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Molecular quantitative analysis of human intestinal microbiota in different age groups

Master´s thesis

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ABBREVIATIONS

CD4+	Cluster of Differentiation 4
CFU	Colony Forming Units
C_P	Crossing Point
C_T	Threshold Cycle
DC	Dendritic Cells
dsDNA	double-stranded DeoxyriboNucleic Acid
FISH	Fluorescent *In Situ* Hybridization
GIT	GastroIntestinal Tract
GM	Germ Free
NOD	Nucleotide-binding Oligomerization Domain
NOD1	Nucleotide-binding Oligomerization Domain 1
NLRs	Nucleotide-binding Oligomerization Domain -Like Receptors
pH	*pondus Hydrogenii*
PPi	PyroPhosphate
qPCR	quantitative Polymerase Chain Reaction
rRNA	ribosomal RiboNucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction
SCFA	Short Chain Fatty Acids
TLRs	Toll-Like Receptors
T-cell	T lymphocyte-cell
T-RFLP	Terminal-Restriction Fragment Length Polymorphism
T-RFs	Terminal Restriction Fragments
T_D	Denaturating Temeperature
T_m	melting Temperature

1. GENERAL INTRODUCTION

The trillions of microbes inhabitant the human intestine. The human gut microbiota is a complex ecological community that through its metabolic activities and interactions with host organism influences normal physiology and pathological processes. The intestinal microbiota performs important vital functions, such as barrier function, metabolic activity, trophic effect and maturation of the adaptive and innate immune responses of the host organism.
Colonization of the human gut starts after birth and it is a dynamic ecosystem. Usually, it stabilizes during the first 2-5 years. During the life gut microbiota composition increases in diversity and richness, and reaches the highest complexity in the human adult. At the late stages of human life diversity of gut microbiota becomes more dynamic. Next factors like alterations in lifestyle, nutritional behavior, inflammatory diseases and infections, ageing of human organism may affect the composition and activity of the gut microbiota, but the course and mechanisms behind these changes are not yet completely understood. Characterization of changes in gut microbial diversity during life-time is the first step in elucidating its role in health and disease. Also, this knowledge is important for promotion effective therapeutic strategy against different diseases. Investigation into structural changes and composition evolution from infants to the elderly has only recently begun. Very little information is available pertaining to possible variations that occurs with ageing.

The most studies published on quantity of intestinal microbiota are associated with different human diseases. Thus, the aim to expand our understanding of the intestinal microbiita including its establishment, composition, and evolution in healthy persons in different age groups and to output the approximate norm of the gut microbial composition in healthy persons. In the future studies, the obtained data gives opportunity to make comparative analysis between human gut microbial composition in health and disease.

This study was performed at the Department of Microbiology, Tartu University.

1.1 Microbiota of human gastrointestinal tract.

The human intestinal tract harbours a large, active and complex community of microbes of different microbial ecosystems that vary according to their location within the gastrointestinal tract (GIT) (Tannock, 2002; Janet *et al*., 2008). Gut microbial diversity in healthy adults varies widely between studies that report between abundance reach 10^{14} cells/g of luminal content, 1000 to 35,000 species depending largely on methods applied (Eckburg *et al*., 2005; Frank *et al.* 2008; Human genome project, 2010). The microbial composition and relative their relative proportion varies with different parts of GIT (Figure 1). Human mouth is colonized by members of *Actinomyces* sp., *Corynebacterium* sp., *Lactobacillus* sp., *Fusobacterium* sp., *Veillonella* sp., *Staphylococcus* sp., *Streptococcus* sp. and one *Archaea* type, and considered to be the major population of upper part of the GIT (10^8 CFU/g) (Zilberstein *et al*., 2007). In addition, the aviability of oxygen varied in different parts of the mouth which allows the growth of anaerobic and aerobic bacteria with the ratio 1:10 (Zilberstein *et al*., 2007, Human genome project, 2010). Majority of bacterial species from human mouth belong to *Streptococcus* sp. and *Veillonella* sp. (Figure 1). They have ability of adherence to various surfaces such as teeth, oral mucosa or tongue. The oral cavity is colonized also by yeasts such as *Candida* sp. (Zilberstein *et al*., 2007).

The microbiota of the esophagus is colonized by allochthonous organisms. Such transitory microbiota does not occupy a niche but pass through the esophagus. Mainly it consists of the same bacteria as those found in the mouth – mostly *Streptococcus* sp. with 40% frequency, *Staphylococcus* sp. with 20% frequency, *Corynebacterium* sp., *Lactococcus* sp. and *Peptococcu*s sp. with 10% frequency respectively (Figure 1) (Zilberstein *et al*., 2007; Janet *et al*., 2008). However, the microbial diversity is smaller in the esophagus in comparison to the mouth.

The secretory activity of the stomach and duodenum affects significantly the number of microorganisms in these parts of the GIT by influence of low gastric pH (pH 1.0-2.0 units). In stomach and duodenum the abundance of microorganisms is less than 10^4 CFU/g (Figure 1). *Lactobacillus* sp., *Veillonella* sp., *Clostridium* sp. and pathogen *Helicobacter pylori*, as the microorganisms colonizing acidic environment, are the main residents of the stomach and duodenum (Zilberstein *et al*., 2007). Moreover, 128 different bacterial phylotypes and few yeast species, such as *C. albicans*, were found in these parts of the GIT. About 50% of 128 bacterial phylotypes is represented by uncultivated bacteria (Bik *et al*., 2006).

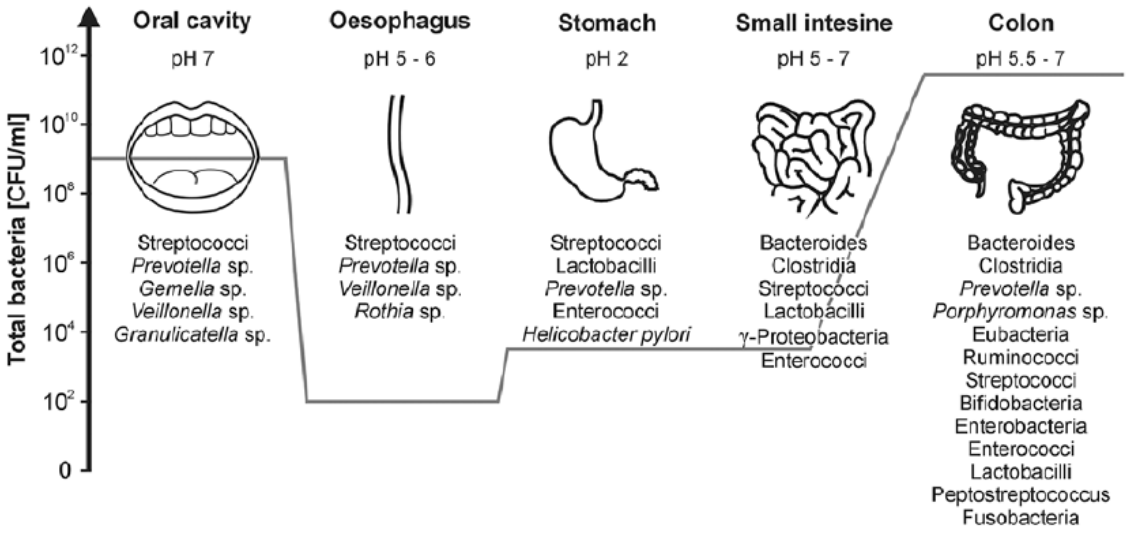


Figure. 1. A bacterial distribution in human gastrointestinal tract, adapted from Janet *et al*., 2008 (bacterial counts are expressed in CFU/ml, colony forming units per ml). With alterations.

Microbiota of the lower digestive tract includes microorganisms colonizing such parts of the GIT as the small intestine (jejunum and ileum), colon and rectum (Figure 1). Microbiota of these parts of digestive system is much more diverse and larger than the population of upper parts of the GIT. Significantly increased pH (gastric pH is 1.0-2.0 units while in the mucosa of the small intestine pH 8.0) positively affect the development of microbial diversity (Slizewska *et al*., 2008). Majority of human gut bacteria is anaerobic (facultative or obligate), however in the intestinal mucus environment favorable for growth of microaerophiles exist. In the adult human gut microbiota *Bifidobacterium* sp., *Bacteroides* sp., *Clostridium* sp. and *Eubacterium* sp. dominate less from the genus *Lactobacillus*, *Escherichia*, *Enterobacter*, *Streptococcus* and *Klebsiella* are found. Only 30% of predominant bacterial species are present in the gut of human, the rest 70% are unique and host-specific microorganisms (Libudzisz *et al*., 2008).

Microbiota of the jejunum resembles the microbiota of the duodenum. There are bacteria of the genera *Bacteroides*, *Lactobacillus* and *Streptococcus*. Also, the yeast *Candida albicans* is present. The abundance is high (up to 10^7 CFU/g) (Stolarczyk *et al*., 2008). Similarly is in the ileum, although except from *Bacteroides* and *Lactobacillus*, bacteria belonging to genus *Clostridium*, *Enterococcus*, and *Veillonella*, and the family *Enterobacteriaceae* are also predominant. Microbial diversity in the cecum mainly consists of facultative anaerobic bacterial populations belonging to *Lactobacillus-Enterococcus* group and *E.coli* species. The count of strict anaerobes such as the *Bacteroides*, *C.leptum* and *C.coccoides* groups are significantly lower than in the colon (Marteau *et al*., 2001).

The population of microorganisms of colon and rectum is the highest in number (Libudzisz *et al*., 2008). In the large intestine, abundance of microorganisms is 10^{11} CFU/g, while in rectum can reach even 10^{14} CFU/g, which represents approximately 30% of its content (Slizewska *et al*., 2008). It has been demonstrated previously that majority of bacteria in colon predominantly belong to *Bacteroides*, *Eubacteria*, *Clostridium*, *Ruminococcus*, *Fusobacterium*, *Bifidobacterium* and *Peptostreptococcus* genus (Figure 1) (Eckburg *et al*., 2005).

1.2 Functions of the human intestinal microbiota

Throughout the human lifetime, the intestinal microbiota performs vital functions, such as barrier function, metabolic reactions (fermentation of nondigestible fibers, salvage of energy as short-chain fatty acids, and production of vitamins), trophic effect and maturation of the innate and adaptive immune responses of the host organism (Stolarczyk *et al*., 2008;; Libudzisz *et al*., 2008; Sekirov *et al*., 2010).

Intestinal microorganisms have a beneficial effect on metabolic activity of the organism. Bacteria belonging to *Firmicutes* and *Bacteroides* phylum, is characterized by a high fermentation activity. The main representatives of type *Firmicutes* in colon belong to classes *Bacilli* and *Clostridia*. Class *Bacilli* produces lactic acid and acetic acid as a result of the saccharide fermentation. Class *Clostridia*, represented mostly by bacteria of the genus *Clostridium* and *Eubacterium*, characterizes in proteolytic and saccharolytic ability producing organic acids such as lactic, butyric, acetic, and formic. The fermentation activity of bacteria belonging to *Bacteroides* type is also very high. These bacteria produce in high counts organic acids such as acetic, succinic, lactic, formic, propionic, and less butyric, isobutyric and isovaleric (Figure 2) (Stolarczyk *et al*., 2008). The metabolic activity leads to the accumulation of energy and absorbable substrates for the host organism, what is important for providing energy and nutrients for life-sustaining activity of bacteria. Fermentation of saccharides is the main source of energy for intestinal epithelial cells (Guarnen *et al*., 2003). The end products of saccarides metabolism are short chain fatty acids (SCFA). SCFA are importaint in stimulating cell proliferation, differentiation of intestinal enterocytes, therefore ensuring the control of integration of the small and large intestine epithelium. In addition, SCFA affect mineral balance and regulate the metabolism of glucose and lipids (Libudzisz *et al*., 2008). Intestinal bacterial community also take part in the synthesis of vitamins (e.g. K, B, D), and absorption of calcium, magnesium and iron (Guarner *et al*., 2003).

Not all microorganisms colonizing the GIT affect positively to the human. Pathogens and microorganisms producing toxins have harmful influence on the host organism. These pathogens include some species of the genus *Enterococcus*, *Streptococcus* and *Escherichia coli* strains, which naturally exists in the human GIT. These pathogen strains can have negative impact when they dominant in this environment. Changes in composition of gut microbiota can be caused by many factors, such as gastrointestinal surgery, lesions of the colon, kidney and liver cancers, impaired immune system, antibiotic and radiological treatment, aging, poor diet, stress, change in body weight, such diseases as faecal pouchitis, ulcerative colitis (Guarner *et al*., 2003; Libudzisz *et al*., 2008). In the case of fecal pouchitis, number of bacteria of the genus *Bifidobacterium* and *Lactobacillus* is significantly reduced, while the number of *Clostridium* sp. increases. Such changes in microbiota composition cause the reduction of the concentrations of protein metabolism products and increase of the pH of intestine content. Ulcerative colitis causes reducing the number of *Bifidobacterium* sp. and increasing the number of bacteria belonging to the *Enterobacteriaceae* family. Such changes induce abnormal immune response to external antigens (allergens and pathogens), and even their own intestinal bacteria (Libudzisz *et al*., 2008). Function abnormalities of intestinal mucosa barrier can lead to the microorganism's translocation from the GIT into other parts of the human body. Microorganisms can enter the lymph nodes, liver or spleen and cause sepsis, shock, organ failure and even death of the human (Berg *et al*., 2001). The diet rich in fats and high intakes of red meat can lead the changes in the gut microbiota composition, and thus even to the tumor. Intestinal microorganisms significantly can be responsible for tumors in the kidneys, liver, ovary, and especially in the colon (Bindgham *et al*., 1999). Through the production and activation of carcinogens and genotoxic compounds, intestinal microorganisms may play an important role in development of neoplasia (Rieger *et al*., 1999).

For many years, several groups of scientists focused their research on the relation between changes in composition of gut microbiota and obesity in children and adults. Two research groups: Backhed et al (Backhed *et al*., 2005) and Ley et al (Ley *et al*., 2006) demonstrated that the number of bacteria from *Bacteroides* phylum is higher in individuals with normal weight. In obese people the percentage of *Firmicutes* is higher than in lean, and thus their intestinal microbiota is characterized by a higher fermentation activity and better efficiency of digestion of food intake (Stolarczyk *et al*., 2008). Abdominal obesity (waist circumference in women >89cm, men >102cm) can lead to the development of metabolic syndrome, also known as insulin resistance syndrome. It is a set of interrelated factors, which cause the

atherosclerosis, type 2 diabetes and vascular complications (Torphy *et al*., 2006; Lewandowska 2010). The risk of the metabolic syndrome increases with age.

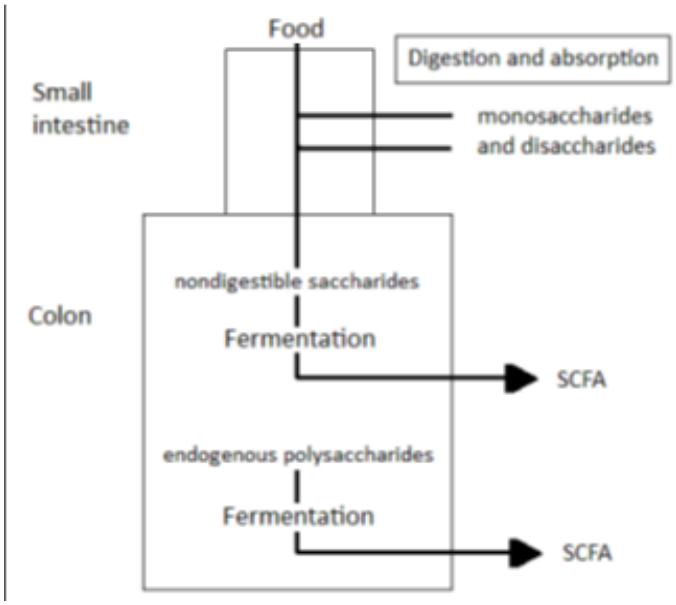


Figure 2. Metabolism of saccharides in the human colon (Stolarczyk *et al*., 2008).

1.3 Gut microbiota and immune system

Gut microbiota plays important role in development of the intestinal mucosal and systemic immune systems. The mucosal immune system is composed of the gut-associated lymphoid tissue (GALT) and small intestinal lymphoid tissue (SILT) in the small intestine, lymphoid aggregates in the large intestine and diffusely spread immune cells in the lamina propria of the GIT. These immune cells are in contact with the rest immune system through the local mesenteric lymph nodes (MLNs) (Rakoff-Nahoum and Medzhittov, 2008). The mucosal immune system has two functions. Firstly, tolerance of overlying microbiota on prevention of detrimental induction and systemic immune response of the host. Secondly, control of the gut microbiota for prevention of overgrowth and translocation to the different parts of the human organism (Sekirov *et al*., 2010). The intestinal mucosa represents the largest surface area of the human body in contact with the antigens of the external environment. Intestinal mucosa contains immune cells with pattern recognition receptors, such as the TLRs (Toll-Like Receptors) and NOD (Nucleotide-binding Oligomerization Domain) -like receptors (NLRs). Intestinal epithelium plays role in generation of immune responses through selection of foreign antigens via TLRs and NLRs receptors (Rakoff-Nahoum and Medzhittov, 2008).

Bouskra et al. has shown that germ-free (GM) animals contain abnormal numbers of several immune system cell types and immune cell products. In adittion, GM animals have deficits in local and systemic lymphoid structures, decreased number of mature isolated lymphoid follicles (Bouskra *et al*., 2008), and reduced number of their IgA and IgG producing plasma cells (Macpherson and Harris, 2004). A major immune deficiency as abnormalities of proliferation of CD4+ and T-cell populations also has been shown. It has been shown that treatment of GM mice with capsular antigen PSA (bacterial PolySAccharide) of *Bacteroides fragilis* has restored the development of lymphocytes-containing spleen white pulp and stimulates proliferation of the CD4+ T-cells (Mazmanian *et al*., 2005). A complex microbial community containing proportion of bacteria from the *Bacteroides* phylum take part in the differentiation of inflammatory Th17 cells (Ivanov *et al*., 2008). Additionally, it has been shown that various *Lactobacillus* sp. differentially regulated DCs with consequent influence on the Th1/Th2/Th3 cytokine balance at the intestinal mucosa (Christensen *et al*., 2002). Peptidoglycans of Gram-negative bacteria have induced formation of isolated lymphoid follicles (ILF) via NOD1 (Bouskra *et al*., 2008).

Prevention of excessive immune response to the bacteria from the gut microbiota can be achieved either through physical separation of bacteria and host cells: by modifications of antigenic moieties of the microbiota or modulation of localized host immune response towards tolerance (Sekirov *et al*., 2010). Resident immune cells of the GIT often have a phenotype distinct from cells of the same lineage found systemically. For instance, DCs found in the intestinal mucosa preferentially induce differentiation of resident T-cells into Th2 and Treg (regulatory T cells) subsets, therefore promoting a more tolerogenic environment in the GIT (Kelsall and Leon, 2005).

1.4 Aging and gut microbiota

The human gut microbiota starts to form after birth and it is a dynamic ecosystem, dominated by *Bifidobacterium* sp. Human gut microbiota usually stabilizes during the first 2-3 years (Koenig *et al*., 2011). During the life gut microbiota composition increases in diversity and richness (Scholtens *et al*., 2012) and reaches the highest complexity in the human adult, mainly composed of the bacteria from two phylas: *Bacteroidetes* and *Firmicutes* (Rajilic-Stojanovic *et al*., 2009).

1.4.1 Intestinal microbiota of newborns, infants and children

Development of the intestinal microbiota in infants is characterized by rapid and large changes in microbial abundance, diversity and composition (Matamoros *et al*., 2012). The formation of the human gut microbiota begins during birth with colonization by microorganisms from mother and the environment (Bezirtzoglou *et al*., 1997). Changes in intestinal microbiota in infants are influenced by medical, cultural and environmental factors such as mode of delivery, type of infant feeding, gestational age, infant hospitalization, and antibiotic use by the infant (Penders *et al*., 2006, Drell *et al*., 2014). The intrauterine environment and newborn infant are sterile until delivery. Infant intestinal environment shows a positive oxidation/reduction potential, so gut is firstly colonized by facultative anaerobes, such as *Escherichia coli, Streptococcus sp., Staphylococcus sp.* and *Enterococcus sp.* The assimilating of oxygen by these bacteria changes the intestinal environment, permitting the growth of strict anaerobes, such as *Bifidobacterium* sp., *Bacteroides* sp. and *Clostridium* sp. (Bezirtzoglou, 1997). During the initial few months of a milk diet, bacteria such as Bifidobacteria can be prevailed (Sela *et al*., 2008). The introduction of solid food causes shift toward similar to adults microbiota (Palmer *et al*., 2007). Dutch study of fecal samples from 1032 infants at 1 month of age were subjected to quantitative real-time PCR assays for the enumeration of total bacterial counts, *Bifidobacterium* sp., *E. coli*, *Clostridium difficile*, *Bacteroides fragilis* group, *Lactobacillus* sp. has showed that infants born through cesarean section had lower numbers of bacteria from *Bifidobacterium* genus from vaginally born infants and *Bacteroides* genus, whereas their gut was more often colonized with *C. difficile*, compared with vaginally born infants (Penders *et al*., 2006). Infants who were born vaginally at home and were breastfed have the most “beneficial” gut microbiota with highest numbers of bacteria from *Bifidobacterium* genus and lowest numbers of *C. difficile,* and *E.coli* (Penders *et al*., 2006). The gut of formula-fed infants was more often colonized with *E.coli*, *C. difficile*, *Bacteroides* sp. and *Lactobacillus* sp. compared with breastfed infants (Penders *et al*., 2006). Hospitalization and preterm birth were associated with higher numbers of *C.difficile* (Penders *et al*., 2006). Using of antibiotics in infants infant was associated with decreased numbers of *Bifidobacterium* sp. and *Bacteroides* sp. feces in comparison to non treated infants. Infants with older brothers and sisters had slightly higher counts of *Bifidobacterium* sp., compared with infants without siblings (Penders *et al*., 2006).

1.4.2 Intestinal microbiota of adults and elderly

Adults gut microbiota composition is dominated by the phyla *Bacteroidetes* and *Firmicutes* (Rajilic-Stojanovic *et al*., 2009). At the late stages of life the microbiota composition becomes less diverse and more dynamic, characterized by a higher *Bacteroides* sp. to *Firmicutes* sp. ratio, increase in *Proteobacteria* sp. and decrease in *Bifidobacterium* sp. (Biagi *et al*., 2010). These changes in the bacterial composition are not clear-cut partially due to the various physiological changes during the life. These include factors such as alterations in lifestyle, nutritional behavior, increase of infection and inflammatory diseases and their medication. All of these factors will affect the composition and activity of the gut microbiota, but the mechanisms behind these changes are not yet completely understood (Ottman *et al*., 2012).

In the study of Harmsen et al. (2000) adults' intestinal microbiota were characterized with fluorescence in situ hybridization (FISH) method. According to the results, intestinal microbiota of adults have hierarchy of dominant (>10^9 CFU/g) anaerobic bacteria represented by the genera *Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, Ruminococcus, Clostidium, Propionibacterium* and sub-dominant bacteria (<10^9 CFU/g) of the *Enterobacteriaceae* family, especially *E.coli*, and the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Fusobacterium, Desulfovibrio* and *Methanobrevibacter* (Harmsen *et al*., 2000). Results obtained by quantitative real-time PCR (qPCR) (Mariat *et al*., 2009) showed that in adults, the Bacteroidetes and Firmicutes are the most prevalent phyla present, the latter of which combines the values obtained for the dominant *Clostridium leptum* and *Clostridium coccoides* groups and sub-dominant *Lactobacillus* group. The *Bifidobacterium* genus is present in eight to ten-fold lower numbers that the two major phyla. *E.coli* was found to be present as a sub-dominant population in adults, and it stabilizes at an intermediate level in seniors (Mariat *et al*., 2009).

1.5 Methods for characterization of the human gut microbiota

1.5.1 Bacteriological and biochemical methods

Traditionally, analysis of the composition of the gut microbiota confide in the use of bacteriological methods such as cultivation on specific medium, microscopy and identification of the bacterial species (Janet *et al*., 2008). After isolation of colonies it is necessary to confirm the genus identity and make characterisation on the species level. For

bacterial species this characterisation requires a group of classical morphological and biochemical tests described in the Bergy's Manual of Systematic Bacteriology (Bergey, 1986). In addition, a bacterial count in the original sample is established by multiplying the number of colonies that develop with the degree of dilution.

The great advantage of cultivation is that the isolates can be recovered and further studied for their ability to metabolise the different substances and also other physiological parameters.

However, cultivation has several drawbacks. Firstly, only a small proportion of the bacterial community residing within intestinal tract can be cultivated with currently available approaches (Tannock *et al*., 2000; Eckburg *et al*., 2005). Secondly, the labour equipment and finances necessary for classical bacteriology are noticeably high thus limiting the effectiveness for analysing a large number of individuals.

1.5.2 Molecular methods

Traditional bacteriological methods recover less than 40% of the total bacterial species of the GIT, and the culturable portion of bacteria is not representative of the total phylogenetic diversity (Eckburg *et al*., 2005). Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on microorganism phenotype rather than genotype. Molecular methods mainly based on ribosomal RNA (rRNA) and the encoded genes have revealed many intestinal bacterial species not previously described or cultivated (Suau *et al*., 1999; Zoetendal *et al*., 2001; Eckburg *et al*., 2005; Frank *et al*., 2007). Molecular methods for identification of comlex microbial ecosystems may are divided to qualitative, such as sequensing of 16S rRNA methods, fingerprints methods terminal restriction fragment length polymorphism (T-RFLP), phylogenetic microarrays, high-through sequencing, and quantitative, such as fluorescence in situ hybridization (FISH), real-time PCR (Figure 3) (Vaughan *et al*., 2000).

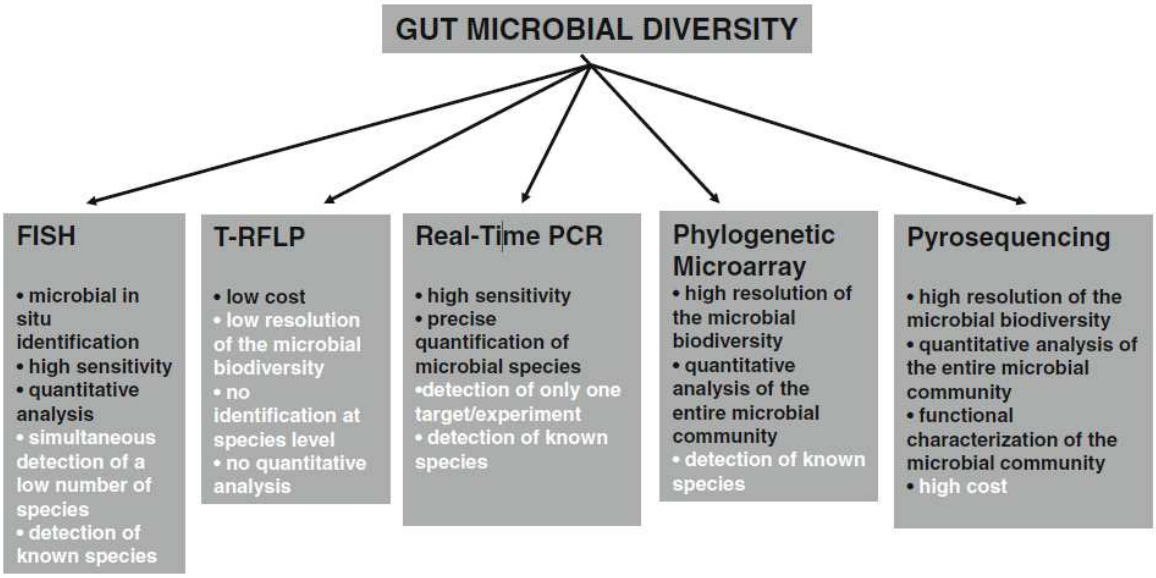


Figure 3. Molecular methods used for characterization of human intestinal microbiota, adapted from Biagi *et al*., 2012

1.5.2.1 Real-time PCR

Most frequently applied molecular approaches to quantify bacterial cells of human intestinal microbiota are real-time polymerase chain reaction (real-time PCR). PCR is the most commonly used nucleic acid amplification technique. The PCR process can be divided into three steps. First step is separation of double-stranded DNA (dsDNA) at temperatures above 90°C. Second is annealing of oligonucleotide primers generally at 50-60°C, and, finally, optimal primers extension occurs at 70-78°C. The temperature at which the primer anneals is usually referred to as the T_m (Mackay *et al*., 2004). This is the temperature at which 50% of the oligonucleotide-target duplexes have formed. In case of real-time PCR, a chemiluminscent fluorescent reaction determines the kinetics of product accumulation during PCR amplification with specific primers for a specific group or species of bacteria. It is possible to use the product accumulation rate curves for calculation of number of original target molecules in a sample. The oligonucleotides are represented as a primer or a labelled probe. Another measure is denaturation temperature T_D which refers to the T_m as it applies to the melting of dsDNA. The rate of temperature change or ramp rate, the length of the incubation at each temperature and the number of times for each cycle of temperatures is repeated and controlled by a programmable thermal cycler.

There are different approaches for real-time PCR. One involves using DNA binding dyes such as SYBR Green I which are easy to design and optimize in an assay. Compared to non

specific chemistries for real-time PCR such as SYBR Green I, a higher level of detection specify is provided by using an internal probe together with a primer set to detect the product of interest. In the absence of the specific target sequence in the reaction, the fluorescence probe does not hybridize and therefore the fluorescence remains quenched. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle (Wittwer *et al*., 2001). Another approach for the real-time PCR is Taqman methodology. It provides high level of quantitation and high specificity. The method uses the 5'nuclease activity of *Taq* polymerase to cleave a nonextendible hybridization probe during the extension phase of PCR. Taqman reaction requires a hybridization probe labeled with two different fluorescent dyes. One dye is a reporter dye (FAM, i.e., 66-carboxyfluorescein), the other is a quenching dye (TAMRA, i.e., 6-carboxy-tetramethylrhodamine) (Heid *et al*., 1997). Once the Taqman probe has bound to its specific piece of the template DNA after denaturation stage and the reaction cools, the primers anneal to the DNA. Taq polymerase then adds nucleotides and removes the Taqman probe from the template DNA. This separates the quencher from the reporter, and allows the reporter to give off it's emit its energy. The more times the denaturing and annealing takes place, the more opportunities there are for the Taqman probe to bind and, in turn, the more emitted light is detected

(http://www.lifetechnologies.com/ee/en/home/brands/molecular-probes.html).

To quantify the amount of template present in a sample, it is necessary to create controls with a fixed number of templates. Standards are used to allow calculation of the amount of template present in a sample, while internal controls (ICs) are mostly used to determine the presence of false-negative reactions, examine the ability to amplify from a preparation of nucleic acids, and, quite rarely in real-time PCR, as a standard for quantification. It is very important to accurately determine concentration of the controls. Finally, the results of quantitation using a molecular control need to be expressed relative to a suitable biological marker, e.g., in terms of the volume of plasma, the number of cells of the mass of tissue or genomic nucleic acid, thus allowing to compare assay results and testing sites (Niesters, 2001). Most commonly, an exogenous control is created using a cloned amplicon, a portion of the target organism's genome, or simply purified amplicon itself (Borson, 1998). This control forms the basis of an external standard curve created from the data produced by the individual amplification of a dilution series of exogenous control. The concentration of unknown, which is amplified in the same reaction, but in a separate vessel, can then be found from the standard curve.

Fluorescence data generated by real-time PCR assays are generally collected from PCR cycles that occur within the linear amplification portion of the reaction, where conditions are optimal and the fluorescence accumulates in proportion to the amplicon (Figure 4) (Mackay *et al*., 2002). The fractional cycle number at which the real-time fluorescence signal mirrors progression of the reaction above the background noise is used as an indicator of successful target amplification (Wilhelm *et al*., 2001). This is called the threshold cycle (C_T), but a similar value is described for the LightCycler, and the fractional cycle is called the crossing point (C_P). The C_T is defined as the PCR cycle in which fluorescence generated by the accumulating amplicon exceeds ten standard deviations of the mean baseline fluorescence, using data taken from cycles 3–15 (Jung *et al*., 2000).The C_T and C_P are proportional to the number of target copies present in the sample (Gibson *et al*., 1996) and are assumed to represent equal amounts of amplicon present in each tube. The C_T and C_P values represent the fractional cycle number for each sample at a single fluorescence intensity value. The final C_T and C_P values are the fractional cycles at which a single fluorescence value (at or close to the noise band) traverses each sample's plotted PCR curve (Wilhelm *et al*., 2001) (Figure 4). The accuracy of the C_T or C_P depends upon the concentration and nature of the fluorescence-generating component, the amount of template initially present, the sensitivity of the platform, and the platform's ability to discriminate.

Real-time PCR is used for detection of nucleic acids from food, vectors used in gene therapy protocols, genetically modified organisms, and areas of human and veterinary microbiology and oncology (Mhlanga and Malmberg 2001; Klein *et al*., 2002; Ahmed, 2002;).

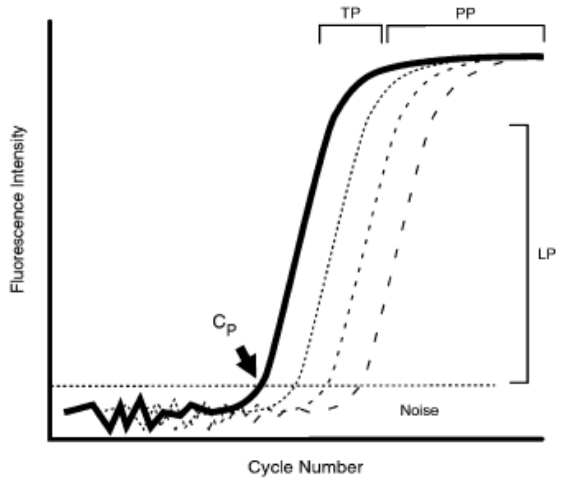


Figure 4. Amplification curve of a real-time PCR adapted from Mackay *et al*., 2004

Amplification curve plotted as fluorescence intensity against the cycle number is a sigmoidal curve. Early amplification cannot be viewed because the emissions are masked by the

background noise. When enough account of amplicon is present, the assay's exponential progress can be monitored as the rate of amplification enters a linear phase (LP). Under ideal conditions, the amount of amplicon increases at a rate of one $\log_{10}$ every 3.32 cycles. As primers and enzyme become limiting, and products inhibitory to the PCR and overly competitive to oligoprobe hybridisation accumulate, the reaction slows, entering a transition phase (TP) and reaching a plateau phase (PP) where there is little or no increase in fluorescence. The point at which the fluorescence surpasses the noise threshold (dashed horizontal line) is called the threshold cycle or crossing point (C_T or C_P; indicated by an arrow), and this value is used in the calculation of template quantity during quantitative real-time PCR. Also shown are curves representing a titration of template (Figure 4, dashed curves), consisting of decreasing starting template concentrations, which produce higher C_T or C_P values. The product accumulation rate curves allow backing calculating the number of original target molecules in a sample (Figure 4, dashed curves). Data for the construction of a standard curve are taken from the linear phase (Mackay, 2004).

Real-time PCR method has some advantages and disadvantages. This method has high sensitivity and precise quantification of microbial species. However, this molecular tool allows detect only one target per experiment and only known microbial species (Biagi *et al*., 2012). Ability to design primers for real-time PCR extends to knowledge of a microorganism´s genome as well as the ability of publicly available sequence databases to suitably represent all variants of that microbe (Mackay, 2004).

Recently the publications about applying of multiplex real-time PCR have been appeared (Haarman and Knol 2006). The difference of this method is that multiple targets are amplified in a single reaction tube. Each target is amplified by a different set of primers, and a uniquely-labeled probe distinguishes each PCR amplicon. Thus, it is possible to measure the expression levels of several targets or genes of interest more quickly. This method uses one or more primer sets to amplify multiple templates within a single reaction. However, multiplex real-time PCR has proven problematic because of the limited number of fluorophores available for detection (Wittwer *et al*., 2001; Mackay *et al*., 2004).

1.5.2.2 Another molecular methods

One more molecular approach for quantifying bacterial cells in complex ecosystems is fluorescence in situ hybridization (FISH). This technique allows nucleic acid sequences to be

examined inside a cell without altering the cell's morphology or integrity of its various compartments (Amann *et al*., 1995; Harmsen *et al*., 1999). The most commonly used target molecule for FISH is 16S rRNA. This method detects nucleic acid by fluorescently labeled probes that hybridize specifically to its complementary target sequence. This method combines the precision of molecular genetics with the visual information from microscopy. The procedure in the following steps: (I) fixation of the specimen, (II) preparation of the sample, possibly including specific pretreatment steps; (III) hybridization with the respective probes for detecting the respective target sequences; (IV) washing steps to remove unbound probes; (V) mounting, visualization and documentation of results (Moter and Göbel, 2000). The nucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5'-end to a single fluorescent dye molecule. Common fluorophors include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5 (Southwick *et al*., 1990). In general, FISH allows quantitative analysis of the bacteria and has high sensitivity. However, this method has some disadvantages, such as false positive results due to autofluorescence of microorganisms themselves; insufficient probe penetration into the bacterial cell; limited number of used genus-or species-specific probes (Moter and Göbel, 2000).

Nowadays, one of the widely used technologies in microbiology is pyrosequencing, a unique sequencing method that was developed as an alternative to classical DNA sequencing for short- to medium-read applications (60-200bp). This method is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides. The cascade starts with a nucleic acid polymerization reaction in which inorganic PPi is released as a result of nucleotide incorporation by polymerase. The released PPi is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light. Because the added nucleotide is known, the sequence of the template can be determined (Ronaghi, 2001). Compared to other, methods, it is highly quantitative and fast. Additional advantages include high accuracy, flexibility and ability to automate sample preparation (Novais and Thorstenson, 2011). The main disadvantage of pyrosequencing is a high cost of this method.

More recently, the microarrays technology has been applied for the study of the diversity of human intestinal microbiota. Phylogenetic DNA microarrays consists of several thousand probes, usually designed from rRNA gene sequence database targeting either specific microorganisms (e.g. pathogenic bacteria) or the whole microbiota at various taxonomic

levels. Using of 16S rRNA microarrays technique provides superior diagnostic power compared to clone library (DeSantis *et al*., 2007). The main advantage of phylogenetic microarrays is a high resolution of microbial biodiversity and quantitative analysis of the entire microbial community (Biagi *et al*., 2012). Several microarrays addressing the gut microbiota have been developed, showing differences in their design and the aims of study. Palmer et al., 2007 have designed an array containing 10,265 probes, each spotted once, and targeting 1,629 species. Another microarray addressing the whole gut microbiota has been spotted with 16,223 probes targeting 775 bacterial species (Paliy *et al*., 2010). The Human Intestinal Tract Chip (HITChip) technology has targeted 1,140 microbial phylotypes (<98% identify) using 4,809 overlapping probes (Rajilic´-Stojanovic´ *et al*., 2009). The HuGChip technolog has been composed of 4,441 probes (2,442 specific and 1,919 explorative probes) targeting 66 bacterial families. This technology is able to detect not only known human gut microbiota species but also not yet described in the human gut microbiota (Tottey *et al*., 2013). It is a high throughput platform used for studying numerous samples and detection thousands of nucleic acids sequences.

Effective method for qualitative characterization of microbial community is terminal restriction fragment length polymorphism (T-RFLP). This approach involves tagging one end of PCR amplicons through using of a fluorescent molecule attached to a primer. Then restriction enzyme cuts the amplified product. Terminal restriction fragments (T-RFs) are separated by electrophoresis and visualized by emission of the fluorophore. T-RFLP analysis provides quantitative data about each detected terminal restriction fragment, including size in base pairs and intensity of fluorescence. T-RF sizes can be compared to a database of theoretical T-RFs derived from sequence information. T-RFLP profiles have been shown to be relatively stable to variability in PCR conditions (Blackwood *et al*., 2003). The disadvantage of this method is impossibility of quantitative analysis and no identification of bacteria at specie level (Biagi *et al*., 2012).

2. AIMS OF THE STUDY

The aim of the study was to quantify the gut microbial compositions of healthy persons in different age groups.

The present study set the following specific objectives:

1. Elaboration of real-time PCR method for quantitative analysing of twelve bacterial groups of the human gut

2. Assessment the counts of dominanat bacterial groups

3. Assessment the ratio of *Bacteroides/Firmicutes*

4. Assessment the counts of beneficial bacterial groups such as *Lactobacillus* and *Bifidobacterium* sp. in different age groups

3. MATERIALS AND METHODS

3.1 Study groups

The study group comprised both twenty five healthy 1- and 5-year-old children who were randomly selected from a large group in which the immune responses to allergens and the develpment of allergy were studied (Julge *et al*., 2001). Adult were recruited from the baseline values of the study assessing the impact of a probiotic product (Mikelsaar *et al*., 2014, submitted). Elderly were selected from the registry of family doctors and orthopaedists of the Tartu University Hospital, Estonia, before performing elective orthopaedic surgery (Mikelsaar *et al*., 2010) (Table 1). The studies were approved by the Ethics Committee of the Medical Faculty of the University of Tartu with approvals no. ISRCTN38739209, 139/16 20.06.2005; 158/10 26.03.2007; ISCRNT53154826. Informed consent was also obtained from the parents of the children.

Table 1. Characterization of the study groups.

	Infants	Children	Adults	Elderly
Subjects (male/female)	25 (17/8)	25 (13/12)	25 (4/21)	23 (9/14)
Age (mean±SD) (months/years old)	11.9±0.6	63.7±2.5	48.2±6.6	72.9±5.0

SD - standard deviation. Age of infants and children present in months; adults and elderly in years.

3.2 Bacterial strains and culture conditions

3.2.1 Sample collection

Fresh stool samples were placed in a plastic cups. The samples collected at home were kept in a domestic refregerator at 4°C for no more than 2h before transportation to the laboratory, where the plastic cups were stored frozen at -70°C until use.

3.2.2 The reference strains

Twelve culture collection strains were purchased from both the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Bacterial fecal isolates identified by MALDI TOF MS spectrophotometer has been used in this study (Table 2).

Table 2. The reference strains used in the study.

Species	Strain
E. coli	ATCC 700336
E.coli	JM109
B. longum	DSM14583
B. fragilis	MALDI-TOF(*B.fragilis,2.3 score)*
C. perfringens	DSM 756
R. gvanus	MALDI-TOF(*R.gvanus,2.3 score*
C. leptum	DSM 753
C. difficile	ATCC 43255
B. bifidum	DSM 20456
L. acidophilus	ATCC 4356
E. faecalis	ATTC 51299
V. parvula	DSM 2007
F.prausnitzii	DSM 17677

3.3 DNA extraction

Bacterial DNA of type strains was extracted using QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to manufacture instructions.

Bacterial DNA from faecal samples was extracted using a QIAamp DNA stool mini kit (QIAgen, Hilden, Germany) with some modifications. 180-220 mg of faeces were resuspended in 200 µl of TE buffer (10 mMTris, 10 mM EDTA pH=8, 20 mg/ml lysozyme, 200 u/ml mutanolysin) and incubated for 1 hour at 37ºC. 0.3 g of 0.1 mm zirconia/silica beads and 1.4 ml of ASL solution from the stool mini kit was added to faecal samples. The tubes were then agitated for 3 min at a speed of 5000 rpm in a mini-bead beater (Biospec Products Inc., USA). The protocol was continued according to manufacture instructions. Extracted DNA was quantified using NanoDrop™ 1000 Spectrophotometer 1.0 (NanoDrop Technologies, Inc., USA) at 260 nm.

3.4 Primers and probes

Primers and probes used in the study were targeted on the 16S rRNA genes (Table 3). The oligonucleotide probe used for the detection of the genus *Bifidobacterium* and *Lactobacillus*

are labeled with the 5 reporter dye VIC and the 3 quencher NFQ-MGB and for total bacteria and *C. difficile* with 6-FAM and TAMRA (Applied Biosystems, The Netherlands).

Table 3. **Table of primers and probes used in the study.**

Target groups (amplicon length, T_m assay)	**Primers/Probes**	**Sequence (5'-3')**	**References**
All bacteria (466bp, 60ºC, TaqMan)	Eub-f Eub-r Eub (Probe)	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT (6-FAM)-CGTATTACCGCGGCTGCTGGCAC-(TAMRA)	Nadkarni *et al*., 2002
Firmicutes phylum (126bp, 60°C, Sybr Green)	Firm934f Firm1060r	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	Guo *et al*., 2008
Bifidobacterium spp.(231bp, 60°C TaqMan)	Allbif-f Allbif-r AllBif(Probe)	GGGATGCTGGTGTGGAAGAGA TGCTCGCGTCCACTATCCAGT (VIC)-TCAAACCACCACGCGCCA-(NFQ-MGB)	Haarman *et al*., 2007
Lactobacillus spp.(92bp, 60°C TaqMan)	AllLacto-f AllLacto-r AllLacto(Probe)	TGGATGCCTTGGCACTAGGAAAATCTCCGGATCAAAGCTTACTTAT (VIC)-TATTAGTTCCGTCCTTCATC-(NFQ-MGB)	Haarman *et al*., 2007
Bacteroides-Prevotella group (140bp, 58ºC, Sybr Green)	Bact-f Bact-r	GGTGTCGGCTTAAGTGCCATCGGACGTAAGGGCCGTGC	Malinen *et al*., 2005
C. perfringens group (120bp, 55ºC, Sybr Green)	Cperf-f Cperf-r	ATGCAAGTCGACCGAKG TATGCGGTATTAAATCTYCCTTT	Malinen *et al*., 2005
C. coccoides-E.rectale group (182bp, 55ºC, Sybr Green)	Ccocc-r Ccocc-f	AGTTTYATTCTTGCGAACG CGGTACCTGACTAAGAAGC	Malinen *et al*., 2005
C. leptum group (239bp, 50ºC, Sybr Green)	Clept-f Clept-r	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	Matsuki *et al*., 2004
C. difficile (177bp,60 ºC, TaqMan)	Cdif398 Cdif399 Cdif(Probe)	GAAAGTCCAAGTTTACGCTCAAT GCTGCACCTAAACTTACACCA (6-FAM)ACAGATGCAGCCAAAGTGGTTG AATT-(TAMRA)	Berg *et al*., 2007
Enterobacteriacea (195bp ,58°C, Sybr)	Eco1457-f Eco1652-r	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	Bartosch *et al*., 2004
Enterococcus spp. (144bp, 61°C Sybr)	Enteroc-f Enteroc-r	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTG	Malinen *et al*., 2005
Veilonella spp. (343bp, 62°C, Sybr Green)	Veilon-f Veilon-r	A(C/T)CAACCTGCCCTTCAGA CGTCCCGATTAACAGAGCTT	Malinen *et al*., 2005
F. prausnitzii (158bp, 61°C , Sybr Green)	Fprau-f Fprau-r	GTCGCAGGATGTCAAGAC CCCTTCAGTGCCGCAGT	Malinen *et al*., 2005

3.5 Plasmids construction and standards.

In order to establish a quantitative assays, we cloned plasmids containing the amplified region of target bacteria using the pGEM-T Easy vector system (Promega, Madison, USA). The PCR amplicon for each reference strain was individually inserted into a separate plasmid vector; the recombinanat vector was transformed into chemically competent *E. coli* JM109 cells. Plasmids were purified with NucleoSpin PlasmidQuick pure Kit according to manufacture instruction (Macherey-Nagel, Germany). The purified plasmids were quantified by spectrofotometry (NanoDrop ND-1000, USA) of multiple dilutions (Bartosch *et al*., 2004; Fite *et al*., 2004). Quantification of target DNA was achived by using serial tenfold dilution from 10^5 to 10^1 plasmid copies of the previously quantified plasmid standards.

3.6 Real-time PCR method (qPCR)

Amplification and detection of DNA by real-time PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems Europe BV, Zug, Switzerland) using optical-grade 96-well plates. Duplicate sample analysis was routinely performed in a total volume of 25 µl using SYBR Green PCR Master Mix (Applied Biosystems). Each reaction included 2µl of template DNA, 12.5µl of SYBR Green Master mix (Applied Biosystems, USA) and 100-400 mM of forward and reverse primers. The end of PCR assays dissociation curve analysis was performed to check for non-specific products and/or contamination for SYBR Green.

For TaqMan assay PCR reaction was performed in a total volume of 25µl using the TaqMan® Universal PCR Master Mix (Applied Biosystems, USA). Each reaction included 2µl of template DNA, 12.5µl of TaqMan® Universal PCR Master Mix (Applied Biosystems, USA), 400 nM of forward and reverse primers, 100 nM of corresponding probe. The real-time PCR conditions consisted of an initial denaturation step 50°C for 2 min and 95°C for 10 min, continued with amplification step followed by 40 cycles consisting of denaturation at 95°C for 15 s, annealing-elongation step at 60°C for 1 min. Standard curves were routinely performed for each real-time PCR run using serial dilutions of control plasmid DNA. Data from triplicate samples were analyzed using the Sequence Detection Software version 1.6.3 (Applied Biosystems, USA).

3.7 Statistical analysis

The statistical analysis was performed using SIGMASTAT 2.0 (Jandel Scientific Corporation, San Safael, CA, USA). Data are present as means and standard deviation or ranges and medians. The prevalence of the species was expressed by as a percentage. According to the data descriptive statistics, Fisher exact test, Bonferron correlation and Mann-Whitney rank sum test were applied to compare the differences in microbiological indices. All differences were considered statistically significant if $p<0.008$.

4. RESULTS

4.1 Elaboration of real-time PCR method

To check the reliability of the QIAamp DNA Stool Mini kit (Qiagen) and to determinate a detection limit of the real-time PCR method, a recovery experiment was performed. Faecal samples, in which *Bifidobacterium* sp. could not be detected, were dosed with aliquots of different dilutions of lactobacilli (DSM 20456; 10^6–0 CFU/ml). DNA isolation was undertaken and real-time PCR analysis of the samples was performed to determine the amount of bifidobacteria. Additionally, electrophoresis on agarose gel was performed to confirm the results.

A standard curve was obtained by using 10-fold serial dilutions of positive plasmids for all target microorganisms. The real-time PCR assay was able to detect $3 \cdot 10^5$-$3 \cdot 10^1$ plasmid copies. To generate a standard curve, the threshold cycle (Ct) of these standard dilutions was plotted against the number of plasmid copies used as input. The copy numbers of the target group for each reaction were calculated from the standard curves. The functions describing the relationship between Ct (threshold number) and x (copy number) for *Firmicutes* phylum were: Ct= -3.17x+37.79; R^2=0.99; *Bacteroides-Prevotella* group: Ct= -2.55x+34.4; R^2=0.99; *C. perfringens* Ct= -2.89x+34.83; R^2=0.98, *C.leptum* group: Ct= -2.81x+41.66; R^2=0.99, *C. coccoides* group: Ct= -3.48x+42.08; R^2=0.99; *C.difficile*: Ct= -3.12x+31.55; R^2=0.99; *Enterobacteriaceae* Ct= -3.62x+39.08; R^2=0.99; *Enterococcus* sp. Ct= -3.07x+38.7; R^2=0.98; *Lactobacillus* sp. Ct= -3.07x+34.99; R^2=0.99, *Bifidobacterium* sp. Ct= -3.87x+46.52; R^2=0.98; *Veillonella* group Ct= -3.08x+36.25; R^2=0.99; *F. prausnitzii* Ct= -2.17x+30.83; R^2=0.98.

Table 4. Most prevalent bacterial groups in human gastrointestinal tract detected by real-time PCR in this study.

RT-PCR assay	Target species
Firmicutes phylum	Bacillaceae, Paenibacillaceae, Staphylococcaceae, Aerococcaceae, Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Acidaminococcaceae, Closridiaceae, Eubacteriaceae, Lachnospiraceae, Peptococcaceae, Peptostreptococcaceae, Erysipelotrichaceae family
Bacteroides-Prevotella-Porphyromonas group	*Bacteroides fragilis, B.stercoris, B.vulgatus, B.eggerthii, B.acidofaciens, B.caccae, B.ovatus, B.uniformis, B.thetaiotaomicron, B.distasonis, B.merdae, B.forsythus, Prevotella tannerae, P.bryantii, P. ruminicola, P. heparinolytica,P. zoogleoformans, P. brevis, P. loescheii, P. buccae, P. oralis, P. enoeca, P. melaninogenica, P. veroralis, P.*

	intermedia, P. albensis, P. nigrescens, P. corpois, P. disiens, P. bivia, P. pallens, P. denticola, Parphyromonas canoris, P. gingivalis, P.asaccharolytica, P.levii, P. cangingivalis, P. macacae, P. circumdentaria, P. catoniae
C. perfringens group	*Clostridium perfringens, C. homopropionicum, C. cadaveris, C. intestinalis, C. putrificum, C. botulinum, C. novyi, C. sporogenes, C. tyrobutyricum, C. kluyveri, C. ljungdahlii, C. scatologenes, C. acetireducens, C. subterminale, C. estertheticum, C. agrentinense, C. sardiniensis, C. paraputrificum, C. longisporum, C. septicum, C. cellulovorans, C. barati, C. absonum, C. chauvoei, C. carnis, C. butyricum, C. beijerinckii, C. kainantoi, C. corinoforum, C. puniceum, C. histolycum, C. proteolyticum, C. limosum, C. paraputrificum, Eubacterium budayi, E. nitritogenes, E. moniliforme, E. multiforme*
C. coccoides-E.rectale group	*Clostridium coccoides, C. proteoclasticum, C. aminophilum, C. symbiosum, C. sphenoides, C. celerecrescens, C. aerotolerans, C. xylanolyticum, C. clostridiiforme, C. fusiformis, C. nexile, C. oroticum, C. populeti, C. aminovalericum, C. indolis, C. herbivorans, C. polysaccharolyticum, Eubacterium xylanophilum, E. ruminantium, E. saburreum, E. fussicatena, E. hadrum, E. rectale, E. ramulus, E. contortum, E. eligens, E. hallii, E. formicigenerans, E. cellulosolvens, Rumminococcus products, R. obeum, R. schinkii, R. hydrogenotrophicus, R. hansenii, R. torques, R. lactaris, R. gvanus, Butyrivibrio fibrisolvens, B. crossotus, B. fibrisolvens, Desulfotomaculum guttoideum, Roseburia cecicola, Pseudobutyrivibrio ruminis, Lachnospira multipara, L. pectinoschiza, Acetitomaculum ruminis, Catonella morbi*
C. leptum group	*Clostridium leptum, C. sporosphaeroides, C. cellilosi, C. viride, R. flavefaciens, R.callidus, R.albus, Eubacterium siraeum, E. plautii, E. desmolans, E.bromii, Butyrate-producing bacterium, Faecalibacterium prausnitzii, Subdoligranulum variable, Anaerofilum agile, A.pentosovorans, Sporobacter termitidis*
C. difficile	*Clostridium difficile*
Enterobacteriacea	*Moellerella wisconsensi, Providencia alcalifaciens, P. rustigianii, P. rettgeri, P.stuartii, Proteus penneri, P. mirabilis, P. vulgaris, Morganella morganii, Leminorella grimontii, Edvardsiella tarda, Escherichia coli, E. fergusonii, Citrobacter koseri, C. amaloonaticus, C. farmeri, C. sedlakii, Serratia marcescens, S. liquefaciens, Klebsiella pneumoniae, K. oxytoca, Citrobacter murliniae, C. amalonaticus, C. farmeri, C. sedlakii, Raoultella planticola, R. Terrigena, Enterobacter aerogenes, E. cancerogenus, E. cloacae, E. asburiae, Pantoea agglomerans, Tatumella ptyseos, Averyella dalhousiensis, Yokenella regensburgei*
Bifidobacterium sp.	*B. longum, B.minimum, B. angulatum, B. catenulatum, B. dentium, B. ruminantium, B. thermophilum, B. subtile, B. bifidum, B.boum, B. lactis, B. animalis, B. choerinum, B. gallicum, B. pseudolongum subsp. globosum, B. pseudolongum subsp. pseudolongum, B. magnum, B. infantis, B. gallinarum, B.*

	pullorum, B. saeculare, B. suis
Lactobacillus group	*Lactobacillus acidophilus, L. amylovorus, L. delbrueckii subsp. bulgaricus, L. delbrueckii subsp. delbrueckii, L. delbrueckii subsp. lactis, L. amylolyticus, L. acetotolerans, L. crispatus, L. amylophilus, L. johnsonii, L. gasseri, L. fermentum, L.pontis, L. reuteri, L. mucosae, L. vaginalis, L. panis, L. oris, L. pentosus, L. plantarum, L. collinoides, L. alimentarius, L. farciminis, L. brevis, L. buchneri, L. kefiri, L. fructivorans, L. mali, L. animalis, L. murinus, L. ruminis, L.agilis, L. salivarius, L. aviarius, L. sharpeae, L. manihotivorans, L. rhamnosus, L. casei subsp. casei, L. casei subsp. fusiformis, L.zeae, L. paracasei subsp. paracasei, L. paracasei subsp. tolerans, L. coryniformis subsp. coryniformis, L. bifermentas, L. perolens, L. sakei, Pediococcus pentosaceus, P. parvulus, P. acidilactici, P. dextrinicus, Weissella halotolerans, W. confusus, W. paramesenteroides, W. hellenica, W. viridescens, W. kandleri, W. minor, Leuconostoc lactis*
Enterococcus sp.	*Enterococcus faecalis, E. faecium, E. asini, E. saccharolyticus, E. casseliflavus, E. gallinarum, E. dispar, E. flavescens, E. hirae, E. durans, E. pseudoavium, E. raffinosus, E. avium, E. malodoratus, E. mundtii, E. azikeevi, E. canis, E. gilvus, E. haemoperoxidus, E. hermanniensis, E. moraviensis, E. pallens, E. phoeniculicola, E. villorum, E. rottae*
Veilonella spp.	*Veilonella parvula, V. dispar, V. atypica, V. ratti, V. criceti, V. rodentium, V. caviae*
F. prausnitzi	*Fusobacterium prausnitzii*

4.2 Quantitative differences between the gut microbiota of infants, children, adults and elderly

Using real-time PCR, a total 12 bacterial groups were quantified from faecal samples of four age groups. Totally 14 families of *Firmicutes* phyla and more than 290 species were detected in faecal samples (Table 4). The large inter-individual variation in the numbers of bacteria detected from faecal samples of the 98 subjects was assessed. Results real-time PCR test are present in Figures 5-9, Table 5. *Firmicutes* phylum, *Enterobacteriaceae, Bacteroides-Prevotella*, *C. perfringens, C.leptum, C. coccoides, Veillonella* groups and *Enterococcus* sp. were detected in all faecal samples.

The most prevalent phyla were *Firmicutes* and *Bacteroides* in all groups. The level of *Firmicutes* phylum increased while *Bacteroides-Prevotella* groups decreased with age (Figure 5 A, B). The highest counts of bacteria belongs to *Firmicutes* phyla were found in elderly in comparison to infants ($1.6 \cdot 10^{11}$-$2.9 \cdot 10^{8}$; median $1.6 \cdot 10^{10}$ *vs*. $4.4 \cdot 10^{9}$-$5 \cdot 10^{8}$; median $1.47 \cdot 10^{9}$; $p<0.001$) (Figure 5A). In opposite, elderly were less colonized with *Bacteroides-Prevotella*

group in comparison to adults and infants ($1.45 \cdot 10^9$-$8 \cdot 10^2$; median $1.33 \cdot 10^7$ *vs*. $1.99 \cdot 10^{11}$-$3.6 \cdot 10^4$; median $3.5 \cdot 10^9$; $5.9 \cdot 10^{11}$-$3.7 \cdot 10^4$; median $1.73 \cdot 10^{10}$; p<0.001, respectively) (Figure 5B).

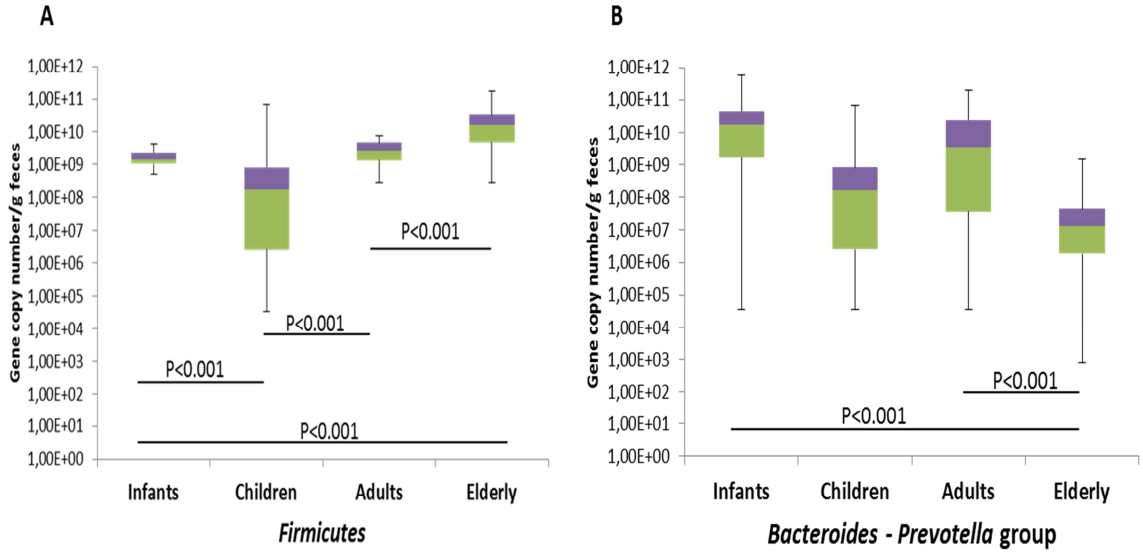


Figure 5. The count of the *Firmicutes* phyla (A) and the *Bacteroides-Prevotella* group (B) in different age groups

4.3 The ratio of *Firmicutes/Bacteroides* genus in different age groups

The *Firmicutes*/*Bacteroides* genus ratio in all studied age groups was observed. The ratio increased with age. The ratio were significant statistically lower for infants in comparison to children and elderly (1.97-0.79, median 0.9 *vs.* 2.39-0.96, median 1.29; *vs*. 3.57-1.12, median 1.41, respectively; p<0.001) and for adults in comparison to elderly (2.2-0.75, median 1.01 *vs*3.57-0.57, median 1.41; p<0.001) (Figure 6).

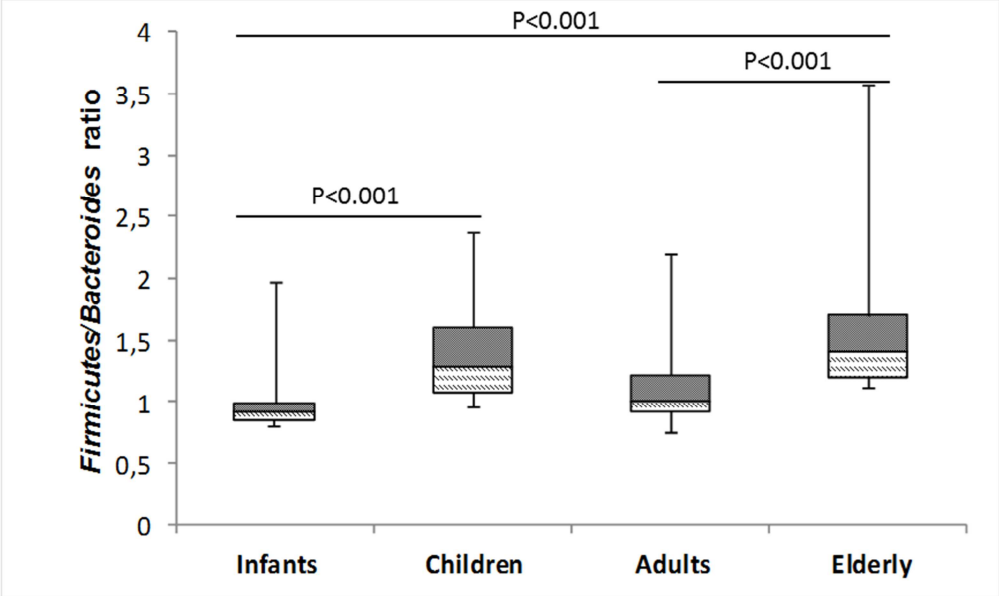
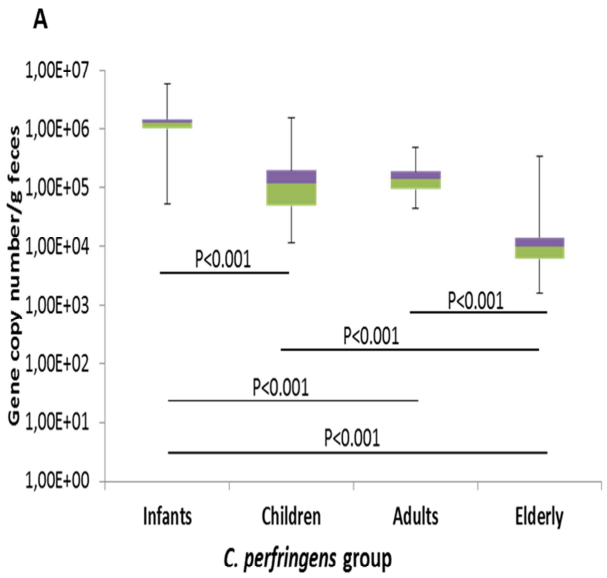


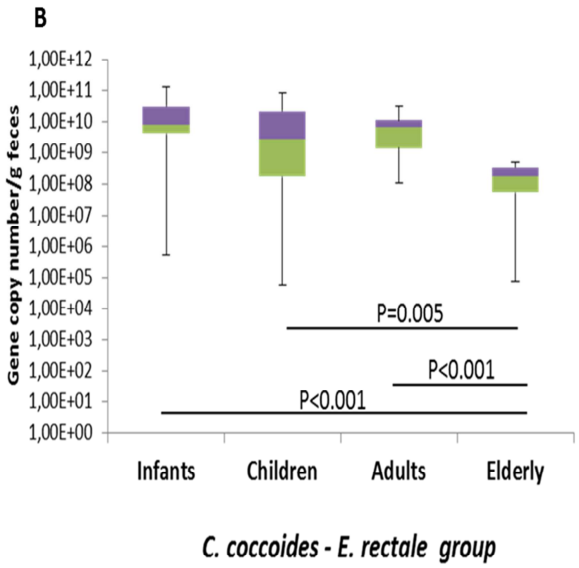
Figure 6. The *Firmicutes*/*Bacteroides* ratio in different age groups. Dot plots indicate max-min, median, and 1st and 3rd quartiles.

In total, four dominant *Clostridium* groups of human intestinal microbiota were detected in this study (*C. leptum, C. perfringens, C. coccoides-E. rectale* and *C. difficile*). *C. leptum* and *C. coccoides-E.rectale* groups were prevalent in all age groups. The counts of *C. perfringens* and C. *cooccoides-E.rectale* group's were found to be significantly lower in elderly ($p<0.001$; $p<0.001$, respectively) (Figure 7 A, B). The data have shown that significant higher numbers of *C. leptum* were observed in adults in comparison to elderly and infants ($2{,}40\cdot10^{12}$-$1{,}83\cdot10^{5}$, median $1{,}82\cdot10^{11}$, $p=0.007$ *vs.* $7{,}77\cdot10^{7}$-$7{,}21\cdot10^{8}$, median $6{,}19\cdot10^{9}$, $p<0.001$ *vs*. $7{,}77\cdot10^{7}$-$7{,}21\cdot10^{8}$, median $6{,}19\cdot10^{9}$, $p=0.007$; respectively) (Figure 7C).

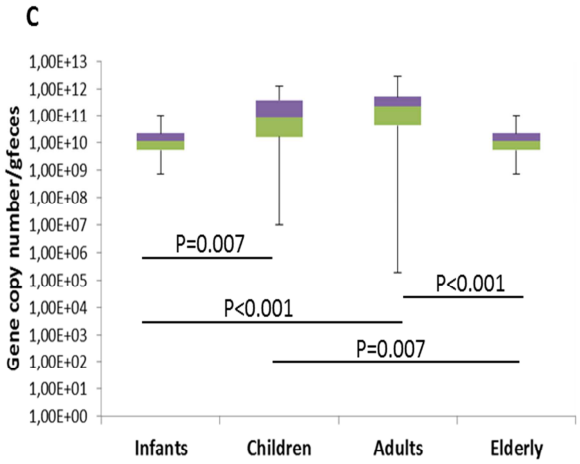
C. difficile was detected only in three age groups (infants, children and elderly). *C. difficile* was more prevalent in elderly and infants than in adults (10/23, 7/25 (28%) vs. 0/25, $p=0.005$; $p<0.001$, respectively) (Table 5). The differences in the *C. difficile* counts between different age groups were not found.



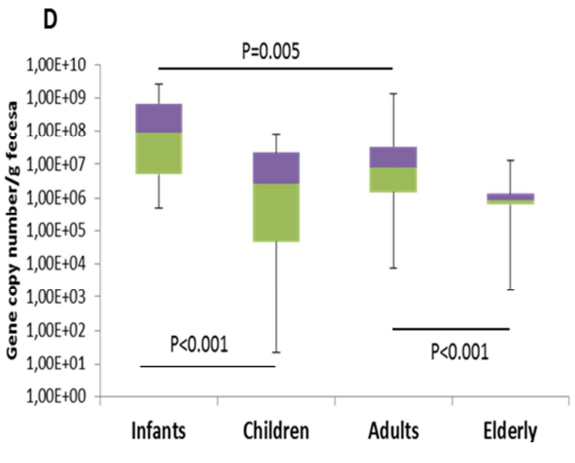
C. perfringens group



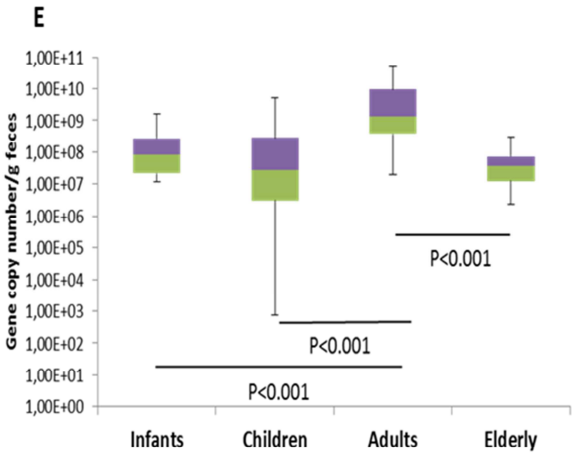
C. coccoides - E. rectale group



Clostridium leptum group



Veillonella group



Fusobacterium prausnitzii

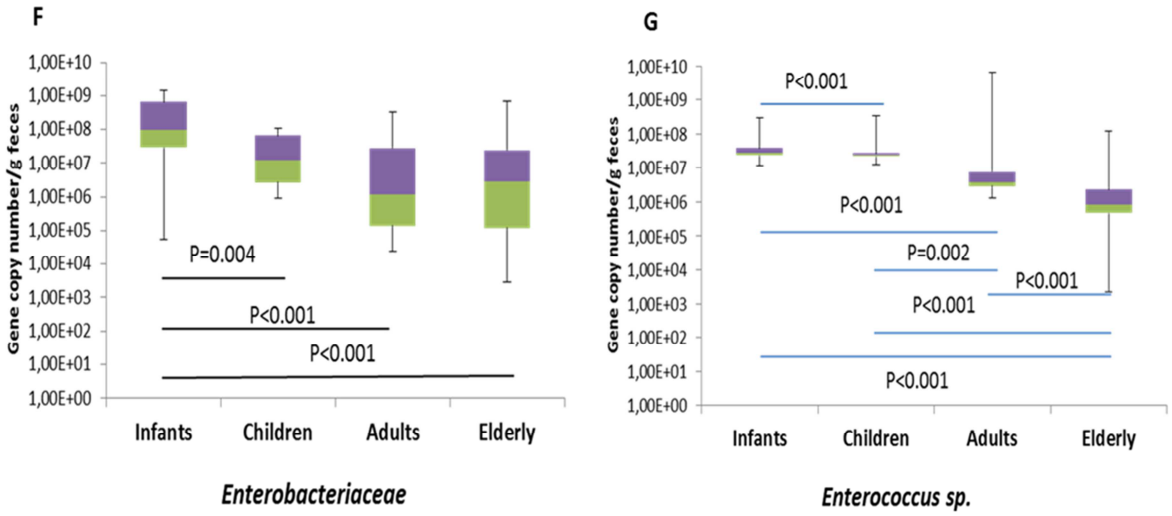


Figure 7. The counts of *C. perfringens group* (A), *C.coccoides-E.rectale* group (B); *C. leptum* group (C), *Veillonella* group (D), *F. prausnitzii* (E), *Enterobacteriaceae* family (F), *Enterococcus* sp. (G) per gram of faeces determined by real-time PCR. Dot plots indicate max-min, median, and 1st and 3rd quartiles

Significant higher numbers of *Enterococcus* sp. were observed in infants versus adults and elderly ($2.88 \cdot 10^8$-$1.2 \cdot 10^7$; median $2.76 \cdot 10^7$ *vs.* $6.37 \cdot 10^9$-$1.27 \cdot 10^6$; median $3.8 \cdot 10^6$; $1.25 \cdot 10^8$-$2.2 \cdot 10^3$; median $8.2 \cdot 10^5$; $p<0.001$, respectively) (Figure 7G).

The highest counts of *Enterobacteriaceae* family are found in infants. Statistical analysis of the data demonstrates progression between age groups with decreasing in children ($p=0.004$) and stabilizing of levels in adults and elderly ($p<0.001$) (Figure 7F).

The highest counts of *F.prausnitzii* were found in adults in comparison to other groups ($5 \cdot 10^{10}$-$1.98 \cdot 10^7$; median $1.34 \cdot 10^9$ *vs.* $3.04 \cdot 10^8$-$2.34 \cdot 10^6$; median $3.6 \cdot 10^7$; $5 \cdot 10^9$-$7.6 \cdot 10^2$; median $2.7 \cdot 10^7$; $1.6 \cdot 10^9$-$1.21 \cdot 10^7$; median $8.18 \cdot 10^7$; $p<0.001$, respectively) (Figure 7E).

Statistical analysis has shown that infants and adults were more harbored *Veillonella* sp. than elderly ($2.6 \cdot 10^9$-$5.1 \cdot 10^5$; median $9.1 \cdot 10^7$; $1.37 \cdot 10^9$-$7.3 \cdot 10^3$; median $7.9 \cdot 10^6$; *vs.* $1.27 \cdot 10^7$-$1.6 \cdot 10^3$; median $8.6 \cdot 10^5$; $p<0.001$, respectively) (Figure 7D).

Table 5. The prevalence and counts of *C. difficile* in different age groups.

	Infants	**Children**	**Adults**	**Elderly**
Prevalence (%)	7/25 (28%)	5/25 (20%)	0/25 (0%)	10/23 (43.4%)
Counts (gene copy number/g feces) (mean±SD)	$2.38 \cdot 10^3 \pm 2 \cdot 10^3$	27.4±7.3	ND	227±37

4.4 Counts of beneficial bacterial groups (*Lactobacillus* and *Bifidobacterium* sp.)

Real-time PCR analysis detected *Bifidobacterium* and *Lactobacillus* species in all fecal samples of different age groups. The significant statistical differences were found between *Bifidobacterium* sp. counts in children and adults groups ($1.12 \cdot 10^9$-$8.6 \cdot 10^5$; median $5.8 \cdot 10^7$ *vs*. $7.2 \cdot 10^8$-$2.08 \cdot 10^4$; median $1.48 \cdot 10^7$; p=0.007) (Figure 9A). The differences in counts of lactobacilli were found between all study groups. Significantly lower amounts were observed in adults in comparison to elderly and children ($1.10 \cdot 10^7$-$4.5 \cdot 10^3$; median $5.0 \cdot 10^5$ *vs*. $9.8 \cdot 10^8$-$4.5 \cdot 10^6$; median $5.6 \cdot 10^7$; *vs*. $6.28 \cdot 10^8$-$1 \cdot 10^6$; median $3.28 \cdot 10^7$, respectively, p<0.001). Infants were less harboured with lactobacilli in comparison to children and elderly ($6.04 \cdot 10^8$-$5.5 \cdot 10^3$; median $7.2 \cdot 10^6$ *vs*. $6.28 \cdot 10^8$-$1 \cdot 10^6$; median $3.28 \cdot 10^7$; *vs*. $9.8 \cdot 10^8$-$4.5 \cdot 10^6$; median $5.6 \cdot 10^7$, respectively, p<0.001) (Figure 9B).

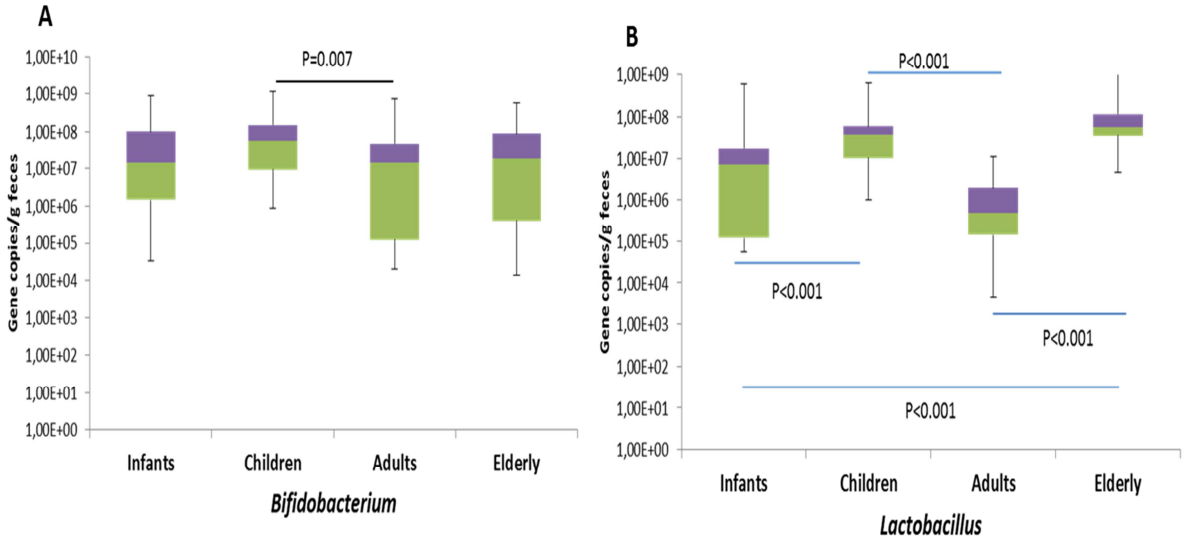


Figure 9. The gene copies numbers of total *Bifidobacterium* sp. (A) and total *Lactobacillus* sp. (B) per gram of faeces determined by real-time PCR. Dot plots indicate max-min, median, and 1st and 3rd quartiles.

5. DISCUSSION

The intestinal microbiota plays an important physiological role in human health and changed with age. Although the knowledge about composition of intestinal microbes is still limited, this study allowed more detailed to describe the complex composition of human intestinal microbiota in different ages. For that the dominant groups of human intestinal microbiota has been quantified by using real-time PCR. Our study have demonstrated that human intestinal microbiota have high individual diversity. Previously has been shown that ageing has been associated with profound changes in the bacterial colonisation as well as metabolic activities in the colonic ecosystem (Zwielehner *et al*., 2009; Claesson *et al*. 2011). Several factors may influence on diversity and stability of the intestinal microbial community such as geographical location, exposure to peristaltic activity, food molecules and gastric secretion (Manson *et al*., 2008).

All samples show very comparable overall structure of intestinal microbiota, that confirms the modern confirmation about increased of *Firmicutes* phylum and decreased of *Bacteroides* phylum among the age groups (Mariat *et al*., 2009). In our study we found that *Firmicutes*/*Bacteroides* ratio increased with age. Previously, increase in the *Firmicutes*/*Bacteroides* ratio was demonstrated for persons with obesity (Ley *et al*., 2005; Cani *et al*., 2009). Larsen et al. (2010) has shown also the positive correlation between the ratios of *Firmicutes/Bacteroides* and plasma glucose. However, the European study of adults and elderly (age groups 20-50 years and >60 years) by using FISH-flow cytometry have demonstrated country specific age related differences in the gut microbiota composition. The level of *Bacteroides–Prevotella* group was higher in elderly in Germany but lower in Sweden and Italy (Mueller *et al*., 2006).

Our study has shown a decrease in *C. perfringens* and *C.coccoides-E.rectale* groups with ageing. Similar results have been demonstrated for Japan, Italian and Finnish elderly (Hayashi *et al*., 2003; Mueller *et al*., 2006; Mäkivuokko *et al*., 2010). The study using a phylogenetic microarray also has demonstrated that members of *Clostridium* sp. and *Bacteroides* genus lower abundant in elderly (Gerritsen *et al*., 2011).

Interesting results have been demonstrated for *F.prausnitzii and C. leptum* group. *F.prausnitzii* belongs to the phylum *Firmicutes* and is the major bacterium of the *C. leptum* group (Cao *et al*., 2014). Our study has demonstrated that the amount of both groups was increased in adults.

There are not studies about quantitative changing of *C.leptum* group during ageing in healthy persons. The interest in studying of *F.prausnitzii* increased recently because this bacterium has anti-inflammatory properties in the gut (Sokol *et al*., 2008). Swedish study has found to have highest numbers of *Fusobaterium prausnitzii* which was associated by high consumption of fish and meat (Mueller *et al*., 2006).

Previous studies suggested that asymptomatic colonization with *C. difficile* early in life is more common in infants who develop allergy diseases (Penders *et al*., 2007). *C.difficile* is a normal gut resident that can cause disease after treatment with antibiotics in stable adults gut.

The quantitative composition of intestinal microbiota in children and adults was a quite similar. It has been shown that the diversity of gut microbiota increases and becomes stable by the first three-five years of life (Koenig *et al*., 2011). In this study we used fecal samples of 5 years old children. However, the significant differences were found in *Bifidobacterium* sp., *Lactobacillus* sp. and *Enterococcus* sp. The detection of *Bifidobacterium* sp. from samples of infants and children is consistent with the earlier findings that gut microbiota is dominated by facultative anaerobes in both groups as compared to adults microbiota and its one of early anaerobic colonizers of infant gut (Ouwehand *et al*., 2001). The *Bifidobacterium* sp. of the gut may differentially exert antimicrobial activity, suggesting their participation in the 'colonization resistance' produced by indigenous microbiota (Lievin *et al*., 2000).

The large changes in the counts of *Lactobacillus* sp. was found for all study groups. Previously, the increase in prevalence in number of *Lactobacilllus* sp. during aging has been described (Tiihonen, 2008). The increase of viable counts of lactobacillus in elderly individuals in comparison with younger adults also has been shown (Mikelsaar *et al*., 2010, Mäkivoukko *et al*., 2010; Štšepetova *et al*., 2011). *Lactobacillus* sp. comprised a minor part of the bacterial community in human gut (Sghir *et al*., 2000). The role of lactobacilli has received much attention, especially due to their putative properties. It has been shown that *Lactobacillus* sp. contribute to digestion, stimulation of immunity and impact on metabolism and energy uptake in the host (Vaughan *et al*., 2004, Valeur *et al*., 2004, Cani and Delzenne, 2009).

6. CONCLUTION

The study presented here we applied real-time PCR method to characterise and examine the human intestinal microbiota of healthy persons in different age groups. Our results confirmed that real-time PCR is powerful technique in the quantitative study of human intestinal microbiota. The statistical analysis allowed obtaining the information about the impact of the ageing process on the gut microbiota composition.

Our results showed that *Firmicutes* phyla and *Bacteroides-Prevotella* goup were prevailed in all age groups. The highest counts of bacteria belong to *Firmicutes* phyla were found in elderly. The *Firmicutes/Bacteroides* sp. ratio undergoes an increase from birth to adults and is further altered with advanced age.

C.leptum and *C.coccoides-E.rectale* groups were highly represented in all groups. *Clostridium* sp. counts decreased with ageing. Mucus-degrading *C.difficile* was detected in three age groups (infants, children, elderly) with highest counts in infants. The counts of *Enterococcus* sp., *Veillonella* sp. and *Enterobacteriaceae* were higher in infants while *F.prausnitzii* in adults.

The microbiota of elderly was generally characterized by high counts of beneficial lactobacilli. Adults and infants were less colonised with lactobacilli comparison to children. The differences in the colonization with *Bifidobacterium* sp. were found between children and adults.

Our study demonstrated that the composition of intestinal microbiota evolves through life, from early childhood to old age with remarkable similarity between children and adults. We tried to output approximately the norms in the counts of bacteria in gut of healthy persons in different ages, but it was quite complicated through high microbial inter-individual diversity. In the future studies it is possible to perform comparable analysis of the gut microbial composition between healthy people and people with different disorders. Thus, by using this data development of the effective treatment and therapies is possible in the case of inflammatory bowel diseases (e.g. Crohn's Disease, ulcerative colitis), metabolic syndrome, autoimmune diseases, anaemia and different infectious diseases.

7. Erinevate vanuserühmade tervete inimeste soolestiku mikroobikoosluse molekulaarne kvantitatiivne analüüs

Natalja Šebunova

Resümee

Antud töös rakendasime real-time PCR meetodit erinevate vanuserühmade tervete inimeste soolestiku mikroobikoosluse iseloomustamiseks. Meie tulemused kinnitasid, et real-time PCR on võimas meetod inimese soolestiku mikrofloora kvantitatiivsel uurimisel. Statistiline analüüs võimaldas hinnata vananemisprotsessi mõju soolestiku mikrofloora koosseisule.

Meie tulemused näitasid, et kõikides vanuserühmades olid kõige kõrgema esinemissagedusega *Firmicutes* hõimkond ja *Bacteroides-Prevotella* rühm. *Firmicutes*/*Bacteroides* sp. suhe suureneb sünniajast täisealisuseni ja muutub kõrges eas.

C.leptum ja *C.coccoides-E.rectale* rühmad olid kõrgelt esindatud kõikides rühmades. *Clostridium* sp. arv vähenes inimese organism vananemisega. Soolestiku epiteeliumi limaskesta lagundav *C.difficile* tuvastasime kolmes rühmas (imikus, lapsed, vanurid), kõige suurema esinemissagedusega imikutel. *Enterococcus* sp., *Veillonella* sp. ja *Enterobacteriaceae* sugukonna hulgad olid kõige kõrgemad imikutel, samas *F.prausnitzii* oli arvukaim täiskasvanutel. Vanurite soolestiku mikroobikooslust iseloomustab kasulike lactobacillide suured arvud. Täiskasvanutel ja imikutel oli koloniseeritus laktobatsillidega kõrgem võrreldes lastega, samuti olid suured erinevused bifidobakteriga koloniseerumisel lastel ja täiskasvanutel.

Meie uuring näitas, et soolestiku mikrofloora areneb läbi elu, alates varasest lapsepõlvest kuni vanaduseni, tähelepanuväärsuse sarnasusega lastel ja täiskasvanutel. Meie proovisime selgitada mikroobide tavapäraseid hulkasid tervete isikute eri vanuses, kuid see oli üsna keeruline mikroobide suure interindividuaalse mitmekesisuse tõttu. Tuleviku uuringutes on võimalik teostada tervete ja erinevate haigustega inimeste soolestiku mikroobse kompositsiooni võrdlevat analüüsi. Seega, kasutades meie saadud andmeid on võimalik arendada efektiivset ravi ja teraapiad selliste haiguste puhul nagu põletikulised soolehaigused (nt Crohni tõbi, haavandiline koliit), metaboolne sündroom, autoimmuunhaigused, anaemia ja erinevad soolenakkushaigused.

8.REFERENCES

Amann R.I., Ludwig W. and Schleifer K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59, 143–169

Ahmed F.E. (2002). Detection of genetically modified organisms in foods. Trends Biotechnol., 20(5):215-23

Berg R.D. (2001) Bacterial translocation from the gastrointestinal tract. Adv. Exp. Med. Biol., 473, 11-30

Berg R.-J., Vaessen N., Endtz P.-H., Schülin T., Vorm E.-R. and Kuijper E.-J. (2007). Evaluation of real-time PCR and conventional diagnostic methods for the detection of Clostridium difficile-associated diarrhoea in a prospective multicentre study. J Med Microbiol., Vol. 56 no. 1, 36-42

Bingham S.A. (1999). High-meat diets and cancer risk. Proc. Nutr. Soc. 58, 243-248

Backhed F., Ley R.E., Sonnenburg J.L., Peterson D.A. and Gordon J.L. (2005). Host bacterial mutualism in the human intestine. Science 307, 1915-1920

Bouskra D., Brézillon C., Bérard M., Werts C., Varona R., Boneca I.G. and Eberl G. (2008). Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature, 456(7221):507-10

Biagi E., Candela M., Fairweather-Tait S., Franceschi C. and Brigidi P. (2012). Ageing of the human metaorganism: the microbial counterpart. AGE, 34:247–267

Biagi E., Nylund L., Candela M., Ostan R., Bucci L., Pini E., Nikki J., Monti D., Satokari R., Franceschi C., Brigidi P. and De Vos W. (2010). Through Ageing, and Beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians. PLoS ONE, 5(5): e10667

Bik E.M., Eckburg P.B., Gill S.R., Nelson K.E., Purdom E.A., Francois F., Perez- Peres G., Blaster M.J. and Relman D.A. (2006). Molecular analysis of the bacterial microbiota in the human stomach. Proc. Nat. Acad. Sci. USA 103, 732-737

Bergey's Manual of Systematic Bacteriology. Krieg NR, Holt JG, eds. Williams and

Wilkins, Baltimore, 1986 Bezirtzoglou E. (1997). The intestinal microflora during the first weeks of life. Anaerobe,

3: 173–177

Borson N.D., Strausbauch M.A., Wettstein P.J., Oda R.P., Johnston S.L. and Landers J.P. (1998). Direct quantitation of RNA transcripts by competitive single-tube RT-PCR and capillary electrophoresis. Biotechnique, 25:130–137

Blackwood C.B., Marsh T., Kim S.-H. and Paul E.A. (2003). Terminal Restriction Fragment Length Polymorphism Data Analysis for Quantitative Comparison of Microbial Communities. Applied and Environmental Microbiology, Vol. 69, №2, p. 926–932

Bartosch S., Fite A., Macfarlane G.-T. and McMurdo M.E.T.(2004). Treatment on the Fecal Microbiota Real-Time PCR and Effects of Antibiotic and Hospitalized Elderly Patients by Using in Feces from Healthy Elderly Volunteers Characterization of Bacterial Communities. Appl. Environ. Microbiol, 70(6):3575

Cao Y., Shen J. and Hua Ran Z. (2014). Association between Faecalibacterium prausnitzii Reduction and Inflammatory Bowel Disease: A Meta-Analysis and Systematic Review of the Literature. Gastroenterology Research and Practice, Vol. 2014

Cani P.D. and Delzenne N.M. (2009). Interplay between obesity and associated metabolic disorders: new insights into the gut microbiota. Curr Opin Pharmacol., 9(6):737-43

Christensen H.R., Frøkiær H. and Petska (2002). Lactobacilli Differentially Modulate Expression of Cytokines and Maturation Surface Markers in Murine Dendritic Cells. The Journal of Immunology, Vol. 168 no. 1 171-178

Claesson M.J., Cusack S., O'Sullivan O., Greene-Diniz R., de Weerd H., Flannery E.., Marchesi .JR., Falush D., Dinan T., Fitzgerald G., Stanton C., van Sinderen D., O'Connor M., Harnedy N., O'Connor K., Henry C., O'Mahony D., Fitzgerald A.P., Shanahan F., Twomey C, Hill C., Ross R.P. and O'Toole P.W. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A., 108 Suppl 1:4586-91

DeSantis T.Z., Brodie E.L., Moberg J.P., Zubieta I.X. and Piceno Y.M.(2007). High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microbial Ecology 53: 371–383

Egert M., de Graaf A.A., Smidt H., de Vos W.M., Venema K. (2006) Beyond diversity: functional microbiomics of the human colon. Trends. Microbiol., 14, 86-91

Drell T., Lutsar I., Štšepetova J., Parm Ü., Metsvaht T., Ilmoja M.-L., Simm J. and Sepp E. (2014). The development of gut microbiota in critically ill extremely low birth weight infants assessed with 16S rRNA gene based sequencing. Gut Microbes, 5(3)

Eckburg P.B., Bik M.B., Bernstein C.N., Purdom E., Dethlefsen L., Sargent M., Gill S.R., Nelson K.E. and Relman D.A. (2005). Diversity of the human intestinal microbial flora. Science,
308: 1635–1638

Frank D.N. and Pace N.R. (2008). Gastrointestinal microbiology enters the metagenomics era. Curr Opin Gastroenterol, 24: 4–10

Frank D.N., St Amand A.L., Feldman R.A., Boedeker E.C., Harpaz N. and Pace N.R. (2007). Molecular phylogenic characterization of microbial community imbalances in human inflammatory bowel diseases. PNAS, 104: 13780–13785

Fink L., L.Z. Zeuthen, Christensen H.R., Morandi B. and F.Guido (2007). Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses. Int. Immunol., 19 (12):1319-1327

Fite A., Macfarlane G.T., Cummings J.H., Hopkins M.J., Furrie E. and Macfarlane S. (2004). Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. Gut, 53:523-529 Intestinal microflora

Guarner F. and Malagelada J-R. (2003). Gut flora in health and disease. Lancet 361, 512-519

Grundy S.M., Brewer H.B., Cleeman J.I., Smith S.C. and Lenfant C. (2004). Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Arteriosclerosis, thrombosis, and vascular biology. J. Am. Heart Associat., 2 (24), 13-18

Gibson U.E.M., Held C.A. and Williams P.M. (1996). A novel method for real time quantitative competitive RT-PCR. Genome Res., 6: 995-1001

Gerritsen J., SmidtGer H., Rijkers T. and de Vos W.M. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr., 6(3): 209–240

Guo X., Xia X., Tang R., Zhou J., Zhao H. and Wang K. (2008). Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. Letters in Applied Microbiology, 47, 367–373

Hayashi H., Sakamoto M., Kitahara M. and Benno Y. (2006). Diversity of the *Clostridium coccoides* group in human fecal microbiota as determined by 16S rRNA gene library. FEMS Microbiology Letters, Vol.257, Issue 2, 202–207

Haarman M. and Knol J. (2006). Quantitative Real-Time PCR Analysis of Fecal Lactobacillus Species in Infants Receiving a Prebiotic Infant Formula. Applied and Environmental Microbiology, Vol. 72, No. 4.,2359–2365

Haarman M. and Knol J. (2005). Quantitative Real-Time PCR Assays To Identify and Quantify Fecal Bifidobacterium Species in Infants Receiving a Prebiotic Infant Formula. Appl. Environ. Microbiol., Vol. 71 no. 5, 2318-2324

Harmsen H.J., Hermie J. M., Wildeboer–Veloo, Alida C. M., Raangs, Gerwin C., Wagendorp, Arjen A., Klijn, Nicolette, Bindels, Jacques G. and Welling Gjalt W. (2000). Analysis of Intestinal Flora Development in Breast-Fed and Formula-Fed Infants by Using Molecular Identification and Detection Methods. Journal of Pediatric Gastroenterology & Nutrition, 30(1):61-7

Heid C.A., Stevens J., Livak K.J. and Williams P.M. (1996). Real time quantitative PCR. Genome Res., 6(10):986–994

Harmsen H.J., Elfferich P., Schut F. and Welling G.W. (1999). A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent in situ hybridization. Microb Ecol Health Dis, 11: 3–12

Ivanov I.I., Fruto L.R., Manel L., Yoshinaga K., Rifkin D.B., Sartor R.B., Finlay B.B. and Littman D.R. (2008). Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine. Cell Host & Microbe, 4, Issue 4:337–349

Jung R., Soondrum K. and Neumaier M. (2000). Quantitative PCR. Clin Chem Lab Med 38: 833–836

Janet M., Rauch M. and Gilmore M.S. (2008). The Commensal Microbiology of the Gastrointestinal Tract. GI Microbiota and Regulation of the Immune System, Landes Bioscience and Springer Science+Business Media.

Julge K., Vasar M. and Björkstén B. (2001). Development of allergy and IgE total serum during the first 5 years of life in Estonian children. Clin Exp Allergy, 31: 1854–1861

Kelsall B.L. and Leon F. (2005). Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. Immunological Reviews, Vol.206, Issue 1:132–148

Koenig J.E., Spor A., Scalfone N., Fricker A.D., Stombaugh J., Knight R., Angenent, L.T. and Ley, R.E. (2011). Succession of microbial consortiain the developing infant gut microbiome. Proc.Natl. Acad.Sci.U.S.A. 108(Suppl.1), 4578–4585

Klein D. (2002). Quantification using real-time PCR technology: applications and limitations.

Trends Mol Med., 8(6):257-60

Liévin V., Peiffer I., Hudault S., Rochat F., Brassart D., Neeser J.R. and Servin A.L. (2000). Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut., 47(5):646-52

Larsen N., Vogensen F.K., van der Berg F.W.J., Nielsen D.S., Andersen A.S., Pedersen B.K., Al-Soud W.A., Sorensen S.J., Hansen L.H. and Jacobsen M. (2010). Gut Microbiota in Human Adults with Type 2 Diabetes Differs from Non-Diabetic Adults. PLoS ONE, 5(2):e9085

Libudzisz Z., Nowak A. (2008). Mikroorganizmy jelitowe człowieka. Standardy Medyczne 2, 372-379

Ley R.E., Tumbaugh P., Klein S. and Gordon J. I. (2006). Human gut microbes associated with obesity. Nature, 444, 1022-1023

Lewandowska M. (2010). Microbiota of human gastrointestinal tract. Food Chemistry and Biotechnology, №1081,Vol. 74

Malinen E., Rinttil T., Kajander K., Matto J., Kassinen A., Kroigus L., Saarela M., Korpela R. and Palva A. (2005). Analysis of the Fecal Microbiota of Irritable Bowel Syndrome Patients and Healthy Controls with Real-Time PCR. American Journal of Gastroenterology, 100:373–382

Marteau P., Pochart P., Dore J., Bera-Maillet C., Bernalier A. and Corthie G. (2001). Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol, 67: 4939–4942

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 Vol. 70, No. 12, 7220–7228

Macpherson A.J. and Harris N.L. (2004). Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology 4, 478-485

Mazmanian S.K., Liu C.H., Tzianabos A.O. and Kasper D.L. (2005). An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell, Vol. 122, Issue 1:107–118

Mäkivuokko H., Tiihonen K., Tynkkynen S., Paulin L. and Rautonen N. (2010). The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. Br J Nutr., 03(2):227-34

Manson J.M., Rauch M. and Gilmore M.S. (2008). The commensal microbiology of the gastrointestinal tract. Adv Exp Med Biol., 635:15-28

Matamoros S., Gras-Leguen C., Le Vacon F., Potel G. and de La Cochetiere M.F. (2012). Development of intestinal microbiota in infants and its impact on health. Trends in Microbiology, Vol.21, No.4

Mackay I.M., Arden K.E. and Nitsche A. (2002). Real-time PCR in virology. Nucleic Acids Res, 30: 1292–1305

Mariat D., Firmesse O., Levenez F.,Guimarães V.D., Sokol H., Doré J.,G Corthier G. and Furet J.-P. (2009). The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiology, 9:123

Mackay I. M. (2004). Real-time PCR in the microbiology laboratory. Clin Microbiol Infect, 10: 190–212

Moter A. and Göbel U.F. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. Journal of Microbiological Methods, 41, 85–112

Mikelsaar M., Štšepetova J., Hütt P., Kolk H., Sepp E., Lõivukene K., Zilmer K. and Zilmer M. (2010). Intestinal Lactobacillus sp. is associated with some cellular and metabolic characteristics of blood in eldrly people. Anaerobe, 16: 240–246

Mueller S., Saunier K., Hanisch C., Norin E., Alm L., Midtvedt T., Cresci A., Silvi S., Orpianesi C., Verdenelli M.C., Clavel T., Koebnick C., Zunft H.J., Dore J. and Blaut M. (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

Mhlanga M.M. and Malmberg L. (2001). Using Molecular Beacons to Detect Single-Nucleotide Polymorphisms with Real-Time PCR. METHODS, 25, 463–471

Niesters H.G.M. (2001). Quantitation of viral load using realtime amplification techniques. Methods, 25:419–429

Novais R.C. and Thorstenson Y.R. (2011). The evolution of Pyrosequencing® for microbiology: From genes to genomes. Journal of Microbiological Methods, 86:1–7

Nadkarni M.-A., Elizabeth Martin F., A. Jacques N. and Hunter N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology, 148, 257–266

Nielsen J.L., Christensen D., Kloppenborg M. and Nielsen P.H. (2003). Quantification of cell-specific substrate uptake by probe-defined bacteria under *in situ* conditions by microautoradiography and fluorescence in situ hybridization. Environ Microbiol, 5:202–211

Ottman N., Smidt H., deVos W.-M. and Belzer C. (2012). The function of our microbiota:who is out there and what do they do? Fronties in cellular and Infection Microbiology, Vol.2, Art. 104

Ouwehand A.C., Isolauri E., He F., Hashimoto H., Benno Y. and Salminen S. (2001). Differences in Bifidobacterium flora composition in allergic and healthy infants. J Allergy Clin Immunol., 108(1):144-5

Palmers C., Bik E.M., DiGiulio D.B., Relman D.A. and Brown P.O. (2007). Development of the Human Infant Intestinal Microbiota. PLoS Biol., 5(7): e177

Penders J., Thijs C., Vink C., Stelma F.F., Snijders B., Kummeling I., van den Brandt P.A. and Stobberingh E.E. (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics, 118: 511–521

Penders J., Stobberingh E.E., van den Brandt P.A. and Thijs C. (2007) The role of intestinal microbiota in the development of atopic disorders. Allergy, 11: 1223–1236

Paliy O., Kenche H., Abernathy F. and Michail S. (2009). High-throughput quantitative analysis of the human intestinal microbiota with a phylogenetic microarray. Applied and environmental microbiology, 75: 3572–3579

Rieger M.A., Parlesak A., Pool-Zobel B.L., Rechkemmer G. and Bode C. (1999). A diet high in fat and meat but low in dietary fiber increases the genotoxic potential of 'fecal water'. Carcinogenesis, 20, 2311-2316

Rakoff-Nahoum S. and Medzhittov R. (2008). Innate immune recognition of the indigenous microbial flora. Mucosal Immunology 1 (Suppl 1), S10–S14

Rajilic´-Stojanovic´ M., Heilig H.G.H.J., Molenaar D., Kajander K. and Surakka A. (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environmental microbiology, 11: 1736–1751

Ronaghi M. (2001). Pyrosequencing sheds light on DNA sequencing. Genome Res., 11:3-11

Slizewska K., Klewicka E. and Motyl I. (2008). Mikroflora człowieka. In: Mikrobiologia Techniczna. Mikroorganizmy i srodowiska ich wystepowania. Red. Libudzisz Z., Kowal K., Zakowska Z. PWN, 240-248

Stolarczyk A., Libudzisz Z., Socha P., Socha J. (2008). Rola probiotyków i prebiotyków w profilaktyce i leczeniu zespołu metabolicznego u dzieci i młodziezy. Standardy Medyczne 2, 175-171

Štšepetova J., Sepp E., Kolk H., Lõivukene K., Songisepp E. and Mikelsaar M.(2011). Diversity and metabolic impact of intestinal Lactobacillus species in healthy adults and the elderly. Br J Nutr., 105(8):1235-44

Sekirov I., Russell S.L., Antunes L.C. and Finlay B.B. (2010). Gut microbiota in health and disease. Physiol Rev., 90(3):859-904

Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P. and Dore, J. (2000). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol., 66, 2263–2266

Scholtens P.A., Oozeer R., Martin R., Amor K.B. and Knol J. (2012). The early settlers: intestinal microbiology in early life. Annu.Rev.Food Sci.Technol. 3, 425–447

Suau A., Bonnet R., Sutren M., Godon J.J., Gibson G.R., Collins M.D., Dorè 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

Southwick P.L., Ernst L.A., Tauriello E.W., Parker S.R., Mujumdar R.B., Mujumdar S.R., Clever H.A. and Waggoner A.S. (1990). Cyanine dye labeling reagents carboxymethylindocyanine succinimudyl esters. Cytometry , 11: 418–430

Sokol H., Seksik, P., Furet, J. P., Firmesse, O., Nion-Larmurier, I., Beaugerie, L., Cosnes, J., Corthier, G., Marteau, P. and Doré, J. (2009). Low counts of Faecalibacterium prausnitziiin colitis microbiota. Inflammatory Bowel Diseases, Vol.15, Issue 8, 1183–1189

Sela D.A., Chapman J., Adeuya A., Kim J.H., Chen F., Whitehead T.R., Lapidus A., Rokhsar D.S., Lebrilla C.B., German J.B., Price N.P., Richardson P.M. and Mills D.A. (2008). The

genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A., 105(48):18964-9

Tannock G.W. (2002). Probiotics and prebiotics: where are we going? In: Tannock GW, edn. Probiotics and prebiotics: where are we going? Wymondham, UK: Caister Academic Press, 1–39

Torphy J. M., Lynm C., Glass R. M. (2006). The metabolic syndrome. JAMA: 295, 850.

Tiihonen K., Tynkkynen S., Ouwehand A., Ahlroos T., Rautonen N. (2008). The effect of ageing with and without non-steroidal antiinflamatory drugs on gastrointestinal microbiology and immunology. Br J Nutr, 100: 130–137

Tottey W., Denonfou J., Jaziri F., Parisot N., Missaoui M., Hill D., Borrel G., Peyretaillade E., Alric M., Harris H. M.B., Jeffer B. I., Claesson M.J., O'Toole P.W., Peyret P., Bruge`re J.-F. (2013). The Human Gut Chip ''HuGChip'', an Explorative Phylogenetic Microarray for Determining Gut Microbiome Diversity at Family Level. PLoS ONE, 8(5):e62544

Vaughan E.E., Schut F., Heilig H.G., Zoetendal E.G., de Vos W.M., Akkermans A.D. (2000). A molecular view of the intestinal ecosystem. Curr Issues Intest Microbiol, 1: 1–12

Vaughan E.E., de Vries M.C., Zoetendal E.G., Ben-Amor K., Akkermans A.D. and de Vos WM. (2002) The intestinal LABs. Antonie van Leeuwenhoek, 82: 341–352

Valeur N., Engel P., Carbajal N., Connolly E. and Ladefoged K. (2004). Colonization and Immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the Human Gastrointestinal Tract. Appl. Environ. Microbiol., Vol. 70 no. 2, 1176-1181

Wittwer C.T., Herrmann M.G., Gundry C.N. and Elenitoba-Johnson K.S. (2001). Real-time multiplex PCR assays. Methods, 25(4):430-42

Wilhelm J., Hahn M., Pingoud A. (2001). Influence of DNA target melting behavior on real-time PCR quantification. Clin Chem, 46: 1738–1743

Zoetendal E.G., Akkermans A.D.L. and de Vos W.M. (2001). Molecular characterization of microbial communitiesbased on 16S rRNA sequence diversity. In: Dijkhoorn L, Towner KJ, Struelens M, eds. New approaches for generation and analysis of microbial typing data. Amsterdam, The Netherlands: Elsevier Science, 267–298

Zilberstein B., Quintanilha A.-G., A Santos M.-A.; Denis Pajecki, G Moura E.,Arruda Alves P.-R.,Filho F.-M.,Ubriaco de Souza J.-A. and Gama-Rodrigues J. (2007). Digestive tract microbiota in healthy volunteers. Clinics, Vol.62 no.1

Zwielehner J., Liszt K., Handschur M., Lassl C., Lapin A. and Haslberger A.-G. (2009). 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. Exp Gerontol., 44(6-7):440-6

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