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OM-X®, a Fermented Vegetables Extract, Facilitates Muscle Endurance Capacity in Swimming Exercise Mice

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The anti-fatigue effect was investigated of the probiotic supplement, OM-X®, on forced swimming capacity in mice. Mice were administered either vehicle (distilled water; DW) or OM-X® (85 mg/kg body weight) by gavage for 4 weeks. Forced swimming tests were conducted weekly using the Ishihara-modified Matsumoto swimming pool. The endurance swimming time of the final forced swimming exercise in mice fed with OM-X® group showed an approximately 2-fold increase compared with the vehicle control group. Biomedical parameters, including blood lactate, blood superoxide dismutase (SOD) activity, serum triacylglycerol (TG), hepatic total lipids (TL), TG and phospholipid (PL) were significantly lower in mice fed with OM-X® than those in the vehicle control group. Furthermore, the mRNA expression levels of carbamoyl phosphate synthetase 1 (Cps1) and arginase 1 (Arg1*)*, in the urea cycle, were increased by OM-X® feeding. Thus, our findings suggest promotion of lipid metabolism and up-regulation of the urea cycle, at least in part, for the anti-fatigue effect mediated by OM-X®.

Keywords: OM-X®, Muscle endurance capacity, Lipid metabolism, Urea cycle, Anti-fatigue.

OM-X® is a probiotic supplement that was manufactured by Dr Iichiroh Ohhira using a unique fermentation method. It is strictly hand-made and is comprised of all natural components. OM-X® is a fermented mixture of vegetables, fruits, seaweeds, and mushrooms, using 12 strains of lactic acid bacteria (LAB) and bifidobacteria. After 5 years of fermentation at room temperature, the fermented mixture contains probiotics, prebiotics such as oligosaccharides and dietary fiber, and trace amounts of vitamins, minerals, short-chain fatty acids, and amino acids. In human clinical studies, OM-X® has shown beneficial effects on bone health [1], oral ulcerations [2], and colitis [3]. Recently we further reported the inhibitory effect of OM-X® on antigen-stimulated type I allergy [4]. The inhibitory mechanisms by OM-X® was due, at least in part, to the suppression of intracellular Ca^{2+} mobilization through inhibition of the translocation of cytosolic subunits of NADPH oxidase to membrane-bound subunits and direct scavenging of ROS that are produced by NADPH oxidase.

Most people are subjected to several stressors in their modern life, resulting in the accumulation of mental and physical fatigues. In the early stage of fatigue, favorable working conditions cannot be maintained, often resulting in impaired performances. Long-term accumulation of fatigue ultimately results in death or life-threatening diseases [5, 6]. Specifically, the recovery from fatigue is crucial for athletes to maintain their high performance in competitions. In order to avoid accumulating fatigue, athletes adopt several dietary and recovery methods that are individually matched. In recent studies, traditional plants [7], green tea [8, 9], astaxanthin [10], fucoidan [11] and deep seawater [12] have demonstrated beneficial effects in the suppression of fatigue accumulation. OM-X is a naturally-fermented plant-based probiotic containing herbs, seaweed, fruits and vegetables. Therefore, the aim of this study was to evaluate the muscle endurance capacity in OM-X®-fed mice, and to prove that OM-X® is a beneficial naturally fermented extract against fatigue.

Changes in body weight, food consumption and water intake: The changes in body weight, food consumption, and water intake were similar between the vehicle control group and OM-X® group during the experimental period (Table 1).

Table 1: Body weight, food consumption, and water intakes in vehicle control mice and OM-X® fed mice for 4 weeks.

Group		water	OM-X
Body weight (g)	0 w	32.1 ± 0.8	32.2 ± 1.6
	1 w	35.1 ± 2.9	34.9 ± 2.1
	2 w	40.0 ± 2.3	39.8 ± 2.3
	3 w	42.1 ± 2.1	40.1 ± 3.0
	4 w	43.5 ± 2.4	44.2 ± 2.4
Food consumption (g/day/mouse)	0-1 w	3.0 ± 0.4	3.0 ± 0.5
	1-2 w	5.8 ± 0.3	5.8 ± 0.4
	2-3 w	5.0 ± 0.4	4.7 ± 0.7
	3-4 w	5.3 ± 0.6	5.4 ± 0.3
Water intake (mL/day/mouse)	0-1 w	5.9 ± 0.6	6.0 ± 0.4
	1-2 w	7.0 ± 0.8	7.4 ± 0.6
	2-3 w	7.0 ± 0.4	6.7 ± 0.4
	3-4 w	7.4 ± 0.5	6.5 ± 0.8

Values are given as mean ± S. E. (each group n=17).

Effect of OM-X® on endurance capacity for swimming: To assess the effect of OM-X® on endurance capacity, we employed the swimming exercise using the Ishihara-modified Matsumoto swimming pool. As shown in Figure 1, intakes of OM-X® over 2 to 4 weeks gradually recovered the endurance swimming time up to the level of 1 week. The extended endurance swimming time in mice fed with OM-X® was significantly higher than that of the control group. Specifically, the endurance swimming time of the final forced swimming exercise in mice fed with OM-X® showed approximately a 2-fold increase compared with the vehicle control group.

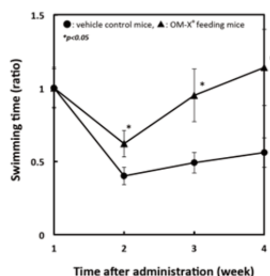

Figure 1: Effects of OM-X® on swimming exercise in mice. ●: vehicle control mice, ▲: OM-X® fed mice. Values are given as mean ± S.E. (each group n=17). $p < 0.05$ (*),

Changes in blood parameters and hepatic lipid profiles in mice fed with OM-X®: Serum components were analyzed after the last forced swimming test. Blood parameters and hepatic lipid profiles are shown in Table 2. The levels of blood lactate (p<0.01), blood SOD activity (p<0.01), serum TG (p<0.05), hepatic TL (p<0.05), TG (p<0.05) and PL (p<0.05) in mice fed with OM-X® were reduced to approximately 48, 25, 33, 26, 38, and 20%, respectively compared with those of vehicle control groups. The levels of BUN, creatinine and ketone bodies, which are the anti-fatigue and renal dysfunction indices, did not change between the vehicle control group and OM-X® group. Additionally, the activity of LDH, which is a myopathy index for over-exertion of muscle, was similar for both groups.

Table 2: Effects of OM-X® on serum biomedical parameters (blood glucose, BUN, creatinine, ketone bodies, LDH activity, lactate, SOD activity), serum lipid profiles, and hepatic lipid contents in mice.

Group	water	OM-X®
Blood glucose (mg/dL)	67.6 ± 4.7	64.0 ± 3.4
BUN (mg/100 mL)	71.9 ± 7.3	86.3 ± 6.3
Creatinine (mg/dL)	13.0 ± 0.9	12.1 ± 0.4
Ketone bodies (µmol/L)	132.6 ± 5.2	126.1 ± 8.3
LDH (Unit/L)	77.3 ± 12.9	82.6 ± 11.6
Lactate (mM)	5.3 ± 0.3	2.8 ± 0.2 **
SOD activity (%)	55.6 ± 3.4	41.5 ± 2.1 **
Serum lipid concentration		
TC (mg/100 mL)	219.3 ± 10.5	166.8 ± 9.2
TG (mg/100 mL)	366.7 ± 36.7	244.7 ± 13.2 *
PL (mg/100 mL)	299.7 ± 20.2	298.8 ± 13.5
NEFA (mEq/L)	1.4 ± 0.1	1.6 ± 0.2
Hepatic lipid profiles		
Liver weight (g)	1.8 ± 0.6	1.7 ± 0.7
TL (mg/g liver)	59.7 ± 3.8	43.9 ± 5.5 *
TC (mg/g liver)	8.8 ± 0.7	8.2 ± 0.5
TG (mg/g liver)	29.1 ± 3.1	17.9 ± 2.9 *
PL (mg/g liver)	21.0 ± 2.0	17.3 ± 1.6 *

(* $p < 0.05$, ** $p < 0.01$ vs. water)

Values are given as mean±S.E. (each group n=17).

Contents of liver and muscle glycogen: Stored glycogen in liver and muscle are very important resources for energy conservation and/or maintaining blood glucose in the physiologic range. During exercise, muscle glycogen is primarily consumed, and the levels of stored glycogen in liver and muscle are low. As shown in Figure 2, there was no change in liver and gastrocnemius muscle glycogen content between mice fed with OM-X® and the vehicle control mice.

The levels of 8-OHdG in liver and kidney: To evaluate the anti-oxidative effects of OM-X®, the levels of 8-OHdG in liver and kidney, as an oxidative damage index, were measured. There were also no differences in 8-OHdG levels between mice fed with OM-X® and the vehicle control mice (Figure 3).

The mRNA expression levels of the ornithine cycle enzyme in liver: The mRNA expression of Cps1, which acts as a rate-limiting enzyme in the ornithine cycle, and Arg1, which catalyzes the hydrolysis of arginine to ornithine and urea, were increased by OM-X® feeding (Figure 4). Specifically, the levels of Cps1 in mice fed with OM-X® significantly increased by approximately 3-fold compared with the vehicle control mice. Furthermore, Arg1 levels in mice fed with OM-X® showed a trend towards an approximately 2-fold increase compared with vehicle control mice (p<0.1), but this was not statistically significant.

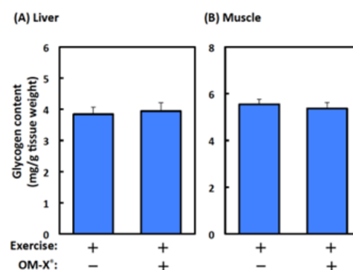

Figure 2: Effects of OM-X® on glycogen contents in exercise loaded-mice liver and muscle. (A) liver (B) muscle. Values are given as mean ± S.E. (each group n=17).

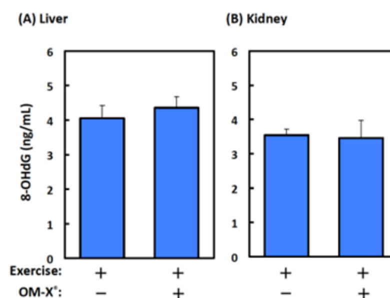

Figure 3: Effects of OM-X® on levels of 8-OHdG in exercise loaded-mice liver and kidney. (A) liver (B) kidney. Values are given as mean ± S.E. (each group n=17).

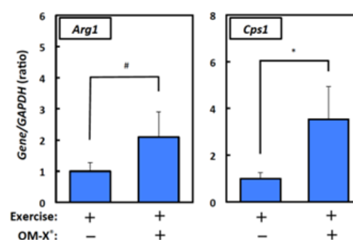

Figure 4: Effects of OM-X® on mRNA expression levels of enzymes of the urea cycle in swimming exercise loaded-mice liver. Values are given as mean ± S.E. (each group n=17). p < 0.05 (*), p<0.1 (#), Student's t-test.

In this study, we examined the anti-fatigue effect (improvement of muscle endurance capacity) of OM-X® using a swimming test, and showed that OM-X® markedly prolonged the swimming time in mice (Figure 1). To delineate the mechanisms underlying this anti-fatigue effect, we further measured several biomedical parameters. The levels of blood lactate, serum TG, hepatic TL, TG, and PL in mice fed with OM-X® were significantly lower than those in the vehicle control group (Table 2). Blood lactate is a glycolysis by-product of carbohydrates under anaerobic conditions [16]. Brooks *et al.* reported that blood lactate, which is produced in fast-twitch oxidative-glycolytic fibers, is translocated to the heart and/or slow-twitch oxidative fibers, and then used as an energy substrate in mitochondria [17,18]. Therefore, the decrease in blood lactate levels in mice fed with OM-X® might be due to its utilization as a mitochondrial substrate for energy production. TG in blood, adipose tissue, and muscle fiber is one of the main building blocks of FFAs. During exercise, FFAs, which are produced from TG by lipoprotein lipase, are oxidized (by β-oxidation) in the mitochondria to supply energy [19, 20]. Thus, it was thought that the reduction of serum TG level in mice fed with OM-X® was due to the production of energy through β-oxidation of FFAs. Terao *et al.* also reported that the concentrations of cholesterol, TG, and PL of very low density lipoprotein (VLDL) in long-distance runners were maintained at

low levels compared with non-athletes, suggesting that VLDL is used as an energy source during running and exercise [21]. VLDL consists of TG (approx. 55%), cholesterol ester (approx. 12%), free cholesterol (approx. 7%), PL (approx. 18%), and protein (approx. 8%; apoprotein B100, -E, C1, -C2, and -C3) and is secreted mainly by the liver. The lowering of hepatic TL, TG and PL in mice fed with OM-X® was likely involved in VLDL formation for the production of energy.

Furthermore, exercise induces free radical formation in the liver and muscles [22]. These free radicals trigger oxidative damage such as lipid peroxidation. Despite the amount of swimming exercise in the OM-X® group being higher than that in the vehicle control group, the levels of 8-OHdG in the liver and kidney were similar between the two. As the almost 2-fold increase in swimming exercise in the OM-X® group did not result in a concomitant increase in oxidative damage, elevated SOD activity was presumed to contribute to the anti-oxidative effect of OM-X®.

The accumulation of ammonia in serum leads to fatigue during exercise. Since ammonia is a by-product of the metabolism of amino acids and other nitrogen compounds, the accumulated ammonia is metabolized via the ornithine cycle (urea cycle) in the liver [23]. Therefore, the up-regulation of mRNA expression and activities of hepatic enzymes involved in ammonia metabolism contributes to the reduction of fatigue. As shown in Figure 4, the expression level of Cps1, which catalyzes the first reaction in the ornithine cycle wherein ammonia and bicarbonate combine to form carbamoyl, in mice fed with OM-X® was approximately 3-fold higher than in vehicle control mice. Although a remarkable difference was not observed in expression of Arg1, which catalyzes the hydrolysis of L-arginine into ornithine and urea in the ornithine cycle, a trend to up-regulation was observed in OM-X® fed mice. These results suggest that intake of OM-X® is capable of regulating the expression of hepatic enzymes involved in ammonia metabolism, resulting in an inhibition of the accumulation of ammonia in blood, eventually leading to lesser muscle fatigue and prolongation of exercise.

Since OM-X® also includes complex carbohydrates, it absolutely contributes to the prolongation of exercise. Recent studies indicated that the up-regulation of glucose transporter type 4 expression through the improvement of insulin sensitivity in muscle and 5' adenosine monophosphate-activated protein kinase activation contributes to prolongation of exercise [24, 25]. We further need to study glucose metabolism in OM-X® fed mice. Based on our results, the mechanisms of anti-fatigue effect by OM-X® were due, at least in part, to (i) catabolism of glucose in OM-X®, (ii) promotion of lipid metabolism, and (iii) up-regulation of the urea cycle. It should be noted that OM-X® is a beneficial natural extract for anti-fatigue.

Experimental

OM-X® preparation: The OM-X® sample was provided by BIOBANK Co., Ltd. (Okayama, Japan). All plant ingredients used in OM-X® are nutritionally rich and safe for human consumption. The general procedure involves loading each ingredient into the manufacturing vats one by one at different stages of fermentation. When each ingredient is added, it is fermented by LAB to produce various organic acids and amino acids. In addition, the solid ingredients are broken down to facilitate absorption of their components through fermentation. Thus, various ingredients that are beneficial to human health are fermented and matured for 5 years [1-4].

Animals and diet: Four-week-old male ddY mice (Japan SLC, Inc., Hamamatsu, Japan), weighing 20-22 g were housed in plastic cages in an air-conditioned room (22 ± 2°C) with a 12-h light and dark cycle (lighting from 0700 to1900 h). All mice were fed commercial MF pellets (Oriental yeast Co., Ltd., Tokyo, Japan) and water *ad libitum* for 1 week to accustom them to the surroundings.

Swimming exercise: First, mice were subjected to a swimming exercise for 15 min to check their swimming performance. The mice that did not perform a self-motion were removed from this study. Thirty-four mice were then divided into 2 groups (n=17 per group) equally based on body weight and swimming activity. They were administrated either vehicle (distilled water; DW) or OM-X® at a dose of 85 mg/kg of body weight by gavage for 4 weeks. Forced swimming tests were then conducted weekly using the Ishihara-modified Matsumoto swimming pool [13-15]. The swimming pool (length×width×depth=90×45×45 (cm^3)) was filled with water to a depth of 38 cm (Figure 5A). A current was generated in the pool using a pump (type C-P60H; Hitachi, Tokyo, Japan), and the current strength was adjusted by a valve. The current speed at the surface was measured with a digital current meter (model SV-101-25S; Sankou, Tokyo, Japan) at the start of every swimming session (10 L/min) and was maintained at a constant speed (14 L/min). The water was maintained at a temperature of 27± 0.5°C using an electric heater. The end point of the swimming test was defined as the time at which the mice could not resist the current and failed to rise to the surface of the pool within 7 s to breathe (Figure 5B). After swimming until the end point, mice were immediately rescued and their bodies wiped with a towel. Upon completion of the test on the final day of the swimming exercise, mice were sacrificed by isoflurane anesthesia, and blood was immediately collected using a 1 mL syringe without heparin to measure blood glucose level, blood urea nitrogen (BUN), creatinine, ketone bodies, lactate, total cholesterol (TC), TG, PL, non-esterified fatty acid (NEFA), transforming growth factor (TGF)-β, lactate dehydrogenase (LDH), and SOD activity. The liver, kidney, and gastrocnemius muscle were then excised immediately, and kept at -80°C until analysis. This animal experiment was approved by the Kindai University animal use committee, and the animals were maintained according to the guidelines of Kindai University for the care of laboratory animals.

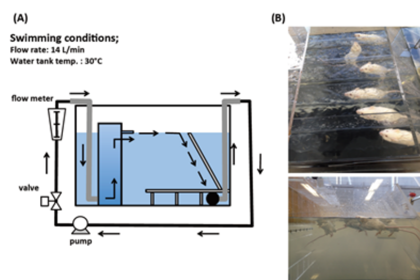

Figure 5: Equipment for forced swimming exercise. (A) Schematic diagram of the Ishihara-modified Matsumoto swimming pool, (B) appearance of swimming exercise.

Analytical procedures: The profile of serum TC, TG, PL, NEFA, blood glucose, BUN, creatinine, and ketone bodies was measured with clinical analysis kits {cholesterol CII, triglyceride G, phospholipid B, non-esterified fatty acid C test, glucose CII test, BUN-B test, creatinine test, and total ketone bodies-test, (Wako Pure Chemical Industries, Ltd., Osaka, Japan)} and lipid contents in the liver, extracted by the method of Folch *et al*., were measured in the same way. The concentration of lactate in serum was measured using a Lactate Colorimetric assay kit (BioVision Inc., CA, USA).

8-Hydroxy-2′-deoxyguanosine (8-OHdG) determination: To determine the anti-oxidative effects of OM-X® in exercised mice, we measured the 8-OHdG levels in either liver or kidney as an oxidative damage index. DNA was extracted from each tissue lysate, which was homogenized using a bead beater-type homogenizer µT-01 (TAITEC, Koshigaya, Saitama, Japan,φ5 mm SUS bead, 3000 rpm/min for 1 min) using the DNA Extractor kit (Wako). The extracted DNA from each tissue was hydrolyzed with 8-OHdG assay preparation reagent (Wako), and then measured using the 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for the Control Aging, NIKKEN SEIL Co., Ltd, Shizuoka, Japan), and normalized to DNA concentration.

Contents of liver and muscle glycogen: The contents of liver or muscle were measured with a glycogen colorimetric assay kit (BioVision Inc.). Frozen liver or gastrocnemius muscle (10 mg) was homogenized with glycogen development buffer in a 2 mL tube using a bead beater-type homogenizer µT-01 (TAITEC,φ5 mm SUS bead, 3000 rpm/min for 1 min). The homogenized sample was centrifuged at 8,000×*g*, 4°C for 5 min, and the supernatant was assayed using the glycogen colorimetric assay kit.

Quantitative real time-PCR: Total RNA was extracted from cells using a RNeasy Mini Kit (QIAGEN Inc, Valencia, CA, USA). cDNA was synthesized from 0.25 mg of total RNA using a PrimeScript reagent kit (Takara Bio, Ohtsu, Japan). cDNA was subjected to quantitative RT-PCR using an Applied Biosystems StepOne™ Real-Time PCR system (Foster City, CA, USA). Primers for arginase 1 (Arg1), carbamoyl phosphate synthetase 1 (Cps1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Takara Bio. The expression level of each gene was determined *using the comparative Ct method* and normalized to that of GAPDH, which was used as an internal control. The PCR reaction consisted of 45 cycles (95°C for 10 s and 60°C for 40 s) after an initial denaturation step (95°C for 10 min).

***Statistical analysis*:** All data were analyzed using the Mac statistical analysis software package for Macintosh, version 2.0 (Esumi Co., Tokyo, Japan). All data are expressed as means ± standard errors (SEs). Statistical analysis was performed using the Student's t-test, and differences were considered significant when *p*-values were less than 0.05.

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