A MULTI-CENTER, DOUBLE-BLIND, RANDOMIZED, PARALLEL GROUP STUDY TO EVALUATE THE EFFECT OF FERMENTED MILK CONTAINING LACTICASEIBACILLUS PARACASEI STRAIN SHIROTA (LCS) ON THE UPPER AIRWAY SYMPTOMS OF TRIATHLETES BOTH BEFORE AND AFTER A COMPETITION

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ABSTRACT

Triathlon, as a sport that combines high-intensity and long duration, imposes the body to an expressive physiological adaptation. However, when the challenge exceeds the physiological adaptation capacity, it is possible to observe the occurrence of several disorders in the athlete's health. In this sense, among several factors that can help in this adaptation, it has been pointed out that the microbiota, which can be modulated by diet, age, medical treatments, lifestyle, and physical exercise, can favor the maintenance of a healthy status. According to the literature, both probiotics, prebiotics, and symbiotics are able to benefit people in different ways, for instance, by favoring immunoregulation, these elements can impact the prevention or decrease in the frequency and/or intensity of infections in the upper airways, both in the population in general and in athletes. However, it is noteworthy to mention that there is still a lack of reports in the scientific literature on the effects of the ingestion of fermented milk containing *Lacticaseibacillus paracasei* strain Shirota (LcS), a well-known probiotic agent, not only on the modulation of the immune/inflammatory responses, but on the manifestation of symptoms of airways and mood state in triathletes. Therefore, in this study, we aimed to investigate, in a general way, the effect of daily intake of fermented milk containing 40 billion LcS in amateur triathletes, emphasizing that our primary endpoint will be to evaluate the concentration of salivary IgA, and the secondary endpoints will be to assess both systemic immune/inflammatory and upper airway responses, as well as the incidence and duration of upper airway symptoms, and also the mood states. In order to respond to these endpoints, samples of saliva, nasal swab, and blood, as well as data regarding quality of life, upper airway symptoms manifestation, and mood states will be obtained on five different occasions: 30 days (1) and 24 hours before the competition (2), and also immediately (3), 72 hours (4), and 14 days after the competition end (5). It is important to highlight that the data related to the quality of life, upper airway symptoms manifestation, and mood states will be obtained by using specific and validated questionnaires, such as SF-36 (Short Form Health Survey 36), WURSS-21 (Wisconsin Upper Respiratory Symptom Survey – 21), and BRUMS (The Brunel Mood Scale), respectively. Finally, it is also paramount to clarify that the triathletes participating in this study will be oriented to ingest a daily bottle of fermented milk containing 40 billion LcS or placebo/unfermented milk throughout the study period, starting the ingestion

30 days before the competition, soon after the first sample collection, until 14 days after the competition.

Keywords: Triathlon; Immunity; Probiotics; Antibodies; Cytokines; Upper Airway´s Symptoms; Mood States.

INTRODUCTION

Since the last century, studies have shown a close association between the immune system and physical exercise (Padilha, 2022). In fact, even though this interaction can promote remarkable mutual benefits, some effects can also lead to the loss of a healthy state (Walsh, 2011). For instance, it was reported that physical exercise presents a modulating effect on the hematological parameters of athletes, particularly a leukocytosis observed after a strenuous and prolonged exercise training session, such as a marathon and triathlon (Costa Rosa &Vaisberg, 2002; Momesso Santos, 2022). Beyond this observation, it was also shown that individuals submitted to sessions of high-intensity and exhausting physical exercises present an increase in the incidence of upper respiratory tract infections (URTI), in special when the exercise training volume exceeds adequate physiological limits (Costa Rosa &Vaisberg, 2002; Pyne, 2000), thus suggesting a possible relationship between training load and the occurrence of infections (Gleeson M. , 2000).

Although it has been pointed out that the URTI incidence rate in amateur athletes practitioners of different sports modalities is similar to those observed in the general population, when assessed in longitudinal studies both retrospectively and prospectively (Keaney, 2021; Orysiak, 2019; Cantó, 2018; Pollastri, 2021), it is noteworthy to mention that when the athletes aim to increase their physical performance, in a general way, they have to increase the training volume at intensities that generate some level of physiological stress, which is often above physiological tolerance. This situation can lead to the development of immunosuppression, which increases the possibility of them presenting URTIs in nonseasonal periods, mainly in periods close to competitions. In this sense, Walsh et al. (2011) reported that swimming athletes tend to present symptoms of URTI in the weeks before competitions, whereas in other sports, such as long-distance running, this set of symptoms tends to be more frequent after competitions, as observed in our previous study (Vaisberg, 2019).

According to Bermon (2007), the symptoms of URTI can be multifactorial and the agents' promoters of such conditions are not only related to infectious conditions but also inflammatory and allergic events. Studies reported that 30% of URTI cases were associated with the presence of infectious agents, confirmed by analysis of secretions obtained from the

throat and nasopharynx, and the other cases were associated with inhalation of cold and dry air (Spence, 2007; Cox, 2008).

Besides the exposure to these different factors, such as pathogens and cold and dry air, as formerly mentioned, the intensity and duration of the physical exercise session, as well as climatic conditions, nutritional aspects, mental stress, and other behavioral factors also can impact the immune response, thus increasing the possibility to the triggering of URTI. It is paramount to highlight that diet is a pivotal element in the modulation of immune and inflammatory responses in all individuals, in special those engaged in exercise training programs, mainly with exhaustive characteristics, since some agents are able to reduce the frequency and magnitude of URTIs (Walsh, 2011).

Among some dietary agents studied in this context, probiotics have drawn attention, since it was observed that, when the probiotic strains are properly applied, they can positively influence the athletes, with remarkable impacts not only in physical capacity but also in the performance of athletes (Smarkusz, 2017; Giron, 2022).

In this respect, the lactobacilli are the probiotic strain most used and it is characterized as non-pathogenic, lactic acid bacteria that are resistant to the action of gastric juice and bile (Baken, 2006; Shida, 2011; Matsuzaki, 2007). Regarding to its action, a benefit of lactobacilli for human health is associated with its corollary ability to regulate the activity of the immune system. In a general way, it has been shown that some lactobacilli strains modulate the immune response to a Th1 profile with evident production of IL-12p70, which favors the improvement of immunity against infections and cancer, whereas other strains mainly induce the production of IL-10 driving to a regulatory profile, demonstrating its anti-inflammatory activity (Shida, 2011). It has been demonstrated that lactobacilli act on cellular responses by increasing not only the recruitment and phagocytic capacity of macrophages but also the activation of Natural Killer (NK) cells and T cell functions, as well as in hormonal responses through the decrease of auto-antibodies (Baken, 2006; Matsuzaki, 2007; Gui, 2020).

According to Shida et al. (2011), specifically in terms of the supplementation with *Lacticaseibacillus paracasei* strain Shirota (LcS), formerly classified as *Lactobacillus casei* strain Shirota, there are three hypotheses to explain their action on the immune response. In the

first hypothesis, the M cells in Peyer's patch, upon uptaking LcS, allowed its recognition through Toll-like receptors (TLRs) by macrophages and dendritic cells, inducing their activation with the consequent secretion of IL-10. A second mechanism proposes that dendritic cells would be able to directly phagocytize bacteria in the gut, thus inducing the secretion of IL-10 and TGF-b, leading to the differentiation of T lymphocytes to the most regulatory profile of the immune response, and increasing the number of regulatory T lymphocytes (Tregs) in the intestinal mucosa. Finally, the M cells presented in the Peyer's patch, by facilitating the recognition of LcS by macrophages and dendritic cells, could induce the secretion of IL-12p70.

In terms of the effects of lactobacilli supplementation in a physical exercise context, although they are still scarce, a longitudinal study carried out with the supplementation of *Lactobacillus rhamnosus* for 3 months found a decrease in duration and severity, but not in the incidence of URTI in athletes (Kekkonen, 2007). Beyond this report, Gleeson et al. (2011), observed a reduction in the frequency of URTI in association with an increase in salivary levels of immunoglobulin A (IgA), in athletes supplemented with LcS for about 4 months. Moreover, our group was able to demonstrate that marathon runners supplemented with fermented milk containing 40 billion of LcS for a period of 30 days before the marathon, not only presented a lower incidence and duration of upper respiratory symptoms (URS) but also highlighted the ability of this bacteria in modulating the immune/inflammatory responses, both systemic and in the upper airway, specifically after the marathon performance (Vaisberg, 2019).

Like the marathon race, the triathlon is recognized as a high-intensity sport, in which the athletes are submitted to a strenuous and prolonged competition with remarkable physiological impacts (O′Toole M.L., 1989). In this sense, triathletes are particularly prone to muscle injuries and fatigue (Kienstra C.M., 2017), not only as a result of mechanical but also inflammatory stress, which can induce muscle damage associated with this sport modality (Howatson G., 2008). Furthermore, triathlon competition also stimulates the production of reactive oxygen species (ROS) due to excessive aerobic respiration, ischemia-reperfusion, and leukocyte activation (He F., 2016) in response to the pattern of long duration and exposure to environmental conditions that favor dehydration, as well as the depletion of carbohydrates can

contribute to the occurrence of fatigue, gastrointestinal (GI) problems, hyperthermia, and hyponatremia that, in conjunction, can drive to a decrease of athletes' performance during this endurance exercise session (Jeukendrup, 2011). Interestingly, it was reported that central fatigue related to the central nervous system (CNS) can be alleviated with the maintenance of glycemia through different energy sources (Nybo, 2003). Based on these pieces of information, it is possible to consider that it has been well established in the literature that performance in the triathlon competition is closely influenced by the state of oxidative stress, inflammation, and energy balance.

Beyond these aspects, it is crucial to cite that in the 80' years of the last century, Morgan (1980) popularized the use of a profile mood state (POMS) in the sports context, since he identified a close association between successful athletic performance and an "iceberg" mood profile, which combined the vigor above-average, with anger, confusion, depression, fatigue, and tension below-average. Based on these facts, a new field of research was provided, and the subsequent reports reinforced the proposal that mood state is one of the most important precompetitive predictive aspects of sports performance and could be a decisive factor in athletes' results (Beedie, PC, & AM., 2000). Recently, Lochbaum and colleagues (2021) suggested that analysis of mood state by the parameters of depression, confusion, and vigor can be useful to define athletic performance when measured prospectively.

Particularly in terms of female athletes, the literature highlights the importance of monitoring not only psychological aspects but also hormonal variation in order to understand their influence on performance, since it has been demonstrated that females in the mid-luteal phase showed reductions in both maximal test (Julian, 2017) as well as in prolonged exercise performance (Xanne, 2003). Moreover, the phase of the menstrual cycle (follicular and luteal phases) and the presence of premenstrual syndrome (PMS) can also impact the inflammatory condition related to mood states and stress hormones in female athletes, as previously reported by our group (Foster, 2019). Corroborating these pieces of information, Gaion and Vieira (2011) reported that the occurrence of PMS leads to negative changes in the mood states of athletes during the premenstrual period, which can be correctly assessed through the determination of circulating levels of sex hormones (estrogen and progesterone).

According to the literature, the mood has been composed of six states: tension, depression, anger, confusion, vigor, and fatigue. Moreover, it can be defined as a set of feelings, ephemeral in nature, with the possibility of varying in intensity and duration and usually involving more than one emotion. In an interesting way, mood presents an evaluative component, particularly related to the degree to which the feelings are perceived such as pleasant or unpleasant, in association with an arousal component characterized by varying degrees of activity (Lane & Terry, 2000).

Among the (patho)physiological mechanisms related to mood disorders, alterations in both hypothalamic-pituitary-adrenal (HPA) axis and in inflammatory/immune responses have been highlighted over the last few decades, besides (Caspi, et al., 2003; Mayberg, 1997; Haroon, Raison, & Miller, 2012; Bao, 2019). There is a growing of pieces of evidence highlighting the role of the association of inflammation in mood modulation, which seems to correlate with all of the other proposed mechanisms, such as the neurotrophin, neurotransmitters, and the HPA axis (Haroon, Raison, & Miller, 2012; de Miranda, 2020).

As suggested by Mucci (2020) the increase in the systemic levels of some proinflammatory cytokines, Interleukin (IL)-1β, IL-6, interferon-γ (IFN-γ), and tumor necrosis factor-alpha (TNF-α), are associated with alterations the mood. In fact, it has been reported that systemic inflammation can elicit neuroinflammation through glial activation (Campbell & MacQueen, 2006) since it is well-known that cytokines can cross the blood-brain barrier and stimulate immune and endothelial cells to produce inflammatory mediators (Haroon, Raison, & Miller, 2012).

It has been suggested that the intestinal microbiota can affect the levels of stress hormones released by the HPA axis, which consequently can also modulate the inflammatory/immune response. In this sense, there is a handful of evidence showing the modulatory effect of probiotics on neurotransmitter signaling and neuronal activation in the brain, as well as in the modulation of hormones and other essential molecules in the body, such as cytokines (Bravo, Julio-Pieper, Forsythe, & al., 2012; Appleton, 2018).

In order to exemplify this prominent modulatory characteristic, particularly in terms of the probiotic LcS, it was reported by Otaka and collaborators that the daily administration of 80

billion LcS to depressed patients was able to improve the symptoms of depression (Otaka, 2021). Corroborating this report, Zhang and collaborators showed that the daily administration of 10 billion LcS to depressed patients not only demonstrated the ability to improve both constipation and depressive symptoms but as well as was able to significantly reduce the systemic IL-6 levels (Zhang, 2021).

In a general way, the scientific literature highlights that alterations in neuroendocrine and neurogenesis pathways, synaptic and plasticity functions, and immune/inflammatory responses are involved in depression pathogenesis (Zhang, 2021; Wu, 2022; Ribeiro, 2022). Specifically in terms of the immune/inflammatory responses, it has been documented that depressive patients present high levels of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and also that this elevation can be an important role in major depression pathogenesis (Zhang, 2021; Rudzki, 2019; Yun, 2021).

As formerly mentioned, alterations in immune/inflammatory responses, which involves a remarkable increase in the systemic levels of some cytokines, such as IL-1β, IL-6, and TNF-α, also can be observed during exercise training performance, preferentially in exercise sessions with high-intensity or strenuous and prolonged, which can lead to an increase in the possibility of those athletes to present URTIs (Walsh, 2011; Vaisberg, 2019). Moreover, it was also cited that the daily administration of 40 billion LcS was able to mitigate the systemic elevation of proinflammatory cytokines in amateur athletes after a performance of a marathon race (Vaisberg, 2019).

Taking these pieces of information together, in which both depressed patients and athletes, particularly after exhaustive physical exercise sessions, presented a systemic proinflammatory state, it is reasonable to consider that the administration of probiotics, such as LcS, not only can affect athletes' mood states, similarly as depressed patients, but also the immune/inflammatory responses after a competition of triathlon, which is classified as a strenuous and prolonged physical exercise session.

Triathlon is a sport that combined different types of physical exercises, such as swimming, cycling, and running in sequence, which are separated by two transition moments, between swimming and cycling, named transition 1, and another between cycling and running, named

transition 2. Of interest, this sport can be performed in different distances, ranging between short and long distances, which are defined and classified by the International Triathlon Union (ITU), the institution that organizes this modality around the world. In order to clarify these differences, a short distance is usually composed of 750 meters of swimming, followed by 20 km of cycling, and, finally, 5 km of running, whereas a standard distance is composed of 1,500 meters of swimming, followed by 40 km of cycling, and 10 km of running, and a long distance is composed of a minimum distance of 1,900 meters of swimming, followed by 90 km of cycling, and 21 km of running.

Based on the previously mentioned, swimming is the first exercise modality in the triathlon competition, being performed in rivers, seas, and lakes, which imposes the athletes to different climatic conditions, such as water salinity levels, turbulence, and temperature. In this last case, it is noteworthy to point out that when the temperature is below 21^{o}C, athletes can wear neoprene clothes up to 5 mm thick, but in situations with water temperature below 14^{o}C the distance can be reduced in order to reduce exposure to the cold and possible occurrence of hypothermia, or even the swimming stage can be canceled (Bentley, McNaughton, Lamyman, & Roberts, 2003). At the end of the swimming stage, the athletes proceed to the transition area, where they will have to equip themselves to start the cycling stage, this time being counted as race time.

In the cycling stage, the athlete must pedal on his own without being behind the other athlete or a group of athletes, in order to avoid the athlete to face less air resistance, which can influence the high economy of movement, generating a lower degree of effort to sustain the same performance that, consequently, will lead to the increase of performance in the next competition stage. Hausswirth et al. (2001) observed that in this stage, the benefits from the vacuum situation are associated with a decrease in oxygen consumption, heart rate, and lactate concentration, when compared to the situation without a vacuum. At the end of the cycling stage, the athletes return to the transition area, where they will store cycling equipment and prepare for the next and last stage, that is, the race.

Therefore, the race is performed with the accumulation of fatigue generated in the previous stages, promoting a high energy cost, which will be superior to the demand necessary to sustain the same speed as compared to an isolated race (Hausswirth C. &., 2001).

It is utmost of importance to clarify that due to the influence of one stage on the others, triathlon competition must be considered a unique modality and not the grouping of three different modalities. Thus, the athletes need to perform an exercise training program that allows them to perform all these modalities with the optimal physical and physiological conditions, which can lead them to achieve the best performance in the competition.

Based on the literature, although several aspects of triathlon competition already having been reported, there are still few studies that aimed to evaluate metabolic changes and immune/inflammatory responses in triathletes.

Among the studies that focused on this issue, Momesso et al. (2022) evaluated the impacts of triathlon competition in extreme conditions, and the authors found significant alterations not only in the systemic lipid profile and inflammatory status but also in whole blood cell culture stimulated or not with lipopolysaccharide (LPS), a pro-inflammatory agent. In view of these findings, the adoption of strategies that could mitigate the negative impacts associated with the performance of a strenuous and prolonged competition on those athletes, such as dietary interventions, for example, using probiotics, can be useful. Corroborating this proposal, Huang et al. (2019) analyzed the effect of supplementation with *Lactobacillus plantarum* PS128 in a group of triathletes, and the authors highlighted that this bacteria strain, by its expressive ergogenic potential, can be a useful strategy to the athletes achieving better exercise training management, physiological adaptations to this physical exercise and health promotion. Moreover, the same authors reported that the *Lactobacillus plantarum* PS128 supplementation was associated with an improvement in athletes' performance through microbiota and metabolic modulation, but not maximal oxygen consumption (Huang W. C., 2020).

Taking these pieces of information together, it is clear that the understanding of the mechanisms involved in the triggering of disorders in systemic and upper airways, particularly in the sport context, is still of great importance, since both in professional and non-professional competitive sports, there is a large increase in the number of practitioners. Furthermore, the evaluation of the effects of supplementation with probiotics, specifically LcS, on athletes submitted to a strenuous and prolonged exercise session, such as a triathlon, not only can improve our knowledge concerning the association between alterations in

immune/inflammatory status and susceptibility to the manifestation of upper airway symptoms, but, depending on the results found, will allow clarifying whether the supplementation with this probiotic can be useful to prevent or even modulate those alterations that can lead to systemic and upper airway disorders in triathletes.

OBJECTIVES

PRIMARY

Investigate the effect of daily ingestion of fermented milk containing 40 billion LcS on the changes in the concentration of salivary IgA in amateur triathletes participating in this study, before and after an official triathlon competition.

SECONDARY

Evaluate the effect of daily intake of fermented milk containing 40 billion LcS on both systemic immune/inflammatory and upper airway responses, as well as the incidence and duration of upper airway symptoms, and also on mood states in amateur triathletes participating in this study, before and after an official triathlon competition.

TRIAL DESIGN

The study is designed as a multi-center, randomized, controlled, researcher and volunteer blinded, with two parallel groups (intervention and placebo). In addition, the randomization will be performed as block randomization with a 1:1 allocation.

MATERIAL AND METHODS

Registration and adherence to SPIRIT standards

This study will follow SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) guidelines for randomized clinical trials, and results will be reported following 2010 CONSORT (Consolidated Standards of Reporting Trials) standards (http://www.consort-statement.org/). In addition, it is important to mention that we intend to register the study on ClinicalTrials.gov as soon as this proposal of study is approved and also perform the sequential dating of each protocol version in order to mitigate potential confusion over which document is the most recent.

Population and design of the study

In order to perform this research project, it is proposed to recruit and select 70-80 amateur triathletes, between 25 to 45 years, both sexes, residing in the city of São Paulo, Brazil, who will be participating in the same triathlon competition.

Regarding the sample size, it was determined using G*Power software (version 3.1.9.6). The analysis was conducted using the F tests - ANOVA: repeated measures, within-between interaction option to accommodate the mixed design of the study, which includes two groups and five repeated measurements. It is important to mention that to carry out the sample size calculation alignment with the study's primary objective, which is associated with the salivary IgA, we used the data presented in the study performed by Vaisberg et al., 2019. In agreement with this data, the following parameters were used to calculate the required sample size: Effect size (f): 0.15 (Vaisberg et al., 2019). Alpha error probability (α): 0.05. Power (1-β error probability): 0.85. Correlation among repeated measures: 0.5. Nonsphericity correction (ε): 1. Based on these inputs, the total sample size required to achieve the desired power of 0.85 is 62 participants. Thus, 70-80 athletes will be included in this study. This sample size will be distributed equally between the two groups (35-40 participants per group).

It is noteworthy to clarify that we intend to separate the volunteers into two groups: male amateur triathletes, which will be composed of 40-45 individuals, and female amateur triathletes, which will be composed of 30-35 individuals. This discrepant number is associated with the fact that the number of male participants in the triathlon competition is higher than the number of female participants. For instance, in 2022, the triathlon competition "IronMan 70.3 São Paulo" presented a total number of 1858 participants, of which 1237 completed the competition. In fact, whereas 1027 men completed the competition, only 210 women conclude this same competition (available in https://www.ironman.com/im703-sao-pauloresults). Based on these pieces of information, we decide to not separate equally the number of volunteers to compose the groups of male and female individuals. In order to achieve the number of volunteers´ groups (male and female) we will invite the athletes subscribed to the triathlon competition through direct contact with the athletes´ consultancies, and also by sending the invitation to their social media.

All individuals will be initially evaluated through clinical examinations, and it will exclude the volunteers who: (1) report intolerance to lactose; (2) are under any type of pharmacological treatment; (3) report chronic rhinopathy; (4) have autoimmune diseases; (5) report the occurrence of non-airway recurrent infections. Besides, (6) whether any data from a volunteer is missing, this participant will be excluded from the study. As an inclusion criterion, it will inquire if the participants already presented the occurrence of any upper airway symptoms after an official triathlon competition. In this sense, according to Andersen et al. (2013), the incidence of diseases in triathletes is similar to other individual sports, such as marathons and swimming, with an incidence of approximately 30% being reported. It is worth clarifying that, according to the same authors, any health problems that are not related to the musculoskeletal system are considered a disease in this context, particularly in terms of the occurrence of similar symptoms of flu post-competition.

It is also important to cite that the participants will be instructed not to consume products containing probiotics or other fermented drinks during the study, and if any volunteer reports the regular habit of consuming such products, he will be oriented to interrupt this consumption for, at least, two weeks before the start of the study (a washout period).

In addition, volunteers invited to perform in this study will receive all the information related to the present study and those to agree in participate will be oriented to sign the Informed Consent Term, which must be previously approved by the Research Ethics Committee of the Federal University of São Paulo (UNIFESP), Brazil. The study is in agreement with both the Ethical Standards and the Declaration of Helsinki.

It is important to clarify that there will be no official data monitoring committee established for this study. Thus, the only institution responsible for following the development of the study is the Ethics Committee, through regular reports.

In order to improve the volunteers´ adherence to the present study we will offer and provide the results concerning the performance of each one in the physical tests, especially concerning the cardiopulmonary test, as well as those related to the laboratory tests. All physical and laboratory tests are following described.

Anamnesis, physical examination, and competition

At the beginning of the study, all participants will undergo an anamnesis with a cardiovascular and otorhinolaryngological analysis to exclude the presence of cardiovascular diseases and upper airway symptoms resulting from rhinosinusitis, rhinopathies, or any other pre-existing alteration in these systems. In addition, the WURSS (Wisconsin Upper Respiratory Symptom Survey) questionnaire will be used to verify the occurrence of upper airway symptoms not only during this period but also throughout the study period (Annex 1). and the presence of premenstrual syndrome will be self-reported by the female volunteers during the anamnesis.

It is noteworthy to cite that the volunteers will be oriented to maintain their usual daily routine, including sleep, diet, and training/physical activity during the study performance. In this respect, to characterize not only the quality of life (Annex 2) but also the level of physical activity (Annex 3), questionnaires will be applied with open questions about the participants' lifestyle and habits, history and current habits of physical activity, as well as participation in competitions. Beyond these questionnaires, it will be also assessed the occurrence of discomfort or gastrointestinal problems related to the daily intake of fermented milk containing LcS or not by using a questionnaire with open questions about gastrointestinal problems will be used (Annex 4). The volunteers who reported any gastrointestinal discomfort during the ingestion of the fermented milk containing or not LcS will be oriented to immediately interrupt this ingestion and seek medical assistance. In case of the volunteer needs to interrupt the ingestion of fermented milk for five consecutive days, following medical recommendation, this volunteer and its data will be excluded from the study.

It is utmost of importance to point out that all athletes will have medical follow-ups by Dr. Mauro Walter Vaisberg, a specialist in Sports Medicine, throughout the study. Besides, through social media, all volunteers will be able to directly contact not only Dr. Mauro Vaisberg but also other researchers involved in the study´s development in order to communicate any adverse events and other unintended effects of trial interventions.

Evaluation of the External Training Load

The external training load will be determined by the sum of time dedicated to each training modality (swimming, cycling, and running), besides the time spent in each of the intensity zones (light, moderate and intense) during the training weeks.

Evaluation of the Internal Load of Training

The internal training load will be established by the relationship between the time dedicated to training and the subjective perception of the effort of the session (PSE-session), which is determined through the use of the subjective effort scale modified by Foster et al. (1998), as proposed by Impellizzeri et al. (2004).

Soon after the exercise session, the Borg CR-10 (Borg Category-Ratio) scale adapted by Foster (1998) will be presented to athletes (Annex 5).

Therefore, the internal training load values obtained from this relationship will be expressed in arbitrary units (AU).

Cardiopulmonary Test

The physical capacity of each volunteer will be characterized through a cardiopulmonary test that will be performed on a treadmill attached to the gas analyzer, with electrocardiography and gas analysis software. This test will be performed at the Research and Training Olympic Center, an institution belonging to the Municipal Secretariat of Sports, Leisure, and Recreation of the City of São Paulo.

In order to carry out the cardiopulmonary test, the patient's preparation rules will be followed, which include hair removal from the place where the electrodes will be placed, cleaning the skin with alcohol, and scarification with water sandpaper number 400. The placement of the electrodes will follow the described by Salient (2019) for the formation of the 12 leads in the Einthoven triangle.

The cardiopulmonary test will be performed with a ramp protocol, with a fixed incline of 1% and load increment (1km/h) every minute until the maximum speed of the treadmill, at which time the increment of 2% incline begins every minute. The protocol should be started

with 10.9 or 8 km/h according to the time for a 10 km race, with the time below 35', between 35' and 46', and above 46', respectively. Tests should be maximum, being interrupted by limiting symptoms / intense physical fatigue.

The data obtained in this test in conjunction with body composition will be used to stratify the volunteers into four different strata. In addition, the randomization of volunteers in each strata will follow the process: firstly, the researcher JBA, will be responsible for creating a computer-generated random list of numbers for the treatment groups of either LcS or placebo, and after each volunteer will be anonymously and sequentially allocated in these groups according to their recruitment ordering number in the program.

LcS ingestion

After stratification, the volunteers will be separated into two groups: (1) LcS Group (LcSG), which will ingest 1 bottle of fermented milk (80 grams) with 40 x 10^9 of LcS per day; and (2) placebo/unfermented milk Group (PLG), which will consume 1 bottle of unfermented milk (80 grams), due to the absence of LcS. Both groups will begin the ingestion 30 days before the competition, maintaining it until 14 days after the competition. As previously mentioned, all volunteers will be oriented to stop drinking immediately and seek medical attention if any symptoms of gastrointestinal disturbance are perceived. To ensure the development of a double-blind study, all bottles used in the study will be kindly provided by YAKULT S/A Industria e Comércio, Brazil. The bottles will be named A and B by the company and will have the same taste, appearance, and smell. Information on the presence or absence of LcS in the bottles will be accessed only after all analyzes have been performed. In addition, to monitor the adherence of volunteers to the study´s protocol, we intend not only to request the return flask fortnightly (empty or not) but also to maintain routine contact via social media with the volunteers. This contact also will allow us to provide responses and orientations to any questions by them, as well as, weekly, to perform auditing concerning the study´s conduction.

It is important to clarify that the Yakult S/A Industria e Comércio, Brazil, will provide the fermented milk containing LcS and also the placebo/unfermented milk. In addition, Yakult Honsha Co., Ltd. Japan, will fund the development of the study, which includes all the materials needed to perform the collection, procedures, storage, and laboratory evaluations of

the biological samples obtained on all occasions described in the study. Besides, it is noteworthy to mention that the design, management, data check and analysis, and preparation of the reports/articles of the study are entirely under the responsibility of the researchers.

Regarding the ingestion period, it is based on the study by Vaisberg et al. (2019), which showed a remarkable capacity of fermented milk containing LcS in modulating several immune/inflammatory parameters, both systemic and in the upper airways, in runners after a marathon.

In order to obtain the best quality in our results, we will follow the experimental design described below (Figure 1), highlighting that some procedures will be performed on the same day of sample collection.

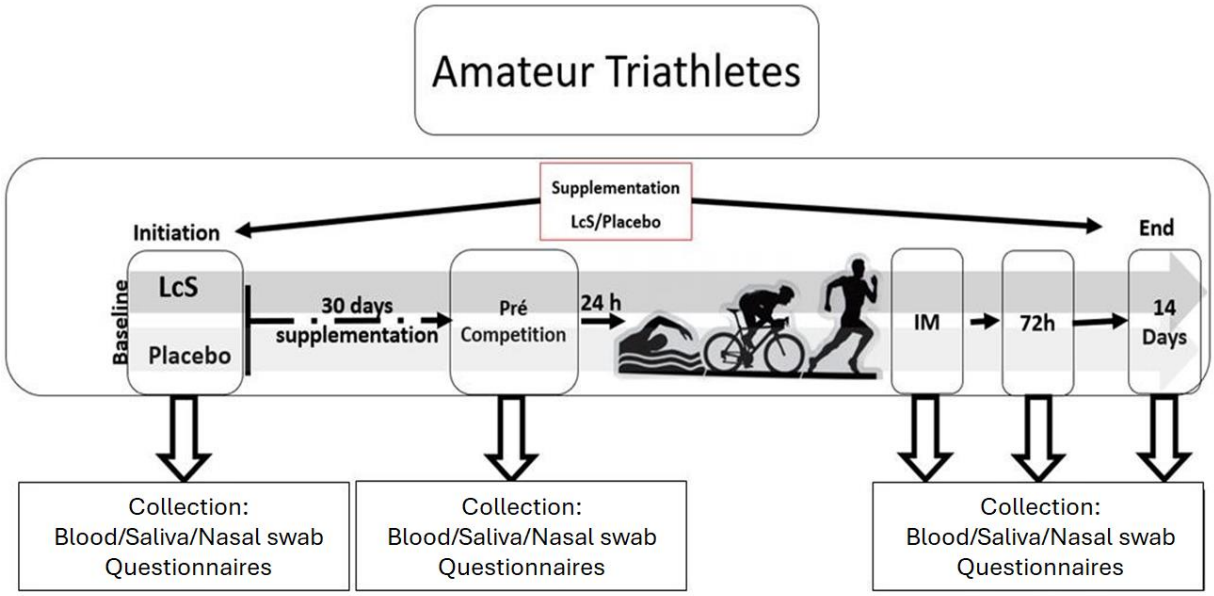

Figure 1 - Study design.

Triathlon competition

It is important to clarify that the volunteers will perform the same triathlon competition, which will be part of the annual calendar for this type of sport. In this respect, it is worth mentioning that we intend to carry out the study by inviting the athletes that will perform the IronMan 70.3 São Paulo in 2025, which, in a general way, occurs in September, and is

composed of 1,900 meters of swimming, followed by 90 km of cycling, and 21 km of running (https://www.ironman.com/im703-sao-paulo).

Besides, to motivate and ensure that all volunteers will perform the competition with maximal effort, the official time of each volunteer to end the competition, obtained through the data available by the organizing committee of the competition, will be used to define their ranking in the present study and, consequently, the best triathletes in this competition.

Sample collection

Blood, saliva, and nasal swab will be collected on five different occasions: (1) before the beginning of the ingestion of fermented milk containing LcS or placebo; (2) 24 hours before the triathlon competition; (3) immediately (IM); (4) 72 hours (post-72h); and (5) 14 days after the competition, as shown in Figure 1. In addition, on the same occasions, the occurrence of upper airway symptoms and mood states will also be assessed by using questionnaires.

Collection of blood samples

Blood samples will be collected in fasting condition, from an antecubital vein in tubes containing or not EDTA anticoagulants. After blood clotting or not, the tube will be centrifuged at 2500 rpm for 10min to obtain 500 µl of serum or plasma, which will be stored at -80^{o}·C for later use to determine circulating cells and serum/plasmatic parameters, as described below.

Blood cell count

Blood cell count will be carried out on the same day using blood obtained in a tube containing EDTA. All subjects must be hydrated at the time of collection. Cell counts (erythrocytes, leukocytes, and platelets) will be performed using the automated system Coulter brand AcTDiff model (Beckman Coulter, USA).

Whole blood culture

A volume of 100µL of the blood sample will be mixed with 900µL of culture medium, containing 10% fetal bovine serum + RPMI 1640, and will be incubated for 48 hours in 48 well plates, maintained in an air-conditioned oven with 95% oxygen and 5% carbon dioxide at

37°C. At the same time point, another well containing the same mixture of blood and medium will be submitted to stimulation with lipopolysaccharide (LPS) and maintained under the same conditions as previously reported. After the incubation period, the culture supernatant will be collected and centrifuged at 1400 rpm for 10 minutes, and a volume of, at least, 500µL will be frozen at 80°C for further evaluation of cytokine concentration.

Saliva sample collection

Two milliliters (2 mL) of saliva samples will be collected from each volunteer directly in 15 mL sterile Falcon tubes, without stimulation. After collection, each sample will be maintained at 4°C until to be centrifuged at 3000 rpm for 10 minutes at 4°C. After that, 500µL of the supernatant will be transferred to 1.5 mL conical tubes and frozen at -80°C for further later determination of the concentration of cytokines, IgAs, and antibacterial peptides, without adding a preservation medium.

Assessment of the nasal mucosa infiltrate

Determination of the percentage of infiltrating cells in the nasal mucosa will be carried out with samples obtained from a swab introduced into each nostril of the volunteer and the cells obtained will be placed on a slide, followed by their fixation with methanol (99% Synth), and then stained in hematoxylin, for later analysis by an experienced pathologist.

Determination of the concentration of IgAs and antimicrobial peptides

The salivary concentration of IgAs and the antibacterial peptides [LL-37 (cathelicidin), defensin-1, lactoferrin, and lysozyme] will be determined using ELISA commercial kits (Biosay Technology Laboratory, Shanghai, China), in accordance with the manufacturer's instructions.

Determination of cytokine concentration

The concentration of cytokines IL-1β, IL-1ra, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL13, IL-17A, and TNF-alpha, both in serum, saliva, as in the whole blood culture supernatant, stimulated or not with LPS (Lipopolysaccharide), will be determined by using LEGENPlex for human cytokines, which is based on a bead system (BioLegend, San Diego, CA, USA), following manufacturer's instructions. The cytokine concentrations will be calculated from the

values of their respective standard curves, obtained at the same time as the evaluation of the samples.

Immune cell immunophenotyping

Evaluation of the number, type, and activation profile of NK cells and T lymphocytes will be carried out with blood samples collected in tubes containing EDTA anticoagulant that will be mixed 1:1 with Ficoll-Paque (density gradient 1077, FicollPaqueTM PLUS, GE Healthcare, Sweden). After centrifugation for 30 minutes at 2000 rpm, the cell pool, corresponding to the fraction of mononuclear cells will be collected, counted, and 1 x 10^5 cells will be added in 1.5 mL conical tubes. Then, these cells will be washed with MACSBuffer [PBS (1x) containing bovine serum albumin (0.5%) and EDTA (2 mM)]. After this step, the cells will be incubated with a mix of monoclonal antibodies to evaluate the type, concentration, and activation profile of these cells, as shown in Table 1, by using the antibodies to the surface molecules: CD3, CD4, CD8, CD16, CD27, CD38, CD45, CD56, CD70, CD197, and HLA-DR.

Table 1. Description of the panel of antibodies to be used in immunophenotyping of NK cells and T lymphocytes.

Type	Profile	Surface Markers				
T Lymphocytes	Activation	CD3+	CD4+	CD38+	HLA-DR+	
			CD8+	CD38+	HLA-DR+	
	Naive	CD3+	CD4+	CD27+	CD45RA+	CD197+
			CD8+	CD27+	CD45RA+	CD197+
	Central Memory	CD3+	CD4+	CD27+	CD45RA-	CD197+
			CD8+	CD27+	CD45RA-	CD197+
	Effector Memory	CD3+	CD4+	CD27-	CD45RA-	CD197-
			CD8+	CD27-	CD45RA-	CD197-
Natural Killer Cells		CD3+	CD16+	CD56+		

Data acquisition will be performed using a BD Canto cytometer (Becton Dickinson Immunodiaginostisc System, San Josem CA, USA). The definition of the gates will be based on the analysis of the FMO (Fluorescent Minus One).

Cytotoxicity Assay of NK cells

The *in vitro* assessment of the activity of NK cells will be carried out by using the commercial kit NKVUE tubes, which allows for evaluating the activity of NK cells through the production of interferon-gamma (Nederby, 2018). In addition, the cytotoxic activity of NK cells will be also assessed by using another test, which will be initiated by the isolation of these cells from peripheral blood using the commercial Invitrogen Dynabeads Untouched Human NK Cell kit (Thermo Fisher Scientific, Invitrogen, Vienna, Austria), which allows the isolation of these cells by negative selection. Then, using the commercial kit NKTEST, we will evaluate the cytotoxic activity of NK cells isolated by mixing these with K562 target cells, in a ratio of 5:1 and 2:1, following the manufacturer's guidelines.

Determination of the systemic concentration of BDNF and hormones

Serum levels of BDNF (pg/mL), cortisol (ngmL-1), and hGH (mLU mL-1) will be determined using ELISA commercial kits [BDNF Quantikine ELISA kit (Catalog number: SBD00); Cortisol ELISA EIA-1887 (Catalog number: SKGE008B; hGH ELISA EIA-3552 (Catalog number: SG100) R&D System, Inc. USA]. In addition, serum levels of sex hormones [progesterone (pg/mL), estrogen (pg/mL), and testosterone (pg/mL)] will also be determined by ELISA commercial kits [Testosterone Parameter Assay Kit (Catalog number: SKGE010), Estradiol Parameter Assay Kit (Catalog number: KGE014), Human Total Progesterone ELISA (Catalog number: DYC5415-5), R&D System, Inc. USA]. It is noteworthy to clarify that these parameters will be evaluated in all volunteers (both in men and women groups), whilst, in particular, the data obtained in the evaluation of the sexual hormones progesterone and estrogen also will be used to define the phase of the menstrual cycle in which each female volunteer of the study will be during the five different occasions proposed in the present study. All evaluations will follow the manufacturers' guidelines.

Assessments of upper airway symptoms

The WURSS-21 questionnaire (Wisconsin Upper Respiratory Symptom Survey – 21 items) (Annex 1) will be used to assess the occurrence of upper airway symptoms. This instrument is composed of 21 questions, in which the first question is associated with the individual's perception of "how sick do you feel today?", followed by 10 questions based on specific

symptoms of the cold condition, more 9 questions related to the impact of cold symptoms in the individual's quality of life, and one last question comparing the current status of the cold symptoms with the previous day. All items will be scored on a Likert scale ranging from 0 to 7 (from 0 = no symptoms to 7 = severe).

The severity of the upper airway symptoms will be measured from the sum of the scores of all the days in which it was evaluated, with the final score divided by the number of days in which there were reports of symptoms. In terms of the severity in relation to the interference in the volunteer's quality of life, it will be obtained the sum of the scores of all the days in which there was a report, and the final score will be divided by the number of days with symptoms. In addition, to analyze the percentage of incidence of the group, the number of days with symptoms of each group will be calculated, adjusting for the number of volunteers in the group. Then, the adjusted values of each group will be added, considering this value as 100%, from which the percentage of each group will be calculated.

Assessment of mood state

The Brunel mood scale (BRUMS, Annex 6) will be used to assess the mood state. The BRUMS involves 24 mood indicators, divided into six subitems, namely anger, confusion, depression, fatigue, tension, and vigor. The volunteer should indicate on a scale from 0 (zero) to 4 (four) what they are feeling on the time-point of application of the BRUMS. (Rohlfs, 2008).

Statistical analysis

All data obtained during the development of this study will be added to a unique file, preferentially in an Excel file, which will be available to all researchers not only to consult and check the data quality/accuracy but also to add any results through GOOGLE-DRIVE. It is noteworthy to clarify that, to perform all the statistical analysis proposed here, we will follow the "intention-to-treat" (ITT) analysis, not only because it is recommended as an analysis strategy for randomized studies but also to be possible to maintain the sample size, as well as to use the data obtained from the participants regardless of whether they complete the study since ITT considers all randomized participants in the analysis, whether they drop out or not. In addition, the multiple imputation (MI) method will be applied to handle missing data since

MI fills in missing values by generating plausible numbers derived from distributions of and relationships among observed variables in the data set. Beyond these analyses, in order to check and increase the reliability of the results obtained in the ITT analysis, we also intend to conduct the statistical analysis regarding the per-protocol (PP) analysis, which will be used exclusively the complete data obtained from the volunteers.

After all data will be added to the Excel file and the data quality/accuracy is checked by the researchers, these data will be initially compared with the Gaussian curve, and normality determined for each variable.

If the variable presents a parametric behavior, the mean and standard deviation will be used, and they will be compared by using the ANOVA test for repeated measures with post hoc tests for intragroup (longitudinal) and intergroup (cross-sectional) analysis.

On the other hand, if the data present a non-parametric behavior, they will be represented by median and interquartile ranges, and they will be compared by using Friedman's test with Müller-Dun post hoc test for intragroup (longitudinal) and the Kruskal-Wallis test with Müller Dunn's post hoc test for intergroup (cross-sectional).

Using Pearson (for parametric values) or Spearman tests (for non-parametric values), the correlation between the variables studied will be evaluated.

In addition, the McNemar Chi-square test (with Yates correction) will be used to determine whether the difference in the number of volunteers who presented or not upper airway symptoms, as well as in the duration of the symptoms, is significantly different in each group (paired analysis). Additionally, Chi-square test will be used to perform the same analysis but between the volunteer groups (unpaired analysis).

A risk of less than to 0.05 ($p<0.05$) will be considered statistically significant for every study.

Researchers´ information and contributions

First of all, it is important to mention that the researchers' names and affiliations are:

- Jônatas Bussador do Amaral (JBA), from the Federal University of São Paulo (UNIFESP) and the sponsor-investigator (Address: Rua Pedro de Toledo, 781, 1o. andar, sala 13, Vila Clementino, 04039-032, São Paulo, Brazil, e-mail: amaraljb@gmail.com).
- César Miguel Momesso dos Santos (CMMS), from United Metropolitan Colleges, Centro Universitário FMU.
- André Luis Lacerda Bachi (ALLB), from Santo Amaro University (UNISA)
- Mauro Vaisberg (MV), from the Federal University of São Paulo (UNIFESP)

In addition, the researcher's contributions are: CMMS, ALLB, and JBA wrote the first draft of the study. CMMS, ALLB, JBA, and MV will conduct the study. CMMS and MV will be responsible for the recruitment and selection of volunteers, for the application of anamnesis, physical examinations, evaluation of the external and internal training load, and cardiopulmonary test. MV will be responsible for the clinical follow-up of the volunteers. JBA will be responsible for the volunteers' randomization. CMMS, ALLB, and JBA will be responsible for the collection, procedure, and storage of the biological samples, as well as for the performance of laboratory tests. CMMS and ALLB will be responsible for conducting the primary statistical analysis. All researchers equally will contribute to the refinement of the study protocol and will participate in the preparation of the report(s) and manuscript(s).

WORK PLAN AND SCHEDULE

For the performance of the present study, we propose a work plan and schedule that will need 12 months to be developed. Thus, a scale of monitoring and collection of materials will be recommended, which initially will depend on the availability of each volunteer recruited to carry out the long-term triathlon competition.

Thus, we propose the following work plan and schedule:

- Recruitment of athletes and performance of clinical evaluations on all volunteers included in the study;

- Collection of blood, saliva, and nasal swab samples before starting the daily intake of fermented milk containing or not the bacteria (LcS group, n=35-40; Placebo group, n=35-40, respectively).

- study volunteers will ingest the fermented milk for 44 days;
- Collection of blood, saliva, and nasal swab samples 24 hours before the competition;
- realization of the competition by all volunteers included in the study;
- Collection of blood, saliva, and nasal swab samples immediately after the end of the competition;
- Collection of blood, saliva, and nasal swab samples 72 hours after the competition;
- Collection of blood, saliva, and nasal swab samples 14 days after the competition;
- Evaluation of the occurrence of upper airway symptoms throughout the study´s period;
- Performing the laboratory evaluations described in the Material and Methods section; - Statistical analysis of the results and preparation of the report.

SCHEDULE OF ACTIVITIES TO BE DEVELOPED

Parameters	Enrollment	Allocation	Intervention				
			Pre-Triathlon		Post-Triathlon		
Timepoint	-60d to -30d		-30d	-1d	0d	3d	14d
Recruitment and selection of volunteers	X						
Informed consent	X						
Anamnesis, anthropometry, cardiopulmonary test	X						
Randomization		X					
Intervention: LcS/Placebo intake			X	X	X	X	X
Questionnaire - WURSS	X			X		X	X

Questionnaire - IPAQ	X			X			
Questionnaire - gastrointestinal problems	X			X		X	X
Questionnaire – SF36	X			X		X	X
Questionnaire – BRUMS	X			X		X	X
Questionnaire - Subjective Perception of Effort Scale	X				X		
Blood, saliva, nasal swab collection			X	X	X	X	X

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ANNEX I – Questionnaire Wisconsin Upper Respiratory Symptom Survey - 21

Wisconsin Upper Respiratory Symptom Survey - 21 --- Daily Symptom Report

Dia:	Data:	Horário:	Identificação:

Por favor, preencha um círculo para cada um dos seguintes itens:

	Não estou doente	Muito levemente		Levemente		Moderadamente		Severamente
	0	1	2	3	4	5	6	7
O quanto você se sente doente/enfermo Hoje?	O	O	O	O	O	O	O	O

Por favor, registre a **severidade média dos seus sintomas de resfriado nas últimas 24 horas**para cada ítem de sintoma

	Não tenho este sintoma	Muito levemente		Levemente		Moderadamente		Severamente
	0	1	2	3	4	5	6	7
Coriza	O	O	O	O	O	O	O	O
Nariz tapado	O	O	O	O	O	O	O	O
Espirrando	O	O	O	O	O	O	O	O
Dor de garganta	O	O	O	O	O	O	O	O
Garganta raspando/pegando	O	O	O	O	O	O	O	O
Tosse	O	O	O	O	O	O	O	O
Rouquidão	O	O	O	O	O	O	O	O
Congestão de cabeça	O	O	O	O	O	O	O	O
Congestão peitoral	O	O	O	O	O	O	O	O
Sentindo cansado	O	O	O	O	O	O	O	O

Nas últimas 24 horas, quanto seu resfriado tem interferido na sua capacidade de:

	Não tem interferido	Muito levemente		Levemente		Moderadamente		Severamente
	0	1	2	3	4	5	6	7
Pensar claramente	O	O	O	O	O	O	O	O
Dormir bem	O	O	O	O	O	O	O	O
Respirar facilmente	O	O	O	O	O	O	O	O
Caminhar, subir escadas, se exercitar	O	O	O	O	O	O	O	O
Cumprir com atividade do dia a dia	O	O	O	O	O	O	O	O
Tarefas dentro de casa	O	O	O	O	O	O	O	O
Tarefas fora de casa	O	O	O	O	O	O	O	O
Interagir com as outras pessoas	O	O	O	O	O	O	O	O
Viver sua vida pessoal	O	O	O	O	O	O	O	O

Comparado com ontem, eu sinto que o meu resfriado está...

Muito melhor	Algo melhor	Um pouco melhor	Igual	Um pouco pior	Algo pior	Muito pior
O	O	O	O	O	O	O

WURSS-21 (Wisconsin Upper Respiratory Symptom Survey) 2004

Criado por Bruce Barret MD PhD et al., UW Department of Family Medicine, 777 S. Mills ST. Madison, WI 53715, USA

ANNEX II – Questionnaire SF36

Versão Brasileira do Questionário de Qualidade de Vida -SF-36

1- Em geral você diria que sua saúde é:

Excelente	Muito Boa	Boa	Ruim	Muito Ruim
1	2	3	4	5

2- Comparada há um ano atrás, como você se classificaria sua idade em geral, agora?

Muito Melhor	Um Pouco Melhor	Quase a Mesma	Um Pouco Pior	Muito Pior
1	2	3	4	5

3- Os seguintes itens são sobre atividades que você poderia fazer atualmente durante um dia comum. Devido à sua saúde, você teria dificuldade para fazer estas atividades? Neste caso, quando?

Atividades	Sim, dificulta muito	Sim, dificulta um pouco	Não, não dificulta de modo algum
a) Atividades Rigorosas, que exigem muito esforço, tais como correr, levantar objetos pesados, participar em esportes árduos.	1	2	3
b) Atividades moderadas, tais como mover uma mesa, passar aspirador de pó, jogar bola, varrer a casa.	1	2	3
c) Levantar ou carregar mantimentos	1	2	3
d) Subir vários lances de escada	1	2	3
e) Subir um lance de escada	1	2	3
f) Curvar-se, ajoelhar-se ou dobrar-se	1	2	3
g) Andar mais de 1 quilômetro	1	2	3
h) Andar vários quarteirões	1	2	3
i) Andar um quarteirão	1	2	3
j) Tomar banho ou vestir-se	1	2	3

4- Durante as últimas 4 semanas, você teve algum dos seguintes problemas com seu trabalho ou com alguma atividade regular, como conseqüência de sua saúde física?

	Sim	Não
a) Você diminui a quantidade de tempo que se dedicava ao seu trabalho ou a outras atividades?	1	2
b) Realizou menos tarefas do que você gostaria?	1	2
c) Esteve limitado no seu tipo de trabalho ou a outras atividades.	1	2
d) Teve dificuldade de fazer seu trabalho ou outras atividades (p. ex. necessitou de um esforço extra).	1	2

5- Durante as últimas 4 semanas, você teve algum dos seguintes problemas com seu trabalho ou outra atividade regular diária, como conseqüência de algum problema emocional (como se sentir deprimido ou ansioso)?

	Sim	Não
a) Você diminui a quantidade de tempo que se dedicava ao seu trabalho ou a outras atividades?	1	2
b) Realizou menos tarefas do que você gostaria?	1	2
c) Não realizou ou fez qualquer das atividades com tanto cuidado como geralmente faz.	1	2

6- Durante as últimas 4 semanas, de que maneira sua saúde física ou problemas emocionais interferiram nas suas atividades sociais normais, em relação à família, amigos ou em grupo?

De forma nenhuma	Ligeiramente	Moderadamente	Bastante	Extremamente
1	2	3	4	5

7- Quanta dor no corpo você teve durante as últimas 4 semanas?

Nenhuma	Muito leve	Leve	Moderada	Grave	Muito grave
1	2	3	4	5	6

8- Durante as últimas 4 semanas, quanto a dor interferiu com seu trabalho normal (incluindo o trabalho dentro de casa)?

De maneira alguma	Um pouco	Moderadamente	Bastante	Extremamente
1	2	3	4	5

9- Estas questões são sobre como você se sente e como tudo tem acontecido com você durante as últimas 4 semanas. Para cada questão, por favor dê uma resposta que mais se aproxime de maneira como você se sente, em relação às últimas 4 semanas.

	Todo Tempo	A maior parte do tempo	Uma boa parte do tempo	Alguma parte do tempo	Uma pequena parte do tempo	Nunca
a) Quanto tempo você tem se sentindo cheio de vigor, de vontade, de força?	1	2	3	4	5	6
b) Quanto tempo você tem se sentido uma pessoa muito nervosa?	1	2	3	4	5	6
c) Quanto tempo você tem se sentido tão deprimido que nada pode anima-lo?	1	2	3	4	5	6
d) Quanto tempo você tem se sentido calmo ou tranqüilo?	1	2	3	4	5	6
e) Quanto tempo você tem se sentido com muita energia?	1	2	3	4	5	6
f) Quanto tempo você tem se sentido desanimado ou abatido?	1	2	3	4	5	6
g) Quanto tempo você tem se sentido esgotado?	1	2	3	4	5	6
h) Quanto tempo você tem se sentido uma pessoa feliz?	1	2	3	4	5	6
i) Quanto tempo você tem se sentido cansado?	1	2	3	4	5	6

10- Durante as últimas 4 semanas, quanto de seu tempo a sua saúde fisica ou problemas emocionais interferiram com as suas atividades sociais (como visitar amigos, parentes, etc)?

Todo Tempo	A maior parte do tempo	Alguma parte do tempo	Uma pequena parte do tempo	Nenhuma parte do tempo
1	2	3	4	5

11- O quanto verdadeiro ou falso é cada uma das afirmações para você?

	Definitivamente verdadeiro	A maioria das vezes verdadeiro	Não sei	A maioria das vezes falso	Definitiva-mente falso
a) Eu costumo obedecer um pouco mais facilmente que as outras pessoas	1	2	3	4	5
b) Eu sou tão saudável quanto qualquer pessoa que eu conheço	1	2	3	4	5
c) Eu acho que a minha saúde vai piorar	1	2	3	4	5
d) Minha saúde é excelente	1	2	3	4	5

CÁLCULO DOS ESCORES DO QUESTIONÁRIO DE QUALIDADE DE VIDA

Fase 1: Ponderação dos dados

Questão	Pontuação	
01	Se a resposta for	Pontuação
	1	5,0
	2	4,4
	3	3,4
	4	2,0
	5	1,0
02	Manter o mesmo valor	
03	Soma de todos os valores	
04	Soma de todos os valores	
05	Soma de todos os valores	
06	Se a resposta for	Pontuação
	1	5
	2	4
	3	3
	4	2
	5	1

07	Se a resposta for 1 2 3 4 5 6	Pontuação 6,0 5,4 4,2 3,1 2,0 1,0
08	A resposta da questão 8 depende da nota da questão 7 Se 7 = 1 e s 'alor da questão é (6) Se 7 = 2 à 6 ‹ › valor da questão é (5) Se 7 = 2 à 6 e se 8 = 2, o valor da questão é (4) Se 7 = 2 à 6 e se 8 = 3, o valor da questão é (3) Se 7 = 2 à 6 e se 8 = 4, o valor da questão é (2) Se 7 = 2 à 6 e se 8 = 3, o valor da questão é (1) Se a questão 7 não for respondida, o escorre da questão 8 passa a ser o seguinte: Se a resposta for (1), a pontuação será (6) Se a resposta for (2), a pontuação será (4,75) Se a resposta for (3), a pontuação será (3,5) Se a resposta for (4), a pontuação será (2,25) Se a resposta for (5), a pontuação será (1,0)	
09	Nesta questão, a pontuação para os itens a, d, e ,h, deverá seguir a seguinte orientação: Se a resposta for 1, o valor será (6) Se a resposta for 2, o valor será (5) Se a resposta for 3, o valor será (4) Se a resposta for 4, o valor será (3) Se a resposta for 5, o valor será (2) Se a resposta for 6, o valor será (1) Para os demais itens (b, c,f,g, i), o valor será mantido o mesmo	
10	Considerar o mesmo valor.	
11	Nesta questão os itens deverão ser somados, porém os itens b e d deverão seguir a seguinte pontuação: Se a resposta for 1, o valor será (5) Se a resposta for 2, o valor será (4) Se a resposta for 3, o valor será (3) Se a resposta for 4, o valor será (2) Se a resposta for 5, o valor será (1)	

Fase 2: Cálculo do Raw Scale

Nesta fase você irá transformar o valor das questões anteriores em notas de 8 domínios que variam de 0 (zero) a 100 (cem), onde 0 = pior e 100 = melhor para cada domínio. É chamado de raw scale porque o valor final não apresenta nenhuma unidade de medida.
Domínio:

- Capacidade funcional
- Limitação por aspectos fisicos
- Dor
- Estado geral de saúde
- Vitalidade
- Aspectos sociais
- Aspectos emocionais

ANNEX III - Questionnaire of Physical Activity (IPAQ)

NOME.....

Data……..

As perguntas a seguir referem-se à quantidade de atividade física que você realizou na semana passada.

Para os 7 dias anteriores, por favor, considere a quantidade, o tipo e a intensidade da atividade física que realizaram. Se isso é mais ou menos do que o normal, isso deve ser refletido em suas respostas.

Este tipo de atividadepodeassumirqualquer forma e podemincluiratividadesdiáriasnormais, comocaminharaté as lojas.

Atividadefísicavigorosarefere-se a atividades que necessitaram de grandeesforçofísico para ser realizado/completado, além de exigir que vocêrespirassemais que o normal.

Atividadesmoderadasreferem-se a atividades que necessitaram de moderadoesforçofísico para ser realizado/completado, além de exigir que vocêrespirasse um poucomais do que o normal.

Com relaçãoaostreinos, tenteclassificarcadasessãointeiracomovigorosaoumoderada.

Marque de forma adequadanascaixasfornecidas.

Seja o maishonestopossível.

1. Nos últimos 7 dias, quantosdiasvocêparticipou de atividadesfísicasvigorosas, comolevantamento de peso, atividadesaeróbias, treinamentointenso, ciclismo, natação**o**u corrida?

   _____ Dias - Se nenhum, responda 00 e passe para a pergunta 3

2. Nos diasem que você fez a **atividadefísicavigorosa**quanto tempo vocêcostumagastarfazendoisso?

   _____Horas_____ Minutos

3. Nos diasem que você fez **atividadefísicamoderada**quanto tempo vocêcostumagastarfazendoisso?

   _____ Dias - Se nenhum, responda 00 e passe para a pergunta 4

   _____Horas_____ Minutos

4. Nos últimos 7 dias, quantosdiasvocê**caminhouporpelomenos 10 minutos** de cadavez?

   _____ Dias - Se nenhum, responda 00 e passe para a pergunta 6

5. Nos diasem que vocêcaminhouporpelomenos 10 minutos de cadavez, no total, quanto tempo vocêgastaandando?

   _____Horas_____ Minutos

6. Quanto tempo você gasta sentado em um dia normal? Pode incluir o tempo de estudo, visitando amigos, lendo ou assistindo televisão.
_____Horas_____ Minutos

ANNEX IV - Questionnaire gastrointestinal problems

Nome.....Data…….....
.

Durante a semana passada você se sentiu incomodado por qualquer um dos seguintes sintomas abaixo listado? Por favor, indique a sua resposta através da marcação ou preenchimento do quadrante para cada um dos seguintes:

SINTOMA	GRAU DE DESCONFORTO						
	Nenhum	Pequeno	Leve	Moderado	Moderadamente intenso	Intenso	Muito intenso
Dor abdominal							
Dor ou desconforto no abdômen aliviado por ação do intestino							
Sensação de inchaço							
Flatulência							
Constipação							
Diarreia							
Evacuação solta							
Fezes duras							
Necessidade urgente de evacuações							
Sentindo que seu intestino não foi completamente esvaziado depois de uma evacuação							
Sentindo-se completo, pouco depois de ter iniciado uma refeição							
Sentindo-se completo, mesmo depois de ter parado de comer							
Inchaço visível do seu estômago							

1. Algum destes sintomas, já afetou sua capacidade de treinar esta semana?

   Sim ( ) Não ( )

   Se sim, indicar qual das seguintes situações:

   Pequena redução do treino, conseguindo manter o mesmo nível ( )

   Treinamento reduzido abaixo do nível normal ( )

   Parou completamente de treinar ( )

2. Você já tomou qualquer medicação (mesmos em receita) nesta semana para aliviar doenças respiratórias ou sintomas de desconforto gastrointestinal?

Sim ( )

Se sim, qual o nome da medicação///

.....

Não ( )

3. Você se consulta com seu médico sobre seus sintomas da doença esta semana?

Sim ( )

Se sim, você tem tomado qualquer medicação prescrita esta semana?

Nome da medicação

.....

Não ( )

4. Você fez alguma alteração na sua dieta normal esta semana?

Sim ( )

Se sim, o que mudou

.....

Não ( )

ANNEX V _ Subjective Perception of Effort Scale

0	Nenhum esforço (Repouso)
1	Muito Fraco
2	Fraco
3	Moderado
4	Um Pouco Forte
5	Forte
6	
7	Muito Forte
8	
9	
10	Esforço máximo

Carga Interna = Valor apontado de PSE x minutos da sessão de treino

888 **ANNEX VI – Brunel Mood Scale (BRUMS)**

A Escala de Humor de Brunel (BRUMS)

Abaixo está uma lista de palavras que descrevem sentimentos. Por favor, leia tudo atenciosamente. Em seguida assinale, em cada linha, o quadrado que melhor descreve **COMO VOCÊ SE SENTE AGORA**. Tenha certeza de sua resposta para cada questão, antes de assinalar.

Escala:

0 = nada **1 = um pouco** **2 = moderadamente**
3 = bastante **4 = extremamente**

	0	1	2	3	4
1. Apavorado	☐	☐	☐	☐	☐
2. Animado	☐	☐	☐	☐	☐
3. Confuso	☐	☐	☐	☐	☐
4. Esgotado	☐	☐	☐	☐	☐
5. Deprimido	☐	☐	☐	☐	☐
6. Desanimado	☐	☐	☐	☐	☐
7. Irritado	☐	☐	☐	☐	☐
8. Exausto	☐	☐	☐	☐	☐
9. Inseguro	☐	☐	☐	☐	☐
10. Sonolento	☐	☐	☐	☐	☐
11. Zangado	☐	☐	☐	☐	☐
12. Triste	☐	☐	☐	☐	☐
13. Ansioso	☐	☐	☐	☐	☐
14. Preocupado	☐	☐	☐	☐	☐
15. Com disposição	☐	☐	☐	☐	☐
16. Infeliz	☐	☐	☐	☐	☐
17. Desorientado	☐	☐	☐	☐	☐
18. Tenso	☐	☐	☐	☐	☐
19. Com raiva	☐	☐	☐	☐	☐
20. Com energia	☐	☐	☐	☐	☐
21. Cansado	☐	☐	☐	☐	☐
22. Mal-humorado	☐	☐	☐	☐	☐
23. Alerta	☐	☐	☐	☐	☐
24. Indeciso	☐	☐	☐	☐	☐

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INFORMED CONSENT FORM

You are being invited to take part in a study as a volunteer. If you agree to take part in the study, please sign at the end of this document, which is in two copies. One copy is yours and the other belongs to the researcher responsible.

RESEARCH INFORMATION: THE EFFECT OF FERMENTED MILK CONTAINING *LACTICASEIBACILLUS PARACASEI* STRAIN SHIROTA (LCS) ON THE UPPER AIRWAY SYMPTOMS OF TRIATHLETES BEFORE AND AFTER A COMPETITION

RESPONSIBLE RESEARCHER: Prof Dr Jônatas Bussador do Amaral.

CONTACT PHONE/EMAIL: (11) 99418-9999 amaraljb@gmail.com

DESCRIPTION OF THE RESEARCH:

Because triathlon is a sport that combines high intensity and long duration, it imposes significant physiological adaptation on the body. However, when the challenge exceeds the capacity for physiological adaptation, it is possible to observe the occurrence of various disorders in the athlete's health, such as the manifestation of symptoms in the upper airways. In this sense, among the many factors that can help with this physiological adaptation is the microbiota, which can be modulated by age, medical treatments, , physical exercise and, in particular, by diet, favoring the maintenance of a healthy state. According to the literature, probiotics, prebiotics and symbiotics can benefit people in different ways. In fact, due to their obvious ability to help regulate the immune/inflammatory system, these agents can have an impact on reducing or even preventing the frequency and/or intensity of upper airway symptoms, both in the general population and in athletes. However, it is worth noting that there is still a lack of reports in the scientific literature on the effects of drinking fermented milk containing *Lacticaseibacillus paracasei* strain Shirota (LcS), a known probiotic agent, not only on the modulation of immune/inflammatory responses, but also on the manifestation of upper airway symptoms and mood in triathletes.

Therefore, in this study, we aim to investigate, in general, the effect of daily intake of fermented milk containing 40 billion LcS in amateur triathletes, emphasizing that our primary endpoint will be to evaluate the occurrence of upper airway symptoms and the secondary endpoints will be to evaluate both systemic and upper airway immune/inflammatory responses, as well as mood states. To meet these objectives, saliva, nasal swab and blood samples will be obtained, as well as data on quality of life, the manifestation of symptoms in the upper airways and mood states on five different occasions: 30 days (1) and 24 hours before the competition (2), as well as immediately (3), 72 hours (4) and 14 days after the end of the competition (5). It is important to note that the data relating to quality of life, the manifestation of symptoms in the upper airways and mood states will be obtained using specific and validated questionnaires, such as the SF-36, WURSS-21 and BRUMS, respectively. Finally, it is also important to clarify that the triathletes taking part in this study will be instructed to drink a bottle of fermented milk containing 40 billion LcS or placebo (unfermented milk) every day for the duration of the study,

starting the intake 30 days before the competition, right after the first sample collection, until the 13th day after the competition.

The risks related to the volunteers' participation in this research are minimal. There may be some discomfort not only when blood samples are taken, with the possible formation of a small hematoma (purple spot), but also when nasal cells are collected using the nasal swab. To reduce these risks, samples should always be taken by trained professionals.

If you agree to participate, you will contribute to an increased understanding of the effects of daily ingestion of fermented milk containing 40 billion LcS on the immune/inflammatory response and mood state of triathletes before and after a session of prolonged and exhausting physical exercise, such as a long-duration triathlon. In addition, it may allow the implementation of new health promotion strategies for triathletes by considering fermented milk containing LcS as an agent capable of positively and beneficially modulating the immunity, inflammatory response and mood of triathletes.

If, after consenting to participate, you withdraw your consent, you are guaranteed the full right and freedom to withdraw your consent at any stage of the research, before or after data collection, regardless of the reason and without any prejudice to you. You will not receive any remuneration. The results of the research will be analyzed and published, and your identity will be preserved and kept confidential. For any further information, you can contact the researcher responsible either in person at the Research Laboratory of the Department of Otorhinolaryngology and Head and Neck Surgery the Federal University of São Paulo (UNIFESP), located at Rua Pedro de Toledo 781, 1º. andar, sala 3, Vila Clementino, CEP: 04039-032, or by telephone (11) 99418-.
9999.

São Paulo, ____of _____2025.

_____	_____
Participant's signature	**Jônatas Bussador do Amaral** Responsible for the research

SUBSTANTIATED OPINION OF THE CEP

Prepared by the Co-participating Institution

RESEARCH PROJECT DATA

Research Title: THE EFFECT OF FERMENTED MILK CONTAINING LACTICASEIBACILLUS PARACASEI CEPA SHIROTA (LCS) ON UPPER AIRWAY SYMPTOMS IN TRIATHLETES BEFORE AND AFTER A COMPETITION

Researcher: Andre Luis Lacerda Bachi

Thematic area:

Version: 3

CAAE: 78576224.9.3001.5505

Proposing Institution: FEDERAL UNIVERSITY OF SAO PAULO

Main Sponsor: Self-financing

OPINION DATA

Opinion number: 7.349.261

Project presentation:

CEP/UNIFESP project n: 0434/2024 (co-participating center)
Project initiated by the researchers themselves Local manager: Jônatas Bussador do Amaral
Research team: Prof. Dr. Mauro Vaisberg, Dr. Jônatas Bussador Do Amaral and Cesar Miguel Momesso Dos Santos
Project linked to the Department of Otorhinolaryngology, São Paulo Campus, UNIFESP.

Multicenter study in Brazil. Two research centers will participate, including UNIFESP.
Coordinating Center: Santo Amaro University (Unisa). Researcher responsible: Andre Luis Lacerda Bachi
Coparticipating Centers: 02

-The information listed in the fields "Presentation of the Project", "Objective of the Research" and "Evaluation of Risks and Benefits" was taken from the file Basic Information of the Research (<PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_ 2321205.pdf> posted on 21/05/2024) and from the file of the detailed project sent (<Projeto_Versao_Portugues_Final.pdf> posted on 24/03/2024).

PRESENTATION:

Because triathlon is a sport that combines high intensity and long duration, it imposes significant physiological adaptation on the body. However, when the challenge exceeds the capacity for physiological adaptation, it is possible to observe the occurrence of various disorders in the athlete's health. In this sense, among the various factors that can help with this adaptation, it has been pointed out that the microbiota, which can be modulated by diet, age, medical treatments, lifestyle and physical exercise, can favor the maintenance of a healthy state. According to the literature, probiotics, prebiotics and synbiotics can benefit people in different ways. For example, by promoting immunoregulation, these elements can have an impact on preventing or reducing the frequency and/or intensity of upper airway symptoms, both in the general population and in athletes. However, it is worth noting that there are still no reports in the scientific literature on the effects of drinking fermented milk containing Lacticaseibacillus paracasei strain Shirota (LcS), a known probiotic agent, not only on the modulation of immune/inflammatory responses, but also on the manifestation of airway symptoms and mood in triathletes. Therefore, in this study, our aim will be to investigate, in general, the effect of daily intake of fermented milk containing 40 billion LcS in amateur triathletes, emphasizing that our primary endpoint will be to assess the occurrence of upper airway symptoms and the secondary endpoints will be to assess both systemic and upper airway immune/inflammatory responses, as well as mood states. To answer these endpoints, saliva, nasal swab and blood samples, as well as data on quality of life, upper airway symptom manifestation and mood states will be obtained on five different occasions: 30 days (1) and 24 hours before the competition (2), as well as immediately (3), 72 hours after the competition (4) and 24 hours after the competition (5).
(4) and 14 days after the end of the competition (5). It is important to note that the data related to
quality of life, the manifestation of upper airway symptoms and mood states will be obtained using specific and validated questionnaires, such as the SF-36, the WURSS-21 and the BRUMS, respectively. Finally, it is also essential to clarify that the triathletes taking part in this study will be instructed to drink a bottle of fermented milk containing 40 billion LcS or placebo/unfermented milk every day for the duration of the study, starting 30 days before the competition, right after the first sample collection, until the 13th day after the competition.

HYPOTHESIS:
Supplementation with Lacticaseibacillus paracasei strain Shirota interferes with immune system modulation and performance in triathlon athletes.

Research Objective:
PRIMARY OBJECTIVES
To investigate the effect of daily intake of fermented milk containing 40 billion LcS on the occurrence of upper airway symptoms in amateur triathletes participating in this study, before and after an official triathlon competition.

SECONDARY OBJECTIVES
To evaluate the effect of the daily intake of fermented milk containing 40 billion LcS on the systemic and upper airway immune/inflammatory responses and also on the mood states of the amateur triathletes who took part in this study, before and after an official triathlon competition.

Assessment of Risks and Benefits:
Regarding the risks and benefits, the researcher declares:
RISKS:
The risks for the research volunteers will be minimal, both for the blood collection, because although it is a routine clinical procedure that is generally safe, there may be minor discomfort (pain) or hematoma at the puncture site, fainting or dizziness, and for the collection nasal cells through the nasal swab. In order to reduce these risks, the collection should always be carried out by trained professionals.

BENEFITS:
Among the benefits for the volunteers is that they can undergo clinical examinations, with the results being given to them free of charge, including relevant information for improving the structure of their physical training.

Comments and Considerations on the Research:

STUDY TYPE: double-blind, randomized, controlled trial with two parallel groups

LOCATION: Otorhinolaryngology Research Laboratory (ORL-LAB) . Rua Pedro de Toledo, 781, 1st floor room 13

PARTICIPANTS:

- Inclusion criteria:

PROCEDURES:

Proposed Methodology:

It will be designed as a double-blind, randomized, controlled trial with two parallel groups:LcS Group (LcSG): Consumes milk fermented with LcS daily for 30 days before and 14 days after a triathlon competition.Placebo Group (PLG): Consumes non-fermented milk daily for the same period.Main characteristics of the study:Participants: 60-70 male and female amateur triathletes aged between 25 and 45, living in São Paulo, Brazil. Interventions: Daily consumption of fermented milk containing SCM or placebo for 56 days. Measures: Upper airway symptoms assessed by the WURSS-21 questionnaire before, during and after competition. Blood, saliva and nasal swab samples taken at five different times for various analyses. Cell count Cell culture with LPS stimulation to assess cytokine production (IL-1¿, IL-1ra, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL13, IL-17A, and TNF-alpha) using the LEGENPlex for human cytokines kit (BioLegend, San Diego, CA, USA). Saliva sample Concentration of IgAs [LL-37 (cathelicidin), defensin-1, lactoferrin, and lysozyme] determined by ELISA commercial kits (Biosay Technology Laboratory, Shanghai, China).Nasal infiltrate Fixation with methanol (99% Synth) and then stained in hematoxylin, for later analysis by an experienced pathologist.Immunophenotyping of immune cells Evaluation of the number, type and activation profile of Natural Killer cells and T lymphocytes Using antibodies to the surface molecules: CD3, CD4, CD8, CD16, CD27, CD38, CD45, CD56, CD70, CD197 and HLA-DR.

Cytotoxicity of Natural Killer Cells The in vitro evaluation of the activity of Natural Killer (NK) cells will be carried out using the commercial NKVUE Tubes kit, which makes it possible to assess the activity of NK cells through the production of interferon-gamma. Determination of the systemic concentration of BDNF and hormonesCommercial ELISA kits [BDNF Quantikine ELISA kit (Catalog number: SBD00);

Cortisol ELISA EIA-1887 (Catalog number: SKGE008B; hGH ELISA EIA-3552 (Catalog number: SG100) R&D System, Inc. USA]Serum levels of sex hormones [progesterone (pg/mL), estrogen (pg/mL) and testosterone (pg/mL) will also be determined by commercial ELISA kits [Testosterone Parameter Assay Kit (Catalog Number: SKGE010), Estradiol Parameter Assay Kit (Catalog Number: KGE014), Human Total Progesterone ELISA (Catalog Number: DYC5415-5), R&D System, Inc. The physical capacity of each volunteer will be characterized by means of a cardiopulmonary test which will be carried out on a treadmill coupled to a gas analyzer with electrocardiography and gas analysis software. The following variables will be determined: The participant's physical capacity. Measurement of maximum oxygen consumption (VO2max). Anaerobic threshold. Pulmonary ventilation. Heart rate. Mood assessed using the Brunel Mood Scale. Occurrence of gastrointestinal discomfort monitored.

INCLUSION CRITERIA:

Inclusion criteria for the study:

1. Participation in the IronMan 70.3 São Paulo competition: The main requirement for taking part in the study is to be registered and participating in the IronMan 70.3 São Paulo competition in 2024.
2. Age group: Participants must be between 25 and 45 years old.
3. Gender: Both men and women can take part in the study.
4. Resident in São Paulo: Participants must reside in the city of São Paulo, Brazil.
5. No pre-existing conditions: Participants must not have: Intolerance to fermented milk. Use of any type of medication. Chronic rhinitis. Autoimmune diseases. Recurrent infections.
6. Agreement to follow the study protocol: Participants must agree to: Consume the fermented milk with LcS or the placebo daily during the study period. Follow the study instructions. Complete questionnaires and provide blood, saliva and nasal swab samples.
7. Sign the Informed Consent Form: All participants must read, understand and sign the Informed Consent Form before starting the study. Observations: The study aims to recruit 60-70 participants, divided into two groups: LcS and placebo. Participants will be selected on the basis of inclusion and exclusion criteria. The research team reserves the right to exclude any participant who does not comply with the study protocol.

EXCLUSION CRITERIA:
(1) reported intolerance to fermented milk;
(2) are under any kind of pharmacological treatment;
(3) report chronic rhinopathy;
(4) have autoimmune diseases;
(5) report the occurrence of recurrent non-airborne infections. In addition,
(6) feeding during the study of foods containing
Lactobacilli
(7) if any data is missing from a volunteer, that participant will be excluded from the study

(for more information, see detailed design).

Consideration of mandatory terms of presentation:

1- The main documents were presented: cover sheet; complete project; copy of the CEP/UNIFESP registration, financial budget and timetable.

2- Other important documents attached to the Brazil Platform:
HSP-HU/UNIFESP CoEPE letter no. 117/24
Forwarding letter (, posted on 21/05/2024) Letter of consent (,
posted on 24/03/2024)
Commitment and confidentiality agreement
Concession agreement

3¿ The model of the ICF was presented by the researcher.

4- The questionnaires / interview scripts are attached at the end of the detailed project.

5 - Project_Portuguese_Version_FINAL_2.docx

6 - Template_TCLE_Unifesp_Triathletes_2.docx

7 - Termo_anuencia_instituicao_URC_2024_Andre_Bachi.pdf

8 - Physiology_Laboratory_Agreement_Term.pdf

9 - Answers_questions_PB_2_completo.doc

Recommendations:

RECOMMENDATION 1- It is the researcher's obligation to develop the research project in full compliance with the proposal submitted to the CEP. Changes and alterations to the protocol must be communicated in the form of an AMENDMENT through Plataforma Brasil.

RECOMMENDATION 2- From the date of approval, it will be necessary to send partial reports (every six months) and the final report (at the end of the study), by means of a NOTIFICATION on the Brazil Platform. Researchers must inform and justify to the CEP any need for temporary suspension or permanent suspension of the research. Incidents and adverse events must also be reported to CEP-Unifesp by means of a NOTIFICATION on the Brazil Platform.

RECOMMENDATION 3- Researchers should take all necessary precautions when collecting and storing data in order to guarantee the secrecy and confidentiality of the research participants' information. It is recommended to download the data collected to a local electronic device, deleting any data from virtual platforms or the cloud. The research protocol documents (files of forms, terms, data and samples) must be kept for at least 5 years after the end of the research.

Conclusions or Outstanding Issues and List of Inadequacies:

Response to opinion no. 7.205.814 of November 5, 2024. PROJECT APPROVED.

RESPONSE TO PENDING ISSUES

PENDENCY 1 - In relation to the timetable provided in the basic information form: we ask that the researchers check whether there is a need to change the start date for the "Recruitment and selection of volunteers" and subsequent stages, so that there is enough time between the responses and approval by the CEP/UNIFESP. We remind you that no study can begin before approval by CEP/UNIFESP (CNS Operational Standard 001 of 2013, item 3.3.f).

ANSWER 1 The dates have been changed in the schedule presented in the Detailed Project and in the Brazil Platform.

PENDENCY ATTENDED TO

PENDENCY 2 ¿ It is requested that the name of Cesar Miguel Momesso Dos Santos (cited in field

"Assistant", in the basic information form of Plataforma Brasil), be included on the front sheet (cover) of the detailed project.
ANSWER 2: The researcher's name has been added
PENDING CARRIED OUT

PENDENCY 3- Inform in the detailed project methodology and in the ICF the average time needed for participants to answer all the questionnaires. ANSWER 3: As requested, the average time needed to answer each questionnaire in this study has been added to both the main text of the study and the ICF. In this sense, it is worth mentioning that it will be necessary: - 5 to 7 minutes to answer the WURSS questionnaire, to check the occurrence of upper airway symptoms not only in this period, but also throughout the study period (Annex 1); - 10 to 12 minutes to answer the quality of life (Annex 2); - 5 to 8 minutes to answer the physical activity level questionnaire (Annex 3); - 5 to 7 minutes to answer the questionnaire with open questions about gastrointestinal problems. (Annex 04).
PENDENCY PARTIALLY FULFILLED CEP/UNIFESP ANALYSIS: The average time needed to answer the questionnaires was not added to the ICF.

Answer 3: The following text was added to the ICF: ¿It is worth mentioning that it will take 5 to 7 minutes to answer the WURSS questionnaire (to check the occurrence of upper airway symptoms), 10 to 12 minutes to answer the quality of life questionnaire, 5 to 8 minutes to answer the physical activity level questionnaire and, finally, 5 to 7 minutes to answer the questionnaire with open questions about gastrointestinal problems¿.
PENDENCY ATTENDED TO

PENDENCY 4- - According to the letter of referral, research participants linked to Hospital São Paulo and UNIFESP will not be involved. We therefore ask for clarification regarding the sending of the COEPE letter and the completion of the CEP registration form stating that UNIFESP outpatient clinics will be used. ANSWER 4: Vale

to clarify that for the development of this study proposal, clinical evaluation will be recommended with emphasis on otorhinolaryngological aspects. Therefore, in order to guarantee reliable data for each volunteer taking part in the study, we will use the outpatient facilities of the Department of Otorhinolaryngology and Head and Neck Surgery at UNIFESP exclusively to carry out this assessment. Finally, it is worth stating that no other UNIFESP sector/ambulatory will be used.
PENDENCY ATTENDED TO

PENDENCY 5- According to the letter of referral, in addition to the competition venues, participants will also be recruited from the Olympic Center and Unisa. Answer 5: With regard to this item, it is worth first clarifying that, as presented in the text of the research project, "In order to reach the number of volunteer groups (male and female), we will invite the athletes registered for the triathlon competition through direct contact with the athletes' advisors and also by sending the invitation to their social media." Therefore, recruitment will be carried out exclusively and directly by those responsible for developing the study and there will be no recruitment from the Olympic Center and Unisa. However, it is worth noting that the premises of the Olympic Center will be used to carry out the cardiorespiratory assessment, while the premises of UNISA will be used to collect, process and store the samples necessary for the development of this study, in addition to carrying certain laboratory assessments. 5a) It will be necessary to send a letter of consent/authorization from the person in charge of the sites regarding the research (one for each site). Answer 5a) As described above, since there will be no recruitment at the Olympic Center and Unisa, it is not necessary to send a letter of consent/authorization from the person in charge of the sites regarding the research. PENDENCY NOT FULFILLED CEP/UNIFESP ANALYSIS: Since the premises of the Olympic Center and UNISA will be used, it is necessary to send the letters of consent and authorization from those responsible for the sites.
Answer 5.a): Letters of consent have been attached to the Brazil Platform system
PENDENCY ATTENDED TO

PENDING 5.b) If recruitment is carried out through the media, social networks, posters, etc., the text of this recruitment must be sent to the CEP for analysis. This information

should be included in the project methodology. GUIDELINE: Invitations to take part in the research should not be made using lists that allow the invitees to be identified or their contact details (e-mail, telephone, etc.) to be viewed by third parties. Any individual invitation sent by e-mail can only have one sender and one recipient, or be sent in the form of a hidden list. (CIRCULAR OFFICE NO. 2/2021/CONEP/SECNS/MS, disponivel em: http://conselho.saude.gov.br/images/Oficio_Circular_2_24fev2021.pdf). Answer 5b) The text of the invitation letter to be sent to the athletes is shown in ANNEX 06. In addition, as requested, this information has been added to the main text of the research project, as shown below.
In order to reach the number of volunteer groups (male and female), we will invite the athletes registered for the triathlon competition through direct contact with the athletes' advisors and also by sending the invitation by e-mail or even to their social media. The text to be sent is shown in ANNEX 05 - PENDENCY PARTIALLY FULFILLED ANALYSIS CEP/UNIFESP: Correct the number of the annex referring to the letter of invitation (annex VII and not VI). Answer 5b) We have changed the numbering of the annex.
PENDENCY ATTENDED TO

PENDENCY 6 - We request that the methodology of the detailed project include the justification for the use of placebo, as CNS resolution 466/2012, item III.3.b., states that research must "have full justification, where appropriate, for the use of placebo, in terms of non-maleficence and methodological necessity, and the benefits, risks, difficulties and effectiveness of a new therapeutic method must be tested, comparing it with the best current prophylactic, diagnostic and therapeutic methods. This does not exclude the use of placebo or no treatment at all in studies in which there are no proven methods of prophylaxis, diagnosis or treatment¿.
ANSWER 6: As requested, the use of placebo in this study is initially justified by the participant's acknowledged belief that the simple use of the bottle containing fermented milk will prevent the manifestation, as well as reduce the duration and airway symptoms associated with the triathlon competition. In addition, the placebo will be useful for understanding and isolating the biological effects, particularly those related immune/inflammatory aspects, considering the non-maleficence, beneficence and justice of

possible improvements in the manifestation of airway symptoms associated with triathlon competition. Finally, it is worth noting that the fermented milk containing SCL is an exclusive product of the company Yakult Honsha Co.,Ltd. and only it can supply the placebo for the development of this research project. The above text has been added to the main text of the research project, specifically in the sub-item "Ingestion of SCL" under "Materials and Methods".

PENDENCY ATTENDED TO

PENDENCY 7- Page 18 of the detailed project reads: ¿To ensure the development of a double-blind study, all vials used in the study will be kindly provided by YAKULT S/A Indústria e Comércio, Brazil.¿ We request that a letter from the company agreeing to provide the vials for research be attached to the Brazil platform.

ANSWER 7: In response to the request, the letter from YAKULT S/A Indústria e Comércio, Brazil stating that it will provide the vials has been attached to the Brazil Platform.

PENDENCY ATTENDED TO

PENDENCY 8- We would like to remind you that, according to Resolution 466/2012:III.3.d, if the treatment proves to be effective at the end of the study, the same treatment should be made available to the control group: please clarify and it will be necessary to include this information in the detailed project and in the ICF. (Resolution 466/2012:III.3.d, proposes.d, proposes: "Ensure that all participants at the end of the study, by the sponsor, have free and indefinite access to the best prophylactic, diagnostic and therapeutic methods that have been shown to be effective.").ICF: inform that if the treatment proves to be effective at the end of the study, the same treatment should be made available to the control group Answer 8: Regarding this item, it is worth clarifying that: 1) the research project will be carried out using a product (fermented milk containing LcS) which is not registered as a medicine but as a foodstuff, according to its registration with the Brazilian Ministry of Agriculture, Livestock and Supply, attached to the Brazil Platform; 2) the purpose of the study is not to evaluate the possible use of fermented milk containing LcS as a treatment for a specific disease or pathological process, but rather to evaluate the possible probiotic effect (immunomodulation) of this fermented milk in a specific context, which involves triathlon competition; 3)

corroborating the item above, as presented in the research project, the study will be carried out with the participation of healthy individuals who do not suffer from a particular disease or pathological process. 4) in addition, the fermented milk to be used in the study is produced and sold commercially in our territory by the company YAKULT S/A Indústria e Comércio, Brazil, and is therefore widely available to the general population; Given this information, which highlights specific points involved in the study, if the use of fermented milk containing SCL proves to be effective at the end of the study, it can be used by any member of the control group by purchasing it commercially, without there being any obligation to make it available to the control group.
PENDENCY ATTENDED TO.

PENDENCY 9- In relation to the collection of blood, saliva and nasal swab, in addition to the information provided, the following information must be included in the project methodology (according to CNS Resolution 441/12 of May 2011):
9.a) and in what quantity (blood);
Answer 9a: As requested, the following text has been added to the project: "Blood will be collected in 4 tubes containing EDTA anticoagulants and in 1 tube without anticoagulants. It is worth noting that each tube can accommodate a volume of 4mL of blood, so we will not have a total volume of 20mL of blood from each volunteer on each occasion."
PENDENCY ATTENDED TO

9.b) which professional will carry out the collection and in which location; Answer 9b: As requested, the following text has been added to the project: "It is important to clarify not only that the collection of biological material will be carried out on the premises of the Santo Amaro University (UNISA) laboratory, under the responsibility of a nursing professional, trained and experienced to carry out this task (...)" PENDENCY ATTENDED TO

PENDENCY 9.c) under what conditions and in what form the biological material will be transported to the analysis laboratory; Answer 9c: As requested, the following text has been added to the draft: "(...) but also that all material collected will be kept refrigerated in a box.

Styrofoam with the addition of regular ice or icex until it is processed." PENDENCY PARTIALLY MET
CEP/UNIFESP ANALYSIS: It is not clear how the samples will be transported from the collection site to the analysis laboratory. Transportation must follow RDC 504/2021
Answer 9.c). The following was added to the project ¿The transportation of biological samples in this research (saliva, nasal swab and blood) will take place in strict compliance with the Resolution of the Collegiate Board (RDC) No. 504, of May 27, 2021, of the National Health Surveillance Agency (ANVISA). Provision is made for collection and packaging procedures, where samples will be collected by a responsible professional and in a suitable environment and with all the necessary care to guarantee the integrity and safety of the biological material and volunteers. The samples will be packaged in watertight primary containers, individually identified and labeled in accordance with technical standards. They will then be placed in absorbent secondary containers, also identified and securely closed. The outer packaging will be resistant to impacts and leaks, and will be duly identified with the name of the research, the name of the technical manager, the recipient's address and contact information. Transportation will be by land, using the researcher's own vehicle. Suitable conditions of temperature, humidity and pressure will be guaranteed throughout transportation, according to the nature of the samples. A transportation manifest will be issued containing all relevant information about the samples, including the biological risk classification. It should be emphasized that all professionals involved in the transportation process will be wearing appropriate PPE and have expertise in good practices for transporting biological materials. In the event of a biological sample leak during transportation, a contingency plan will be activated immediately. This plan includes notifying the relevant authorities, isolating the contaminated area and using appropriate personal protective equipment. Containment of the leak will be carried out with specific absorbent materials, followed by rigorous cleaning and decontamination. All actions will be recorded and a full incident report will be drawn up.
PENDENCY ATTENDED TO

PENDENCY 9d- under what conditions the biological material will be stored, in what location, for how long before analysis;
Answer 9d: It is worth noting that we have revised the description of the items mentioned and the following text

was added to the project: "After processing, the samples will be kept, without the addition of preservatives, in a -80o.C freezer on the premises of the UNISA Research Laboratory (URC) until they are used in the laboratory procedures recommended in the study. The samples will be stored for a maximum of 6 months before being used in (...), and will then be disposed of as soon as the study is completed."
PENDENCY ATTENDED TO

PENDENCY 9.e) where (laboratory) the analysis will be carried out and what will be analyzed;
Answer 9e: All laboratory evaluations will be carried out at the UNISA Research Center and/or at the Research Laboratory of the Department of Otorhinolaryngology and Head and Neck Surgery of UNIFESP.
PENDENCY PARTIALLY MET
ANALYSIS CEP/UNIFESP: The response did not mention what will be analyzed.
Answer 9.e). The following has been added to the project: "In these sectors, the saliva, nasal swab and blood samples collected from the volunteers will be processed.
PENDENCY ATTENDED TO

PENDING 9.f)- what will be done with the material after the analysis (will it be discarded, stored until the end of the research, or stored even after the research is finished?). If it will be stored after the end of the research, state where it will be stored and for what reason (repetition of the analyses to verify the results, and/or for possible new analyses).
Answer 9f: All samples will be kept in a -80o C freezer until the end of the research project, after which they will be disposed of in accordance with biosafety regulations. After the end of the experiments and publication of the results, all the samples will be discarded. The biological material will be packed in white bags and collected by the São Paulo City Hall and incinerated (following UNIFESP's solid waste disposal rules).
PENDENCY ATTENDED TO

PENDENCY 10- - According to Resolution 441/11 Art °1 item II - ¿Biorepository: collection of human biological material, collected and stored during the execution of a specific research project, in accordance with technical, ethical and operational regulations or standards prior to the implementation of the project.

defined, under the institutional responsibility and management of the researcher, without commercial purposes¿, It is requested to include information about the biorepository in the detailed project methodology:

10.a) Researcher Responsible and email Answer) Researcher Jônatas Bussador do Amaral (email: jbamaral@unifesp.br)

10.b) Department/Sector Answer) Department of Otorhinolaryngology and Head and Neck Surgery -EPM/Unifesp.

10.c) Head of Department and email Reply) Prof. Dr. Onivaldo Cervantes (email: ocervantes@uol.com.br)

10.d) Address where the Biorrepository will be located Answer) Located in Unifesp's Research Building 1 (Rua Pedro de Toledo 781)-São Paulo-SP

10.e) Telephone number of the laboratory where the biorepository will be located Answer) The ORL Lab's telephone number is 11 5576-4848 (VOIP 1372).

10.f) Type of biological material stored Answer) Whole blood and its derivatives (serum and plasma), saliva and nasal swab

10.g) How the samples will be stored Answer) After collection, the biological material will undergo processing consisting of: 1-Centrifugation at 800xG 2-Removal of the supernatant 3-Storage in 2mL conical tubes 4-Labeling and identification of tubes and boxes 5-Storage in an ultrafreezer - 80º.C In this biorepository, whole blood and its derivatives (serum and plasma), saliva and human tissues are stored. All the samples in the biorepository are discarded after the end of the projects. They are removed and then disposed of via the infectious solid waste route (¿white waste¿) and sent for incineration via the São Paulo city hall.

10.h) How the samples will be collected Answer) Blood, saliva and nasal swab samples will be obtained by health professionals. The blood will be obtained via venipuncture. The saliva will be collected in appropriate tubes. Finally, the nasal swabs will be inserted into the nasal cavity and used to make the smear on glass slides, which will then be stored in the ORL Lab.

COPY OF THE AMENDED TEXT OF THE ENTIRE ITEM 10 E(TEXT INSERTED IN THE DETAILED DESIGN AND TCLE):

Biorepository

The ORL Lab's ultrafreezer keeps biological samples at a temperature of -80oC and is used as a Biorepository. All sample preparation and control of the flow of

storage is done at the ORL Lab under the responsibility of researcher Jônatas Bussador do Amaral (email: jbamaral@unifesp.br). Located in Unifesp's Research Building 1 (Rua Pedro de Toledo 781), this biorepository belongs to the Department of Otorhinolaryngology and Head and Neck Surgery and is headed by Prof. Dr. Onivaldo Cervantes (email: ocervantes@uol.com.br). The ORL Lab's telephone number is 11 5576-4848 (VOIP1372). This biorepository stores whole blood and its derivatives (serum and plasma), saliva and human tissues. All samples from the biorepository are discarded after the end of the projects. In this study, saliva, blood and its derivatives (serum and plasma) will be stored. PENDENCY ATTENDED TO

PENDING 11- TFIC ATTENTION: The TFIC attached is inadequate. Please prepare the TCLE again, using the TCLE template on the CEP/UNIFESP website, link: UNIFESP - Pesquisa - Comitê de Ética em Pesquisa ¿ Projeto envolvendo seres humanos -Plataforma Brasil: Modelo de TCLE, or at: https://cep.unifesp.br/linksuteis#modelos)
11.a) In the attached TCLE, in addition to the CEP/UNIFESP data, the data (telephone, e-mail and address) of the CEP of the coordinating center should be inserted with the indication that it can be contacted if there is any doubt about the ethics of the study; ANSWER 11: In response to the request, the TCLE has been revised and prepared according to the suggested model.
PENDENCY PARTIALLY MET
CEP/UNIFESP ANALYSIS: The CEP data for the coordinating center has not been added. We request that this be done.
Answer 11): The following was added to the ICF: ¿If necessary, you can also contact the CEP of Universidade Santo Amaro (UNISA), at the address: Rua Prof. Enéas de Siqueira Neto, 340 ¿ Jardim das Imbuias ¿ Prédio F1 ¿ Térreo ¿ Pós-Graduação do Campus Interlagos ¿ CEP: 04829-300 ¿ São Paulo ¿ SP, telephone (011) 2141-8687 and email: pesquisaunisa@unisa.br. The UNISA CEP's opening hours are from Monday to Friday, from 8am to 12pm, and from 1pm to 6pm, except on Friday until 5pm. You can also contact the National Research Ethics Council (CONEP) by e-mail:conep@saude.gov.br or by telephone: (61) 3315-5878 to resolve doubts and ask for clarification. PENDENCY ATTENDED TO

NEW PENDENCY 12- ICF: Add the data of the researcher Jônatas Bussador do Amaral (UNIFESP) next to the data of André Luiz de Lacerda Bachi

The following excerpts have been added to the ICF:

Investigators: Dr. André Luis Lacerda Bachi (UNISA) and Dr. Jônatas Bussador do Amaral (UNIFESP)¿.

If you have any questions about the research, you can contact the researchers responsible: André Luiz de Lacerda Bachi and Jônatas Bussador do Amaral , on the following telephone numbers: 11 5576-4848 / Voip 1372, cell phone (11)99891-7767, Rua Pedro de Toledo, 781, 1º andar sala 13, São Paulo ¿ SP and e-mail miguelmomesso@gmail.com¿

PENDENCY ATTENDED TO

Final considerations at the discretion of the CEP:

In view of the above, the Research Ethics Committee - CEP, in accordance with the attributions defined in CNS Resolution No. 466, of 2012 and/or CNS Resolution No. 510, of 2016, and in CNS Operational Standard No. 001, of 2013, expresses its approval of the research protocol.

This opinion has been drawn up on the basis of the documents listed below:

Document Type	Archive	Post	Author	Situation
Basic Information Project	PB_BASIC_INFORMATION_OF_PROJETO_2321205.pdf	11/12/2024 09:33:01		Accepted
Others	Answers_questions_PB_2_completo.doc	11/12/2024 09:31:48	Jônatas Bussador do Amaral	Accepted
Declaration of Institution and Infrastructure	Physiology_Laboratory_Agreement_Termogia_.pdf	22/11/2024 09:50:39	Jônatas Bussador do Amaral	Accepted
Declaration of Institution and Infrastructure	Institution_annulment_term_URC_2024_Andre_Bachi.pdf	22/11/2024 09:49:43	Jônatas Bussador do Amaral	Accepted
TCLE / Terms of Assent / Justification for Absence	Template_TCLE_Unifesp_Triathletes_2.docx	22/11/2024 09:45:39	Jônatas Bussador do Amaral	Accepted
Detailed Design / Brochure Researcher	Project_Portuguese_Version_FINAL_2.docx	22/11/2024 09:45:22	Jônatas Bussador do Amaral	Accepted

Others	Ministry_of_Agriculture_Registry.pdf	12/10/2024 16:46:37	Jônatas Bussador do Amaral	Accepted
Declaration of Sponsor	Collaboration_letter_YAKULT.pdf	12/10/2024 16:40:06	Jônatas Bussador do Amaral	Accepted
Others	CADASTRO_CEP_UNIFESP_assinado.pdf	21/05/2024 12:04:00	Jônatas Bussador do Amaral	Accepted
Others	Signed_forwarding_letter.pdf	21/05/2024 12:01:19	Jônatas Bussador do Amaral	Accepted
Others	COEPE.pdf	21/05/2024 11:58:45	Jônatas Bussador do Amaral	Accepted
Others	Confidentiality_Covenant_Signed.pdf	24/03/2024 09:16:37	Andre Luis Lacerda Bachi	Accepted
Others	Signed_Concession_Term.pdf	24/03/2024 09:15:43	Andre Luis Lacerda Bachi	Accepted
Others	Signed_Agreement_Letter.pdf	24/03/2024 09:15:06	Andre Luis Lacerda Bachi	Accepted
TCLE / Terms of Assent / Justification for Absence	ECLE_Project_Triathlon_LcS.pdf	24/03/2024 09:14:01	Andre Luis Lacerda Bachi	Accepted
Detailed Design / Brochure Researcher	Project_Portuguese_Version_Final.pdf	24/03/2024 09:13:51	Andre Luis Lacerda Bachi	Accepted

Status of opinion:
Approved

Needs CONEP appraisal:
No

SAO PAULO, January 29, 2025

Signed by:
Paula Midori Castelo Ferrua (Coordinator)