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Assessment of human 4-hydroxynonenal, 8-isoprostane concentrations and glutathione reductase activity after synbiotics administration

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ABSTRACT

Purpose: Probiotics and prebiotics have become an object of intense research, to identify methods of mitigating oxidative stress. Over the past few years, the number of *in vitro* and *in vivo* studies, related to antioxidant properties of probiotics/prebiotics has significantly increased. The aim of the present study was to assess whether probiotic in combination with prebiotic influences the level of human 4-hydroxynonenal, 8-isoprostane and glutathione reductase activity.

Material/methods: Experiments were carried out on healthy volunteers (male and female). All oxidative stress markers were measured in blood plasma pre- and post-administration of synbiotic.

Results: The administration of synbiotic resulted in a significant decrease in 4-hydroxynonenal in the female-synbiotic group ($p < 0.05$), 8-isoprostanes in the female-synbiotic group and male-synbiotic group ($p < 0.05$) and non-significant increase in the activity of glutathione reductase ($p > 0.05$) *vs.* control.

Conclusions: The present results show that supplementation of synbiotics contributed to the decrease in oxidative stress parameters in the female patients.

1. Introduction

Reactive oxygen species (ROS) are generated as a result of incomplete one-electron reduction of molecular oxygen and can be divided into two groups. The first one includes compounds called radicals, whereas the other one - compounds which are non-radical forms. Both groups can demonstrate highly reactive properties. The main feature differentiating radical and non-radical forms is their electron structure [1,2].

It is proved that there are three basic sources of free radicals generation [3,4]. The first source includes: metabolic processes in mitochondria and other organelles, redox reactions taking place in the mitochondria, carried out by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, superoxide dismutase and flavoprotein oxidase. The second source of ROS is the external environment which contains both natural and artificial toxins that either are free radicals themselves or they produce free radicals. This negative environment consists of air pollutants, toxic wastes, pesticides, solar radiation and ionizing radiation, bacteria or viruses. The third source is chain reactions, initiated by other radicals.

ROS are involved in pathological conditions [3,5]. Their targets include lipids, proteins and nucleic acids. Lipid components of biological membranes (side chains of fatty acids) are particularly susceptible to effects of free radicals. They may be damaged in the process of lipid peroxidation - free radical oxidation of unsaturated fatty acids and other lipids. Mainly residues of polyunsaturated fatty acids are involved in this process. They build phospholipids, which results in forming lipid peroxides, which, in turn, contributes to lipid membrane damage. End-products of lipid peroxidation are α, β-unsaturated aldehydes (mainly malondialdehyde and human 4-hydroxynonenal) which are considered second messengers of lipid peroxidation [5,6].

Probiotics are living microorganisms which have beneficial health effects if they are administered in appropriate amounts. Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth or/and activity of one/or a limited number of bacteria in the colon [7]. Many authors suggest that oxidative stress (OS) protection is another beneficial property of probiotics/prebiotics. *In vitro* studies, many parameters can be taken into account and for numerous strains can be analyzed. The authors investigate antioxidant properties of a wide range of bacterial species *e.g.* 11 strains of *Lactobacillus*, 7 strains of *Bifidobacterium*, and 6 strains of *Lactococcus* [8]. *Lactobacillus* strains have been found to have higher total antioxidant activity than other examined strains [9]. Animal experiments are highly useful in identifying the antioxidant properties of probiotics. An interesting study of the antioxidative effect of *L. casei* spp. in Wistar rats was performed over a 90-day period [10]. A significantly higher

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CAT activity and lower levels of lipid peroxidation were observed in the liver, after probiotic supplementation. In recent years, the antioxidant properties of probiotics have also been tested in humans. Such studies are necessary, because they provide direct guidance about effectiveness of their actions. Thankfully, it is now possible to evaluate a large number of strains and conduct studies on many subjects. A potential role of probiotics in the improvement of antioxidant status in type 2 diabetic patients was a subject of one study [11]. The results were very promising: probiotic yogurts can significantly increase the total antioxidant status, GPx and SOD level compared with the control group.

The aim of the study was to evaluate the concentration of: 8-isoprostane (isoP), human 4-hydroxynonenal and glutathione reductase (GR) activity after administration of synbiotic in the human plasma of healthy volunteers. There are no studies on HNE and isoprostanes concentrations after *L.casei* + inulin administration in the plasma of healthy volunteers. To develop novel synbiotic products with the potential one (s) for preventing oxidative stress, the search for specific probiotic strains which offer the most effective prevention and mitigation of oxidative stress needs to be continued. Other studies are also needed to reveal the complete antioxidative properties of potential synbiotics.

2. Materials and methods

Thirty-two healthy volunteers (20–35 years old) were recruited for the study. There were 16 males and 16 females. The study was carried out in Poland in years 2014-2015. Subject were asked to fill in a questionnaire regarding their medical condition. Subjects with a history of gastrointestinal disease, food allergy, acute infections, alcoholism, addiction to cigarettes, administration of antimicrobial, anti-inflammatory or nonsteroidal drugs over last three months or administration of vitamins/probiotics were excluded from the study. Healthy subjects who were not on special diets which might affect antioxidant properties of plasma, or subject who were not taking antioxidant vitamins/yogurts were included in the study. Blood samples from the forearm veins were collected pre- and post-administration of synbiotics (after 7-weeks). The study was approved by the Ethical Committee of the Medical University of Lodz (number RNN/801/14/KB). All subjects gave their informed consent. A synbiotic was purchased from ICN Polfa Rzeszow S.A., Poland. The capsule contained 4×10^8 CFU lyophilized *Lactobacillus casei* plus 400 mg of inulin. Subjects were administered one capsule of synbiotic per day for 7 weeks [12].

2.1. Measurement of 4-HNE concentration in human plasma

To measure 4-HNE concentration, Human 4-Hydroxynonenal ELISA Kit (Item No. MBS006597), manufactured by MyBioSource (P.O. Box 153308, San Diego, CA 92195-3308, USA), was used. This method is based on 4-HNE antibody - 4-HNE antigen reactions (immunosorbency) and HRP colorimetric detection system to detect 4-HNE antigen targets in samples [13]. Human 4-Hydroxynonenal ELISA Kit (Item No. MBS006597) consisted of: Standards, Sample Diluent, HRP - Conjugate Reagent, Wash Solution (20x), A and B Chromogen Solution and Stop Solution. At first, 50 µl of Standard, sample or Sample Diluent were added to respective wells. Then, 100 µl of HRP-conjugate reagent was added to wells. The cuvette was incubated for 60 min at 37 °C. Next step was to wash the microtiter plate 4 times. Then, 50 µl of Chromogen Solution A and Chromogen Solution B were added to each well. The cuvette was incubated for 15 min at 37 °C. Finally, 50 µl of Stop Solution was added to each well. The color in the wells changed from blue to yellow. Absorbance was read at 450 nm with a plate reader (TECAN Sunrise with software Magellan Standard).

2.2. Measurement of 8-isoprostanes concentration in human plasma

To measure 8-isoprostanes concentration, 8-Isoprostane ELISA Kit (Item No. 516351) manufactured by Cayman Chemical Company, Ann Arbor, MI (BIOKOM, Ul. Wspolna 3, 05-090 Janki, Poland), was used. This method is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites [14]. 8-Isoprostane ELISA Kit (Item No. 516351) consisted of: 8-isoprostane Antiserum, 8-isoprostane-AchE Tracer, 8-isoprostane ELISA Standard, ELISA Buffer Concentrate (10x), Wash Buffer Concentrate (400x), Polysorbate 20, Mouse anti-rabbit IgG coated plate, Ellman's reagent, ELISA Tracer Dye and ELISA Antiserum Dye. A suggested plate format (Blk- blank; TA – total activity; NSB – non- specific binding; B0 – maximum binding; S1-8 – standards 1-8) was used. At first, 100 µl of ELISA Buffer was added to NSB wells and 50 µl of this buffer was added to B0 wells. After preparation of 8-isoprostane Standard, 50 µl of ELISA Standard was added to S1-8 and 50 µl of sample was added to wells. Then, 50 µl of AChE Tracer was added to wells except TA and Blk wells. 50 µl of 8-isoprostane ELISA Antiserum was added to each well except TA, NSB and Blk wells. The plate was incubated for 18 h at 4 °C. The next step was to wash the microtiter plate 5 times. Then, 200 µl of Ellman's regent was added to each well and 5 µl of tracer was added only to TA wells. The assay developed in 90-120 min. Absorbance was read at 405 nm.

2.3. Measurement of glutathione reductase (GR) activity in human plasma

To measure GR activity, the Glutathione Reductase Assay Kit (Item number 703202), manufactured by Cayman Chemical Company, Ann Arbor, MI (BIOKOM, Ulica Wspolna 3, 05-090 Janki, Poland), was used. This kit determinates GR activity by measuring the rate of NADPH oxidation. Oxidation of NADPH to $NADP^+$ is related with decrease in absorbance at 340 nm [15]. Glutathione Reductase Assay Kit Item number 703202 consisted of: GR Assay Buffer (10x), GR Sample Buffer (10x), GR glutathione reductase-Control, GR NADPH and GR glutathione disulfide (GSSG). To prepare the background or non-enzymatic wells, 120 µl of final Assay Buffer and 20 µl of GSSG to three wells were added. To prepare positive control wells 100 µl of final Assay Buffer, 20 µl of GSSG and 20 µl of diluted GR to three wells were added. To prepare sample wells, 100 µl of final Assay Buffer, 20 µl of GSSG and 20 µl of sample to three wells were added. To initiate the reaction, 50 µl of NADPH was added to all used wells and the plate was carefully mixed for a few seconds. Absorbance was read every minute at 340 nm with a plate reader to obtain readings at 5 time points.

2.4. Statistical analysis

The data are presented as MEAN ± SEM in each group. Groups were compared using the Student's t-test and Mann-Whitney test. The selection of appropriate tests depended on the distribution of the obtained data. A p value below 0.05 was considered significant.

3. Results

3.1. Evaluation of 4-HNE concentrations

Levels of 4-HNE in the female-control group and male-control group were no significantly higher than those of the control group before experiment. A significant decrease in 4-HNE levels was observed in the female-synbiotic ($p < 0.05$) group but insignificant in male-synbiotic group ($p > 0.05$) in comparison to the their control groups before experiments (Fig. 1).

3.2. Evaluation of 8-isoprostane concentrations

Levels of 8-isoprostanes in the female-control group and male-control group were no significantly higher than those of the control group before experiment ($p > 0.05$). Administration of synbiotic significantly reduced 8-isoprostanes levels in the female-synbiotic group

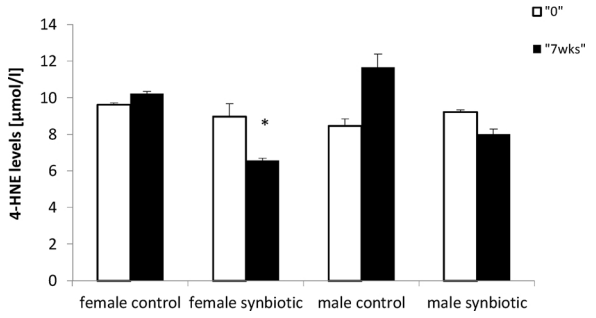

Fig. 1. Plasma 4-HNE concentrations in subjects. Data is shown as mean ± S.E.M. *p < 0.05 *vs*. control group.

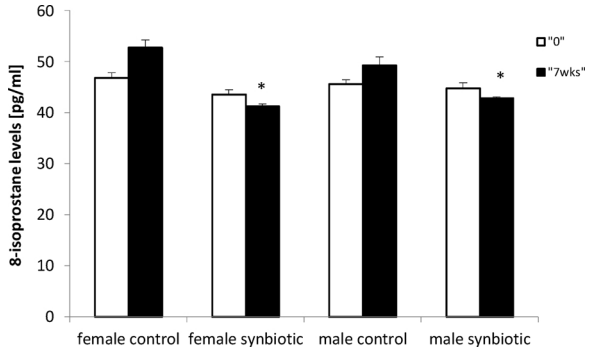

Fig. 2. Plasma 8-isoprostane concentrations in subjects. Data is shown as mean ± S.E.M.

and male-synbiotic group (p < 0.05) in comparison to the their control groups before experiments (Fig. 2).

3.3. Evaluation of GR activity

A nonsignificant increase in GR activity was observed in the control groups of subjects. The level of GR activity increased also in the female-synbiotic and male-synbiotic groups. However, no significant changes were observed (Fig. 3).

4. Discussion

Numerous studies investigate probiotic antioxidant activities, but the exact mechanism of action still remains unclear. However, there are many theories regarding the mechanisms of modulation of antioxidation by probiotics. The first one assumes that probiotics chelate metal ion. Transition metal ions are able to initiate decomposition of hydrogen peroxide into peroxyl and alkyoxyl radicals or to start lipid peroxidation, thus increasing oxidative stress [20]. Highest antioxidant

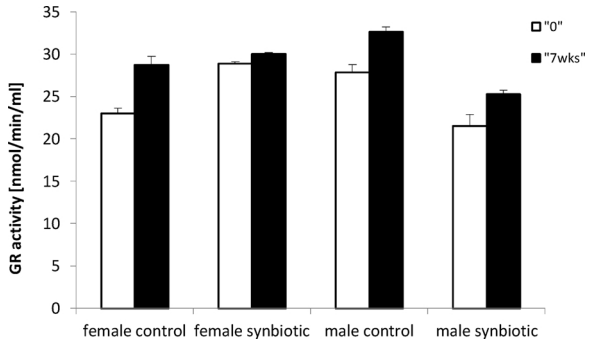

Fig. 3. Plasma glutathione reductase activity in subjects. Data is shown as mean ± S.E.M.

activity from selected *Lactobacillus* strains was demonstrated by *L. casei* KCTC 3260 [21,44]. The researchers assumed that it may be caused by very strong chelating activity for $Cu^{(2+)}$ and $Fe^{(2+)}$ ions. Another study [22] demonstrated that free radical-scavenging ability of particular *lactobacilli* is connected with the modulation of redox state in gut chyme and "free" ferrous ion chelating activity. *L. rhamnosus* GG (LGG) and *L. paracasei* Fn032 significantly inhibited production of hydroxyl radicals induced by ferrous ions. Additionally, together with *Lactobacillus*, Fn001 successfully inhibited growth of *E. coli* and *Enterococcus*, related to ferrous ion presence.

The second theory says that probiotics possess their own antioxidant enzymatic systems or produce antioxidant metabolites. SOD is one of the best known of antioxidant enzymes. The authors have proved that *Lactobacillus fermentum* are able to express Mn-SOD to combat oxidative stress [45]. Probiotics can also generate various metabolites with antioxidant activity; *e.g.* glutathione, stimulate the antioxidant system of the host and increase the activities of antioxidases efficiently. Peptides, which are released during yoghurt fermentation and their expected antioxidative properties, can play an important role in search of probiotic antioxidative mechanism [23]. *In vitro* tests revealed that lower molecular weight protein fractions were characterized with stronger antioxidative potential than high molecular weight fractions. The second part of this study [24] indicates that beneficial antioxidant properties are conditioned by the presence of certain free amino acids and peptides. In one final study, probiotic pre-treatment was found to successfully prevent oxidative stress in rats with the involvement of mucosal glutathione biosynthesis mechanisms [26]. Probiotic administration caused increased levels of mucosal glutathione (GSH) and stimulated GSH biosynthesis, which contributed to attenuated oxidative mucosal damage.

Another authors described that probiotics regulate signaling pathways (Nrf2-Keap1-ARE, NF*k*B, MAPK and PKC). Nrf2 activation upregulates a series of genes, *e.g.* those involved in ROS detoxification. *L. plantarum* SC4 and *L. plantarum* CAI6 were investigated in hyperlipidemic mice [25]: the *Lactobacillus* antioxidative defense mechanism was found to be associated with stimulation of Nrf2 expression in the liver. ROS can mediate the activation of redox-sensitive transcription factor NF*k*B (during inflammation) and the subsequent expression of inflammatory cytokines. Diao et al. [46] proved that the extracellular polysaccharide from *Bacillus sp*. strain LBP32 prevented LPS-induced inflammation in RAW 264.7 macrophages by inhibiting NF*k*B and ROS production. The mechanisms by which oxidative stress (OS) evokes an antioxidant responsive element (ARE) response has been studied. On stimulation, Nrf2 dissociates from its cytoplasmic negative regulator Keap1 and translocates to the nucleus, where it forms dimmers with basic region leucine zipper proteins and binds to the antioxidant responsive element sequence. Itoh et al. [52] and McMahon et al. [53] have described that, under unstimulated conditions, Keap1 increases proteasomal degradation of Nrf2 through its direct interaction with the N-terminal Neh2 domain within Nrf2. The redox-sensitive interaction is disrupted on stimulus, allowing nuclear accumulation of the Nrf2 protein.

Probiotics can also stimulate the immune system, which then reduces inflammation and prevents cytokine-induced oxidative stress. Another important strategy is the inhibition of intestinal pathogens, and its consequent reduction of inflammation and oxidative damage. Additionally, probiotics reduce postprandial lipids involved in oxidative damage or enhance micro- and macronutrient absorption [16,17]. Among many possible mechanisms of oxidative stress reduction, researchers identified exopolysaccharides (EPS) that are released by probiotic bacteria and may potentially play a role in the reduction of oxidative stress [18,19]. This long-chain polysaccharides are released by probiotic bacteria and are constructed from branched sugar units, which protect probiotics under extreme temperature, pH or starvation conditions. The study also compares the antioxidant properties of EPS synthesized by *Bacillus coagulans* RK-02 with those of vitamin E and C as

a reference standard [18]. EPS exhibited significant free radical scavenging and strong antioxidant activities analyzed with the use of various methods. Another experimental study [19] evaluated the effect of EPS producing probiotics on colitis model in rats. *L. delbrueckii* ssp. *bulgaricus* B3 characterized by high production of EPS was compared to low-EPS producing *L. delbrueckii* ssp. *bulgaricus* A13. The study confirmed a significantly higher level of oxidative biomarker MPO in a low-EPS A13 strain group than in high-EPS B3 group, after induction of oxidative stress.

The present work shows that administration of synbiotics resulted in a decrease in the 4-HNE and 8-isoprostane concentrations in the human plasma. These findings are consistent with recent studies. Stancu et al. [27] assessed antioxidant effects of a probiotic mix (*Lactobacillus acidophilus* plus *Bifidobacterium animalis*) in hyperlipidemic hamsters. Probiotics decreased plasma 4-HNE levels compared to control. Loguercio et al. [28] evaluated whether chronic therapy with probiotics *VSL#3* affects oxidative stress parameters levels in patients with various types of chronic liver disease. Treatment with *VSL#3* exerted different effects in various groups of patients – it also significantly improved plasma levels of 4-HNE. The aim of the next study [29] was to examine the effect of the goats' milk with *Lactobacillus fermentum* ME-3 on oxidative stress markers in human blood and urine. Administration of probiotics lowered levels of 8-isoprostanes and enhanced total antioxidative activity. Vassalle et al. [30] described that 8-isoprostane concentrations were higher in CAD patients with multivessel disease compared to patients with single-vessel disease. Similarly, Gross et al. [31] presented an association between increased concentrations of circulating 8-isoprostanes and coronary calcification.

Our results indicate that the level of GR activity was no significantly higher in women than in men after synbiotic administration. As we described previously [12], daily consumption of synbiotic also resulted in higher activity of SOD, CAT and GPx, which are important antioxidant enzymes in the human body. GR catalyzes the reduction of GSSG to GSH and is essential for the glutathione redox cycle (maintains adequate levels of cellular GSH). Glutathione acts directly by the catalytic action of GPx and is considered to be involved in the regeneration of the vitamins E and C. GSH is considered to regulate redox, signaled by changes in both level of GSHt and in ratio of its forms (GSSG/GSH). It has been proven, that low levels of cellular GSH inhibit autophosphorylation of PDGF receptor. GSH is believed to participate in regulation of several transcription factors activation such as AP-1 and NF-κB. Recent report [32] has shown that probiotic *Bacillus subtilis* fmbJ (BS fmbJ) significantly increased GR activity.

In the present study, we proved that the administration of the *Lactobacillus casei* in combination with inulin is more effective than administration of a single probiotic strain in preventing oxidative stress. Some authors have described the implication of prebiotic use in the prevention and treatment of various diseases [47,48,49,50]. In a study conducted by Pourghassem Gargari et al. [47], inulin was used as intervention for 8 weeks (10 g) in women with type 2 diabetes. The results demonstrate that prebiotics are effective compounds that protect the human body from oxidative stress damage. Inulin administration resulted in a significant decrease in the serum concentration of malondialdehyde (MDA) and increase in antioxidant enzymes activity *e.g.* SOD. Rault-Nania et al. [48] have proved that supplementation with inulin-type fructans is efficient against fructose induced hypertension. The antihypertensive effect of inulin could be associated with the reduction of the high fructose induced oxidative stress. Recent reports [49] have shown that inulin protects the human colon mucosa from LPS-induced damage and this effect appears to be related to the protective effect of inulin against LPS-induced oxidative stress. Prebiotic supplementation significantly reduced serum concentration of MDA and increased SOD activity in a trial on patients with chronic viral hepatitis [50]. Animal studies also show the effectiveness of inulin in treating Crohn's disease *via* beneficial modulation of the gut microbiota [51].

To be effective, probiotic bacteria must survive in the highly acidic human gastrointestinal system, a location which represents a high level of physiological stress for bacteria. Therefore, tolerance to an acidic environment is a desirable trait for potentially probiotic strains [33]. Lactobacillus strains, often considered probiotic, to a certain extent are intrinsically acid-resistant. Afterwards, if probiotics reach the small intestine, they must demonstrate resistance to bile, which may affect the fatty acid and lipid composition of their cell membranes and reduce the survival of bacteria [34]. After overcoming these challenges probiotics can affect the composition and hence, the behavior of intestinal microflora. Although many variables are known to have an influence on the survival of probiotics in the gastrointestinal tract, the most significant are stomach acidity and length of exposure, concentration and length of exposure to bile salts, and individual properties of the probiotics strains [35].

Some *in vitro* methods are suitable for the simulation of gastro-intestinal conditions and selection of probiotic strains [33]. A very important factor for the future transit through the adverse conditions of the stomach and small intestine is the viability of probiotics at the point of consumption [36]. This is strongly supported by study demonstrating that survival rate of lactic acid bacteria placed in simulated duodenal fluid is correlated with the initial count of bacteria [37]. An analysis of probiotic products confirmed the poor survival of probiotic microorganisms in traditionally fermented dairy products [38,39].

Probiotic survival in products may be affected by a broad range of factors, including pH, hydrogen peroxide production, storage temperatures, stability in dried or frozen form, post-acidification in fermented products or finally oxygen toxicity, which is one of major reasons for poor survival of probiotic bacteria in products [36,38,39,40]. In response to these challenges, protective technologies to keep probiotics active and alive during processing and storage are under development. One such technique is encapsulation, which significantly increases probiotic survival. The goal of this process is to create a specific, favorable micro-environment in which probiotic bacteria survive processing and storage and will be released in an appropriate place. Studies have demonstrated the benefits of encapsulation regarding protection against adverse conditions [41,42].

A study conducted on 20 healthy volunteers confirms that *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus from commercial yogurt can be recovered after passage through the gut. Yogurt bacteria, which were found in human feces, evidenced that they can survive the transit through the gastrointestinal tract. However, permanent selection of improved functional probiotic strains and the adoption of new methods to enhance survival are needed to achieve better results on the field of probiotic survival and viability [43].

5. Conclusions

The synbiotic described in this work caused a decrease in 4-HNE and 8-isoprostanes concentrations in the female-synbiotic groups in comparison to the their control groups before experiments. The present results have shown that the administration of the *Lactobacillus casei* in combination with inulin contributed to the decrease in oxidative stress parameters in the female.

Financial disclosure

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Conflict of interests

The authors declare no conflict of interest.

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