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ORIGINAL PAPER

# Bacterial Microbiota and Fatty Acids in the Faeces of Overweight and Obese Children

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Abstract

The growing number of children with overweight and obesity constitutes a major health problem of the modern world and it has been suggested that intestinal microbiota may influence energy intake from food. The objectives of this study were to determine quantity and proportions of dominant genera of *Bacteroides, Prevotella* (phylum *Bacteroidetes*); *Clostridium*, *Lactobacillus* (phylum *Firmicutes*) and *Bifidobacterium* (phylum *Actinobacteria*) in the intestines and to determine the content of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) in the stool of 20 obese children and 20 children with normal body weight. Strains classified as *Firmicutes* (*Clostridium* and *Lactobacillus*) predominated in stool microbiota of obese children, while those of *Bacteroidetes* (*Prevotella* and *Bacteroides*) were in minority ($p < 0.001$). Concentration of SCFAs in the stool of obese children was lower in comparison to the stool of normal weight children ($p = 0.04$). However, these differences were significant only in obese children, not in overweight children in comparison with the lean ones. Therefore, in our study obesity was associated with intestinal dysbiosis and a predominance of phylum *Firmicutes*. Secondly, stool of obese children contained lower amounts of SCFAs.

Key words: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, BCFAs, obesity, SCFAs

## Introduction

Obesity is considered a global epidemic (Report WHO, 2008; Report WHO, 2009). The causes of obesity are very complex, from bad habits such as intake of high amount of fats and simple sugars, via environmental conditions, stressogenic and genetic factors, as well as the influence of intestinal microbiota and short-chain fatty acids. The pathologically increased amount of fat causes a number of disorders in the proper functions of the systems, organs and tissues. Complications of the cardiovascular, respiratory, endocrine system and psychosocial problems proved to be particularly dangerous. Obesity is not only a medical problem, which directly contributes to 10–13% of premature deaths in Europe, but it is also associated with a significant economic burden because of the costs associated with obesity which in Europe range from 2% to 7%. A major problem is the phenomenon of transferring childhood obesity to adulthood (Maziak et al. 2007; Report WHO 2008; Report WHO 2009).

In 2004, Gordon hypothesized that intestinal microbiota is associated with body mass control of the host (Backhed et al. 2004). According to his assumptions, certain groups of bacteria are capable to absorb nutrients and energy more efficiently, and individuals, whose intestinal microbiota can rapidly metabolize nutrients, absorb more calories and increase body weight much easier; therefore, they are prone to obesity. It is believed that the specific relative ratio between *Bacteroidetes*

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and *Firmicutes*, which both constitute 90% of bacteria colonizing the intestines, is associated with obesity. The overgrowth of bacteria of phylum *Firmicutes* with simultaneous reduction of bacteria from phylum *Bacteroidetes* was observed in the intestines of both obese mice and humans (Backhed et al. 2004; Ley et al. 2006; Sanz et al. 2008; De Filippo et al. 2010; Diamant et al. 2011; Everard et al. 2013, Canfora et al. 2015; Lu et al. 2016; Raza et al. 2017).

The specific ratio of *Bacteroidetes* to *Firmicutes* is important for a healthy body weight; and beside this, the gut microbiota have many beneficial functions, such as: the influence on immunity, stimulation of the microvilli development, fermentation of dietary fiber and prebiotics: short-chain fatty acids (SCFAs) (butyric, propionic and acetic acids) and lactic acid that are very beneficial to humans (Neish 2002; Stewart et al. 2004; Cani et al. 2008; Cani et al. 2009; Walker and Lawley 2013). This observation has recently been questioned since different studies failed to confirm differences in the *Bacteroidetes*/*Firmicutes* ratio between lean and obese humans (Mondot and Lepage 2016; Lin et al. 2016; Bouter et al. 2018). Metabolism of glucose is regulated by the gut microbiota. It was shown that the high number of bacteria belonging to the *Bacteroidetes* phylum significantly affects glucose intolerance, which was caused by the consumption of a high-fat diet. However, the high *Bacteroidetes* content had little impact on mouse obesity (Rabot et al. 2016). The individual's microbiota composition may cause phenotypic variation correlated to mice feeding with high-fat diet (Rabot et al. 2016).

SCFAs have many functions; among them, propionic acid has a positive effect on the growth of hepatocytes and acetic acid on the development of peripheral tissues. Butyric acid stimulates the growth of intestinal epithelial tissue, nourishes intestinal cells and affects their proper maturation and differentiation. SCFAs regulate also glucose and lipid metabolism, stimulate proliferation and differentiation of intestinal enterocytes, contribute to decrease in the pH of intestinal content and promote the absorption of minerals by increasing their solubility (Blaut and Clavel 2007; Lin et al. 2012).

The objective of this study was to determine the prevalence and proportions of dominant bacteria of genera *Bacteroides*, *Prevotella* (phylum *Bacteroidetes*); *Clostridium*, *Lactobacillus* (phylum *Firmicutes*) and *Bifidobacterium* (phylum *Actinobacteria*) in the intestines as well as to determine the content of short-chain fatty acids (acetic, butyric, formic, propionic, valeric) and branched-chain fatty acids (isovaleric and isobutyric) in the stool of 20 obese children and 20 children with normal body weight.

## Experimental

### Materials and methods

**Biological material.** The stool of 20 children: 10 overweights (BMI 25,68–29,48) and 10 obese children (BMI 31,71–41,18) aged between 6 and 15 years, and stool of 20 lean children (BMI 18,5–22,38) in the same age range was collected. As many as 55% of overweight and obese children lived in the city, while others (45%) were from rural areas. Children with normal body weight lived mostly in the city (70%) while 30% were from rural areas. In the group of overweight and obese children, girls and boys accounted for 60% and 40%, respectively. In the group of children with normal body weight, girls and boys constituted 65% and 35%, respectively. In the close family of overweight and obese children, one could report overweight or obesity in over 95% cases; while in children from family where normal body weight was reported – only 15% of family members were overweight or obese. Definitions of overweight and obesity were based on definition of the International Obesity Task Force (IOTF) criteria developed by Cole et al. (2012). Immediately after the samples were taken into sterile containers, the faeces were frozen and transported on the same day for further analysis.

**Determination of the number of bacteria.** The number of bacteria was determined with fluorescence *in situ* hybridization (FISH). Hybridization procedure was prepared based on the method described by Barczyńska et al. (2017). Hybridization was conducted in a humid chamber at a temperature and time specific to the oligonucleotide probes applied. Table I shows the sequences of probes and hybridization conditions used

Table I
The sequence of oligonucleotide probes and hybridization conditions used in FISH procedure for the identification of the bacteria present in children faeces.

| Probe | Identified microorganisms | Sequence (5'→3') | Fluorescent label | Temp [°C] | Time [h] |
|---|---|---|---|---|---|
| Lab 158 | *Lactobacillus-Enterococcus* | GGT ATT AGC A(T?C)CTGT TTC CA | 5'Fluo | 46 | 24 |
| Bif 164 | *Bifidobacterium* spp. | CAT CCG GCA TTA CCA CCC | 5'Cy3 | 58 | 18 |
| Bac 303 | *Bacteroides-Prevotella* | CCA ATG TGG GGG ACC TT | 5'Cy3 | 55 | 3 |
| Erec 484 | *Clostridium coccoides* | GCT TCT TAG TCA GGT ACC G | 5'Cy3 | 57 | 16 |
| Prov | *Prevotella* | ATCTTGAGTGAGTTCGATGTTGG | 5'Fluo | 57 | 18 |

for the identification of bacteria by FISH. Microscopic observations were performed using Eclipse E-400 fluorescence microscope (Nikon, Japan), combined with COHU 4910 camera (Cohu Inc., USA) and coupled with a computer. Enumeration of microbial cells was performed using NIS Elements BR version 3.2 computer program (Nikon, Japan).

**Isolation, amplification, RFLP and sequencing of DNA.** Isolation of bacteria genomic DNA was performed using kit Genomic Mini (A&A Biotechnology) according to the manufacturer's protocol. The isolated DNA was amplified by PCR using oligonucleotides specific to bacterial 16S RNA fragments. For sequencing purposes, the PCR products were purified using Exonuclease I (EURx) and Fast Polar-BAP kit (EURx). For restriction enzyme length polymorphism analysis (RFLP), the PCR products were digested using Taq I restriction enzyme (Thermo Scientific) and compared to control DNA mass marker. Automated sequencing of purified DNA fragments was performed in the Laboratory of DNA Sequencing and Oligonucleotides Synthesis at the Institute of Biochemistry and Biophysics of Polish Academy of Sciences at Warsaw using 3730 XL DNA Analyzer (Applied Biosystem). Sequence data of the selected isolates were analyzed and compared to sequences of reference strains obtained from Gen Bank NCBI base using BLASTN 2.2.32+ program.

**Analysis of the content of SCFA and BCFA acids by high performance liquid chromatography, HPLC.** Determination of the concentrations of lactic acid, SCFAs (acetic, propionic, butyric, formic, and valeric acids) and BCFAs (isobutyric and isovaleric acids) was conducted using high performance liquid chromatography (HPLC) with Surveyor liquid chromatograph (Thermo Scientific). Aminex HPX-87H (300 × 7.8 mm) (Bio-Rad Aminex®) column filled with styrene-divinylbenzene sulfonated copolymer bed were used. The following parameters were used in the analyses: Aminex HPX-87H column, mobile phase 0.005 M $H_2SO_4$, UV detector at a wavelength of 210 nm, rheodyne type injection valve, injection of sample – 10 µl, analysis temperature –60°C, flow rate 0.6 µl $min^{-1}$, analysis time of a single sample 35 min. HPLC analysis was performed for the samples with known concentrations of appropriate acids, as follows: 0, 0.125, 0.25, 0.50, 0.75 and 1% to plot calibration curves, i.e., the concentration of the acid in the function of surface area of the peak shown in the chromatogram (area). The equations developed were based on calibration curves and enabled calculation of the concentration of short-chain fatty acids in the analyzed stool samples.

**Statistical analysis.** The results were evaluated with W-Shapiro Wilk test in order to assess the normality of the distribution of the results. Due to the deviation from the normal distribution, further analyses were performed with use of U Mann-Whitney test. The data obtained from the questionnaire were analysed using the $\chi^2$ test. Statistical significance was established at $p < 0.05$. The statistical analysis was performed using STATISTICA 10.0 software (StatSoft, Inc.).

## Results

**Determination of the number of bacteria of the main genera from overweight, obese and normal weight children.** It was found that in the stool of overweight and obese children, among of the five bacteria genera tested, those classified as *Clostridium* predominated and their number was from 7.21 to 8.96 $\log_{10}$ cells $g^{-1}$, average 8.03 $\log_{10}$ cells $g^{-1}$. In the stool of children with normal weight, the average amount of these bacteria was lower by about 14% ($p < 0.01$), and their number was from 5.42 to 7.92 $\log_{10}$ cells $g^{-1}$. In the stool of children with normal body weight the dominant genera were *Bacteroides* (their number was from 6.58 to 10.06 $\log_{10}$ cells $g^{-1}$, average 8.57 $\log_{10}$ cells $g^{-1}$) and *Bifidobacterium* (their number was from 6.78 to 9.52 $\log_{10}$ cells $g^{-1}$, average was 9.07 $\log_{10}$ cells $g^{-1}$). In overweight and obese children, the number of *Bacteroides* was lower by about 20% ($p < 0.01$) (6.67 to 8.0 $\log_{10}$ cells $g^{-1}$), and *Bifidobacterium* by about 18% ($p = 0.036$) (6.51 to 8.48 $\log_{10}$ cells $g^{-1}$). The number of *Prevotella* was higher on average by 30% ($p < 0.01$) in children with normal body weight (the number was from 3.25 to 8.98 $\log_{10}$ cells $g^{-1}$) compared to children with obesity (the number was from 3.19 to 5.18 $\log_{10}$ cells $g^{-1}$). The average number of *Lactobacillus* strains in the stool of overweight, obese and normal weight children was similar and accounted for 7.81 $\log_{10}$ cells $g^{-1}$ (6.61 to 9.52 $\log_{10}$ cells $g^{-1}$), and 7.77 $\log_{10}$ cells $g^{-1}$ (7.16 to 8.65 $\log_{10}$ cells $g^{-1}$), respectively ($p = 0.913$) (Fig. 1).

Systematizing the strains tested to phyla, it was found that in the stool of overweight and obese children, bacteria of phylum *Firmicutes* (*Lactobacillus* and *Clostridium* genera) accounted for the majority of the bacteria tested, on average 45.9%. In the faeces of overweight and obese children, the bacteria belonging to genera *Lactobacillus and Clostridium* was similar and accounted for 23% of bacteria population from the five tested bacteria genera. However, bacteria belonging to phylum *Bacteroidetes* (*Prevotella and Bacteroides* genera) accounted on average for 32.4% of bacteria. Within this phylum *Bacteroides* dominated (19.89%), while *Prevotella* was in the minority (12.5%). *Bifidobacterium* (phylum *Actinobacteria*) accounted for 21.7% of the bacteria. In the stool of children with normal weight, the relative proportion of the major types of bacteria was different in comparison to overweight and obese children. The number of bacteria classified to

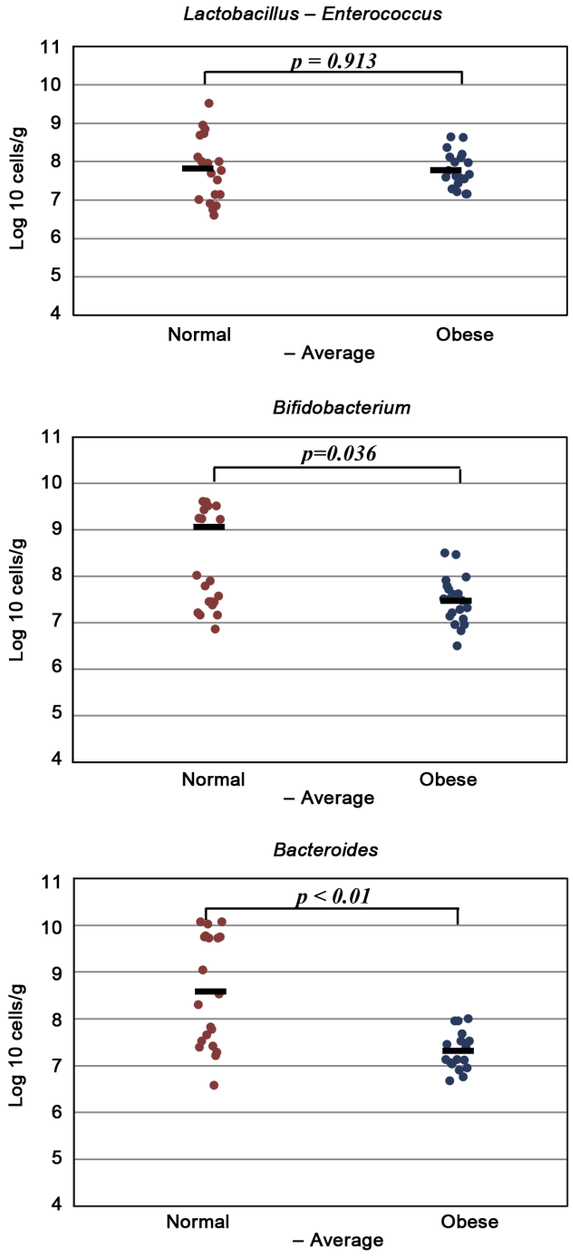


Fig. 1. The number of bacteria isolated from stool of overweight, obese and normal weight children

*Firmicutes* phylum (*Lactobacillus* and *Clostridium* genera) was lower when compared to the previous group and accounted for on average 39.1% of the bacterial population investigated. Bacteria from genera *Clostridium* and *Lactobacillus* accounted for 18.4% and 20.7% of bacterial population from the five bacteria genera tested. The 39% of bacteria was classified to *Bacteroidetes* phylum, 22.7% to *Prevotella,* and 16.3% to *Bacteroides.* The *Bifidobacterium* strains (*Actinobacteria*) represented 21.9% of total bacterial population in the stool of overweight, obese and normal weight children (Fig. 2a, 2b).

Among the group of obese children, three of them have a high BMI equal to 40.1. In this group of children, a high abundance of *Firmicutes* (53.8%, among them *Lactobacillus* 26.4% and *Clostridium* 27.4%) and low of *Bacteroidetes* (25%, among them *Prevotella* 11.3% and *Bacteroides* 13.6%) as well as *Bifidobacteria* (21%) when compared to slim children. However, the number of individuals in this group is too small to perform a statistical analysis (Fig. 2c).

**RFLP and sequencing analysis.** RFLP analysis of the 16S rRNA amplicons digested with Taq I restriction enzyme showed that the bacteria investigated in this study belong to various groups, as: *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus and Prevotella.* Bacteria classified to one strain had identical RFLP pattern. It was confirmed by sequencing analysis of bacterial 16S rRNA amplicons, whose sequences were compared to nucleotide sequences of the reference bacterial strains obtained from National Center for Biotechnology Information (NCBI).

**SCFA and BCFA in the stool of overweight, obese and normal weight children.** In the stool of overweight, obese and lean children, lactic acid dominated. The concentration of lactic acid in the stool of overweight and obese children ranged from 0.017 to 4.351 mg $g^{-1}$ of stool (1.35 ± 0.19 mg $g^{-1}$ of stool), and in children with normal body weight it ranged from 0.093 to 4.909 mg $g^{-1}$ of stool (2.24 ± 0.32 mg $g^{-1}$ of stool; $p = 0{,}014$) (Table II). The concentration of SCFAs in the stool of overweight and obese children ranged from 0.342 to 7.521 mg $g^{-1}$ of stool (3.59 ± 0.49 mg $g^{-1}$ of stool), and BCFAs from 0.022 to 0.896 mg $g^{-1}$ of stool (0.36 ± 0.05 mg $g^{-1}$ of stool) (Table II). The concentration of SCFAs in the stool of children with normal body weight was higher, ranged from 0.424 to 10.18 mg $g^{-1}$ of stool (5.44 ± 0.76 mg $g^{-1}$ of stool, $p = 0{,}040$), and the BCFAs concentration fluctuated from 0.001 to 1.151 mg $g^{-1}$ of stool (0.44 ± 0.06 mg $g^{-1}$ of stool, $p = 0{,}741$). Concentrations of formic, valeric and butyric acids in stool did not differ between groups. Similarly, concentrations of acetic and propionic acids were similar in both groups.

In the stool of overweight and obese children isovaleric acid occurred at the highest concentration among BCFAs. Its concentration was equal to 0.20 mg $g^{-1}$ of stool, while the average isobutyric acid concentration was equal to 0.16 mg $g^{-1}$ of stool (Table II). In the stool of children with normal body weight the concentration of BCFA isovaleric and isobutyric acids was similar and was equal to 0.20 mg $g^{-1}$ of stool ($p = 0.913$) and 0.23 mg $g^{-1}$ of stool ($p = 0.640$), respectively.

The results presented in Table II showed that there are significant differences in intestinal microbiota between obese and lean children. Secondly, overweight and obese children have significantly disturbed composition of fatty acids in stool.

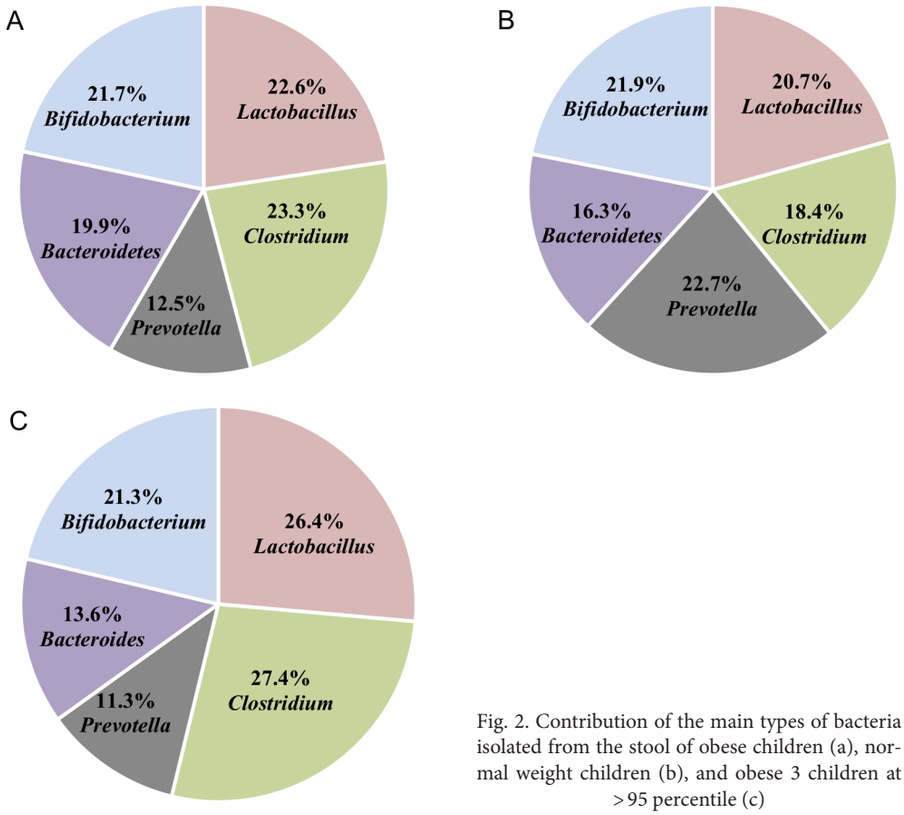


Fig. 2. Contribution of the main types of bacteria isolated from the stool of obese children (a), normal weight children (b), and obese 3 children at >95 percentile (c)

## Discussion

The development of obesity is hypothesized to be closely related to changes in intestinal microbiota and the current results of both experimental and clinical studies indicate significant differences in microbiota and bacterial gut metagenomes of obese adults and children compared with lean individuals (Backhed et al. 2004; Kimm et al. 2005; Ley et al. 2006; Sanz et al. 2008; De Filippo et al. 2010; Shen et al. 20013). It was demonstrated that modifications of the intestinal microbiota towards the dominance of *Bacteroidetes* over *Firmicutes* correlated with decreased body weight in overweight and obese individuals (Backhed

Table II
SCFAs and BCFAs in the stool of overweight, obese and normal weight children.

| Acid | Obese children | | | Children with normal weight | | | |
|---|---|---|---|---|---|---|---|
| | Acid concentration [mg $g^{-1}$ stool] | Average [mg $g^{-1}$ stool] | Median [mg $g^{-1}$ stool] | Acid concentration [mg $g^{-1}$ stool] | Average [mg $g^{-1}$ stool] | Median [mg $g^{-1}$ stool] | $p$ |
| Lactic | 0.017–4.351 | 1.35 | 1.126 | 0.093–4.909 | 2.24 | 1.804 | 0.014 |
| Acetic | 0.111–1.289 | 0.71 | 0.650 | 0.026–3.269 | 1.38 | 1.122 | 0.279 |
| Propionic | 0.085–1.232 | 0.67 | 0.594 | 0.050–2.128 | 1.08 | 0.985 | 0.354 |
| Butyric | 0.014–0.543 | 0.40 | 0.381 | 0.030–0.948 | 0.33 | 0.299 | 0.446 |
| Formic | 0.008–0.660 | 0.20 | 0.199 | 0.010–0.484 | 0.21 | 0.154 | 0.645 |
| Valeric | 0.012–0.412 | 0.26 | 0.254 | 0.063–0.472 | 0.20 | 0.187 | 0.164 |
| Total SCFA | 0.342–7.521 | 3.59 | 2.708 | 0.424–10.18 | 5.44 | 3.974 | 0.040 |
| **BCFA** | | | | | | | |
| Isobutyric | 0.048–0.341 | 0.16 | 0.100 | 0.020–0.545 | 0.23 | 0.200 | 0.640 |
| Isovalerian | 0.017–0.528 | 0.20 | 0.120 | 0.001–1.180 | 0.20 | 0.121 | 0.913 |
| Total BCFA | 0.022–0.896 | 0.36 | 0.255 | 0.001–1.151 | 0.44 | 0.421 | 0.741 |

Analysis was based on U Mann-Whitney test. Statistical significance was established at $p < 0.05$.

et al. 2004; Ley et al. 2006; Sanz et al. 2008; Turnbaugh et al. 2008; De Filippo et al. 2010; Diamant et al. 2011; Everard et al. 2013).

The composition of intestinal microbiota depends on many factors such as age, sex, diet, used medicines but also the region of the world and regional cuisine. The number of bacteria belonging to the main genera *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Bacteroides*, *Cliostridium* in the selected population of Polish children was determined for the first time in this work.

We found that the stool of normal weight Polish children contained higher number of bacteria (*Prevotella* and *Bacteroides*) classified to *Bacteroidetes* phylum than stool of overweight and obese children. However, when we analyzed obese children separately, we found clear dominance of bacteria of *Clostridium* and *Lactobacillus* genera classified to *Firmicutes* in comparison to *Bacteroidetes* (*Prevotella* and *Bacteroides*) observed only in three children, who have highest BMI and were eating food rich in animal fat.

A consequence of changes in the proportion of types of bacteria in the gut microbiota ecosystem is the modification of the proper production of SCFAs, which may also indirectly participate in the development of obesity (Canfora et al. 2015; Lu et al. 2016). The results of this study indicated that the concentration of SCFAs in the stool of overweight and obese children was lower in comparison to the stool of normal weight children. This phenomenon was also demonstrated for BCFAs.

In the stool of normal weight children, the decreasing concentrations of fatty acids (excluding lactic acid) were observed in the following order: acetic, propionic and butyric acid. In stool of overweight and obese children, these proportions were not so clear, and the propionic acid occurred at a similar concentration as acetic acid. The lower concentration of lactic acid in the stool of overweight and obese children then in the faeces of children with normal body mass correlated with the variable number of the main bacterial phyla. The higher concentration of lactic acid in the faeces of children with normal body weight was associated with a higher number of *Bifidobacteria* and comparable number of *Lactobacillus*, which is an effective producer of this acid. A similar relationship was demonstrated for propionic and acetic acid, the concentration of which was significantly higher in the faeces of children with normal body weight. This observation corresponded to the higher number of *Prevotella* and *Bacteroides* strains. On the other hand, the lower concentration of butyric acid was associated with a lower abundance of *Clostridium* in the faeces of children with normal body mass when compared to overweight and obese children. Pekmez et al. (2018) have shown that in faeces of obese children the higher concentrations of butyrate and propionate could be noticed when compared to lean children.

SCFAs are also ligands for the two G protein-coupled receptors – GPR41 and GPR43; however, they exhibit a different affinity for these receptors. GPR41 has a highest affinity toward propionic acid, followed by butyric and acetic acids, while the GPR43 receptor reacts similarly with each of these three acids (Arora et al. 2011). Binding of SCFAs, especially the propionic acid to GPR41 receptors, results in increased expression of leptin. Similarly, the binding of acetic acid to GPR43 receptors results in the increased secretion of leptin in the fatty tissue of the mice's mesentery and sodium propionate increases leptin secretion by 80% (Backhed et al. 2004; Arora et al. 2011; Nie et al. 2018).

The clarification of the mechanisms by which short-chain fatty acids (SCFAs) reduce body weight could help in the development of an strategy to effectively control the body weight. The body weight gain induced by a high-fat diet might be significantly inhibited by supplementation of diet with acetate, propionate, butyrate or their mixture. Significant changes in the expression of GPR43 and GPR41, characterized by increase of the adipose tissue mass and reduction in the colon, occurred due to the supplementation of SCFAs. Additionally, short-chain fatty acids influenced the change in microorganisms proportion in faeces by decreasing the abundance of *Firmicutes* and increasing the abundance of *Bacteroidetes* (Lu et al. 2016).

In the present study, we found that the concentration of propionic acid was lower in the stool of overweight and obese children than in the stool of normal weight children. The interpretation of this finding is difficult. The lower concentrations of propionic acid may be due its increased binding to G protein-coupled receptors as a ligand, and thus promoting inhibition of lipolysis and enhanced accumulation of lipids. On the other hand, lower concentrations of propionic acid may be associated with lower leptin secretion. Unfortunately, we did not measure leptin and other adipokines. The other interpretation of lower concentration of this SCFA in obese children is as a counterregulatory mechanism for increased caloric intake (Arora et al. 2011).

Our study has several limitations. First, we used conventional bacteriological methods and we do not assess bacterial metagenome. Second, our study group was small and we did not correlate our findings with metabolic abnormalities such as insulin resistance, dyslipidemia and immune activation. However, there are also few potential perspectives. It is interesting whether the interventions such as dietary modifications and/or increased physical activity may affect intestinal microbiome. Furthermore, it is important to analyze relation between intestinal microbiota and metabolic abnormalities typical for obesity and metabolic syndrome.

Concluding, we found that intestinal microbiome of overweight and obese children was significantly differ-

ent in comparison with lean children. It was accompanied by significant change in SCFAs and BCFAs content in stools of overweight and obese children. This research has been carried out on a selected population of Polish children using conventional microbiological methods. The recent scientific reports indicate that the role of intestinal microbiota in the pathogenesis of obesity is ambiguous and therefore it is justified to repeat the study on metabolome and metagenome of faecal samples from Polish children.

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