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Regular paper

Impact of diet and synbiotics on selected gut bacteria and intestinal permeability in individuals with excess body weight – A Prospective, Randomized Study

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Overweight and obese individuals may have leaky intestinal barrier and microbiome dysbiosis. The aim of this study was to determine whether body mass reduction with diet and synbiotics in an adult person with excess body mass has an influence on the gut microbiota and zonulin concentration. The study was a single blinded trial. 60 persons with excess body mass were examined. Based on randomization, patients were qualified either to the intervention group (Synbiotic group) or to the control group (Placebo group). Anthropometric measurements, microbiological assessment of faecal samples and zonulin concentration in the stool were performed before and after observation. After 3-months, an increase in the variety of intestinal bacteria (increase in the Shannon-Weaver index and the Simpson index) and a decrease in concentration of zonulin in faecal samples were observed in the Synbiotic group. Also, statistically significant correlation between zonulin and *Bifidobacterium spp*. (Spearman test, R=–0.51; *p*=0.0040) was noticed. There were no significant relationships between the body mass, BMI and changes in the intestinal microbiota or zonulin concentrations. The use of diet and synbiotics improved the condition of the microbiota and intestinal barrier in patients in the Synbiotic group.

Key words: gut bacteria; obesity; zonulin; tight junction; synbiotics

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Abbreviations: BF, Body Fat; BMI, Body Mass Index; BMR, Basal Metabolic Rate; FM, Fat Mass; LBM, Lean Body Mass; PBF, Percent of Body Fat; TBW, Total Body Water

INTRODUCTION

The growing prevalence of overweight and obesity is an important problem that heightened in recent years. Excess weight gain applies not only to residents of the developed, but also to the developing countries. The main reasons for this phenomenon are changes in lifestyle, physical activity reduction and incorrect eating habits, and above all the over consumption of high-energy products (Swinburn *et al.*, 2004). The epidemiological data of the World Health Organization (WHO) show that obesity is the sixth most important cause of death in the world, affecting nearly 20% of people worldwide (Heymsfield *et al.*, 2019). Moreover, WHO indicated that in 2016, 39% of women and the same percentage of men aged 18 and over were overweight (Heymsfield *et al.*, 2019).

The definition of overweight and obesity refers to an excessive or abnormal fat accumulation, the consequence of which is an increase in body weight above certain cut-off values (Hruby & Frank, 2015). Body fat content exceeding 25% of women's and 15% of men's body weight indicates obesity (Bray & Bouchard, 2003). The most widely used criterion to classify overweight and obesity is the body mass index (BMI). Several studies suggested a relationship between BMI and body fat content and the gut microbiome (Abenavoli *et al.*, 2019; Heeney *et al.*, 2019; Stephens *et al.*, 2018; Hiippala *et al.*, 2018).

Intestinal microbiota is currently one of the fastest growing scientific topics. Many global projects, such as MetaHIT (China and EU), MicrOBESE (France) or the Human Microbiome Project (HMP, USA) that started in 2007, focused on determining the role of intestinal bacteria in maintaining a good state of human health. The evaluation of mutual connections and interactions between individual components of the intestinal ecosystem is now a necessary element to understand the causes of occurrence of many disease entities and implementation of targeted treatment (Malla *et al.*, 2019). The interaction between human microbes and the immune system affects several human metabolic functions, and plays a role in determining the well-being or disease status of the human body (Malla *et al.*, 2019).

Results of animal model studies indicate significant differences in the composition of intestinal bacteria between overweight or obese animals when compared to lean ones (Turnbaugh *et al.*, 2008; Le Roy *at al.*, 2019; Ley *et al.*, 2019). A mice study highlighted the fact that the gut microbiota contribute to more efficient energy harvesting from food, which may constitute a link combining overweight and obesity with changes in the composition of the intestinal microbiome (Tilg *et al.*, 2009; Tran *et al.*, 2015). It is suggested that changes in intestinal microbiome may affect the permeability of the intestinal barrier, and in 2000, zonulin was discovered by Fasano as a marker used to assess its efficiency (Fasano, 2000).

Studies (Vancamelbeke, 2017; Fasano, 2020; Żak-Goląb *et al.*, 2013; Moreno-Navarrete *et al.*, 2012) indicate that zonulin is a physiological modulator of intercellular tight junctions, is responsible for the transepithelial transport of ions or fluids between the lumen of the intestine and the circulatory system, thereby regulating the intestinal permeability. Gliadins and pathogenic intestinal bacteria are considered to be the most potent activating

substances. Zonulin protein levels are therefore measurable not only in the blood but also in the faeces. They reflect the state of intercellular connections in the intestinal barrier, and their increase is considered to be a marker of intestinal barrier stability (Fasano, 2001; Vancamelbeke, 2017; Fasano, 2020; Żak-Goląb *et al.*, 2013; Moreno-Navarrete *et al.,* 2012).

The gut microbiota has been recently seen as a factor contributing to overweight and obesity (John *et al.*, 2016). To prevent and treat obesity, manipulation of the gut microbiota with probiotics has been considered. Results of some studies showed that probiotic supplementation effects on metabolism and body weight are strain specific. On the other hand, the long-term effects of probiotics, the dosage and even the duration of therapy to prevent overweight or obesity are not known well-enough (Brusaferro *et al.*, 2018; Dror *et al.*, 2017).

Aims of this Study

The aim of this study was to determine whether body mass reduction accomplished with a diet and synbiotics in adult persons with excess body mass has an influence on selected gut bacteria and zonulin concentration.

MATERIALS AND METHODS

This study was a single blinded trial with 12 weeks observation period. Among the group of 60 subjects with BMI ≥25 kg/m², 56 (44 F and 12 M, mean age 40.8±14 years) met criteria of inclusion and were enrolled into two groups based on randomisation: Placebo or Synbiotic. Subjects were patients of the Outpatient Ambulatory of the Department of Clinical Nutrition, Medical University of Gdansk. The study obtained the consent of the Ethics Committee of the Medical University of Gdansk (no. NKBBN/379/2013). Criteria for inclusion in the study were: BMI ≥25 kg/m²; exclusion of secondary overweight/obesity; patient's consent for study; at least 18 years of age. Exclusion criteria from the study were: BMI <25 kg/m²; patient's lack of consent; under 18 years of age; antibiotic or probiotic therapy up to 3 months before the study; history of gastrointestinal disorders. The scheme of the study is shown in Fig. 1.

Diet intervention. Dietary habits were assessed using the FFQ-6 (*Food Frequency Questionnaire*) prior to the intervention. The FFQ-6 is a semi-quantitative tool, validated for the Polish population, allowing to assess the frequency and amount of food eaten customarily in the last 12 months.

Next, a reduction diet, based on the Polish Society of Dietetics recommendations, was applied in all subjects (Gajewska *et al.,* 2015).

Methodology of reduction diet planning included determination of the energy demand by measuring the basal metabolic rate (BMR) based on bioimpedance weight indications. Then, the patient's total energy requirement was calculated, using appropriate physical activity coefficients (PAL=1.4, for people with low physical activity; 1.6, for people with medium physical activity; 2.0, for people with high physical activity). The amount of weight reduction per unit of time was set at 0.5–1.0 kilograms per week – a deficit of 500 kcal per day was assumed for uniformity. Nutrient requirements were determined, i.e.: protein 20–25%, fats 25–30% and carbohydrates 45–55% of daily energy requirements, while mineral requirements were determined based on the dietary standards for the Polish population. The patients' daily diet defined 4–5 meals a day, and the whole dietary

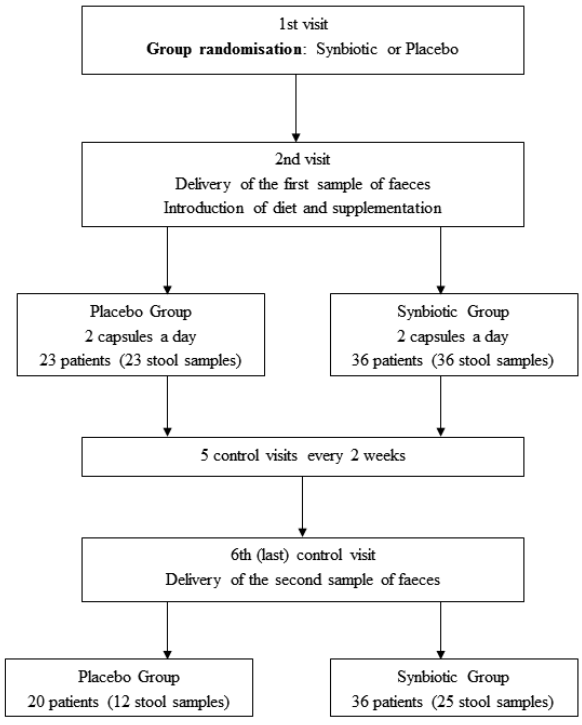

Figure 1. Scheme of the study.

therapy was scheduled for 12 weeks during which the patients had a control visit every 2 weeks.

During the study, participants met with a dietitian for nutrition advice every 2 weeks. Before the 12-week dietary intervention, the patients were instructed how to use a reduction diet. All patients were instructed to maintain the level of physical activity throughout the study and all participants were offered the same level of dietary advice, regardless of the study group. At each control meeting, a 24-hour interview was conducted and patients were re-educated on the correct use of the reduction diet. Each time at the meeting, the patients were subjected to bioimpedance measurements on the basis of which BMR and the patient's total energy requirements were determined as the starting point for determining energy reduction in the diet, in accordance with the Polish Society of Dietetics recommendations. Additionally, the regularity of the recommended probiotic intake was checked among the patients, and for this purpose used packs of the supplement were taken from patients. Patients reported how many capsules of the preparation they used since the last visit, which was recorded.

Anthropometry. Following anthropometric measurements were done in all subjects: body mass measurement and assessment of body composition, including: Lean Body Mass (LBM), Body Fat (BF), Total Body Water (TBW), Percent of Body Fat (PBF) and Basal Metabolic Rate (BMR), were done using the bioimpedance method (Jawon, Model – Contact 350F); height was assessed using a measuring rod (SECA height gauge).

BMI (*Body Mass Index*) was calculated based on the formula: current body mass/height².

The BMI classification was adopted as follows: <18.5 =underweight; 18.5–24.9=normal body weight; 25.0–29.9 = overweight; ≥30.0=obesity.

Microbiology. Microbiological assessment of faecal samples included analysis of the presence and number

of selected 12 types of bacteria (KyberKompact test, Poland).

Microbiological examination is a quantitative and qualitative analysis of faecal samples towards selected types of bacteria present in the gastrointestinal tract and analysis of parameters, such as the pH and stool consistency. Stool samples, which were provided by subjects, were immediately sent to the laboratory. There, the samples were diluted in a special buffer (pH 7.2) and seeded on suitable microbiological agar media. Bacteria were cultured under aerobic and anaerobic conditions.

A number of appropriate dilutions of the faeces samples were spread on selective and multiplying media for determination of total bacterial count – agar medium with 5% sheep's blood (bioMerieux); anaerobic bacteria *Bacteroides* (selective culture) – Schaedler's agar (Heipha); anaerobic bacilli of the *Bifidobacterium* genus – culture on a selective medium and sticks of the genus *Clostridium*, DIC agar and SPM medium (Heipha), respectively; *Enterococcus* bacteria and *Escherichia coli* – CPS (bioMerieux) chromogenic medium; potentially pathological form of *Escherichia coli Biovare* – Endo (Heipha) inoculation; lactobacilli of the *Lactobacillus* genus and lactic acid sticks producing hydrogen peroxide H_2O_2 *Lactobacillus* – cultivation on Rogos medium with peroxidase and TMB (Heipha) compound; the *Enterobacteriaceae* sticks (*Enterobacter spp., Proteus spp.*) and *Pseudomonas* sticks – culture on chromogenic CPS medium. The microbiological stool sample analyses have been described in previous reports (Grąt *et al.*, 2016; Grąt *et al.*, 2015; Żak-Goląb *et al.,* 2013).

To assess possible correlations between patients' bowels and the number of microorganisms in the gastrointestinal tract, colony-forming units (CFU) per 1 g of wet faeces were established for bacteria present in the culture and divided into three groups, depending on their functions in the body – protective microbiota (*Bifidobacterium spp., Bacteroides spp., Lactobacillus spp.,* H_2O_2 *Lactobacillus*), immunostimulatory microbiota (*Escherichia coli, Enterococcus spp.*) and proteolytic microbiota (mucilage, lactose-negative strains of *Escherichia coli Biovare, Proteus spp., Pseudomonas spp., Clostridium spp.*, other proteolytic bacteria) (Grąt *et al.*, 2016).

Faecal samples came from healthy patients, who met all inclusion criteria. In the whole group of patients qualified for the study, 3 patients suffered from asthma, 3 from reflux, 6 patients had hypertension. At the time of inclusion in the study and during its duration, the patients did not take any additional drugs. Subjects suffering from acute or chronic diseases, using any drugs and supplements, including antibiotics and oral contraceptives, and persons with hormonal disorders, i.e. hyperthyroidism and hypothyroidism, Cushing's syndrome and polycystic ovary syndrome, were excluded from the study.

Characteristics of the synbiotic preparation used. The study employed a commercially available synbiotic (Sanprobi Super Formula®, Sanprobi Sp. z o.o., Sp. k., Szczecin, Poland; owner of the probiotic strains – NIZO, Ede, Netherlands), containing seven live strains of probiotic bacteria and two prebiotics. Table 1 presents the list of bacteria and prebiotics contained in the preparation, as well as the quantitative composition of active substances contained in the maximum portion recommended for daily consumption (2–4 capsules). The total number of colony forming units of bacteria that are present is at a concentration of 1×10^9/g. All probiotic strains contained in the synbiotic used have detailed molecular characteristics and are registered in the NIZO collection of probiotic cultures, according to the NIZO Report 2009/216 (Fijan *et al.,* 2018).

We decided to use this product because presence of both, *Bifidobacterium spp.* and *Lactobacillus spp.* promotes intestinal microbial biodiversity. A balanced microbiota supports the functioning of the intestinal barrier, and thus provides protection against the entry of antigens and toxins from the digestive tract into the body. Prebiotics are, among others, a source of energy for intestinal bacteria and intestinal epithelial cells.

Authors of available publications indicate that administration of multistrain probiotics combined with lifestyle changes can modify the microbiome of obese people and helps to restore it to the state of the microbiome that occurs in lean people (Angelakis *et al.* 2013; Kobyliak *et al.*, 2016; Palacios *et al.*, 2014).

Bacterial strains contained in the used synbiotic exhibit a number of pro-health properties. The probiotic bacteria strains *Bifidobacterium lactis* W51 (NIZO 3680) and W52 (NIZO 3882) inhabit the mucosa of the end section of the ileum and large intestine, favourably affecting the digestive tract and the intestinal defence system. They protect the body against invasion of pathogenic bacteria, and are involved in the production of B vitamins and vitamin K. Their ability to stimulate secretion of anti-inflammatory interleukin 10 and to inhibit synthesis of pro-inflammatory interleukin 8 by intestinal epithelial cells has been also demonstrated (Niers *et al.,* 2005). The presence of bifidobacteria in the digestive tract ensures a rich diversification of the intestinal microbiota and proper obtaining of energy from food. Probiotic bifidobacteria also regulate gastrointestinal motility (Waller *et al.,* 2011). The modulating effect of the *Lactobacillus acidophilus* W22 (NIZO 3674) strain on functioning of the immune system has been demonstrated. Moreover, *Lactobacillus acidophilus* W22 increases production of the anti-inflammatory cytokines in peripheral blood immune cells and participates in regulation of the lipid metabolism (De Roock *et al.,* 2010). The *Lactobacillus casei* W20 strain (NIZO 3672) occurs naturally in the digestive tract and produces various bacteriocins that inhibit growth of pathogenic bacteria. Moreover, the stabilizing effect of this strain on the intercellular junctions of the intestinal epithelium and strengthening of the intestinal barrier has been shown (Endo *et al.,* 2011). *Lactobacillus plantarum* W21 (NIZO 3673) bacteria play a major role in regulation of metabolic processes and ensure proper fermentation of carbohydrates found in food. *Lactobacillus plantarum* stimulates production of the occludin and zonulin

Table 1. Composition of the synbiotics used.

Probiotics		
Species	Strain	Amount (CFU)
Bifidobacterium lactis	W51 (NIZO 3680)	$\geq2.8*10^8$
Bifidobacterium lactis	W52 (NIZO 3882)	
Lactobacillus acidophilus	W22 (NIZO 3674)	$\geq1.2*10^8$
Lactobacillus paracasei	W20 (NIZO 3672)	$\geq0.9*10^8$
Lactobacillus plantarum	W21 (NIZO 3673)	$\geq1.1*10^8$
Lactobacillus salivarius	W24 (NIZO 3675)	$\geq0.9*10^8$
Lactococcus lactis	W19 (NIZO 3671)	$\geq1.1*10^8$
Prebiotics		mg
Fructooligosaccharides (FOS)		9.6
Inulin		110.4

proteins, which are part of tight junctions (Ahrne *et al.,* 2011). *Lactobacillus salivarius* W24 (NIZO 3675) is involved in regulation of pH in the oral cavity and other parts of the digestive tract, and provides an appropriate environment for the growth of a normal, richly diversified intestinal microflora. In turn, *Lactococcus lactis* W19 (NIZO 3671) is a strain of probiotic bacteria with strong immunomodulatory properties. It regulates production of cytokines in the cells of the immune system. The safety of *Lactococcus lactis* W19 has been demonstrated, among others, in randomized studies conducted in infants, older children and pregnant women (Rotten *et al.,* 2011).

Taking into account the available research results, using the therapeutic properties of several strains of probiotic bacteria at the same time may support obesity therapy to a much greater extent than using a single strain of bacteria, by regulating metabolism, concomitant inflammation and control of nutrient intake (Kobyliak *et al.* 2016; Cani *et al.*, 2014).

Zonulin concentration in faeces. Zonulin concentration was examined in the stool samples before and after observation, by the ELISA method (kit K5600, Immundiagnostik AG, Germany). The assay sensitivity was less than 0.01 ng/ml. The ELISA kit used to measured zonulin concentration detects only the active form of zonulin. According to the results presented by others, we established that the mean concentration of zonulin in faeces ≥61 ng/ml, was the cut-off for classification of increased permeability of the intestinal barrier (Malíčková *et al.*, 2017; Moreno-Navarrete *et al.*, 2012).

Statistical analysis. All statistical calculations were carried out using the STATISTICA 12.0 software (StatSoft, Krakow, Poland). Quantitative variables were characterized by means of arithmetic mean, standard deviation, median, minimum and maximum value (range) and 95% CI (confidence interval). On the other hand, the qualitative type variables were presented by means of numbers and percentages (percentage). The W Shapiro-Wilk test was used to assess whether the quantitative variable was from a population with a normal distribution, whereas Leven's (Brown-Forsythe test) was used to check the hypothesis of equal variances. Using the Grubbs test, the outliers were determined in the data, which were then excluded in further statistical analyses. Significance of differences between the two groups (unrelated variables model) was tested by the significance tests, Student's t (or in the absence of homogeneity of variance) or Mann-Whitney U test (in case of nonfulfilment of Student's t-test conditions or for variables measured on the scale). Chi² independence tests were used for qualitative variables (using the Yates correction for cell numbers below 10, checking the Cochran conditions, Fisher's exact test, respectively). In order to establish the relationship between the variables, the Pearson and/or Spearman tests were used to calculate the correlation coefficients. In all calculations, $p=<0.05$ was assumed as the level of significance.

In addition, the Shannon-Weaver and Simpson indexes were calculated, which can be used to describe the diversity of populations in the samples (Kim *et al.,* 2017).

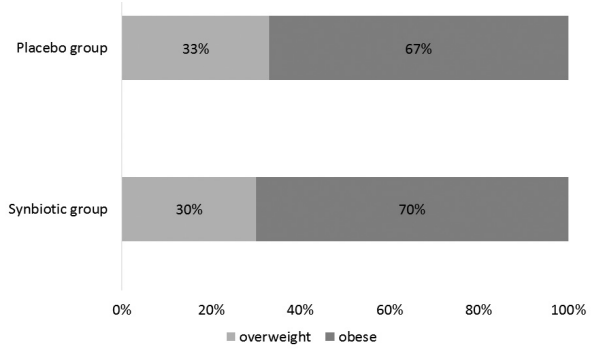

Figure 2. The percentage of obese and overweight patients before onset of the study.

RESULTS

Anthropometry

Prior of the study, the diet used by all patients did not differ significantly. Statistical analysis did not show significant differences in the frequency of consumption of food products in both groups.

In all study groups, the mean BMI was 34.0±6.9 kg/m², range 25.0–56.4 kg/m²; 31.5% of subjects presented obesity and 68.5% were overweight. The percentage of obesity in the Synbiotic group was 70% (17 women and 7 men) and overweight was 30% (10 women and 2 men), and in the Placebo group it was 67% (11 women and 3 men) and 33% (4 women and 2 men), respectively (see Fig. 2).

Table 2 presents changes in body composition before and after onset of the study in each group.

As presented in Table 2 in both groups, after 12 weeks of observation, a statistically significant decrease

Table 2. The anthropometrical results obtained in study groups. Data are presented as mean±SD (there were no statistical differences between groups).

Parameters	Synbiotic group n=36 (mean± S.D.)		Placebo group n=20 (mean± S.D.)	
	before	after	before	after
Age (years)	42.8±13.5		37.1±14.3	
Females/Males (n/%)	27/9 75.0%/25.0%		27/9 75.0%/25.0%	
BMI before (kg/m²)	33.4±6.5	32.3±6.7*	34.4±8.0	32.4±7.1*
BM before (kg)	94.1±18.6	88.8±16.9*	99.2±28.9	93.4±26.7*
FFM before (kg)	57.9±11.3	56.6±11.0	63.0±19.4	60.8±18.0
TBW before (kg)	41.6±8.2	40.9±7.8	45.4±14.3	42.3±11.6
FM before (kg)	35.7±9.4	31.9±9.9*	36.2±13.9	32.6±13.0*
BMR before (kcal)	1 483.0±277.7	1440.6±270.5	1 566.4±376.4	1599.8±597.9

Table 3. Comparison of the selected gut bacteria in both groups.

	Synbiotic group (CFU/g) (mean±S.D.)		Placebo group (CFU/g) (mean±S.D.).	
	before	after	before	after
Protective group				
Bifidobacterium spp. (*10^7)	67.50±103.6	58.07±114.7	80.000±106.729	41.875±76.488
Bacteroides spp. (*10^7)	171.22±133.1	121.86±140.0	179.900±122.443	182.500±174.642
Lactobacillus spp. (*10^4)	2965.78±16640.8	20803.72±111395.6*, 2	139.800±225.597	60.000±152.817[2]
H_2O_2 Lactobacillus (*10^4)	732.22±3335.2	2147.10±11128.6*, 2	53.900±132.371	53.000±154.055
Immunostimulatory group				
Escherichia coli (*10^4)	3131.22±6982.6	3274.34±6172.2	3161.3±5990.449	1119.647±1679.463
Enterococcus spp. (*10^4)	816.84±1703.5	895.03±1640.5	745.800±2282.557	358.125±739.296
Proteolytic group				
E. coli Biovare (*10^4)	707.28±3376.7	287.31±1483.9*, 2	303.300±781.403	332.250±996.119
Proteus spp. (*10^4)	2.00±0.0	1.66±0.8*, 2	2.00±0.0	1.50±0.89
Pseudomonas spp. (*10^4)	2.00±0.0	1.66±0.8*, 2	2.900±4.025	1.500±0.89
Clostridium spp. (*10^4)	23.67±73.3	19.17±73.8	29.910±88.158	26.250±67.831
Total bacteria (*10^4)	8.07±8.8	17.67±16.0[2]	10.62±12.66	13.60±12.59
Shannon Index	0.7710	1.046	0.707	0.538
Simpson Index	0.4449	0.5658	0.4451	0.3159

*p S *vs* P after treatment; [1]U-Mann-Whitney test; [2]Wilcoxon test

in BM, BMI and FM was noticed (p=0.0001) in comparison to the baseline parameters.

Mean body mass reduction in the Placebo group was 5.8 kg, and the subjects from the Synbiotic group lost 5.6 kg body mass on average. This was not a statistically significant difference between the groups.

Gut bacteria composition

The baseline analysis of the selected gut bacteria and the diversity indexes (Shannon-Weaver index and Simpson index) are presented in Table 3.

No statistically significant differences were observed between the three functional groups of bacteria (protective, proteolytic and immunostimulating) in both study groups before and after treatment (Wilcoxon's test, p>0.05). However, there was a statistically significant (p=0.02) increase in the number of *Lactobacillus spp.* and H_2O_2 *Lactobacillus* in the Synbiotic group (Table 3) after the diet onset (the Δ values (difference between end and baseline results) for *Lactobacillus spp.* was IQR=0.8 and for H_2O_2 *Lactobacillus* IQR=0.48)), when compared to the Placebo group. Similarly, a statistically significant decrease in the number of proteolytic bacteria (*E. coli Biovare*, p<0.01; *Proteus spp.*, p=0.04; *Pseudomonas spp.*, p=0.04) was observed in the Synbiotic group, when compared to the Placebo group.

However, an increase in the number of *E. coli* and *Enterococcus spp.* in the Synbiotic group was shown, contrary to the Placebo group, where the number of indicated bacteria decreased. Moreover, in both groups the changes described were not statistically significant.

It is also interesting that in both groups the number of *Proteus spp.* and *Pseudomonas spp.* decreased, but in the Placebo group the number of these bacteria was lower after treatment than in the Synbiotic group.

At the end of the treatment, there was a statistically significant increase in the total number of bacteria in the Synbiotic group (Wilcoxon test, p=0.01841) when compared to the Placebo group. The Shannon-Weaver and Simpson indexes indicated an increase in bacterial diversity in the faeces samples after diet and supplementation.

Zonulin concentration

Faecal zonulin concentration ranged from 7.0 to 249.00 ng/ml in all participants (n=56). The mean concentration of zonulin in all subjects was 67.9±53.82 ng/ml (median (IQR)=35.5 (72.5) ng/ml) before onset of the study. High intestinal permeability with regard to the zonulin cut-off point was noticed in the Placebo group in 6 subjects (30%) and in the Synbiotic group in 12 subjects (33%) at the beginning of the observation.

After 12 weeks, the mean zonulin concentration in the Placebo group was 67.2±42.0 ng/ml and in the Synbiotic group it was 45.8±38.0 ng/ml (Placebo *vs* Synbiotic p=0.15). After treatment, the mean concentration of zonulin was nonsignificantly reduced in patients in the Placebo group (72.6 *vs* 67.2 ng/ml, p=0.54), whereas in the Synbiotic group the decrease was significant (mean before treatment was 63.2, and after – 45.8 ng/ml, p=0.024) (see Fig. 3).

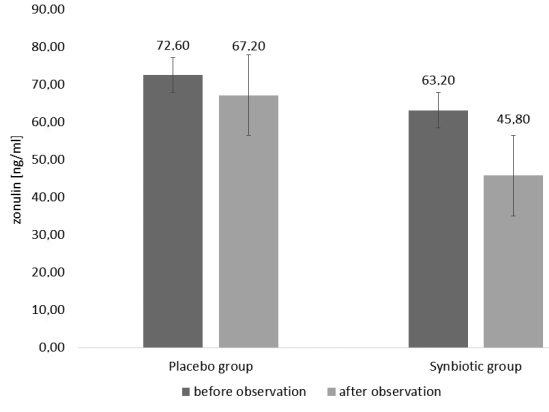

Figure 3. Changes in zonulin concentrations in both groups.

No statistically significant relationships between BMI and zonulin concentrations were found in both groups.

After 12 weeks of the study, the improvement of intestinal barrier tightness was noted in the Synbiotic group, from the initial 12 subjects (33% of the group) where the zonulin concentration was high (>61ng/ml), after the study only in 5 subjects (13.9% of the group) zonulin concentration was not decreased, whereas in the Placebo group the zonulin concentration decreased in 1 out of 6 initially indicated subjects (25% of the group still had an elevated zonulin concentration).

The correlation between *Bifidobacterium spp.* (protective group) and the concentration of zonulin was observed (see Fig. 4). At baseline, the *Bifidobacterium spp.* negatively correlated with zonulin concentration (Spearman test, R=–0.51; p=0.0040).

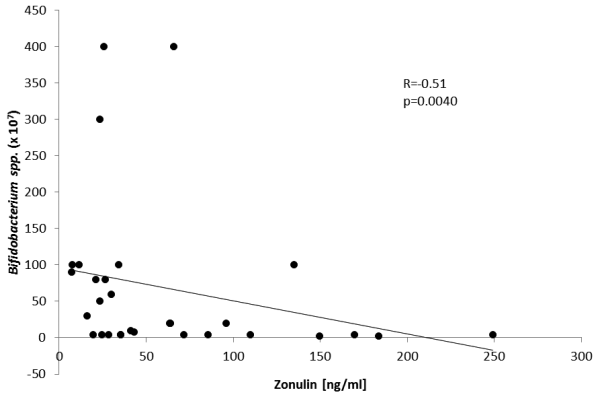

Figure 4. The correlation of zonulin with protective bacteria (*Bifidobacterium spp.*) in Synbiotic group before onset of the study.

DISCUSSION

Due to the fact that the treatment of overweight and obesity based on a reduction diet may be insufficient, in our study the therapy was enriched with a synbiotic therapy, which was hypothesized to affect modification of the selected intestinal bacteria composition along with the diet. Regular body weight analysis provided full control over changes in fat mass and lean body mass.

It is well known that a properly balanced diet allows for a permanent reduction of body mass by an average of about 0.5–1 kg per week (Sacks *et al.,* 2009). In our study mean reduction of the body mass was 5.7 kg/12 weeks, and we did not observe any differences in body mass decrease independently to synbiotic supplementation.

At the beginning of this study, an abnormal concentration of zonulin was observed in 18 subjects, which was probably related to their high intestinal permeability. The analysis of the relationship between zonulin and microbiome in our study showed a negative correlation of zonulin with *Bifidobacterium spp.* at baseline. The positive effects of diet and synbiotics are visible, especially due to an increase in the number of *Bifidobacterium spp.*, which play a protective role in the gastrointestinal tract. At the same time, a decrease in the concentration of zonulin in faecal samples was observed, presumably improving the intestinal tightness of individuals in the Synbiotic group. In adults, different strains of *Lactobacillus* and *Bifidobacterium*, individually or in combination, can help to significantly reduce body mass, BMI, waist circumference and fat mass, as it is shown by the results from different studies (Gomes *et al.*, 2017; Minami *et al.*, 2018; Pedret *et al.*, 2018). Żak-Goląb *et al.* also measured the changes in zonulin concentration in obese individuals. A slight decrease in zonulin concentration in the Placebo group in our study may be related to the effect of diet; perhaps a longer treatment time than 3 months would improve the tightness of the intestinal barrier more clearly, as can be seen in the group of Synbiotics, because here the element supporting the diet was a synbiotic. Hence, probably only a positive protective effect in the group of Synbiotics was observed.

Exposure to the presence of bacteria in the small intestine has been identified among several stimuli that may trigger the release of zonulin. Research results prove that changes in the intestinal microbiome are associated with increased secretion of the intestinal permeability marker, i.e. zonulin (El Asmar *et al.*, 2002). Exposure of small intestine cells to the bacteria of the *Enterobacteriaceae* family, regardless of the species or virulence of microorganisms, is associated with greater secretion of zonulin (El Asmar *et al.*, 2002). Liu et al. hypothesized that the total number of bacteria in the faeces correlating with circulating zonulin is a sign of inflammation of the intestinal mucosa (Liu *et al.*, 2013). The positive role of probiotic supplementation on intestinal permeability may be supported by some findings. It was shown that increased zonulin levels were found in septic patients, which is potentially reflecting an increased intestinal permeability in sepsis (Suzuki, 2013). Additionally, abundant growth of gut microbiota may be the consequence of high energy consumption by the obese subjects, related to high dietary fat intake. That is why, the increased intestinal permeability in the obese is the effect of long-lasting inappropriate dietary habits (Klaus *et al.*, 2013).

In our study, the diversity of the selected bacterial genera increased after treatment applied in the Synbiotic group (see table 3). The reduction diet resulted in a slight increase in *Bacteroides spp.* The results of the study so far indicate an increase in the number of *Bacteroides spp.* in the gastrointestinal tract, which is a result of excess body mass, proportional to the number of reduced kilograms (Balamurugan *et al.*, 2010; Ley *et al.*, 2015; Furet *et al.*, 2010; Qin *et al.*, 2010). The fact of changes in the number of proteolytic bacteria due to diet and supplementation seems to be equally interesting. The results indicate that the *Proteus spp.* are more often isolated from faecal samples, as compared to slim subjects (Löwik *et al.*, 2019). Moreover, it is assumed that the presence of *Proteus spp.* and *Pseudomonas spp.* may be associated with the occurrence of inflammation in obesity and endotoxaemia due to the bacterial lipopolysaccharide (LPS) endotoxin, which is an essential molecule of the cell walls of Gram-negative bacteria (such as *Proteus spp.*). LPS stimulates the adipose tissue deposition, increases the degree of inflammation and promotes insulin resistance (Hamilton *et al.,* 2018).

The results indicate that modification of intestinal microbiota by pre- and probiotics may be a preventive and therapeutic goal in obesity (Żak-Goląb *et al.,* 2014). A randomized, single-blind study showed that administration of the *Lactobacillus gasseri* SBT2055 (LGG2055) strain over 12 weeks to obese patients resulted in a significant reduction in body weight and body fat, when compared to patients in the placebo group (Kadooka *et al.,* 2010). The latest results of a study using a multi-graft probiotic also indicate a positive effect of these preparations in the treatment of obesity. In the group of 32 obese women, after 8 weeks of observation, a significant correlation be-

tween BMI, body weight, fat mass and intestinal microbiome was noted (Gomes *et al.*, 2019).

Limitation of the study

The main limitation of our study is the small group of subjects. In spite of this limitation, it should be highlighted that the study was a single-blind and randomized study. The patients included in the intervention formed homogeneous groups with very similar reduction diet and with similar eating habits before onset of the study. However, good compliance with the diet and synbiotic/placebo treatment was noticed.

CONCLUSION

The reduction diet and the use of synbiotics were associated with positive changes in the number of selected intestinal bacteria genera forming intestinal microbiota, and improved intestinal barrier tightness, which was related to the decrease of zonulin concentration in the faecal samples from patients in the Synbiotic group.

Author Contributions

A.J. did the research, literature search, contributed to interpretation of the data and wrote the draft of the manuscript. E.A-W. made zonulin determinations. Z.K. critically reviewed the document and added valuable information. S.M. designed and conceptualised the study, contributed to interpretation of the data, and revised the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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