

## DIFFERENCES IN GUT MICROBIOTA ACTIVITY (ANTIMICROBIALS, POTENTIAL MUTAGENS, AND STEROLS) ACCORDING TO DIET

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(Received: 15 May 2016; accepted: 13 July 2016)

The human endogenous intestinal microbiota is an essential “organ” in providing nourishment, regulating epithelial development, and instructing innate immunity. Even though lots of scientists have evaluated the content of gut microbiota from various points of view, we examined the content of intestinal microbes in the group of healthy middle aged volunteers (40–60) from Slovakia. We have compared faecal cultivable microbiota of vegetarians and omnivores. We have found that the composition of the human microbiota is fairly stable, and it seems that the major microbial groups on species level that dominate the human intestine are conserved in all individuals regardless of dietary habits. Beside the microbial content we have examined the faecal samples also for the presence of antimicrobial active compounds, potential mutagens, and faecal sterols.

**Keywords:** intestinal microbiota, dietary habits, antimicrobial active metabolites, potential mutagenic compounds, faecal sterols

The composition of human intestinal microbiota in the population was first studied by METCHNIKOFF at the beginning of the last century (1903, 1907). Since that time, the composition of intestinal microbiota and its impact on human health became the research subject of several scientific groups worldwide. According to the microbial lifestyle, most cultivable intestinal microorganisms are anaerobes or facultative anaerobes (STEPHEN & CUMMINGS, 1980; SEARS, 2005). The compositions of microbiota rely on several factors, such as host diet, colonization history, and immune status. Microbial composition dramatically changes in the course of life (SEGAL & BLASER, 2014). Changes in the colon during aging may be caused by the decreasing resistance of autochthone bacteria to colonization by other bacteria. The number of anaerobic bacteria is increasing with increasing age. The genus *Eubacterium* is one of the arising species. The resident gut microbiota mediates metabolic effects, such as the syntheses of vitamins like biotin and folate, as well as absorption of ions including magnesium, calcium, and iron, and contributes to the lipid metabolism (O’HARA & SHANAHAN, 2006). Intestinal microbiota has

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recently been linked to intestinal cholesterol reduction, due to microbial conversion of cholesterol (*Eubacterium coprostanoligenes*, lactobacilli and bifidobacteria) to its non-absorbable form coprostanol. Coprostanol represents approximately 60% of total sterols accumulated in human faeces. The conversion of cholesterol to coprostanol is seen as a natural method of reducing serum cholesterol in humans (REN et al., 1996).

The objective of our work was study of qualitative and quantitative changes in the microbial population in middle aged volunteers of 40–60 years, with different diet habits (vegetarians and omnivores). We have also focused on the occurrence monitoring of substances with antibacterial activity as well as potential mutagenic components located in the colon. Finally, we were interested in the representation of individual sterols in the colon.

## 1. Materials and methods

### 1.1. Volunteers recruitment and faecal sample collection

Healthy human subjects were recruited in Bratislava and its surrounding area. The studied subjects were born and grown up in Slovakia. The group of volunteers consisted of 120 middle aged persons (40–60 years old), with BMI>16 (BMI=25.4±4.4). Volunteers were divided into two groups according to their dietary habits that were taken from fulfilled questionnaires. The group of vegetarians consisted of lactovegetarians (n=6), vegans (n=2), lacto-ovovegetarians (n=12), and semi-vegetarians (n=47). The other volunteers were omnivorous (total no=51). Lacto-ovovegetarians have not consumed meat in any form, but ate animal products such as milk, cheese, and eggs. A vegan diet was considered for those who did not consume animal products. Semi-vegetarians have stated that they consume animal products and also poultry and fish meat. The omnivores consumed all type of meat (pork, red meat, poultry, and fish) a minimum of 5 times a week. All volunteers were healthy and had not undergone antibiotic or other medical therapy in the last 6 months. Faecal samples were collected during the spring month from April to May. Distribution of the studied participants according to age, sex, and diet is shown in Table 1.

Table 1. Distribution of study group participants according to age, sex, and diet

Age group (Year)	Mean age in years (range) (n)			
	Vegetarians		Omnivores	
	Female	Male	Female	Male
41–50	44 (41–49) (19)	45 (41–50) (14)	45 (43–50) (11)	44 (42–46) (11)
51–60	55 (51–59) (24)	56 (31–40) (13)	57 (52–60) (16)	55 (51–60) (12)

### 1.2. Sample collection and microbiological analysis

Faecal samples were collected in sterile tubes by a single application and subsequently transported to laboratory. Quantitative and qualitative composition of microbiota was analysed by cultivation on selective diagnostic media according to MITSUOKA and HAYAKAWA (1972) and ŠAKOVÁ and co-workers (2015).

### 1.3. Assessment of potential mutagenicity

Assessment of potential mutagenic activity was performed using classical incorporation method (MARON & AMES, 1983) without metabolic activation. Filtrated sample was applied in Ames test (ŠAKOVÁ et al., 2015).

### 1.4. Antibacterial activity assessment

Presence of antimicrobial compounds was qualitatively investigated by the disc diffusion method using eleven model bacterial strains obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). The experiment was carried out three times and evaluated by the mean values (ŠAKOVÁ et al., 2015).

### 1.5. Analysis of faecal sterols

All individual stools were collected in plastic containers, weighed. Individual stools were lyophilized and then stored frozen at  $-20^{\circ}\text{C}$  until analysis. Faecal sterols were extracted with cyclohexane, reconstituted in 500  $\mu\text{l}$  decane, and without further derivatization these were injected in GC-MS in duplicate described by KELLER and JAHREIS (2004).

## 2. Results and discussion

### 2.1. Assessment of cultivable microbiota

Comparing the cultivable intestinal microbiota of middle aged volunteers (40–60 years) with different diet habits revealed no significant difference in the microbial content. Though culture dependent techniques for evaluation of gut microbiota has been considered inadequate, the most recent study with a complete novel workflow observed a strong correlation between culture dependent and independent approaches at the species level. The results of BROWNE and co-workers (2016) demonstrate that a considerable proportion of the bacteria within the faecal microbiota can be cultured even if using only one complex growth medium.

Studies that evaluated the composition of gut microbiota using cultivation dependent and independent methods showed that the most predominant microorganisms are the spore formers (*Clostridium* cluster XIVa and *Clostridium* cluster IV) (HOLD et al., 2002; BROWNE et al., 2016) and strict anaerobe *Bacteroidetes*. Our study shows the predominant presence of *Clostridia* spp. (Table 2). Some of these may possess health risks. *Clostridium perfringens* and *Clostridium tetani*, with lecithinase activity and toxin production, are the most redoubtable species. However, most of the clostridia have a commensal relationship with the host and are promoting gut health (LOPETUSO et al., 2013).

The highest CFU number of lecithinase positive (LP) clostridia was observed in volunteers aged 50–60 years, preferring meaty food. Their content represented 14% of the total clostridia number. The number of LP clostridia in faecal samples of vegetarians was lower, and represented only 9% of the total clostridia. The number of *Bacteroides* spp. was not as high as it is usually reported in literature (Table 2). The most prevalent were *B. ovatus* and *B. vulgatus*. Even though no significant differences in the CFU numbers of *Enterobacteriaceae*, *Staphylococci*, *Veilonella* spp. were detected, the greatest difference in facultative anaerobes was found in the number of enterococci, between vegetarians aged 40–50 and vegetarians aged 51–60 years. In the group of vegetarians aged 51–60 years

approximately one logarithmic order increase of enterococci was observed. Regarding the presence of staphylococci, their content was balanced in all assayed groups. Almost one quarter of the faecal samples was positive for *Staphylococcus aureus*.

Table 2. Quantitative data on groups of microorganisms in vegetarians and omnivores obtained after cultivation of faecal samples on selected diagnostic agar media

Group of microorganisms	Microbial content (average log <sub>10</sub> CFU±SE per gram of wet stool)			
	Vegetarians		Omnivores	
	41–50 years		51–60 years	
Total aerobes	8.43±0.20	7.94±0.28	8.94±0.25	8.07±0.51
Total anaerobes	7.84±0.42	8.22±0.21	9.29±0.20	8.37±0.43
<i>Bacteroidetes</i>	3.56±0.33	3.34±0.43	3.18±0.44	2.93±0.52
<i>Bifidobacterium</i> spp. and <i>Lactobacillus</i> spp.	5.81±0.31	5.58±0.50	5.29±0.54	5.90±0.50
Total clostridia	8.55±0.33	8.05±0.26	8.27±0.53	8.45±0.28
<i>Enterobacteriaceae</i>	7.59±0.21	7.22±0.25	7.13±0.36	7.19±0.42
<i>Enterococcus</i> spp.	6.78±0.34	6.05±0.42	7.60±0.47	7.0±0.32
LP <i>Clostridium</i> spp.	1.11±0.32	1.18±0.44	0.45±0.27	1.55±0.50
<i>Staphylococcus</i> spp.	5.94±0.25	5.18±0.66	5.19±0.62	4.58±0.77
<i>Veillonella</i> spp.	7.62±0.33	7.58±0.40	7.69±0.46	6.28±0.55
Yeasts and moulds	3.38±0.37	3.28±0.37	3.00±0.37	3.97±0.39

SE: standard error; LP: lecithinase positive

## 2.2. Antimicrobial compounds in faecal samples

Numerous antimicrobially active compounds such as defensins, cathelicidins, and C-type lectins, are produced in the human gastrointestinal tract; they are a diverse group of compounds that act by disrupting surface structures of both commensal and pathogenic bacteria (SALZMAN et al., 2007; HOOPER, 2009). The main function of these compounds is the regulation of composition and numbers of the intestinal microbiota (SALZMAN et al., 2007). These compounds with antimicrobial activity are also able to stimulate the intestinal immune system and some are displaying anti-carcinogenic and anti-mutagenic activity (DELORME, 2008). We have focused on the determination of antibacterially active metabolites in heated and filtered faecal samples using disc diffusion method (Table 3). Approximately 39% of obtained omnivore stool samples were able to inhibit Gram-positive (*M. luteus*, *S. pyogenes*) as well as Gram-negative bacteria (*E. faecalis*, *P. aeruginosa*). Same antibacterial activity was detected in 23 vegetarian stool samples. In the group aged 51–60 years, 24 samples displayed antibacterial activity and number of positive samples was comparable in both groups (Table 3). These antimicrobial substances often tend to exhibit antimicrobial activity against bacterial groups similar to the producer, possibly in a strategy aimed at keeping potential competitors out of the producer's favoured intestinal niches, but also benefiting the host along the way (SEKIROV et al., 2010).

Table 3. Presence of thermostable antimicrobial compounds in faecal samples

Type of diet	Age (year)	Percentage of samples with antibacterial activity (%)	Sensitive model bacterial strains
Vegetarian	41–50	39	<i>E. faecalis</i> , <i>S. pyogenes</i> , <i>M. luteus</i> , <i>P. aeruginosa</i>
Omnivorous	41–50	23	<i>E. faecalis</i> , <i>P. aeruginosa</i> , <i>M. luteus</i>
Vegetarian	51–60	12	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>B. cereus</i> , <i>E. cloacae</i> , <i>P. aeruginosa</i> , <i>S. Enteritidis</i> , <i>S. Typhimurium</i> , <i>M. luteus</i> , <i>S. aureus</i>
Omnivorous	51–60	12	<i>P. aeruginosa</i> , <i>S. pyogenes</i>

### 2.3. Presence of potential mutagens in faecal samples

While the gut microbiota is an essential partner in health, some aspects of its presence can induce highly unwanted processes in the host. Metabolism of some nutrients by particular members of the microbiota can elaborate by-products that are toxic to the intestinal epithelium. Imperfect repair of the injured epithelium can then result in neoplastic transformations (SEKIROV et al., 2010). Additionally, some microbes could produce toxic metabolites at the gastric mucosa, which have the potential to serve as mutagens and are able to cause DNA damage. Diet seems to be involved in the risk for the presence of potential mutagenic compounds (BURKITT, 1978). Presence of mutagenic compounds in human faeces was examined by Ames incorporation test. The highest percentage (13%) of subjects with potential mutagenic compounds present in stool was detected in meat eaters group aged 41–50 years. On the other hand, slightly higher percentage of positive samples was observed in the vegetarian group aged 51–60 years compared to omnivores (Fig. 1). Our results are showing that not only the diet but also the age is the factor that influences the presence of potential mutagen compounds in GIT.

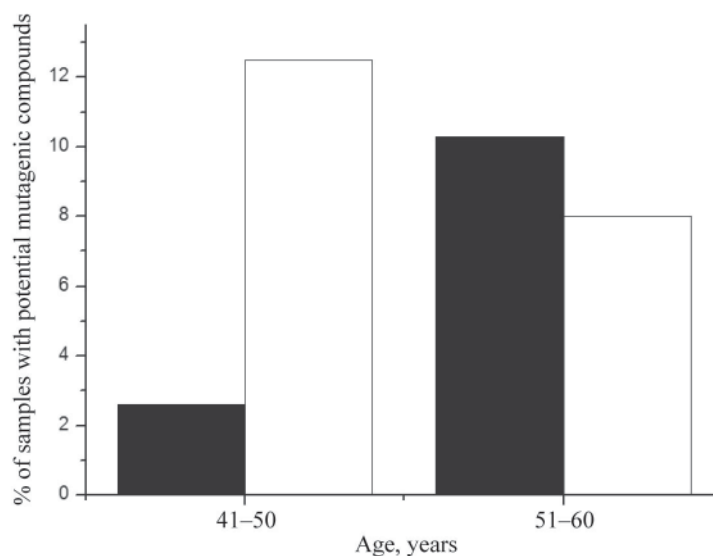


Fig. 1. Faecal samples with detected potential mutagenic activity  
 ■: Vegetarians; □: omnivores

#### 2.4. Detection of faecal sterols

There is no doubt that resident microbes perform several useful functions, including maintaining barrier function, synthesis and metabolism of nutrients, drug and toxin metabolism, and behavioural conditioning (SCALDAFERRI et al., 2012). Gut microbiota is also involved in the digestion of energy substrates, production of vitamins and hormones (SEKIROV et al., 2010), and also involved in the conversion of cholesterol to the saturated product coprostanol. The conversion of cholesterol to coprostanol has been considered as a natural means of lowering serum cholesterol in humans, and thus reducing the risk of cardiovascular disease. However, high faecal concentrations of coprostanol might be associated with colon carcinogenesis in humans (PEUCHANT et al., 1987). One way of reducing cholesterol is its conversion to coprostanol in the presence of the enzyme cholesterol-reductase produced by lactic acid bacteria (LYE et al., 2010). Furthermore, phytosterols, such as sitosterol and campesterol, are predominantly converted to the analogous 5-cholestanes by colonic bacteria. Intermediate products (4-cholesten-3-one and 9 coprostanone) were occasionally observed, suggesting an indirect pathway for cholesterol to coprostanol conversion (KELLER & JAHREIS, 2004). Our work shows the incidence of sterols of animal and plant origin in the faecal samples of vegetarians and omnivores. The total sterol content in the faeces of studied subjects ranged from  $8.3 \pm 1.75$  mg g<sup>-1</sup> of dry weight for vegetarians to  $11.0 \pm 1.75$  mg g<sup>-1</sup> for omnivores (Table 4). The highest concentration of all sterols was registered for coprostanol ( $6.3 \pm 1.53$ ), the main product of bacterial cholesterol degradation. Its value was higher in omnivores compared to vegetarians. In samples of vegetarians the amount of coprostanol was very similar to the amount of cholesterol (Table 4).

Our results show no significant differences between vegetarian and omnivore samples in the contents of animal origin sterols. Slight differences were found in sterols of plant origin reflected in the sitosterol content. The value of sitosterol was higher in the group of vegetarians. The levels of cholesterol, coprostanol, and the content of minor products of cholesterol transformation, cholestenone, and also the conversion degree of cholesterol to coprostanol, were comparable in these two groups (Table 5). A wide inter-individual range in conversion rate of cholesterol into degradation products (coprostanol, coprostanone, cholestanol) was observed (conversion: 14–90%, in both groups). These differences led to high standard deviations of the mean of faecal sterol concentrations and the conversion rate. Higher amounts of lactic acid bacteria (lactobacilli+bifidobacteria  $10^8$ – $10^{10}$  cell/g faeces) were observed in case of samples with higher sterol conversion (conversion 85–90%). This finding agrees with the data in literature that the level of cholesterol reducing bacteria must be at least  $10^6$  cell/g of wet stool for efficient conversion of cholesterol in the human gut, while a population of more than  $10^8$  cell/g of wet stool leads to nearly complete conversion (VEIGA et al., 2005). Taking into account that most of isolated bacterial converters belong to *Bacteroidetes*, whose number was low (on average  $10^3$  cell/g faeces) in studied faecal samples, these data correlate well. In the same way, the number of lactic acid bacteria (the second largest group of converters) was not so high, the average number of bacteria ranged from  $10^5$ – $10^6$  cell/g faeces.

Table 4. Sterol content in faecal samples (mg g<sup>-1</sup> wet weight)

Sterol	Vegetarians		Omnivores	
	Mean value	SE	Mean value	SE
Coprostanol	3.7	0.95	6.3	1.56
Coprostanon	1.1	0.57	1.8	1.20
Cholesterol	3.6	1.30	2.8	0.99
Methylcoprostanol	0.13	0.05	0.15	0.10
Methylcoprostanon	0.71	0.26	1.60	0.40
Campestreol	0.04	0.03	0	0
Cholestenon	0.06	0.03	0.1	0.09
Ethylcoprostanol	0.94	0.31	1.7	0.44
Sitosterol	2.8	0.99	1.7	0.64
Stigmasterol	0.19	0.10	0.25	0.11

SE: standard error

Table 5. The conversion of faecal cholesterol to coprostanol (mg g<sup>-1</sup> wet weight)

Sterol	Vegetarians		Omnivores	
	Mean	SE	Mean	SE
Coprostanol	3.7	0.95	6.3	1.56
Coprostanone	1.1	0.57	1.8	1.20
Cholesterol	3.6	1.30	2.8	0.99
Cholestenone	0.06	0.03	0.16	0.09
Sterol sum	8.3	1.75	11.0	2.41
Conversion	69%	8%	67%	13%

SE: standard error

### 3. Conclusions

Given the plethora of factors that can affect microbial composition in the human gut, it is perhaps surprising that the composition of the human microbiota is fairly stable. The similarity in the gut microbial content of vegetarians and omnivores could hang also with the spring season of sample collection, when omnivores consumed a lot of local vegetables combined with animal products. Another factor contributing to the obtained result is also that the majority of vegetarian group was semi-vegetarian and the nutrition value of their food is comparable to the food consumed by omnivores (GILL et al., 2006).

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This study was conducted in the frame of research project: "Health effects of plant food and the possibility of reduction of health risks", ITMS code: 26240220022. We thank also to Barbora Kaliňáková for excellent help with the experiments.

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