

ARTICLE III

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# Diversity and temporal stability of fecal bacterial populations in elderly subjects consuming galacto-oligosaccharide containing probiotic yoghurt

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## Abstract

Denaturing gradient gel electrophoresis was applied to study the effect of 3-week consumption of probiotic yoghurt containing galacto-oligosaccharides (GOS) on intra-individual diversity and temporal stability of predominant bacterial, bifidobacterial, *Lactobacillus*-group, and Erec-group *Eubacterium rectale*–*Clostridium coccooides* fecal populations of elderly subjects. Diversity and temporal stability of the selected bacterial groups of elderly subjects were compared with those obtained from younger adults in our previous studies. In the present study, GOS-yoghurt consumption did not significantly affect diversity or temporal stability of the selected bacterial groups. On average, the Erec-group and the predominant bacterial and bifidobacterial populations remained almost stable, and the *Lactobacillus*-group was unstable in elderly subjects. No differences were found between elderly and younger adults in temporal stability of studied bacterial populations. However, the difference in diversity of predominant bacterial population and Erec-group bacteria was significantly higher in elderly subjects of this study as compared with younger adults from our previous studies.

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**Keywords:** Elderly; Fecal bacteria; DGGE; Yoghurt; GOS

## 1. Introduction

It was shown several decades ago that the gastrointestinal microbiota evolves with age (Gorbach, Nahas, Lerner, & Weinstein, 1967). As life expectancy in the western world has rapidly risen, interest in the gastrointestinal microbiota of elderly subjects has increased. Asian elderly subjects have been reported to have a lower level of bifidobacteria and higher levels of clostridia, lactobacilli, streptococci, and Enterobacteriaceae in culture-based studies (Benno et al., 1989; Mitsuoka, 1992). In European culture-based and molecular biology based studies, however, results have been partly contradictory (He, Harmsen, Raangs, &

Welling, 2003; Hopkins, Sharp, & Macfarlane, 2001, 2002; Mueller et al., 2006; van Tongeren, Slaets, Harmsen, & Welling, 2005; Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004). Moreover, the difference in several bacterial groups between elderly and younger adults has been shown to be dependent on geographical location (Mueller et al., 2006). The number of bifidobacteria were found to be lower in elderly subjects compared with younger adults in European studies (Hopkins et al., 2001, 2002; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004).

Bifidobacteria are generally considered beneficial for health, and have been widely used, both individually and in combination with lactobacilli, in probiotic foods and products to increase the probiotic content in the human gastro-intestinal tract (Salminen, Deighton, Benno, &

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Gorbach, 1998). The absence of bifidobacteria, or their low numbers in the elderly, may have metabolic and health consequences for the host, affecting immune system function and a multiplicity of other functions, e.g., synthesis of vitamins and protein, and supplementation in digestion and absorption (Hopkins et al., 2001; Mitsuoka, 1992). In addition, bifidobacteria are involved in colonization resistance in the bowel (Hopkins et al., 2001). The number of bifidobacteria may be increased in the gut either by continuous supplementation of probiotic bifidobacteria or by adding prebiotics to food products. Prebiotics are non-digestible food ingredients that positively affect the host by selectively stimulating the growth, activity, or both of one or a limited number of beneficial bacterial species already resident in the colon (Gibson & Roberfroid, 1995). The prebiotics that currently fulfill the definition criteria are fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). GOS, derived from lactose in milk, have received less attention than the other prebiotics, although they are considered to have bifidogenic effects in humans. GOS is a collective term for a group of semi-synthetic non-digestible carbohydrates made using galactosidases as catalysts. GOS contain zero to one glucose units and one to six galactose units bound to each other by different glycosidic bonds ( $\beta$ 1–2,  $\beta$ 1–3,  $\beta$ 1–4,  $\beta$ 1–6). During production of GOS, a mixture of GOS of different chain length are formed (Alander et al., 2001; Ito et al., 1993; Tannock et al., 2004).

The aim of this study was to evaluate the impact of yoghurt containing GOS on the diversity and temporal stability of predominant fecal bacterial population and selected bacterial groups—namely the clostridial cluster XIVa (*Eubacterium rectale*–*Clostridium coccooides*-group, called the Erec-group), bifidobacteria, and lactobacilli—of elderly subjects suffering from constipation. In addition, the diversity and temporal stability of selected bacterial groups of elderly subjects were compared with those of younger adults.

## 2. Materials and methods

### 2.1. Study design

A double-blind, placebo-controlled, randomized crossover study was performed on 41 elderly subjects with self-

reported constipation (10 male and 31 female subjects, 60–79 years of age; mean 68 years). All the subjects had either difficulties in defecation most of the time or defecated less than five times weekly. Additional inclusion criteria were: aged between 60 and 80 years and a successful completion of the Mini-Mental State questionnaire (Folstein, Folstein, & McHugh, 1975). The Barthel index (Mahoney & Barthel, 1965) was used to evaluate the functional capacity of the subjects, and all participants received the full score 100. The exclusion criteria for both groups were daily use of laxatives, other gastrointestinal disorders (celiac disease, inflammatory bowel disease, gastrointestinal tumors, diverticulitis, or irritable bowel syndrome), thyroid dysfunction, use of strong psychopharmaceuticals or opioids, in addition to use of antimicrobials during the preceding month. The subjects defecated into a plastic container, and placed the samples immediately after defecation into their own freezer ( $-20^{\circ}\text{C}$ ). Within 1 week, the samples were retrieved from the subjects and placed into a  $-70^{\circ}\text{C}$  freezer to await further analysis.

All subjects gave their written informed consent to participate in the study. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS, Finland) approved the study protocol.

Schematic representation of the study design is presented in Fig. 1. During the first intervention, elderly subjects of the GOS group consumed GOS-yoghurt whereas elderly subjects of the placebo group consumed placebo-yoghurt. After a wash-out period, during which neither group consumed test yoghurts, the second intervention was started. During this intervention, the elderly subjects of the GOS group consumed placebo-yoghurt whereas the elderly subjects of the placebo group consumed GOS-yoghurt. The GOS were produced enzymatically in situ in condensed milk, which was then used to prepare the GOS-yoghurt. Both the GOS- and placebo-yoghurts were fermented using a commercial starter culture containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and *Bifidobacterium lactis* strains, and yoghurts were flavored with vanilla. The yoghurts were low in lactose ( $<1\text{ g per }100\text{ g}$  yoghurt), which was confirmed by high-pressure liquid chromatography (HPLC) analysis. The test yoghurts were

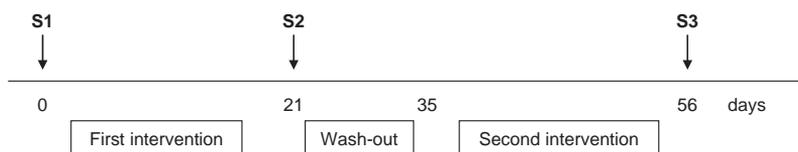


Fig. 1. Schematic representation of the study design. The arrows (S1–S3) indicate the time points for fecal sample collection (S1, baseline; S2, sample after 1st intervention; S3, sample after 2nd intervention). During the first intervention (sample S2), the GOS group consumed daily two portions (150 g per portion) of test yoghurt containing 5 g galacto-oligosaccharides (GOS) per portion and the placebo-group consumed daily two portions of test yoghurt (150 g per portion; no GOS addition). During the second intervention (sample S3), the GOS group consumed daily two portions of test yoghurt (150 g per portion; no GOS addition) and the placebo-group consumed daily two portions (150 g per portion) of test yoghurt containing 5 g galacto-oligosaccharides (GOS) per portion. During wash-out, elderly subjects of both groups consumed their normal diet, which did not include probiotic test yoghurt.

prepared at Valio Ltd. (Helsinki, Finland) and sent to the coordinator of the clinical trial as coded specimens. The daily GOS consumption for the GOS-yoghurt group was 10 g of GOS per day.

## 2.2. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) of predominant microbiota

DNA was extracted from 300 mg of fecal material using FastDNA Spin Kit for Soil (QBIogene, Carlsbad, CA, USA), as described previously (Maukonen et al., 2006a). Partial 16S rRNA gene was PCR-amplified for the detection of predominant bacteria as described by Mättö et al. (2005) using primers U968-f+GC (CGCCCCGGGGCGCCCCGGGGCGGGGGCGGGGGCACGGGGGAACGGAAGAACCTTA) and U1401-r (CGGTGTGTACAAGACCC) of Nübel et al. (1996). Bifidobacteria were PCR-amplified with primers Bif164-f (GGGTGGTAATGCCGGATG) and Bif662-GC-r (GCCCGCCGCGCGCGGGGGCGGGGGCGGGGGCACGGGGGGCCACCGTTACACCGGGAA) to evaluate the diversity and temporal stability of bifidobacteria according to Satokari, Vaughan, Akkermans, Saarela, and de Vos (2001a). The *Lactobacillus*-group, which comprises the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella*, was amplified as described by Vanhoutte, Huys, De Brandt, and Swings (2004) using primers Lac1 (AGCAGTAGGGAATCTTCCA) and Lac2GC (CGCCCGCGCGCCCCGCGCCCCGCGCCCCCGCCCCATTYACCGCTACACATG; Walter et al., 2001). The Erec-group (Clostridial phylogenetic clusters XIVa; Collins et al., 1994) was PCR-amplified using primers Ccoc-f (AAATGACGGTACCTGACTAA; Matsuki et al., 2002) and Ccoc-r+GC (CGCCCCGGGGCGCGCCCCGGCGGGGGCGGGGGCGGGGGGCTTTGAGTTTCATTCTTGCAGAA), as described previously (Maukonen et al., 2006b).

The PCR products were separated in polyacrylamide gels with denaturing gradients of 38–60% (predominant bacteria and Erec-group), 45–55% (bifidobacteria), and 30–60% (*Lactobacillus*-group), where 100% is 7 M urea and 40% (v/v) deionized formamide, as described by Mättö et al. (2005). Similarity of the PCR-DGGE profiles of the samples obtained from a single subject at different sampling points was compared to evaluate the temporal stability of the investigated bacterial populations. The comparison of the profiles was performed by calculating the similarity percentage using BioNumerics software version 4.50 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Clustering was performed with Pearson correlation and the unweighted-pair group method. Amplicons with the total surface area of at least 1% were included in the similarity analysis. In addition to analysis of each different type of DGGE, a composite dataset including all the performed DGGE-analyses was created in the BioNumerics software.

## 2.3. Adult study group

In our previous studies we have analyzed adult microbiota diversity and stability (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). The results from previous studies were used in the present study to evaluate the difference between diversity and temporal stability between elderly subjects and younger adults. All the previous younger adult control study groups consisted of healthy Finnish adults with normal intestinal balance (self-reported, no medical examination). The exclusion criteria for all control study groups were regular GI-tract symptoms, lactose-intolerance, celiac disease, and antimicrobial therapy during the last 2 months prior to each sampling time point. All the study groups were approved by the ethical committee of VTT Technical Research Centre of Finland, Espoo, Finland. All subjects gave their written informed consent for participation of the study in question.

The control study group of Maukonen et al. (2006a) consisted of 16 healthy adult volunteers (12 females, 4 males, age 26–63 years; mean 45 years) and the study group of Maukonen et al. (2006b) consisted of 12 healthy adult volunteers (nine females, three males, age 34–63 years; mean 47 years) who did not receive any additional supplementations to their normal Finnish diet. The control study group of Saarela et al. (2007) consisted of 10 healthy adult volunteers (nine females, one male, age 34–57 years; mean 41 years), who, after baseline sampling (the sample during normal Finnish diet, before the probiotic consumption started), were consuming a probiotic capsule containing *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. The two subjects from study groups of Maukonen et al. (2006a) and Maukonen et al. (2006b), whose age was overlapping with the present study (age 60 or more) were excluded from the previous study groups.

Only baseline samples from all our previous healthy adult study groups (Maukonen et al., 2006a, 2006b; Saarela et al., 2007) were included in the comparison of bacterial diversity. For comparison of temporal stability of different bacterial groups, only the control study group of Saarela et al. (2007) was included, since this study group consumed the same probiotics and yoghurt starter bacteria for 2 weeks, as did the elderly groups of this study. In the previous studies, DGGE-analyses were performed and analyzed similarly as have been described in this study.

## 2.4. Statistical analysis

Mean and standard deviation were calculated for each experiment. Student's *t*-test (two-sample, assuming unequal variances) was used for the statistical analysis of the results obtained from a single DGGE-analysis. Multivariate analysis of variance (MANOVA) and principal component analysis (PCA), included in the BioNumerics

software (Applied Maths BVBA), were used for statistical analysis of the composite datasets. In addition, discriminant analysis including similarity between baseline and first intervention (Fig. 1; from all different DGGE analyses) and difference in band numbers (also from all different DGGE analyses) was performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

### 3. Results

#### 3.1. Diversity and temporal stability of the predominant bacteria, *Erec*-group, *Lactobacillus*-group, and bifidobacteria of elderly subjects

A carry-over effect was observed after the wash-out period (Surakka et al., unpublished data). A carry-over effect is such an effect that “carries over” from one treatment period to another. Whenever study participants perform in more than one treatment period, there is a possibility of carry-over effects. When using a cross-over design, one should test for possible carry-over by statistical methods. If carry-over is detected, as was unfortunately the case in the current trial in regard to, for example, fecal short-chain fatty acids, it is not advisable to analyze the data from the second treatment period. Therefore, only results which were obtained after the first intervention period (Fig. 1) are included in the results and in the statistical analysis.

#### 3.2. Predominant bacteria

DGGE analysis targeted to the predominant bacterial population showed considerable intra-individual diversity

as well as uniqueness of fecal microbiota before and after intervention (Fig. 2A). The intra-individual fecal samples clustered together in all of the cases (one distinct cluster per subject), irrespective of GOS- or placebo-yoghurt administration. Predominant fecal microbiota was temporally rather stable after both placebo (mean similarity  $81.6 \pm 10.5\%$ ) and GOS-yoghurt consumption (mean similarity  $80.9 \pm 11.9\%$ ) (Table 1). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

#### 3.3. *Erec*-group

The intra-individual diversity and uniqueness of *Erec*-group DGGE in the fecal samples were similar to the case of predominant bacteria (Fig. 2B). *Erec*-group bacteria remained temporally mostly stable after both placebo (mean similarity  $91.1 \pm 6.1\%$ ) and GOS-yoghurt consumption (mean similarity  $87.7 \pm 7.9\%$ ) (Table 1). In addition, the number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

#### 3.4. *Lactobacillus*-group

*Lactobacillus*-group targeted DGGE analysis showed intra-individual diversity as well as uniqueness of *Lactobacillus* microbiota before and after intervention (Fig. 2C). The intra-individual fecal samples clustered together in only 11 out of 37 subjects (one distinct cluster per subject, six placebo subjects and five GOS subjects, data not

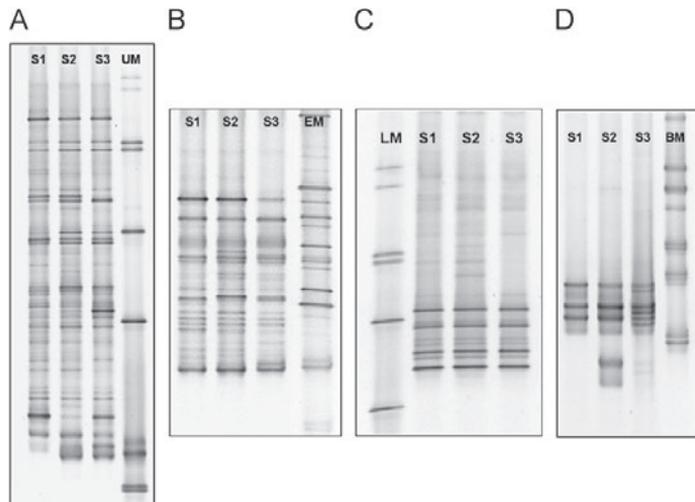


Fig. 2. Fecal denaturing gradient gel electrophoresis (DGGE) profiles of one elderly subject (placebo group, male, age 76 years) as detected with predominant bacterial polymerase chain reaction (PCR)-DGGE (A), *Clostridium coccooides*-*Eubacterium rectale*-group specific PCR-DGGE (B), *Lactobacillus*-group PCR-DGGE (C), and bifidobacteria-specific PCR-DGGE (D). The samples were obtained from fecal samples at time points baseline (S1), 3 weeks placebo yoghurt consumption (S2), and 3 weeks galacto-oligosaccharide (GOS) yoghurt consumption after 2 weeks wash-out period (S3); UM, universal marker; EM, *Erec*-marker; LM, lactobacilli-marker; BM, bifidobacteria-marker.

Table 1

Temporal stability (= similarity values) of denaturing gradient gel electrophoresis (DGGE) profiles of human fecal samples obtained at baseline and after consumption of test yoghurt with or without GOS supplementation from 41 elderly subjects with primers specific to *Clostridium coccoides*–*Eubacterium rectale* (Erec) group of clostridia, *Lactobacillus*-group, and bifidobacteria, in addition to primers that target predominant bacterial microbiota<sup>a</sup>

Analysis	Similarity (%) <sup>b</sup> (baseline vs. 1st intervention)			Similarity (%) <sup>d</sup> in adults <sup>c</sup>
	GOS	Placebo	All together <sup>d</sup>	
Predominant bacteria				
Mean ± SD	80.9 ± 11.9	81.6 ± 10.5	81.2 ± 11.2	81.0 ± 7.6
Range	51.4–95.5	58.6–94.9	51.4–95.5	70.0–90.6
Erec-group of clostridia				
Mean ± SD	87.7 ± 7.9	91.1 ± 6.1	89.1 ± 7.3	92.3 ± 4.7
Range	69.7–96.6	73.9–96.9	69.9–96.9	81.6–97.5
<i>Lactobacillus</i> -group				
Mean ± SD	59.1 ± 25.6	67.2 ± 28.3	62.6 ± 26.7	68.2 ± 11.9
Range	0.0–98.3	8.1–98.8	0.0–98.8	50.0–85.7
Bifidobacteria				
Mean ± SD	82.3 ± 21.7	82.0 ± 19.8	82.2 ± 20.6	87.1 ± 10.8
Range	0.5–99.3	17.3–97.8	0.5–99.3	68.2–97.8

<sup>a</sup>Temporal stability of DGGE profiles from an adult study group is also presented for the above-mentioned bacterial groups.

<sup>b</sup>Similarity values were counted with BioNumerics 4.50 software. Amplicons with the total surface area of at least 1% were included in the similarity analysis.

<sup>c</sup>Similarity values were counted from a healthy Finnish adult study group, which was consuming *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* (Saarela et al., 2007; Maukonen et al., unpublished results).

<sup>d</sup>All together = GOS-group + placebo-group.

Table 2

Diversity of denaturing gradient gel electrophoresis (DGGE) profiles of human fecal samples obtained at baseline and after first intervention from 41 elderly subjects with primers specific to the Erec group of clostridia, *Lactobacillus*-group, and bifidobacteria, in addition to primers that target predominant bacterial microbiota<sup>a</sup>

Analysis	Diversity (number of bands) <sup>b</sup> in elderly subjects			Difference in diversity <sup>c</sup> (between 1st intervention and baseline)		Diversity (number of bands) <sup>b</sup> in adults <sup>d</sup>
	Baseline	GOS	Placebo	GOS	Placebo	
Predominant bacteria						
Mean ± SD	42.1 ± 5.0*	42.0 ± 4.6	43.6 ± 5.0	0.14 ± 3.27	0.65 ± 4.53	32.4 ± 4.7*
Range	35–57	35–51	35–52	–4–7	–8–11	24–40
Erec-group of clostridia						
Mean ± SD	20.7 ± 3.6*	20.8 ± 3.5	21.0 ± 3.0	–0.23 ± 2.81	0.29 ± 1.86	14.7 ± 2.1*
Range	14–28	14–26	16–26	–6–5	–3–3	11–19
<i>Lactobacillus</i> -group						
Mean ± SD	10.7 ± 4.0	11.9 ± 5.6	11.8 ± 2.8	0.57 ± 4.93	2.19 ± 2.69	8.2 ± 3.9
Range	2–17	5–30	7–18	–7–13	–2–7	4–17
Bifidobacteria						
Mean ± SD	8.6 ± 3.2	8.5 ± 2.7	9.5 ± 3.2	0.19 ± 1.03	0.71 ± 2.31	9.1 ± 2.8
Range	3–14	4–14	5–17	–2–2	–4–6	6–15

<sup>a</sup>The difference in diversity between baseline sample and a sample after first intervention is also presented. In addition, diversity of DGGE profiles from previous adult study groups is also presented for the above-mentioned bacterial groups.

<sup>b</sup>Diversity is presented as the number of bands detected by the BioNumerics 4.50 software (Applied Maths BVBA).

<sup>c</sup>Difference in diversity was calculated as (number of bands after 1st intervention)–(number of bands at baseline), therefore negative results indicate that there were less bands after the 1st intervention than at baseline, and positive results indicate that there were more bands after the 1st intervention than at the baseline. \*Significant differences ( $P < 0.05$ ) in diversity between baseline samples of elderly subjects and adults.

<sup>d</sup>Diversity of DGGE-profiles in adults was calculated by combining the diversity of the baseline samples from healthy Finnish adult study groups of Maukonen et al. (2006a, 2006b, unpublished results) and Saarela et al. (2007). None of the subjects in the adult study groups had any supplementation to their normal Finnish diet at the time of the baseline sample.

shown). The subjects whose samples clustered together had an intra-individual temporal stability similarity value of (approx.) greater than 75%. All the other subjects

had lower intra-individual temporal stability similarity values (data not shown). The samples of one subject did not amplify at all with the *Lactobacillus*-group PCR,

although the procedure was repeated with different amounts of DNA template for several times (data not shown).

The fecal lactobacilli population was temporally unstable (mean similarity  $59.1 \pm 25.6\%$ ) after GOS-yoghurt consumption and temporally rather unstable (mean similarity  $67.2 \pm 28.3\%$ ) after placebo consumption (Table 1). However, the difference in temporal stability between the study groups was not significant ( $P = 0.19$ ). The number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at the baseline (Table 2).

### 3.5. *Bifidobacteria*

The intra-individual diversity and uniqueness of bifidobacteria DGGE in the fecal samples were similar to the case of predominant bacteria and Erec-group bacteria (Fig. 2D). However, the intra-individual fecal samples clustered together in only 26/38 subjects (one distinct cluster per subject; data not shown). The subjects whose samples clustered together had an intra-individual temporal stability similarity value of (approx.) greater than 85%. The fecal bifidobacterial population was temporally mostly rather stable after both GOS-yoghurt consumption (mean similarity  $82.3 \pm 21.7\%$ ) and after placebo-yoghurt consumption (mean similarity  $82.0 \pm 19.8\%$ ) (Table 1). The

number of amplicons after GOS- and placebo-yoghurt consumption was similar to the number of amplicons at baseline (Table 2).

### 3.6. Composite DGGE dataset

A composite dataset containing all different DGGE analyses was created in the BioNumerics software. According to a PCA plot, none of the sample groups (GOS, placebo, baseline) was distinguishable from the other groups (Fig. 3). However, the group of GOS samples was more concise (the samples more similar to each other) than the other sample groups (Fig. 3). The group of baseline samples was the most diverse (biggest ellipse; Fig. 3). Significant differences were not found in discriminant analysis or MANOVA on temporal stability and bacterial diversity on composite datasets.

### 3.7. Difference in diversity and temporal stability of the predominant bacteria, Erec-group, Lactobacillus-group, and bifidobacteria between elderly subjects and younger adults

In the comparison between elderly subjects of this study and younger adults of our previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007), only the placebo-yoghurt group was included in the comparison of temporal stability. For comparison of bacterial diversity, the baseline

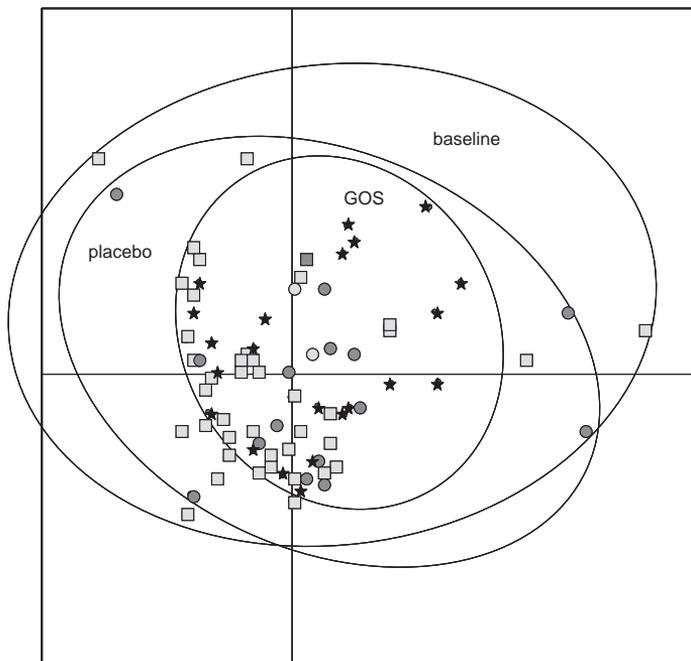


Fig. 3. A PCA plot of the composite denaturing gradient gel electrophoresis (DGGE) dataset (containing predominant bacterial, Erec-group, *Lactobacillus*-group, and bifidobacteria specific DGGE analyses) of baseline samples (light gray squares), samples after galacto-oligosaccharide (GOS) intervention (black stars), and samples after placebo intervention (dark gray dots).

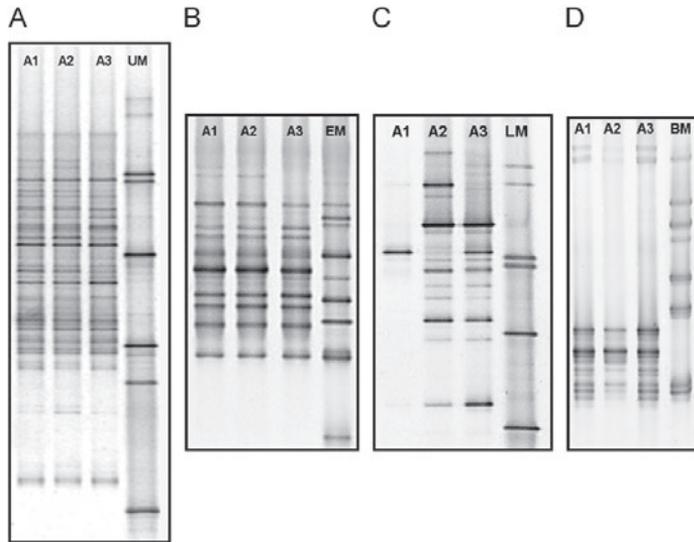


Fig. 4. Fecal denaturing gradient gel electrophoresis (DGGE) profiles of one healthy adult subject (female, 41 years old) as detected with predominant bacterial polymerase chain reaction (PCR)–DGGE (part of the study group of Saarela et al., 2007) (A); *Clostridium coccooides*–*Eubacterium rectale*-group specific PCR–DGGE (Maukonen et al., unpublished data) (B); *Lactobacillus*-group PCR–DGGE (Maukonen et al., unpublished data) (C); and bifidobacteria-specific PCR–DGGE (Maukonen et al., unpublished data) (D). The samples were obtained from fecal samples at time points baseline (A1), after 1-week probiotic capsule (containing *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *Lactobacillus acidophilus* LaCH-5, in addition to yoghurt starter bacteria *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) consumption (A2), and 2 weeks probiotic capsule consumption (A3); UM, universal marker; EM, Erec-marker; LM, lactobacilli-marker; BM, bifidobacteria-marker.

samples of both GOS- and placebo-yoghurt groups were included. Temporal stability of studied bacterial groups did not differ between elderly and younger adults (Table 1). Diversity of predominant bacterial population was found to be significantly higher ( $P < 0.001$ ) in elderly subjects as compared to younger adults ( $42 \pm 5$  amplicons vs.  $32 \pm 5$  amplicons, respectively; Table 2, Fig. 4A). In addition, diversity of Erec-group bacteria was significantly higher ( $P < 0.001$ ) in elderly subjects than in younger adults ( $21 \pm 4$  amplicons vs.  $15 \pm 2$  amplicons, respectively; Table 2, Fig. 4B). Diversity of *Lactobacillus*-group of elderly subjects ( $11 \pm 4$  amplicons) was somewhat higher than that of younger adults ( $8 \pm 4$ ;  $P = 0.09$ ; Fig. 4C), whereas diversity of bifidobacteria was similar in both elderly subjects and younger adults ( $9 \pm 3$  amplicons; Table 2, Fig. 4D).

#### 4. Discussion

The present study was designed as a cross-over study to minimize the effect of inter-individual variation on fecal microbiota. However, the 2-week wash-out period was not long enough for the present elderly subjects—probably due to their mild constipation—even though according to literature (Alander et al., 2001; Tannock et al., 2004) a 2-week wash-out period should have been sufficient for ingestion of GOS-supplemented foods. Therefore, we could not use the samples after the wash-out, which substantially

reduced the number of subjects in both study groups. In addition, since our new study groups composed of different subjects, we cannot exclude possible minor changes to the investigated bacterial populations, which could have been seen at a person level, but could not be seen at a group level. The possible side effects of GOS were not investigated in this study, but according to earlier studies, the amount of GOS used in this study (10 g per day) is well-tolerated (Sairanen, Piirainen, Nevala, & Korpela, 2007; Teuri & Korpela, 1998). In addition, only one GOS concentration was used, but no differences regarding the bifidogenic effect were found between different GOS doses in an earlier study by Bouhnik et al. (2004). The effect of age on gastro-intestinal microbiota has been investigated mainly by enumeration techniques, either by cultivation, fluorescent in situ hybridization, or by real-time PCR (e.g., Bartosch, Fite, Macfarlane, & McMurdo, 2004; He et al., 2003; Hopkins et al., 2001, 2002; Mitsuoka, 1992; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004), whereas stability and diversity of fecal microbiota of elderly subjects has received less attention (Blaut et al., 2002; Hayashi, Sakamoto, Kitahara, & Benno, 2003; He et al., 2003; Hopkins & Macfarlane, 2002). We found only one study in which DGGE was applied to follow the stability and diversity of human fecal microbiota—predominant bacteria and bifidobacteria—during GOS ingestion (Tannock et al., 2004). However, the ages of the subjects (15 subjects) belonging to the study

group were not defined. Therefore, we are not able to make correct comparisons between our study group and that of Tannock et al. (2004).

We did not observe any changes in the predominant fecal bacterial microbiota of elderly subjects after the GOS-yoghurt intervention period. The similarity percentages remained similar in both placebo- and GOS-yoghurt groups. This has been observed also by Tannock et al. (2004), who found that GOS-containing biscuits had an effect in the RNA-derived predominant bacterial DGGE profiles but not in the DNA-derived DGGE profiles in New Zealand subjects. In addition, it has been shown on younger adults that the probiotics, which were included in the test yoghurt, did not have a great effect on the stability of the fecal predominant bacteria (Saarela et al., 2007). Species diversity in fecal microbiota increases with age (Blaut et al., 2002). The increase in predominant bacterial species diversity in elderly subjects was observed also in our study. The average number of amplicons found from elderly subjects was significantly higher than the average number of amplicons in younger adults in our previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). In addition, the predominant bacterial DGGE profiles of elderly subjects in our study were different according to visual inspection as compared with DGGE profiles of younger adults in previous studies (Maukonen et al., 2006a, 2006b; Saarela et al., 2007). There were amplicons throughout the DGGE profile (from top to bottom) derived from feces of elderly subjects (Fig. 2A), whereas in the DGGE profiles of younger adults (Fig. 4A) there usually has been a small empty gap between the upper part of the DGGE gel (low-medium GC-content; most of the adult fecal microbiota) and the lower part of the gel (high GC-content; bifidobacteria and sulfate-reducing bacteria) (Maukonen et al., 2006a, 2006b; Saarela et al., 2007).

We did not find any difference in the stability or diversity of bacteria belonging to the Erec-group between the GOS- and placebo-yoghurt groups. The number of bacteria belonging to the Erec-group—as detected with FISH (fluorescent in situ hybridization)—has been shown to be lower in Dutch and Italian elderly subjects compared with younger adults (He et al., 2003; Mueller et al., 2006). On the contrary, the number of bacteria belonging to the Erec-group was higher in the French, German, and Swedish elderly subjects when compared with younger adults (Mueller et al., 2006). Since Finnish diets are close to Swedish diets, it could be expected that the number of bacteria belonging to the Erec-group would be higher in the elderly subjects than in the younger adults. We did not quantify the number of Erec-group bacteria, but we found significantly more amplicons from the elderly subjects in this study than we did from younger adults in our previous study (Maukonen et al., 2006b). In addition, Maukonen et al. (2006b) showed that each amplicon corresponded to a phylogenetic cluster, and therefore the diversity of fecal Erec-group of elderly subjects was higher than that of

younger adults in our study. Contradictory to our findings, Hayashi et al. (2006) found as many operational taxonomic units (OTUs) of Erec-group bacteria in Japanese elderly and younger adults.

Since the number of bifidobacteria (Hopkins et al., 2001, 2002; Mitsuoka, 1992; Mueller et al., 2006; van Tongeren et al., 2005; Woodmansey et al., 2004) and the diversity of bifidobacterial population (Hopkins & Macfarlane, 2002; Woodmansey et al., 2004) have been reported to decrease with age, we wanted to study the effect of GOS-yoghurt on the bifidobacterial population. We did not find any difference in stability or diversity between the GOS- and placebo-yoghurt groups in agreement with Tannock et al. (2004) and Satokari, Vaughan, Akkermans, Saarela, and de Vos (2001b). In addition, the average number of amplicons that we found from elderly subjects was similar to the number of amplicons in healthy younger adults (Maukonen, unpublished results), which is not in agreement with the earlier culture based studies (Hopkins & Macfarlane, 2002; Woodmansey et al., 2004). We used a PCR–DGGE which did not target the ingested *B. animalis* subsp. *lactis* (Satokari et al., 2001a, 2001b), so this strain was not seen in the bifidobacterial DGGE-profiles, and therefore did not affect the diversity and temporal stability of the fecal bifidobacterial profiles.

Lactobacilli population has been shown to be temporally unstable in adults (Vanhoutte et al., 2004). We found the fecal lactobacilli population of elderly subjects to be unstable as well. Since the ingested *L. acidophilus* produced only a single band in the lactobacilli DGGE-profile, it did not constitute a considerable change of the similarity values between the samples. The lactobacilli population of the GOS-yoghurt group subjects was more unstable than that of the placebo-yoghurt group in our study. However, the difference was not statistically significant due to high standard deviation between subjects. Species diversity of fecal lactobacilli was similar in both study groups. In addition, we did not find a significant difference between the number of amplicons in elderly subjects and in younger adults of our previous studies (Maukonen, unpublished results).

## 5. Conclusions

Consumption of probiotic yoghurt containing galacto-oligosaccharide (GOS) for 3 weeks did not significantly affect the diversity or temporal stability of predominant bacterial, bifidobacterial, *Lactobacillus*-group or *Eubacterium rectale*—*Clostridium coccoides* group (Erec-group) fecal populations in elderly subjects. On average, the Erec-group bacteria remained stable, the predominant bacterial and bifidobacterial populations remained reasonably stable and the *Lactobacillus*-group was unstable in both study groups. Moreover, the difference in temporal amplicon diversity of the GOS-yoghurt group was smaller in all studied bacterial groups as compared to the placebo-yoghurt group.

We did not find any differences between elderly and younger adults in temporal stability of studied bacterial populations. However, the difference in diversity of predominant bacterial population and Erec-group bacteria was found to be significantly higher in elderly subjects as compared to younger adults.

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