

## The Effects of Swimming Exercise and Probiotic VSL#3 on Zonulin and Some Inflammatory and Oxidative Parameters in Rats <sup>[1]</sup>

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### Abstract

Moderate exercise stimulates immune system whereas intensive exercise may display immune-suppressive effect associated with the disruption of intestinal barrier. With this study, we tested the effects of moderate and intensive swimming exercises on some cytokines and oxidant variables and zonulin, an intestinal barrier marker, in rats. We also tested possible ameliorative effects of probiotic VSL#3 in both moderate and intensive exercise regimens. Twenty eight rats were randomly divided into 4 equal groups: Control-C, Probiotic-P, Exercise-E, Probiotic+Exercise-PE. The rats in group E and PE underwent moderate swimming exercise for 5 weeks. Following this period, intensive swimming exercise was performed for 5 days. The rats in group C and group P were sedentary. Probiotic VSL#3 was given to group P and PE in the water. At the end of the experiments, serum zonulin, TNF- $\alpha$ , IL-6, IL-10, TGF- $\beta$ , MDA, and protein carbonyl levels were determined. Evidences obtained from present study indicate that moderate swimming exercise improves barrier integrity of intestine and decreases oxidative stress. During the moderate swimming experiment, probiotic VSL#3 supplementation may also improve inflammatory response. On the other hand, intensive exercise does not led changes in the inflammatory response and oxidative stress, but beneficial responses of moderate exercise on the selected parameters probably disappear due to the intense exercise-induced mild stress.

**Keywords:** Cytokine, Oxidative parameters, Probiotic, Rat, Swimming exercise, Zonulin

## Ratlarda Yüzdürme Egzersizi ve Probiyotik VSL#3'ün Zonulin İle Bazı Yangısal ve Oksidatif Değişkenlere Etkileri

### Özet

Yoğun egzersizler intestinal bariyerin bozulmasıyla ilişkili olarak immun-supresif etki oluşturabilirken, ılımlı egzersizler immun sistemi stimule eder. Bu çalışma ile ratlarda ılımlı ve yoğun yüzdürme egzersizlerinin bazı sitokinlere, oksidan değişkenlere ve intestinal bariyer belirteci zonuline etkilerini değerlendirdik. Ayrıca probiyotik VSL#3'ün hem ılımlı hem de yoğun yüzdürme periyodundaki olası iyileştirici etkilerini test ettik. Yirmi sekiz rat, rasgele 4 eşit gruba ayrıldı: Kontrol-C, Probiyotik-P, Egzersiz-E, Probiyotik+Egzersiz-PE. E ve PE grubundaki ratlara 5 hafta süreyle ılımlı yüzdürme egzersizi ve ılımlı yüzdürme periyodunu takiben 5 gün süreyle yoğun yüzdürme egzersizi yaptırıldı. C ve P grupları ise sedanter grupları oluşturdu. P ve PE gruplarına içme suyu ile probiyotik VSL#3 verildi. Deneylerin sonunda serumda zonulin, TNF- $\alpha$ , IL-6, IL-10, TGF- $\beta$ , MDA ve protein karbonil düzeyleri belirlendi. Sonuçlar ılımlı yüzdürme egzersizinin bağırsak bariyer bütünlüğünü koruduğunu ve oksidatif stresi azalttığını göstermektedir. İlimli egzersiz sürecinde probiyotik VSL#3 alınması yangısal yanıtı da olumlu etkileyebilir. Diğer taraftan yoğun egzersiz herhangi bir yangısal yanıtı ve oksidatif strese neden olmamıştır. Fakat ılımlı egzersizin ortaya çıkardığı faydalı yanıtlar muhtemelen yoğun egzersizin oluşturduğu hafif şiddeteki stres nedeniyle ortadan kalkmıştır.

**Anahtar sözcükler:** Sitokin, Oksidatif değişkenler, Probiyotik, Rat, Yüzdürme egzersizi, Zonulin

### INTRODUCTION

Cytokines are involved in the maintaining of many physiological processes besides fighting pathogens entering the body. They have significant roles against the

changes in internal environment caused by various stress factors such as exercise <sup>[1]</sup>. Moderate exercise stimulates immune system, but intense or exhaustive exercise may cause immune-suppressive effect <sup>[2]</sup>. Intensive exercise may also cause upper respiratory infections and some



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gastrointestinal complaints such as cramp, diarrhea, bloating, nausea or bleeding [3,4]. The prevention of such disorders occurring after strenuous training or competition are among priorities of exercise scientists, athletes, and trainers [3,5]. These problems created by intense exercise are associated with the weakness of intestinal barrier and the alterations in the intestinal permeability [4]. Intestinal ischemia-induced oxidative damage and some pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 activated by oxidative damage and nuclear factor kappa (NF $\kappa$ B) are responsible for the alterations in the barrier integrity during and after intensive exercise [6,7]. Intestinal barrier weakness, also called leaky gut, can lead to absorption of pathogens and toxins into the blood and tissues. One of the main components of gastrointestinal permeability is the tight junctions controlling the passage of the molecules from paracellular area among the intestinal epithelial cells. The integrity of the tight junctions is regulated by complex interactions between the gut microbiota and their products, intestinal epithelial cells, and immune cells [8]. Zonula occludens toxin (ZOT) derived from *Vibrio cholera* causes reversible disruption in the structure of tight junction and increases intestinal permeability. An analogue of this protein, zonulin also known pre-haptoglobin 2, has recently been discovered and isolated from fetal and adult human gut. When intestinal barrier is disrupted, zonulin levels increase in serum, gut and stool, leading to reversible deterioration of tight junction [9].

Probiotics have favorable effects on the intestinal barrier integrity and immune system. They are major supplements for intestinal and overall health among athletes [4,10]. Probiotics reduce the incidence and the severity of respiratory infections [11-13], and duration of gastrointestinal complaints in athletes [13,14]. Probiotics also attenuate the inflammatory response [15] and enhance the plasma antioxidant levels [16]. Probiotic supplementation for 14 weeks leads to a reduction in fecal zonulin and plasma TNF- $\alpha$  levels and this also ameliorates the exercise-induced protein oxidation in training athletes [15]. However, information on the effects of probiotic on intestinal barrier, inflammatory, and the oxidative response in moderate and intensive exercise is limited [4]. We aimed to investigate whether moderate and intensive swimming exercises affect intestinal barrier and the exercise-associated oxidative and inflammatory processes. We also investigated the ameliorative effects of probiotic VSL#3 on these processes both in sedentary and exercised rats.

## MATERIAL and METHODS

### Animals and Experimental Design

The present study was approved by the Adnan Menderes University Animal Ethics Committee (ADÜ-HADYEK-Approval No: 64583101/2013/096). A total of 28 early adult male Sprague-Dawley rats were housed in individual cages at 22 $\pm$ 2°C and 55-65% humidity. Standard mouse chow and tap water were given *ad libitum*. The rats were divided into 4 groups equally (n=7). Two groups underwent moderate swimming exercise on weekdays for the first 5 weeks. They were then subjected to intensive swimming exercise on weekdays for one additional week following moderate swimming. The other two groups of the rats were not subjected to exercise (sedentary groups). Probiotic VSL#3 (Sigma Tau Pharmaceuticals, Inc. MD, USA) in tap water was given to one of sedentary groups and one of exercise groups. The rats were then swum with a lead load of 5% body weight attached to the tail in water at 32 $\pm$ 1°C and 70 cm depth in plastic bucket, which was 80 cm in length, 50 cm in width and 90 cm in depth between 10:00 a.m. and 12:00 p.m. for 1 h a day, 5 days a week for 5-week moderate swimming exercise program [17,18]. Adaptation to swimming with load was performed by gradually increasing swimming time (10 min. every day). At day 5 of first week of moderate exercise, the rats were swum for 10 min. free and for 50 min. with load. The swimming exercise procedure continued as 10 min. free and for 50 min. with load for the next 5 weeks. The intensive swimming program was performed as 3 times (1 h each) a day for the next 1 week following the moderate swimming exercise. In this period, rats were allowed to rest for 150 min between exercise sessions. To eliminate the effects of water stress, sedentary groups left into shallow water without load at the same temperature for 30 min once a day during moderate exercise and for 30 min. 3 times a day during intensive exercise. After the rats were taken out from the water, they were dried with towel and returned to their cages. For adaptation to water, the rats were allowed to rest in the buckets filled with water for 30 min before the experiments started (Table 1).

### Probiotic Supplementation

Probiotic VSL#3 in tap water (20 mg dissolved in 75 mL tap water/rat/day) was given on weekdays for 6 weeks [19]. Probiotic was prepared daily. Each unflavored VSL#3 sachets

Table 1. Experimental design

Tablo 1. Deney dizayni

Groups [Sedantary Groups; Control (C) and Probiotic (P), Swimming Exercise Groups; Exercise (E) and Probiotic+Exercise (PE)]				
Weeks (0-6)				
Water Adaptation	Moderate Swimming Exercise	Blood collection	Intensive Swimming Exercise	Blood collection
0	5 weeks		6 <sup>th</sup> week	
30 min/a day	1 h/ a day for 5 days/ a week		3 times, 1 h/a day with 150 min interval for 5 days/a week	

(2.5 g) contain corn starch and 450 billion live freeze-dried bacteria; one strain of *Streptococcus Thermophilus*, three strains of *Bifidobacterium* and four strains of *Lactobacillus*. The rats consumed mean 60 mL water per day (Fig. 3), corresponding to about 16 mg probiotic or 2.88 billion bacteria.

#### **Blood Collection and Analysis**

Body weight, food and water consumption were recorded weekly throughout the experiment. Blood samples were collected from the tail vein at the end of week 5 (immediately after last moderate exercise) and week 6 (immediately after intensive exercise) under ether anesthesia. Serum was separated by centrifugation at 3.500 rpm for 15 min and stored at -20°C until analyzed. Zonulin, TNF- $\alpha$ , IL-6, IL-10, TGF- $\beta$ , and MDA and protein carbonyl concentrations were measured by ELISA except MDA. A microplate spectrophotometer (Thermo Scientific, Multiscan GO, Finland) was used for determining the absorbance of all ELISA tests.

#### **Cytokine Analysis**

TNF- $\alpha$ , IL-6, IL-10, and TGF- $\beta$  levels were measured by ELISA method using commercial kits (eBioscience Rat Platinum Sandwich ELISA kits). All test protocols described by the manufacturer for the cytokine analyses were similar. Each cytokine present in sample or standard coating specific anti-rat cytokine antibodies was adsorbed onto microwells. A biotin conjugated anti-rat cytokine antibody was added and bond to rat cytokine captured by the first antibody. Following incubation, unbound biotin conjugated anti-rat cytokine antibody was removed with washing steps. Streptavidin-HRP was added and bond to the biotin-conjugated anti-rat cytokine antibody. Following incubation, the unbound Streptavidin-HRP was removed with washing steps and substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of rat TNF- $\alpha$ , IL-6, IL-10 or TGF- $\beta$  present in sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from 7 specific standard dilutions and each cytokine concentration was determined. Concentrations of cytokines in serum were expressed as pg/mL.

#### **Zonulin Analysis**

Zonulin analysis was assessed using the Rat Zonulin ELISA kit (MyBioSource). Principle of the assay based on the competitive enzyme immunoassay technique utilizing a monoclonal anti-ZON antibody and a ZON-HRP conjugate in pre-coated plate for 1 h. After the incubation period, the wells are decanted and washed 5 times. Wells were incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. Finally, a stop solution was added and blue colored solution turned to yellow. The intensity of the

color was measured at 450 nm. The intensity of color was inversely proportional to the ZON concentration since ZON from samples and ZON-HRP conjugate compete for the anti-ZON antibody binding site. A standard curve was prepared relating the intensity of the color to the concentration of standards and ZON concentration in each sample was determined. Results were expressed as ng/mL.

#### **Analysis of Protein Carbonyl and Malondialdehyde**

Protein Carbonyl was measured using the Oxiselect™ Protein Carbonyl ELISA Kit (Cell Biolabs, Inc). BSA standards or protein samples (10  $\mu$ g/mL) were adsorbed onto micro-wells at 4°C overnight. The protein carbonyl present in sample or standard was derivatized to DNP hydrazine and probed with an anti-DNP antibody, followed by HRP conjugated secondary antibody. Reaction was stopped after incubation with the substrate solution. Absorbance of each well was measured at 450 nm. The protein carbonyl content in unknown sample was determined by comparing with a standard curve that was prepared from predetermined reduced or oxidized BSA standards. Results are expressed as nmol carbonyl content/mg protein.

Serum MDA levels were determined thiobarbituric acid (TBA) method described by Ohkawa et al.<sup>[20]</sup>. Principle of analysis based on the measurement of pink-colored pigment produced with reaction between TBA and MDA in acidic pH and hot environment. The absorbance was measured spectrophotometrically at 532 nm and results were expressed as  $\mu$ mol MDA/mL.

#### **Statistical Analysis**

Statistical analysis was performed using SPSS for Windows (Version 22.0, SPSS Inc., Chicago, IL, USA). Data are expressed as mean  $\pm$  standard error of mean (SEM). Distribution of data was assessed by the Shapiro-Wilk test. The data distributed normally were analyzed by ANOVA with Duncan *post hoc* test for multiple comparisons after moderate and intensive swimming exercises. The data violated normality assumptions were analyzed by Kruskal-Wallis ANOVA with Bonferroni-adjusted Mann-Whitney-U *post hoc* test. Body weight, food and water consumption were analyzed by repeated measures test. P-values <0.05 were considered to be statistically significant.

## **RESULTS**

Body weight, food and water consumption increased over time ( $P < 0.001$ ), but there was no intervention effect (Fig. 1, 2 and 3).

It was determined that moderate exercise or probiotic separately has reducing effect on zonulin concentration after 5 weeks. Zonulin concentration decreased in exercise and sedentary probiotic groups when compared with the control group ( $P = 0.011$ ). Moderate exercise and probiotic

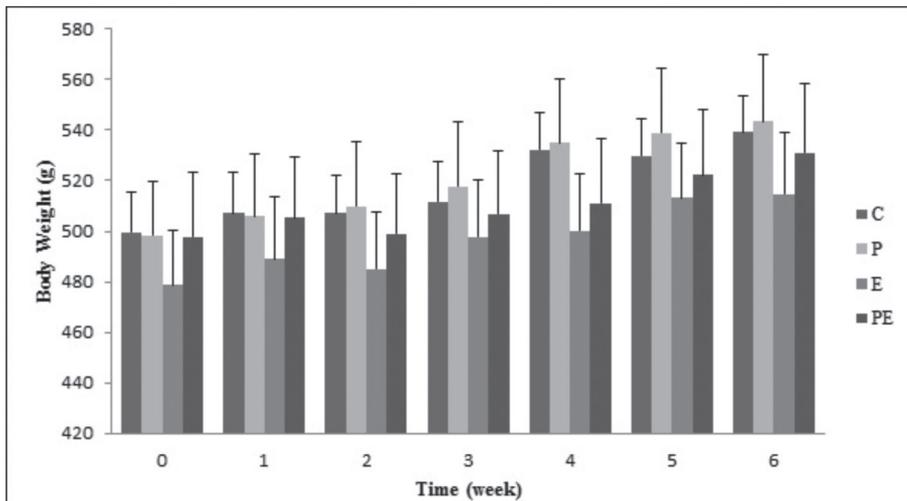


Fig 1. Body weight of rats

Şekil 1. Ratların vücut ağırlıkları

Fig 2. Food consumption of rats  
Şekil 2. Ratların yem tüketimleri

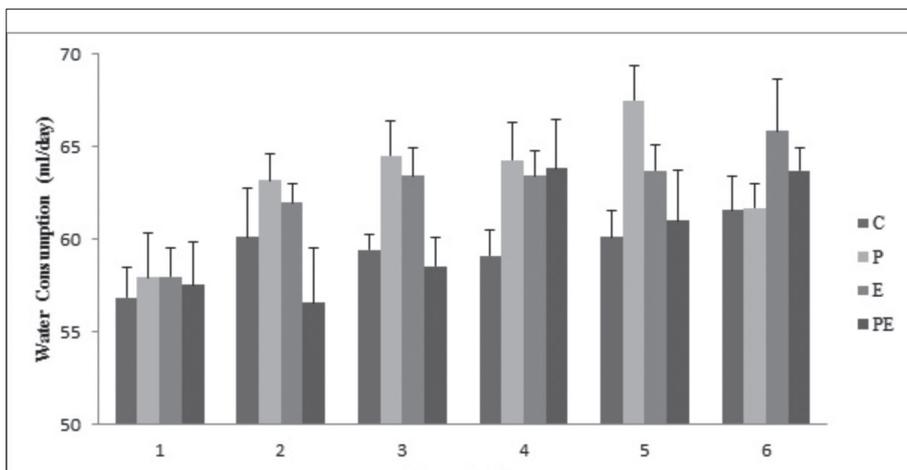
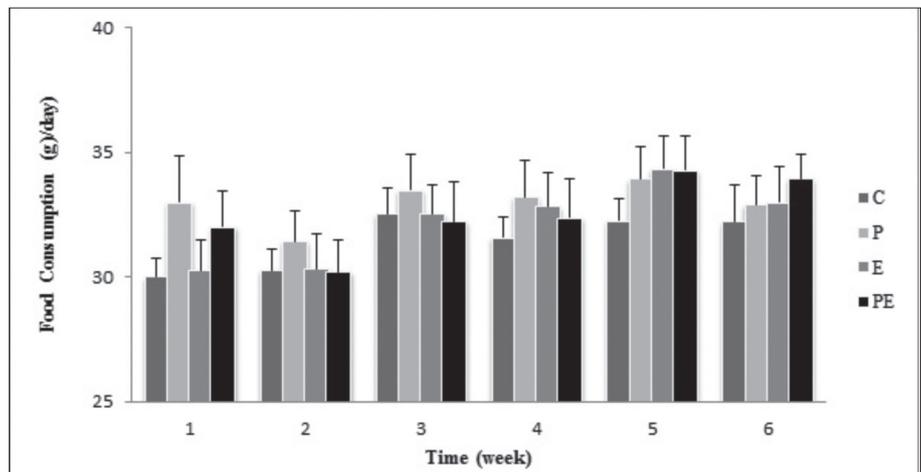


Fig 3. Water consumption of rats

Şekil 3. Ratların su tüketimleri

VSL#3 supplementation together led to non-significant reduction in zonulin levels, in contrast to the expectations, indicating that no synergistic effect between exercise and probiotic VSL#3 supplementation. After intense exercise, zonulin levels were still low in the exercise group, but this was not statistically significant ( $P=0.072$ ) (Table 2).

The effects of moderate exercise on the cytokine and

oxidant variables were found to be more prominent than the intense exercise (Table 2 and Table 3, respectively). Moderate exercise and probiotic VSL#3 supplementation together decreased IL-6, TNF- $\alpha$ , and TGF- $\beta$  levels compared with the one or more of other groups ( $P=0.022$ ,  $P=0.003$  and  $P=0.040$  respectively) (Table 2). However, the mean levels of IL-6, TNF- $\alpha$ , and TGF- $\beta$  in sedentary probiotic or exercise groups were not different from the control group.

**Table 2.** Zonulin and cytokine levels after moderate (M) and intensive (I) swimming exercise periods**Tablo 2.** İlimli (M) ve yoğun (I) yüzdürme egzersizi sonrası zonulin ve sitokin düzeyleri

Zonulin and Cytokines	Groups				P
	Control	Probiotic	Exercise	Probiotic + Exercise	
Zonulin/M (ng/mL)	321.26±15.43 <sup>a</sup>	217.01±31.48 <sup>b</sup>	198.05±29.73 <sup>b</sup>	271.88±24.53 <sup>ab</sup>	0.011
Zonulin/I (ng/mL)	334.94±30.81	308.46±37.61	235.76±40.29	383.84±39.18	0.072
IL6/M (pg/mL)	107.82±4.15 <sup>ab</sup>	112.03±3.27 <sup>ab</sup>	114.35±2.93 <sup>a</sup>	97.60±4.07 <sup>b</sup>	0.022
IL6/I (pg/mL)	110.39±3.75	114.53±5.10	111.98±6.54	105.49±4.56	NS
TNF-α/M (pg/mL)	151.67±4.29 <sup>b</sup>	140.39±2.37 <sup>ab</sup>	151.99±6.06 <sup>b</sup>	128.86±3.37 <sup>a</sup>	0.003
TNF-α/I (pg/mL)	141.76±4.98	134.64±5.80	139.63±3.85	131.34±6.87	NS
TGF-β/M (pg/mL)	520.22±24.08 <sup>ab</sup>	554.63±31.83 <sup>b</sup>	576.62±32.09 <sup>b</sup>	449.31±31.31 <sup>a</sup>	0.040
TGF-β/I (pg/mL)	488.53±40.60	540.88±53.20	519.39±43.40	493.68±30.38	NS
IL10/M (pg/mL)	193.97±20.57	188.05±34.66	188.44±19.12	180.47±22.69	NS
IL10/I (pg/mL)	127.91±11.09	106.76±12.39	149.79±12.51	139.70±20.50	NS

Data are shown as means ± SEM; <sup>a,b</sup> The means in the different row with differing letters differ from each other, NS: Not significant

**Table 3.** The levels of oxidant variables after moderate (M) and intensive (I) swimming exercise periods**Tablo 3.** İlimli (M) ve yoğun (I) yüzdürme egzersizi sonrası oksidan değişkenlerin düzeyleri

Oxidant Parameters	Groups				P
	Control	Probiotic	Exercise	Probiotic + Exercise	
PC/M (nmol/mg prot)	0.62±0.03	0.78±0.08	0.83±0.07	0.62±0.07	NS
PC/I (nmol/mg prot)	0.71±0.03	0.55±0.11	0.70±0.10	0.73±0.03	NS
MDA/M (μmol/mL)	0.40±0.05 <sup>ab</sup>	0.43±0.01 <sup>b</sup>	0.37±0.01 <sup>a</sup>	0.36±0.03 <sup>a</sup>	0.010
MDA/I (μmol/mL)	0.47±0.01 <sup>b</sup>	0.48±0.02 <sup>b</sup>	0.43±0.01 <sup>a</sup>	0.44±0.01 <sup>ab</sup>	0.035

Data are shown as means ± SEM; <sup>a,b</sup> The means in the different row with differing letters differ from each other, NS: Not significant

There were no significant changes for IL-10 levels between groups after both moderate and intensive exercises (Table 2).

Oxidant variables including protein carbonyl and MDA showed different responses to exercise (Table 3). Protein carbonyl levels tended to increase in exercise groups and probiotic supplementation returned protein carbonyl levels to the control levels (P=0.097). Both moderate and intensive exercises led to a reduction in MDA levels. MDA levels in the both exercise groups were lower than the sedentary groups after 5-week moderate exercise, but differences were confirmed only for between exercise groups and sedentary probiotic group (P=0.010). MDA levels of sedentary probiotic group were not different from those of the control group. After intense exercise, MDA levels significantly decreased only in exercise group compared with the control and probiotic groups (P=0.035).

## DISCUSSION

Exercise leads to several endocrine, immunological and oxidative responses. Regular and moderate exercise regimens induce immune response beneficially and are effective in prevention and in the treatment of various diseases. In contrast, intensive or strenuous exercise can

have immune suppressive effect and cause some upper respiratory and gastrointestinal complaints which are associated with disruption of the intestinal barrier [3,21,22]. Based on these evidences, we hypothesized that moderate exercise and/or probiotic supplementation decrease intestinal barrier marker, zonulin, levels and selected cytokines such as pro-inflammatory (e.g.TNF-α), inflammation responsive (e.g. IL-6), and mechanical stress-induced (e.g. TGF-β1) whereas increase anti-inflammatory cytokines (e.g. IL-10). We also hypothesized that intense swimming exercise causes the opposite effects of moderate exercise on cytokine and zonulin levels and the usage of probiotics can improve this situation.

We preferred cocktail VSL#3 as probiotic supplementation because VSL#3 is one of the most beneficial recommendations for ulcerative colitis therapy [23] and leads to a reduction in colonic expression of pro-inflammatory cytokines and mucosal damage and stimulates barrier integrity in different cases of colitis [24] or in common bile duct ligation induced inflammatory response [25]. We here show that 5-week moderate exercise and probiotic VSL#3 supplementation together led to reductions in some cytokine levels whereas only moderate exercise or probiotic VSL#3 supplementation does not, suggesting that usage

of probiotic VSL#3 during moderate exercise improves immune system, at least in part with these experimental conditions. In general, exercise can exclusively affect cytokine levels depending on exercise type, duration, and intensity [21,26]. Several exercise protocols performed in animals or humans have reported different findings regarding cytokine levels [15,27-31]. Mechanical stimulation of some tissues such as muscle, vascular smooth muscle, connective tissue, and bone leads to change in TGF- $\beta$ 1 production. It seems like TGF- $\beta$ 1 levels transiently increase at the beginning of the exercise regimen and then normalization occurs due to the adaptation [32]. It has also been determined that exercise led to a reduction in TNF- $\alpha$  by stimulating IL-6 levels and increase in IL-1ra and IL-10 and antioxidant levels [22,27-30]. On the other hand, there is evidence that moderate and overtraining swimming exercise does not induce any significant differences in TNF- $\alpha$  and IL-6 levels, but moderate exercise decreases TBARS levels in rats, suggesting that the intensive exercise periods or overtraining may not cause detrimental effects which consistent with our results [31]. Together, our results obtained from exercise group are partly consistent with previous studies. More importantly, probiotic VSL#3 and moderate exercise together make significant changes in immune response which may be important or more effective for curing some diseases such as colitis.

Many studies indicate that moderate endurance exercises decrease oxidative stress whereas improved anti-oxidative mechanisms [28,33-35]. Our results support these evidences that moderate and intensive exercise exert to decrease oxidative stress, but probiotic supplementation neither improve nor exacerbate this notion which suggests that there is no synergistic effect between probiotic and exercise in terms of MDA levels. Probiotics have anti-oxidative effects by reducing oxidative stress induced by mutagen, doxorubicin, diabetes or common bile duct ligation in different animal models [25,36-38]. However, this effect does not appear in healthy state [37,38]. The administration of probiotic fermented beetroot juice [37] or kefir [37] does not have effect on MDA levels in rats which is consistent with our result. In addition, it seems like protein oxidation does not affect from probiotic supplementation, exercise or both as evidenced by no change in protein carbonyl (a product of protein oxidation) levels which is supported by the estimates of Wadley et al. [39]. The effects of exercise on protein carbonyl levels may vary depending on intensity and duration of exercise due to balance between production and clearance of protein carbonyl product [39].

Probiotics are live micro-organism that regulates gut microbiota, mucosal barrier, and immune system. There is limited number of studies regarding relationship between probiotics, exercise, and zonulin levels [15,35]. Zonulin is an important marker that modulates the permeability of the intestinal barrier [9]. Probiotic supplementation causes a reduction in zonulin levels both *in vivo* and *in vitro* [15,40-42].

Chronic exercise training may cause a mild increase in the intestinal permeability by slightly enhancing zonulin levels in human [15,43]. On the other hand, there is evidence that suggest 10-day treadmill exercise (60 min/day) may led to alterations in zonulin mRNA expression and this could be related to intestinal barrier disruption [35]. Probiotic supplementation and chronic exercise together also caused to a reduction in zonulin levels in feces [15] which suggest that exercise and probiotic supplementation can beneficially affect the intestinal barrier. In the present study, we show that either probiotic supplementation or moderate exercise improve intestinal barrier integrity by decreasing blood zonulin levels. However, we did not determine additive effect between probiotic supplementation and exercise, as evidenced by non-significant reduction in zonulin levels of the rats that subjected to exercise and probiotic supplementation.

In conclusion, moderate swimming exercise decreases oxidative stress and zonulin levels that are important for the integrity of the intestinal barrier. Moderate swimming exercise and probiotic supplementation together improve immune and oxidative response and display beneficial tendencies regarding barrier integrity. However, these beneficial changes in moderate exercise and/or probiotic supplementation disappear in case of intensive swimming exercise possibly due to a mild stress in this period. The lack of any significant alterations in barrier disruption and inflammatory response suggest that exercise-adapted rats could tolerate this intensive regimen.

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