

# A Probiotic Mixture Including Galactooligosaccharides Decreases Fecal $\beta$ -Glucosidase Activity but Does Not Affect Serum Enterolactone Concentration in Men during a Two-Week Intervention<sup>1–3</sup>

Riina A. Kekkonen,<sup>4,5</sup> Reetta Holma,<sup>5,7\*</sup> Katja Hatakka,<sup>4</sup> Tarja Suomalainen,<sup>8</sup> Tuija Poussa,<sup>9</sup> Herman Adlercreutz,<sup>6</sup> and Riitta Korpela<sup>5</sup>

<sup>4</sup>Valio Research Centre, FIN-00039 Helsinki, Finland; <sup>5</sup>Institute of Biomedicine, <sup>6</sup>Folkhälsan Research Center, and Division of Clinical Chemistry, Biomedicum, FIN-00014 University of Helsinki, Helsinki, Finland; <sup>7</sup>Navidia Ltd, Kallioliinantie 7, FIN-00140 Helsinki, Finland; <sup>8</sup>Helsinki University of Technology, Department of Biotechnology and Chemical Technology, FIN-02015 TKK, Finland; and <sup>9</sup>STAT-Consulting, Vahverokatu 6, FIN-37130 Nokia, Finland

## Abstract

A high serum concentration of enterolactone, an enterolignan produced by colonic microbiota from precursors in cereals, vegetables, and fruits, is associated with reduced risk of acute coronary events. Probiotics and prebiotics modify colonic metabolism and may affect the serum enterolactone concentration. The effects of a probiotic mixture alone and with galactooligosaccharides (GOS) on serum enterolactone concentration and fecal metabolism were investigated in 18 healthy men. Participants received 3 interventions, each for 2 wk: 1) probiotics [*Lactobacillus rhamnosus* strains GG (LGG) and LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99, for a total amount of  $2 \times 10^{10}$  CFU/d]; 2) probiotics and GOS 3.8 g/d; 3) probiotics, GOS, and rye bread (minimum 120 g/d). Serum enterolactone and fecal dry weight, enzyme activities, pH, SCFA, lactic acid bacteria, bifidobacteria, propionibacteria, and the strains LGG and LC705 were determined. The serum enterolactone concentration (nmol/L) tended to be decreased from baseline [mean (95% CI) 18.6 (10.8–26.4)] by probiotics alone [15.2 (7.8–22.7);  $P = 0.095$ ], was not significantly affected by probiotics with GOS [21.5 (13.2–29.8)], and was increased by probiotics with GOS and rye bread [24.6 (15.4–33.7);  $P < 0.05$ ]. Probiotics alone did not affect fecal  $\beta$ -glucosidase activity and bifidobacteria, but probiotics with GOS decreased  $\beta$ -glucosidase activity and increased bifidobacteria compared with baseline ( $P < 0.05$ ) and with probiotics alone ( $P < 0.01$ ). In conclusion, this probiotic mixture with or without GOS does not significantly affect serum enterolactone concentration. Because probiotics with GOS decreased fecal  $\beta$ -glucosidase activity but not serum enterolactone, the reduced fecal  $\beta$ -glucosidase, within the range of activities measured, does not seem to limit the formation of enterolactone. J. Nutr. 141: 870–876, 2011.

## Introduction

Increased serum enterolactone concentrations have been associated with reduced risk of acute coronary events and death from coronary heart disease and from cardiovascular disease (1–3).

Enterolactone is produced by colonic bacteria from plant-derived precursor lignans, such as matairesinol, secoisolariciresinol, pinoresinol, and lariciresinol (4,5), and possesses various biological activities (6). Though consumption of lignan-containing foods such as whole-grain cereals, beans, other vegetables, and some fruits and berries (6) and constipation appear to be among the most important determinants of serum enterolactone concentration, they account for only a small part of the differences in serum enterolactone concentrations between individuals (7,8). This suggests, along with the effects of oral antimicrobials in decreasing serum enterolactone concentration (9), that intestinal microbiota is very important in the metabolism of lignans. The bacteria species that produce enterolactone are beginning to be clarified. Formation of enterolactone from one of the most abundant dietary lignans, secoisolariciresinol diglucoside, involves phylogenetically di-

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<sup>3</sup> Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at [jn.nutrition.org](http://jn.nutrition.org).

\* To whom correspondence should be addressed. E-mail: [reetta.holma@helsinki.fi](mailto:reetta.holma@helsinki.fi).

verse bacteria, most of which belong to the dominant human intestinal microbiota (10).

The intestinal microbiota of humans predominantly consist of the phyla *Bacteroidetes* and *Firmicutes*, the latter including genera *Dorea*, *Eubacterium*, *Ruminococcus*, *Clostridium*, *Lactobacillus*, and *Streptococcus* as well as the species *Faecalibacterium prausnitzii* (11). Also, members of *Actinobacteria*, including the genus *Bifidobacterium*, are abundant in the intestine. Several species of lactobacilli and bifidobacteria fulfill the criteria of probiotics (12), “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (13). They may modify fecal enzyme activity (14–20). Galactooligosaccharides (GOS)<sup>11</sup> are nondigestible carbohydrates that stimulate the growth of colonic bifidobacteria and, to a lesser extent, lactobacilli (21) and are considered prebiotics. They have a history of safe commercial use (21). Theoretically, a combination of probiotics and prebiotics may enhance survival and function of probiotics in addition to resident beneficial microbes (12).

Our primary aim in this study was to investigate the effects of a probiotic mixture alone and together with GOS on serum enterolactone concentration. The secondary aim was to investigate their effects on fecal metabolic activity. We used a probiotic combination of *Lactobacillus rhamnosus* GG (LGG), *L. rhamnosus* LC705 (LC705), *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99 (Bb99). This combination alleviates symptoms in irritable bowel syndrome (IBS) and during *Helicobacter pylori* eradication therapy (22,23). Also, when administered together with GOS, it reduces eczema and increases resistance to respiratory infections in infants (24,25). Rye bread was included in this study, because rye has the highest concentration of total lignans among cereal species (26) and rye foods are known to increase the serum enterolactone concentration (27). We hypothesized that the changes in fecal enzyme activity caused by probiotics and prebiotics could alter the production of enterolactone from its precursors.

## Methods

### Participants

Eighteen healthy Finnish men aged 30–60 y (mean 45 y) volunteered to participate in this study. Before entering the study, the participants were interviewed for illness, medication, diet, and smoking. Exclusion criteria included antibiotic treatment for 4 wk before the intervention, chronic gastrointestinal diseases, and the use of chemotherapeutics. Two participants used medication for high blood pressure, 1 for heart disease, 1 for asthma, and 5 of the participants smoked. All participants consumed an omnivorous diet. The study protocol was approved by the Ethics Committee of the Foundation for Nutrition Research, Helsinki, Finland, conforms to the provisions of the Declaration of Helsinki in 1995, and was carefully explained to the participants, who then gave their written informed consent.

### Study design

The study lasted for 11 wk and consisted of a 3-wk run-in period, a 6-wk intervention period, and a 2-wk follow-up. Intervention consisted of three 2-wk periods: 1) probiotics: juice with probiotic bacteria (65 mL/d, resulting in  $2 \times 10^{10}$  CFU daily); 2) probiotics+GOS: juice with probiotic bacteria (65 mL/d) and GOS (3.8 g/d); and 3) probiotics+GOS+rye bread: juice with probiotic bacteria (65 mL/d), GOS (3.8 g/d), and a minimum of 120 g/d whole-grain rye bread in addition to participants'

normal diet, which also included rye bread. The 3-wk run-in period and the 2-wk follow-up period did not include any special treatment. The 2-wk intervention was considered long enough based on a previous study showing that enterolactone concentrations in plasma reflect dietary change within 2 wk (28). Because of the substantial variation in serum enterolactone concentrations between individuals (7), we chose a study design with sequential interventions, where each participant served as his own control. Wash-out periods were not included because of the “additive” sequential interventions. During the study, the participants were not allowed to eat seeds, nuts, and products containing probiotic bacteria. The participants were instructed not to change their ordinary diet during the study other than according to the interventions.

The probiotic juice (Valio) contained 2 *Lactobacillus rhamnosus* strains, LGG (ATCC 53103,  $5.9 \times 10^{10}$  CFU/L) and LC705 (DSM7061,  $1.3 \times 10^{11}$  CFU/L), 1 bifidobacterium strain, Bb99 (DSM 13692,  $5.7 \times 10^9$  CFU/L), and 1 propionibacterium strain, *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM7067,  $1 \times 10^{11}$  CFU/L). After the probiotics period, 10.3 g GOS syrup (Valio) was added to the probiotic juice. The syrup contained 37% pure GOS, resulting in a daily amount of 3.8 g GOS/participant. The extra rye bread (Reissumies, Fazer Bakeries) specially delivered to the participants for this study contained 62% rye flour and 12% fiber. The mean daily amount of the extra rye bread consumed during the probiotics+GOS+rye bread period was 143 g (range 78–210 g), resulting in a mean daily total (extra + usual) amount of 211 g (range 78–351 g).

### Questionnaires

Participants estimated the severity of gastrointestinal symptoms (stomachache, abdominal distension, flatulence, heartburn, loose stools, hard stools) during the previous 2 wk by using a visual analogue scale (100 mm, 0–100) at the end of each intervention period and at the end of follow-up. During the probiotics+GOS+rye bread period, the participants recorded each day the amount of rye bread they consumed. Use of nonpermitted food products (seeds, nuts, and products containing probiotic bacteria) and medication during the previous 2 wk was evaluated by the participants at the end of each intervention period and at the end of follow-up. In addition, such use during the study was controlled by a frequency questionnaire conducted at the end of the follow-up period. Finally, the participants were asked at the end of the follow-up period if there were changes in their body weight, alcohol use, intake of fat, and exercise habits during the study.

### Serum sample analysis

Blood samples were collected 5 times during the experiment at the end of each period. The sampling was performed in the morning and always on the same day of the week. Serum was separated from the blood samples not more than 2 h after sampling. The samples were centrifuged and stored at  $-20^{\circ}\text{C}$  until analysis. Enterolactone analyses were made by time-resolved fluoroimmunoassay (29,30). At baseline, the blood sample was taken after an overnight fast, and serum cholesterol and TG as well as blood glucose were analyzed by a biochemical analyzer (Reflotron IV, Boehringer Mannheim). LDL-cholesterol was calculated according to the following formula: total cholesterol – HDL-cholesterol –  $(0.45 \times \text{TG})$ .

### Fecal sample analysis

Fecal samples were collected 5 times during the experiment, at the end of every period. The participants were asked to freeze the samples immediately and to keep them frozen until taken in a cooler with ice packs to the study center. In the laboratory, the fecal samples were stored at  $-70^{\circ}\text{C}$  until further analysis.

**Fecal pH and dry weight.** The pH of the fecal samples was measured with the Mettler Toledo InLab 427 electrode. For dry matter determination, ~1 g of fecal sample was weighed and dried in the oven ( $105^{\circ}\text{C}$  17 h), cooled down in an exicator to room temperature, and reweighed. Fecal dry weight was expressed as a percent of the wet weight.

**Fecal enzyme activities.** Fecal activities of  $\beta$ -glucosidase and  $\beta$ -glucuronidase were analyzed by a method described by Goldin et al.

<sup>11</sup> Abbreviations used: Bb99, *Bifidobacterium breve* Bb99; GOS, galactooligosaccharides; IBS, irritable bowel syndrome; LC705, *Lactobacillus rhamnosus* LC705; LGG, *Lactobacillus rhamnosus* GG.

(31). For the determination of fecal urease activity, the enzyme reaction was conducted at 37°C (pH 7.4) in a total volume of 1.0 mL containing a final concentration of 0.02 mol/L potassium phosphate buffer, 10 mmol/L urea, and fecal extract (0.2 mL). The reaction was terminated by the addition of 0.1 mol/L sulfuric acid (9.0 mL). After the addition of 1.0 mL of 10 mol/L sodium hydroxide, the ammonia content was determined with an automated analyzer (Mira S, Roche) using a Boehringer Mannheim Ammonia kit. Enzyme activities were expressed per gram protein and consequently the protein content was determined with a protein test kit (Bio-Rad Protein Assay, Bio-Rad Laboratories).

**SCFA.** For the SCFA and enzyme analyses, fecal samples were diluted 1:10 with 0.1 mol/L phosphate buffer and homogenized with a Stomacher blender, filtered, sonicated 1 min at 4°C, and centrifuged at 450 × g for 15 min at 4°C. The supernatant fraction was used for analysis. The samples were stored at -70°C. The concentrations of acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, capronic acid, and isocaproic acid were determined with the capillary gas chromatograph (HP-6890, Hewlett-Packard) by adapting the method of Hoverstad et al. (32).

**Microbiological analyses.** Fecal samples were homogenized 1:10 in Wilkins-Chalgren broth (Oxoid Ltd) in an anaerobic chamber and serial dilutions were plated on MRS-agar (LAB M, International Diagnostics Group) for total lactic acid bacteria, raffinose agar (33) for bifidobacteria, and yeast extract lactose agar (34) supplemented with 1% (wt:v) of β-glycerolphosphate (Merck) for propionibacteria. MRS plates were incubated at 37°C for 3 d, raffinose agar plates at 37°C for 2 d, and yeast extract lactose agar plates at 30°C for 7 d, all anaerobically. LGG and LC705 strains were analyzed by plating the diluted samples on MRS-vancomycin (50 mg/L) agar and anaerobically cultivating for 2–3 d at 37°C. In LGG and LC705 analyses, 20 isolates of every sample were purified further on MRS agar with vancomycin and tested for lactose utilization by anaerobically incubating overnight at 37°C on MRS agar (pH 7.2) containing bromocresol purple 0.005% and 2% lactose, resulting in a yellow (LC705) or white (LGG) color due to lactose utilization.

#### Statistical analysis

Serum enterolactone was the primary outcome variable studied. Fecal enzyme activities, fecal bacterial counts, fecal pH and dry weight, SCFA, and gastrointestinal symptoms were the secondary variables. The variables were measured during the study at the end of each study period, the run-in period, the 3 intervention periods, and the follow-up period. The run-in period was considered as baseline and it was taken as the reference period in the post hoc analyses. Gastrointestinal symptoms were not assessed before the interventions started and therefore post hoc comparisons to baseline were replaced by comparisons to the follow-up period. ANOVA for repeated measurements or Friedman test, when appropriate, were conducted to analyze the within-subject changes in the primary and secondary variables during the study periods. ANOVA for repeated measurements with Greenhouse-Geisser adjustment was applied when the sphericity assumption was violated. In the case of a significant global *P*-value, the post hoc comparisons were performed

using paired samples *t* test or Wilcoxon's Signed Rank test, when appropriate. In general, 5 post hoc comparisons were conducted: the 3 intervention periods were compared with baseline, and probiotics+GOS was compared with probiotics, and probiotics+GOS+rye bread was compared with probiotics+GOS. Only in the analysis of secondary variables were the post hoc comparisons Bonferroni corrected. The association between serum enterolactone and the secondary variables was analyzed using the Spearman rank correlation. Secondary variables, such as fecal enzyme activities, were divided into 2 groups (median as a cutoff point) and the Mann-Whitney U test was used to compare the serum enterolactone concentrations between the groups. *P* < 0.05 was considered significant. Statistical analyses were performed with SPSS (release 15.0) software. Values in the text are means ± SD unless otherwise noted.

## Results

**Participant characteristics and compliance.** At baseline, the serum concentration in fasting participants of total cholesterol was 5.29 ± 1.15 mmol/L, of HDL cholesterol, 1.15 ± 0.32 mmol/L, of LDL cholesterol, 3.53 ± 0.92 mmol/L, of TG, 1.39 ± 0.77 mmol/L, and of fasting glucose, 5.34 ± 0.70 mmol/L. None of the participants used antibiotics during the study. Three used forbidden probiotic products once and 1 ate nuts once during the study. The rest of the participants did not consume any of the excluded products. One participant lost 1.5 kg during the study, and 1 participant reported that his intake of fat and alcohol decreased during the study. All the participants completed the study.

**Gastrointestinal symptoms.** Of individual symptoms, only flatulence and abdominal distension changed during the study (*P* < 0.02). These symptom scores were higher during the period of probiotics+GOS+rye bread [median (IQR)] [49.5 (17.3–73.3) and 23.0 (13.0–49.9)] than during the follow-up period [23.0 (10.8–41.3) and 12.5 (9.0–27.0)] (*P* < 0.01). Consequently, the total symptom score changed during the study (*P* = 0.027), being significantly higher during the period of probiotics+GOS+rye bread [mean (95% CI)] [153.6 (108.1–199.1)] than during the follow-up [96.5 (65.8–127.2)] (*P* = 0.025). There were no significant changes in other gastrointestinal symptoms.

**Serum enterolactone.** The serum enterolactone concentration tended to be lower during the probiotics period than at baseline [mean difference (95% CI) -3.4 nmol/L (-7.4–0.7)] (*P* = 0.095) but did not differ from baseline during the probiotics+GOS period [mean difference (95% CI) 2.9 nmol/L (-4.1–9.9)] (*P* = 0.39) (Table 1). On the other hand, during the period of probiotics+GOS+rye bread, the serum enterolactone concentration was higher than at baseline [mean difference (95% CI)

**TABLE 1** Men's serum enterolactone concentrations during a run-in period, sequential 2-wk interventions with probiotics, probiotics+GOS, and probiotics+GOS+rye bread, and a follow-up period

Serum enterolactone, <sup>1</sup> nmol/L	<i>n</i>	Probiotics+GOS +				Follow-up	<i>P</i> <sup>2</sup>
		Run-in	Probiotics	Probiotics+GOS	rye bread		
All participants	18	18.6 (10.8–26.4)	15.2 (7.8–22.7)	21.5 (13.2–29.8)	24.6 (15.4–33.7) <sup>a</sup>	23.2 (13.5–32.8)	0.045
Subgroups by run-in enterolactone							
<20 nmol/L	9	5.0 (0.4–9.5)	3.2 (1.0–5.5)	10.5 (1.8–19.2)	9.6 (2.3–16.9)	6.8 (0.4–13.1)	0.19
≥20 nmol/L	9	32.3 (25.9–38.6)	27.3 (17.9–36.6)	32.6 (21.9–43.2)	39.5 (31.0–48.1)	39.5 (30.9–48.2)	0.096

<sup>1</sup> Values are means (95% CI). <sup>a</sup>Different from run-in, *P* < 0.05.

<sup>2</sup> ANOVA for repeated measures. *P*-value refers to the global test.

6.0 nmol/L (0.3–11.6)] ( $P = 0.042$ ). There were no significant differences in serum enterolactone concentration between intervention periods in subgroups by run-in serum enterolactone concentration below and above 20 nmol/L.

**Fecal pH and dry weight.** Fecal pH was not significantly changed during the study (Table 2). Fecal dry weight (percent) was significantly lower during the probiotics+GOS and probiotics+GOS+rye bread periods than at baseline (Table 2). Fecal dry weight was also lower during the probiotics+GOS period than during the probiotics period.

**Fecal enzyme activities.** Fecal activity of  $\beta$ -glucosidase was lower during the probiotics+GOS period than at baseline [mean difference (95% CI)  $-5.4$  nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$  ( $-9.3$  to  $-1.5$ )] ( $P = 0.045$ ) and during the probiotics period [ $-7.2$  nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$  ( $-11.3$  to  $-3.1$ )] ( $P = 0.008$ ) (Table 2).  $\beta$ -Glucosidase activity during the probiotics and probiotics+GOS+rye bread periods did not significantly differ from baseline. Activities of urease and  $\beta$ -glucuronidase were not significantly affected during the study (Table 2).

**Fecal bacterial counts.** Fecal counts of both *Lactobacillus* strains administered, LGG and LC705, as well as of total propionibacteria were significantly higher during each intervention period than at baseline (Table 3). Total bifidobacteria counts were significantly higher during the probiotics+GOS period than at baseline and during the probiotics period. There was no significant difference in total lactic acid bacteria during the probiotics, probiotics+GOS, or probiotics+GOS+rye bread periods from baseline.

**Fecal SCFA.** Total fecal SCFA content as well as fecal acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, isovaleric acid, and caproic acid contents were not significantly different between the intervention periods (Supplemental Table 1).

**Associations between serum enterolactone and fecal markers.** We counted correlations between baseline values of serum enterolactone (nmol/L) and several fecal markers. Serum enterolactone concentration was not correlated with fecal pH or fecal dry weight. Serum enterolactone was positively correlated with  $\beta$ -glucosidase activity (nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$ ) ( $r = 0.57$ ;  $P = 0.013$ ) and tended to be positively correlated with urease ( $r = 0.46$ ;  $P = 0.055$ ) and  $\beta$ -glucuronidase ( $r = 0.45$ ;  $P = 0.064$ ) activity (nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$ ).  $\beta$ -Glucosidase

activity was categorized according to the median (20.2 nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$ ). The median serum enterolactone was 2.2 and 29.5 nmol/L ( $P = 0.024$ ) when the  $\beta$ -glucosidase activity was below or above the median, respectively (Fig. 1A). Serum enterolactone tended to be positively correlated with 2 fecal bacterial counts (CFU/g dry weight): total bifidobacteria ( $r = 0.44$ ;  $P = 0.067$ ) and total lactic acid bacteria ( $r = 0.42$ ;  $P = 0.086$ ). Total bifidobacteria count was categorized according to the median (44.9 CFU/g dry weight). The median serum enterolactone was 2.2 and 29.5 nmol/L ( $P < 0.01$ ) when the count of total bifidobacteria was below or above the median, respectively (Fig. 1B). Correlations between serum enterolactone and other fecal bacteria were not significant. Serum enterolactone was positively correlated with 3 SCFA ( $\mu$ mol/g dry weight): isobutyric ( $r = 0.56$ ;  $P = 0.021$ ), isovaleric ( $r = 0.56$ ;  $P = 0.020$ ), and caproic acids ( $r = 0.57$ ;  $P = 0.019$ ).

## Discussion

Our aim in this study was to investigate the effects of a probiotic mixture alone and together with GOS on the serum enterolactone concentration and fecal metabolic activity. We hypothesized that GOS would enhance the colonic metabolism of probiotics and resident microbiota and thus lead to a larger change in the serum enterolactone concentration than probiotics alone. However, consumption of probiotics alone or with GOS did not have a significant effect on the serum enterolactone concentration. This is in line with the results of a previous in vitro study where LGG as well as 3 other *Lactobacillus* strains, *L. casei* Shirota, *L. johnsonii* La1, and *L. bulgaricus*, and *Bifidobacterium lactis* Bb12 did not convert a plant lignan 7-hydroxymatairesinol to enterolactone or to other metabolites (36). However, rye bread consumption increased the serum enterolactone concentration, consistent with a previous study (27), probably due mainly to rye bread's high lignan content (26).

Lignans occur in plants as glucosides (10,37). In the present study, the activity of fecal  $\beta$ -glucosidase was positively correlated with the serum enterolactone concentration at baseline, consistent with a previous in vitro study where there was a trend toward positive correlation between  $\beta$ -glucosidase activity and enterolactone production by human fecal bacteria (38). Activity of fecal  $\beta$ -glucosidase was not significantly affected during the period of probiotics alone but decreased during the probiotics together with GOS treatment period compared both to baseline

**TABLE 2** Men's fecal pH, dry weight and enzyme activities during a run-in period, sequential 2-wk interventions with probiotics, probiotics+GOS, and probiotics+GOS+rye bread, and a follow-up period

Variable <sup>1</sup>	Run-in	Probiotics	Probiotics+GOS	Probiotics+GOS +		$P^2$
				rye bread	Follow-up	
Fecal pH	6.90 $\pm$ 0.40	6.83 $\pm$ 0.23	6.67 $\pm$ 0.35	6.65 $\pm$ 0.40	6.85 $\pm$ 0.40	0.12
Fecal dry weight, %	19.6 $\pm$ 0.77	19.4 $\pm$ 1.12	18.4 <sup>a,b</sup> $\pm$ 1.31	18.4 <sup>c</sup> $\pm$ 1.20	18.9 $\pm$ 1.36	0.007
Urease, nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$	32.5 $\pm$ 26.6	32.6 $\pm$ 23.1	23.0 $\pm$ 12.5	30.9 $\pm$ 21.9	35.9 $\pm$ 18.3	0.20
$\beta$ -glucuronidase, nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$	8.94 $\pm$ 3.12	8.47 $\pm$ 4.08	8.33 $\pm$ 5.03	6.80 $\pm$ 3.23	9.53 $\pm$ 4.90	0.26
$\beta$ -glucosidase, nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$	22.0 $\pm$ 8.58	23.9 $\pm$ 10.1	16.6 <sup>a,d</sup> $\pm$ 5.32	18.2 $\pm$ 4.72	23.3 $\pm$ 7.86	0.001

<sup>1</sup> Values are means  $\pm$  SD,  $n = 18$  except pH,  $n = 16$ . <sup>a</sup>Different from run-in,  $P < 0.05$ ; <sup>b</sup>different from probiotics,  $P < 0.05$ ; <sup>c</sup>different from run-in,  $P < 0.01$ ; <sup>d</sup>different from probiotics,  $P < 0.01$ .

<sup>2</sup> Friedman's 1-way ANOVA for fecal dry weight and ANOVA for repeated measures for other variables.  $P$ -value refers to the global test. Bonferroni corrected comparisons.

**TABLE 3** Men's fecal bacterial counts during a run-in period, sequential 2-wk interventions with probiotics, probiotics+GOS, and probiotics+GOS+rye bread, and a follow-up period<sup>1</sup>

Fecal bacteria <sup>2</sup>	Run-in	Probiotics	Probiotics+GOS	Probiotics+GOS + rye bread	Follow-up	P <sup>3</sup>
	<i>log<sub>10</sub> CFU/g dry weight</i>					
Total lactic acid bacteria	31.4 (27.1–35.2)	31.8 (28.5–33.8)	32.3 (30.1–38.3)	30.6 (24.4–32.9)	25.0 (16.1–30.7)	0.001
Total bifidobacteria	44.9 (40.7–46.6)	46.6 (43.5–51.1)	51.0 <sup>a,b</sup> (48.6–55.1)	50.9 (44.5–54.3)	47.1 (34.7–51.4)	0.001
Total propionibacteria	10.4 (10.1–12.1)	30.5 <sup>c</sup> (25.9–35.4)	32.6 <sup>c</sup> (27.9–35.9)	33.6 <sup>c</sup> (27.2–37.0)	10.7 (10.2–11.5)	<0.001
LC705	10.2 (9.9–10.6)	26.7 <sup>c</sup> (23.6–30.3)	30.4 <sup>c</sup> (24.8–34.0)	29.4 <sup>c</sup> (23.9–30.2)	11.1 (10.4–20.8)	<0.001
LGG	10.2 (9.9–10.5)	24.5 <sup>c</sup> (20.1–29.1)	29.4 <sup>c</sup> (26.5–33.8)	24.1 <sup>c</sup> (16.9–28.6)	10.7 (10.2–21.7)	<0.001

<sup>1</sup> Values are medians (IQR), *n* = 18 during each period. <sup>a</sup>Different from run-in, *P* < 0.05; <sup>b</sup>different from probiotics, *P* < 0.01; <sup>c</sup>different from run-in, *P* < 0.01.

<sup>2</sup> Fecal counts of total lactic acid bacteria, total bifidobacteria, and total propionibacteria (expressed as log<sub>10</sub> CFU/g wet weight) during run-in, Probiotics and Probiotics+GOS treatments have been published (35).

<sup>3</sup> Friedman's 1-way ANOVA. *P*-value refers to the global test.

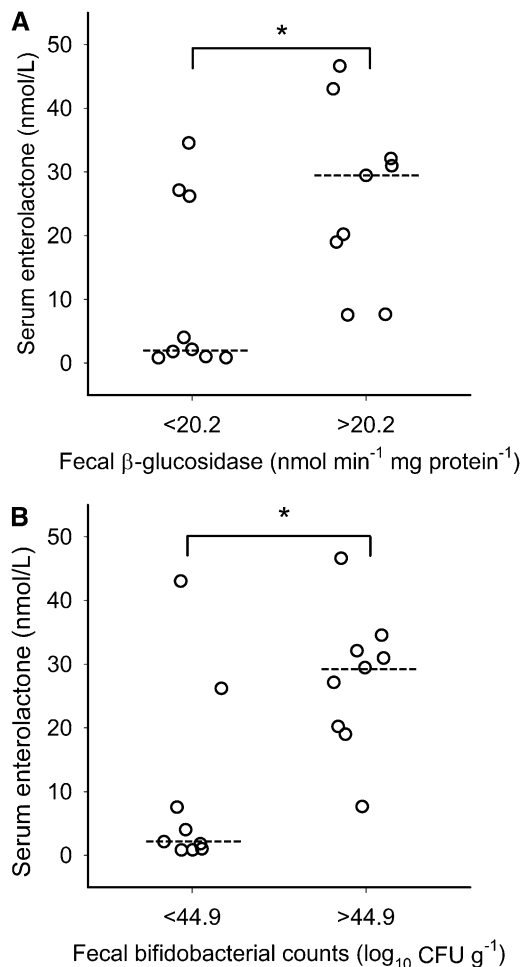
and to the period of probiotics alone, suggesting that GOS either directly inhibited  $\beta$ -glucosidase activity or reduced the number or metabolism of bacterial species with high  $\beta$ -glucosidase activity. GOS addition led to very large and rapid reductions in the activity of this enzyme in a previous study using an in vitro human gut bacterial ecosystem, suggesting a direct inhibition

(39), and GOS reduced fecal  $\beta$ -glucosidase activity in healthy men (40). However, in the present study, the decreased activity of fecal  $\beta$ -glucosidase during the consumption of probiotics together with GOS did not lead to decreased serum enterolactone concentrations, suggesting that despite a significant correlation with serum enterolactone, within the range of activities measured in the present study, fecal  $\beta$ -glucosidase does not have a central role in modifying serum enterolactone concentration.

Fecal counts of total propionibacteria, LC705, LGG, and total bifidobacteria were increased by the interventions, whereas counts of total lactic acid bacteria were not. The result that total bifidobacteria counts were increased during probiotics with GOS, compared with both baseline and probiotics alone, is in line with a previous finding that GOS alone increased the bifidobacterial population in healthy adults (41). In addition, trials using the same probiotic mixture as the present study indicated that fecal bifidobacteria counts significantly increased only when administered with GOS in infants (24,42) and slightly decreased when administered without GOS in IBS in adults (43). Total lactic acid bacteria counts in these studies, on the other hand, increased in infants given with or without GOS (24,42), although lactobacilli counts have not changed in adults with IBS (43).

Certain bifidobacteria species have been shown to synthesize high levels of  $\beta$ -glucosidase (44) and the administration of *B. breve* increases fecal  $\beta$ -glucosidase activity in healthy adults (45). In the present study, the increase in total bifidobacteria counts during probiotics with GOS did not increase  $\beta$ -glucosidase activity or serum enterolactone concentration. This suggests that the positive correlation between fecal bifidobacteria counts and serum enterolactone at baseline is explained by some other variable than fecal  $\beta$ -glucosidase activity, such as the amount of rye bread consumed, which has the potential to increase both (27,46,47). It is also possible that those bifidobacteria strains that increased during probiotics with GOS (*Bb99* in the probiotic mixture or resident strains) do not synthesize high levels of  $\beta$ -glucosidase or that  $\beta$ -glucosidase activity is reduced by a direct inhibition by GOS (39).

Fecal pH or SCFA concentrations did not significantly change during the study. These findings are supported by previous intervention studies. The same probiotic mixture (without GOS) as that used in the present study had no significant effect on fecal SCFA concentrations in adults with IBS (43). GOS reduced pH and increased lactic acid and acetic acid concentrations in the proximal, but not distal, colon of pigs, without a significant effect on propionic acid and butyric acid concentrations in ei-



**FIGURE 1** Serum enterolactone concentrations at baseline in men with fecal  $\beta$ -glucosidase activity less than or greater than the median of 20.2 nmol·min<sup>-1</sup>·mg protein<sup>-1</sup> (A) and with fecal bifidobacteria counts less than or greater than the median of 44.9 log<sub>10</sub> CFU/g dry weight (B). Horizontal lines are medians (*n* = 9). \**P* < 0.03.

ther (48). In healthy men, fecal pH and SCFA concentrations remained unaltered during GOS administration, except for the acetic acid concentration, which increased (40). In the present study, rye bread together with probiotics and GOS appeared to increase concentrations of several SCFA compared with probiotics alone or with GOS. In our previous study, rye bread increased acetic acid, propionic acid, and butyric acid concentrations in feces and reduced fecal pH in constipated adults (49). SCFA are challenging to measure from feces, because they are efficiently absorbed from the colon, with only 10–20% being excreted in the feces (50).

A previous study showed that enterolactone concentrations in plasma reflected dietary change within 2 wk of the introduction of a new diet but they continued to increase for 4–6 wk (28). On the basis of this previous study, perhaps the intervention of 2 wk was too short to detect the maximal effect of interventions on serum enterolactone concentration. This is suggested by the fact that serum enterolactone concentrations were high during the follow-up.

In conclusion, the consumption of probiotics alone or with GOS in the present study did not have a significant effect on the serum enterolactone concentration, although probiotics with GOS, but not alone, decreased fecal  $\beta$ -glucosidase activity. This indicates that fecal  $\beta$ -glucosidase, at the activities measured, does not have a major role in modifying the serum enterolactone concentration.

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R.A.K. and R.H. analyzed the data and wrote the paper; R.H. had primary responsibility for final content; R.K., K.H., H.A., R.A.K., and T.S. designed and conducted the research; T.P. conducted the statistical analyzes; and R.K. directed the research. All authors read and approved the final manuscript.

### Literature Cited

1. Vanharanta M, Voutilainen S, Lakka TA, van der Lee M, Adlercreutz H, Salonen JT. Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. *Lancet*. 1999;354:2112–5.
2. Vanharanta M, Voutilainen S, Rissanen TH, Adlercreutz H, Salonen JT. Risk of cardiovascular disease-related and all-cause death according to serum concentrations of enterolactone: Kuopio Ischaemic Heart Disease Risk Factor Study. *Arch Intern Med*. 2003;163:1099–104.
3. Peterson J, Dwyer J, Adlercreutz H, Scalbert A, Jacques P, McCullough ML. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutr Rev*. 2010;68:571–603.
4. Borriello SP, Setchell KDR, Axelson M, Lawson AM. Production and metabolism of lignans by the human faecal flora. *J Appl Bacteriol*. 1985;58:37–43.
5. Heinonen S, Nurmi T, Liukkonen K, Poutanen K, Wähälä K, Deyama T, Nishibe S, Adlercreutz H. In vitro metabolism of plant lignans: new precursors of mammalian lignans enterolactone and enterodiols. *J Agric Food Chem*. 2001;49:3178–86.
6. Adlercreutz H. Lignans and human health. *Crit Rev Clin Lab Sci*. 2007;44:483–525.
7. Kilkkinen A, Stumpf K, Pietinen P, Valsta LM, Tapanainen H, Adlercreutz H. Determinants of serum enterolactone concentration. *Am J Clin Nutr*. 2001;73:1094–100.
8. Milder IE, Kuijsten A, Arts IC, Feskens EJ, Kampman E, Hollman PC, Van't Veer P. Relation between plasma enterodiols and enterolactone and dietary intake of lignans in a Dutch endoscopy-based population. *J Nutr*. 2007;137:1266–71.
9. Kilkkinen A, Pietinen P, Klaukka T, Virtamo J, Korhonen P, Adlercreutz H. Use of oral antimicrobials decreases serum enterolactone concentration. *Am J Epidemiol*. 2002;155:472–7.
10. Clavel T, Henderson G, Engst W, Doré J, Blaut M. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS Microbiol Ecol*. 2006;55:471–8.
11. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*. 2010;464:59–65.
12. Preidis GA, Versalovic J. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology*. 2009;136:2015–31.
13. FAO/WHO. Report of a joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. 2002 [cited 2010 20 Nov]. Available from: <ftp://ftp.fao.org/esn/food/wgreport2.pdf>.
14. Ling WH, Korpela R, Mykkänen H, Salminen S, Hänninen O. *Lactobacillus* strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults. *J Nutr*. 1994;124:18–23.
15. Pedrosa MC, Golner BB, Goldin BR, Barakat S, Dallal GE, Russell RM. Survival of yoghurt-containing organisms and *Lactobacillus gasseri* (ADH) and their effect on bacterial enzyme activity in the gastrointestinal tract of healthy and hypochlorhydric elderly subjects. *Am J Clin Nutr*. 1995;61:353–9.
16. Isolauri E, Kaila M, Mykkänen H, Ling WH, Salminen S. Oral bacteriotherapy for viral gastroenteritis. *Dig Dis Sci*. 1994;39:2595–600.
17. Guérin-Danan C, Chabanet C, Pedone C, Popot F, Vaissade P, Bouley C, Szyllit O, Andrieux C. Milk fermented with yogurt cultures and *Lactobacillus casei* compared with yogurt and gelled milk: influence on intestinal microflora in healthy infants. *Am J Clin Nutr*. 1998;67:111–7.
18. Spanhaak S, Havenaar R, Schaafsma G. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *Eur J Clin Nutr*. 1998;52:899–907.
19. Brigidi P, Vitali B, Swennen E, Bazzocchi G, Matteuzzi D. Effects of probiotic administration upon the composition and enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. *Res Microbiol*. 2001;152:735–41.
20. Rochet V, Rigottier-Gois L, Sutren M, Kremetscki MN, Andrieux C, Furet JP, Tailliez P, Levenez F, Mogenet A, et al. Effects of orally administered *Lactobacillus casei* DN-114 001 on the composition or activities of the dominant faecal microbiota in healthy humans. *Br J Nutr*. 2006;95:421–9.
21. Macfarlane GT, Steed H, Macfarlane S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *J Appl Microbiol*. 2008;104:305–44.
22. Kajander K, Hatakka K, Poussa T, Färkkilä M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. *Aliment Pharmacol Ther*. 2005;22:387–94.
23. Myllyluoma E, Veijola L, Ahlroos T, Tynkkynen S, Kankuri E, Vapaatalo H, Rautelin H, Korpela R. Probiotic supplementation improves tolerance to *Helicobacter pylori* eradication therapy: a placebo-controlled, double-blind randomized pilot study. *Aliment Pharmacol Ther*. 2005;21:1263–72.
24. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol*. 2007;119:192–8.
25. Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Long-term safety and impact on infection rates of postnatal probiotic and prebiotic (synbiotic) treatment: randomized, double-blind, placebo-controlled trial. *Pediatrics*. 2008;122:8–12.
26. Smeds AI, Eklund PC, Sjöholm RE, Willför SM, Nishibe S, Deyama T, Holmbom BR. Quantification of a broad spectrum of lignans in cereals, oilseeds, and nuts. *J Agric Food Chem*. 2007;55:1337–46.
27. McIntosh GH, Noakers M, Royle PJ, Foster R. Whole-grain rye and wheat foods and markers of bowel health in overweight middle-aged men. *Am J Clin Nutr*. 2003;77:967–74.
28. Jacobs DR, Pereira MA, Stumpf K, Pins JJ, Adlercreutz H. Whole grain food intake elevates serum enterolactone. *Br J Nutr*. 2002;88:111–6.
29. Adlercreutz H, Wang GJ, Lapcik O, Hampl R, Wähälä K, Mäkelä T, Lusa K, Talme M, Mikola H. Time resolved fluorimmunoassay for plasma enterolactone. *Anal Biochem*. 1998;265:208–15.

30. Stumpf K, Uehara M, Nurmi T, Adlercreutz H. Changes in the time-resolved fluoroimmunoassay of plasma enterolactone. *Anal Biochem.* 2000;284:153–7.
31. Goldin BR, Swenson L, Dwyer J, Sexton M, Gorbach SL. Effect of diet and *Lactobacillus acidophilus* supplements on human fecal bacterial enzymes. *J Natl Cancer Inst.* 1980;64:255–61.
32. Hoverstad T, Fausa O, Bjørneklett A, Bohmer T. Short-chain fatty acids in the normal human feces. *Scand J Gastroenterol.* 1984;19:375–81.
33. Hartemink R, Kok BJ, Weenk GH, Rombouts FM. Raffinose-Bifidobacterium (RB) agar, new selective medium for bifidobacteria. *J Microbiol Methods.* 1996;27:33–43.
34. Malik AC, Reinbold GW, Vedamuthu ER. An evaluation of the taxonomy of *Propionibacterium*. *Can J Microbiol.* 1968;14:1185–91.
35. Tiihonen K, Suomalainen T, Tynkkynen S, Rautonen N. Effect of prebiotic supplementation on a probiotic bacteria mixture: comparison between a rat model and clinical trials. *Br J Nutr.* 2008;99:826–31.
36. Lahtinen S, Saarinen NM, Ämmälä J, Mäkelä SI, Salminen S, Ouwehand AC. Interactions between lignans and probiotics. *Microb Ecol Health Dis.* 2002;14:106–9.
37. Xie LH, Akao T, Hamasaki K, Deyama T, Hattori M. Biotransformation of pinoreosin diglucoside to mammalian lignans by human intestinal microflora, and isolation of *Enterococcus faecalis* strain PDG-1 responsible for the transformation of (+)-pinoreosin to (+)-lariciresinol. *Chem Pharm Bull (Tokyo).* 2003;51:508–15.
38. Possemiers S, Bolca S, Eeckhaut E, Depypere H, Verstraete W. Metabolism of isoflavonoids, lignans and phenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol Ecol.* 2007;61:372–83.
39. McBain AJ, MacFarlane GT. Modulation of genotoxic enzyme activities by non-digestible oligosaccharide metabolism in in-vitro human gut bacterial ecosystems. *J Med Microbiol.* 2001;50:833–42.
40. van Dokkum W, Wezendonk B, Srikumar TS, van den Heuvel EG. Effect of nondigestible oligosaccharides on large-bowel functions, blood lipid concentrations and glucose absorption in young healthy male subjects. *Eur J Clin Nutr.* 1999;53:1–7.
41. Depeint F, Tzotzis G, Vulevic J, l'Anson K, Gibson GR. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: a randomised, double-blind, crossover, placebo-controlled intervention study. *Am J Clin Nutr.* 2008;87:785–91.
42. Viljanen M, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy.* 2005;60:494–500.
43. Kajander K, Krogius-kurikka L, Rinttilä T, Karjalainen H, Palva A, Korpela R. Effects of multispecies probiotic supplementation on intestinal microbiota in irritable bowel syndrome. *Aliment Pharmacol Ther.* 2007;26:463–73.
44. McBain AJ, MacFarlane GT. Ecological and physiological studies on large intestinal bacteria in relation to production of hydrolytic and reductive enzymes involved in formation of genotoxic metabolites. *J Med Microbiol.* 1998;47:407–16.
45. De Preter V, Raemen H, Cloetens L, Houben E, Rutgeerts P, Verbeke K. Effect of dietary intervention with different pre- and probiotics on intestinal bacterial enzyme activities. *Eur J Clin Nutr.* 2008;62:225–31.
46. Oikarinen S, Heinonen S, Karppinen S, Mättö J, Adlercreutz H, Poutanen K, Mutanen M. Plasma enterolactone or intestinal *Bifidobacterium* levels do not explain adenoma formation in multiple intestinal neoplasia (Min) mice fed with two different types of rye-bran fractions. *Br J Nutr.* 2003;90:119–25.
47. Crittenden R, Karppinen S, Ojanen S, Tenkanen M, Fagerström R, Mättö J, Saarela M, Mattila-Sandholm T, Poutanen K. *In vitro* fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. *J Sci Food Agric.* 2002;82:781–9.
48. Tzortzis G, Goulas AK, Gee JM, Gibson GR. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. *J Nutr.* 2005;135:1726–31.
49. Holma R, Hongisto SM, Saxelin M, Korpela R. Constipation is relieved more by rye bread than wheat bread or laxatives without increased adverse gastrointestinal effects. *J Nutr.* 2010;140:534–41.
50. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol.* 2006;40:235–43.