

The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition

Harri Mäkiyuokko^{1,2}, Kirsti Tiihonen¹, Soile Tynkkynen³, Lars Paulin⁴ and Nina Rautonen^{1*}

¹Danisco Finland Oy, Sokeritehtaantie 20, FIN-02460 Kantvik, Finland

²Finnish Red Cross Blood Service, Research and Development, Kivihaantie 7, FIN-00310 Helsinki, Finland

³Valio Ltd, Research and Development, Meijeritie 4, FIN-00370 Helsinki, Finland

⁴Institute of Biotechnology, University of Helsinki, Viikinkaari 9, FIN-00014 Helsinki, Finland

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Ageing has been suggested to cause changes in the intestinal microbial community. In the present study, the microbiota of a previously well-defined group of elderly subjects aged between 70 and 85 years, both non-steroidal anti-inflammatory drugs (NSAID) users (n 9) and non-users (n 9), were further compared with young adults (n 14) with a mean age of 28 years, by two DNA-based techniques: percentage guanine + cytosine (%G + C) profiling and 16S rDNA sequencing. Remarkable changes in microbiota were described with both methods: compared with young adults a significant reduction in overall numbers of microbes in both elderly groups was measured. Moreover, the total number of microbes in elderly NSAID users was higher than in elderly without NSAID. In 16S rDNA sequencing, shifts in all major microbial phyla, such as lower numbers of Firmicutes and an increase in numbers of Bacteroidetes in the elderly were monitored. On the genus level an interesting link between reductions in the proportion of known butyrate producers belonging to *Clostridium* cluster XIVa, such as *Roseburia* and *Ruminococcus*, could be demonstrated in the elderly. Moreover, in the Actinobacteria group, lower numbers of *Collinsella* spp. were evident in the elderly subjects with NSAID compared both with young adults and the elderly without NSAID, suggesting that the use of NSAID along with age may also influence the composition of intestinal microbiota. Furthermore, relatively high numbers of *Lactobacillus* appeared only in the elderly subjects without NSAID. In general, the lowered numbers of microbial members in the major phyla, Firmicutes, together with changes in the epithelial layer functions can have a significant effect on the colon health of the elderly.

Non-steroidal anti-inflammatory drugs: Ageing: Percentage guanine + cytosine: Microbiota

The human gastrointestinal tract harbours a highly diverse population of commensal microbes, estimated as 10^{14} bacterial cells, participating in the breakdown of indigested food^(1,2). The composition of the microbiota develops during the first years of life and remains relatively stable throughout adulthood⁽³⁾. Interestingly, after birth the maturation of immune responses reflects the microbial colonisation in the intestine^(4,5). It has been suggested that towards seniority specific changes in the microbial community⁽⁶⁾ and immune defence occur again⁽⁷⁾. In the elderly, immune responses are weakened, predisposing seniors to a higher incidence of morbidity and mortality by infectious diseases^(8,9). Furthermore, the common use of non-steroidal anti-inflammatory drugs (NSAID) among the elderly is known to increase gastrointestinal ailments^(10–12). However, the elderly immune responses can be enhanced by supplementing the diet with probiotics, implying that not only in children are the immune responses modified by intestinal bacteria⁽¹³⁾. With improved life expectancy the share of the population over the age of 60 years is estimated to reach 2 billion by 2050⁽¹⁴⁾. In order to improve the quality of life of the growing elderly population and

decrease health-care costs, the role of intestinal microbes in health necessitates deeper understanding.

Age- and country-specific differences in intestinal microbiota have been reported by several studies. Most commonly, decreasing numbers of bifidobacteria and increasing numbers of enterobacteria have been suggested^(2,6,15,16) in the elderly. Some reports indicate changes also in the *Lactobacillus* and *Bacteroides* groups⁽¹⁷⁾. Recently, also a reduced microbial activity has been described in elderly subjects^(17,18). It is noteworthy that changes in the microbial composition have not only been linked to age but also to a condition with multiple health risk factors, namely obesity⁽¹⁹⁾, and several gastrointestinal diseases such as Crohn's disease, irritable bowel disease and irritable bowel syndrome (IBS)^(20,21). This underlies the importance of increasing knowledge of the intestinal microbial composition.

The availability of microbial DNA sequences has revealed that a great proportion of the gastrointestinal microbes belong to unknown and uncultivable genera and species⁽²²⁾. Thus it is important to include DNA-based methods that are independent of prior knowledge of bacteria when evaluating

Abbreviations: %G + C, percentage guanine + cytosine; IBS, irritable bowel syndrome; NSAID, non-steroidal anti-inflammatory drug; RDP, Ribosomal Database Project; S_ab-score, RDP sequence match score.

* **Corresponding author:** Dr Nina Rautonen, fax +358 9 298 2203, email nina.rautonen@danisco.com

the overall changes in the microbial communities. The percentage guanine + cytosine (%G + C) profiling technique has been previously used to reveal changes in the microbial community structure^(20,21,23–25). The technique exploits the diverging weight of microbial genomes due to different proportions of guanidine and cytosine bases in their genomes, and thus needs no prior information of specific sequences, allowing analysis of previously uncultivated microbes. The 16S rDNA sequencing technique, however, requires some prior information of the microbes present in the sample, since the broad-range primers used by the technique are common for known cultivated bacteria⁽²⁶⁾. The combination of these two methods can provide useful information from samples with mixed populations containing hundreds of different species. In fact, the two methods have been used successfully previously in describing differences in the microbiota between IBS patients and healthy subjects⁽²¹⁾.

In the present study we have characterised the intestinal microbiota of a subgroup of previously well-defined elderly subjects, with or without NSAID, and young adults⁽¹⁷⁾ using %G + C profiling and 16S rDNA sequencing techniques in order to expose differences caused by age and NSAID use.

Materials and methods

Study subjects

A total of nine elderly NSAID users (mean age 80 (range 77–85) years) and nine elderly non-users (mean age 78 (range 70–83) years) living in a nursing home and fourteen young adults (mean age 28 (range 21–39) years) were recruited to the study in the city of Kuopio, Finland. The number of study subjects per group was originally planned to be fifteen but due to an unexpected high number of drop-outs, such as use of antibiotics, or diarrhoea, the number of especially elderly study subjects decreased. Significant differences in microbial composition and metabolites were found for the two elderly groups⁽¹⁷⁾, prompting the separate analysis of the sequence data for the elderly NSAID users and non-users. The elderly were defined as NSAID users when they used the medication three or more times per week. The NSAID used included indomethacin, ibuprofen, ketoprofen, diclofenac, piroxicam, celecoxib, nimesulid and aspirin. The exclusion criteria were critical illnesses, inflammatory bowel disease, coeliac diseases and major malignancies in the gastrointestinal tract. Subjects using antibiotics during the study or within 2 months before the screening were excluded. The use of probiotic products was not allowed during the study. The inclusion and exclusion criteria were monitored during the run-in period before randomisation.

Throughout the sampling both the elderly and the young study subjects followed their habitual diet and most of them used the uniform meal service provided at least once per d. The calculated dietary intakes of the elderly living in the nursing home, based on the diets provided, were: energy, 6.17 MJ/d (6.5 MJ/d fibre included); protein, 66 g/d (18% energy); fat, 52 g/d (32% energy); cholesterol, 146 mg/d; carbohydrates, 181 g/d (50% energy); dietary fibres, 20 g/d.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the

Research Ethics Committee of the Hospital Districts of Northern Savo, Finland. The purpose of the study was explained to the participants, and written consent to the study was given by all of the subjects. Information on the age, sex, bowel function, diseases, allergies, current medication and pre-trial use of probiotics of the study subjects was obtained at the beginning of the study. The pre-trial characteristics of the study groups are presented in Table 1.

Faecal samples

Spot samples of faeces were obtained three times from each subject over 3 consecutive weeks. Subjects were personally instructed to take the faecal samples according to the written protocol. All the equipment and documentation papers were provided to the subjects before sampling. All faecal samples were initially stored at -20°C , and, transferred to the laboratory within 12 h of defecation and stored at -70°C until analysed. The three samples obtained from each study subject were pooled to result in a representative individual sample.

Percentage guanine + cytosine profiling

Total genomic DNA of faecal microbes was extracted as previously described⁽¹⁷⁾. In short, 1 g of faecal material was washed, microbes were collected and freeze-thawed to loosen their cell membranes, after which the microbes were lysed, the cell debris was removed and genomic DNA was precipitated.

The composition of microbiota in the faecal samples was monitored by %G + C profiling⁽²⁴⁾. The method enables fractionation of total microbial DNA into bacterial subpopulations by their genomic G + C content. For fractionation, three individual DNA samples were pooled for each elderly donor pool (five pools in total; three pools of elderly without NSAID and two with NSAID) and four or five individual DNA samples in each of the young adult donor pools (three pools in total). One pool of three subjects for the elderly with NSAID group had to be removed from the %G + C-profile analysis due to breakdown of genomic DNA into smaller fragments during the extraction phase, making %G + C-profiling of that particular pool inaccurate compared with pools with intact DNA. However, this fragmented pool was included in the sequencing procedure, in which only intact 16S rDNA was needed. Each of the pools contained 300 μg genomic DNA.

Microbial enumeration

The total bacteria cell counts in faecal samples were determined by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA) as described previously⁽²⁴⁾. The bacterial fractions were recovered by suspending faecal samples in a buffer, followed by centrifugation and washing. The cell samples were diluted, fixed and stained with the fluorescent nucleic acid-binding dye SYTO 24 (Molecular Probes, Leiden, The Netherlands). The results are expressed as the quantity of bacteria/g faeces.

Sequencing procedure

For sequencing, six fractions containing %G + C areas of 20–30, 30–40, 40–50, 50–60, 60–70 and 70–80 %G + C

Table 1. Sex, age, bowel function and medication of the thirty-two study subjects

	Elderly, no NSAID (n 9)	Elderly, with NSAID (n 9)	Young adults (n 14)
Subjects (n)			
Men	3	3	9
Women	6	6	5
Age (years)			
Mean	77.8	80.2	28.1
SD	4.1	2.2	6.5
Defecation frequency (times/d)			
Mean	0.5	0.5	1.6
SD	0.2	0.2	0.7
Quality of faeces (n)			
Very loose	–	–	–
Loose	–	1	1
Normal	5	5	13
Hard	4	3	–
Very hard	–	–	–
Medication use (n)*			
Laxatives†	5	5	–
For CVD‡	6	6	–
For alimentary canal and urinary tract disorders§	6	5	–
For psychological disorders	8	4	–
For neurological disorders¶	5	3	–

NSAID, non-steroidal anti-inflammatory drugs.

* Several drugs can have gastrointestinal side effects. No side effects by the medications used by study subjects were observed.

† Macrocol, sodium bicarbonate, sodium chloride, potassium chloride, sodium sulfate, lactulose, sodium picosulfate (common (> 1/100) gastrointestinal side effects: diarrhoea; others – flatus).

‡ Enalapril, bisoprolol, spironolactone, losartan, aspirin, warfarin, metoprolol, dipyridamole + aspirin, bisoprolol, clopidogrel, dipyridamole, dipyridamole, furosemide, isosorbide dinitrate, indapapamide, propranolol (common (> 1/100) gastrointestinal side effects: diarrhoea; others – flatus, vomiting, gastrointestinal bleeding).

§ Magnesium hydroxide, finasteride, ranitidine, macrocol, sodium bicarbonate, sodium chloride, potassium chloride, sodium sulfate, lactulose, sodium picosulfate (common (> 1/100) side effects: diarrhoea, constipation; others – flatus).

|| Olanzapine, mirtazapine, oxazepam, risperidone, melperone, thioridazine, chlorprothixene, temazepam, zopiclone, quetiapine, citalopram (very common (> 1/10) gastrointestinal side effects: constipation; others – flatus, diarrhoea, vomiting).

¶ Galantamine, oxcarbazepine, levodopa + benserazide, entacapone, pramipexole, donepezil, memantine, sodium valproate, memantine, rivastigmine (very common (> 1/10) gastrointestinal side effects: vomiting, diarrhoea; others – constipation).

were collected from the pools. All corresponding %G + C fractions in one study group (i.e. young adults, elderly and elderly with NSAID) were pooled, resulting in six %G + C fractions for each study group, and totally eighteen fractions. The DNA of all the eighteen fractions were used as templates to amplify 900 bp 16S rDNA regions for sequencing. The PCR amplification was performed using a Dynazyme II polymerase kit (Finnzymes, Espoo, Finland) according to the manufacturer's instructions and broad-range primers pA 5'-AGAGTTTGATCCTGGCTCAG-3' and pE/5'-CCGTC AATTCCTTTGAGTTT-3'^(26,27) corresponding to the 8–28 and 928–908 positions in the *Escherichia coli* 16S rDNA sequence. Amplification products were purified with Sephadryl S-400 colon (Amersham Biosciences, Little Chalfont, Bucks, UK) and cloned in pDrive cloning vector (Qiagen, Hilden, Germany) in *E. coli*. Colonies were picked and sequenced from both directions with T7 and SP6 primers using BigDye Chemistry and analysed on an ABI 3730 Capillary DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were assembled and edited using Gap4 from the Staden package⁽²⁸⁾.

Spermidine analysis

The spermidine concentrations in faeces were analysed using the method described by Saarinen⁽²⁹⁾.

Phylogenetic and statistical analysis of sequence data

%G + C fractions were analysed with ANOVA applying linear mixed models⁽³⁰⁾ inside each fraction, and then computing *post hoc t* tests whenever a significant difference between the groups was found. Spermidine data were analysed with ANOVA applying a linear model and *post hoc t* tests. The sequences were phylogenetically aligned with the Ribosomal Database Project (RDP) II My RDP on-line-tool (<https://rdp.cme.msu.edu/login/myrdp>) and statistically compared using the RDP II Library Compare Tool Analysis on-line-tool (<http://rdp.cme.msu.edu/seqmatch/>) with a 95 % confidence threshold for genus determination, which uses a RDP-naive Bayesian classifier to provide rapid classification of library sequences into the bacterial taxonomy. The equality of the sequence proportions between the groups in Fig. 1 was tested with a standard multi-population proportions test whenever all the frequencies were larger than five, and if statistically significant non-equality was found, the proportions were analysed further with a pair-wise proportions test. If the proportions were smaller than five, a special proportions test for small counts was applied as explained in Wang *et al.*⁽³¹⁾, and first introduced for digital gene expression profiles in Audic & Claverie⁽³²⁾. The comparison analysis enabled the calculation of the most abundant strain of each genus using a similarity score or a sequence match score (S_ab) comparison reporting the number of (unique) seven-base oligomers shared between an input sequence

	Elderly with NSAID	Elderly without NSAID	Young adults		Elderly with NSAID	Elderly without NSAID	Young adults
Firmicutes	501	545	696	Actinobacteria	13	70	69
<i>Ruminococcus</i>	82	85	152	<i>Eggerthella</i>	4	10	8
<i>Faecalibacterium</i>	49	55	83	<i>Collinsella</i>	2	42	47
<i>Roseburia</i>	34	18	69	<i>Bifidobacterium</i>	1	2	0
<i>Subdoligranulum</i>	21	29	44	<i>Corynebacterium</i>	1	0	0
<i>Oscillospira</i>	19	24	4	<i>Actinomyces</i>	0	3	4
<i>Eubacterium</i>	16	19	25	<i>Olsenella</i>	0	1	0
<i>Streptococcus</i>	13	19	4	<i>Slackia</i>	0	1	0
<i>Coprococcus</i>	11	7	16	<i>Propionibacterium</i>	0	0	1
<i>Dorea</i>	10	16	36	Unclassified Actinobacteria	5	11	9
<i>Coprobacillus</i>	8	7	34	Bacteroidetes	71	72	35
<i>Lactobacillus</i>	7	50	0	<i>Bacteroides</i>	28	44	24
<i>Sporobacter</i>	5	8	1	<i>Prevotella</i>	7	2	4
<i>Papillibacter</i>	4	1	2	<i>Alistipes</i>	6	6	3
<i>Phascolarctobacterium</i>	2	5	2	<i>Parabacteroides</i>	6	4	1
<i>Sporacetigenium</i>	2	0	0	<i>Rikenella</i>	1	2	0
<i>Acetanaerobacterium</i>	1	0	0	Unclassified Bacteroidetes	23	14	3
<i>Acetivibrio</i>	1	0	0	Proteobacteria	8	3	1
<i>Anaerostipes</i>	1	2	6	<i>Desulfovibrio</i>	3	0	0
<i>Anaerotruncus</i>	1	0	0	<i>Bilophila</i>	1	0	0
<i>Anaerovorax</i>	1	3	1	<i>Citrobacter</i>	1	0	0
<i>Butyrivibrio</i>	1	0	4	<i>Shigella</i>	1	1	0
<i>Catenibacterium</i>	1	0	1	Unclassified Proteobacteria	2	2	1
<i>Clostridium</i>	1	6	0	Defembacteres	9	5	0
<i>Dialister</i>	1	0	16	<i>Synergistes</i>	9	5	0
<i>Lachnospira</i>	1	0	1	Venemicrobia	0	0	1
<i>Mogibacterium</i>	1	2	0	<i>Akkermansia</i>	0	0	1
<i>Holdemania</i>	0	1	1	Unclassified sequences	8	7	1
<i>Lactococcus</i>	0	1	6	Total query sequences	610	702	803
<i>Leuconostoc</i>	0	1	0	Unclassified/total sequences	40 %	31 %	25 %
<i>Peptococcus</i>	0	1	0				
<i>Syntrophococcus</i>	0	1	0				
<i>Veillonella</i>	0	0	3				
<i>Enterococcus</i>	0	1	0				
Unclassified Firmicutes	207	183	185				

Fig. 1. Numbers of partial 16S rRNA gene sequences obtained from the three different study groups organised into bacterial phyla by the Ribosomal Database Project (RDP) II My RDP on-line-tool (<https://rdp.cme.msu.edu/login/myrdp>). ↔, Statistical significance determined by standard proportions test ($P < 0.001$) between two study groups. NSAID, non-steroidal anti-inflammatory drugs.

and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences. In addition to RDP software (Center for Microbial Ecology at Michigan State University, East Lansing, MI, USA), the statistical programming language R was used to perform analyses (<http://www.r-project.org/>).

Results

The total microbial numbers and the corresponding %G + C-profiles were compared to reveal the proportional differences

in the total microbiota profiles of the three study groups (Fig. 2). The young adult group had statistically higher numbers of microbes in all cumulated 10 %G + C-fractions between the 30–69 %G + C area when compared with either of the elderly groups ($P < 0.001$ and $P < 0.0003$, respectively, in each 5 % fraction) and in the 20–29 %G + C area when compared with the elderly group without NSAID ($P < 0.006$). The two elderly groups differed from each other in the 20–59 %G + C area ($P < 0.0002$, respectively, in each 5 % fraction), the microbial numbers being higher in the elderly group with NSAID.

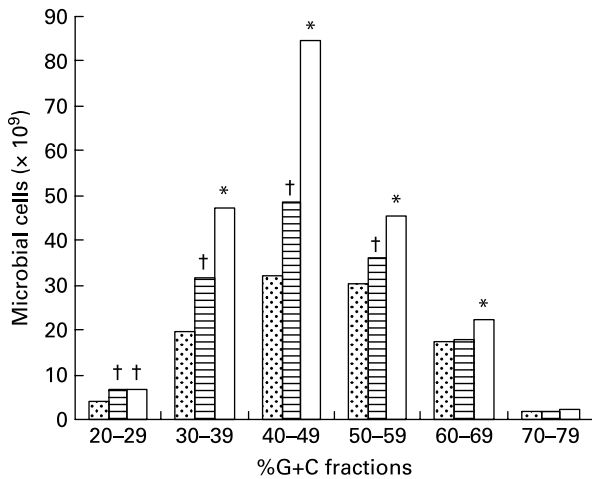


Fig. 2. Average number of microbes in the percentage guanine + cytosine (%G + C) fractions in the three study groups: elderly subjects without non-steroidal anti-inflammatory drugs (NSAID) (▨); elderly subjects with NSAID (▨); young adults (□). Due to problems in DNA extraction (see Materials and methods), the final number of subjects included in the DNA pool of the elderly with NSAID was six, and nine from the elderly without NSAID. Statistically significant differences were determined by ANOVA and *post hoc t* tests. * Mean value was significantly different from those of the two elderly groups ($P < 0.01$). † Mean value was significantly different from that of the elderly group without NSAID ($P < 0.01$).

A total of 2112 high-quality sequences were obtained by sequencing of the six fractionated DNA samples of each study group. The total sums of sequences obtained were 803 sequences in the young adults, 702 sequences in the elderly without NSAID and 610 sequences in the elderly with NSAID. In Fig. 1 these aligned sequences are divided into six phyla and fifty-one presently known microbial genera; the bacterial genera differing significantly ($P < 0.001$) between the study groups are marked with arrows. The majority of the microbes in each study group were found to be associated to the Firmicutes phylum followed by the Bacteroidetes and Actinobacteria phyla. Comparison of young adults with either of the elderly groups revealed more differences in the number of genus-specific sequences than the comparison of the two elderly groups. The young adults exhibited higher faecal numbers of *Rumicoccus*, *Roseburia*, *Coprobacillus* and *Dialister* spp., all belonging to Firmicutes, than the elderly without NSAID, suggesting an age-dependent shift in genera. *Lactobacillus*, *Streptococcus* and *Bacteroides* were present in higher numbers in the elderly. Compared with the elderly NSAID users, the samples obtained from the young adults exhibited more *Dialister*, *Coprobacillus* and *Collinsella*. The significant differences between the elderly groups were the higher amounts of *Lactobacillus* and *Collinsella* in the elderly without NSAID and *Roseburia* in the NSAID users.

In order to indicate the degree of match of study sequences to each named phylotype in the RDP-II database (<http://rdp.cme.msu.edu/>), S_{ab} -scores were calculated on single species in each genus with statistical significance between study groups present in Fig. 1; species with S_{ab} -scores higher than 0.97 were considered significant. The S_{ab} -scores for the *Ruminococcus* and *Coprobacillus* genera had no significant matches, while the *Roseburia*, *Lactobacillus*, *Streptococcus*, *Dialister*, *Bacteroides* and *Collinsella* genera

each had one or two matching strains with the RDP-II database; the most abundant bacterial species were, respectively, *Roseburia intestinalis*, *Lactobacillus ruminis*, *L. sobrius*, *Streptococcus lutensis*, *Dialister invisus*, *Bacteroides coprocola*, *B. dorei* and *Collinsella aerofaciens*.

The concentrations of spermidine in the faeces of the elderly (median 76.7 and 67.6 nmol/g faeces, respectively, in the elderly without NSAID and with NSAID) were monitored to be significantly lower when compared with the concentrations in samples obtained from the younger adults (median 160.4 nmol/g faeces; $P = 0.004$ and $P = 0.007$, respectively).

Discussion

In the present study we characterised the intestinal microbiota of a subgroup of previously well-defined elderly subjects, with or without NSAID drugs, and young adults⁽¹⁷⁾ using %G + C profiling and 16S rDNA sequencing techniques in order to expose differences caused by age and NSAID use. Our previous study⁽¹⁷⁾ focused mainly on the microbial metabolism and intestinal immunological status but analysed also differences in faecal microbiota. The study showed that specific immune responses as well as generally the microbial metabolism were decreased, and furthermore that, for example, the proportion of facultative anaerobic microbes was increased in the elderly.

The %G + C-profiling technique has recently been used as a tool for describing the total microbial community in human^(21,25), mouse⁽²³⁾ and piglet⁽³³⁾ intestines. The %G + C-profiling enables microbiota analysis that includes previously unknown or presently unculturable microbial genera since it exploits only the different proportion of guanidine and cytosine bases in their genomes. Moreover, %G + C fractioning of microbiota DNA before 16S rRNA gene sequencing enhances the possibility of less abundant microbial species to be revealed in specific fractions.

The obtained %G + C-profiles support the sequencing data well by depicting that young adults have the highest numbers of microbes especially in the Firmicutes-rich %G + C area (low %G + C bacteria). In the young adults 87% of sequences were classified to Firmicutes. Similarly, in the elderly groups, 78–82% of the sequences grouped to this phylum; however, the significantly higher number of bacteria in the NSAID users shown in %G + C profiling did not result in higher numbers of sequences. Also in the high %G + C areas (>50%G + C), a difference between the %G + C and sequencing data can be noted. In the elderly NSAID users the 50–59%G + C area was significantly higher compared with that of the non-users, but our sequencing data do not reveal an increase in bacterial species. As %G + C profiling is a totally culture- and probe-independent analysis method portraying only the concentration differences of the microbial genomic DNA, this discrepancy is most probably due to higher numbers of bacteria belonging to the same genera or problems in amplifying high-G + C sequences, which affected especially the *Bifidobacterium* genus. One explanation for the lack of bifidobacteria in the present study is due to the primer used in PCR. The pE' primer 5'-CCGTCAATTCCTTTGAGTTT-3' has a mismatch C/T (highlighted in bold) in the middle. This mismatch can abolish the amplification of bifidobacteria 16S rRNA genes. Others have also reported the lack

of bifidobacteria in 16S rRNA libraries, possibly due to mismatches in primers or imperfect DNA denaturation during PCR^(15,34).

The %G + C profiling showed that the young adults had statistically higher numbers of microbes in the %G + C area of Firmicutes, Bacteroidetes and Actinobacteria, the major phyla reported in the human intestine, compared with either elderly group. The most prominent difference dependent on age, however, was a clear reduction in numbers of *Ruminococcus* and *Roseburia* spp., the known butyrate producers in *Clostridium* cluster XIVa, in the elderly. This is consistent with our earlier studies showing a decrease in the *Clostridium coccoides*–*Eubacterium rectale* group in the elderly⁽¹⁷⁾. Similarly, the elderly with NSAID had higher numbers of bacteria in most %G + C fractions compared with the non-users. Therefore, both ageing and the use of specific medication can deeply alter the abundance and composition of the microbial community. The sequencing revealed higher amounts of lactobacilli in the elderly groups compared with the young adults, which is in accordance with our earlier findings⁽¹⁷⁾.

The major anaerobic microbes in the gastrointestinal tract are *Bacteroides* species. These organisms can function as symbiotic partners in the host physiology or they can cause serious diseases⁽³⁵⁾. Recent studies have indicated the link between levels of the *Bacteroides* and Firmicutes and obesity^(36–38). Ley *et al.*⁽³⁶⁾ showed that obesity alters gut microbiota by increasing the relative abundance of Firmicutes over Bacteroidetes. Evidently in mice with diet-induced obesity the Western diet nutrient milieu provides a competitive advantage to the Firmicutes⁽³⁸⁾.

Interestingly, also in the present study, major shifts in the microbiota occurred in the levels of Bacteroidetes and Firmicutes, suggesting perhaps differences in the diet of the elderly and young adults and also in the physiology of the intestinal tract. This is consistent with our earlier finding of a higher proportion of anaerobic bacteria in faecal samples from elderly subjects^(17,18). Furthermore, some of the major differences between the microbiota of healthy elderly and young adults reported previously in German⁽²⁾ and Japanese populations⁽¹⁵⁾ correlate with those presented in the present study, that is, a higher proportion of *Bacteroides* in the elderly and a higher proportion of *Ruminococcus* in the young adults. However, Italian elderly were reported to have lower levels of *Bacteroides* when compared with young adults⁽²⁾. These results indicate strong country specificity in diversity of the elderly microbiota.

The metabolic activity of the intestinal microbes has been reported by us to be decreased in elderly subjects^(17,18). Changes in the microbial community structure described in the present study may offer some explanations or hypotheses to the detected differences. *R. intestinalis* and *Eubacterium rectale* are known butyrate-producing species belonging to Firmicutes^(39,40). *Roseburia* sp. can convert acetate to butyrate, which is the major energy source for colonocytes and has been reported to reduce risk for developing colon cancer and colitis⁽⁴¹⁾. During a low-carbohydrate diet, the levels of *Roseburia* and *Eubacterium rectale* decrease, causing a drop in butyrate concentrations⁽⁴²⁾, which emphasises the importance of the diet. In the present study, reduction of *Roseburia* was described in the elderly, and it may partially explain the described reduced concentrations of butyrate in elderly

subjects compared with young adults as reported by Tiihonen *et al.*⁽¹⁷⁾. *C. aerofaciens* is abundantly found in the human intestine⁽⁴³⁾. Kassinen *et al.*⁽²¹⁾ reported that the amount of *Collinsella* in IBS patients was significantly reduced. In the present study the number of *C. aerofaciens* was reduced in the elderly subjects with NSAID, while elderly subjects without NSAID and young adults had high numbers of *C. aerofaciens*. The use of NSAID in the study with IBS patients was not recorded but due to common gastrointestinal discomfort and pain in subjects with IBS, NSAID may have been used by the subjects.

Ruminococci are abundant in the human gastrointestinal tract, comprising 2.5 % of total bacteria⁽⁴⁴⁾. These *Clostridium coccoides* group XIVa-belonging bacteria are cellulolytic, found on solid particles in faeces⁽⁴⁵⁾ and have been linked to acetate and butyrate production⁽⁴⁶⁾. Furthermore, *R. intestinalis* has been suggested to be a polyamine producer⁽⁴⁷⁾. Putrescine, spermidine and spermine are important for cell proliferation and differentiation and mucosal barrier function (for reviews, see Thomas & Thomas⁽⁴⁸⁾ and Larque *et al.*⁽⁴⁹⁾). In the present study we found that the concentration of spermidine in the faecal samples was decreased in the elderly individuals when compared with the young adults. The reduced production of this polyamine, which is essential for the maintenance and function of rapidly renewing gastrointestinal epithelia^(50,51), can most probably alter the structure of the epithelial layer, thus affecting the adherence of the commensal intestinal microbiota, and therefore reducing the overall microbial numbers in the gut. Lowered microbial numbers, together with an altered epithelial layer surface, can have a significant effect on the digestibility and breakdown of food components entering the colon, enhancing development of constipation and reducing nutrient intake.

In conclusion, further research using a larger set of faecal donors of different ages could confirm the relationship between changes in shares of specific species and the consequences in their activity, especially regarding metabolism of important compounds for colon health, such as butyrate or spermidine. We propose that in future research, measurements of, for example, specifically two prominent intestinal strains, *R. intestinalis* and *C. aerofaciens*, should be included as potentially useful biomarkers for monitoring changes in the intestinal conditions.

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References

- Hooper LV, Midtvedt T & Gordon JI (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu Rev Nutr* **22**, 283–307.
- Mueller S, Saunier K, Hanisch C, *et al.* (2006) Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol* **72**, 1027–1033.
- Ouwehand A, Isolauri E & Salminen S (2002) The role of the intestinal microflora for the development of the immune system in early childhood. *Eur J Nutr* **41**, Suppl. 1, I32–I37.
- Bauer E, Williams BA, Smidt H, *et al.* (2006) Influence of dietary components on development of the microbiota in single-stomached species. *Nutr Res Rev* **19**, 63–78.
- Rautava S & Walker WA (2007) Commensal bacteria and epithelial cross talk in the developing intestine. *Curr Gastroenterol Rep* **9**, 385–392.
- Benno Y, Endo K, Mizutani T, *et al.* (1989) Comparison of fecal microflora of elderly persons in rural and urban areas of Japan. *Appl Environ Microbiol* **55**, 1100–1105.
- Gill HS, Darragh AJ & Cross ML (2001) Optimizing immunity and gut function in the elderly. *J Nutr Health Aging* **5**, 80–91.
- Kumar R & Burns EA (2008) Age-related decline in immunity: implications for vaccine responsiveness. *Expert Rev Vaccines* **7**, 467–479.
- van Duin D & Shaw AC (2007) Toll-like receptors in older adults. *J Am Geriatr Soc* **55**, 1438–1444.
- Bjarnason I, Hayllar J, MacPherson AJ, *et al.* (1993) Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. *Gastroenterology* **104**, 1832–1847.
- Gabriel SE, Jaakkimainen RL & Bombardier C (1993) The cost-effectiveness of misoprostol for nonsteroidal antiinflammatory drug-associated adverse gastrointestinal events. *Arthritis Rheum* **36**, 447–459.
- Laine L, Connors LG, Reicin A, *et al.* (2003) Serious lower gastrointestinal clinical events with nonselective NSAID or coxib use. *Gastroenterology* **124**, 288–292.
- Gill HS, Rutherford KJ, Cross ML, *et al.* (2001) Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *Am J Clin Nutr* **74**, 833–839.
- Cohen JE (2003) Human population: the next half century. *Science* **302**, 1172–1175.
- Hayashi H, Sakamoto M, Kitahara M, *et al.* (2003) Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. *Microbiol Immunol* **47**, 557–570.
- Bartosch S, Fite A, Macfarlane GT, *et al.* (2004) Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl Environ Microbiol* **70**, 3575–3581.
- Tiihonen K, Tynkynen S, Ouwehand A, *et al.* (2008) The effect of ageing with and without non-steroidal anti-inflammatory drugs on gastrointestinal microbiology and immunology. *Br J Nutr* **100**, 130–137.
- Woodmansey EJ, McMurdo ME, Macfarlane GT, *et al.* (2004) Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Appl Environ Microbiol* **70**, 6113–6122.
- Turnbaugh PJ, Ley RE, Mahowald MA, *et al.* (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031.
- Malinen E, Rinttilä T, Kajander K, *et al.* (2005) Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* **100**, 373–382.
- Kassinen A, Krogius-Kurikka L, Makivuokko H, *et al.* (2007) The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* **133**, 24–33.
- Hayashi H, Sakamoto M & Benno Y (2002) Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiol Immunol* **46**, 535–548.
- Apajalahti JH, Kettunen H, Kettunen A, *et al.* (2002) Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Appl Environ Microbiol* **68**, 4986–4995.
- Apajalahti JH, Sarkilahti LK, Maki BR, *et al.* (1998) Effective recovery of bacterial DNA and percent-guanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. *Appl Environ Microbiol* **64**, 4084–4088.
- Mäkiuokko HA, Saarinen MT, Ouwehand AC, *et al.* (2006) Effects of lactose on colon microbial community structure and function in a four-stage semi-continuous culture system. *Biosci Biotechnol Biochem* **70**, 2056–2063.
- Lane DJ, Pace B, Olsen GJ, *et al.* (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* **82**, 6955–6959.
- Edwards U, Rogall T, Blocker H, *et al.* (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.
- Staden R, Beal KF & Bonfield JK (2000) The Staden package, 1998. *Methods Mol Biol* **132**, 115–130.
- Saarinen MT (2002) Determination of biogenic amines as dansyl derivatives in intestinal digesta and feces by reversed phase HPLC. *Chromatographia* **55**, 297–300.
- Pinheiro JC & Bates DM (2000) *Mixed-Effects Models in S and S-PLUS*. New York: Springer-Verlag.
- Wang Q, Garrity GM, Tiedje JM, *et al.* (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**, 5261–5267.
- Audic S & Claverie JM (1997) The significance of digital gene expression profiles. *Genome Res* **7**, 986–995.
- Fava F, Makivuokko H, Siljander-Rasi H, *et al.* (2007) Effect of polydextrose on intestinal microbes and immune functions in pigs. *Br J Nutr* **98**, 123–133.
- Suau A, Bonnet R, Sutren M, *et al.* (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* **65**, 4799–4807.
- Wexler HM (2007) Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev* **20**, 593–621.
- Ley RE, Backhed F, Turnbaugh P, *et al.* (2005) Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A* **102**, 11070–11075.
- Ley RE, Hamady M, Lozupone C, *et al.* (2008) Evolution of mammals and their gut microbes. *Science* **320**, 1647–1651.
- Turnbaugh PJ, Backhed F, Fulton L, *et al.* (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. *Cell Host Microbe* **3**, 213–223.

39. Barcenilla A, Pryde SE, Martin JC, *et al.* (2000) Phylogenetic relationships of butyrate-producing bacteria from the human gut. *Appl Environ Microbiol* **66**, 1654–1661.
40. Duncan SH, Hold GL, Barcenilla A, *et al.* (2002) *Roseburia intestinalis* sp. Nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *Int J Syst Evol Microbiol* **52**, 1615–1620.
41. Louis P, Scott KP, Duncan SH, *et al.* (2007) Understanding the effects of diet on bacterial metabolism in the large intestine. *J Appl Microbiol* **102**, 1197–1208.
42. Duncan SH, Belenguer A, Holtrop G, *et al.* (2007) Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl Environ Microbiol* **73**, 1073–1078.
43. Kageyama A, Sakamoto M & Benno Y (2000) Rapid identification and quantification of *Collinsella aerofaciens* using PCR. *FEMS Microbiol Lett* **183**, 43–47.
44. Zoetendal EG, Ben-Amor K, Harmsen HJ, *et al.* (2002) Quantification of uncultured *Ruminococcus obeum*-like bacteria in human fecal samples by fluorescent *in situ* hybridization and flow cytometry using 16S rRNA-targeted probes. *Appl Environ Microbiol* **68**, 4225–4232.
45. Walker AW, Duncan SH, Harmsen HJ, *et al.* (2008) The species composition of the human intestinal microbiota differs between particle-associated and liquid phase communities. *Environ Microbiol* **10**, 3275–3283.
46. Duncan SH, Holtrop G, Lopley GE, *et al.* (2004) Contribution of acetate to butyrate formation by human faecal bacteria. *Br J Nutr* **91**, 915–923.
47. Matsumoto M & Benno Y (2007) The relationship between microbiota and polyamine concentration in the human intestine: a pilot study. *Microbiol Immunol* **51**, 25–35.
48. Thomas T & Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* **58**, 244–258.
49. Larque E, Sabater-Molina M & Zamora S (2007) Biological significance of dietary polyamines. *Nutrition* **23**, 87–95.
50. Wang X, Feith DJ, Welsh P, *et al.* (2007) Studies of the mechanism by which increased spermidine/spermine N1-acetyltransferase activity increases susceptibility to skin carcinogenesis. *Carcinogenesis* **28**, 2404–2411.
51. Seiler N & Raul F (2007) Polyamines and the intestinal tract. *Crit Rev Clin Lab Sci* **44**, 365–411.