of short and long term intake of RS and RS+WB on faecal parameters (study 2; $n = 10$)

	Baseline	Short term intake		Long term intake	
		RS	RS+WB	RS	RS+WB
Faecal weight (g/day)	173.2 ± 50.8	162.2 ± 29.5	170.8 ± 36.7	176.0 ± 37.2	152.0 ± 40.2
Faecal dry weight (%)	24.2 ± 9.6	22.1 ± 6.7	24.5 ± 9.0	26.4 ± 7.6	19.5 ± 7.8
Transit (recovery of ^{3}H in percentage)	61.35 ± 22.30	71.00 ± 13.87	64.47 ± 22.50	49.28 ± 26.05	48.00 ± 28.87
^{15}N-excretion (percentage adm dose/^{3}H)	21.09 ± 7.99	30.32 ± 11.55	25.28 ± 8.64	27.34 ± 12.61	24.48 ± 9.17

Values are means ± SD, $n = 10$

fermentation mainly occurs in the proximal colon. Since SCFA are rapidly absorbed, fermentation of RS does not significantly contribute butyrate to the distal colon. However, the presence of butyrate in the distal colon may be highly desirable in view of the reduction of colon cancer risk or the treatment of ulcerative colitis. Several studies in rats or in humans attempted to shift the fermentation of RS to more distal parts by addition of dietary fibres such as psyllium [17], chitosan [25] or WB [12, 13, 20]. Most of these studies used an increased concentration of SCFA in faeces as an indication of more distal fermentation. We have investigated whether ^{15}N-LU, a biomarker to assess the colonic ammonia metabolism, could serve as a non-invasive and elegant alternative allowing to estimate the site of fermentation from measurements in urine.

Previous studies had already shown a decreased urinary ^{15}N-excretion upon administration of lactulose or oligofructose-enriched inulin [6, 7, 10]. In the first part of the present study, a similar decrease in urinary ^{15}N-excretion, significantly different from placebo, was observed in the presence of RS. After 2 weeks intake of RS, the colonic ammonia metabolism was assessed again in the absence of the substrate. In this way we were able to investigate whether changes in microbiota composition were associated with changes in microbial activity. It had previously been shown that administration of the same type of RS at a dose of 10 g/day for 7 days significantly increased the faecal bificobacteria counts [1]. Although the urinary ^{15}N-excretion after 2 weeks RS intake was still significantly different from the ^{15}N-excretion at baseline, this decrease was not different from the placebo effect. As a consequence, it must be concluded that changes in microbial composition have limited effects on microbial activity and that mainly the actual presence of the fermentable carbohydrate in the colon, which provides an energy source to the microbiota thereby expanding bacterial mass, is important.

In the second part of the study, the effect of RS as such was compared to the effect of RS+WB. In this study protocol, urinary collections were extended in view of the expected effect in later urine fractions and faecal collections with transit measurements were added. As expected, the observed decrease in urinary ^{15}N-excretion was accompanied with an increase in faecal ^{15}N-excretion, suggesting indeed a stimulation of the bacterial activity through administration of RS. In addition, the effect of RS on the cumulative urinary ^{15}N-excretion (0–72 h) was similar to that of RS+WB.

When looking at the kinetics of the urinary ^{15}N-excretion, it was found that the major part of ^{15}N was excreted in the 6–24 h urine fraction and that the effect of RS and RS+WB on the cumulative ^{15}N-excretion was mainly due to an effect on the 6–24 h excretion for both treatments. Effects in the 6–24 h urine fraction most likely reflect effects occurring in the proximal part of the colon since residence times in the right colon have been estimated at approximately 11.3 ± 1.1 h [16]. It was hypothesized that a shift of the fermentation of RS to more distal parts of the colon by addition of wheat bran to the test meal would be reflected in a decreased ^{15}N-excretion in a later urine fraction (24–48 or 48–72 h). Although a significantly smaller effect in the 6–24 h fraction was observed after administration of RS+WB suggesting less proximal fermentation, no decrease of ^{15}N-excretion in the 24–48 or 48–72 h fraction was found. Hence, no indications for more distal fermentation of RS+WB were found. This observation can not be attributed to an acceleration of colonic transit caused by the addition of WB since transit times were not influenced. Our results as well as others indicate that RS tends to delay rather than to hasten intestinal transit [4, 19]. Although addition of WB to the test meal was expected to increase transit due to faecal bulking and its water-holding capacity [24], no increase in faecal weight nor in transit was observed. A possible explanation might be the relatively low dose of WB although the same dose was used by Muir et al. [20]. However, it is important to note that, contrary to the experiments performed by Muir, the tests to determine the long term effects were performed after the last day of intake of the substrates. This means that no RS (+WB) was consumed during the days of stool collection which might contribute to explain the discrepancy between our results and those by Muir.

These results thus only suggest that RS in the presence of WB is less fermented in the proximal part of the colon as compared to RS as such but do not allow to conclude that RS+WB is fermented more distally. However, almost 80% of the cumulative 72 h ^{15}N-excretion appears in urine within 24 h which indicates that the metabolism (hydrolysis and absorption) of the biomarker ^{15}N-LU proceeds quite rapidly. Therefore, it is possible that the labelled NH_3 itself does not reach the distal colon and hence, that this biomarker only allows to evaluate the processes occurring in the proximal colon. Perhaps, fractionation of the 6–24 h urine fraction into smaller fractions would reveal subtle differences in urinary ^{15}N-excretion pattern.

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