Michalickova, Danica, Rajna Minic, Nenad Dikic, Marija Andjelkovic, Marija Kostic-Vucicevic, Tamara Stojmenovic, Ivan Nikolic, and Brizita Djordjevic. 2016. '*Lactobacillus Helveticus* Lafti L10 Supplementation Reduces Respiratory Infection Duration in a Cohort of Elite Athletes: A Randomized, Double-Blind, Placebo-Controlled Trial'. *Applied Physiology, Nutrition, and Metabolism* 41 (7): 782–89. https://doi.org/10.1139/apnm-2015-0541.

# *Lactobacillus helveticus* Lafti® L10 supplementation reduces respiratory infection duration in a cohort of elite athletes: a randomized double-blind placebo-controlled trial

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**Abstract**

A randomized, double-blind, placebo-controlled study was conducted, in order to evaluate if *Lactobacillus helveticus* Lafti® L10 supplementation during 14 weeks in winter can influence the duration, severity and incidence of URTI, as well as to monitor different immune parameters in the population of elite athletes. Before and after the treatment, cardiopulmonary testing and self-rated state of moods evaluation (by Profile of Mood States questionnaire) were performed and blood samples were collected. Thirty-nine elite athletes were randomized either to the placebo (n=19) or the probiotic (n=20) group. The probiotic group received *L. helveticus* Lafti® L10, 2 x $10^{10}$ Colony Forming Units (CFU). Lafti® L10 significantly shortened the URTI episode duration (7.25±2.90 vs. 10.64±4.67 days, p=0.047) and decreased the number of symptoms in the probiotic group (4.92±1.96 vs. 6.91±1.22, p=0.035). Severity and incidence of URTI did not differ between the treatments. There were no significant changes in leukocyte subpopulation abundance, TGF-β serum levels, level of IL-10 secreted from peptidoglican stimulated peripheral blood mononuclear cells (PBMCs), IFN-γ level secreted from concanavalin A stimulated PBMCs or viability/proliferation of PBMCs upon antigen stimulation. Group effect for CD4+/CD8+ ratio was significant $F(1,37)=6.99$, $p=0.020$, $\eta^2=0.350$ and this difference was not significant at baseline, but was evident after 14 weeks (p=0.02). A significant interaction effect was noted for self-rated sense of vigor $F(1,37)=11.76$, $p=0.009$, $\eta^2=0.595$. Self-rated sense of vigor increased in the probiotic group (18.5±4.1 vs. 21.0±2.6, p=0.012). Probiotic strain Lafti® L10 can be a beneficial nutritional supplement for the reduction of URTI length in elite athletes.

**Keywords:** Lactobacillus, upper respiratory tract illness (URTI), CD4+/CD8+ ratio, POMS

## Introduction

There is an increasing amount of studies which support the potential health benefits of probiotics, such as the stimulation of immunity, reduction of oxidative stress (Lamprecht et al. 2012; Sharma et al. 2014), improvement of gastrointestinal barrier (Lamprecht et al. 2012), promotion of antitumor activity (Thomas and Ockhuizen, 2012) etc. *Lactobacillus* and *Bifidobacterium* are the most widely used and studied probiotic bacteria, due to their safety, and abundance in food preparations (Fijan 2014).

On the other hand, physical activity can have both positive and negative effects on immunity, depending on its intensity and volume. In fact, the link between the frequency of upper respiratory tract illness (URTI) and intensity of physical activity is a “U”-shaped curve (Walsh et al. 2011). The modification of immune variables may cause the increased URTI incidence within the population of elite athletes (West et al. 2009; Walsh et al. 2011; Gleeson 2007).

Although the immunology of athletes has been studied for a long time, still the evidence of suppressive impact of physical activity of high intensity on immune system is a challenge to overcome. It is generally considered that chronic intense physical activity leads to the disturbance of cellular and humoral aspects of immunity, such as reduced number and activity of NK cells, decreased activity of neutrophils, impaired proliferation of T-lymphocytes, increased levels of anti-inflammatory cytokines, decreased levels of pro-inflammatory cytokines and decreased levels of salivary IgA (West et al. 2009; Walsh et al. 2011; Gleeson 2007; Shepard 2000). The most likely causes of the above mentioned changes in the immune system are increased serum levels of circulating "stress hormones" cortisol and catecholamine

which are excessively produced during vigorous physical activity (Walsh et al. 2011; Rehm et al. 2015).

The increased susceptibility of athletes to infections occurs especially in a period of intensive training before the competition and during the cold months of the year (West et al. 2009; Gleeson 2007). Considering how health issues can affect the performance of athletes, certain intervention studies have been conducted in recent years, in order to improve the immunity of the susceptible population. However, satisfactory results are few. The increasing number of proven beneficial effects indicates that probiotics could be the nutrition supplement of choice for preventing the illness of the respiratory and the GI tract. Certain strains may reduce the incidence of URTI (Cox et al. 2010; Haywood et al. 2013), the severity of symptoms (West et al. 2011; Lamprecht et al. 2012), and shorten the duration of URTI episodes (West et al. 2013).

The probiotic strain *Lactobacillus helveticus* Lafti® L10 was chosen as its inherent immunity enhancing properties and nonpathogenic nature were previously reported (Paturi et al. 2007; Paturi et al. 2008). Furthermore, *L. helveticus* Lafti® L10 caused the reversal of decreased secretion of the pro-inflammatory IFN-γ in fatigued athletes (Clancy et al. 2006), so it was of interest to explore this further and particularly to test the effect on clinical outcomes of URTI. Therefore, the aim of this study was to evaluate the clinical and immunomodulatory effects, as well as sport performance enhancement of *Lactobacillus helveticus* Lafti® L10 in the population of elite athletes.

## Materials and methods

### Experimental design

The study was conducted as a randomized, double-blind, placebo-controlled parallel-groups design procedure, following the guidelines laid down in Declaration of Helsinki and was approved by the Ethics committee of Sports medicine association of Serbia. Written consent of the participants was obtained before the beginning of the study.

The recruitment of the athletes began in March 2014 and was finished in November 2014. One hundred ten volunteers were enrolled in eligibility checking (anthropometric measurements, blood panel, clinical check-up and extensive health questionnaire). A total of 50 athletes were involved in the study, while the rest of the athletes enrolled in the initial check-up declined or were not eligible to participate.

After the recruitment of athletes, they were randomly allocated to one of the groups, according to maximal aerobic capacity (evaluated by cardiopulmonary testing, CPT). The experimental group received the probiotic capsules of *L. helveticus* Lafti® L10 ($2 \times 10^{10}$ CFU) daily for 14 weeks. The control group received placebo capsules, which were identical in taste and appearance as the probiotic capsules. The placebo capsules contained 1% magnesium stearate and 99% maltodextrin and the probiotic capsules contained 72.2% of the bacterial mass, 26.7% maltodextrin and 1% magnesium stearate. Capsules were composed of hydroxypropylmethylcellulose (HPMC) and covered by titanium dioxide ($TiO_2$). Both probiotic and placebo capsules were kept in the fridge (2˚C to 8˚C). To enhance the compliance, athletes were asked to take the capsules every day at the same time after breakfast. Also, the researchers were communicating daily with athletes by phone, reminding them to take the capsules and fill in the health and training load questionnaires.

Athletes and the study team were blinded to the intervention until the statistical analyses

were finished. The exercise and medical eligibility testing started in December. The supplementation started in the middle of January and lasted for 14 weeks. Before the study, athletes kept food diaries for three days. Also, they were asked to avoid supplements which are intended for promotion of the immune system (e.g.): Echinacea, caffeine, Ginseng panax, propolis, multivitamins and multiminerals. During the study, the subjects had a steady training regimen and a diet without eating yogurt and fermented milk products.

**Subjects**

A total of 50 elite athletes were recruited: 36 men (VO2max ranged from 49.50 to 82 ml/kg/min) and 14 women ($VO_2$max ranged from 45 to 57 ml/kg/min), aged 18-28 years, non-smokers, with training >11 hr/week (considered for high training load) (Gleeson et al. 2013).

Exclusion criteria were: sensitivity to the ingredients of probiotics, the use of probiotics and antibiotics a month before the beginning of the study, recent surgical intervention (in the previous year), the presence of chronic diseases (diabetes mellitus, rheumatoid arthritis, neurological, renal, pulmonary, psychiatric diseases etc.).

Athletes included in the study were training: badminton, triathlon, cycling, alpinism, athletics, karate, savate, kayak, judo, tennis and swimming. Unlike similar studies, which included university or recreational athletes, the participants in this study were the winners of the national or European and world championships in their categories and sport.

A total of 39 athletes completed the study. Physical and anthropometric characteristics of the participants were similar (Table 1).

**Sample collection**

Blood samples (10 mL per serum tube and 9 mL per $K_3$EDTA tube) were taken out of the antecubital vein, prior to the cardiopulmonary testing. All the samples were collected twice: before the study and after the study, at the same time (between 9:30 and 10:30), in order to avoid diurnal changes. Blood samples were allowed to clot for thirty minutes at room temperature. Serum was separated by centrifugation (1500 x g, 15 min, 4°C) and stored frozen at -20 °C until analysis. Blood from the $K_3$EDTA tubes was used for phenotypic characterization of the leukocyte population and in vitro antigen stimulation of cytokine production.

**Blood leukocyte and lymphocyte subpopulations counts**

Resting blood from the $K_3$EDTA Vacutainer was used for phenotypic characterization of the leukocyte population of peripheral blood. FACS tubes were filled with 100 µL of blood and 4 µL of antibodies was added. Antibodies against CD3 (OKT-3 Mouse IgG2a FITC), CD4 (MEM-241 Mouse IgG1 PE), CD8 (HIT8a Mouse IgG1,k PE), CD19 (LT19 Rat IgG1 FITC), CD56 (MEM-188 Mouse IgG2a PE), CD11b (MEM-174 Mouse IgG2a FITC) and CD45RO (UCHL1 Mouse IgG2a FITC) were used. All antibodies were used according to the manufacturer's recommendations (Immunotools, Friesoythe, Germany). After incubation (20 min, 20 °C, dark), 2 mL of lysis solution were added, vortexed and incubated for 10 min at 20 °C. The suspension was centrifuged at 400 g for 10 min, supernatant was aspirated and washed with 3 ml 2% BSA in PBS. After washing, 200 µL PBS with 0.4% FA were added. The signal was analyzed using FACSVerse (BD Biosciences, San Jose, CA, USA).

Evaluation of absolute CD3+CD4+ (T helper cells), CD3+CD8+ (T cytotoxic cells), CD4+CD45RO+ (memory helper cells), CD8+CD45RO+ (memory cytotoxic cells), CD19+ (B cells) cell counts were obtained from the total lymphocyte counts and CD11b+ (cells exposing CR3, or Mac-1, which is involved in cellular adhesion, phagocytosis and leukocyte migration) cell count was obtained from the total leukocyte count. Percentages of lymphocyte subpopulations were derived from the respective results obtained in flow cytometry analyses.

**The determination of cytokine response**

Peripheral blood mononuclear cells (PBMCs) were separated on Histopaque 1077 (Sigma Aldrich), resuspended in complete RPMI 1640, 50 μM β-mercaptoethanol with 10% FCS, and washed twice. Cells were counted, and 1 x $10^6$ PBMCs was stimulated with either ConA (Concanavalin A from *Canavalia ensiformis*, Sigma-Aldrich) 10μg/mL, LPS (Lipopolysaccharides from *Salmonella minnesota*, Sigma-Aldrich) 10μg/mL, PGN (Peptidoglycan from *Staphylococcus aureus*, BioChemika, Fluka) 10μg/ml, or with 1 x $10^7$/mL heat killed *L. helveticus* Lafti® L10. The cells were co-cultured at 37°C and 5% $CO_2$ for 24 h. After centrifugation at 400 x g for 10 min at 4°C, supernatants were collected and stored frozen at -80 °C. Levels of IFN-γ, IL-10 and IL-4 were measured with commercial ELISA kit (R&D Systems®, USA), according to manufacturers' recommendations, in duplicates.

TGF-β1 level was measured directly from the serum. Samples were diluted 7x and determined by commercial ELISA kit (Human/Mouse TGF beta 1 ELISA Ready-SET-Go, eBioscience, San Diego, USA). Samples were assayed in duplicates; the intra-assay coefficient of variation was 11.06%.

**MTT test**

For measurements of cell proliferation/viability, the cells were prepared in the same manner and stimulated with LPS and ConA, but were grown in 96 well plates and were incubated for 48h. After centrifugation (400 x g, 10 min) the RPMI 1640 was discarded and phenol red free RPMI with 0.5 mg/ml MTT was added and incubated for 4h, at 37°C and 5% $CO_2$. Crystals were dissolved with equal amount of 10% SDS, 10mM HCl, and the absorbance was measured at 570 nm in ELISA plate reader (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA).

**Respiratory and gastrointestinal infections: incidence, severity and duration**

All the symptoms of upper respiratory tract illness (URTI) were measured daily. WURSS-21® (Wisconsin Upper Respiratory Symptom Survey) was used for this purpose (Barrett et al., 2002). This questionnaire contains 10 items (runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, hoarseness, head congestion, chest congestion, feeling tired), whose severity is self-rated by a 8-point Likert scale (the absence of symptom was scored as 0 and extreme severity as 7). The total symptom severity score of each episode was counted as previously described (Gleeson et al. 2011). The symptom-severity score for each subject was obtained by multiplying the total number of days each symptom was recorded by the self-scored symptom severity rating. The total symptom-severity score represented the sum of all symptom-severity scores. Only episodes whose scores were >24 were considered as URTI (Gleeson et al. 2011), meaning that athlete had to experience at least 3 moderate symptoms (severity rating was scored as 4) for 2 days, or 2 moderate symptoms

for 3 days.

Furthermore, the participants were asked to record gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain, abdominal bloating, flatulence, "stomach rumbles" and loss of appetite) and to rate them by 8-point Likert scale (West et al. 2011).

Athletes were also asked to report all the medications and dietary supplements which they used during the illness, as well as the duration of their consumption and visits to the doctor.

**Exercise performance**

Cardiopulmonary tests were conducted twice (before the study and after the study). Maximum oxygen consumption was determined by a graded cardiopulmonary test on a treadmill (Quarck b2-Cosmed). The exercise intensity was progressively increased, while oxygen and $CO_2$ concentration of the inhaled and exhaled air were measured. A test was considered maximal if participants achieved 90% or more of predicted maximal heart rate for age and gender, a plateau in oxygen consumption was reached despite increased workload, a respiratory exchange ratio was grater then 1.00, and subjects reached volitional exhaustion.

**Physical activity and training loads**

Athletes were obliged to report their training loads weekly, filling in the standard short form of International Physical Activity Questionnaire (IPAQ; http://www.ipaq.ki.se/downloads.htm). Training loads in metabolic equivalents (MET-hr/week) were counted on the basis of completed questionnaires, according to Ainsworth (Ainsworth et al. 2011). Moreover, athletes were asked to rate the influence of illness on their

ability to train on 8-point Likert scale (no influence was scored as 0 and total disruption of training was rated as 7). The score of illness influence on training ability was obtained by multiplying the total number of days impairment of training was recorded by self-scored rating of impairment. The total score of illness influence on training ability represented the sum of all scores reported in a group during the period of study. The total number of days which athletes didn't train was also counted.

**Profile of mood and state (POMS) questionnaire**

Profile of Mood States questionnaire (POMS) is a validated psychological questionnaire invented by McNair (McNair et al. 1971). The questionnaire consists of 65 statements that describe feelings and moods in the past week, including the moment questionnaire was performed. Athletes were required to rate each item as "Not at All", "A Little", "Moderately", "Quite a Lot" or "Extremely". According to the instructions of the manual, tension, depression, anger, confusion, vigor, fatigue, as well as total mood disturbance scores were assessed. The questionnaire was filled 10 minutes before the cardiopulmonary testing at two time points: before and after the study.

**Evaluation of clinical outcomes**

Primary endpoint of the study was to determine whether probiotic supplementation leads to a reduction in the incidence of respiratory infection, the severity of symptoms and shortening of illness duration, as well as total of infected days in athletes. Secondary endpoints were to determine whether systemic (leukocyte subpopulations and antigen stimulated cytokine production, immune cells proliferation/viability), as well as sport

performances and mood profiles scores are enhanced by probiotics supplementation.

**Statistical analyses**

All statistical analyses were performed with SPSS software (SPSS v. 20.0; SPSS Inc, Chicago, IL, USA). The normality of the data was checked by Shapiro-Wilk test. The proportion of subjects who experienced URTI symptoms and reported impaired training, as well as compliance to the study between groups and number of infected days were statistically analyzed by chi-square test. The comparisons between groups for severity, URTI episode duration, number of used medicines/supplements, number of symptoms per episode, training loads, illness influence of training ability, total number of days without training, adverse effects were performed by independent (unpaired) T-test. A mixed (between-within subjects) analysis of variance (ANOVA) was used to determine the main effects, as well as interaction effect between the two independent variables of time (baseline and after 14 weeks; within subjects factor) and group (placebo and probiotic; between subjects factors) on immunological variables and POMS scores. Partial eta squared ($\eta^2$) was used to estimate the magnitude of the difference within each group, while the thresholds for small, moderate and large effects were defined as 0.01, 0.06, and 0.14, respectively (Cohen, 1988). T test with Bonferroni correction was applied for any significant main effect or interaction effect. Correlated assumptions of sphericity in the data, as well as homogeneity of variances were checked. Cytokine data for IFN-γ and IL-10 was found to be significantly nonnormal, so it was transformed using a log transformation prior to analysis. $P<0.05$ was considered significant. The results for normally distributed variables data are expressed as mean values and standard deviation (SD). Where distribution was not normal, medians and 95% confidence intervals were given.

In order to detect 30% reduction of infected days with $\alpha$=0.05 and $\beta$=0.80, a total of 37 subjects were needed (Haywood et al. 2013). A total of 50 athletes were recruited at the beginning of the study, accounting for the dropout.

## Results

### Compliance to the study

The athletes were asked to return the remaining capsules when coming to the final testing after the intervention. The researchers counted the remained capsules; the compliance in the probiotic group was 94.7% and in the placebo group was 93.6% (p=0.74).

### Respiratory and gastrointestinal infections: severity, incidence, duration

Contrary to our basic hypothesis, neither the incidence, nor the severity of respiratory infection differed between the treatments (Table 2), although a trend for decreasing severity in the probiotic group emerged (p=0.078). However, the duration of an URTI episode was shorter in the probiotic group than in the placebo group (7.25±2.90 versus 10.64±4.67 days). Moreover, there were less reported symptoms of URTI in the probiotic group. There were no substantial differences in the number or the duration of consumption of medicine or dietary supplement used to relieve the URTI symptoms. The number of GIT infections was too low to make any comparison between the groups.

### Exercise performance, training loads and impact of illness on training

There were no significant differences in exercise performance: $VO_2$ max, treadmill performance time, HR max, recovery of HR in the first, second or third minute (data are not shown). Neither recorded training loads, nor the self-rated influence of illness on training

ability showed substantial difference between treatments. Also, the total number of days when athletes skipped the training was similar in both groups (Table 3). However, a trend towards reduction of proportion of athletes reporting impaired training emerged for the probiotic group (p=0.054).

**Blood leukocyte and lymphocyte subpopulations counts**

There were no significant group, time or interaction effects for lymphocytes, monocytes and granulocytes counts, as well as lymphocyte subpopulations: CD3+CD4+, CD3+CD8+, CD4+CD45RO+, CD8+CD45RO+, CD19+ and CD11b+ cells (Table 4). There was a significant group effect for CD3-CD56+ ($F=11.57$, $p=0.006$, $\eta^2 =0.449$), but without significant time and interaction effects. Changes between groups were substantial at baseline ($p= 0.041$), but were not after 14 weeks ($p= 0.082$). On the other hand, there were significant interaction $F(1,37)=28.77$, $p=0.001$, $\eta^2 =0.489$, time $F(1,37)=25.74$, $p=0.001$, $\eta^2 =0.462$ and group effects $F(1,37)=6.99$, $p=0.020$, $\eta^2=0.350$ for CD4+/CD8+ ratio. Following up interaction indicated that there was no significant difference between groups at baseline ($p=0.775$), while the difference was evident after 14 weeks ($p=0.02$). Furthermore, a significant change in values at baseline and after 14 weeks was observed only in the probiotic group ($p<0.001$).

**Cytokine response**

Levels of IL-10, as well as IFN-γ obtained after PBMCs' stimulation with each of the stimulants (ConA, PGN, LPS and heat killed Lafti®) were compared by one way-ANOVA. The results indicated that the optimal stimulation of IFN-γ and IL-10 was achieved by ConA and PGN, respectively. Therefore, only these data are presented in the paper and considered

for statistical analyses. The intra-assay coefficients of variation for IL-10 and IFN-γ were 8.66%, and 7.35%, respectively.

There was a significant interaction effect ($F(1,37) = 62.99$, $p = 0.003$, $\eta^2 = 0.471$) for the level of IFN-γ, secreted from PBMCs stimulated with ConA, but without observed group effect. Moreover, we noted a strong time effect $F(1,37) = 22.35$, $p<0.001$, $\eta^2 = 0.818$), with higher values at the end of the study for both probiotic (p=0.011) and placebo group (p=0.025). Significant difference was observed between the values at baseline (p=0.005), but not after 14 weeks (p=0.09). There were no interaction, time or group effects for level of IL-10 secreted from PBMCs stimulated with PGN. Time effect for TGF-β1 level measured in serum was significant $F(1,37)=6.14$, $p=0.030$, $\eta^2 = 0.178$), but without noted time and interaction effects (Table 5). However, there were no differences for both probiotic (p=0.22) and placebo (p=0.06) groups at baseline and after 14 weeks.

IL-4 level was under the limit of detection in both pre- and post-intervention samples.

**POMS**

There were no interaction, time or group effects observed for the total mood disturbance, anger, confusion, depression, fatigue and tension scores (Table 6). However, a significant interaction effect was noted for vigor $F(1,37) = 11.76$, $p=0.009$, $\eta^2 = 0.595$. There was a significant difference for vigor scores at baseline and at the end of supplementation in the probiotic group (p=0.012). There were no significant differences between the values in the probiotic and placebo group at baseline (p=0.706) and at the end of supplementation (p=0.087).

**Cell proliferation/viability**

No interaction, time or group effects were observed in mixed ANOVA for the viability/proliferation of PBMCs stimulation with ConA or LPS. Table 7 shows proliferation coefficients, obtained by dividing the absorbance of stimulated cells with the absorbance of same sample cells left unstimulated.

### Adverse effects

There was no substantial difference between the number of adverse effects between the groups. No serious adverse events occurred during the study.

## Discussion

The present study investigated the effects of *Lactobacillus helveticus* Lafti® L10 on URTI, sport performances, state of mood profile, as well as on systemic immunity parameters in the population of highly active athletes during the period of 14 weeks in winter. The background profiles of the participants in the probiotic and placebo group (maximal aerobic capacity, training loads, demographic characteristics, compliance) were similar. Also, both the number and the utilization length of supplements, and/or medications reported by athletes during the study were similar between the groups. Furthermore, no difference in adverse effects between groups was noted.

The most important finding was that the probiotic significantly reduced the length of URTI episodes and lowered the number of symptoms per episode. Although not statistically relevant, a trend for decreased total symptom severity scores of URTI episodes in the probiotic group occurred. These results are consistent with similar studies in rugby players

(Haywood et al. 2013) and endurance runners (Cox et al. 2010), where the effect of probiotics on URTI severity was trivial, while the number of infected days was substantially lower when probiotics were used. These positive clinical outcomes could support the previous findings of beneficial effects of probiotics in highly active individuals. However, there are other diverse subgroups of the general population which were affected by probiotics in a similar way. In a study conducted on elderly (Guillemard et al. 2010), *L. casei* shortened the duration of common infectious diseases, especial URTI, such as rhinopharyngitis. Other studies showed reduction of length of common colds in children (Hojsak et al. 2010; Kloster et al. 2008) and academically stressed students (Langkamp-Henken et al. 2015).

On the other hand, the proportion of athletes who reported URTI was similar in both groups. There is a vast number of studies reporting that probiotics reduce the incidence of URTI and some of them were conducted in highly active individuals (Gleeson et al. 2011; West et al. 2013; Haywood et al. 2013). In a large study in physically active subjects, *Bifidobacterium lactis* supplementation was linked to 27% reduction of risk of URTI (West et al. 2013). In the above mentioned study conducted in rugby players, supplementation by multi-strain probiotic product resulted in a significant reduction in the number of URTI and GIT infections (Haywood et al. 2013). However, we didn't observe a substantial decrease of the incidence of URTI.

There are several possible reasons for missing the incidence and severity reduction by Lafti® L10 administration: a relatively small cohort of athletes included into the supplementation, late onset of the trial (the middle of January) and too low daily dose of probiotic. Finally, the fact that athletes were not required to refrain from use of over the counter medication and other medications for relief of URTI symptoms, could also contribute to the lack of some positive outcomes.

The observed benefits of the probiotics in the present study might be related to an augmentation of the systemic immune system. Namely, *Lactobacilli* belong to the group of Gram-positive bacteria and exert microbe-associated molecular patterns (MAMPs), such as polysaccharides and peptidoglycans bound to the cell wall and lipoteichoic acids attached to the cytoplasmic membrane (van Baarlen et al. 2013). The receptors exposed at the surface of different epithelial and immune cells in the GIT can communicate with the MAMPs and induce different immune responses, like antigen presentation and immune stimulation (van Baarlen et al. 2013). In such a way, van Baarlen et al. reported modulation of mucosal transcriptomes of healthy adults after oral administration of Lafti® L10 (van Baarlen et al. 2011). Specifically, several IFN-γ-responsive genes were up-regulated, pointing out that *L. helveticus* Lafti® L10 triggers a Th1 shift. Indeed, these findings were corroborated with animal studies (Paturi et al. 2007; Paturi et al. 2008): the oral administration of Lafti® L10 in mouse elicited increase of IFN-γ secreted both from splenocytes and in serum.

Furthermore, Clancy et al. 2006 reported an enhancement of antigen stimulated IFN-γ after a month of *L. helveticus* Lafti® L10 supplementation, but only in a cohort of fatigued athletes suffering recurrent viral infections, while this effect was not noted in healthy athletes. However, direct parallels with our trial cannot be drawn, since the discussed study didn't involve placebo-controlled design. Nevertheless, we failed to provide such an evidence of Th1 shift induced by Lafti® L10 supplementation; to be exact, we observed much higher levels of IFN-γ secreted from ConA stimulated PBMCs at the end of the supplementation in both probiotic and placebo groups. In other words, this significant change of IFN-γ secretion after the study was irrespective of treatments. In addition, the hypothesis of impaired pro-inflammatory cytokines response to be a risk factor for higher incidence of URTI is controversial: some authors indicated that higher IFN-γ responses correlated with subjects

more prone to URTI (Gleeson and Bishop, 2013).

Furthermore, we failed to detect IL-4 in all the samples, while Clancy et al. 2006 reported the level of secreted IL-4 remaining steady during the study. This finding could be ascribed to the lower sensitivity of ELISA kit we used for IL-4 level determination.

We report a significant increase of CD4+/CD8+ (T helper/T suppressor) cells ratio in the probiotic group. Apparently, CD4+/CD8+ ratio was noted as an index sensitive to high training loads and was found to be decreased after strenuous physical activity (Shepard 2000; Gleeson 2007). Moreover, low CD4+/CD8+ cell ratio is usually related to acute viral diseases and hemophilia (Chakravarti 1995). Therefore, improvement of this immunological parameter could contribute to the favorable effects of Lafti® L10 on URTI.

However, further evidences of immunity response augmentation upon Lafti® L10 supplementation are still lacking: blood leukocyte counts, lymphocyte subpopulations, levels of cytokines secreted from ConA and PGN stimulated PBMCs, as well as PBMCs proliferation/viability upon ConA and LPS stimulation didn't change during the study. This is in accordance with other studies researching effects of probiotics in athletes. Changes in leukocytes subsets could, possibly, be noted in animal studies, with no genetic diversity.

Interestingly, a significant increase of subjective feeling of vigor was noted in the probiotic group, although total mood disturbance score was not changed during the study, as well as other self-rated states (tension, depression, anger, confusion, fatigue). Some previous studies reported an association between elite athletes marathon training with significantly decreased vigor, increased fatigue and non-significant impairment of total mood state (Hassmen and Blomstrand 1991; Achten et al. 2004). Psychological health assessed by POMS questionnaire tightly correlated with the estimated physical health: it is reported that illness

influence immunity either in physical, or psychological way (Konig et al. 2000; Strasner et al. 2001; Talbott and Talbott 2013). Therefore, self-rated sensation of increased vigor could give a remote contribution to overall health improvement.

The weakness of the study could be that isolated PBMCs were used as a source of cytokines to be measured. It is noted that isolated PBMCs are sequestered from the normal communication with other cells and hormones and thus do not reproduce the natural environment, as whole blood cultures do (Svendsen et al. 2014). Furthermore, cytokines stimulation was performed by ConA, LPS and PGN and not by multigen vaccine, as in similar studies, which would imitate the situation of naturally occurring URTI in athletes (Svendsen et al. 2014). However, despite of the fundamental cause, the magnitude in absolute cytokine production in response to antigen challenge is presumed to have involvement into higher susceptibility of athletes to URTI (Svendsen et al. 2014). Another limitation of the present study is, as previously mentioned, a relatively small sample of athletes that possibly decreased the chance of finding significant differences in some clinical outcomes. However, our aim was to include only athletes with high training loads (>11 h training per week) into the study, since this cohort is reported to be in high risk of URTI and to have an impaired immunity (Gleeson et al. 2013). Unlike the majority of similar studies, the participants in this one were the best national athletes. This could be a particular strength of the study, since they are very difficult to be either recruited or monitored during the period of few months, taking into account their frequent voyages out of the country and constant psychological pressure. Another advantage of the study is the use of validated questionnaires for URTI symptoms and profile of mood states and by our knowledge, this is the first study to monitor psychological effects of probiotics in highly active athletes and to note self-rated increase of vigor, possibly linked to probiotic supplementation.

In conclusion, supplementation of *L. helveticus* Lafti® L10 reduced the duration of URTI episodes and decreased the number of symptoms in elite athletes. Future clinical trials should be focused on larger samples of athletes and higher daily dose of Lafti®, in order to observe possible positive clinical outcomes of Lafti® supplementation (reduction of frequency and/or severity of URTI, debilitation of impact of URTI on training) and provide further clarification of mechanism of Lafti® interaction with immune system in athletes.

## Acknowledgements

The authors gratefully acknowledge material and scientific support of Lallemand Health Solutions, Montreal, Canada. This work is supported by Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. III 46009 and OI 172049). The present study does not constitute endorsement of the product by the authors or any conflict of interest.

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## Tables

**Table 1.** Physical and anthropometric characteristics of the participants

| | *Probiotic* | *Placebo* | *p value* |
|---|---|---|---|
| Number | 20 | 19 | |
| Males/females | 15/5 | 14/5 | |
| Age (years) | 23.5±2.7 | 22.8±2.5 | 0.86 |
| $VO_2$ max | 56.2±9.3 | 57.1±10.0 | 0.79 |
| Weight (kg) | 74.1±10.7 | 74.0±10.9 | 0.97 |
| Height (cm) | 180.2±10.0 | 180.1±7.7 | 0.95 |
| BMI | 22.9±2.2 | 22.9±2.2 | 0.95 |
| % fat | 14.22±7.16 | 14.70±6.46 | 0.75 |

Note: BMI, Body-Mass Index. Results are expressed as means ± standard deviations. Significance: $p<0.05$ was obtained by unpaired T-test.

**Table 2.** The effect of probiotic on respiratory symptoms

| | *Probiotic* | *Placebo* | *p value* |
|---|---|---|---|
| Proportion of athletes reported URTI episode | 12/20 | 11/19 | 0.897 |
| Duration (number of days) | 7.25±2.90 | 10.64±4.67* | 0.047 |
| Episode severity | 110.92±96 | 129.73±40.33 | 0.078 |
| Number of symptoms per episode | 4.92±1.96 | 6.91±1.22* | 0.035 |
| Number of medications/supplements per episode | 1.17±1.11 | 1.91±0.94 | 0.101 |
| Number of days of medications per episode | 3.67±4.33 | 7.55±5.84 | 0.166 |
| Total number of infected days | 88 | 132* | 0.000563 |

Note: Results are expressed as means ± standard deviations.
*Significance: $p<0.05$ (unpaired T-test, except for incidence and total number of days: chi-square test)

**Table 3.** Training loads and influence of illness on training

| | *Probiotic* | *Placebo* | *p value* |
|---|---|---|---|
| Training loads (MET-hr/week) | 98.89±54.93 | 100.40±56.95 | 0.98 |
| Illness influence on training ability score | 22.92±26.41 | 28.82±22.58 | 0.57 |
| Total number of days without training | 2.0±3.1 | 1.7±2.3 | 0.48 |
| Proportion of athletes reporting impaired training | 40% | 42% | 0.054 |

Note: MET, metabolic equivalence. Results are expressed as means ± standard deviations and significance: $p<0.05$ (unpaired T-test, except for proportion of athletes, significance was obtained by chi-square test).

**Table 4**. Blood leukocyte and lymphocyte subpopulations absolute counts

| | | *Treatment time* | | | | |
|---|---|---|---|---|---|---|
| | | Baseline | *14 weeks* | Interaction effect (p, $\eta^2$) | Time effect (p, $\eta^2$) | Treatment effect (p, $\eta^2$) |
| Lymphocytes | probiotic | 1.96±0.59 | 2.35±1.24 | 0.849, 0.003 | 0.147, 0.144 | 0.504, 0.033 |
| (cells ^$10^9$/L) | placebo | 2.13±0.70 | 2.64±0.87 | | | |
| Monocytes | probiotic | 0.40±0.12 | 0.52±0.49 | 0.434, 0.044 | 0.615, 0.019 | 0.712, 0.010 |
| (cells ^$10^9$/L) | placebo | 0.40±0.12 | 0.43±0.17 | | | |
| Granulocytes | probiotic | 4.42±1.04 | 4.31±1.61 | 0.420, 0.047 | 0.226, 0.103 | 0.352, 0.062 |
| (cells ^$10^9$/L) | placebo | 4.37±1.96 | 4.60±1.91 | | | |
| CD3+CD4+ | probiotic | 0.82±0.32 | 0.89±0.26 | 0.330, 0.073 | 0.679, 0.014 | 0.073, 0.227 |
| (cells ^$10^9$/L) | placebo | 0.77±0.12 | 0.89±0.40 | | | |
| CD3+CD8+ | probiotic | 0.63±0.34 | 0.46±0.07 | 0.711, 0.012 | 0.100, 0.209 | 0.296, 0.090 |
| (cells ^$10^9$/L) | placebo | 0.58±0.13 | 0.70±0.21 | | | |
| CD3-CD56+ | probiotic | 0.16±0.09 | 0.12±0.07 | 0.198, 0.124 | 0.520, 0.033 | 0.006*, 0.449 |
| (cells ^$10^9$/L) | placebo | 0.24±0.20 | 0.13±0.14 | | | |
| CD4+CD45RO+ | probiotic | 0.43±0.30 | 0.39±0.27 | 0.785, 0.006 | 0.535, 0.030 | 0.394, 0.056 |
| (cells ^$10^9$/L) | placebo | 0.44±0.09 | 0.52±0.19 | | | |
| CD8+CD45RO+ | probiotic | 0.12±0.07 | 0.13±0.09 | 0.745, 0.008 | 0.811, 0.005 | 0.396, 0.056 |
| (cells ^$10^9$/L) | placebo | 0.15±0.10 | 0.17±0.14 | | | |
| CD19+ | probiotic | 0.18±0.07 | 0.21±0.17 | 0.214, 0.116 | 0.550, 0.028 | 0.420, 0.051 |
| (cells ^$10^9$/L) | placebo | 0.19±0.04 | 0.28±0.14 | | | |
| CD11b+ | probiotic | 3.81±1.52 | 2.96±1.17 | 0.764, 0.007 | 0.230, 0.101 | 0.140, 0.149 |
| (cells ^$10^9$/L) | placebo | 4.00±1.59 | 4.52±1.88 | | | |
| CD4+/CD8+ratio | probiotic | 1.30±0.04 | 1.41±0.07 | 0.001†, 0.489 | 0.001†, 0.462 | 0.020†, 0.350 |
| | placebo | 1.28±0.05 | 1.29±0.06 | | | |

Note: Results are expresed as means± standard deviations; Significance was considered for $p<0.05$. Results are derived from mixed ANOVA analyses. T test with Bonferroni correction was applied for any significant main effect or interaction effect.

* Changes between groups were substantial at baseline (p= 0.041), but were not significant after 14 weeks (p= 0.082).

† No significant difference between groups at baseline (p=0.78) was observed, while the difference was evident after 14 weeks (p=0.02). A significant change in values at baseline and after 14 weeks was observed only in probiotic group (p<0.001).

**Table 5.** The levels of cytokynes response at baseline and after 14 weeks

| | | *Treatment time* | | | | |
|---|---|---|---|---|---|---|
| | | Baseline | *14 weeks* | Interaction effect (p, $\eta^2$) | Time effect (p, $\eta^2$) | Treatment effect (p, $\eta^2$) |
| IFN-γ (pg/ml) | probiotic | 283.5 (111.5-333.7) | 1635 (510.0-2696) | 0.003*, 0.471 | <0.001*,0.818 | 0.494, 0.034 |
| | placebo | 577.8 (130.7-962.7) | 1249 (179-2308) | | | |
| IL-10 (pg/ml) | probiotic | 379.5 (214.2-554.8) | 365.2 (34.7-624.5) | 0.317, 0.072 | 0.732, 0.009 | 0.833, 0.003 |
| | placebo | 396.3 (109.91-471.97) | 434.8 (22.4-587.8) | | | |
| TGF-β (ng/ml) | probiotic | 5.03±2.42 | 6.27±2.36 | 0.315, 0.045 | 0.030†, 0.178 | 0.526, 0.055 |
| | placebo | 4.71±3.70 | 6.53±2.87 | | | |

Note: IL-10- level of interleukin-10 secreted from peptidoglycan stimulated PBMCs; IFN-γ - level of interferon-gamma secreted from concanavalin A stimulated PBMCs; TGF- β1- level of transforming growth factor - β1 measured in serum. Results are expressed as means ± standard deviations or medians (95% confidence interval). Significance was considered for $p<0.05$. Results are derived from mixed ANOVA analyses. T test with Bonferroni correction was applied for any significant main effect or interaction effect.

*There were significant changes for values at baseline and after 14 weeks in probiotic (p=0.011) and placebo group (p=0.025); Significant difference was observed between values at baseline (p=0.005), but not after 14 weeks (p=0.09).

† No differences for both probiotic (p=0.22) and placebo (p=0.06) group at baseline and after 14 weeks.

**Table 6**. POMS scores at baseline and after 14 weeks

| | | *Treatment time* | | | | |
|---|---|---|---|---|---|---|
| | | Baseline | *14 weeks* | Interaction effect (p, $\eta^2$) | Time effect (p, $\eta^2$) | Treatment effect (p, $\eta^2$) |
| Total mood disturbance | probiotic | 7.4±19.2 | 5.9±18.1 | 0.578, 0.015 | 0.547, 0.020 | 0.457, 0,012 |
| | placebo | 4.4±17.0 | 9.8±15.9 | | | |
| anger | probiotic | 6.6±3.1 | 7.4±5.6 | 0.457, 0.045 | 0.125, 0.074 | 0.465, 0.054 |
| | placebo | 5.6±3.9 | 7.1±4.4 | | | |
| confusion | probiotic | 5.2±5.8 | 4.8±3.4 | 0.145, 0.056 | 0.178, 0.087 | 0.633, 0.008 |
| | placebo | 3.0±2.9 | 5.1±3.0 | | | |
| depression | probiotic | 2.2±2.7 | 2.9±4.2 | 0.987, 0.007 | 0.875, 0.008 | 0.566, 0.055 |
| | placebo | 3.4±2.5 | 3.8±3.6 | | | |
| fatigue | probiotic | 4.8±4.1 | 4.9±2.8 | 0.544, 0.045 | 0.315, 0.074 | 0.325, 0.085 |
| | placebo | 6.4±2.3 | 6.0±4.0 | | | |
| tension | probiotic | 7.4±3.5 | 6.2±4.0 | 0.106, 0.095 | 0.145, 0.088 | 0.345, 0.088 |
| | placebo | 5.2±4.0 | 6.3±4.5 | | | |
| vigor | probiotic | 18.5±4.1 | 21.0±2.6 | 0.009*, 0.595 | 0.286, 0.140 | 0.608, 0.034 |
| | placebo | 19.6±4.8 | 18.0±3.2 | | | |

Note: Results are expressed as means ± standard deviations. Significance was considered for $p<0.05$. Results are derived from mixed ANOVA analyses. T test with Bonferroni correction was applied for any significant main effect or interaction effect.

* There was a significant difference for vigor scores at baseline and at the end of supplementation in the probiotic group (p=0.012). There were no significant differences between the values in the probiotic and placebo group at baseline (p=0.706) and at the end of supplementation (p=0.087).

Table 7. MTT assay of PBMC's measuring metabolic activity of cells, upon stimulation with antigens (ConA, PGN, LPS)

| | | *Treatment time* | | | | |
|---|---|---|---|---|---|---|
| | | Baseline | *14 weeks* | Interaction effect (p, $\eta^2$) | Time effect (p, $\eta^2$) | Treatment effect (p, $\eta^2$) |
| ConA | probiotic | 1.10±0.23 | 1.06±0.20 | 0.658, 0.020 | 0.281, 0.115 | 0.391, 0.074 |
| | placebo | 1.12±1.04 | 1.21±0.24 | | | |
| LPS | probiotic | 1.07±0.09 | 1.17±4.17 | 0.280, 0.115 | 0.425, 0.065 | 0.399, 0.072 |
| | placebo | 1.14±0.40 | 1.31±0.06 | | | |

Note: ConA – ConA stimulated cells, LPS – LPS stimulated, PGN – PGN stimulated cells Results are expressed as means ± standard deviations. Significance was considered for $p<0.05$. Results are derived from mixed ANOVA analyses.