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Inactivated Probiotic *Bacillus coagulans* GBI-30 Demonstrates Immunosupportive Properties in Healthy Adults Following Stressful Exercise

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Abstract

Objective: Probiotics have been associated with many health benefits and have an established safety record. The purpose of this pilot study was to determine the safety and efficacy of inactivated *Bacillus coagulans* GBI-30, 6086 (Staimune®) in supporting the immune system at rest, in response to an *in vitro* bacterial challenge, and in response to strenuous exercise.

Methods: In a randomized double-blind placebo-controlled pilot study, 16 healthy adults received either Staimune® or Placebo over 28-d. Basal immune system was assessed (salivary IgA, complete blood count with differential, helper T-cell ($CD3^+/CD4^+$), cytotoxic T-cells ($CD3^+/CD8^+$), and natural killer cells ($CD3-/56^+$ or $CD3-/16^+/56^+$) percentages/proportions. In addition, cytokine (IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IFN gamma, GM-CSF, and TNF-a) in response to an *in vitro* lipopolysaccharide challenge as well examining basal serum cortisol values. Subjects performed 60-min treadmill running at 60-80% of their Karvonen predicted max heart rate. Blood and salivary samples were also collected at 10, 60, and 120-min post-exercise. Data analysis was based upon changes over time relative to baseline for within group changes as well as between-group differences ($P<0.05$), 95% CI.

Results: Both conditions supported mucosal immunity. Ten minutes after exercise WBC was significantly greater in the Staimune group vs. Placebo, no difference between groups at 60 or 120-min. Neutrophils and Monocytes were significantly greater in the Study Product group. There were variable impacts on eosinophils and basophils. Markers of non-specific and innate immunity were significantly impacted in Staimune® as were the impacts on pro and anti-inflammatory cytokines. There was significantly enhanced recovery for cortisol in the Staimune® group in the post-exercise period.

Conclusion: Inactivated *Bacillus coagulans* GBI-30, 6086 supports the immune system at rest and after strenuous exercise. The study product may lessen the window of immune system suppression post-exercise, and enhance stress recovery supporting immunity.

Keywords: Immunity; *Bacillus coagulans*; Nutrition; Exercise; Stress; Recovery; Probiotics

Introduction

The human microbiota is formed throughout infancy and it reaches its adult composition by the age of 2. It has been suggested that it develops and adapts with the host throughout the lifespan and alters its own composition and gene expression in response to our changing environmental conditions [1]. The functions and pathways encoded in the core microbiome are essential for the correct functioning of the gut. Therefore, maintaining a balance that favors beneficial bacteria is key to overall health. Some well-studied benefits to maintaining gastrointestinal microbial balance include protection against potential pathogens, digestion of polysaccharides, production of essential vitamins, stimulation of angiogenesis, regulation of fat storage, maintenance of a healthy body weight and modulation of the host's immune system [2]. It is widely recognized that the immune system is regulated by the microbiota [3]. Thus, any disruption in the balance of beneficial and pathogenic bacteria not only disturb the function of the gastrointestinal tract but impact host immunity and thus overall health. There is growing evidence that disruptions in the microbiota can contribute to the development of chronic diseases as well as inflammation and an overall decline in health and well-being. While antibiotic use and the Western diet have been linked to an imbalance in microbial diversity, a healthy diet and exercise have been shown to favorably alter the microbiota [4]. While it is difficult to separate one from the other, exercise appears to improve the balance of the microbiota along with diet [5]. In further support of this Estaki et al showed that subjects with higher cardiorespiratory fitness had increased microbial diversity compared to those with lower fitness [6]. Although, more studies specifically on exercise and the microbiota are needed. It is established that it is a complex two-way relationship, for instance, rat models have shown that the balance of the microbiota may improve exercise performance [7]. In addition, it is well recognized that regular moderate exercise leads to improved immune

function and decreased risks of disease susceptibility [8]. Research has identified a correlation between moderate level physical activity and lower incidence of bacterial and viral infections as well as decreased cancer incidence and mortality [9,10]. In contrast, exhaustive exercise and overtraining may result in immune suppression and increased susceptibility to many diseases [8]. Therefore, a connection between gut microbial balance, exercise and immune system function is well supported and modulating one aspect of this connection will most likely influence the function of the other.

One such method for modulating gut health and its' related physiological systems are probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [11]. Consuming probiotic bacteria either through diet or supplementation has been shown to improve gut microbial diversity, therefore providing a wide range of health benefits mostly related to anti-inflammatory and immune system regulation [12-14]. Intake of most commonly used probiotics have an established safety record for most populations, the exception being severe immune compromised patients. However, there are benefits for inactivated probiotic strains, such as increased shelf life, food quality and for immune-compromised individuals [15].

Inactivated bacteria, such as *Lactobacillus plantarum* L-137 (HKL-137) is produced by heating, leaving the outer bacterial cell wall as the main mechanism of interaction with host immune cells [15]. It has been studied in animals [16] and humans and has shown to protect against influenza virus infection and to support healthy immune function [17].

A group of lactic-acid producing probiotics with unique strains is *Bacillus coagulans* (*B. coagulans*, previously classified as *L. sporogenes*). The teichoic acid from *B. coagulans* walls has a higher lipid content than most Gram-positive bacteria, and is a glycerophosphate polymer substituted with two neutral sugars, glucose and galactose. It is unique because it lacks amino acid substituents, which is considered a characteristic of teichoic acids (TAs) [18]. Teichoic acids, which are molecules on the external surface of the PGN layer, have been shown to be involved in immune responses [19]. It was found that lipoteichoic acids (LTAs) from *L. casei* induced elevated levels of TNF-α in mouse macrophages through NF-κB activation mediated by TLR2 [20].

Inactivated *B. coagulans* GBI-30, 6086 cells have been recently produced for oral consumption. In a recent study, Jensen et al. showed that that the inactivated bacterial cells demonstrated similar immune activation and anti-inflammatory benefits compared to the live [15]. Inactivated *B. coagulans* GBI-30 was added to peripheral blood mononuclear cell cultures at doses from 0.78 × 106-100 × 106 inactivated cells/mL cell culture. In the study, it was found that the study product triggered activation of the CD69 marker on human immune cell types as well as increased production of multiple cytokines, chemokines, and growth factors [15]. Thus, potent-immune-activating properties of inactivated *B. coagulans* were determined. Additionally, in a clinical study testing the effects of inactivated *B. coagulans* on immunological markers, it was found that $CD3^{+}CD69^{+}$cells, IL-6, IL-8, interferon-γ (IFN-γ), and TNF-α levels increased from subjects' baseline after 28 days from consumption of 1 capsule/day. One capsule per day containing 500 million CFU of the study product was suggested to enhance immunological response associated with respiratory tract infections (500 million CFU) [21]. In mice with Clostridium difficile it helped to reduce symptoms and decrease death [22,23]. Clinical studies showed that consuming BC30 increased the numbers of beneficial bacteria [24] and *ex vivo* testing of blood from elderly humans who had consumed BC-30 for 28 days reported an increase in anti-inflammatory cytokines [25]. Jager et al reported that the consumption of BC30 supports exercise performance and helps reduce exercise-induced muscle damage [26].

Although studies to date show promise for inactivated bacterial cells, few studies have reported on the effects of consuming these on immune system function alone or after stressful exercise, known to compromise immune function or on consumption while exposed to a bacterial challenge.

Materials and Methods

The purpose of this study was to determine the safety and efficacy of an inactivated *Bacillus coagulans* GBI-30, 6086 (Staimune®) in supporting the immune system at rest, in response to an *in vitro* bacterial challenge, and in response to undergoing strenuous exercise in healthy adults. Within this paper, the Staimune® is referred to as Study Product.

Study subjects and recruitment

In a randomized, double-blind, placebo-controlled manner, 16 healthy males with a Body Mass Index of 18.0 to 34.9 kg/m^2 and aged 18 to 30 years of age were recruited to participate in this prospective exploratory early stage dietary supplement study. All subjects read, reviewed and signed an Informed Consent consistent with the Declaration of Helsinki prior to starting any of the study related procedures. The study was approved by the Bio-Kinetic Clinical Applications, LLC. Institutional Review Board (Springfield, MO). Recruited subjects had to be physically active (a minimum of three times per week of structured exercise for the previous month, or a minimum of twice per week over the past three months), have no orthopedic limitations per medical history or in-person screening physical exam, be a non-smoker and have the ability to exercise for a minimum of 60 minutes (min). In addition, this study excluded potential subjects with a significant medical and surgical history, a first degree relatives who experienced a myocardial infarction before the age of 50, evidence of metabolic disorders (via physical exam and blood work), an abnormal screening electrocardiogram, a recent (prior 12 months) history of substance or alcohol abuse, an inability to wash out any vitamin, mineral or any probiotic containing product for a minimum of 14 days, recently donated blood or plasma (prior seven days) and those who had participated in another clinical trial within 30 days of this trial.

Screening visit: All subjects underwent a screening visit, whereby medical and surgical history were assessed, anthropometrics obtained, blood pressure and heart rate recorded, a physical exam undertaken, a 12-lead electrocardiogram conducted, the Physical Activity Readiness Questionnaire (PAR-Q) administered and scored, and screening blood work to assess that the subject was free of communicable diseases (HIV, HCV, HBS) as well as having healthy hepatic, renal and metabolic systems, along with an intact immune system and no abnormities with components of the complete blood count. Blood tests included chemistry metabolic panel, complete blood count and serology testing.

Randomization baseline visit: At this study visit, subjects were randomized to the Study Product (Staimune®-50 mg of inactivated *Bacillus coagulans* GBI-30, 6086) or a matching Placebo. Subjects also had saliva and blood taken in order to establish basal levels of the

biomarkers of interest. Subjects received education about the study, the study products and all general questions were answered. Standard monitoring of concomitant medications occurred. Subjects were next scheduled for the Day 28 (one month) end of study visit and were informed between visit phone calls would occur in order to remind them of next study visit, no strenuous exercise for the 24 hours prior to a visit, no alcohol for the 24 hours and to limit caffeine intake for the 12 hours prior to the next visit, during this time, any questions that the subject had were addressed by the PI or by the study staff.

End of study visit (Day 28): All subjects came in to the research offices and were queried about any medication or other product usage, assessed for adverse events, unused product was collected (for compliance assessment), vitals (blood pressure and heart rate) were obtained (pre-exercise), and safety laboratory blood work was taken (metabolic panel, CBC with differential). Subjects also had pre-exercise saliva and blood samples taken, underwent the exercise test bout for 60 minutes and then had repeat blood draws along with a saliva sampling with repeat sampling at 10 minutes, 60 minutes and 120 minutes post-exercise testing completion. Subjects were permitted a standardized snack after they completed the end of study visit.

Exercise testing and intervention (Day 28)

Exercise testing and parameters: Subjects completed the exercise test after 28 days of supplementation. Exercise was conducted on a standard motorized treadmill under normal environmental conditions. Subjects were instructed to exercise (i.e., jog or run) at a speed and intensity that would elicit at least a 60 to 80% heart rate maximum zone as calculated by the Karvonen method (Target Heart Rate=((max HR-resting HR) × %Intensity)+resting HR example. After familiarization and determination of the subject's target heart rate zone, subjects were fitted with a Polar Heart rate monitor in order to monitor heart rate during exercise (Polar Electro Inc., Bethpage, NY). Subjects warmed up on the treadmill for five minutes at a self-selected pace in order to raise the core body temperature and get the person ready for exercise. Next, subjects were instructed to start to jog and then run in order to obtain an exercise pace and intensity that allowed them to stay within the Karvonen target heart rate zone of 60 to 80% of maximum. Once subjects obtained their steady-state exercise in the Karvonen target heart range, subjects were asked to maintain their pace and intensity for 60 minutes of cumulative exercise. Once the subject completed 60 minutes of high energy exercise, they were asked to stop and cool down with a five-minute walk on the assigned treadmill. For each subject, a total of five blood samples (approximately 29.5 mL of blood) were collected during the study. Blood and saliva were collected at the baseline visit and again on Day 28 pre-exercise, 10 minutes post, with additional measurement at one and two hours post-exercise cessation.

Measuring mucosal immunity: Changes in mucosal immunity were determined by measuring salivary IgA. Salivary IgA samples were collected at baseline, pre-dosing and again on Day 28 pre-exercise and 10 minutes, 1 hour and 2 hours post-exercise. Salivary IgA analysis was undertaken by a commercial academic laboratory (Salimetrics. Carlsbad, CA. website: www.salimetrics.com). Briefly, Salivary IgA is measured using an enzyme immunoassay technique that has been validated [27] with a commercially available kit (indirect enzyme immunoassay kit with a coefficient of variation of 4.49-6.99%, greater than 100% recovery and sensitivity to a minimal concentration of 0 to 2.5 ug/ml).

CBC with differential response: This parameter was measured as complete blood cell count with differential (focusing on the Absolute and Percent of differential), blood samples were collected in a 4.0 mL K2EDTA vacutainer tube, BD No. 367861 and processed by the local hospital laboratory (Mercy Laboratory Services, Mercy Hospital, Springfield, MO)

Cortisol response: Cortisol was obtained at baseline (pre-intervention) and again on the Day 28 visit, pre-exercise. Cortisol was collected via standard phlebotomy techniques in a 4.5 mL lithium heparin vacutainer tube (BD#367962). Processing was per protocol. Serum cortisol was processed and analyzed by the local hospital laboratory (Mercy Laboratory Services, Mercy Hospital; Springfield, MO).

Non-specific immune response: This aspect of immunity measured by helper T-cell (i.e., $CD3^+/CD4^+$), cytotoxic T-cells (i.e., $CD3^+/CD8^+$), and natural killer cells (i.e., $CD3^-/56^+$ or $CD3^-/16^+/56^+$) percentages/proportions. These measurements were made using three-color flow cytometry on a calibrated instrument (Millipore Sigma, easy Cyte 12HT). Measurements were performed on a contract basis by the University of North Texas, Applied Physiology Laboratory, using previously published, validated methods (28, 29).

Cytokine production response to an LPS challenge: Cytokine production following an *in vitro* lipopolysaccharide (LPS) challenge included was measured in duplicate from the cell-free supernatant using a commercially-available bead-based multiplex assay (Millipore Sigma Milliplex). Completed assays were acquired on a calibrated analyzer (Luminex MagPix). The specific cytokines measured were: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IFN gamma, GM-CSF, and TNF-α. Measurements were performed on a contract basis by the University of North Texas, Applied Physiology Laboratory, using previously published, validated methods [28, 29].

Dietary and lifestyle controls: All study participants were asked not to change their typical diet or eating style as well as exercise routines. Throughout the study, subjects were queried regarding dietary habits. Subjects were required to fast (no food or beverage other than water) for 4 hours prior to the baseline and end of study visit. In addition, subjects were asked to restrict as follows: to not consume alcohol for at least 24 hours prior to each visit and caffeine-containing beverages and products for at least 12 hours prior to each visit. However, subjects were allowed to drink water or flavored non-caloric water during the study exercise intervention in ad libitum fashion.

Statistical approaches

The study groups were analyzed at baseline to determine heterogeneity. Outcome analysis was based on changes over time (from time-point 0) for within-group changes as well as between-group differences. P value for significance was set at $P<0.05$ for significance and included 95% Confidence Intervals where appropriate. As this was a pilot study, no corrective factors for multiple sets of analysis were employed. All acquired variables are summarized by time point and by-product. Numerical variables are presented as mean, standard deviation, count, median, and range (minimum to maximum value). Changes from the baseline are summarized and presented in the same way. Time-matched differences between the products (Active and placebo) were also summarized and presented in the same way. Time-wise comparisons include changes from baseline values for all tests of interest from Visit 1 to Final Test Visit 2 (for changes from pre-exercise intervention to post-exercise intervention). For each continuous

variable, the mean change from baseline to each subsequent time point was tested for nominal significance by the paired Student t-test, or by the non-parametric Wilcoxon signed-rank test if data is non-normally distributed. For each continuous variable at each time point, the mean differences in the variable, or in the change in that variable from baseline, within and/or between the different products was tested for nominal significance by the paired Student t test or by Wilcoxon signed-rank test if data were non-normally distributed for within-group comparison, and independent t-test or Wilcoxon rank-sum test for between-group comparison. For each categorical variable, the difference in the distribution of categories between the different product groups was tested for nominal significance by the Fisher Exact test if possible, or by the Chi-Square test if necessary. Excel 2003 (Microsoft Corp, Redmond WA), was used for data entry. Statistical software utilized for this study included SAS Studio (Basic edition) v3.6 (SAS Institute Inc., USA). SAS Studio (Basic edition) v3.6 was used to perform the descriptive and inferential tests. The proposed enrollment of 16 subjects was pre-specified by the sponsor. The study subject number was based upon other similar published studies and by examining the subject load enrolled. The study sample size is considered a "Convenience Sample".

Results

Study subjects were 22.63 ± 3.36 years of age, had a body weight of 84.60 ± 12.09 kg and had a mean body mass index of 25.71 ± 2.77 kg/m^2. All parameters were within normal limits and ranges at baseline. Thirty-nine (39) subjects were screened in order to obtain the 16 who were randomized. Using an Intent to Treat approach, all 16 completed the study, and Per Protocol, 14 subjects completed all of the study tests and procedures. One subject was dropped due to an unrelated adverse event (syncope, nausea; placebo group) and one subject was lost to follow up from the Study Product group.

There were no serious adverse events reported or observed. Minor adverse events included nausea and syncope for one subject in the placebo group (which was considered unlikely to be related to the study product; subject did not finish the study) and syncope as well for one person on the Study Product group (considered unrelated to the study product).

Effects on mucosal immunity

The results of this study demonstrate that the Study Product did not have an impact on resting mucosal immunity (as measured by basal levels of Salivary IgA pre-intervention to Day 28 values for this biomarker of immunity). In addition, it does not appear that exercise of high intensity alone impacts this biomarker of immunity as no significant changes occurred in the Placebo control group nor in the Study Product group after stressful exercise.

Effects on cortisol

The effects or impacts of the dietary supplement on serum cortisol were varied and of interest. Initially, serum cortisol trended for elevation (p=0.085) for change from pre-intervention levels to Day 28 (over the 28 days and sample took pre-exercise testing on Day 28) in the Study Product group, however, this was not significant between groups. On the exercise test day (Day 28), the cortisol response was such that 10 minutes after exercise cessation, the Study Product group was higher than the Placebo group (16.53 ± 7.95 vs. 6.88 ± 2.74; p=0.006), however the changes in cortisol by product over the two-hours of post-exercise data collection revealed trends for significance and a significant decrease in exercise induced elevated cortisol in the Study Product group (change over the first hour; -4.22 ± 6.46; p=0.069), coupled with a significant reduction through the second post-exercise hour (-5.19 ± 6.98; p=0.043) cortisol, this was trending for between group significance as well (p=0.107). Acutely, the Study Product group also achieved a significant reduction in cortisol in the exercise recovery time period. Examining the change from 10 minutes post-exercise to the one hour time point reveals a strong cortisol drop (3.48 ± 3.14; p=0.006), which was also significant when compared to Placebo (p=0.019). Further, when examined as change in cortisol from 10 minutes post-exercise to the two-hour time point, the directional change was significant for the Study Products group (-3.48 ± 3.14 vs. 7.33 ± 5.88; p=0.016), the same significance is true when looking at change from the one to two hour recovery time period (-0.97 ± 4.60 vs. 7.13 ± 4.58; p=0.026). Overall, the signals indicate that the Study Product may impact post-exercise cortisol recovery.

Effects on overall immunity

The effects and impacts of the Study Product on overall immunity were varied. The White Blood Cell count significantly increased from the pre-exercise on Day 28 to the 10-minute post-exercise time point (1.09 ± 1.06; p=0.010) and also at the two-hour post-exercise time period (3.59 ± 2.46; p=0.001) for the Study Product group (data not shown). No other changes of potential importance are noted. It is also reported that there were no significant changes in Red Blood Cell Count between the groups (data not shown). In terms of Absolute Neutrophils, the Study Product group experienced significant changes from pre-exercise to 10 minutes post (1.28 ± 1.14; p=0.006), at the one-hour mark post (2.11 ± 2.62; p=0.031) and at the two-hour post-exercise time-point (2.99 ± 2.70; p=0.006). With respect to Absolute Lymphocytes, the Study Product group experienced statistically significant changes at the post-exercise one-hour time-point (-0.60 ± 0.57; p=0.009) and trended for significance at the two-hour post-exercise mark (-0.32 ± 0.48; p=0.062). Absolute Monocytes testing revealed that the Study Product group tended to elevate this immune biomarker by Day 28 over Placebo (resting, pre-exercise values) (0.55 ± 0.24 vs. 0.40 ± 0.04; p=0.079). No other impacts on Absolute Monocytes were noted. Impacts of the Study Product on Absolute Eosinophils indicate a strong trend for an impact over the first 10 minutes post-exercise (0.06 ± 0.09; p=0.057) with significance for change achieved at the one-hour post-exercise mark (0.09 ± 0.11; p=0.028) which also trended for significance over Placebo (p=0.095). The Study Product group also experienced a significant change from pre-exercise to the two-hour post-exercise time period (0.10 ± 0.12; p=0.0290) which also trended towards significance versus Placebo (p=0.067). In addition, the change from 10 minutes post to both one and two-hours revealed significance (at hour one; p=0.012: at two-hours; p=0.056). Finally, Absolute Basophil response to the Study Product revealed the effect 10 minutes after exercise until the one-hour mark an effect (0.02 ± 0.01 vs. 0.01 ± 001; p=0.044), with no other differences between the groups observed.

Non-specific immunity

The Study Product had variable impacts on Non-Specific Immunity. There was a significant impact on % NK cells as a change from pre-exercise values on Day 28 to the one and two-hour post-exercise time-points (2.52 ± 1.61; p=0.001, and 2.63 ± 1.35; p=0.0004 respectively). There was also significant within Study Group activity for the %

CD56$^+$/16$^-$ with a strong rise in basal levels (from Day 1 to Day 28 pre-exercise (3.60 ± 2.82; p=0.005) with continued significance one and two hours post-exercise (-1.89 ± 2.62; p=0.004 and 2.10 ± 1.36; p=0.001) respectively. The %CD56$^+$/16$^+$ experienced some changes in the Study Product group, namely an increase 10 minutes after exercise cessation (0.39 ± 0.55; p=0.050) along with a significant increase from pre-exercise to two hours post-exercise time-point (0.42 ± 0.38; p=0.007). The % CD3$^+$ impacted the Study group to where from pre-dosing (Day 1) to Day 28 (pre-exercise value), there was a significant decrease in this biomarker (-16.00 ± 12.13; p=0.004), in fact the differences between the Study Product and Placebo for Day 28 pre-exercise approached significance (p=0.054). On Day 28, 10 minutes after exercise the Study Product group significantly increased -9.90 ± 12.95; p=0.038) with the one and two-hour post-exercise values also being significant for change (14.55 ± 10.80; p=0.002 and 16.61 ± 11.51; p=0.001) respectively. The % CD3$^+$/CD4$^+$ activity impacted the Study Product group from the change from Day 1 to Day 28 pre-exercise (-7.96 ± 8.06; p=0.018). The Study Product group also tended to exhibit an increase in these cells one hour after exercise on Day 28 (5.34 ± 8.67; p=0.083). The % CD3$^+$/CD8$^+$ cells experienced a significant effect one-hour after exercise on Day 28 (8.33 ± 10.28; p=0.030) as well as two hours after exercise intervention (11.83 ± 10.81; p=0.007). Finally, in terms of the CD4/CD8 ratio, this appeared to only be impacted on Day 28 in the post-exercise period. The change from Pre-exercise to the two-hour post-exercise period trended for difference (0.51 ± 0.86; p=0.097).

Effects on cytokine production response to *in vitro* LPS challenge

The Study Product group experienced a decrease from Day 1 to Day 28 pre-exercise in GM-CSF (-30.13 ± 36.21; p=0.0273), while experiencing a significant increase on Day 28 10 minutes post-exercise (28.36 ± 25.39; p=0.0064), with the one-hour post-exercise change trending for significance (11.61 ± 18.59; p=0.0798) and the two-hour post-exercise change also significant (13.09 ± 17.14; p=0.0389) and the difference between the Study Product and Placebo significant (13.09 ± 17.14 vs. -7.36 ± 6.56; p=0.0073). Another cytokine to express activity was IL-13. Il-13 in the Study Product group rose significantly on Day 28 from pre-exercise to the two-hour post-exercise time period (2.76 ± 3.23; p=0.0243) The change over the two-hours post-exercise time period was also significant between the groups (2.76 ± 3.23 vs. -0.84 ± 1.66; p=0.0196). Interleukin-2 (IL-2) activity in the Study Product group trended for differences two hours after exercise on Day 28 (1.63 ± 2.57; p=0.0751), IL-5 mirrored this effect at two-hours post-exercise on Day 28 (0.39 ± 0.57; p=0.0630), as IL-7 tended to be increased also one-hour after exercise on Day 28 (5.06 ± 8.48; p=0.0919) and became significant two-hours post-exercise (7.02 ± 8.28; p=0.0251) which also was a strong trend for difference versus the Placebo (7.02 ± 8.28 vs. 0.90 ± 5.84; p=0.0778), IL-8 for the Study Product group also appeared to be elevated significantly two-hours after exercise on Day 28 (406.70 ± 553.18; p=0.0451). TNF-alpha trended for elevation in the Study Product group one hour after exercise on Day 28 (416.20 ± 705.36; p=0.0949) as well as at two hours post-exercise (436.30 ± 513.54; p=0.0249).

Discussion

The benefits of consuming probiotics have been reported extensively in the literature [12-14]. Although the viability of live bacterial cells in probiotics offer beneficial effects, such as treatment of inflammatory, auto-immune and gastrointestinal diseases, researchers have sought to study inactivated bacterial cells in probiotics (termed "para-probiotic") in order to provide a safer alternative among individuals with weaker immune systems and/or inflammatory processes [19].

In this study, significant differences (p ≤ 0.05) in a declining trend were observed for T-cell measurements in the Study Product (inactivated Bacillus coagulans GBI-30, 6086) group. Specifically, significant differences were observed for Total T-cell (CD3) concentration from pre-exercise to 10-min post-exercise as well as from pre-exercise to 1 and 2-hours post-exercise. Additionally, significant differences were observed for both Helper T-cell (CD3/CD4) and Cytotoxic T-cell (CD3/CD8) from pre-exercise to 1-hour post-exercise in the Study Product group. This declining trend supports the interpretation that Study Product increased transmigration of T-cells from the blood to the tissue, thus reflecting an increase in peripheral immunity. T-cells mediate and control immune responses. Two types of T-cells include Helper T-cells and Cytotoxic T-cells. Helper T-cells provide overall control for innate and humoral immune response, whereas Cytotoxic T-cells are responsible for a primarily T-cell innate response. Another measurement of innate immunity was NK cells (CD3$^-$/56$^+$), which are the primary cells responsible for killing tumor and other cells. In observation of the Study Product group, a significant decline was prominent at 1 and 2-hours post-exercise, compared to baseline. Therefore, since the Study Product group experienced a more rapid decline post-workout compared to the Placebo group, it is reasonable to speculate that the inactivated Bacillus coagulans GBI-30, 6086 reduced the magnitude of the "open window" of immune suppression at the systemic level due to circulating responses.

Stimulated cytokine production presented outcomes that were similar to improvements observed with T-cell and NK cell. IL-12 (p70) is a Th1 cytokine that is responsible for promoting Th1 cell production from Th0 cells. As well, IL-2 is a Th1 cytokine that promotes T-cell differentiation and diversity. There was a statistical trend for difference (0.0751, and 0.0876, respectively) observed in the Study Product group for both IL-2 and IL-12, respectively, from pre-exercise to 2-hours post-exercise, in which there was a greater decline in the cytokine levels. These findings suggest that formation of Th1 cells was reduced. Accordingly, it is possible that the movement of T-cells was driven by Th1 cells, as the formation of additional Th1 cells was reduced. Another cytokine that yielded similar trends was IL-7, the cytokine responsible for the production of Th0 cells in the bone marrow. The significant difference (decline) from baseline to 2-hours post-exercise in the Study Product group indicates that there was an increase in the formation of the cells during the "open window" period and explains the observed migration of T-cells and reduction in Th1 cytokine signals in the presence of inactivated *Bacillus coagulans* GBI-30, 6086.

It is also important to note trends observed between pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α, did not show significant differences nor statistical trends for differences for the Study Product group. As pro-inflammatory cytokine levels did not show any change, it can be inferred that pro-inflammation was not influenced by inactivated *Bacillus coagulans* GBI-30, 6086. However, it is possible that anti-inflammatory cytokines were involved. IL-13, both a pro- and anti-inflammatory cytokine in the respiratory tract, was reduced from pre-exercise to 2-hours post-exercise in the Study Product group as well as reduced (in a greater amount) from baseline to 1-hour post-exercise in the Placebo group. Therefore, given the duality of the IL-13

mechanism, it is difficult to determine if the observed effect is dampening the inflammatory response. It is suggested that future clinical studies focus on IL-13 levels in relation to other pro- and anti-inflammatory cytokines.

Although some significant differences were observed within the Study Product group, lack of significant differences or statistical trend for differences were found comparing the Study Product group to the Placebo group. Considering the small sample size per study group and the fact that this was designed as a pilot proof of concept study, it is suggested that future clinical studies should include larger sample sizes. By doing so, a more accurate representation of the mechanistic pathways that inactivated *Bacillus coagulans* GBI-30, 6086 has on the immune system can be uncovered.

In a study examining the effects of alcohol on post exercise LPS-stimulated production of cytokines, it was found that heavy resistance exercise increased production of IFNγ, TNF-α, IL-1β, and Il-6 and decreased production of IL-10. Also, after alcohol ingestion, IL-8 was reduced at 5 hours post-exercise and IL-6 was reduced from pre-exercise to 3-hours [30]. In contrast, in our study, exercise was executed aerobically and without alcohol. However, as heavy resistance exercise is an anaerobic process, more pronounce changes in such cytokine levels could be observed. Additionally, it is important to consider a variety of ingestion conditions, such as ingestion with alcohol. By doing so, effects of inactivated *Bacillus coagulans* GBI-30, 6086. could be either enhanced or diminished. A study by McFarlin et al. tested the effects oral supplementation with bioavailable curcumin has on Exercise-Induced Muscle Damage (EIMD) and delayed onset muscle soreness (DOMS). Curcumin acts via modification of the COX-2 pathway, which reduces inflammatory cytokines IL-1β, IL-6, IL-8, and/or TNF-α. Results showed that curcumin supplemented significantly increased creatine kinase, TNF-α, and IL-8 concentration following EMID compared to placebo [29]. In this study, IL-1β, IL-6, IL-8, and TNF-α did not have significant changes between pre-exercise and post-exercise. However, the inflammatory cytokines also did not increase post-exercise, meaning inactivated *Bacillus coagulans* GBI-30, 6086 could have inhibiting or dampening effects on pro-inflammatory cytokines, as does bioavailable curcumin. In addition, subjects evaluated in the study by McFarlin included individuals who had osteoarthritis and as such, the disease or condition itself may alter responses to any anti-inflammatory agent. Therefore, in hopes to uncover the mechanistic pathway of inactivated *Bacillus coagulans* GBI-30, 6086 expanding the study population to particular inflammatory conditions may be required.

Conclusion

Inactivated *Bacillus coagulans* GBI, 6086, a para-probiotic has immune supportive properties in healthy adults, which deserve further development and evaluation.

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