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Effects of probiotics supplementation and circuit training on immune responses among sedentary young males

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ABSTRACT

BACKGROUND: Growing evidence suggests that probiotics may have positive benefits on immune responses following endurance exercise. However, little attention has been given to its possible beneficial effects on immune responses following resistance exercise.
METHODS: Forty-one healthy sedentary males were recruited and randomised into four groups: sedentary control with placebo (C), probiotics (P), circuit training with placebo (Ex), and circuit training with probiotics (PEx) groups. Participants in the Ex and PEx groups performed a progressive load of circuit training at 3 times/week for 12 weeks. Each circuit comprised 10 exercises with work to rest ratio of 1:2. Participants consumed either multi-strain probiotics or placebo twice daily for 12 weeks. Body height and weight, blood pressure, resting heart rate, saliva and blood samples were collected at pre- and post-tests.
RESULTS: Saliva flow rate and salivary IgA, α-amylase, lactoferrin and lysozyme responses were not significantly different ($P>0.05$) between groups and also between pre- and post-test within each group. Similarly, total leukocytes, total lymphocytes, T lymphocytes, T-helper, T-cytotoxic, B lymphocytes, and natural killer cells counts were not significantly affected ($P>0.05$) by the probiotics and/or circuit training. However, circuit training significantly increased ($P<0.05$) immune cells count at post-test as compared to pre-test. Yet, a combination of circuit training and probiotics showed no significant ($P>0.05$) effects on immune cells count.
CONCLUSIONS: This study did not provide enough support for the positive effects of probiotics on immune responses among sedentary young males following resistance exercise. However, 12 weeks of circuit training enhanced immune cells count.

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Key words: Probiotics - Resistance training - Lymphocytes - Leukocytes

Probiotics, also known as "friendly bacteria," is a Greek word which means "for life." [1] It is defined as "*live microorganisms which when administrated in adequate amounts confer a health benefit on the host*." [2] These non-pathogenic microorganisms can be found in fermented food products such as yoghurt, tempeh, kefir, sauerkraut, cabbage kimchee, soybean-based miso, natto and probiotic drink. Documented health benefits of probiotics include treatment of travellers' diarrhea, relief of milk allergy in infants, reduction in the risk of atopic diseases, treatment of some inflammatory conditions, increased resistance to enteric pathogens, promotion of anti-tumor activity, and alleviation of some allergic and respiratory disorders in children.[3]

To date, growing evidence support that regular consumption of probiotics can modify the population of gut microbiota and subsequently influence immune responses of the host.[4, 5] Furthermore, it has been reported that probiotics consumption increase host resistance to upper respiratory tract infection (URTI) among the general population [6, 7] and also athletes.[8, 9] It has been proposed that probiotics exert its effects in the body by modulation of the intestinal immune system and displacing the potential pathogens by involving a competitive elimination or by producing the antimicrobial agents.[10]

It is well established that exercise-induced immunodepression depends on the intensity and duration of exercise.[11] While regular moderate-intensity exercise enhances immune responses above those having a sedentary lifestyle, prolonged exercise and periods of intensive training and competition may impair immune responses.[12] Nevertheless, despite numerous research carried out to investigate the effects of endurance exercise on immune responses, the effects of resistance exercise (for example, circuit training) on immune responses are limited to date. In addition, evidence on the combined effects of resistance exercise and probiotics supplementation on immune responses is also scarce. Therefore, the present study was proposed to investigate the combined effects of probiotics supplementation with circuit training on immune responses, *i.e.* salivary antimicrobial proteins and immune cells counts in young males following 12 weeks of intervention period.

Materials and methods

Research design

A randomized, placebo-controlled, double-blinded and parallel study design was employed for the present study. Measurements were conducted at pre and post 12-weeks intervention period.

Participants

Participation in this study was on a voluntary basis and the sampling method used was an opportunistic or convenient sampling. The inclusion criteria included healthy young males with a sedentary lifestyle, age between 19 to 26 years old, did not consume probiotics supplementation or any supplements that are known to affect immune responses prior to the study, did not engage in any physical training program and did not exercise more than once per week. On the other hand, the exclusion criteria included smoking/vaping, on medication and having chronic diseases.

Research procedures

The procedure for this study has been approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM), Kelantan (JEPeM Code: USM/JEPeM/15040132). The committee adopts research ethics guidelines outlined by the Helsinki Declaration agreed by the World Medical Association and Council for International Organizations of Medical Sciences (CIOMS).

After recruitment of participants, pre-test measurements which included measurements of body height and weight, blood pressure, and resting heart rate were carried out. In addition, 8 mL of blood and 5 mL of saliva samples were also collected. Then, participants were randomly divided into four groups: sedentary with placebo supplementation group (C), sedentary with probiotics supplementation group (P), circuit training with the placebo group (Ex), and circuit training combined with probiotics supplementation group (PEx).

During the intervention period, participants were given a form to record their weekly activity. They were also given a checklist to assess their adherence to the supplementation regimen. Following the 12 weeks of the intervention period, post-test measurements which were similar to the pre-test measurement were carried out.

Circuit training programme

The prescribed exercise regimen used in this study was designed by Chen [13] and has been used in several previous studies.[14-16] Participants in the Ex and PEx groups performed circuit training at 3 times/week for 12 weeks with 2 circuits of exercises from week 1-8 followed by 3 circuits of exercises from week 9-12. Each circuit comprised 10 stations (one type of exercise per station) with work to rest ratio of 1:2 (participants exercised for 30 seconds in one particular station, and rested for one minute before continuing with the next station). The resting period between circuits was 5 minutes.

The type of exercises prescribed in each circuit training program from station 1 to station 10 was heel raise with dumbbell, side lateral raise with elastic band, leg abduction with elastic band, shoulder extension and flexion using elastic band, rope skipping, triceps extension with dumbbell, half squat with elastic band, standing chest fly with dumbbell, leg curl with elastic band and biceps curl with dumbbell respectively. The weight of the dumbbells (range from 3.5-10 kg; depending on individual's capacity) and elasticity of the elastic band used were progressively decreased after 6 weeks of intervention.

The intensity of circuit training was estimated by referring to the post-exercise heart rates of the partici-

pants. It was measured by using a heart rate monitor (Polar watch, S710, USA) worn by each participant throughout each training session. Participants in the C and P groups were not involved in this circuit training programme where they were asked to continue their sedentary lifestyle.

Probiotics and placebo supplementation

Participants in the C and Ex groups consumed placebo while participants in the P and PEx groups consumed probiotics at a dosage of 2 sachets per day. All the supplements were prepared, packaged, and labelled by the supplier (B-Crobes) to ensure double blinding on the type of supplements given to the participants. The un-blinding was carried out after the data collection procedure was completed.

Probiotics used in this study was Hexbio© granule and was registered with the Malaysian Ministry of Health and was certified Halal. Each 3-g sachet probiotics has a concentration of $3x10^{10}$ colony forming unit (CFU) of *L. acidophilus*, *L. lactis*, *L. casei*, *B. longum*, *B. bifidum* and *B. infantis*. As for the placebo, it was identical in shape, taste and colour to the probiotics but contains no bacteria.

Saliva and blood samples collection and analysis

Saliva and blood samples were collected before and after the 12 weeks of intervention. The samples collection were carried out in the morning at 8.30 am after an overnight fast (drinking plain water was permitted). Saliva sample (2 mL) was obtained by 5 min un-stimulated dribbling into a pre-weighed tube. During saliva collection, participants sat on a chair, with the head leaned forward while letting the saliva passively dribble into the tube; without using their tongue or any mouth movement. Following that, the tube with saliva was weighed and recorded.

Saliva samples collected were analyzed by using a commercially available reagent kit to determine the concentration of salivary antimicrobial proteins (AMPs) which included salivary IgA, lysozyme, lactoferrin and α-amylase. The saliva flow rate and secretion rate of salivary AMPs were calculated by using the following formula:

Saliva volume (mL) = Difference in weight (g) of bijou tube after collection of saliva (assuming a saliva density of 1.0 g/mL)

$$\text{Saliva flow rate (mL/min)} = \text{Saliva volume (mL)} / \text{Collection time (min)}$$

$$\text{Salivary AMPs secretion rate (μg/min)} = \text{Saliva flow rate} \times \text{Salivary AMPs concentration}$$

As for blood sample (8 mL), it was collected and separated into two different test tubes: 2 mL into a K_3EDTA tube and 6 mL into a plain tube. Blood sample in the K_3EDTA tube was used for analysis of full blood count by using an automated hematology analyzer (Sysmex XS-800i, Norderstedt, Germany) whereas; blood sample in the plain tube was used for immunophenotyping analysis by using a flow cytometer (BD FACS Cantor II, USA).

Statistical analysis

Statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) version 24.0. Mixed-factorial analysis of variance (ANOVA) was performed to determine the significance of differences between and within groups. Differences were considered significant at $P<0.05$. Results are reported as mean±standard deviation (SD).

Results

The mean age and body composition of the participants are tabulated in Table I. Mean body weight, Body Mass Index (BMI) and body fat percentage of participants between the four groups were not significantly different at pre-test. Moreover, there were also no significant changes in these measured parameters after 12 weeks of intervention (post-test) in each group.

In general, the attendance of the participants during training sessions was good with the lowest percentage of training attendance of 80% in 2 participants. Similarly, participants' adherence to the supplementation regimen was also good, where participants missing probiotics or placebo consumption were not more than 13 sachets (8%) out of 168 sachets in total.

Table II shows the mean of saliva flow rate and concentration, and secretion rate of salivary IgA, lactoferrin, lysozyme and α-amylase. There were no significant differences between the four groups on the salivary AMPs measured in the present study. There were also no significant differences in the salivary AMPs responses between pre- and post-tests in each group.

The mean of immune cells count at pre- and post-tests

TABLE I.—*Participants' mean age and body composition.*

Variables		C (N.=10)	P (N.=10)	Ex (N.=12)	PEx (N.=9)
Age (years)		22±2.0	23±1.0	21±2.0	22±3.0
Body height (cm)		170.4±7.2	169.8±5.0	169.3±7.0	170.2±5.1
Body weight (kg)	Pre-test	61.4±10.4	63.0±10.3	60.6±9.9	64.1±10.2
	Post-test	61.8±10.5	62.4±11.5	60.9±10.0	63.7±11.4
Body mass index (kg/m^2)	Pre-test	21.1±2.8	21.8±3.4	21.1±2.7	22.1±3.4
	Post-test	21.3±2.9	21.5±3.8	21.2±2.8	21.9±3.9
Body fat percentage (%)	Pre-test	18.6±7.3	20.5±6.4	18.3±6.8	21.1±6.4
	Post-test	19.8±7.2	20.1±6.7	19.3±6.7	20.7±6.8

C:sedentary with placebo control group; P: probiotics group; Ex: exercise with placebo group; PEx: combined probiotics with exercise group.

TABLE II.—*Mean salivary AMPs concentration and secretion rate.*

Variables		Groups	Pre-test	Post-test	Percentage of differences (%)
Saliva flow rate (mL/min)		C	0.85±0.45	0.82±0.92	-3.53
		P	2.13±1.32	1.55±1.54	-27.23
		Ex	1.31±0.92	0.79±0.73	-39.69
		PEx	1.12±0.65	0.56±0.40	-50.00
Salivary IgA	Concentration (µg/mL)	C	68.91±39.26	68.92±37.36	+0.01
		P	64.47±54.04	59.41±32.31	-7.85
		Ex	80.36±46.66	77.34±54.07	-3.76
		PEx	75.20±17.81	69.30±23.32	-7.85
	Secretion rate (µg/min)	C	27.69±15.28	29.66±16.22	+7.11
		P	27.89±8.40	36.21±9.20	+29.83
		Ex	24.33±20.81	24.51±15.33	+0.74
		PEx	28.41±14.27	30.19±16.63	+6.27
Salivary α-amylase	Concentration (µg/mL)	C	120.58±59.56	122.73±50.29	+1.78
		P	103.02±46.18	132.12±77.64	+28.25
		Ex	122.48±68.28	102.86±70.16	-16.02
		PEx	135.29±60.63	100.81±37.35	-25.49
	Secretion rate (µg/min)	C	44.56±20.03	41.23±29.40	-7.47
		P	53.82±33.89	60.28±43.61	+12.00
		Ex	41.41±38.36	32.07±28.05	-22.55
		PEx	46.74±29.79	24.75±18.73	-47.05
Salivary lactoferrin	Concentration (µg/mL)	C	13.08±6.23	12.78±9.36	-2.29
		P	21.68±13.85	16.17±13.12	-25.42
		Ex	18.33±14.31	16.25±11.65	-11.35
		PEx	19.42±11.75	12.04±3.95	-38.00
	Secretion rate (µg/min)	C	5.37±2.91	4.71±5.06	-12.29
		P	9.90±7.51	7.07±6.74	-28.59
		Ex	5.83±3.67	3.11±1.96	-46.66
		PEx	6.15±4.66	3.03±1.52	-50.73
Salivary lysozyme	Concentration (µg/mL)	C	3.94±2.83	3.72±2.62	-5.58
		P	4.48±2.33	3.83±2.97	-14.51
		Ex	4.33±2.42	3.73±2.39	-13.86
		PEx	4.55±2.59	3.91±2.57	-14.07
	Secretion rate (µg/min)	C	0.85±0.45	0.82±0.92	-3.53
		P	2.13±1.32	1.55±1.54	-27.23
		Ex	1.31±0.92	0.79±0.73	-39.69
		PEx	1.12±0.65	0.56±0.40	-50.00

C: sedentary with placebo control group; P: probiotics group; Ex: exercise with placebo group; PEx: combined probiotics with exercise group.

in each group are presented in Table III. There were no significant differences in immune cells count between the four groups. However, at post-test, total lymphocytes, total T lymphocytes ($CD3^+$) and T-helper ($CD4^+$) count were significantly higher compared to pre-test in Ex group. On the other hand, T-cytotoxic ($CD8^+$) count was significantly higher at post-test compared to pre-test in the P, Ex and PEx groups.

Discussion

Body composition

In the present study, sedentary males with mean age of 22.0±2.0 years old were recruited. Overall, they were healthy with body mass index (BMI) within the normal range for Asian population; *i.e.* between 18.5 and 22.9 kg/m^2.[17] The 12 weeks of circuit training has no effect on body weight and fat loss among the participants. However, previous studies reported that probiotics have a potential to reduce body weight.[18, 19] For example, in one study, consumption of single strain probiotics containing *L. gasseri* appeared to reduce body weight.[20] In the present study, participants were given multi-strain probiotics (*L. acidophilus, L. lactis, L. casei, B. longum, B. bifidum and B. infantis*) with no *L. gasseri*. Thus, a different strain of bacteria and mixture of strains of bacteria may have caused different results. To our knowledge, there are still glaring gaps to clarify the mechanism of actions of probiotics in reducing body weight and fat.

Salivary AMPs

Overall, there were no significant differences between the four groups on the salivary AMPs measured in the present study: salivary IgA, lactoferrin, lysozyme and alpha-amylase. Similarly, within group effects also showed that the exercise and/or probiotics intervention for 12 weeks did not elicit any significant effects on salivary AMPs among the sedentary population. To date, reports on the effects of exercise, especially resistance exercise, on salivary AMPs are scarce. With regards to endurance exercise, previous researchers found that it could affect salivary AMPs. For example, it was reported that salivary lactoferrin and lysozyme concentrations were higher among rowers compared to control. In addition, it was found that salivary α-amylase [21] and lactoferrin and lysozyme responses [22] were significantly increased during exercise but then significantly declined at post-exercise. In contrast, another study found that 12 weeks of exercise training causes decreased in the

TABLE III.—*Mean immune cells count.*

Variables	Groups	Pre-test Mean±SD	Post-test Mean±SD	Percentage o f differences (%)
Total leukocytes count	C	5.13±0.87	5.32±0.69	+3.70
	P	5.97±1.10	6.38±1.49	+6.87
	Ex	6.26±0.88	6.61±1.47	+5.59
	PEx	4.63±1.56	5.29±1.10	+14.25
Total lymphocytes count	C	2.16±0.60	2.20±0.68	+1.85
	P	2.13±0.48	2.30±0.41	+7.98
	Ex	2.38±0.71	2.67±0.83*	+12.18
	PEx	2.01±0.74	2.26±0.82	+12.44
Total T lymphocytes ($CD3^+$) count	C	1.35±0.37	1.39±0.37	+2.96
	P	1.45±0.34	1.63±0.34	+12.41
	Ex	1.49±0.34	1.74±0.50*	+16.78
	PEx	1.18±0.42	1.26±0.51	+6.78
T-helper ($CD4^+$) count	C	0.62±0.14	0.63±0.19	+1.61
	P	0.67±0.18	0.76±0.13	+13.43
	Ex	0.70±0.17	0.84±0.24*	+20.00
	PEx	0.68±0.24	0.78±0.23	+14.71
T-cytotoxic ($CD8^+$) count	C	0.47±0.06	0.60±0.14	+27.66
	P	0.63±0.17	0.80±0.19*	+26.98
	Ex	0.70±0.20	1.05±0.35*	+50.00
	PEx	0.47±0.22	0.71±0.30*	+51.06
B lymphocytes ($CD19^+$) count	C	0.24±0.06	0.24±0.06	0.00
	P	0.28±0.04	0.29±0.11	+3.57
	Ex	0.29±0.09	0.33±0.10	+13.79
	PEx	0.27±0.13	0.28±0.12	+3.70
Natural Killer (NK) cells count	C	0.31±0.13	0.33±0.13	+6.45
	P	0.36±0.17	0.40±0.16	+11.11
	Ex	0.35±0.16	0.40±0.21	+14.29
	PEx	0.38±0.17	0.43±0.20	+13.16

C: sedentary with placebo control group; P: probiotics group; Ex: exercise with placebo group; PEx: combined probiotics with exercise group.
*Significantly different from pre-test ($P<0.05$).

α-amylase activity compared to the control groups.[23] However, similar to our findings, a few other studies reported no effects of exercise on salivary IgA,[22, 24] salivary flow rate and α-amylase activity.[25]

Reports on salivary AMPs responses to exercise are inconsistent to date due to several factors which include methods of collecting the saliva samples, differences in individual fitness, methods of expressing the results, saliva collection time, nutritional status of the individuals, psychological stress, age, gender and the exercise protocol employed.[26] However, its measurement is important because it is the first line of defence against infection [27] and its lower concentration has been associated with increased risk of getting an upper respiratory tract infection.[28] Generally, it was postulated that moderate exercise may provide better maintenance of immune responses compared to a sedentary lifestyle and high intense exercise training. The secretion and composition of saliva were related to the activity of the sympathetic and parasympathetic nervous systems where, physical activity causes stimulation of the autonomous nervous system, which may reduce the amount of saliva production.[29] It may induce the increment of the total protein content in the saliva which causes an increase in salivary secretion rate during exercise. Therefore, the result found may only reflect on the total protein content of the saliva sample, rather than the increment in IgA.[30, 31]

In the present study, probiotics supplementation alone and the combination of exercise and probiotics also showed no effects on salivary AMPs responses among the sedentary population. This was consistent with some previous studies [8, 32-34] but also inconsistent with a few other studies.[9, 32] These inconsistent findings may be attributed to the difference in the strains of bacteria used, dosage, population of participants and environmental condition.

Humans have numerous microorganisms that significantly affect metabolic functions, host nutrition, maturation of the immune system and gut development.[35] It appeared that the functions carried out by these species were similar in everybody's gastrointestinal tract. The gut microbiota promotes digestion and food absorption for host energy production, whereas, in the colon, complex carbohydrates are digested and subsequently fermented into short chain fatty acids. The gut microbiota also plays a fundamental role in the induction and function of the host immune system, protection from pathogens, and stimulation and maturation of epithelial cell.[36] This beneficial effect of probiotics administration may initiate in the gut as it provides an effective protection at distal mucosal sites.[37] Therefore, it is believed that regular consumption of probiotics resulted in enhanced regional immunity of the respiratory tract to promote the synthesis of AMP).[38]

Immune cells count

Similar with previous studies,[39, 40] this present study found that exercise significantly increased the cells count of total lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ after the 12 weeks of intervention. It is well known that intensive endurance exercise induces a biphasic perturbation of the circulating leukocytes count.[41] Immediately after exercise, the total leukocytes increase 50-100%. The lymphocyte count starts to decline to 30-60% below baseline levels within 30 minutes of recovery and remaining low for 3 to 6 hours. However, if the exercise is moderate, the lymphocyte count does not decline in the recovery period.[41] A similar pattern of leukocyte concentration changes can also be seen after strenuous strength exercise.[42] It was reported that resistance exercise induces leukocytosis due to an increase in circulating lymphocytes where exercise-induced leukocytosis and lymphocytosis was essentially signified by the increase in the number of total lymphocytes, monocytes, $CD3^+$, $CD8^+$ and $CD3^-CD16/CD56^+$ cells.[43] This leukocytosis can result from increased cell traffic (mobilization) from bone marrow to blood, demargination from the blood vessel walls (*e.g.* after intense physical exercise), and decreased exit to tissues.

The exercise training in the present study was conducted based on time rather than repetition counts. The weight that the participants lifted during the exercise was not based on 1-RM, but according to the participant's maximal ability to repeatedly lift the provided dumbbell within a specific time. Previously, it has been reported that even though the individuals performing the same exercise intensity of resistance exercise protocol, distinctive immune responses may arise from differences in absolute total work performance.[44]

In the present study, probiotics alone (P group) and the combination of probiotics and exercise (PEx group) showed increment trend in immune cells count after the 12 weeks of intervention. But, the increments were not statistically significant, except for the $CD8^+$. Other re-

searchers have postulated that different microbiota composition and structure might affect exercise performance by modifying the activity of the antioxidant enzymes.[45] Thus, the gut microbiota may promote increased enzyme's activity and thus reduce exercise-induced fatigue.

To our knowledge, studies on the combination effect of probiotics supplementation and exercise on immune responses among sedentary individual are scarce in the literature. In a previous study, there was a significant increment of T cytotoxic, T suppressor and T helper cells counts after a long-term consumption of probiotics during winter and spring periods (3 and 5 months) among 479 healthy adults.[7] Similarly, in elite athletes, the increased in $CD4^+/CD8^+$ ratio was observed after 14 weeks of probiotics supplementation.[46] In contrast, there were other studies which reported differently. For instance, one study reported no significant changes in total leukocyte in neutrophils, eosinophil, monocytes, $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD16^+/56^+$ after 3 weeks of probiotics consumption.[47] Similarly, Gleeson *et al*.[34] also reported no significant changes in blood leukocyte, neutrophil, monocyte, and lymphocyte-following 16 weeks of probiotics supplementation.

Overall, the combination of probiotics supplementation and exercises may reduce the duration of the immune suppression that resulted in faster recovery rate. In addition, the consumption of probiotics alone also potentially provides a regulation of immunomodulatory responses in the innate and adaptive immune response in the intestine through the luminal conversion process. The production of soluble factors and metabolites, for example short-chain fatty acids (SCFAs) and vitamins, from the diet affect the function of intestinal epithelium and mucosal immune cells, therefore, producing the cytokine and related factors such as a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) for better body protection.[48]

Conclusions

In summary, immune responses were not significantly different between control, probiotics alone, exercise alone, and the combination of probiotics and exercise groups. However, within-group effects (comparing between pre- and post-tests measurement in each group) showed that control group did not elicit any significant effects on all the parameters measured. However, exercise alone (Ex) had significantly increased most of the immune cells count measured, probiotics alone (P) significantly increased $CD8^+$ count, the combination of exercise and probiotics (PEx) also significantly increased the $CD8^+$ count. Nevertheless, no significant changes were observed on salivary antimicrobial proteins. Hence, it can be concluded that exercise alone (Ex), probiotics alone (P) and the combination of probiotics and exercise (PEx) are better than control (C) on enhancing immune functions.

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