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RESEARCH ARTICLE

Combined effect of *Bacillus coagulans* GBI-30, 6086 and HMB supplementation on muscle integrity and cytokine response during intense military training

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Gepner Y, Hoffman JR, Shemesh E, Stout JR, Church DD, Varanoske AN, Zelicha H, Shelef I, Chen Y, Frankel H, Ostfeld I. Combined effect of *Bacillus coagulans* GBI-30, 6086 and HMB supplementation on muscle integrity and cytokine response during intense military training. *J Appl Physiol* 123: 11–18, 2017. First published April 13, 2017; doi:10.1152/japplphysiol.01116.2016.— The purpose of this study was to compare the coadministration of the probiotic *Bacillus coagulans* GBI-30, 6086 (BC30) with β-hydroxy-β-methylbutyrate (HMB) calcium (CaHMB) to CaHMB alone on inflammatory response and muscle integrity during 40 days of intense military training. Soldiers were randomly assigned to one of two groups: CaHMB with BC30 (CaHMBBC30; $n = 9$) or CaHMB with placebo (CaHMBPL, $n = 9$). A third group of participants served as a control (CTL; $n = 8$). During the first 28 days soldiers were garrisoned on base and participated in the same training tasks. During the final 2 wk soldiers navigated 25–30 km per night in difficult terrain carrying ~35 kg of equipment. All assessments (blood draws and diffusion tensor imaging to assess muscle integrity) were conducted before and ~12 h after final supplement consumption. Analysis of covariance was used to analyze all blood and muscle measures. Significant attenuations were noted in IL-1β, IL-2, IL-6, CX3CL1, and TNF-α for both CaHMBBC30 and CaHMBPL compared with CTL. Plasma IL-10 concentrations were significantly attenuated for CaHMBBC30 compared with CTL only. A significant decrease in apparent diffusion coefficients was also observed for CaHMBBC30 compared with CaHMBPL. Results provide further evidence that HMB supplementation may attenuate the inflammatory response to intense training and that the combination of the probiotic BC30 with CaHMB may be more beneficial than CaHMB alone in maintaining muscle integrity during intense military training.

NEW & NOTEWORTHY β-Hydroxy-β-methylbutyrate (HMB) in its free acid form was reported to attenuate inflammation and maintain muscle integrity during military training. However, this formulation was difficult to maintain in the field. In this investigation, soldiers ingested HMB calcium (CaHMB) with *Bacillus coagulans* (BC30) or CaHMB alone during 40 days of training. Results indicated that CaHMB attenuated the inflammatory response and that BC30 combined with CaHMB may be more beneficial than CaHMB alone in maintaining muscle integrity during intense military training.

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β-HYDROXY-β-METHYLBUTYRATE (HMB) is a derivative of the branched-chain amino acid leucine and has been demonstrated to enhance recovery and attenuate muscle damage from high-intensity exercise (15, 16, 25, 44, 45). The mechanism of action supporting HMB's role in accelerating recovery has not been fully elucidated, but evidence does suggest that HMB may inhibit muscle degradation during catabolic events (14, 23) and/or enhance the anabolic response of muscle (1, 6, 50). Part of this response has been suggested to be related to HMB's role in attenuating inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (7, 8, 18). Additional evidence has also demonstrated that HMB administration can enhance the anabolic response of muscle tissue by activating mammalian target of rapamycin (mTOR) signaling, enhancing protein synthesis (38).

In studies examining intense physical activity with minimal recovery, such as that encountered by soldiers during sustained combat operations, the use of HMB supplementation may mitigate the deleterious effects associated with this physical stress. Significant decrements in body mass, strength, and power have been reported in soldiers during sustained military operations (27, 30). These stresses are also associated with significant elevations in inflammatory cytokine markers (29, 31). A recent field study demonstrated that when HMB was supplemented by soldiers for 3 wk during intense training, including simulated combat, the inflammatory response was attenuated and accompanied by a maintenance of muscle integrity as determined through diffusion tensor imaging (DTI) (18). These results were consistent with other investigations, albeit not in military personnel, reporting that short (e.g., 4 days) (16, 44)- and long (e.g., 12 wk) (24)-duration HMB supplementation can attenuate the cytokine response to a muscle-damaging protocol.

The use of probiotics as a dietary supplement has become very popular in the past few years for the prevention and treatment of a variety of diseases (13, 41). Probiotics are live bacteria that are suggested to be beneficial for improving digestive health and immune function while decreasing inflammation (13). It is thought that probiotics, such as *Bacillus*

coagulans GBI-30, 6086 (BC30), can enhance enzymatic digestion of foods within the gut, resulting in greater absorption of nutrients (48). Considering that BC30 has been reported to enhance protein absorption, it is possible that if combined with HMB in its calcium salt form it may enhance HMB uptake and potentially provide a greater benefit for soldiers supplementing with HMB during intense military training. As efficacy for probiotics is strain specific, BC30 was chosen as it is the only *Bacillus coagulans* that has been shown to have this effect. Thus the purpose of this study was to compare the coadministration of the probiotic BC30 with HMB calcium (CaHMB) to CaHMB alone on muscle damage and the inflammatory response during 40 days of highly intense military training.

MATERIALS AND METHODS

Participants. Twenty-six male soldiers from an elite combat unit of the Israel Defense Forces (IDF) volunteered to participate in this double-blind, parallel-design study. After an explanation of all procedures, risks, and benefits, each participant provided his informed consent to participate in the study. The Institutional Review Board of the IDF Medical Corps and the Medical Ethics Board and the Helsinki Committee of Soroka Medical Center approved this research study (1504-2015). Participants were not permitted to use any additional dietary supplements and did not consume any androgens or other performance-enhancing drugs. Screening for performance-enhancing drug use and additional supplementation was accomplished via a health questionnaire completed during participant recruitment. Soldiers were from the same unit and were randomly assigned to one of two groups: CaHMB with BC30 (CaHMBBC30, $n = 9$; 20.5 ± 0.8 yr, 1.75 ± 0.09 m, 75.4 ± 9.6 kg) or CaHMB with placebo (CaHMBPL, $n = 9$; 19.1 ± 3.4 yr, 1.73 ± 0.05 m, 71.4 ± 6.4 kg). A third group of participants from the same unit, who were interested in participating in the study but were not interested in consuming a supplement, agreed to serve as a control group (CTL, $n = 8$; 20.4 ± 0.7 yr, 1.73 ± 0.05 m, 68.6 ± 5.3 kg). A CONSORT schematic outlining the overall study sample is presented in Fig. 1.

Study protocol. During the 40-day intervention period, all participants performed the same daily protocol. This training period was part of the soldiers' advanced military training. During the first 28 days soldiers were garrisoned on base and participated in the same advanced military training tasks that included combat skill development and conditioning including 90 min of intense hand-to-hand combat (krav-maga) training five times a week. In addition, all soldiers consumed their meals together, maintaining a similar dietary intake throughout the study duration. The physical training included on average two 5-km runs per week. During *weeks 5* and *6* soldiers were in the field and navigated between 25 and 30 km per night in difficult terrain carrying ~35 kg of equipment on their back (equating to ~40% of participant's body mass). The duration of the navigational exercise lasted between 5 and 8 h per evening. During the last evening of training (*day 40*), the soldiers also performed an additional 5-km stretcher carry after the navigational training. During the final 2 wk of training soldiers slept between 5 and 8 h per night. All assessments [blood draws and magnetic resonance imaging (MRI)] were conducted in a single day before (PRE) and ~12 h after the final supplement consumption (on *day 40*) (POST). All assessments were performed in the same order at both PRE and POST.

Supplementation protocol. Participants in both CaHMBBC30 and CaHMBPL ingested 1.0 g of CaHMB three times per day for a total daily consumption of 3 g. Each serving consisted of four capsules (250 mg CaHMB) consumed during morning, noon, and evening meals. CaHMB was obtained from Metabolic Technologies (Ames, IA). The probiotic supplement (BC30) was provided by Ganeden Biotech (Mayfield Heights, OH). Each serving contained 1.0×10^9 colony-forming units. Participants consumed one serving per day (morning meal). The placebo was provided by the manufacturer and matched in appearance, weight, and taste to the active product. Both the placebo and the active product were provided in powder form and mixed in water (~250 ml) before ingestion. Participants in CaHMBBC30 and CaHMBPL were provided with two 20-day supplies of BC30 and placebo. Participants were required to return all used and unused packets at the end of each 20-day period.

Blood measurements. Resting blood samples were obtained before each testing session. All blood samples were obtained after a 15-min equilibration period with the participant in a seated position. These blood samples were obtained from an antecubital arm vein with a 20-gauge disposable needle equipped with a Vacutainer tube holder

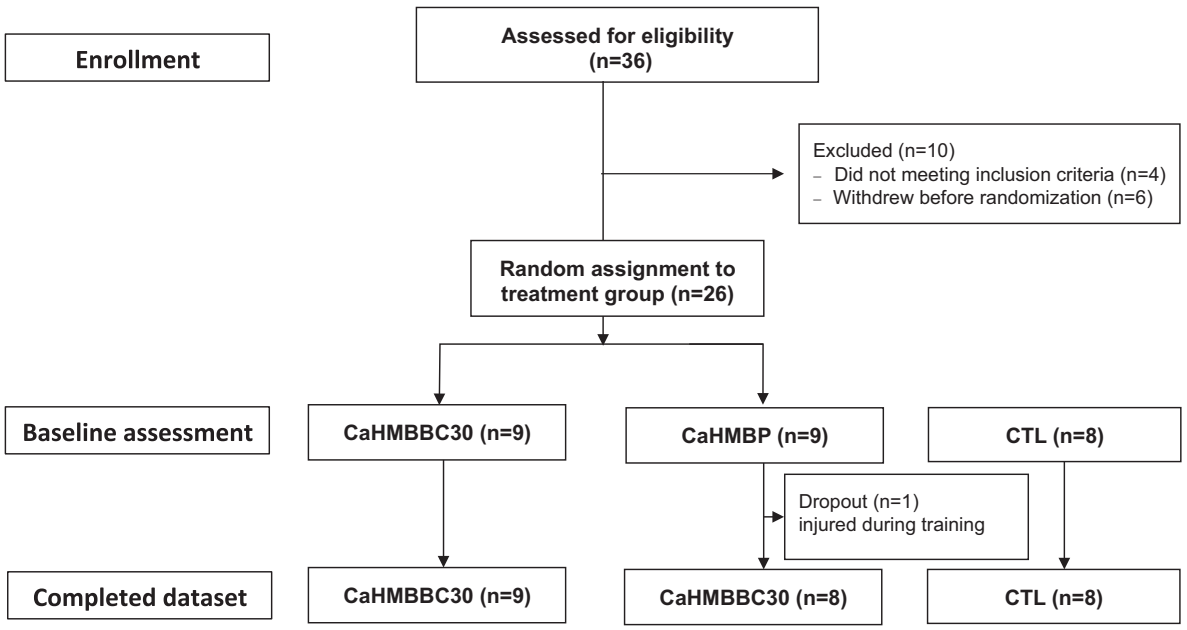

Fig. 1. CONSORT diagram. Participant screening through study completion is shown for all study participants. CaHMBBC30, calcium HMB and *Bacillus coagulans*; CaHMBP, calcium HMB and placebo; CTL, control.

(Becton Dickinson, Franklin Lakes, NJ). Participants' blood samples were obtained at the same time of day during each session after an overnight fast. All blood samples were collected into two Vacutainer tubes, one containing no anticlotting agent for plasma and the second containing K_2EDTA for serum. The blood in the first tube was allowed to clot at room temperature for 30 min and subsequently centrifuged at 1,400*g* for 15 min along with the remaining whole blood from the second tube. The resulting plasma and serum were placed into separate 1.8-ml microcentrifuge tubes and frozen at −80°C for later analysis.

Biochemical analysis. Serum concentrations of creatine kinase (CK) and lactate dehydrogenase (LDH) were analyzed with a commercially available kinetic assay (Sekisui Diagnostics, Charlottetown, PE, Canada; Sigma-Aldrich, St. Louis, MO) per manufacturer's instructions. Plasma concentrations of cytokines and chemokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), fractalkine (CX3CL1), interferon (INF)-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, and TNF-α were analyzed via multiplex assay using Human Cytokine/Chemokine Panel I (EMD Millipore, Billerica, MA). In addition, plasma HMB concentrations were analyzed by gas chromatography-mass spectrometry and performed by Metabolic Technologies with methods previously described (32). All samples were thawed once and analyzed in duplicate by the same technician using a BioTek Eon spectrophotometer for CK and LDH concentrations (BioTek, Winooski, VT) and MagPix for cytokine and chemokine concentrations (EMD Millipore). Mean intra-assay variability for all assays was <10%.

Magnetic resonance imaging. Changes in muscle integrity of the rectus femoris (RF) and vastus lateralis (VL) were assessed with MRI. Because of logistical considerations (element of time), it was determined a priori that the primary focus of the study was to compare the effect of BC30 coadministered with CaHMB to CaHMB only; therefore only soldiers in CaHMBBC30 and CaHMBPL were assessed with MRI. Muscle integrity was determined through DTI. DTI is a sensitive MRI technique to assess subclinical signs of muscle injury (10). DTI assessment is predicated on cell membranes and other structures constraining water diffusion. Water movement can be evaluated by determining the three orthogonal directions of water diffusion, called eigenvectors, and their intensities, called eigenvalues (5). From the three eigenvalues (λ1, λ2, and λ3), parameters such as fractional anisotropy (FA) and apparent diffusion coefficient (ADC) can be calculated to evaluate the character of water diffusion in a voxel. These measures have been demonstrated to provide information about the integrity of skeletal muscle (4).

The MRI data were obtained with a 3.0-T whole body imager (Ingenia, Philips Medical Systems, Best, The Netherlands). During each measure participants were placed supine in the scanner and imaged with phased-array surface coils. A position 20 cm above the patella was chosen as the image center and marked with a fish oil capsule. All scans were planned axially and consisted of 40 slices of 4-mm width for a foot-head coverage of 160 mm and a field of view of 290 × 280 mm (RL × AP). Three image acquisitions were performed. A T1w Dixon was used for anatomical reference, a T2w turbo spin-echo to assess any structural damage to the muscle, and a DTI sequence for muscle fiber tracking. The sequence parameters that were used have been previously published (18).

Fat suppression (spectrally selective adiabatic inversion recovery) was used for the T2-TSE and DTI scans. The DTI sequence was a two-dimensional EPI sequence imaged in two packages. The *b* value was 400 s/mm^2 and imaged in 15 unique directions. Muscle fiber tracking analysis was calculated with Philips FiberTrak software. A region of interest was hand drawn for RF and VL on *slices 15* and *25*. The software then was allowed to delineate the muscle fibers with an algorithm that eliminated tracks if the FA was <0.1, if the change in angle was >27°, or if the fiber length was <10 mm. The same investigator performed all assessments.

Statistical analyses. Analyses of covariance (ANCOVAs) were used to analyze all MRI and blood-dependent variables (muscle damage markers and cytokines) based on the recommendations of Huck and McLean (21). PRE and POST values were used as the covariate and dependent variable, respectively, after verification of the homogeneity of regression (47). In the event of a significant *F* ratio, least significant difference post hoc pairwise comparisons were used to examine the differences among the groups. In accordance with Vickers (47), results of the ANCOVA were also converted to change from PRE. An α level of $P \leq 0.05$ was considered statistically significant for all comparisons. All data are reported as means ± SD unless otherwise noted. Statistical analysis was performed with SPSS (IBM Statistics for Windows, version 23.0; IBM, Armonk, NY).

RESULTS

Of the 26 soldiers who participated in this trial, 25 completed the intervention. The only participant who withdrew from the study was injured during training. No changes in body mass were noted between groups ($P = 0.22$). No side effects associated with supplementation were reported during the study. Medical personnel involved in the study were attached to the soldiers' unit and monitored compliance on a daily basis. Based on CaHMB and BC30 consumption (determined by the number of capsules and BC30 packets returned) compliance for supplementation was 95.0 ± 3.0% for the two CaHMB groups. An additional measure for study compliance was conducted by analyzing plasma HMB concentrations at PRE and POST for participants in both supplement groups. Significant elevations ($P = 0.010$) were noted in plasma HMB concentrations from PRE (3.28 ± 0.73 nmol/l) to POST (34.1 ± 43.9 nmol/l) assessments.

Blood data. Comparisons of the individual response change from PRE for each participant within the groups are depicted in Fig. 2. Significant differences were observed for changes in circulating TNF-α ($F = 6.48$, $P = 0.006$), CX3CL1 ($F = 4.70$, $P = 0.025$), IL-1β ($F = 6.93$, $P = 0.006$), and IL-2 ($F = 4.96$, 0.019) concentrations. Plasma concentrations at POST for these cytokines were significantly attenuated for both CaHMBBC30 and CaHMBPL compared with CTL ($P < 0.05$). In addition, significant differences were also noted at POST in plasma IL-6 ($F = 6.27$, $P = 0.012$) and IL-10 ($F = 3.72$, $P = 0.041$) concentrations. Plasma IL-6 and IL-10 concentrations were significantly attenuated in CaHMBBC30 compared with CTL ($P < 0.01$). No significant differences were noted between CAHMBBC30 and CAHMBPL. Furthermore, no significant differences were observed between any of the groups in INF-γ ($F = 1.25$, $P = 0.31$), IL-8 ($F = 1.49$, $P = 0.25$), or GM-CSF ($F = 0.71$, $P = 0.50$) concentrations.

Analysis of muscle damage markers revealed no significant differences between the groups for plasma LDH ($F = 0.15$, $P = 0.86$) or CK ($F = 0.17$, $P = 0.84$) concentrations. No changes in LDH concentrations were noted from PRE (537.7 ± 86.1 IU/l) to POST (567.5 ± 87.4 IU/l) in the groups combined. In addition, no change was noted in CK concentrations from PRE (225.4 ± 79.8 IU/l) to POST (377.6 ± 230.2 IU/l) in the groups combined.

DTI. Comparisons of FA and ADC assessments between CaHMBBC30 and CaHMBPL can be observed in Table 1. In addition, comparisons of the change from PRE between groups are depicted in Fig. 3. No significant difference ($F = 0.315$, $P = 0.587$) in FA was observed between CaHMBBC30 and CaHMBPL in the RF; however, when collapsed across groups

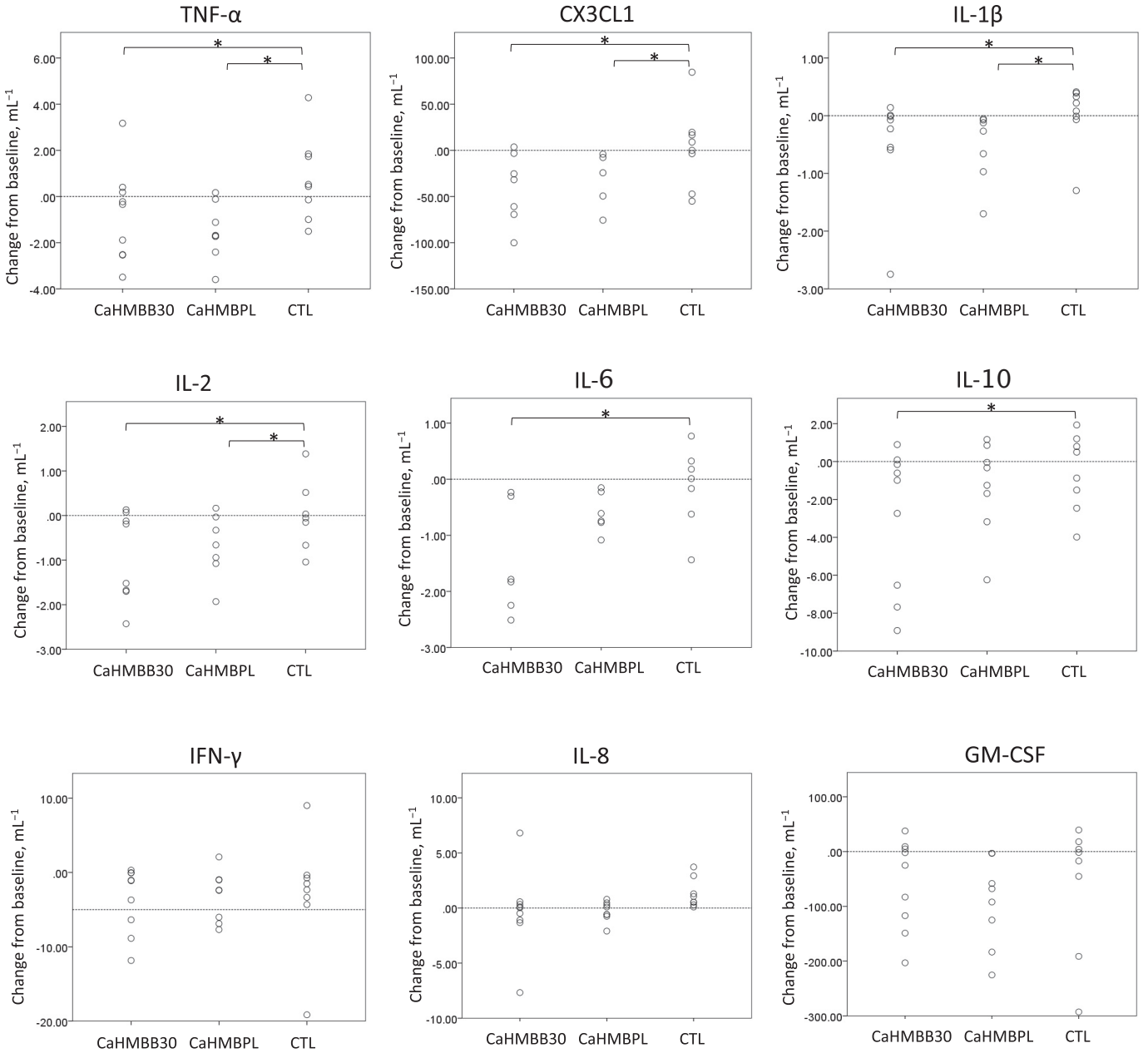

Fig. 2. POST-PRE comparisons of the individual change in the inflammatory cytokine response to intense military training following 40 days of supplementation. CaHMBBC30, calcium HMB and *Bacillus coagulans*; CaHMBPL, calcium HMB and placebo; CTL, control. Results are reported as means ± SD. $^*P < 0.05$ for main effect for group.

a significant decrease was noted from PRE to POST. A significant difference ($F = 7.198$, $P = 0.023$) in ADC was noted between the groups in RF. Participants in CaHMBBC30 experienced a decrease in ADC, while participants in CaHMBPL experienced an increase. No significant differences between the groups were noted in the VL for either FA ($F = 2.95$, $P = 0.117$) or ADC ($F = 1.886$, $P = 0.200$).

DISCUSSION

The results of this study indicate that 40 days of HMB supplementation with and without BC30 can attenuate inflammatory cytokine markers during highly intense military training. This is consistent with a previous study examining the potential benefits of HMB ingestion in soldiers during intense training (19) and provides further evidence supporting attenuation of the cytokine response to highly intense exercise protocols when supplementing with HMB (16, 31, 44). A novel aspect to this investigation was examining the combined ingestion of a probiotic with CaHMB compared with CaHMB ingestion alone. This combination appeared to attenuate the IL-6 and IL-10 response compared with CTL, while no difference was noted in the comparison between CaHMBPL and CTL. In addition, the combination of CaHMB and BC30 provided a significant benefit compared with CaHMB alone in maintaining muscle integrity, as indicated by a decrease in ADC for the RF.

Table 1. *MRI and DTI measures in β-hydroxy-β-methylbutyrate with and without probiotics (BC30) in response to intense military training*

		PRE	POST	*P* Value
Rectus femoris				
Fractional anisotropy	CaHMBBC30	0.24 ± 0.05	0.20 ± 0.02	0.014
	CaHMBPL	0.23 ± 0.02	0.19 ± 0.02	
Apparent diffusion coefficient ($\times 10^{-3}$ mm^2/s)	CaHMBBC30	1.79 ± 0.09	1.72 ± 0.09	0.69
	CaHMBPL	1.68 ± 0.06	1.77 ± 0.04	
Vastus lateralis				
Fractional anisotropy	CaHMBBC30	0.20 ± 0.03	0.21 ± 0.02	0.23
	CaHMBPL	0.20 ± 0.01	0.19 ± 0.01	
Apparent diffusion coefficient ($\times 10^{-3}$ mm^2/s)	CaHMBBC30	1.73 ± 0.05	1.71 ± 0.05	0.86
	CaHMBPL	1.73 ± 0.06	1.75 ± 0.12	

Data are reported as nonadjusted means ± SD. CaHMBBC30, calcium HMB and *Bacillus coagulans*; CaHMBPL, calcium HMB and placebo.

To the best of our knowledge, this appears to be the first investigation to examine the combined use of BC30 and HMB supplementation. Although previous studies have suggested that BC30 can enhance protein absorption (13, 48), this appears to be the first examination of the potential benefit of the coingestion of these two supplements. However, the design of this study does not provide any clarity regarding enhanced absorption capability when CaHMB is combined with BC30. Soldiers last ingested CaHMB ~12 h before the final blood draw; however, final ingestion of BC30 or placebo occurred 24 h before POST measures. Furthermore, the timing of ingestion was not tightly controlled as to allow for any discussion regarding differences in absorption rates. Nevertheless, results of plasma HMB concentrations at POST were interesting. Plasma HMB concentrations for CaHMBBC30, CaHMBPL, and CTL were 50.6 ± 15.6 nmol/l, 15.6 ± 28.0 nmol/l, and 3.3 ± 0.9 nmol/l, respectively. The greater HMB concentrations observed for CaHMBBC30 do suggest that BC30 may have enhanced absorption capability; however, additional research is still warranted. Previous research has reported that plasma HMB concentrations peak ~2 h after ingestion of CaHMB with a half-life of 3.17 h from a single 1-g dose (11). Previously, the ingestion of HMB in its free acid form resulted in a doubling in the area under the curve for 24-h plasma HMB concentrations compared with ingestion of CaHMB (11). However, if HMB absorption is enhanced through the use of a probiotic, the potential for greater HMB availability from CaHMB ingestion may exist. The absorption of CaHMB is thought to occur in the duodenum and proximal jejunum (2). At this location along the digestive tract the spores of BC30 have been shown to withstand gastric acids and bile salts, with a theoretical survival of the spores demonstrated to be >80% at the terminal ileum (12). In addition, BC30 appears to offer a more favorable medium in the intestinal tract by enhancing germination of the spores producing antimicrobial compounds and converting genotoxic compounds to unreactive products (20). Thus when CaHMB is combined with BC30 it may provide for greater absorption capability and potentially for a greater increase in circulating HMB.

Supplementation with HMB has been previously suggested to enhance recovery and attenuate muscle loss by inhibiting the ubiquitin-proteasome-mediated proteolytic pathway and reducing the proteolytic effects of various muscle-degrading stimuli (7, 8). A reduction in the cytokine response to intense training has often been used to indicate a more favorable recovery from high-intensity training (9, 18, 31, 39, 44). The attenuation observed in TNF-α, IL-6, and CX3CL1 concentrations for both CaHMBBC30 and CaHMBPL is consistent with studies examining both resistance exercise (7, 8, 44), and intense military training (18). In addition, 40 days of HMB ingestion also

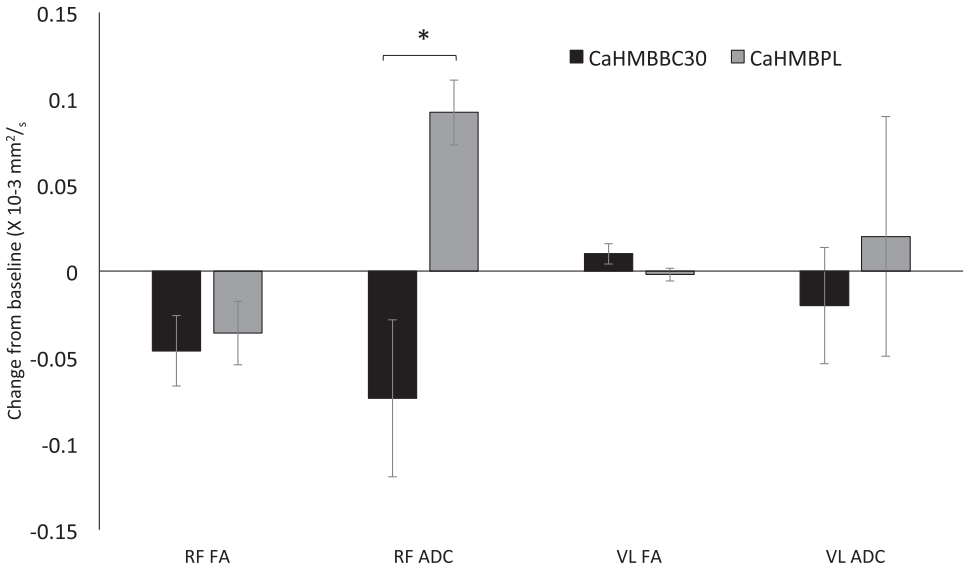

Fig. 3. POST-PRE changes in diffusion tensor imaging (DTI) measures following 40 days of supplementation and intense military training. CaHMBBC30, calcium HMB and *Bacillus coagulans*; CaHMBPL, calcium HMB and placebo; RF, rectus femoris; VL, vastus lateralis; FA, fractional anisotropy; ADC, apparent diffusion coefficient. Results are reported as means ± SD. *Significantly different between CaHMBBC30 and CaHMBPL.

appeared to attenuate IL-1β and IL-2 responses to the high-intensity military training.

Only a limited number of investigations have investigated the effect of HMB supplementation and changes in circulating IL-6 concentrations during intense physical activity (18, 31, 39). IL-6 is considered to be a proinflammatory cytokine (49), and changes in IL-6 concentration are often used as a marker of muscle recovery or an indicator of training stress (9). IL-6 has also been suggested to have anti-inflammatory effects (9, 49), which are attributed to a negative feedback loop mechanism in which elevations in IL-6 production will result in a suppression of IL-6 release (49). Elevations in IL-6 have also been associated with decreases in TNF-α and promotion of other anti-inflammatory cytokines such as IL-1ra, IL-10, and C-reactive protein (9). Previously, significant elevations in IL-6 have been reported during short (7 days)-, moderate (23 days)-, and long (~60 days)-duration military training (17, 18, 28, 38). The physical stress (35-kg load carriage and 250- to 300-km total marching distance) experienced by the soldiers of the present study was most similar to that previously reported by Henning et al. (17). In that study, IL-6 concentrations were reported to increase 2.7-fold above baseline. Although the participants in this study began at a relatively higher level, likely related to the intense training of the unit before study enrollment, the supplementation period resulted in a significant attenuation of the IL-6 response. This was consistent with a previous investigation examining 23 days of HMB ingestion in soldiers during intense training (18).

The attenuation of both plasma TNF-α and CX3CL1 concentrations in both HMB groups during intense military training was consistent with that recently reported by Hoffman and colleagues (18). Both TNF-α and CX3CL1 are proinflammatory cytokines. TNF-α is part of the signaling mechanism that directs immune cells to sites of damage and initiates degradation of tissue (37), while CX3CL1 is involved in promoting leukocyte binding and adhesion as well as activation of target cells (51). In addition, significant reductions in the proinflammatory cytokines IL-2 and IL-1β were also noted in both HMB groups as well. The attenuated response of IL-1β contrasted with that previously reported after 23 days of HMB supplementation during high-intensity training in soldiers (18) but was consistent with the response observed by Kraemer and colleagues (24) during an intense resistance training session (6 sets of 10 repetitions at 80% of maximal strength in squat exercise) following 12 wk of HMB supplementation. Although differences in the stressor (e.g., chronic highly intense military training vs. an acute bout of resistance exercise) likely result in different response patterns, longer periods of supplementation may be required to alter certain cytokine responses. IL-1β has also been shown to be a very sensitive marker to stress associated with intense military training (28, 46) and has been suggested to be a sensitive marker of extreme fatigue following deployment (46). van Zuiden and colleagues (46) reported a dose-response association of IL-1β-induced IL-8 production in cultures of whole blood collected in soldiers 6 mo after deployment. In the present study, the significant attenuation observed in IL-1β from HMB ingestion coincided with 7.5% and 12.0% decrease in circulating concentrations of IL-8 in CaHMBBC30 and CaHMBPL, respectively. Interestingly, a 51.4% increase in plasma IL-8 concentrations was observed in CTL during the study period. Although these results were not statistically different, the response pattern does appear to support the results of van Zuiden et al. (46).

No significant differences were observed between the groups in the GM-CSF and INF-α response to intense military training. Previously, HMB ingestion was reported, with the use of magnitude-based inferences to analyze results, to possibly attenuate GM-CSF but likely attenuate INF-γ after a 23-day ingestion protocol during intense training (18). Although studies examining these specific inflammatory cytokines appear to be limited, especially during intense military training, the GM-CSF response does appear to be consistent with that previously reported after 12 wk of HMB supplementation and resistance exercise (31) but differs from the INF-γ response reported in the same study. Although INF-γ is a proinflammatory cytokine, it has been suggested to be attenuated by elevations in cortisol (42). Although speculative, considering that cortisol concentrations were not analyzed in this study, the response pattern of INF-γ across all groups (38.5%, 28.2%, and 15.4% decrease in CaHMBBC30, CaHMBPL, and CTL, respectively) does raise the specter of a cortisol-induced attenuation.

The results of this study provide further evidence that HMB ingestion can lead to attenuation of the cytokine response to intense physical activity. However, the results of this study may have also been influenced by a synergistic effect of BC30. Recently, 28 days of BC30 supplementation in older adults was reported to attenuate IL-6 and increase IL-10 concentrations (33). IL-10 is considered to be an anti-inflammatory marker and has been suggested to be stimulated by elevations in IL-6 (40) and/or by a downregulation of IL-1β and TNF-α (26). However, the relationship between IL-6 and IL-10 has been reported only during an extreme inflammatory response such as sepsis (31). The attenuation of IL-10 observed in CaHMBBC30 is not consistent with these reports but likely reflects the lower inflammation associated with military training compared with a life-threatening infection. Furthermore, the physiological relevance of increasing anti-inflammatory cytokines during exercise has been questioned (36). The proinflammatory response is generally thought to directly stimulate the anti-inflammatory response (43). However, if the proinflammatory cytokine response is attenuated, this would also blunt the anti-inflammatory cytokine response as well. These contrasting responses are difficult to explain but are consistent with the complexity associated with the cytokine response to muscle-damaging events (35).

No significant interaction or main effect was noted in either of the muscle damage markers. Both LDH and CK concentrations at PRE appeared to be high and likely reflect the stress of the units training. The relationship between inflammatory markers and muscle damage markers appears to be unclear, as significant elevations in CK and myoglobin have been reported without any change in inflammatory cytokine markers (22). In addition, the lack of any change in the muscle damage markers may reflect the experience level of the soldiers. Soldiers involved in advanced military training likely have a degree of sensitization or resiliency to the physical stress of training (18, 19).

Measures of muscle integrity were conducted with DTI, which is considered to be a sensitive method of assessing subclinical signs of muscle injury (3, 10). DTI measures the diffusion of water molecules and the direction of their move-

ment in a three-dimensional muscle microstructure (5). In healthy tissue, the integrity of the structure results in a barrier to diffusion (3). FA represents the increase in diffusivity into tissue following trauma, while ADC reflects the degree of diffusion in each direction of the muscle by the length of its axis (3). A decrease in FA and an increase in ADC represent a disruption to the integrity of the muscle indicating greater diffusion (3, 5). Previously, we reported significant decreases in FA in both the RF and semitendinosus for the placebo group only and a likely increase in ADC of the VL in the supplement group, indicating that HMB provided in its free acid form may enhance muscle integrity during intense military training (18). This present study focused only on examining whether the addition of BC30 to CaHMB can enhance the degree of protection of muscle integrity greater than CaHMB alone. Although a significant decrease in FA for the RF was observed in both CaHMBBC30 and CaHMBPL, the combination of CaHMB and BC30 resulted in a significantly lower ADC in the RF compared with CaHMB only. The decrease in FA across time with both groups is consistent with the extent of muscle damage associated with intense military training. However, the data also indicate that the addition of BC30 to CaHMB may have provided a synergistic effect for maintaining muscle integrity, resulting in a greater degree of muscle protection than that offered by CaHMB alone.

A limitation of this study was the lack of performance and body composition measures. Because of the logistical requirements of the soldiers, these measurements could not be performed. In addition, the lack of MRI measures in the control group limited our interpretation of the effect of CaHMB on changes of muscle integrity. Although dietary intake was similar among all participants, dietary consumption was not recorded and is an additional limitation of this study. This needs to be acknowledged in consideration that previous research has indicated that dietary intake can affect the inflammatory response in soldiers (34). In summary, the results of this study provide further evidence that CaHMB supplementation may attenuate the inflammatory response to intense military training. In addition, the results of this study also indicate that the combination of the probiotic *Bacillus coagulans* with CaHMB may be more beneficial than CaHMB alone, specifically for maintaining muscle integrity during intense military training. Although this benefit may be related to an enhanced rate of absorption, this will require additional research.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.G., J.R.H., E.S., Y.C., H.F., and I.O. conceived and designed research; Y.G., J.R.H., E.S., D.D.C., A.N.V., H.Z., I.S., and I.O. performed experiments; Y.G., J.R.H., J.R.S., D.D.C., A.N.V., and I.S. analyzed data; Y.G., J.R.H., J.R.S., and I.S. interpreted results of experiments; Y.G. and J.R.H. prepared figures; Y.G. and J.R.H. drafted manuscript; Y.G., J.R.H., E.S., J.R.S., D.D.C., A.N.V., H.Z., I.S., Y.C., H.F., and I.O. edited and revised manuscript; Y.G., J.R.H., E.S., J.R.S., D.D.C., A.N.V., H.Z., I.S., Y.C., H.F., and I.O. approved final version of manuscript.

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