



Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of , bifidobacteria and clostridium cluster IV in institutionalized elderly

Jutta Zwielehner, Kathrin Liszt, Michael Handschur, Alexander Lapin,
Alexander G. Haslberger

► To cite this version:

Jutta Zwielehner, Kathrin Liszt, Michael Handschur, Alexander Lapin, Alexander G. Haslberger. Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of , bifidobacteria and clostridium cluster IV in institutionalized elderly. *Experimental Gerontology*, 2009, 44 (6-7), pp.440. 10.1016/j.exger.2009.04.002 . hal-00493104

HAL Id: hal-00493104

<https://hal.science/hal-00493104>

Submitted on 18 Jun 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and clostridium cluster IV in institutionalized elderly

Jutta Zwielehner, Kathrin Liszt, Michael Handschur, Alexander Lapin, Alexander G. Haslberger

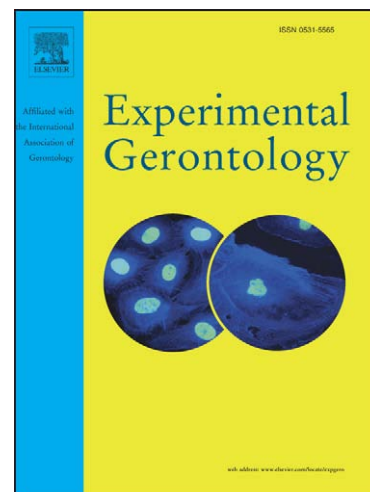
PII: S0531-5565(09)00079-5
DOI: [10.1016/j.exger.2009.04.002](https://doi.org/10.1016/j.exger.2009.04.002)
Reference: EXG 8601

To appear in: *Experimental Gerontology*

Received Date: 24 September 2008
Revised Date: 7 April 2009
Accepted Date: 8 April 2009

Please cite this article as: Zwielehner, J., Liszt, K., Handschur, M., Lapin, A., Haslberger, A.G., Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and clostridium cluster IV in institutionalized elderly, *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.04.002](https://doi.org/10.1016/j.exger.2009.04.002)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of *Bacteroides*, bifidobacteria and clostridium cluster IV in institutionalized elderly.

Jutta Zwielehner¹, Kathrin Liszt¹, Michael Handschur¹, Alexander Lapin³, Alexander G. Haslberger^{1*}.

Affiliations: ¹Department of Nutritional Sciences, University of Vienna, Austria.

³Sozialmedizinisches Zentrum Sophienspital der Stadt Wien

*Corresponding Author:

Univ.-Doz. Dr. Alexander Haslberger

Department of Nutritional Sciences

Althanstrasse 14, UZAll; 2D541

1090 Vienna

phone: +431427754996

fax : +43142779549

mail: alexander.haslberger@univie.ac.at

running title : Bacterial diversity in elderly.

Keywords: microbiota, PCR-DGGE, qPCR, *Bacteroides*, bifidobacteria, *Clostridium*, feces, ageing

Abbreviations:

APS ammonium-persulfate

TEMED N,N,N',N'-tetramethylethylenediamine

GIT gastro-intestinal tract

ABSTRACT

Aims: This study aimed at determining ageing-related shifts in diversity and composition of key members of the fecal microbiota by comparing institutionalized elderly (n=17, 78-94 yrs) and young volunteers (n=17, 18-31 yrs).

Methods and Results: A combination of molecular methods was used to characterize the diversity and relative abundance of total gastro-intestinal flora, along with relevant subsets within the genera *Bacteroides*, bifidobacteria and *Clostridium* cluster IV. The institutionalized elderly harbored significantly higher numbers of *Bacteroides* cells than control ($28.5 \% \pm 8.6\%$; $21.4 \% \pm 7.7\%$; $p=0.016$) but contained less bifidobacteria ($1.3 \% \pm 0.9$, $2.7 \% \pm 3.2\%$, $p=0.026$) and *Clostridium* cluster IV ($26.9\% \pm 11.7\%$, $36.36\% \pm 11.26\%$, $p=0.036$). The elderly also displayed less total *Bacteria* diversity and less diversity with the *Clostridium* cluster IV ($p<0.016$) and *Bacteroides*.

Conclusion: Despite high individual variations, our analyses indicate the composition of microbiota in the elderly comprises a less diverse subset of young healthy microbiota.

Significance and Impact of the Study: A better understanding of the individual composition of the human microbiota and the effects of ageing might result in the development of specifically targeted supplementation for elderly citizens in order to support healthy ageing.

INTRODUCTION

It is estimated that between 60-80 % of the microbial diversity resident within the human gastro-intestinal tract (GI) has yet to be cultivated (Suau *et al.* 1999). However, the use of culture-independent molecular methods has enabled researchers to identify important characteristics of this community. We now know that the composition of GI microbiota is highly distinct between individuals (Dethlefsen *et al.* 2006). Despite this variability, some general features are apparent. There appear to be 'core species' present in a majority of humans which are quite resilient to external influences (Dore 2007). In addition to this group, there are also 'passengers' or transients, sometimes in great numbers, sometimes below detection limit (Favier *et al.* 2003). However, the majority of sequences seem to be unique to some individual and not one single phylotype may be present in all humans (Turnbaugh *et al.* 2009). A number of factors have been identified which influence community composition. The type of bacteria introduced to the environment is determined by those associated with food sources (Duncan *et al.* 2007; Pryde *et al.* 2002). The nature and composition of non-digestible carbohydrates in a diet can further stimulate different types of bacteria (Kolida and Gibson 2007), whilst hereditary dispositions and personal gut environmental factors may account for unique personal characteristics (Eckburg and Relman 2007). Correlations between the GI microbiota and diseases such as allergenicity (Shreiner *et al.* 2008), inflammatory bowel diseases (Sokol *et al.* 2006) and individual dispositions such as obesity (Zhang *et al.* 2009) have been discussed. The colon harbors butyrate-producing species of several genera such as *Clostridium*, *Eubacterium* and *Fusobacterium* (Pryde *et al.* 2002). Among strains that produce

high levels of butyrate *in vitro* are those related to *Faecalibacterium prausnitzii*, an ubiquitous member of *Clostridium* cluster IV (Pryde et al. 2002). *Bacteroidetes* species have been reported to show high variations between individuals, although *Bacteroides thetaiotaomicron* is found in all human beings (Layton et al. 2006). Both *Bacteroidetes* and *Clostridium* cluster IV are known to be involved in beneficial functions, including nutrient absorption, production of short chain fatty acids (SCFAs) and epithelial cell maturation and maintenance (Woodmansey 2007).

Another important subgroup of the human GI microbiota is the bifidobacteria. Stimulation of these bacteria has previously been shown after prebiotic intervention with inulin and fructo-oligosaccharides (Kolida and Gibson 2007). Furthermore, they have been shown to be involved in prevention of atopic disease (Ouwehand et al. 2007), obesity and insulin resistance via enhanced barrier function of the gut epithelium (Cani et al. 2007).

With ageing, a decrease in beneficial organisms such as *Lactobacilli* and bifidobacteria, amongst other anaerobes, and an increase in the number of facultative anaerobes (Guigoz et al. 2008) have been reported. Population cross-sectional studies with relevant numbers of healthy elderly also show age-related changes in GI microbiota. These include a consistent global increase in nonpathogenic Gram-negative bacteria (mainly *Enterobacteria*), as well as country-specific changes in bifidobacteria (Guigoz et al. 2008). This, along with a general reduction in species diversity within most bacterial groups, changes to diet and altered digestive physiologies such as intestinal transit time, may result in increased putrefaction in the colon and a greater susceptibility to disease. The aged gut is characterized by increased proteolytic activity, decreased amylolytic activity and reduced levels of SCFA (Woodmansey 2007). Ageing is associated with reduced

levels of prostaglandins such as PGE₂ and PGF_{2α} as shown in specimens of stomach and duodenum biopsies (Tiihonen *et al.* 2008). Subclinical intestinal inflammation in elderly populations has been detected and is believed to contribute to impaired immune functions, the underlying cause of mortality beyond 75-80 years of age (Guigoz *et al.* 2008).

Analyses of individual dispositions associated with changes of the microbiota should consider quantitative and qualitative aspects of gut community structure. Analysis of stool samples can target changes in colonic microbiota, since feces are representative of inter-individual differences (Turnbaugh *et al.* 2007).

The aim of this work was to investigate shifts in GI microbiota associated with aging, by comparing institutionalized elderly with young healthy volunteers. To analyze changes in total bacterial community composition, along with specific compositional changes within the *Bacteroidetes*, bifidobacteria and *Clostridia* cluster IV, we used the polymerase chain reaction (PCR) based community fingerprinting method Denaturing Gradient Gel Electrophoresis. The resolution of this method allows for the characterization of the dominant members of a targeted microbial community.

Further, we used quantitative-PCR (q-PCR) to determine the relative load of *Bacteroidetes*, bifidobacteria and *Clostridia* cluster IV groups within our samples.

Thus, use of these methods in combination allowed the characterization of both diversity and relative abundance of our targeted organisms.

MATERIAL AND METHODS

Probands

Seventeen institutionalized elderly aged 86 ± 8 years, BMI 21.75 ± 5.08, from a geriatric department in Vienna and 17 students from Vienna joined the study.

Probandes were interviewed following a questionnaire assessing: age; gender; body length and weight; individual health status, including chronic or acute diseases and blood lipid levels; and life-style aspects, such as physical activity and dietary habits. Five percent of geriatric patients suffered from manifest diabetes mellitus type 2. Ten patients were bed-ridden and seven mobile. Causes for loss of mobility were Parkinson's disease, dementia and osteoporosis. Nursing staff reported the application of NSAIDs (non-steroidal anti-inflammatory drugs) on demand. Seventeen young healthy volunteers were aged 24 ± 2.5 years, BMI 22.68 ± 3.41 and their dietary habits were typical for Central Europe. Study populations were gender balanced, with 55% females in the group of elderly and 50% in the young group. Only non-pregnant probands with no diagnosed gastrointestinal disease and no antibiotic or chemotherapeutic treatment three months prior to sampling were included in the study. All probands agreed to participate in the study and gave their informed consent.

Sampling and DNA extraction from Stools and Type Strains

From each proband, three stool samples were taken within the course of a week and immediately stored at -70°C . Portions of the three samples from each patient were pooled, a 200 mg aliquot was treated twice for 45 sec in a bead-beater (Mini-Beadbeater-8) and DNA extracted with the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol and then immediately stored at -20°C .

Type strains known to be associated with GI microbiota were grown, and DNA extracted, for use as part of the markers in DGGE analysis (see below). Type strains *L. casei* DSM 20011^T, *L. delbrueckii* subsp. *lactis* DSM 20072^T, *Bacteroides fragilis* DSM 2151^T, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum*

DSM20219^T, *B.longum* DSM 20211, *B.pseudolongum* DSM 20099 and
B.thermophilus DSM 20210 were anaerobically cultivated on blood agar, *E.coli* IMBH
252/07 and clones were aerobically cultivated on LB-agar (liquid broth medium). The
biomass was resuspended in sterile phosphate buffered saline (1x PBS from 10x
PBS Roti®stock, ROTH). Tenfold dilutions from these suspensions in sterile 1x PBS
were plated in duplicate on blood agar or LB-agar, colony forming units (CFU/ml)
were counted and DNA extracted from serial dilutions. DNA was extracted from
clones using the Wizard® Plus.SV Minpreps DNA Purification System (Promega).
The DNA of all Gram-negative bacteria was extracted with the DNA Mini Kit
(QIAGEN), for Gram- positive bacteria, the FastDNA Spin Kit for Soil (MP-
Biomedicals) was used following the instructions of the manufacturer.

Polymerase Chain Reaction (PCR)

PCR was used to amplify 16S ribosomal RNA gene sequences from type strains and
Bacteria in stool samples for use in DGGE analysis and manufacture of clone
libraries (see below). All reactions were carried out using a ready-to-use mastermix
(Promega) with 1.5mM MgCl₂. Bovine serum albumin (10mg/ml, Fermentas) was
added to a final concentration of 400µg/ml, primer concentration in the reaction
volume was 0.5µM. Amplifications were carried out in a Robocycler (Stratagene).

Clone libraries

Clone libraries were constructed from stool samples to identify dominant members of
the *Clostridium* cluster IV and the *Bacteroidetes*. Selected clones were then used,
along with the cultured type strains, to generate appropriate reference markers for
DGGE analysis. Amplifications were carried out using primer pair 27f Edwards et al.

1989) and sg-Clep-R (Matsuki *et al.* 2004), which are specific for members of the *Clostridium* cluster IV, and 32f and 708r (Bernhard and Field 2000) which are specific for members of the *Bacteroides*. Amplified products were cloned into a p-GEM Easy Vector (Promega) following the instructions of the manufacturer. Clone libraries were screened as previously described (Schabereiter-Gurtner *et al.* 2001). Clone inserts were sequenced by 'DNA confidence' (Vienna). Nucleotide sequences were corrected for vector and primer sequences in CodonCode Aligner (www.codoncode.com) and taxonomically identified by comparison to previously published sequences using the online tools of the ribosomal database project (<http://rdp.cme.msu.edu/>).

DGGE

PCR amplifications of 16S rRNA gene fragments from total DNA extracted from stool samples were carried out using the specific primer sets outlined in Table 2. Separate DGGE gels were run to analyse samples for patterns in a) total *Bacteria*, b) *bifidobacteria*, c) *Clostridium* cluster IV and d) *Bacteroides*. DGGE gels were prepared as described previously (Muyzer and Smalla 1998) with a linear gradient of 25-65% for *Bacteria* (general), 30-65% for *bifidobacteria*, 20-50% for *Bacteroides* and 30-50% for *Clostridium* cluster IV, using a gradient mixer (Hoefer SG 30) and a peristaltic pump. We generated reference markers appropriate for each set of DGGE analyses (i.e. a different reference marker was used for each of a,b,c and d above) to enable meaningful comparisons across multiple gels and to provide putative identification of some bands. These reference markers contained fragments of 16S rRNA genes from cultured bacteria and clones generated from fecal material (as described above). Each marker was loaded in triplicate on each gel to allow gel-to-

gel comparison. The reference marker for DGGE fingerprinting of general *Bacteria* consisted of *E.coli* IMBH 252/07, *Enterococcus faecium* DSM 20477^T, *Bacteroides thetaiotaomicron* DSM 2079^T, *Bifidobacterium longum* DSM 20219^T, *Clostridium perfringens* (laboratory isolate), clone BT11 (98.8% similarity with *Bacteroides uniformis* JCM 5828^T), clone BT17 (96.0% similarity with *Bacteroides vulgatus*^T and bacterium LY88 previously identified in human feces (Eckburg *et al.* 2005)) and clone CL16 (98.4% similarity with an uncultured bacterium from human feces (Turnbaugh *et al.* 2006) and 96.7% similarity with *Faecalibacterium prausnitzii*^T). The reference marker for bifidobacteria specific DGGE analysis consisted of *Enterococcus faecium* DSM 20477^T, *E.coli* IMBH 252/07, *Bifidobacterium longum* DSM 20219^T, *B. longum* DSM 20211, *B. thermophilus* DSM 20210^T and *B. pseudolongum* DSM 20099^T. The reference marker for *Bacteroides* specific DGGE analysis was composed of *Bacteroides thetaiotaomicron* DSM 2079^T, fecal clones Bt 17 and Bt11 and *Enterococcus faecium* DSM 20477^T. The reference marker for *Clostridium* cluster IV DGGE analysis was constructed from 7 clones all representing previously uncultured species from cluster IV: Similarities with type strains from this cluster were 81.8%, 96.6%, 96.7% and 97.8% similarity with *Faecalibacterium prausnitzii*^T, 92.1% and 92.9% similarity with *Eubacterium desmolans*^T and 95.9% similarity with *Subdoligranulum variabile*^T.

TaqMan RTQ-PCR

The TaqMan-assay was carried out in a Rotorgene 3000 (Corbett Life Science) in duplicate in a volume of 10 µl containing 5 µl TaqMan SensiMix DNA Kit (Quantace), 1 µl of each primer and probe (final concentrations table 1) and 2 µl of the 100-fold dilution of the template. All probes were labeled with 6-FAM at the 5' end and carried

a BHQ-1 quencher at the 3' end. TaqMan Probe (Clep-P) for *Clostridium* cluster IV was designed with CLC DNA Workbench (www.clcbio.com). Analysis with ProbeMatch (rdp.9.58) indicated that Clep-P binds to all members of *Clostridium* cluster IV. The PCR program for bifidobacteria, *Bacteroides* and universal bacteria consisted of denaturing at 95°C for 3min and 45 cycles of 95°/60°C for 15/ 45 sec. The amplification program for clostridium cluster IV was: denaturation at 95° for 5 min and 45 cycles at 95°/ 55°C for 30/ 45 sec.

DNA of *Bacteroides thetaiotaomicron*^T and *Bifidobacterium longum*^T, clone CL16 and one fecal sample were used to construct standard curves for comparison of PCR reaction efficiencies among different experiments and enumeration of all bacterial groups. Reaction efficiency (E) was estimated using the slope of the standard curve and the formula $E=10^{(-1/\text{slope})} - 1$ as described elsewhere (Penders *et al.* 2005).

Quantification was done using standard curves obtained from known concentrations of organisms containing the respective amplicons for each set of primers. The percentage of bacterial group rRNA gene copies in relation to total rRNA gene copies (relative abundance) was calculated for each individual, and the mean was determined for each subject group. Relative quantification (% of bacteria) was performed using Rotor-Gene 3000 calculation software (Corbett operator manual) and Excel. Cross reactivity with non-target strains was tested using the Probe-Match tool at the RDP website and using the strains mentioned above.

Statistical analysis.

Food frequency data were analyzed based on Chi-square approximation as implemented in SPSS. P values <0.05 were considered significant. Band comparison tables were analyzed with principal component analysis (PCA) using the default

settings in 'R-software environment for statistical computing' (www.r-project.org) until 100% variance was explained. Transformed data were plot in a bi-plot as a function of the first two principal components. Clustering was applied to DGGE fingerprinting data as implemented in the GelComparII environment (www.applied-maths.com). Clustering was performed based on Dice coefficient as well as based on Pearson correlation. Those methods are supplementary to each other, clustering after Dice takes band positions into account, whereas Pearson correlation based clustering analyzes the densitometric curves of each fingerprint. UPGMA dendrograms were generated and Jackknife analysis was performed using average similarities and 100 resamplings. Jackknife analysis is a leave-one-out method that tests the reliability of the clustering similar to bootstrap analysis. Shannon and Simpson's diversity index were calculated on binary band information (presence-absence) with the default settings implemented in the 'vegan' package in 'R'. Shannon index is defined as $H = -\sum p_i \ln p_i$, where p_i is the proportional abundance of species i . For Simpson's index D , this is $1 - \sum ((p_i)^2)$, where p_i is the relative frequency of the i -th species. Prior to application of Student's t-test for diversity indices and qPCR data, equality of variances of the three datasets was tested using the F-test as implemented in Microsoft Excel.

RESULTS

Dietary aspects

Analysis of the participant's dietary habits indicated similar consumption patterns of fruits, vegetables and milk products in both groups. Young volunteers stated significantly less frequent (χ^2 Test; $p < 0.04$) consumption of meat than elderly

probands and regular consumption of whole grain products several times a week.

The institutionalized elderly of this study did not consume any whole grain products at all but received supplements with soluble fiber (Benfiber®, Novartis).

TaqMan-quantification

TaqMan assays were set up quantifying bacterial sub-populations as percentage of the total bacterial DNA. We detected no cross-reactivity of group-specific primers and probes with non-target strains. Test-retest variations were between 2.7% and 5.2%, values after relative quantification varied by less than 4%. The sensitivity was corresponding to a 100 000 fold dilution of DNA from feces, that is 20 copies of 16S rRNA gene per reaction.

The elderly harbored only $69\% \pm 21.6\%$ of the total bacterial load in their faeces compared to control (figure 1). *Bacteroides* were found to represent a larger percentage than bifidobacteria in all samples (figure 1) and the institutionalized elderly harbored significantly more *Bacteroides* than young volunteers ($p=0.016$). Although relative levels of bifidobacteria were highly variable among samples, the differences between young and elderly probands were statistically significant ($p=0.026$). Furthermore, elderly citizens had significantly less members of *Clostridium* cluster IV (figure 1) in their fecal microbiota than young volunteers ($p=0.036$).

DGGE bandpattern analysis

DGGE fingerprinting with primer pair 341GC-518, which amplified the total microbial community, showed high inter-individual variations (figure 2). The mean numbers of bands per individual were 16.6 ± 3 bands for institutionalized elderly and 20 ± 3 bands in control. Two bands, highlighted in figure 2, occurred significantly less

frequently in elderly probands. Shannon and Simpson indices of diversity were significantly lower for elderly than control (figure 3). The highly diverse dataset was subjected to principal component analysis (PCA). Extraction of underlying components within the dataset indicated grouping of samples according to their variance (figure 4) along the first two principal components. Cluster analysis could separate the fingerprints of young and elderly (figure 5). Jackknife analysis suggested that the dominant bacteria of the elderly microbiota are a subpopulation of the microbiota of young individuals: Predictability of groupings was 100% for young and only 13.33% for elderly.

Bifidobacteria

An average of thirteen bands were observed in individual DGGE bandpatterns obtained with the primer pair specific for bifidobacteria. Two bands with the same melting behaviour as the type strains *B.longum* DSM 20219^T and *B.pseudolongum* DSM 20099^T in our marker were abundant in the majority of probands. PCA was performed and the first two PC's explained 23.64% of variance (figure 4). Although cluster analysis showed high similarity of young and elderly bandpatterns, jackknife testing demonstrated greater similarity of bandpattern for control than for elderly (expressed in predictability of groupings: 91.67% for control and 35.72% for elderly). PCA supported these results and could not separate young and elderly according to variances in the dominant bifidobacteria of their microbiota.

Bacteroides

DGGE fingerprints obtained with the *Bacteroides* specific primers contained an average of 7.6 ± 2.5 bands for the elderly, whilst young individuals averaged 9.5 ± 3

bands. Diversity indices (figure 3) showed a tendency to be lower for elderly than for control. PCA results suggest a tendency for less *Bacteroides* diversity with ageing (figure 4). Elderly subjects grouped along the first principal component. Clustering of Pearson correlations could separate young and elderly individuals according to DGGE fingerprinting. Jackknife analysis using average similarities could predict groupings of young bandpattern with 76.92% reliability and 75% for elderly.

Clostridium cluster IV

DGGE fingerprints obtained with the *Clostridium* cluster IV specific primers yielded an average of 10 ± 3.5 bands per elderly individual and 13 ± 3 bands per young individual. Diversity indices (figure 2) were significantly ($p=0.02$) lower for aged citizens than for the young. One band that occurred more frequently in the young than in the elderly had the same melting characteristics as a band in our marker. This band was generated from a clone from our library related to the genus *Faecalibacterium*. This clone had the highest similarity (99.2%) to an uncultured bacterium EF403698 and also displayed 96.6% similarity to *Faecalibacterium prausnitzii*^T. Three more bands occurred more frequently in the young than in elderly. These bands were identified from our clone libraries as relating to the genera *Ruminococcus* (clone had 95.9% similarity to *Ruminococcus bromii*^T, 99% similarity to uncultured AJ408987 from human colon) and *Subdoligranulum* (clones had 94.7%/ 97.5% similarity to *Subdoligranulum variabile*^T, and 97.9%/ 98% similarity to uncultured bacterium DQ793301). PCA (figure 4) indicated separation of young and elderly according to their *Clostridium* cluster IV DGGE fingerprints along PC1.

Clustering and Jackknife analysis did not result in distinct clustering of elderly and young individuals. *Clostridium* cluster IV representatives in the elderly microbiota are most likely to be a subset of the species present in young individuals: Only in 35.71% of resamplings elderly bandpattern grouped with each other, whereas Jackknife value for young individuals was 100%.

DISCUSSION

Metagenomic analysis of the human GI microbiota is presently the subject of large research consortia and has already substantiated the concepts of a 'core microbiome' and inter-individual variations (Turnbaugh *et al.* 2007). However, the collection of data on 'reference microbiota' is far from complete and thus no definition of a healthy microbiota is available yet. Important information comes from analyses addressing the abundance and diversity of specific bacterial populations with relevance to disease, diet or probiotic intervention. We used a combined molecular approach to compare patterns in several target GI microbial groups between the institutionalized elderly and young healthy volunteers. The community fingerprinting method PCR-DGGE was used to compare the diversity present in total *Bacteria* and also specifically within the *Clostridium* cluster IV, bifidobacteria and *Bacteroides*, whilst q-PCR was used to quantify the relative population abundance of these same bacterial groups in all samples.

Our results demonstrate some significant shifts in patterns in GI microbiota between our study groups. Faeces from the institutionalized elderly had less total Bacterial abundance and lower total Bacterial diversity than that from the young subjects, However, samples from the elderly displayed an increase in the relative abundance of *Bacteroides*, although this group tended to display less diversity than *Bacteroides*

in the young. The relative abundance of the bifidobacteria and *Clostridium* cluster IV were significantly higher in the young, and the *Clostridium* cluster IV also displayed greater diversity in the young. Furthermore, cluster analysis revealed that for all microbial groups analysed, the members of the GI microbiota in the elderly could be considered a subset of that present in the young.

The results of our bifidobacteria analysis are in agreement with previous studies which have identified a reduction and loss of diversity of bifidobacteria associated with aging (Woodmansey et al. 2007, Hopkins et al. (2002)) or hospitalization of aged citizens (Bartosch et al. 2004). Our study population of elderly was supplemented with soluble fiber. This prebiotic intervention alone was apparently not able to antagonize ageing-related changes in the bifidobacteria. In this respect Ouwehand et al. (2008) recently reported that supplementation with lactitol and a probiotic *Lactobacillus* led to an increase in bifidobacteria in the microbiota of elderly. Bartosch et al (2004) also reported a marked reduction in the abundance of *Faecalibacterium prausnitzii*, a member of the *Clostridium* cluster IV which decreased in our aged group. However several studies (Hopkins et al. 2002, Woodmansey et al. 2007, Bartosch et al. 2004) have reported a decrease in the relative abundance of the *Bacteroides*, whereas our aged study population displayed a relative increase in abundance of this group. Like us, all the previous studies reported a decrease in *Bacteroides* diversity. Increased levels of *Bacteroides* have been found in individuals with infectious colitis (Sokol et al. 2006). Discrepancies in changes of the *Bacteroides* abundance might be due to country-specific differences in this bacterial subgroup as indicated by Mueller et al. (2006). Mueller et al. (2006) also observed gender effects within *Bacteroides*, with levels being generally higher in males than in females. Our study populations were gender balanced with volunteers being 55% females in the

group of elderly and 50% in the young. The results presented here do not support gender differences in *Bacteroides* abundance. Reductions in amylolytic activity observed in a healthy elderly population have been correlated with the occurrence and diversity of *Bacteroides* (Woodmansey 2007).

Reduced numbers of *Bacteria* in the fecal content of elderly reflect the physiological alterations associated with ageing. These include prolonged colonic transit time and reduced dietary energy requirement and food uptake (Morley et al. 2007). Further reductions in the relative abundance of important sub-populations such as *Clostridium* cluster IV and bifidobacteria might result in reduced formation of SCFAs, altered epithelial cell maturation and maintenance, and altered barrier function of the gut epithelium in elderly probands. Those changes in the GI microbiota have previously been linked to impaired immune functions prevalent in individuals of advanced age and may result in a greater susceptibility to disease.

Improved analytical concepts for the characterization of the microbiota of consumers and patients might become important as a rationale for individualized probiotic intervention. Probiotic supplementation is a promising concept in restoring impaired functions or enhancing specific desirable functions of the microbiota. Encouraging effects of probiotic supplementation have been reported for aspects such as direction of host immunity, pathogen defense, maintenance of integrity of the gut epithelium, alleviation of lactose intolerance symptoms and immune effects, such as in atopic disease. For further insight into the relationships between phylogenetic information and metabolic activities, sequence information in addition to 16S rRNA based fingerprinting will be mandatory.

CONCLUSION

Studies comparing elderly and young volunteer microbiota with PCR-DGGE fingerprinting and qPCR are still rare. We found that ageing is associated with less overall bacteria and significantly decreased *Clostridium* cluster IV and bifidobacteria and an increase of *Bacteroides*. Diversity of dominant bacteria, *Bacteroides* and *Clostridia* cluster IV were reduced. Those changes in the GI microbiota are suggested to be cause and effect of impaired immune functions in individuals of advanced age and may result in a greater susceptibility to disease.

Tables:

Table 1: Primers and probes used for quantification of fecal bacteria using TaqMan assays targeting 16S rRNA coding regions.

Target organism	Primer or probe	Sequence (5' - 3')	Fragment Size (bp)	Conc. [nM]	Reference
bifidobacteria	Fwd primer	GCG TGC TTA ACA CAT GCA AGT C	125	300	(Penders <i>et al.</i> 2005)
	Rev primer	CAC CCG TTT CCA GGA GCT ATT		300	
	Probe	(FAM)- TCA CGC ATT ACT CAC CCG TTC GCC -(BHQ-1)		150	
<i>Bacteroides</i>	AllBac296f	GAG AGG AAG GTC CCC CAC	106	300	(Layton <i>et al.</i> 2006)
	AllBac412r	CGC TAC TTG GCT GGT TCA G		300	
	AllBac375Bhq	(FAM)-CCA TTG ACC AAT ATT CCT		100	
	r	CAC TGC TGC CT-(BHQ-1)			
Bacteria (general)	BAC-338-F	ACT CCT ACG GGA GGC AG	468	1000	
	BAC-805-R	GAC TAC CAG GGT ATC TAA TCC		1000	

		(FAM)-TGC CAG CAG CCG CGG TAA			
	BAC-516-P			200	(Yu 2005)
		TAC-(BHQ-1)			
	<i>Clostridium</i>				
	sg-Clep-F	GCA CAA GCA GTG GAG T	239	400	
	cluster IV				
					(Matsuki <i>et al.</i>
	sg-Clep-R	CTT CCT CCG TTT TGT CAA		400	2004)
	Clep-P ^a	(FAM)-AGG GTT GCG CTC GTT-		200	This study
		(BHQ-1)			

^a position of target site (numbering corresponding to *E. coli* 16S rRNA gene) 1082 to 1107.

Table 2: Primers applied for PCR-DGGE fingerprinting of 16S rRNA coding regions.

Target	Primer	Sequence (5'-3')	Ann.temp	Reference
organism	pairs		(°C)	
bacteria	27f	GTGCTGCAGAGAGTTTGA	57	(Edwards <i>et al.</i>
		TCCTGGCTCAG		1989)
	985r	GTAAGGTTCTTCGCGTT	57	(Heuer <i>et al.</i> 1999)
	341f-GC	CCT ACG GGA GGC AGC	55	(Muyzer <i>et al.</i>
		AG		1993)
	518r	ATT ACC GCG GCT GCT	55	(Neefs <i>et al.</i> 1991)
		GG		
<i>Bacteroides</i>	32f			(Bernhard and
		AACGCTAGCTACAGGCTT	56	Field 2000)
	708r			(Bernhard and
		CAATCGGAGTTCTTCGTG	56	Field 2000)

bifidobacteria	g-BifidF			(Matsuki <i>et al.</i>
		CTCCTGGAAACGGGTGG	58	2002)
	g-BifidR	GGTGTTCCTTCCCGATATC		(Matsuki <i>et al.</i>
		TACA	58	2002)
<i>Clostridia</i>	sg-Clep-			
cluster IV	F-GC	see table 1	55	
	sg-Clep-			
	R			

Figures

Figure 1: Proportions of *Bacteroides*, *bifidobacteria* and *Clostridium* cluster IV of the total bacterial load in stool samples of institutionalized elderly and young healthy volunteers. Total amount of bacteria is depicted relative to the mean counts for healthy young young.

Figure 2: PCR-DGGE bandpattern of 16S rRNA coding regions of dominant bacteria amplified with primer pair 341GC-518. Bands that were observed more frequently in young than in elderly are indicated with arrows. Organisms and sequences listed were used for the construction of the reference lanes.

A, G, I, J, young; B, C, E, F, elderly; D, H, reference lanes

Figure 3: Diversity indices derived from DGGE fingerprinting of 16S rRNA coding regions.

Y, young; E, elderly; Cl.IV, *Clostridium* cluster IV; B, *Bacteroides*, Bif, bifidobacteria;
*, $p < 0.05$.

Figure 4: PCA of DGGE fingerprints of 16S rRNA coding regions of dominant
bacteria in fecal samples.

Y, young; O, elderly

Figure 5: UPMGA dendrogram showing clustering (Dice) based on the similarities of
DGGE fingerprints of dominant bacteria obtained with primer pair 341GC-518.

□, elderly; ■, young

Acknowledgements:

We would like to thank all study participants and Dr.Viviana Klose and Mag.Verity-
Ann Sattler of IfA Tulln for their cooperation in the analysis of DGGE fingerprinting.
The Hochschuljubiläumsfond of the Austrian national bank supported this work.

References:

- Bartosch, S., Fite, A., Macfarlane, G.T. and McMurdo, M.E. 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. *Applied and Environmental Microbiology*. **70**: 3575-3581.
Bernhard, A.E. and Field, K.G. 2000 A PCR assay To discriminate human and
ruminant feces on the basis of host differences in *Bacteroides*-*Prevotella* genes
encoding 16S rRNA. *Appl Environ Microbiol*. **66**: 4571-4574.
Dethlefsen, L., Eckburg, P.B., Bik, E.M. and Relman, D.A. 2006 Assembly of the
human intestinal microbiota. *Trends Ecol Evol*. **21**: 517-523.
Dore, J. 2007 Gut microbiota in health and disease. *Report of the 2nd International
Workshop in Amsterdam, April 26-28*.
Duncan, S.H., Louis, P. and Flint, H.J. 2007 Cultivable bacterial diversity from the
human colon. *Lett Appl Microbiol*. **44**: 343-350.
Eckburg, P.B. and Relman, D.A. 2007 The role of microbes in Crohn's disease. *Clin
Infect Dis*. **44**: 256-262.

- Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., *et al.* 2005 Diversity of the human intestinal microbial flora. *Science*. **308**: 1635-1638.
- Edwards, U., Rogall, T., Blocker, H., Emde, M. and Bottger, E.C. 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.
- Guigoz, Y., Dore, J. and Schiffrin, E.J. 2008 The inflammatory status of old age can be nurtured from the intestinal environment. *Curr Opin Clin Nutr Metab Care*. **11**: 13-20.
- Heuer, H., Hartung, K., Wieland, G., Kramer, I. and Smalla, K. 1999 Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints. *Appl Environ Microbiol.* **65**: 1045-1049.
- Hopkins, M.J., Sharp, R. and Macfarlane, G.T. 2002 Variation in human intestinal microbiota with age. *Dig Liver Dis.* **34 Suppl 2**: S12-18.
- Kolida, S. and Gibson, G.R. (2007) Prebiotic capacity of inulin-type fructans. *J Nutr.* **137**: 2503S-2506S.
- Layton, A., McKay, L., Williams, D., Garrett, V., Gentry, R. and Sayler, G. 2006 Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. *Appl Environ Microbiol.* **72**: 4214-4224.
- Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T. and Tanaka, R. 2004 Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. *Applied and Environmental Microbiology.* **70**: 7220-7228.
- Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., *et al.* 2002 Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl Environ Microbiol.* **68**: 5445-5451.
- Morley, J.E. 2007 The aging gut: physiology. *Clin Geriatr Med.* **23**: 757-767, v-vi.
- Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., *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.
- Muyzer, G. and Smalla, K. 1998 Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek.* **73**: 127-141.
- Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. 1993 Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol.* **59**: 695-700.
- Neefs, J.M., Van de Peer, Y., De Rijk, P., Goris, A. and De Wachter, R. 1991 Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* **19 Suppl**: 1987-2015.
- Ouwehand, A.C. 2007 Antiallergic effects of probiotics. *J Nutr.* Mar;137(3 Suppl 2):794S-7S.
- Ouwehand, A.C., Tiihonen, K., Saarinen, M., Putaala, H. and Rautonen, N. 2008 Influence of a combination of Lactobacillus acidophilus NCFM and lactitol on healthy elderly: intestinal and immune parameters. *Br J Nutr.* Jul 18:1-9.
- Penders, J., Vink, C., Driessen, C., London, N., Thijs, C. and Stobberingh, E.E. 2005 Quantification of Bifidobacterium spp., Escherichia coli and Clostridium difficile in

- faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett.* **243**: 141-147.
- Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S. and Flint, H.J. 2002 The microbiology of butyrate formation in the human colon. *FEMS Microbiol Lett.* **217**: 133-139.
- Schabereiter-Gurtner, C., Pinar, G., Lubitz, W. and Rolleke, S. 2001 An advanced molecular strategy to identify bacterial communities on art objects. *J Microbiol Methods.* **45**: 77-87.
- Shreiner A, Huffnagle GB, Noverr MC. 2008 The "Microflora Hypothesis" of allergic disease. *Adv Exp Med Biol.* ;635:113-34.
- Sokol, H., Seksik, P., Rigottier-Gois, L., Lay, C., Lepage, P., Podglajen, I., *et al.* 2006 Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis.* **12**: 106-111.
- Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D. and Dore, J. 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.
- Tiihonen, K., Tynkkynen, S., Ouwehand, A., Ahlroos, T. and Rautonen, N. 2008 The effect of ageing with and without non-steroidal anti-inflammatory drugs on gastrointestinal microbiology and immunology. *Br J Nutr.* **100**: 130-137.
- Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R. and Gordon, J.I. 2006 An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* **444**: 1027-1031.
- Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. and Gordon, J.I. 2007 The human microbiome project. *Nature.* **449**: 804-810.
- Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Dunan, A., Ley, R.E., *et al.* 2009 A core gut microbiome in obese and lean twins. *Nature* 457:480-485
- Woodmansey EJ, McMurdo ME, Macfarlane GT, Macfarlane S. 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.* Oct;70(10):6113-22.
- Woodmansey, E.J. 2007 Intestinal bacteria and ageing. *J Appl Microbiol.* **102**: 1178-1186.
- Yu Y, Lee C, Kim J, Hwang S. 2005 Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time Polymerase Chain Reaction. *Biotechnol Bioeng.* Mar 20;89(6):670-9.
- Zhang, H., Dibaise, J.K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y. *et al.* 2009 Human gut microbiota in obesity and after gastric bypass. *PNAS Early Edition* Jan 21.

