Thirty days of double-strain probiotic supplementation increases monocyte phagocytosis in marathon runners

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Summary

Marathon runners, subjected to intense training regimes and prolonged, exhaustive exercises, often experience a compromised immune response. Probiotic supplementation has emerged as a potential remedy to mitigate the impact of prolonged exercise on athletes. Consequently, this study sought to assess the influence of probiotic supplementation on monocyte functionality both before and after the official marathon race. Twenty-seven runners were randomly and double-blindly assigned to two groups: Placebo-PLA (n=13) and Probiotic-PRO (n=14). Over thirty days, both groups received supplements—PLA sachets containing maltodextrin (5g/day) and PRO sachets containing 1 x 1010 CFU Lactobacillus acidophilus and 1 x 1010 CFU Bifidobacterium bifidum subsp. lactis. Blood samples were collected, and immunological assays, including phagocytosis, hydrogen peroxide production, cytokine levels, and monocyte immunophenotyping, were conducted at four different intervals: Baseline (start of supplementation/thirty days pre-marathon), 24h-Before (one-day pre-marathon), 1h-After (1h post-marathon), and 5d-After (five days post-marathon). Monocyte populations remained consistent throughout the study. A notable increase in phagocytosis was observed in the PRO group after thirty days of supplementation. Upon LPS stimulation, both PRO and PLA groups exhibited decreased IL-8 production. However, after the marathon race, IL-15 stimulation demonstrated increased levels of 5d-After, while IL-1-β, IL-8, IL-10, IL-15, and TNF-α varied across different intervals, specifically within the PRO group. Probiotic supplementation notably enhanced the phagocytic capacity of monocytes. However, these effects were not sustained post-marathon.

Keywords: Probiotic Supplementation, Monocytes, Phagocytosis, Cytokines, Strenuous Exercise and Marathon.

Introduction

Probiotics are selected live microorganisms that can be ingested as supplements in order to achieve an effect on health [(1)]. Probiotics are commercially available in capsules or tablets and powder sachets. Although some foods contain probiotics, they are not classified as probiotics as they lack studies on their health benefits as stipulated by the definition of probiotics [(2)]. Studies have shown that probiotics maintain the intestinal barrier, accelerate energy metabolism, prevent the adhesion of pathogens from hosting cells, improve neurological diseases related to oxidative stress and improve the production of vitamins, chain fats, short acids (SCFAs) and neurotransmitter molecules involved in gut communication with various physiological systems [(2)].

It is stated that the consumption of probiotics is beneficial to the body according to strain, time, and supplementation dose [(3)]. Probiotic supplementation produces effects on the gastrointestinal tract, such as improving barre function and on the immune system by modulating the innate and adaptive immune response [(4)], intensifying communication between both. For instance, a study carried out in humans demonstrated that the supplementation of *Bifidobacterium animals* subsp. lactis HN019 with 1 x 10^{11} CFU twice daily for six weeks increases innate cell number and functionality [(5)]. Monocytes and neutrophils showed greater phagocytic capacity after supplementation of *Lactobacillus acidophilus* 74-2 (9 x 10^{8} CFU) and *Bifidobacterium animalis* subsp lactis DGCC 420 (3 x 10^{6} CFU) for five weeks, without changing in the release of the large amounts of reactive oxygen species (oxidative burst) (Klein et al. 2008). Moreover, Roessler and collaborators demonstrated the supplementation with *Lactobacillus paracasei* Lpc-37, *Lactobacillus acidophilus* 74-2, and *Bifidobacterium animalis* subsp. lactis DGCC 420 for eight weeks increases the phagocytic activity; that is, it enhances the biological process by which phagocytes, such as monocytes and neutrophils, engulf and digest solid particles, such as bacteria, viruses, cellular debris or inanimate particles [(6)].

Monocytes are heterogeneous, highly malleable cells present in the bloodstream [(7)]. Depending on the environment in which they are inserted, they modify their phenotype and functionality. The main functions are related to phagocytosis and microbicidal actions [(8)]. Despite the evidence demonstrating the effectiveness of probiotic supplementation, the mechanisms involved in enhancing innate immune function still need to be well elucidated. It is believed that the benefits of probiotic supplementation occur through improved intestinal transit, re-establishment of the microbiota, preservation of intestinal epithelial integrity, and increased immune functionality [(3; 9)].

Acute exercise reduces IL-1β and TNF-α production when Lipopolysaccharide (LPS) challenges monocytes since the sensitivity of membrane receptors of the TLR-4 to LPS is lower after exercise. This result shows a possible effect of exercise in reducing the ability of monocytes to produce pro-inflammatory cytokines since LPS is a molecule that is part of the cell wall of Gram-negative bacteria and has a solid ability to stimulate monocytes to produce pro-inflammatory cytokines. Moderate and intense acute exercise can modify phagocytosis, antitumor activity, reactive oxygen and nitrogen species production, and chemotaxis [10]. On the other hand, the immunomodulatory effect of physical exercise is recognized [11; 12; 13]. Exercise performed at a moderate intensity of 50-75% of VO_2max and lasting up to 45 min is immunostimulatory, while prolonged strenuous exercise exceeding 90 minutes in length, such as marathon running, can be immunosuppressive [12; 14]. In this regard, dietary supplementation is used to reduce the stress generated by physical exercise on the body and improve the immune response of athletes [15].

In a recent publication by our research group, multi-strain probiotic supplementation, consisting of 1 billion CFU of each of *Lactobacillus acidophilus* LB-G80, *Lactobacillus paracasei* LPc-G110, *Lactococcus subp*. lactis LLL-G25, *Bifidobacterium animalis* subp. lactis BL-G101, and *Bifidobacterium bifidum* BB-G90 for thirty days attenuated the incidence of URTI symptoms and preserved cytokine production by monocytes [16]. However, little is known about the effects of supplementation with other strains and other critical cellular functions of monocytes, such as phagocytosis and hydrogen peroxide production.

The present study aimed to evaluate the effect of 30 days of probiotic supplementation on phagocytosis and H_2O_2 production in monocytes after a marathon race. The study hypothesizes that probiotic supplementation of 1 x 10^{10} CFU of Lactobacillus acidophilus and 1 x 10^{10} CFU of Bifidobacterium lactis can increase phagocytosis and H_2O_2 production and reduce the immunosuppressive effects of the marathon on these monocyte functions.

Methods

Ethical Protocols: This study was approved by the Research Ethics Committee of UNIFESP/Hospital São Paulo (REC #000.007 - 2017) and complied with the norms established by Brazilian legislation in resolution nº 466/12 of the National Health Council and is following the guidelines established in the Declaration of Helsinki adopted in 1964. All participants agreed with the study by signing the Free and Informed Consent Form.

Sample: An online screening (via e-mail) was performed to verify the inclusion and exclusion criteria of the study. The inclusion criteria were as follows: age between 25 - 45

years, history of at least one marathon race, and at least two years of training. The exclusion criteria were as follows: consumption of alcoholic beverages more than twice a week, smokers, diagnosis of cardiovascular, pulmonary, or metabolic disease, use of Probiotics or consumption of foods enriched with Probiotics, and use of drugs that could interfere with the results of the study, such as anti-inflammatories and antibiotics in the last six months. Finally, the sample consisted of twenty-seven male marathon runners divided into two distinct groups: placebo (PLA, n = 13) and probiotic (PRO, n=14). The anthropometric characterization of the runners was previously described [(17)]. Based on a study that assessed the alteration in IL-6 production by monocytes following probiotic supplementation (or placebo) in conjunction with a marathon, we conducted a sample calculation for the repeated measures ANOVA test (2 groups X 5 time points) using the G*Power software. The significance level (alpha) was set at <0.05, and the statistical power at >0.80. The effect size calculated for internal factors was 0.47, and for between groups, it was 0.62. The correlation between repeated measurements was found to be 0.25. Consequently, it was recommended that a total of 10 volunteers (5 in each group) would be adequate to detect pre- and post-supplementation differences. Additionally, 12 volunteers in total (6 in each group) were suggested to identify distinctions between the groups. Considering the experimental design's nature and the potential for volunteer discrepancies, we initiated the study with a larger sample size, concluding with 13 volunteers in the placebo group and 14 in the probiotic group. The sample size calculation drew inspiration from the study conducted by Tavares-Silva et al [(16)].

Experimental Design: This is a randomized, controlled, and double-blind study (Figure 1). As shown in Figure 2, athletes were first attended to the laboratory for registration, which included a body composition analysis (BOD POD® body composition system; Life Measurement Instruments, Concord, CA, USA). Whether eligible for the study, athletes were allocated to their respective groups. Then, at Baseline, it was collected a peripheral blood sample and provided supplements (probiotics or placebo). After thirty days of supplementation, athletes returned to the laboratory, and a new blood sample was collected 24 hours before the competition (24h-Before). The supplementation was stopped at this time. On the day of the official marathon (42.195m), a blood sample was collected one hour after the end of the race (1h-After). Another blood sample was collected five days after the marathon race (5d-After).

Supplementation: The study was double-blind concerning supplementation. A researcher outside the project offered the placebo or probiotic to the participants and monitored their

intake. The researchers became aware of the division of groups and supplementation only after the statistical analysis had been carried out. Supplementation was ingested daily in sachets. All participants were informed to consume one sachet per day with water for thirty days at night before sleeping. The PRO group received 30 sachets containing 1 x 10^{10} CFU *Lactobacillus acidophilus* and 1 x 10^{10} CFU *Bifidobacterium bifidum* subsp. lactis + 5 g of maltodextrin. The PLA group received 30 sachets containing 5 g of maltodextrin, with a similar colour, smell and taste of probiotic supplementation. The amount of maltodextrin consumed by the placebo group does not affect the variables studied. PRO and PLA supplements were manipulated in a pharmacy (Drogaderma – São Paulo – BR). The probiotics were approved and certified by reports before the supplementation period, confirming the validity and identification of the species. The certification is produced by Lemma Supply Solutions Ltda, Jardinópolis, São Paulo/BR.

Exercise Protocol: Data was collected in three Official Marathon races - São Paulo International Marathon (42.195 m) in 2017 and 2018. The environment temperature before the race was 18.33 ± 3.05 Celsius degree, with a relative humidity of 72.66 ± 15.94, and it started between 6 and 7 a.m. The athletes were instructed to complete the route in the shortest possible time (Time Trial).

Blood Collection: To measure the biochemical parameters, 30 ml of blood was collected at each moment following a 4-hour fasting period, except on the day of the marathon when the athletes were instructed to eat as they usually do before long runs. After collection, 10 ml of whole blood was used for cellular function assays. The remaining 20 ml of blood was centrifuged at 400 x g for 15 minutes at 4ºC. Then, serum were stored at –80ºC for further analysis.

Monocyte isolation: Peripheral blood mononuclear cells (PBMC) were isolated from fresh EDTA blood samples by centrifugation (400 g, 25ºC, 30 min) with 3 ml of Histopack 1077 and 3 ml of Histopack 1119. Washed two times with PBS (400 x g at 4ºC for 10 minutes) and counted. 500ul of diluted cells were plated per well with 2 ml of RPMI-1640 culture medium enriched with 2 mM glutamine, 1 ml of the volunteer's serum, and 650 µl of penicillin in a six-well culture plate. After 1h of incubation at 37°C, the supernatant containing lymphocytes was collected, leaving the attached monocytes in the plate. 1.8 ml of enriched RPMI-1640 medium and 0.2 ml of LPS (5 µg/ml) were added to each well, and the plate was incubated for 24 hours at 37ºC and 5.0% CO_2. One billion cells were plated per well. All supernatants were aliquoted into 2 ml tubes and frozen in a -80°C freezer until analysis. Brand reagents Sigma-Aldrich (Merck group), San Luis, MI, USA.

Cytokine dosage: The concentration of the cytokines IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-15 and TNF-α in cell supernatant and serum were evaluated by the Multiplex assay with Millipore kits. Darmstadt, Germany. Simultaneous analysis of multiple cytokines is performed using Luminex technology with magnetic Beads in 96-well plates. The test steps contained in the manufacturer's manual were respected. The Luminex 200™ Analyzer was used with the Magpix® system using Milliplex® Analyst 5.1 Software.

Hydrogen Peroxide (H_2O_2) Production: Hydrogen peroxide production was measured using the modified method described by Pick and Mizel [(18)]. Incubations were carried out in previously siliconized glass flasks (1% silicone in acetone, v/v) and subsequently washed with distilled water. Monocytes were incubated at 37ºC with shaking for a final volume equal to $1x10^6$ cells in 1 ml of incubation medium. The incubation medium was composed (for final volume) of PBS with calcium, 2% BSA (v/v), 5 Mm glucose, 2 mM glutamine, 0.56 Mm phenol red, 10 µl PMA (100 µg/ml PBS), and 100 µl Peroxidase (1 mg/2 ml PBS). The formation of hydrogen peroxide (H2O2) was interrupted after 1 hour of incubation with the addition of 20 µl of 1N NaOH. After 15 minutes, the total amount produced was quantified in a spectrophotometer at 620nm.

Phagocytosis: To assess phagocytic capacity, The Vybrant® Phagocytosis Assay Kit – Molecular Probe's – ThermoFisher - USA was used. The Vybrant® Phagocytosis Assay Kit allows researchers to observe and quantify the process of phagocytosis following the internalization of an antigen–dead E. coli (strain K-12) cell that has been labelled with fluorescent dye. In addition to the lyophilized E. coli BioParticles® component, the kit contains a trypan blue solution (to quench the fluorescence of particles that have not been internalized). The instructions contained in the kit were followed, as well as the step-by-step instructions for carrying out the phagocytosis assay on a fluorescence microplate reader.

Immunophenotyping: 100 µl of whole blood was separated in a test tube, and 2 ml of RBC Lysis Solution, obtained from the company Quiagem®, USA, was added. The tube was kept at 37°C for 10 minutes and centrifuged at 400 x g for 10 minutes. The cell washing process was carried out twice with PBS (with centrifugation between washes). After the last centrifugation, whole blood was stained with the following monoclonal antibodies: 1 µl of CD14(BL-2) and 1.8 µl of CD16 (BL-3). The tubes were incubated in the absence of light for 30 min, washed with PBS, centrifuged to 400 x g for 20 min, and the final pellet was resuspended with PBS 2% BSA. Samples were acquired in an Attune NxT flow cytometer (Thermo Fisher Scientific®, USA). Data were analyzed using FlowJo software version V10 (Treestar Inc, Ashland, USA).

Statistical Analysis: Normality was verified using the Shapiro-Wilk`s test, and normally distributed data are expressed as mean ± standard deviation. Homogeneity was analyzed with the Levene test, and the sphericity of the data was verified using the Mauchly test and Greenhouse-Geiser correction. Analysis of repeated measures by the General Linear Model (GLM) was used according to the design, followed by Tukey's posthoc. The significance level adopted was less than 5%. Statistical analyses were performed using IBM SPSS Statistics 20 software.

Results

Both PLA and PRO showed a significant increase in total leukocytes and circulating monocytes 1h-after the race when compared to their Baseline ($p<0.01$) and 24h-before ($p<0.01$), and a significant decrease when compared to 5d-after ($p<0.01$). No difference was found between the groups (Figure 3). Immunophenotyping results demonstrated homogeneity between groups in the total number of classic ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and non-classical ($CD14^{+}CD16^{++}$) monocytes (Figure 4).

Regarding cell function, the PRO group showed an increase in phagocytosis 24h before compared to Baseline ($p<0.01$), followed by a decrease 1h after compared to 24h before ($p=0.03$). At the 5d-after, there was a significant increase compared to the 1h-after ($p<0.01$). In the PLA group, only an increase was observed in 5d after compared to 1 h after ($p=0.03$) (Figure 5A). The hydrogen peroxide concentration did not differ significantly at any evaluated point-time (Figure 5B).

In both the PRO and PLA groups, IL-8 production by monocytes decreased 5d-after compared to their respective 24h-before ($p<0.001$) and 1h-after ($p<0.001$). In the PRO group, IL-15 production increased 5d-after compared to Baseline ($p<0.001$), 24h-before ($p<0.01$), and 1h-after ($p<0.01$), while the PLA group showed a significant increase in 5d -after compared to Baseline ($p<0.001$). Besides, only the PLA group showed an increase in the production of TNF-α 5d-after compared to the Baseline ($p=0.02$) (Table 2).

The serum concentration of IL-1β in the PRO group differed in 24-before ($p<0.01$), 1h-after ($p<0.01$), and 5d-after ($p<0.001$) compared to Baseline. Whereas the PLA group showed a decrease at 1h-after ($p<0.001$) and 5d-after ($p<0.001$) compared to Baseline and 5d-after compared to 1h-after ($p=0.03$). IL-6 levels were lower 5d-after compared to Baseline in both PRO ($p=0.04$) and PLA groups ($p=0.03$). A significant reduction in IL-8 concentrations was observed 5d-after in the PRO group compared to Baseline ($p<0.001$), 24h-before ($p<0.01$), and 1h-after ($p<0.04$). PLA group showed a decrease in 5d-after compared to

Baseline ($p<0.001$) and 24h-before ($p<0.01$). The anti-inflammatory cytokine, IL-10, from samples of the PRO group, showed an increase 1h-after compared to Baseline ($p<0.01$) and 24h-before ($p<0.01$) and reduced 5d-after compared to Baseline ($p<0.001$), 24h-before ($p<0.02$) and 1h-after ($p<0.01$). In the PLA group, this cytokine increased 1h-after ($p<0.02$) and reduced 5d-after compared to 1h-after ($p<0.02$) and Baseline ($p<0.001$). Finally, both IL-15 and TNF-α decreased at 24h-before ($p<0.01$), 1h-after ($p<0.001$), and 5d-after ($p<0.001$) times compared to Baseline on both groups (Table 3).

Discussion

The main finding of the study was that 30 days of probiotic supplementation increased phagocytosis in monocytes compared to the placebo group. The increase in phagocytic capacity represents an improvement in the essential function of monocytes. It may shed light on the possibility of endurance athletes using probiotics to mitigate the immunosuppressive effects of strenuous exercise. Probiotics have been widely studied as dietary supplements capable of improving immune functions in endurance athletes, mainly runners [19; 20].

The phagocytosis results agree with previous research carried out in humans without exercise. Schiffrin et al. [21] demonstrated that consumption of *Bifidobacterium bifidum* Bb 12 1 x 10^{10} or *Lactobacillus acidophilus* LA1 7 x 10^{10} CFU for three weeks in fermented milk three times a day (120mL dose) increases monocyte phagocytosis. This same result was found after daily supplementation with 300 g of yoghurt enriched with *Lactobacillus acidophilus* 74-2, 9.3x10^{8} CFU and *Bifidobacterium a*nimalis subsp lactis DGCC 420, 3 x 10^{6} CFU, for five weeks [22] and *Lactobacillus supplementation paracasei* Lpc-37 3.9 x 10^{8} CFU, *Lactobacillus acidophilus* 74-2 2.9 x 10^{4} and *Bifidobacterium animalis* subsp lactis DGCC 420 5.9 x 10^{4} for eight weeks [6].

The increase in the phagocytic rate in the supplemented group was not accompanied by an increase in hydrogen peroxide (H_2O_2) and cytokines production. H_2O_2 comes from cellular metabolism by oxidizing organic substrates [23]. During infectious conditions and in the presence of inflammatory cytokines, there is an increase in the combination of H^+ ions with O_2 in immune cells, exhibiting antimicrobial and antitumor functions [24; 25; 26]. Our results corroborate the study by Klein et al. [22], in which they did not observe an increase in the cellular oxidative burst of monocytes after daily supplementation with *Lactobacillus acidophilus* 74-2, 9.3 x 10^{8} CFU and *Bifidobacterium animalis* subsp lactis DGCC 420, 3 x 10^{6} UFC for five weeks. However, Roessler et al. [6] observed an increase in the oxidative burst after eight weeks with probiotic supplementation of *Lactobacillus paracasei* Lpc-37 3.9

x 10^8 CFU, *Lactobacillus acidophilus* 74-2 2.9 x 10^4 and *Bifidobacterium animalis* subsp lactis DGCC 420 5.9 x 10^4 CFU. Furthermore, we did not observe significant changes in cytokine concentrations that could stimulate an increase in H_2O_2 production. Thus, the time of supplementation and dose of probiotics should be considered [3], suggesting in future studies the inclusion of *Lactobacillus paracasei*, as in the Rossler study, in the probiotic supply to assess the oxidative response of monocytes.

In order to verify the cellular functionality, during the monocyte incubation, we used the volunteers' serum to reproduce the cellular medium. This protocol has the characteristic of mimicking *in vitro* what may be happening *in vivo*, demonstrating the mechanisms that may be involved in the benefits of probiotics supplementation by maintaining the concentrations of energy substrates, hormones, metabolites, and cytokines under the same conditions as they were *in vivo* at the time of collection [27]. For this reason, our results should be compared with endurance exercise studies. Races of shorter duration and different intensities, such as 5 or 10 km, represent different physiological stress than marathon running and, therefore, different immunometabolic responses to supplementation and exercise. Interestingly, our results showed that the significant differences in the concentrations of IL-8 and IL-15 produced by monocytes were accompanied by changes in the concentrations of plasmatic cytokines after the marathon race.

Cytokine production by monocytes after LPS stimulation is closely related to pattern recognition receptors (PRR), mainly Toll-like 4 receptor (TLR-4) TLR-4 and activation of the NF-κB pathway [28]. It is acknowledged that physical exercise promotes a decrease in the sensitivity of cell membrane receptors of the TLR-4 to LPS, both in short-term and high-intensity exercise [29] and in prolonged exercise [30]. In the study by Oliveira and Gleeson [30], the expression of TLR-4 by monocytes was evaluated before and after an acute amount of prolonged physical exercise. Despite an increase in the number of circulating monocytes, there was a decrease in the expression of TLR-4 receptors. As in acute exercises, training also promotes a decrease in the expression of PRR and the production of inflammatory cytokines [31].

In both groups, lower plasma concentrations of immunostimulatory cytokines were observed throughout the experiment (IL-1β, IL-15, and TNF-α) and a specific increase in IL-10 one hour after the marathon compared to Baseline. Plasma alterations were accompanied by a reduction in the production of IL-8, an increase in IL-15 five days after the marathon race, and a reduction in the phagocytic rate in the probiotic group after the race. The

imbalance in the concentrations of pro- and anti-inflammatory cytokines can affect cellular functionality [32; 33; 34].

The mechanism of action of monocyte activation involves both LPS and cytokine receptors [28; 35]. In the presence of inflammatory cytokines or LPS, monocytes are activated, triggering a cascade response via JAK-STAT or NF-κB to carry out gene transcription and initiate the immune response [36]. Each cytokine activates a specific JAK-STAT associated with the respective receptor, such as IL-2 (JAK3-STAT5), IL-4 (JAK3-STAT6), IL-10 activating (JAK1-STAT3) [37]. Even in the presence of LPS, if there is an increase in the concentration of IL-10, there is activation of JAK1-STAT3, inhibiting the cellular response via NF-κB [37].

It turns out that after binding of IL-10 to the receptor and activation of JAK1 and phosphorylation of STAT3, the NF-κB pathway is blocked by suppression of the IKK complex, thus impairing the immune response[38; 39]. Furthermore, five days after the marathon race, we observed a significant decrease in plasma concentrations of IL-10 and a consequent increase in the production of inflammatory cytokines and the phagocytic capacity of monocytes. However, the mechanisms involved in the monocyte response after probiotic supplementation are still unclear.

The literature describes some non-exclusive hypotheses, which include the interaction of bacteria into Peyers' patches and GALT, stimulating cell proliferation and maturation [22]. Probiotics are also associated with increased sensitivity of PRR and antigen presentation by dendritic cells, resulting in the maturation and proliferation of lymphocytes through the production of cytokines such as IFN-γ, IL-2, and IL-12 that induce a cellular immune response [40], in addition to increased production of serotonin by enterochromaffin (EC) cells [41; 42]. Moreover, it is known that monocytes exhibit a high ability to stimulate cytokine production and increase phagocytic and oxidative capacity on exposure to serotonin [43; 44; 45; 46]. Therefore, probiotics act through different mechanisms, modulating the intestinal microbiota with the production of vitamins, increasing the bioavailability of nutrients, and assisting in the homeostatic development of the resident microbial environment by stimulating immune functions [47].

Strenuous exercises such as marathons are potential stressors, causing changes in the concentrations of hormones such as cortisol and catecholamines, glucose, amino acids and fatty acids that are important for the metabolism of monocytes and other cells of the immune system. On the other hand, several nutritional actions may have immunonutrition actions in exercise [3; 4]. The relationship between microbiota, probiotics and the immune system during

exercise still needs to be explored. Our results show that 30 days of probiotic supplementation had effects on phagocytosis. As for the production of cytokines by monocytes and cytokines in the bloodstream, the effects of exercise overlap with those of probiotics. Interestingly, the influence of exercise and supplementation was not able to change H_2O_2 production by monocytes. The regulation of immune system cells and inflammatory mediators occurs through several mechanisms, which are only sometimes detected in studies.

This study was carried out with young adults aged between 25 and 45 years old, as in this age group, there are few physiological differences due to age and because most of the population runs marathons and does strenuous exercise. Therefore, the results cannot be extrapolated to other age groups. In addition, this study was carried out only with men to avoid the influence of female and male sex hormones, as it was not possible to adequately control the phases of the menstrual cycle and the concentration of estrogen and progesterone at the time of collection. The results should be extrapolated to women with great caution.

In conclusion, the probiotic supplementation of *Lactobacillus acidophilus* 1 x 10^{10} CFU and *Bifidobacterium animalis* subsp lactis 1 x 10^{10} CFU for 30 days prior to a marathon race was effective in increasing monocyte phagocytosis in marathon runners. However, changes in the concentration of plasma cytokines, including an increase in IL-10, impacted the cellular response. These outcomes reinforce the importance of investigating probiotics supplementation and probiotic food in the immunologic field.

Further studies with endurance athletes, including other immune cells and mechanisms not evaluated here, like plasma serotonin concentration, the sensitivity of RRP, maturation rate and cell proliferation, as well as adjusting for time, dose, and composition of probiotics, are needed. Studies in this context with female runners should also be carried out in the future.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Authorship

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List of Abbreviations

Bovine Serum Albumin (BSA)

Classic Monocytes ($CD14^{++}CD16^{-}$)

Cluster of Differentiation (CD)

Colony Forming Units (CFU)

Enterochromaffin (EC)

G force (G)

Galactose-1-phosphate uridyltransferase (GALT)

General Linear Model (GLM)

Hydrogen Peroxide (H_2O_2)

IkappaB kinases (IKK)

Interleukin-1 (IL-1)

Interleukin-10 (IL-10)

Interleukin-15 (IL-15)

Interleukin-2 (IL-2)

Interleukin-4 (IL-4)

Interleukin-6 (IL-6)

Interleukin-8 (IL-8)

Intermediate Monocytes ($CD14^{++}CD16^{+}$)

Janus Kinase 1 (JAK1)

Janus Kinase 3 (JAK3)

Lipopolysaccharide (LPS)

Maximal Oxygen Consumption ($VO2_{max}$)

Milliliters (ml)

Non-classical Monocytes ($CD14^{+}CD16^{++}$)

Nuclear Factor NF-kappaB

Pattern Recognition Receptors (PRR)

Peripheral Blood Mononuclear Cells (PBMC)

Phorbol Myristate Acetate (PMA)

Phosphate-Buffered Saline (PBS)

Placebo (PLA)

Probiotic (PRO)

Signal Transducer and Activator of Transcription 5 (STAT5)

Signal Transducer and Activator of Transcription 6 (STAT6)

Sodium Hydroxide (NaOH)

Toll Like 4 Receptor (TLR-4)

Tumor Necrosis Factor -α (TNF-α)

Upper Respiratory Tract Infection (URTI)

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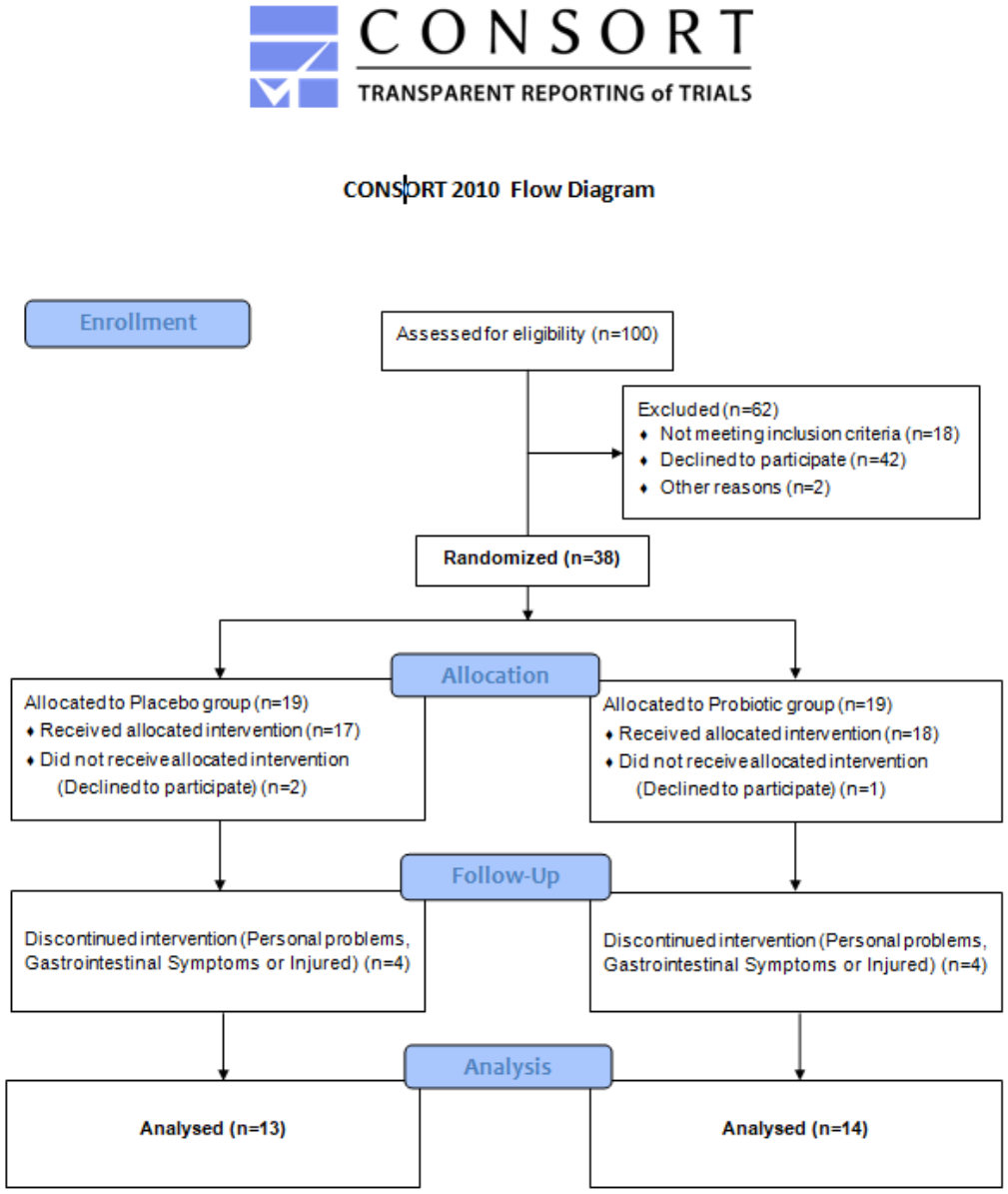

Figure 1. CONSORT 2010 Flow Diagram

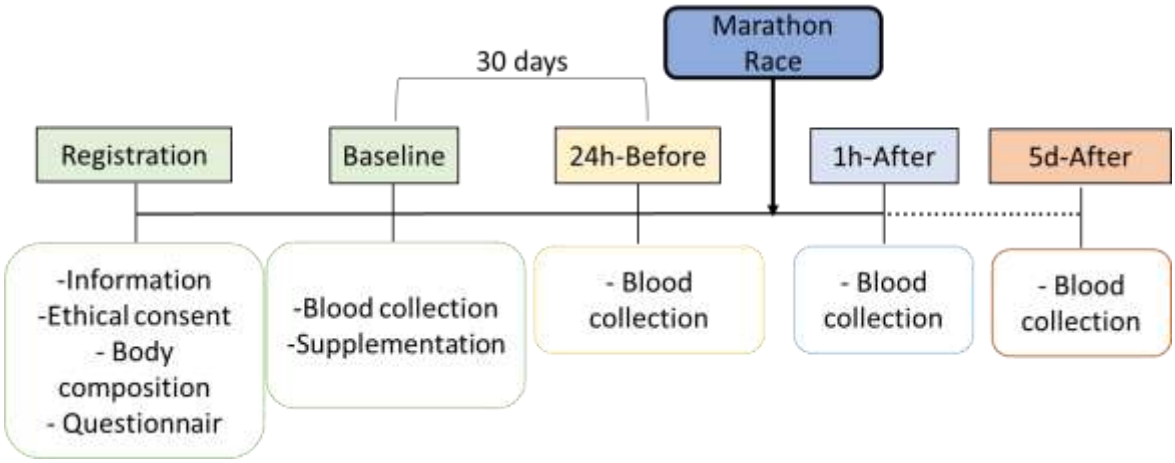

Figure 2. Experimental Design.

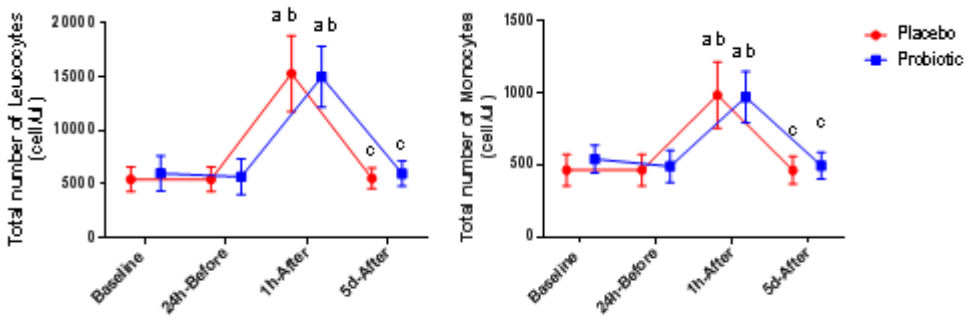

Figure 3. Total number of Leucocytes and Monocytes. Data presented in mean ± standard deviation. Placebo (n=13) and Probiotic (n=14). The comparison between groups and moments was performed through the general Linear Model (GLM) with Post-hoc Tukey. 'a' differs from the baseline condition of the same group. 'b' differs from the 24h-before of the same group. 'c' different from 1h-after of the same group.

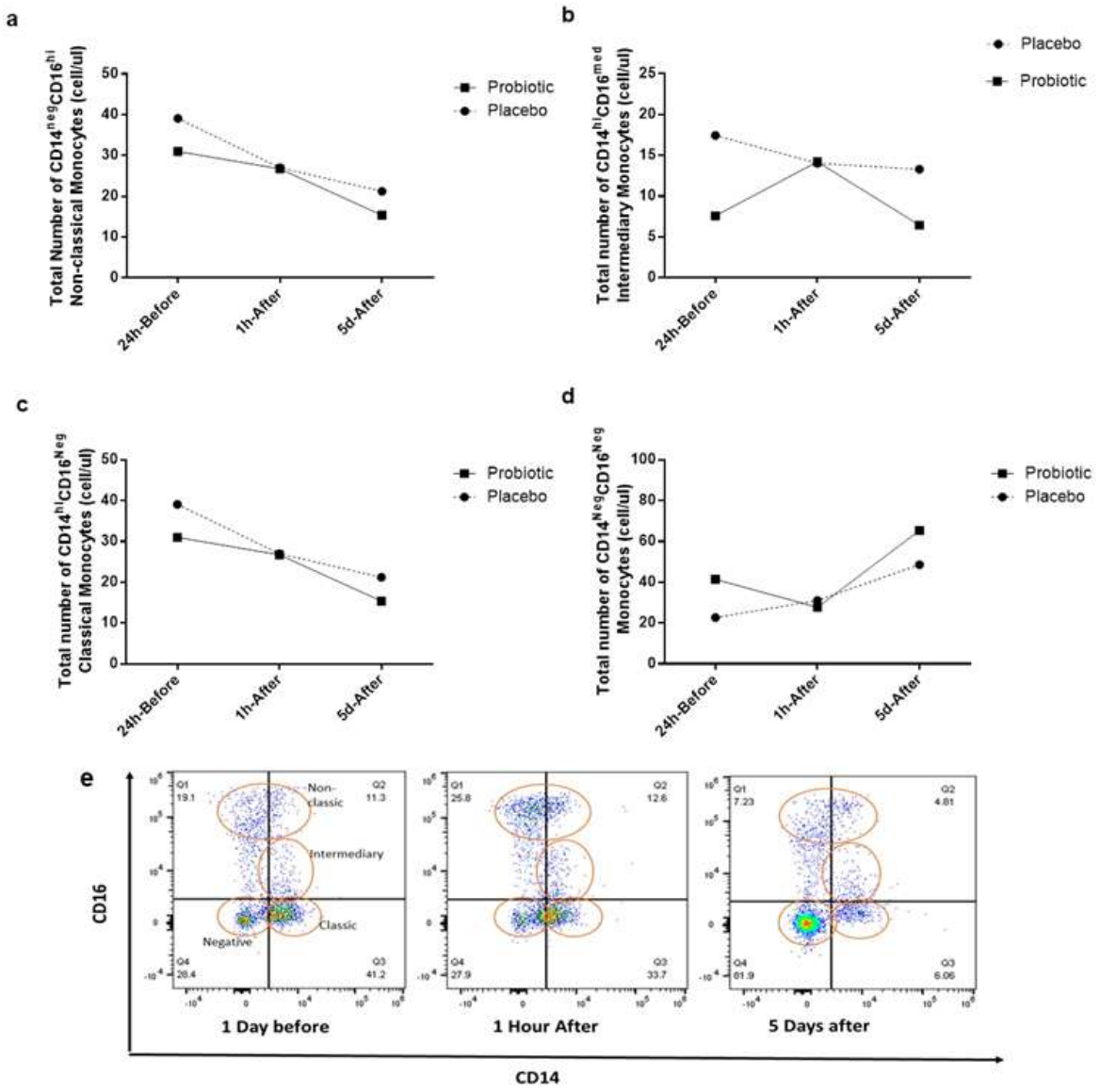

Figure 4. Immunophenotyping. Data presented as mean ± standard deviation. Significance $p<0.05$. Absolute amount of non-classical monocytes CD14pos CD16pos graph A, intermediate CD14pos CD14med graph B, classic CD14pos CD16neg graph C and double negative CD14pos CD16neg graph D of both groups; in the Pre-Test, Post-Test and Recovery moments. Graph E refers to the representation of the monocyte population from flow cytometry. Positive Y axis CD16 and positive X axis CD14. Total of 8 participants (Placebo 4 and Probiotic 4).

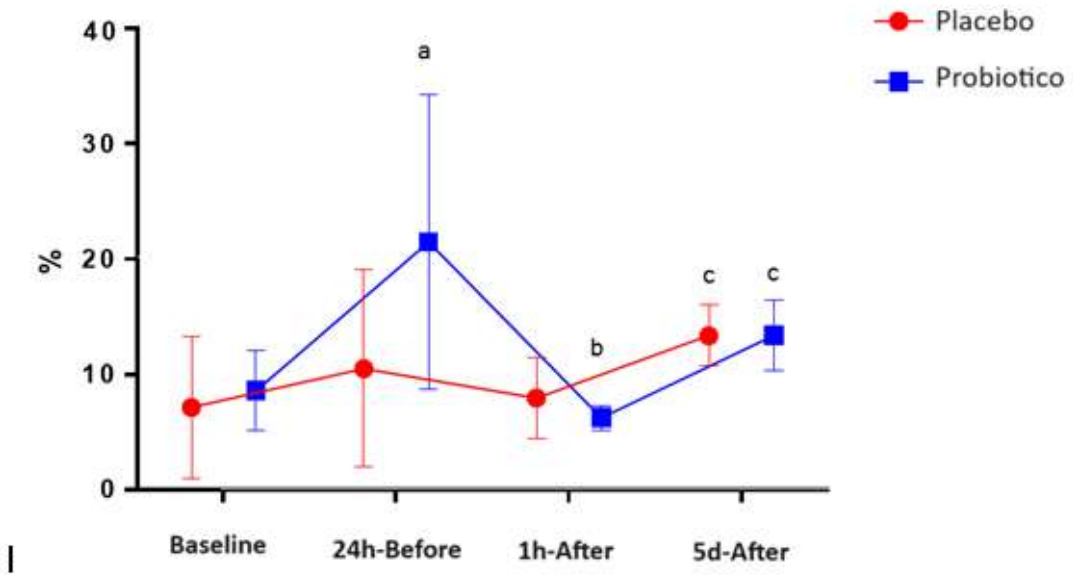

Figure 5b

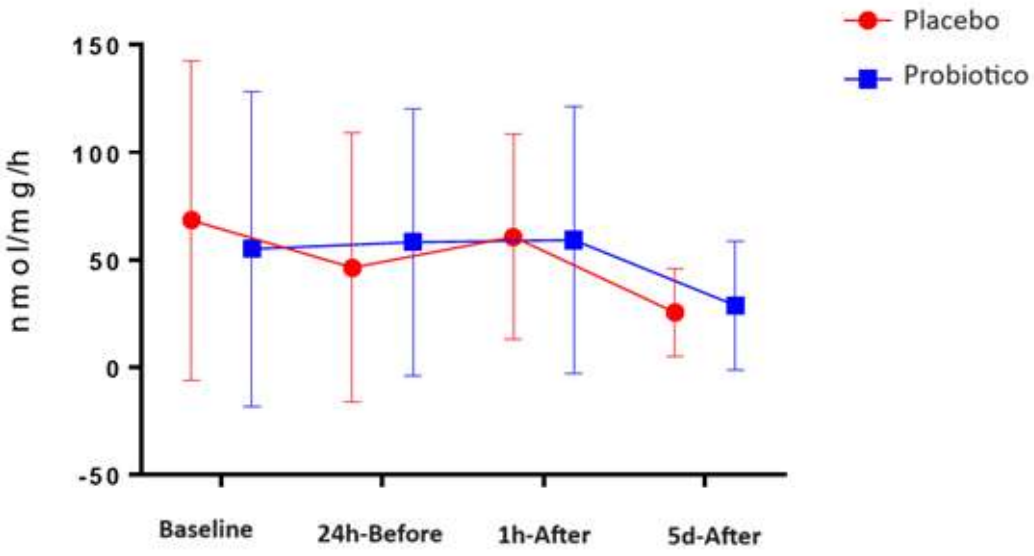

Figure 5. Phagocytosis expressed in % (Figure 5a) and H2O2 production expressed in nmol/mg/h (Figure 5b). Data presented in mean ± standard deviation. Placebo (n=13) and Probiotic (n=14). The comparison between groups and moments was performed through the general Linear Model (GLM) with Post-hoc Tukey. 'a' differs from the baseline condition of the same group. 'b' differs from the 24h-before of the same group. 'c' different from 1h-after of the same group.

Table 2. Cytokine production by monocytes.

Cytokines	PLACEBO-PLA				PROBIOTIC-PRO			
	Baseline	**24h-Before**	**1h-After**	**5d-After**	**Baseline**	**24h-Before**	**1h-After**	**5d-After**
IL-1β	16.05 ± 6.34	21.71 ± 10.86	22.97 ± 12.46	25.13 ± 9.50	27.97 ± 23.49	31.55 ± 23.60	31.12± 25.87	18.59 ± 5.06
IL-6	38.02 ± 28.84	59.71 ± 33.24	82.07 ± 31.15	56.40 ± 21.98	47.88 ± 33.92	59.81 ± 34.55	77.91± 32.82	50.14 ± 21.48
L-8	115.13± 58.05	156.84 ± 40.58*	172.52 ± 40.75*	92.12 ± 33.78*	143.54± 73.26	163.01 ± 42.40*	174.48 ± 41.19*	84.52 ± 35.04*
IL-10	7.97 ± 7.06	19.05 ± 15.88	18.70 ± 9.09	11.41 ± 3.65	11.58 ± 17.03	15.85 ± 12.03	25.35 ± 18.21	16.80 ± 12.09
IL-15	1.76 ± 3.18*	4.77 ± 2.49	4.94 ± 2.58	9.29 ± 3.94*	1.66 ± 2.94*	3.13 ± 2.66*	3.30 ± 2.05*	10.32 ± 3.17*
TNF-α	26.99 ± 20.04	76.21 ± 75.42	71.75 ± 81.12	93.93 ± 58.18*	49.65 ± 50.23	78.93 ± 68.99	80.07 ± 82.69	73.55 ± 54.65

Placebo-PLA (n=13) and Probiotic-PRO (n=14) group. Values presented as mean ± standard deviation. Production of IL-1β, IL-6, IL-8, IL-10 and TNF-α were divided by 100. *difference between time points.

Table 3. Serum cytokines

Cytokines	PLACEBO-PLA				PROBIOTIC-PRO			
	Baseline	**24h-Before**	**1h-After**	**5d-After**	**Baseline**	**24h-Before**	**1h-After**	**5d-After**
IL-1β	9.22 ± 3.30*	6.20 ± 2.22	4.47 ± 1.83*	3.47 ± 2.08*	8.01 ± 2.51*	5.29 ± 2.07*	4.72 ± 2.27	3.40 ± 1.13*
IL-6	9.75 ± 13.54*	3.64 ± 4.20	5.02 ± 7.44	2.25 ± 5.44*	6.07 ± 4.80*	2.71 ± 2.73	3.80 ± 7.09	1.17 ± 3.72*
L-8	9.15 ± 5.53*	5.44 ± 3.55*	5.41 ± 3.32	2.13 ± 2.61*	7.95 ± 3.33*	4.99 ± 3.07*	7.03 ± 8.42*	1.67 ± 2.08*
IL-10	20.34 ± 6.98*	9.06 ± 8.67	154.15 ± 184.43*	1.53 ± 1.38*	20.11 ± 3.79*	11.76 ± 10.63*	261.39 ± 225.75*	1.94 ± 1.26*
IL-15	11.39 ± 2.46*	4.84 ± 4.11*	1.64 ± 0.65*	2.20 ± 1.77*	10.97 ± 1.72*	5.29 ± 5.03*	1.81 ± 1.06*	1.68 ± 0.62*
TNF-α	27.81 ± 5.37*	14.60 ± 5.56*	13.72 ± 3.67*	11.28 ± 7.06*	25.85 ± 4.65*	15.34 ± 8.70*	14.52 ± 5.01*	12.05 ± 4.51*

Placebo-PLA (n=13) and Probiotic-PRO (n=14) group. Values presented as mean ± standard deviation. *difference between moments.