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Probiotic supplementation has little effect on peripheral blood regulatory T-cells

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Discussion

The main finding of this study was that circulating CD4⁺ T-cells, CD4⁺ T-regs (CD127^{lo}Foxp3⁺ or CD127^{lo}CD25⁺) and CD4⁺ T-cells and T-regs expressing the chemokine receptors CXCR3 or CCR9 were not significantly different between subject groups following 150 days of supplementation with probiotic or placebo. These outcomes are in contrast with other research examining the effect of probiotic supplementation on the number of T-regs in peripheral blood [1]. However, comparisons of results from studies using different probiotic strains, dosages, methods of administration must be treated with caution. In the case of the current investigation, the selection of healthy individuals undertaking regular physical activity, which may alter the frequency of T-regs, may have masked a probiotic-induced change in the CD4⁺ T-cell or CD4⁺ T-reg subsets. The lack of induction of a probiotic-induced response mediated via T-regs in a cohort of healthy, active individuals provides important information, given that probiotics are showing promise for administration in chronic inflammatory conditions [2]. While this study suggests that probiotics consumed as a drink do not alter circulating CD4⁺ T-cell subsets or their expression of mucosal and viral response-associated chemokine receptors, modulation of immune responses local to the respiratory tract cannot be excluded as the mediator of the observed reduction in URTI in this cohort.

Interest in the use of probiotic supplements leverages off the role of the microbiota in human health [3]. A growing body of evidence indicates that probiotic bacteria are able to induce T-regs in various animal models of inflammation, including food allergy, experimental autoimmune encephalomyelitis, colitis and rotavirus vaccination [4-7]. The ability of probiotics to induce T-regs is strain-specific and depends on interaction effects between ingested and autochthonous species. The degree of induction can also vary dramatically between different anatomical locations. For example, a mixture of *L. acidophilus*, *L. casei*, *L. reuteri*, *B. lactis* and *S. thermophilus* fed to normal healthy BALB/c mice for 20 days significantly increased CD4⁺Foxp3⁺ cells in mesenteric lymph nodes compared with each of the strains individually or control [8]. Another study comparing feeding of *L. plantarum* WCFS1, *L. salivarius* UCC118, and *L. lactis* MG1363 on the intestinal regulatory immune phenotype in healthy mice reported that *L. salivarius* UCC118 skewed towards an immune regulatory phenotype in the small intestinal lamina propria. In the large intestine *L. salivarius* UCC118 induced activation

of both CD4⁺ and CD8⁺ T-cells while *L. plantarum* WCFS1 induced a more regulatory phenotype [9]. These studies provide mechanistic evidence that probiotic bacteria are able to induce T-regs in the intestine.

The present findings are in contrast to the observations of Konieczka et al, who reported an increase in peripheral blood T-regs, from 8.14 $\pm$ 0.25 (SD 1.0) to 9.19 $\pm$ 0.34 (SD 1.36) of total CD4⁺ T-cells, with eight weeks of supplementation with the probiotic *B. infantis* in 17 healthy volunteers [1]. In the current study, we also saw a significant increase in circulating T-regs over the course of the study, from 5.69 $\pm$ 0.21 (SD 1.99) to 6.38 $\pm$ 0.18 (SD 1.76) (n=93), but there was no significant effect of probiotic supplementation compared to placebo. There are a number of key differences between the two studies, including the study duration (56 days versus 150 days), the strains under investigation, the size of the population cohort (n=17 versus n=93) and the method of delivery of the probiotics, all of which may have altered the effect of probiotic supplementation. Consumption as a drink may have limited the opportunity for passage through, or transient colonisation by, the bacteria of the small intestine, although it has been found to alter faecal recovery of ingested species. Thus *L. acidophilus* NCFM consumed with skim milk or water reaches the colon and is associated with a significant increase in the faecal recovery of NCFM [10]. Passage through the intestine may be important given that *in-vitro* research indicates that induction of T-regs by the probiotic bacterium *Bifidobacterium infantis* 35624 is mediated by dendritic cell (DC) subsets [1]. Identifying whether local induction of T-regs in the respiratory passage occurs may shed further light on the mechanisms of probiotics taken in powder form.

We observed a non-specific increase in circulating T-regs in all supplement groups over the five months of the study. Regular exercise is recognised to exert an anti-inflammatory effect on the immune system, mediated via increased secretion of IL-10 and IL-4, down-regulation of co-stimulatory molecules, and lower absolute lymphocyte counts at rest [11]. These anti-inflammatory effects are similar to those reported in animal and *in-vitro* investigations of the effects of lactic acid bacteria [12]. A number of studies indicate that regular exercise increases the frequency of circulating T-regs at rest [13, 14]. A comparison of 40 males equally matched to either sedentary, recreationally active, sprint- or endurance-trained athlete groups indicated that, as a proportion of total lymphocytes, those in the endurance-trained group had a significantly higher T-reg count compared to the sedentary group. Moreover T-regs were a significantly lower proportion of CD4⁺ T cells in the sedentary

group compared to all the physically active groups [13]. A 12 week tai chi chuan exercise programme in middle aged participants was associated with an increase in absolute circulating T-reg numbers [14]. More recently, Weinhold et al reported a causal link between endurance exercise and an increase in circulating T-regs in elite Olympic athletes [15]. The cohort under investigation in the present study undertook, on average, four exercise sessions per week for a total of six hours. It may be that the anti-inflammatory effect of regular physical activity masked any effects of probiotics in altering basal circulating T-reg frequency. Furthermore, such an effect may be responsible for the significant increase over the course of the study when the groups were pooled.

Evidence showing strain-specific immunomodulatory effects of probiotics has strengthened calls for rigorously designed studies to elucidate the specific mechanistic, physiological and clinical effects in population cohorts before recommendations on usage can be issued. On the basis of this study it appears that the reduction in risk of URTI associated with supplementation utilising *Bifidobacterium animalis* subsp. *lactis* Bl-04 (Bl-04) or a combination of *Bifidobacterium animalis* subsp. *lactis* (Bi-07) & *Lactobacillus acidophilus* NCFM in healthy active adults is not associated with changes in the frequency of circulating T-regs, or in the expression of the chemokine receptors CCR9 and CXCR3. These findings are consistent with a growing number of human studies that have been unable to identify peripheral blood biomarkers that underpin the beneficial effects of probiotics for URTI. Immune factors that are strain specific and local to the respiratory mucosa may yet hold promise for future investigations.

Materials and methods

Design

Full methodological information and results of the primary clinical trial are published elsewhere [16]. Fifty participants from each of the two experimental groups and the control group provided blood samples for assessment of immune function. The trial consisted of three phases, including a 14 d run-in period in which participants' ceased consumption of purported immunomodulatory supplements. At the end of the run-in period

participants in the sampling group provided blood, were allocated to a treatment group, were provided with detailed instructions on completing an internet-based physical activity and illness log, had their height and weight measured, and completed the Connor-Davidson Resilience Questionnaire. The second phase of the study comprised 150 d of probiotic supplementation, in which participants filled out the daily physical activity and illness log, and completed the Connor-Davidson Resilience Questionnaire and a 4-d food diary at day 75 and day 150. The final phase was a 14 d monitoring period to ensure that no adverse events occurred following the cessation of probiotic supplementation. The trial was registered on the Australia New Zealand Clinical Trials Registry (ACTRN12611000130965). Ethics approval was obtained from the Griffith University Human Research Ethics Committee and the Australian Institute of Sport Human Research Ethics Committee.

Subjects

Participants were recruited from Canberra, Australia and surrounding regions. Inclusion in the study required a body mass index below 25 kg.m² and a minimum of three sessions of exercise of at least 30 min duration per week. Participants were excluded if they were on immunomodulating drug therapy, suffered from an autoimmune or gastrointestinal disease, or were unwilling to adhere to the study protocol. 241 males (age 35 ± 12 y; mean ± SD) and 224 females (age 36 ± 12 y) were allocated to one of the three supplement groups.

Characteristics for the sub-group providing a blood sample for assessment of immune function are presented in the results section.

Treatment

Participants consumed one sachet daily containing 1 g sucrose base plus either 2.0×10^9 CFU per day of *Bifidobacterium animalis* subsp. *lactis* Bl-04 (Bl-04; Danisco USA, Madison, WI) or *Lactobacillus acidophilus* NCFM and *Bifidobacterium animalis* subsp. *lactis* Bi-07 (Danisco USA) 1.0×10^{10} CFU per day (5.0×10^9 CFU of each strain) or no additives (placebo group) dissolved in a cold non-alcoholic beverage during the intervention period of 150 days. The placebo was identical in packaging, appearance and taste to the probiotic supplement.

Collection of PBMCs

Venous blood was collected in the morning at both time-points via venepuncture into heparinised tubes (Greiner Bio-one; Frickenhausen, Germany) from an antecubital vein. Following collection an automated cell count was performed using a Sysmex XT-2000i Haematology Analyser (Sysmex Corporation, Kobe, Japan) to determine the concentration of white blood cells in venous blood. Methods for PBMC isolation are published elsewhere [17]. Briefly, PBMCs were isolated by Ficoll (GE Healthcare, UK) density gradient separation, washed twice with PBS at 4°C, suspended at a concentration of $1-2 \times 10^6$ cells/mL in medium containing 10% DMSO and cooled to -70°C at a rate of -1°C per min before transfer to liquid nitrogen for storage until assay.

Flow cytometric analysis

Cryopreserved PBMCs were thawed and analysed for phenotypic characterisation of T-regs using flow cytometry. Antibodies used were CD4-PE-Cy7 (Biolegend, CA, USA), CD25-APC (Becton Dickinson, NJ, USA), CD127-BV421 (Biolegend), biotinylated CD45RO (Biolegend) followed by streptavidin Pacific Orange (Invitrogen, CA, USA), CCR9-PE (Biolegend) and CXCR3-PerCP-Cy5.5 (Biolegend). For intracellular staining, part of the sample was fixed after surface staining. Permeabilisation and staining with Foxp3-Alexa Fluor 488 (Biolegend) was performed according to the manufacturers instructions. Data from both surface and intracellular stained samples was acquired on a Canto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ USA).

Flow cytometry data were analysed using FlowJo software (Tree Star Inc., San Carlos, CA, USA). The data were collected in an uncompensated digital FCS3 file format and a software compensation matrix was calculated from single-colour compensation control samples and applied to the study sample files.

To determine the percentage of T-regs, CD4⁺ T-cells were gated on for the surface-stained samples and on CD127^{lo}FoxP3⁺ for the intracellular-stained samples (Supplementary Figure (SF) 1A). Since CD4⁺ T-cells were a stable proportion of PBMCs, T-regs were expressed as a percentage of total CD4⁺ T-cells (SF 1). Expression of CD45RO was used as a marker of effector/memory CD4 T-cells and T-regs. T-cells and T-regs not expressing CD45RO (CD45RO⁻) were termed naïve T-cells /T-regs (SF 1). The expression of the mucosal

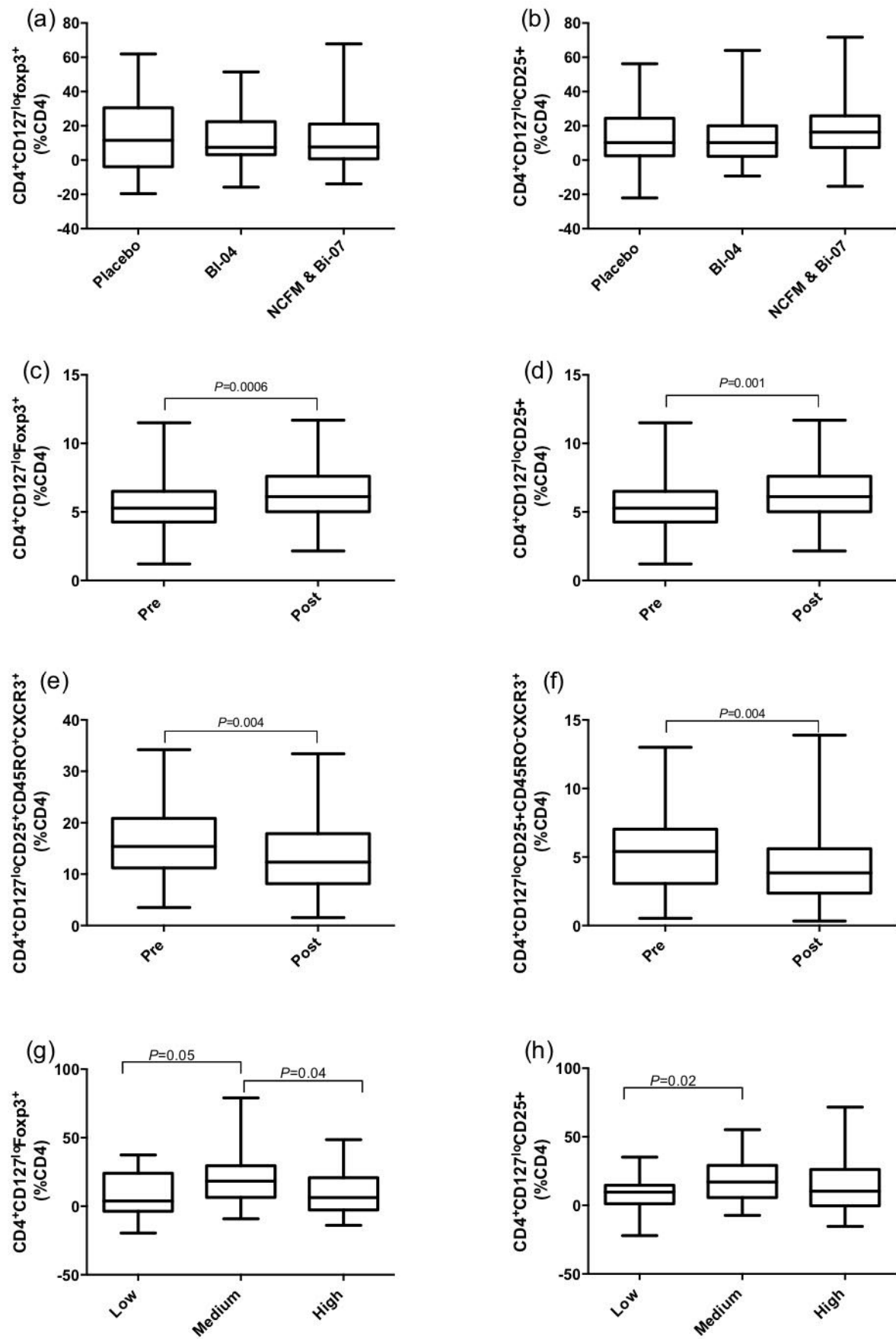
homing markers CCR9 and CXCR3 in both the effector/memory (CD45RO⁺) and naïve (CD45RO⁻) CD4 T-cells and Tregs was determined and expressed as a percentage of total Tregs and total CD4⁺ T cells (SF 1). Only surface stained samples were used to determine expression of chemokine receptors, as detection of the relevant PerCP-Cy5.5-conjugated antibodies was compromised by the fixing and permeabilisation procedure (SF1), as we have previously reported [17].

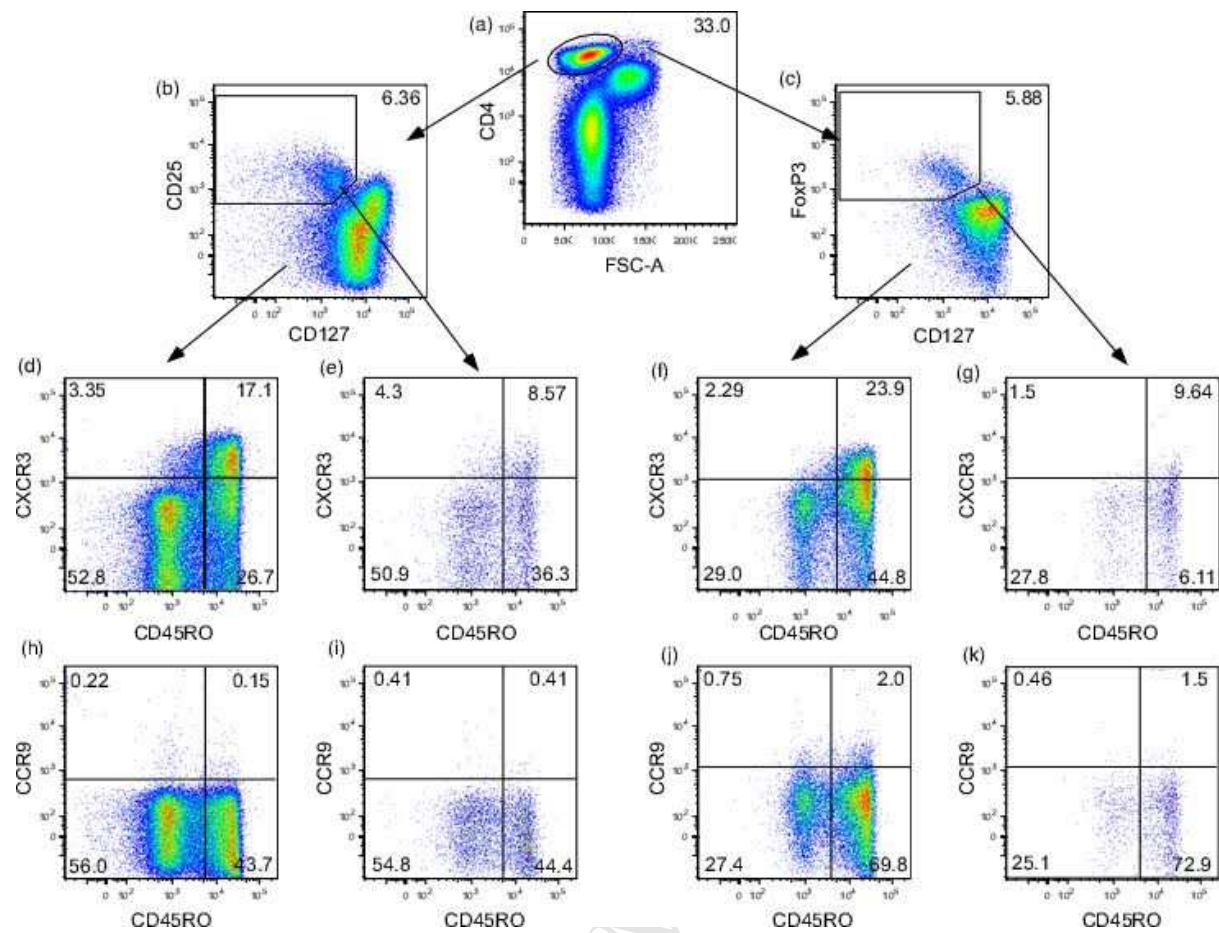
Statistical analysis

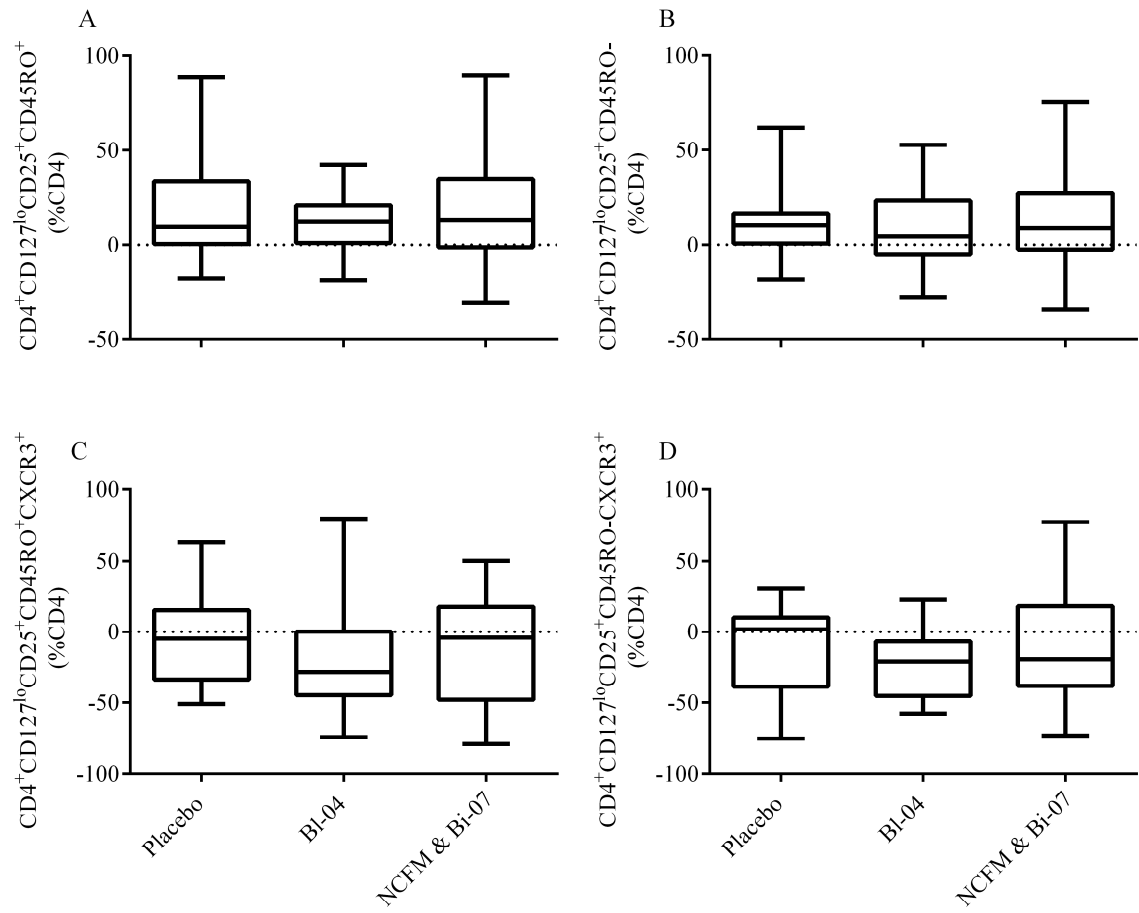
All statistical analysis was conducted using the Statistical Analysis System (SAS Institute, Cary, NC). All data were log-transformed before analysis to permit the effect of the treatments to be analysed as a percent. We analysed the differences in T-reg subsets between groups (BI-04 and placebo; NCFM & Bi-07 and placebo) with the t-statistic for independent samples with unequal variances. Post-hoc analyses were used for further analysis of significant differences. Adjustment for baseline values, URTI load (symptom duration × symptom intensity), age, sex and physical activity was undertaken. The effects of supplementation are displayed as mean ± 95% confidence interval or mean ± standard deviation. Statistical significance was set at $P < 0.05$.

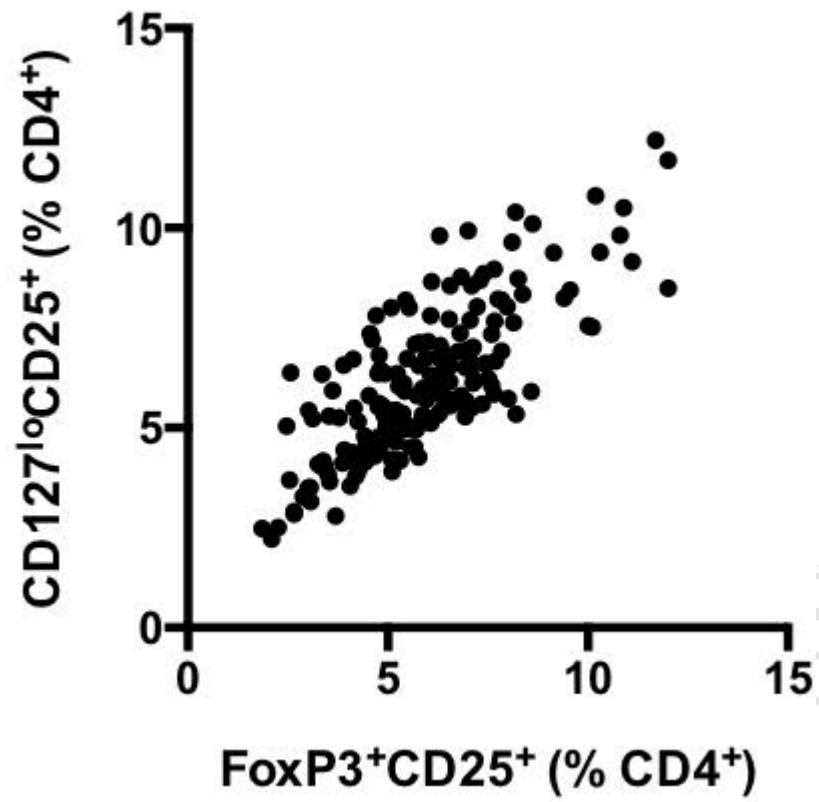
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Supplementary Table 1: Total CD4⁺ T cells as a percentage of live cells and the percentage of total effector/memory and naïve CD4⁺ T cells and effector/memory and naïve CD4⁺ T cells expressing CXCR3 from participants receiving BI-04, NCFM & Bi-07 or a placebo supplement for 150 d. There were no significant differences between the groups from pre- to post-supplement.

	Placebo		BI-04		NCFM & Bi-07		% Difference BI-04 to Placebo		% Difference Placebo to NCFM & Bi-07	
	Pre	Post	Pre	Post	Pre	Post	Mean (95% CI)	<i>P</i> -value	Mean (95% CI)	<i>P</i> -value
	Mean ± SD %	Mean ± SD %	Mean ± SD %	Mean ± SD %	Mean ± SD %	Mean ± SD %				
CD4 ⁺ (% live)	37 ± 7.2	38 ± 7.3	34 ± 7.2	36 ± 6.7	39 ± 8.4	39 ± 6.8	0.2 (-4.5 to 5.1)	0.95	0.4 (-4.8 to 5.8)	0.91
CD4 ⁺ CD45RO ⁺ (%CD4 ⁺)	38 ± 12	41 ± 11	45 ± 16	47 ± 16	38 ± 11	41 ± 12	-2.1 (-7.8 to 3.9)	0.56	-3.6 (-9.6 to 2.7)	0.34
CD4 ⁺ CD45RO ⁻ (%CD4 ⁺)	62 ± 12	58 ± 11	55 ± 16	53 ± 16	62 ± 11	59 ± 12	-0.9 (-3.2 to 4.2)	0.71	0.4 (-4.4 to 5.4)	0.89
CD4 ⁺ CD45RO ⁺ CXCR3 ⁺ (%CD4 ⁺)	18 ± 6.6	16 ± 6.4	20 ± 8.0	18 ± 7.5	17 ± 6.9	17 ± 7.1	-3.3 (-19 to 15)	0.76	7.0 (-12 to 30)	0.56
CD4 ⁺ CD45RO ⁻ CXCR3 ⁺ (%CD4 ⁺)	4.8 ± 3.6	4.1 ± 3.6	4.8 ± 2.5	4.0 ± 2.7	3.8 ± 3.2	3.0 ± 2.3	-0.7 (-16 to 17)	0.94	1.4 (-22 to 31)	0.93