Effects of dietary fiber preparations made from maize starch on the growth and activity of selected bacteria from the *Firmicutes, Bacteroidetes,* and *Actinobacteria* phyla in fecal samples from obese children*

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Currently, there is a search for substances that would be very well tolerated by an organism and which could contribute to the activation of the growth of *Bacteroidetes* and *Actinobacteria* strains, with simultaneous inhibition of the growth of *Firmicutes*. High expectations in this regard are raised with the use of fiber preparations from starch — resistant corn dextrins, branched dextrins, resistant maltodextrins and soluble corn fiber. In this paper, the influence of fiber preparations made from corn starch was evaluated on growth and activity of *Bacteroidetes*, *Actinobacteria* and *Firmicutes* strains isolated from obese children. It was demonstrated that in the stool of obese children *Firmicutes* strains predominate, while *Bacteroidetes* and *Actinobacteria* strains were in the minority. A supplementation of fecal culture with fiber preparations did not cause any significant changes in the number of strains of *Bacteroidetes* and *Firmicutes*. Addition of fiber preparations to the fecal samples of obese children increased the amount of short-chain fatty acids, especially acetic ($p < 0.01$), propionic, butyric ($p = 0.05$) and lactic acid ($p < 0.01$).

Key words: Microbiota, obesity, dietary fiber, SCFA

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INTRODUCTION

Microorganisms present in the gastrointestinal tract of humans form a complex ecosystem constituting about 10^{14}/g of microbial cells, classified up to 1000 of different species (Havenaar, 2011). The most abundant bacterial species belong into 3 phyla among which *Firmicutes*' amount is in the range between 46 and 60%, while *Bacteroidetes* and *Actinobacteria* jointly represent 8 to 28% of the total microbial population (Egert *et al.,* 2006). In the past several years, a key role of microbiota in the proper functioning of the human organism has been indicated. Among three major functions of the intestinal microbiota, one can distinguish metabolic, trophic and protective functions, which account for homeostasis maintenance, regulation of processes of absorption of micro- and macronutrients, vitamin synthesis, digestion support, influence on the immune system of the organism, stimulation of the development of microvilli, fermentation of undigested food and hence the production of short-chain fatty acids (SCFA) important for a given organism (Eckburg *et al.,* 2005; Donovan *et al.,* 2009; Fujimura *et al.,* 2010; Guinane *et al.,* 2013; Tojo *et al.,* 2014). Microbiota may play a beneficial role in the metabolism of potentially harmful substances, such as nitrosamines, heterocyclic amines, and bile acids (Neish, 2002; Stewart *et al.,* 2004; Walker *et al.,* 2013). The most preferred state for a human being is a state of natural balance of the intestinal microbiota (Walker *et al.,* 2013).

As a result of fermentation of undigested food components, in particular dietary fibers (prebiotics), microbiota form such SCFA as butyric acid, which stimulates the development of intestinal epithelial tissue, nourishes the intestinal cells, affects their proper maturation and differentiation. Propionic acid has a positive effect on the growth of hepatocytes, while acetic acid affects the development of peripheral tissues. SCFAs are involved in regulation of the glucose and lipid metabolism, stimulation of proliferation and differentiation of intestinal enterocytes, affecting the reduction in pH of intestinal contents and thereby promoting the absorption of minerals by increasing their solubility (Blaut & Clavel 2007; Lin *et al.,* 2012). Studies conducted by Bäckhed, Gordon, and De Filippo indicate another important function of the intestinal microbiota relating to the influence and maintenance of normal body weight (Bäckhed *et al.,* 2004; Ley *et al.,* 2006; Backhed *et al.,* 2007; De Filippo *et al.,* 2010).

It has been found that the ratio of bacteria form the *Bacteroidetes* and *Firmicutes* phyla is associated with obesity. Increase in the amount of *Firmicutes* with simultaneous reduction in the amount of *Bacteroidetes*, as well as an increase in body weight were observed during both, *in vitro* and *in vivo* studies (Bäckhed *et al.,* 2004; Ley *et al.,* 2006; Sanz *et al.,* 2008; De Filippo *et al.,* 2010).

Prebiotics are defined as substances not digested by the host (human) enzymes which stimulate the development of beneficial bacteria of the large intestine, such as *Actinobacteria* and *Bacteroidetes*. A prebiotic is defined as a substance responsible for "the selective

stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host" (Roberfroid *et al.,* 2010). It has been shown that the increase of beneficial microbiota, and thereby sealing of the intestinal barrier, may be modulated by the addition of prebiotics to the diet (Everard *et al.,* 2013). Great expectations in this regard are raised with the use of starch products such as fiber preparations — resistant dextrins, branched dextrins, resistant maltodextrins and soluble corn fiber. The objective of the present study was to determine whether prebiotic dietary fiber preparations made from maize starch can stimulate the growth of selected strains of bacteria of the *Bacteroidetes* and *Actionbacteria* type, while inhibiting that of selected strains of bacteria of the *Firmicutes* type, in fecal microbial consortia obtained from obese children.

MATERIALS AND METHODS

Materials. The raw material for the production of potentially prebiotic preparations was maize starch (Sigma-Aldrich). It was subjected to simultaneous thermolysis and chemical modification in the presence of a volatile inorganic acid (hydrochloric acid) as a dextrinization catalyst, and an organic acid (citric acid in the K1 fiber preparation and tartaric acid in the K2 fiber preparation) as a modifying agent (according to the method of preparation and characterization of destrins reported by Jochym *et al.,* 2011).

The study material consisted of fecal samples from 5 obese children, aged 5 to 15, who were patients at the Children's Memorial Health Institute in Warsaw. The study group was selected based on the criteria of the International Obesity Task Force (IOTF), developed by Cole *et al.* (2000).

Methods. Sample culture. Immediately after the samples were taken into sterile containers, the feces were frozen and transported on the same day for further analysis. On the following day, 10 grams of feces, with addition of 1% dietary fiber made from corn starch, were homogenized (Bionovi, Bagmixer 400 P) for 10 minutes and then incubated for 24 h at 37°C under anaerobic conditions. These conditions were ensured by incubating the samples in the anaerostat with addition of Anaerogen (Oxoid). Fecal samples without addition of fiber preparations were considered as controls. The following groups of bacteria were examined: *Lactobacillus, Clostridium (Firmicutes), Bacteroides, Prevotella (Bacteroidetes), Bifidobacteria (Actinobacteria).*

Determination of the number of bacteria by fluorescence *in situ* hybridization (FISH). To 0.5 g of stool sample (after incubation), 4.5 ml of PBS (pH = 7.5) and glass beads (4 mm in diameter) were added. The samples were vortexed, which was followed by centrifugation at 2000 rpm for 5 min 4%

(final concentration in the sample) paraformaldehyde was added to the supernatant at a ratio of 1:3 and the samples were incubated for 18 h at 4°C. Then, the precipitate was centrifuged (10000 rpm, 10 min, 4°C) and washed 3 times with PBS. The precipitate was stored in 1 ml of 50% ethanol (in PBS), at 4°C, until further analysis. For hybridization, a 50 µl sample was transferred into a PCR tube, followed by the addition of 20 µl of lysozyme in TRIS-EDTA. After vortexing, the samples were incubated at 37°C for 30 min. The supernatant was removed and the precipitate was washed with 100 µl of PBS (Harmsen *et al.,* 1999).

Then, 50 µl of hybridization buffer and 10 µl of the appropriate probe were added (Table 1). Hybridization was conducted in a humid chamber (80%) at a temperature and time specific for the molecular probe applied. In addition, the total number of microorganisms was determined by DAPI staining. After the hybridization step, the samples were centrifuged, and the supernatant was removed. 150 µl of the wash buffer was added to the pellet and incubated for 30 minutes at a temperature suitable for a given probe (Table 1). The precipitate was washed in 100 µl PBS, centrifuged (14000 rpm, 5 min, 4°C), and followed by the removal of supernatant. Subsequently, the precipitate was suspended in 50 µl of PBS and stored at 4°C until further preparation of microscopic slides.

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

Analysis of the content of SCFA and BCFA acids -by high performance liquid chromatography, HPLC. Determination of lactic acid, short-chain fatty acids (acetic, propionic, butyric, formic, and valeric acids) and branched-chain fatty acids (isobutyric and isovaleric acids) was conducted using high performance liquid chromatography, utilizing the Surveyor liquid chromatograph (Thermo Scientific).

Aminex HPX-87H (300 × 7.8 mm, Bio-Rad Aminex®) column filled with styrene-divinylbenzene sulfonated copolymer bed was 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 10 µl sample volume, analysis temperature 60°C, flow rate — 0.6 µl/min, analysis time of a single sample — 35 min.

Calibration curves for organic acids were performed with standard substances with known concentrations: 0, 0.125, 0.25, 0.50, 0.75 and 1% acid/ml, in order to plot calibration curves describing the concentration of the acid in the function of surface area of the peak shown in the chromatogram (area). Equations were

Table 1. The sequence of oligonucleotide probes and hybridization conditions used in FISH procedure for the identification of bacteria from fecal samples (Metabion).

| 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
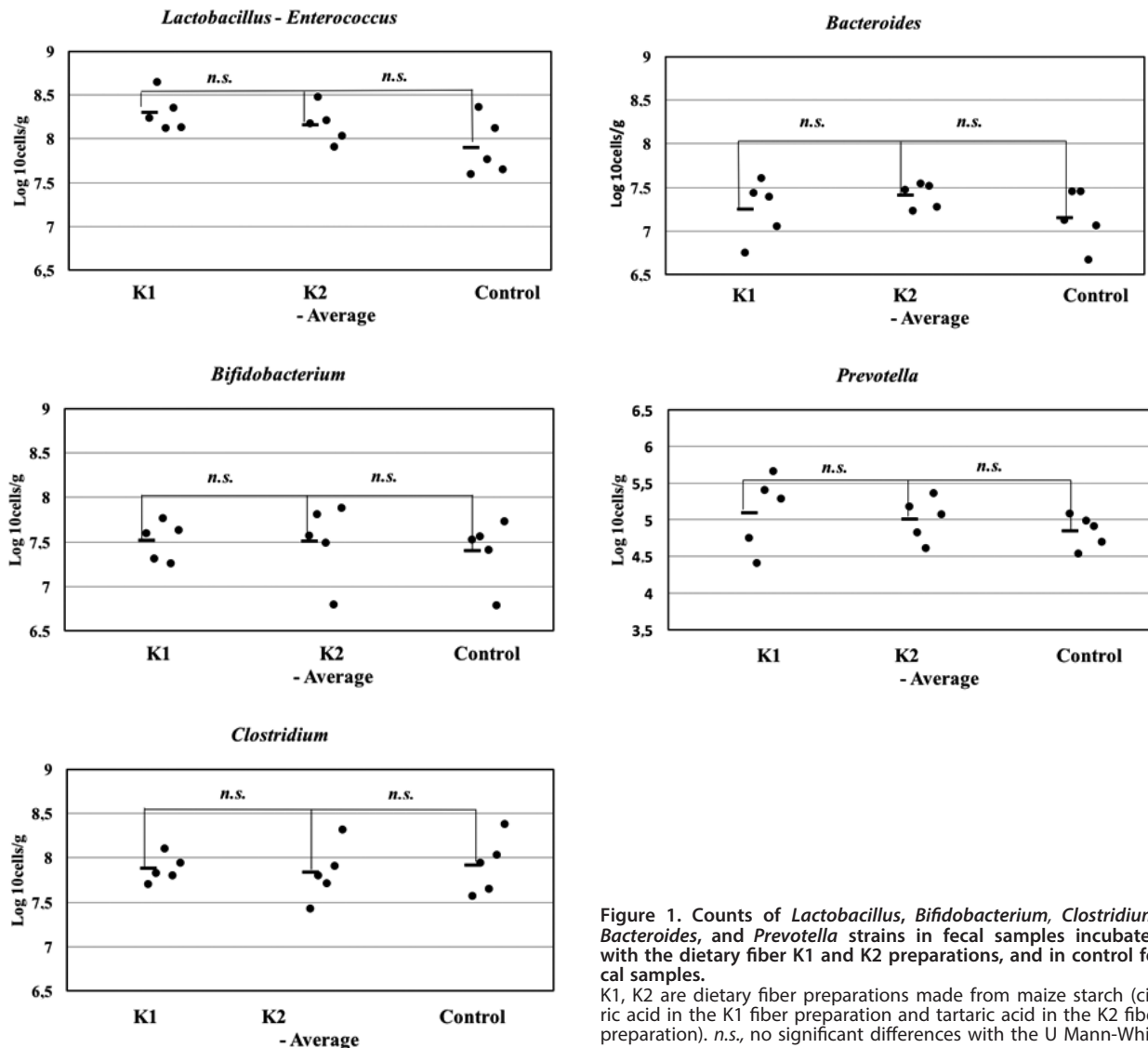
Figure 1. Counts of *Lactobacillus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, and *Prevotella* strains in fecal samples incubated with the dietary fiber K1 and K2 preparations, and in control fecal samples.

K1, K2 are dietary fiber preparations made from maize starch (citric acid in the K1 fiber preparation and tartaric acid in the K2 fiber preparation). *n.s.*, no significant differences with the U Mann-Whitney test.

developed based on calibration curves and enabled to calculate the concentration of short-chain fatty acids in the analyzed stool samples.

pH of fecal samples. pH measurements were performed by using the CP-411 manual Elmetron electrode EPS-1 pH-meter.

Statistical analysis. The results were evaluated with W-Shapiro Wilk test assessing normality of the distribution of the results. Due to the deviation from the normal distribution, further analysis was based on U Mann-Whitney test. Statistical significance was established at $p < 0.05$. Statistical analysis was performed using the STATISTICA 10.0 software (StatSoft, Inc.).

RESULTS AND DISCUSSION

Five fecal samples containing the dietary fiber K1 and K2 preparations were incubated for 24 h in order to check whether they stimulate the growth of the bacterial strains present in most normal-weight individuals, and reduce the growth of the bacteria characteristic of the microbiota of obese children. It was found that the addition of K1 and K2 preparations had no significant effects on *Lactobacillus* strains as compared to fecal sam-

ples incubated without those preparations. Furthermore, in cultures with K1 and K2, the quantities of *Prevotella*, *Bacteroides* and *Bifidobacterium* genera were not significantly different relative to fecal samples without those preparations. Similarly, the addition of dietary fiber preparations to fecal samples did not change the growth of *Clostridium* strains (Fig. 1).

Systematizing chosen strains that were tested to the types, it was found that in the stool of obese children, bacteria classified to the *Firmicutes* type accounted for the majority, particularly 46% of the tested bacteria, while those of *Bacteroidetes* and *Actinobacteria* type constituted 33% and 21% on average, respectively, which is in accordance with the results published by Filippo and coworkers (2010) who demonstrated the predominance of *Firmicutes* over *Bacteroidetes* in obese children in Florence.

The addition of fiber preparations to stool samples induced slight changes in the proportions of the main types of bacteria. The number of bacteria classified to *Firmicutes* was decreased and was estimated at 43% and 44%, on average, in cultures with K2 and K1, respectively, in the tested bacterial populations. While the number of bacteria classified to the *Bacteroidetes* type was higher and accounted for 35% and 36%, on average,
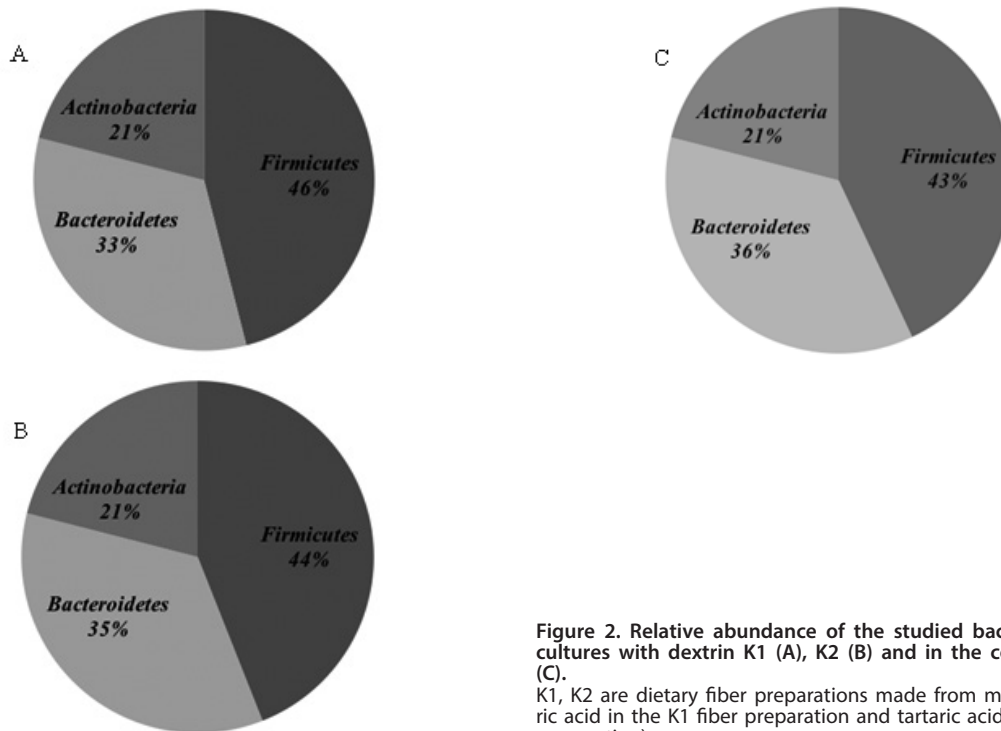
Figure 2. Relative abundance of the studied bacterial phyla in cultures with dextrin K1 (A), K2 (B) and in the control samples (C).
K1, K2 are dietary fiber preparations made from maize starch (citric acid in the K1 fiber preparation and tartaric acid in the K2 fiber preparation)

for K1 and K2, respectively, the number of *Actinobacteria* strains did not change and accounted for 21% of the population in all cultures (Fig. 2). These changes appear not to be significant.

Prebiotic substances exhibiting bifidogenic effects should also have an antagonistic effect towards unfavorable microbiota, such as *Clostridium* (Leeman *et al.,* 2006; FAO Technical Meeting on Prebiotics, 2007; Abell *et al.,* 2008; Roberfroid *et al.,* 2010). It has been reported that the consumption of prebiotic substances stimulates the growth of not only *Bifidobacteria*, but also of strains belonging to the *Bacteroidetes* and *Actinobacteria* phyla, while

inhibiting *Firmicutes* strains (especially *Clostridium*) (Martinez *et al.,* 2010), which is consistent with the presented results on the dietary fiber K1 and K2 preparations.

SCFA and BCFA profile of fecal samples from obese children incubated with the addition of the dietary fiber K1 and K2 preparations

In the culture of a stool sample without the addition of fiber preparations, lactic acid concentration ranged from 0.083 to 1.165 mg/g of stool (0.82 mg/g of stool on average). The addition of K1 and K2 preparations

Table 2. SCFA and BCFA in the stool samples of obese children.

Acid	K1			K2			Control	
	Acid concentration (mg/g feces)	Average (mg/g feces)	$P*$ K1/C	Acid concentration (mg/g feces)	Average (mg/g feces)	$P*$ K2/C	Acid concentration (mg/g feces)	Average (mg/g feces)
Lactic	5.11–9.06	6.46	<0.01	3.94–14.0	6.90	<0.01	0.08–1.16	0.82
SCFA								
Acetic	0.94–5.32	2.67	<0.01	1.21–6.13	3.65	<0.01	0.09–0.85	0.47
Propionic	0.33–0.64	0.48	0.05	1.12–8.26	3.62	0.04	0.28–0.70	0.83
Butyric	0.27–0.82	0.44	n.s.	0.24–0.86	0.64	0.05	0.01–0.54	0.36
Formic	0.11–0.47	0.31	n.s.	0.09–0.52	0.30	n.s.	0.03–0.65	0.24
Valeric	0.01–0.63	0.27	n.s.	0.08–0.41	0.30	n.s.	0.01–0.48	0.22
Total SCFA	0.01 - 5.32	0.74	n.s.	0.08–8.26	1.70	0.03	0.01–0.85	0.42
BCFA								
Isovaleric	0.04–0.37	0.23	n.s.	0.04–0.41	0.26	n.s.	0.05–0.37	0.18
Isobutanoic	0.02–0.10	0.07	0.05	0.02–0.31	0.21	n.s.	0.04–0.24	0.15
Total BCFA	0.02–0.37	0.15	n.s.	0.02–0.41	0.23	n.s.	0.04–0.37	0.16
SCFA:BCFA%	96:4–94:6	83:17	–	97:3–95:5	88:12	–	81:19–84:16	72:28

Analysis was based on U Mann-Whitney test. Statistical significance was established at $p<0.05$. n.s., not significant difference. K1, K2 — dietary fiber preparations made from maize starch (citric acid in the K1 fiber preparation and tartaric acid in the K2 fiber preparation); SCFA — short-chain fatty acids; BCFA — branched-chain fatty acids

resulted in an increased concentration of lactic acid by 88% on average ($p<0.01$), (Table 2).

The concentration of SCFA in cultures with K1 preparation ranged from 0.01 to 5.33 mg/g of stool (0.74 mg/g of stool on average), and was not significantly higher in comparison to the control samples ($p>0.05$), BCFA in culture with K1 ranged from 0.03 to 0.37 mg/g of stool (0.15 mg/g of stool on average), and was comparable to that obtained in the control sample ($p>0.05$, Table 2). The concentrations of valeric and formic acids were established at 0.27 and 0.31 mg/g of stool and were not significantly affected ($p>0.05$), while the concentration of propionic and butyric acids was comparable and accounted for 0.44–0.48 mg/g of stool. Among SCFA, the highest concentration was established for acetic acid, particularly 2.67 mg/g of stool, and was higher by 82% when compared to the control sample ($p<0.01$, Table 2). Among BCFAs, isovaleric acid predominated and its concentration was 0.23 mg/g of stool, while the concentration of isobutyric acid was 0.07 mg/g of stool and lower on average (Table 2). The average amount of BCFA was comparable to the amount of these acids in the control sample ($p>0.05$).

The concentration of SCFA in cultures with K2 preparation ranged from 0.081 to 8.260 mg/g of stool (1.7 mg/g of stool on average), and was higher in comparison to control samples by about 75% ($p=0.03$), while the concentration of BCFA in the culture with K2 ranged from 0.021 to 0.418 mg/g of stool (0.23 mg/g of average) and was not significantly higher in comparison to the control sample ($p>0.05$, Table 2). In the culture where the stool with K2 preparation was used, the concentrations of valeric and formic acids were established at 0.30 mg/g of stool for each ($p>0.05$), and the amount of butyric acid was two-fold higher and was established at 0.64 mg/g of stool ($p=0.05$). Among SCFA, the highest concentration was demonstrated for acetic and propionic acids, achieving 3.65 and 3.62 mg/g of stool, respectively. The amounts of acetic and propionic acids in the culture with K2 preparation were higher by about 87% and 77%, respectively, in comparison to the control sample ($p<0.01$ and $p=0.04$, Table 2). The amount of BCFA was comparable, and accounted for 0.21–0.26 mg/g.

A significant difference in pH of fecal samples was observed in this research. The average value of fecal pH without the addition of dextrins was 5.9, however, by adding the dietary fiber K1 and K2 formula, it was lowered to 4.2 and 4.3, respectively. The difference appeared to be due to a higher content of lactic acid in the stool specimens with the addition of dextrins.

In the human body, SCFAs are formed as a result of anaerobic degradation of dietary fiber and starch; in other words, they are the product of fermentation conducted by anaerobic bacteria residing in the cecum and colon (Cummings *et al.,* 1987). The concentration and relative proportions of the SCFAs produced, depend not only on the composition and quantity of microbiota in the colon, but also on the diet the type of dietary fiber available for fermentation (Havenaar, 2011). The addition of the dietary fiber K1 and K2 preparations to fecal samples obtained from obese children led to an increase in SCFA content in the feces, which proves that these preparations provide a beneficial fermentation substrate for the fecal microbiota.

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