Effects of bovine colostrum supplementation on upper respiratory illness in active males

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Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

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ABSTRACT

Bovine colostrum (COL) has been advocated as a nutritional countermeasure to exercise-induced immune dysfunction and increased risk of upper respiratory illness (URI) in athletic populations, however, the mechanisms remain unclear. During winter months, under double-blind procedures, 53 males (mean training load ± SD, 50.5 ± 28.9 MET-h·week^{-1}) were randomized to daily supplementation of 20 g of COL (N = 25) or an isoenergetic/isomacronutrient placebo (PLA) (N = 28) for 12 weeks. Venous blood was collected at baseline and at 12 weeks and unstimulated saliva samples at 4-week intervals. There was a significantly lower proportion of URI days and number of URI episodes with COL compared to PLA over the 12 weeks ($p < 0.05$). There was no effect of COL on *in vitro* neutrophil oxidative burst, salivary secretory IgA or salivary antimicrobial peptides ($p > 0.05$), which does not support previously suggested mechanisms. In a subset of participants (COL = 14, PLA = 17), real-time quantitative PCR, targeting the 16S rRNA gene showed there was an increase in salivary bacterial load over the 12-week period with PLA ($p < 0.05$) which was not as evident with COL. Discriminant function analysis of outputs received from serum metabolomics showed changes across time but not between groups. This is the first study to demonstrate that COL limits the increased salivary bacterial load in physically active males during the winter months which may provide a novel mechanism of immune-modulation with COL and a relevant marker of *in vivo* (innate) immunity and risk of URI.

Keywords: URTI, innate immunity, mucosal immunity, microbiome, 16S rRNA, metabolomics

1. Introduction

It is now well established that exercise of a strenuous and/or prolonged nature can lead to significant transient perturbations of immune function (commonly referred to as immunodepression) which includes, but is not limited to, decreases in both cell-mediated and mucosal parameters (Nieman, 2007). This may result in an 'open window' during which risk of illness is increased (Nieman, 2000). Hence, if such exercise is performed on a regular basis, as with endurance athletes, and particularly in combination with other life stressors (e.g. inadequate nutrition, psychological stress) the overall risk can be substantially higher (Gleeson, 2007). The increase in the frequency and severity of symptoms of upper respiratory illness (URI) in athletes (e.g. sore throat, runny nose) has been attributed to such periods of heavy exertion (Walsh et al., 2011).

Bovine colostrum (COL) may be effective at alleviating recurrent URI in situations of immune deficiency (Patel and Rana, 2006). Previous evidence has shown that 8-10 weeks of COL supplementation can reduce the incidence of URI in physically active populations but the mechanism(s) behind such effects remains unclear (Brinkworth and Buckley, 2003; Crooks et al., 2010). Animal and *in-vitro* culture studies demonstrate that COL has a mediating effect on cell-mediated immunity by influencing the production of cytokines (Biswas et al., 2007; Boudry et al., 2007; Shing et al., 2009). Increasing concentrations of COL, *in-vitro*, has been shown to modulate cytokine production in peripheral blood mononuclear cells from resting, healthy individuals, to promote a Th1 profile (cell-mediated immunity) (Shing et al., 2009), which may suppress the binding of pathogens (e.g. rhinovirus) (Sethi et al., 1997). Direct effects of COL on leukocyte capacity are also supported by evidence of an

70 enhancement of phagocytosis and oxidative burst of polymorphonuclear cells (i.e.
71 neutrophils) following short term culture with COL (Sugisawa et al., 2001, 2002, 2003).
72 Sugisawa et al. (2003) proposed that in the presence of COL leukocytes become
73 primed for subsequent activation by low-molecular weight substances (< 10 kDa) such
74 as protease peptones.

75 Given the aforementioned effects of COL within inflammatory *in vitro* culture
76 conditions, it may be expected that COL can act as a nutritional countermeasure to
77 exercise-induced immunodepression. Our previous work suggests that 4 weeks of
78 COL (20 $g \cdot day^{-1}$) supplementation can limit the immunodepressive effects of an acute
79 physical stressor (2 h of cycling) by enhancing neutrophil function (stimulated
80 degranulation/elastase release) post-exercise (Davison and Diment, 2010). Within this
81 study the modulatory effects of COL also extended to innate mucosal immunity by
82 preventing the exercise-induced decrease of salivary lysozyme concentration and
83 secretion.

84 Such findings may provide support to proposed mechanisms that some of the immune-
85 modulatory effects of COL are due to bioactive components that become biologically
86 available upon digestion of COL and prime leukocyte capacity (Davison, 2013). It is
87 currently unclear whether longer term supplementation of COL and exposure to these
88 priming agents also leads to changes in innate markers in athletes at rest. Crooks et
89 al. (2006) demonstrated that longer periods of COL supplementation (i.e. 12 weeks)
90 may be associated with significant increases in resting concentrations of salivary
91 secretory IgA (sIgA), which is the only immune measure to date that has been
92 consistently related to risk of URI in exercising populations (Walsh et al., 2011). Other
93 studies have also seen improvements in resting sIgA concentrations with COL
94 supplementation but have not monitored URI (Appukutty et al., 2010; Mero et al.,

2002). To date, the majority of both longitudinal and cross-sectional exercise training studies have focused on changes in salivary sIgA (Walsh et al., 2011). Although the importance of other salivary antimicrobial peptides (AMPs) (e.g. lysozyme, lactoferrin) for host defense have been recognized, they have received limited attention (West et al., 2006).

In addition to the presence of inducible factors such as AMPs at mucosal surfaces, protection from invading microorganisms is also provided by the diverse community of commensal microbes which colonize the upper respiratory tract (Blaser and Falkow, 2009; Bosch et al., 2013). Subsequently, disturbance of this respiratory microbial community can contribute to acquisition of new pathogens which may result in respiratory illness, particularly if host immunity is compromised (Murphy et al., 2009). However, the effects of exercise and nutritional interventions on changes in the salivary microbiome have not previously been investigated.

The aims of this study were to investigate the effects of 12 weeks of COL supplementation on innate and mucosal immunity as well as the salivary microbiome in a population of males who engage in exercise training during the winter months. The study also aimed to determine whether any effects of COL on these parameters would also lead to a change in the incidence of URI. Given the potential involvement of a diverse array of biological pathways, we also undertook a metabolomic profiling approach on serum in an attempt to gain a more detailed understanding of any modulation of the immune system by COL.

2. Methods

2.1. Participants

Following both verbal and written details of the procedures, 57 male participants provided written informed consent for their inclusion within the study. The study was conducted in accordance with the Declaration of Helsinki principles and all procedures were approved by the Research Ethics Committee of Aberystwyth University. Participants were non-smokers, not taking medication or other supplements, free from any infectious illness for 4 weeks prior to the study and completing at least 3 h of moderate-vigorous endurance exercise per week. Participants were not limited in their use of mouthwash before and during the study period.

2.2. Supplementation

All 57 participants were randomly allocated into COL or placebo (PLA) groups with stratification by age and type of exercise training only. In a double blind manner, participants were asked to consume 20 $g \cdot day^{-1}$ (10 g prior to morning and evening meal) of COL (Neovite UK, London) or an isoenergetic/isomacronutrient PLA (as used in Davison and Diment, 2010) for 12 weeks. Four participants (COL = 3, PLA = 1) were lost due to lack of compliance with the study protocol (e.g. lack of training or supplement consumption due to injury, family bereavement or air travel). All participants who successfully completed the study (COL group, n = 25, age: 30.5 ± 13.8 years, height: 179.9 ± 6.4 cm, body mass: 77.2 ± 8.9 kg); PLA group, n = 28, age: 31.5 ± 13.2 years, height 178.4 ± 6.6 cm, body mass 74.5 ± 8.7 kg) commenced the study between September and December.

2.3. Monitoring of upper respiratory illness and training volume

Participants completed a health questionnaire (Gleeson et al., 2011, 2012) on a daily basis. This involved participants indicating if they were suffering from any of the illness symptoms listed on the questionnaire: sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains, weakness, headache, and loss of sleep. Upon reporting of any of the above symptoms, participants were asked to provide a subjective rating of the severity of symptoms (light, moderate, severe). As used previously (Fricker et al., 2005; Gleeson et al., 2011, 2012), these ratings of light, moderate and severe were given numerical scores of 1, 2 and 3 respectively for data analysis. At any given point during the 12 weeks, a total symptom score of ≥12 was used to indicate that an URI was present. Each week, participants were asked to complete a standard short-form International Physical Activity Questionnaire (http://www.ipaq.ki.se/downloads.htm) to provide quantitative data of training loads in metabolic-equivalent (MET)-h·week^{-1} (Craig et al., 2003). Participants were allowed unrestricted use of medication during episodes of URI but were asked to report such use and report how their training was affected by the URI (1 – training maintained, 2- training reduced, 3 – training discontinued).

2.4. Blood sampling

Blood samples were drawn from an antecubital vein into 4 ml K_3EDTA (BD, Oxford, UK) and 6 ml plain (BD, Oxford, UK) vacutainers at baseline and 12 weeks following COL or PLA supplementation. All participants avoided strenuous exercise for 24 h prior to each visit and arrived at the laboratory following an overnight fast of at least 10 h. Blood collected in the K_3EDTA vacutainer was used for determination of total and differential leukocyte counts (Pentra 60C+, Horiba, Montpellier, France) and

neutrophil function. Blood collected in the 6 mL plain vacutainers was allowed to clot at room temperature for 1 h 20 min. Following centrifugation (1300 g for 10 min at 4°C), serum was stored at -80°C for later metabolomic analysis.

2.5. Neutrophil function

Whole blood from the EDTA vacutainers was stored at room temperature (no longer than 2 h) prior to measurement of *in-vitro* stimulated neutrophil oxidative burst activity response to formyl-leucyl-methionyl-phenylalanine (fMLP) using a commercially available chemiluminescence (CL) kit (ABEL, Knight Scientific Ltd, Plymouth, UK). The CL per well was measured by a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). The contents of each microplate well that contained an fMLP stimulated sample were as follows: 20 µL of diluted whole blood (blood and Hank's balanced salt solution; HBSS, without calcium and magnesium, at ratio of 1:100), 90 µL assay buffer (HBSS with calcium and magnesium), 50 µL Pholasin and 20 µL adjuvant K (substance that enhances the luminescence of Pholasin).

These mixtures were gently shaken and incubated at 37°C for 30 s in the luminometer, prior to the addition of 20 µL fMLP (10 µM) to provide an end total volume of 200 µL per well, a 1:1010 final blood dilution and a fMLP concentration of 1µM respectively. The unstimulated state was calculated as the mean CL of the well prior to addition of fMLP (45 s). Following addition of fMLP, area under the curve (over 300 s), above the stable unstimulated state was used to determine stimulated CL. To calculate responses on a per cell basis, it was assumed that the stimulated CL is entirely from neutrophils (Morozov et al., 2003). Thus, fMLP-stimulated area under the CL curve was divided by the number of neutrophils present in each well to give CL in RLU (i.e. oxidative burst) per neutrophil.

2.6. Serum metabolomics

To remove proteins before mass spectrometry, 20 µL of each serum sample was mixed with 30 µL of ice-cold ultrapure water and vortexed. 50 µL of ice-cold HPLC grade acetone (Fisher Scientific UK Ltd, Loughborough, UK) was then added and the mixture vortexed. Samples were left on ice for 30 min to allow protein precipitation, after which, they were centrifuged at 16 000 g for 10 min in a micro-centrifuge. After centrifugation, 50 µL of the supernatant was removed and transferred to a glass insert placed in a glass auto-sampling vial, to which 250 µL of ice-cold 70% (v/v) methanol (made up using HPLC grade methanol (Fisher Scientific UK Ltd.) and ultrapure water) was added. Seven control serum samples, from human male AB plasma (Sigma-Aldrich, Dorset, UK), were run simultaneously using the same protocol to act as machine and protocol controls.

Analysis was carried out using Direct Injection Electrospray Ionisation Mass-Spectrometry (DI-ESI-MS) on a Micromass LCT mass-spectrometer (Micromass/Waters Ltd., UK) in negative ionization mode where metabolites are singly ionized by the loss of H^+. The polar extracts were reconstituted in 0.25 mL 30% [v/v] methanol: H_2O and 50 µL added to 200 µL inserts in 2 mL (Waters Ltd. UK) and introduced by direct-infusion (DI) at a flow rate of 0.05 mL min^{-1} in 30% [v/v] methanol: H_2O running solvent. Data were acquired over the *m/z* range 100-1400 Th and were imported into MATLAB, binned to unit mass and then normalized to percentage total ion as stated in Johnson et al. (2007). Tentative identification of metabolites of interest was achieved through interrogation of the HMDB Serum Metabolome database (Psychogios et al., 2011).

2.7. Saliva sampling

Participants reported to the laboratory for a saliva sample at baseline and 4, 8 and 12 weeks following supplementation. All participants avoided strenuous exercise 24 h prior to each visit and arrived at the laboratory after an overnight fast of at least 10 h. For all saliva samples the mouth was rinsed with plain water at least 10 min before the collection period. To obtain the sample, the participant remained seated with the head tilted slightly forward and passively dribbling into a pre weighed 7 mL sterile bijou tube while keeping orofacial movement to a minimum. The final duration of collection was recorded and the tube was weighed again to allow for estimation of saliva flow rate when the density of saliva was assumed to be 1.0 $g \cdot mL^{-1}$ as used in previous studies (Chicharro et al., 1998; Davison et al., 2009). With the use of a freezing point depression osmometer (Osmomat 030, Gonotec, GbBH, Berlin, Germany), saliva osmolality was determined to allow for concentration of salivary immunological parameters to be expressed relative to saliva osmolality and flow rate. Saliva samples were centrifuged for 5 min at 16000 g to allow separate storage (at -80°C) of the pellet and supernatant for later microbiome analysis and enzyme-linked immunosorbent assay (ELISA) respectively. Aliquots of saliva samples were thawed at room temperature only once prior to each ELISA. Following the thawing of saliva, samples were again centrifuged for 5 min at 16000 g to precipitate mucins and other debris and allow for the resulting clear supernatant to be analyzed.

2.8. Blood contamination

Aliquots of saliva were screened for blood contamination by the determination of salivary transferrin concentration using an ELISA kit (Salimetrics, State College, Pennsylvania, USA). If salivary transferrin concentration was greater than 1 $mg \cdot dL^{-1}$,

the sample was considered to be contaminated with blood. If a sample at any timepoint was found to be contaminated, all salivary lysozyme (s-Lys) and lactoferrin (s-Lys) data for that participant were excluded from the study (this was not done for IgA as the ELISA was specific to sIgA, see below).

2.9. Saliva sIgA

The concentration of s-IgA ($mg \cdot L^{-1}$) was determined in all saliva supernatants following the protocol of Leicht et al. (2011). The sandwich ELISA approach was slightly modified to include a capture antibody specific to the secretory component of human IgA (Mouse anti-human IgA secretory, Merck Millipore, Darmstadt, Germany).

2.10. Saliva antimicrobial peptides

All participants who were free from any contaminated samples (COL = 22, PLA = 24) were assessed for changes in s-Lys and s-Lac. Measurements were performed according to manufacturer's instructions using commercial available ELISA kits (Assaypro LLC, St-Louis, MO).

2.11. Extraction of microbial DNA

Microbial DNA was extracted from 200 µL of the salivary pellet at baseline and 12 weeks of 31 participants (COL = 14, PLA = 17) who all commenced the study late September/early October and completed the study late December/early January. Extraction was performed using a FastDNA SPIN Kit (MP Biomedical, Santa Ana, USA) following the manufacturer's guidelines, except that bead beating was carried out using a FastPrep24 (MP Biomedical) machine with three cycles at speed setting 6.0 for 30 s, with cooling on ice for 60 s between each cycle. Extracted DNA was quantified using Epoch (BioTek, Winooski, USA) spectrophotometry. All extractions

were confirmed to have a 260/280 nm ratio of between 1.8 and 2.0 for quality control purposes.

2.12. 16S rRNA gene terminal restriction fragment length polymorphisms (T-RFLP)

Amplification of the 16S rRNA gene was accomplished through PCR using primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3' with FAM labelled on 5' end) and 1389r (5'-ACG GGC GGT GTG TAC AAG-3') as described by Huws et al. (2011). All PCR products were verified using a 1.0% agarose gel. Triplicate reactions for each sample were pooled and purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) following the manufacturer's guidelines. 25 ng of purified PCR product for each sample was digested for 5 h at 37°C with restriction enzymes *Hae*III and *Msp*I (Promega, Madison, USA), in separate reactions. Restriction products were separated through size using an ABI PRISM1 377 Automated DNA Sequencer (Applied Biosystems, Warrington, UK). As there was an inability to sequence the 16S rRNA genes at one of the timepoints for 2 participants, peak profiles and Shannon Diversity Indexes were determined on 29 participants only (COL= 12, PLA= 17). Peak profiles were examined using Genemapper software (Version 3.7, Applied Biosystems). Those peaks with an estimated fragment size below 50 nucleotides were removed and the remaining data were modelled using FingerPrinting II software (BioRad, Hercules, USA). Shannon Diversity Indexes were determined using Fingerprint Analysis with Missing Data software (Version 1.2) (Schluter and Harris, 2006).

2.13. 16S rRNA Gene Quantitative PCR

Quantitative PCR was carried out on neat extracted DNA against standards created by amplifying the 16S rRNA gene of 5 randomly selected baseline samples. This used

1 µl of each sample in a PCR reaction using 27f and 1389r primers, as detailed above, except that the 27f primer did not have FAM on the 5' end, to amplify the gene. The resulting PCR product was purified and quantified, as previously detailed, to estimate the total number of 16S rRNA gene copies and serial dilutions made to a 10^{-10} level. Serial dilutions of 10^{-0}, 10^{-2}, 10^{-4}, 10^{-6}, 10-8, and 10^{-10} were used in subsequent qPCR reactions using a C100 thermal cycler (BioRad) and CFX96 optical detector (BioRad), with data captured using CFX Manager software (BioRad). qPCR reactions were completed in 25 µl volumes consisting of 1X SYBR Green Mastermix (Applied Biosystems), 400 nM of each of the EubF forward (5'-GTG STG CAY GGT TGT CGT CA-3') and EubR reverse (5'-ACG TCR TCC MCA CCT TCC TC-3') primers, as detailed by Kim et al. (2008) and 3 µl of neat DNA. The final volume was made up with PCR grade water (Roche, Hertfordshire, UK).

2.14. Microbial Growth Curve Analysis

To ascertain the antimicrobial properties of each supplement**,** 10 µl of a 50 $g \cdot L^{-1}$ (w/v) solution of COL or PLA (made using autoclaved ultrapure water), were cultured with 200 µL of artificial saliva medium and 10 µL of a salivary microbial culture as previously described by McBain et al. (2003). In addition, 10 µL of the COL and PLA solutions were incubated with 200 µL of artificial saliva medium, without the addition of the salivary microbial culture, to determine the level of microbial load for each solution. These cultures, alongside appropriate positive and negative growth controls, were incubated in a CellStar tissue culture 96 well plate with flat bottom and lid (Greiner Bio-one, Nürtingen, Germany) in a BioTek ELx808 microplate reader (BioTek Instruments, Winooski, USA) set at 37°C for 72 h. An optical density reading was taken every 20 min at a 630 nm wavelength, before which, the plate was shaken for 5 s.

Kinetic read data was exported from the Gen5 software package (BioTek Instruments) and corrected to a baseline (the first reading taken for each of the 96 wells). To allow for log transformation of the raw data, all data points, after baseline correction, were added to 10. The Log_{10} value for each data point was then calculated. After 3 replicate 96 well plates were completed, the mean and standard deviation for each growth condition, across all 3 plates, was calculated.

2.15. Statistical analyses

Data shown in the text, tables and figures are presented as mean values and standard deviation unless stated otherwise. Statistical analysis of all data were performed via the statistical computer software package SPSS (v20.00; SPSS Inc., Chicago, IL, USA) unless stated otherwise. Statistical significance was accepted at $P < 0.05$. All immunological parameters were checked for normal distribution using the Shapiro-Wilk test. Data not normally distributed (leukocyte counts, stimulated neutrophil oxidative burst, sIgA and sLys) were normalized with log transformation before further analysis. Initially, a 2 factor mixed model ANOVA (group × time) was carried out on all immunological measures,16S rRNA (salivary bacterial load) and total peak number for *Hae*III and *Msp*I (salivary bacterial diversity) to determine if the effect of time was different between COL or PLA groups. Any significant main effects identified in the ANOVA were further analyzed by post-hoc paired t-tests with Holm-Bonferroni correction. Independent t-tests were used to determine differences between groups at baseline and 12 weeks in Shannon Diversity Indexes for TFRLP (*Hae*III *and Msp*I). Data for the proportion of reported URI days and proportion of participants who suffered URI over the 12 weeks between the COL and PLA groups were assessed by

chi-squared test. To also examine the time-course of any effect of COL, chi-squared analyses of URI at 4 week intervals were performed, in accordance with the timing of saliva collections in the current study and the methods of Crooks et al. (2006, 2010). Chi-squared analysis was also used to assess proportion of participants within COL or PLA who reported use of medication or a negative effect on training load (reduced or prevented) during URI episodes. Comparisons between COL and PLA groups for mean number of self-reported URI episodes, mean duration of URI, mean severity of URI and mean weekly training loads were performed with an independent *t*-test. Metabolite data was analyzed through multivariate statistics, including principal component analysis and discriminant function analysis (DFA), using PyChem software (Jarvis et al., 2006) and following accepted Metabolomics Standard Initiative procedures (Sansone et al., 2007). Those whole mass unit bins which had a DFA loading of more than 2 standard deviations from the mean were selected for tentative identification. Determination of change from baseline for each whole mass unit bins was determined using 1-way ANOVA.

3. Results

3.1. Training load

Analysis of IPAQ questionnaires showed no significant differences between COL (53.1 ± 6.0 MET-h·week^{-1}) and PLA (48.2 ± 5.4 MET-h·week^{-1}) groups for weekly training volume at moderate-vigorous intensity (t (51) = -0.61, p = .546).

3.2. Upper respiratory illness

Chi-squared analysis showed a significantly lower proportion of days with URI during the 12 weeks in the COL group (5%) compared to the PLA group (9%) ($\chi^2(1) = 40.52$,

p < .001). Further chi-squared analyses of URI at 4 weeks intervals showed a significantly lower proportion of days with URI in the COL group at 1-4 weeks (COL = 6 %, PLA = 8%; χ^2 (1) = 4.56, p = .021) and 5-8 weeks (COL = 2 %, PLA = 13% ; χ^2 (1) = 64.12, p < .001) but not 9-12 weeks (COL = 6%, PLA =7%; χ^2 (1) = 0.29, p = .348) (Figure 1). Independent *t*-test showed a significantly lower mean number of URI episodes in the COL group compared to PLA group over the 12 weeks (COL, 0.4 ± 0.7; PLA, 0.8 ± 0.7; t (51) = 1.88, p = .033). The proportion of participants who reported URI during the study period (all 12 weeks combined) was not lower in the COL group (40%) than PLA group (64%) (χ^2 (1) = 3.13, p =.067). There were, however, a significantly lower proportion of participants who reported URI in the COL group (12%) compared to PLA (36%) at 5-8 weeks (χ^2 (1) = 0.29, p = .044) which was not evident at 1-4 weeks (COL = 16 %, PLA = 25%; χ^2 (1) = 0.65, p = .322) or 9-12 weeks (COL = 20 %, PLA =18%; χ^2 (1) = 0.04, p = .559) (Figure1). When URI episodes were reported by participants, the severity (COL, 35.3 ± 26.9; PLA, 42.0 ± 27.4; t (32) = 0.68, p = .250) and duration of symptoms (COL, 7.9 ± 4.4 days; PLA, 9.9 ± 5.6; t (32) = 1.02, p = .159) were similar between groups. During episodes of URI, there were no differences between groups for the proportion of participants who used medication (COL = 50%, PLA =78%; χ^2 (1) = 2.27, p =.139) or had training negatively affected (i.e. reduced or prevented) (COL = 60%, PLA =54%; χ^2 (1) = 1.15, p =.249). All of the above patterns/effects were similar in the subset of participants used for microbiome analysis (see section 2.11).

3.3. Cell counts and neutrophil function

Results for total and differential leukocyte counts, and stimulated neutrophil oxidative burst, at baseline and 12 weeks following supplementation in COL and PLA groups are shown in Table 1.

3.4. Salivary sIgA and antimicrobial peptides

The analyses of salivary antimicrobial peptides across the 12 week period are shown in Table 2 and 3. One participant was removed from sIgA analysis due to a lack of clear supernatant upon centrifugation of saliva samples. Final comparison for sIgA was conducted on 52 participants (COL = 25, PLA = 27). This participant had been excluded from sLac and sLys for contamination purposes as previously mentioned (see above). There were no significant differences between groups in salivary measures during the 12 weeks (P > .05) (Table 2 and 3).

3.5. Salivary bacterial load and diversity

The ANOVA to analyze log of estimated copy number of 16S rRNA gene revealed a significant time (F (1,29)= 15.38, p < .001) and interaction effect (F (1,29)= 4.90, p = 0.035) but no group effect (F (1,29)= 0.28, p = .602) (Figure 2). Post-hoc analysis of interaction revealed a significant increase in bacterial load over the 12 weeks in the PLA group (t (16)= -6.64, p < .001) that was not present in COL group (t (16)= -0.90, p = 0.386) (Figure 2). ANOVA on total TRFLP peak number obtained from the restriction enzyme *Msp*I revealed a main time effect (F (1,27)= 21.80, p < .001), with decreased bacterial diversity at 12 weeks, but no interaction (F (1,27) = 0.43, p = .515) or group effect (F (1,27) = 0.01, p = .917). However, analysis of total TRFLP peak number using *Hae*III revealed no time (F (1,27)= 2.54, p = .123), interaction (F (1,27)

396 = 0.13, *p* = .912) or group effects (*F* (1,27) = 0.29, *p* = .866). Analysis of Shannon
397 Diversity Indexes for TFRLP using *Msp*I showed no differences between groups at
398 baseline (*t* (29)= 0.27, *p* = .789) or at 12 weeks (*t* (28)= 0.33, *p* = .746) . There were
399 also no differences at baseline (*t* (28)= 0.41, *p* = .683) or 12 weeks (*t* (28)= 0.23, *p* =
400 .823) in Shannon Diversity Indexes for TFRLP using *Hae*III. Microbial growth analysis,
401 shown in Supplementary Figure 1, showed no indication of a direct antimicrobial effect
402 of either the COL or PLA supplement.

403 **3.6. Metabolomics**

404 Sugisawa et al. (2003) have suggested that low molecular weight (< 10 kDa)
405 substances may be responsible for the immunological effects of COL. We therefore
406 used a metabolomics approach to determine whether COL increased the
407 concentration of compounds at the lower end of this range (<1.4 kDa) with the
408 expectation that any such effects would be due to bioactive metabolites. Thus,
409 metabolite profiles of serum derived using DI-ESI-MS were analysed using
410 multivariate approaches. DFA of serum profiles at baseline and at 12 weeks (Figure
411 3), showed no separation, at either time point, between the COL and PLA groups.
412 However, there was clear separation between the two time points. Interrogation of the
413 loading vectors selected by the algorithm to derive DF1 suggested that 13 m/z were
414 calculated to be the major sources of variation. Interrogation of the HMDB Serum
415 Metabolome database allowed the tentative identification of these 13 metabolites
416 (Table 4). Within the tentative identifications, there are several possibilities for each
417 m/z but no particular biochemical pathways or immunological metabolite markers
418 appeared to have been targeted.

4. Discussion

This study aimed to determine the effects of COL on innate and mucosal markers of immunity and the subsequent incidence of URI in regularly exercising males. Compared to the PLA group, there was a significantly lower number of URI episodes and subsequently less proportion of URI days in the COL group over the 12 weeks. This supports previous evidence in studies of both active and immune-deficient populations (Brinkworth and Buckley, 2003; Crooks et al., 2010; Patel and Rana, 2006). Beneficial effects of COL were most evident during periods of greater prevalence of URI within the study population by reducing the proportion of participants who report URI (hence a greater scope for intervention). As not all participants commenced the study at the same time of year, it is important to recognize that these findings reflect the effects of COL within the timeframe and the incidence of illness in participants of the current study rather than proposing a specific seasonal window where COL affects URI. There was, however, no effect of COL on severity and duration of URI episodes, which supports some previous studies (Brinkworth and Buckley, 2003; Crooks et al., 2006, 2010) but not the beneficial effects reported by Patiroglu and Kondolot (2011).

In contrast to the effect of COL on URI, there was no significant effect of COL on salivary sIgA and AMPs or fMLP-stimulated blood neutrophil oxidative burst. The lack of effect on immune parameters despite differences in URI is in accordance with previous evidence (Crooks et al., 2006; Shing et al., 2007). Although previous studies have found beneficial effects in mucosal protection (sIgA/AMPs) following COL supplementation, either a blunting of the exercise-induced immune dysfunction (Davison and Diment, 2010) or increased resting salivary sIgA (Crooks et al., 2006),

443 they did not measure URI (Davison and Diment, 2010) or failed to see simultaneous
444 effects of COL on URI (Crooks et al., 2006). This has led to proposals that the effect
445 of COL occurs through a combination of mechanisms (Shing et al., 2007). In the
446 current study we proposed the use of a novel *in-vivo* marker (salivary microbiome) that
447 may have greater sensitivity to changes in innate mucosal defense following an
448 intervention. This study shows for the first time that during regular training in the winter
449 months, COL limits the increase in salivary bacterial load that was observed in the
450 PLA group. However it is currently unclear whether the significant increase in bacterial
451 load in the PLA group played a role in the greater episodes of URI observed in this
452 group or whether the increased bacterial load occurred as result of a greater number
453 of illnesses, or compromised immunity (due to training and/or seasonal effects) .
454 Future studies should investigate salivary bacterial load at the taxonomic level with
455 next generation sequencing methods to determine whether changes reflect
456 predisposed interactions with viruses which are the common cause of URI (Bosch et
457 al., 2013).

458 Evidence from respiratory health research has suggested that bacterial colonization
459 of tissues contiguous to the oral cavity (e.g. airway) can trigger an increase in the
460 frequency of disease exacerbations (Wedzicha and Donaldson, 2003). Furthermore,
461 the interaction between viruses and bacteria which colonize the upper respiratory tract
462 has been highlighted to affect the risk of illness (Bosch et al., 2013). Of relevance to
463 this study are suggestions that the balance of microbes involved in colonization can
464 be perturbed when host immunity is compromised (Murphy et al., 2009). It is apparent
465 that the magnitude of change in immunity immediately following each bout of
466 strenuous exercise may have more clinical significance than training-induced
467 alterations in resting immunity (Abhassi et al., 2013; Nieman et al., 1994; Pedersen

and Bruunsgaard, 1995). It is reasonable to suggest that over the course of the 12 weeks the COL group suffered fewer incidences of transient immune perturbations, and/or smaller or shorter disturbance, (in response to training and/or seasonal variation) which may have limited conditions for changes in the salivary microbiome to occur. COL has been shown to prevent exercise-induced decreases in salivary AMPs and improve recovery of the capacity of neutrophils (a source of AMPs) following strenuous exercise (Davison and Diment, 2010). Deficiencies or decreases in AMPs expressed in mucosal secretions have been shown to be related to greater bacterial invasion and/or cases of infection (Bals et al., 1998, 1999; Daele and Zicot, 2000; Smith et al., 1996; Goldman et al., 1997). It is worthy to note, however, that the present study has only investigated a narrow range of AMPs that are present in mucosal secretions. Therefore, the effects of COL on the resting concentrations of other AMPs such as cathelicidins, which have broad anti-bactericidal effects (Bals, 2000), remain unclear and cannot be excluded. In addition, there were no apparent effects of COL on *in-vitro* microbial culture in this study which supports that the aforementioned effects on bacterial load are not due to components of COL having direct anti-microbial effects in the oral cavity during consumption.

Of particular note was the lack of simultaneous increase in bacterial diversity within the PLA group during the study. This suggests that it was not the acquisition of new bacteria that caused the significant increase in bacterial load in the PLA group but rather amplification of bacteria resident at baseline. The oral cavity is exposed to a constant array of exogenous and endogenous factors, thus measurement via saliva has been recognized to provide a ‘fingerprint’ of the whole oral microbiota (Dewhirst et al., 2010; Fabian et al., 2008; Li et al., 2005; Boutaga et al., 2007). We propose that, rather than causing URI *per se*, an increase in bacterial load is indicative of

493 compromised innate immune status, and as such is a relevant marker of *in-vivo*
494 (innate) immune status. Furthermore, if COL supplementation does limit increases in
495 salivary bacterial load, it will be important to determine whether this is a general
496 reduction in bacteria, or whether it is biased towards certain bacterial taxonomies.

497 A further aim of this study was to determine any metabolomic changes as a result of
498 COL supplementation. Metabolomics provides an unbiased biochemical “snap shot”
499 of samples which due to the use of high resolution MS-based (as here) or nuclear
500 magnetic resonance (NMR)-based approaches simultaneously and accurately
501 measure hundreds of metabolites. Exercise immunologists view that high throughput
502 laboratory methods such as metabolomics will provide greater understanding of the
503 mechanisms behind modulations of the immune system with exercise and/or nutrition
504 (Walsh et al., 2011). Crucially, in this study, we did not observe any changes in serum
505 metabolome linked to COL supplementation, indicating that either no major changes
506 were occurring or that these were occurring below the detection limits of the Mass
507 Spectrometer ($<10^{-12}$ mol, Sumner et al., 2003). There was, however, a clear
508 separation of metabolome profiles obtained at baseline and at the 12 week timepoint
509 of the study. The timepoint effect could reflect a combination of a seasonal effect and
510 accumulation of training stress. As seasonal and/or exercise effects alone on the
511 immune system were not a primary aim of the study, future studies should investigate
512 whether changes in metabolome are involved in the greater incidence of URI seen in
513 the winter months. Confirmatory identifications of the 13 m/z which appear to be
514 responsible for the majority of the temporal separation were not accomplished as
515 changes in the serum metabolome were not as a result of COL supplementation and
516 thus fell outside of the scope of this project.

517 Previous *in-vitro* culture studies suggested that low molecular weight (≤ 10 kDa)
518 components of COL (e.g. proteose peptones) rather than larger growth factors or
519 cytokines may be responsible for the effects on human immune function (e.g.
520 leukocyte capacity) (Sugiswa et al., 2003). Given that our metabolomics approach
521 provides data on (<1.4 kDa) metabolites involved in biological pathways (Holmes et
522 al., 2008), it may have been expected to identify traces of such bioactive metabolites
523 in the circulation following 12 weeks of supplementation. Future studies could
524 additionally examine components between 1.4-10 kDA to identify whether the above
525 mentioned components of COL investigated within *in-vitro* culture become
526 bioavailable to affect human immune function following periods of supplementation.
527 Such studies will help identify the bioactive components and eradicate discrepancies
528 found between studies of COL that are due to the source/quality of the supplement
529 (Davison, 2013). It is plausible, however, to suggest that in the present study the
530 proposed priming effects may have been localised to immune parameters in the
531 mucosae rather than having systemic effects. Another proposed mechanism behind
532 the effects on URI is the ability of COL to truncate the increase in gut permeability
533 following strenuous exercise (Marchbank et al., 2011). This effect on intestinal integrity
534 may prevent additional stress on the immune system via the translocation of luminal
535 bacteria into systemic circulation. This hypothesis, however, requires further study in
536 relation to episodes of URI.

537 Although previous studies have shown inconsistencies in isolated immune markers at
538 rest, the present study suggests that the effects of COL are apparent when an
539 integrated and interactive holistic immune marker is taken (e.g. salivary bacterial load).
540 It should be noted that participants in this study were not limited in their use, either
541 before or during the study, of mouthwash. This could be considered a potential

limitation of the study and an important consideration for future studies using the salivary bacterial load as an in-vivo marker. Given the presence of null findings in the present study compared to previous studies of investigated immune parameters (e.g. sIgA), it is important to acknowledge methodological limitations not yet considered. The present study involved participants who were involved in a range of sports training. Although these were all endurance based activities, previous studies have reported that effects of COL on sIgA may not be universal in all groups of regular exercisers (Crooks et al., 2010). In addition, it is unclear whether the use of participants who had a higher mean weekly training load and hence potentially a greater number of immunodepressive bouts would have produced different findings for these measures. Indeed, when highly trained cyclists completed 5 consecutive days of high-intensity training, COL supplementation was found to prevent the decreases in cytotoxic/suppressor T cells observed at the end and in the recovery period from the training in the PLA group (Shing et al., 2007).

In summary, we have shown for the first time that COL supplementation can limit microbial changes in the oral cavity during the winter months. This may be due to COL acting as a nutritional countermeasure to seasonal effects on salivary microbiome or immune perturbations following acute exercise. The clinical implications of this may be a reduction in the number of URI episodes. Future research should include responses to acute training during the monitoring period to determine the effects on the salivary microbiome and whether COL influences these (and other, e.g. AMPs) responses.

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Fig. 1. URI during 4 week intervals in bovine colostrum (COL)/placebo(PLA) groups. Columns reflect proportion of participants in each group who report URI. Line and symbol represent proportion of URI days in each group during 4 week intervals. **Significantly lower proportion of URI days in COL group at 1-4 weeks and 5-8 weeks ($p<.05$). * Significantly lower proportion of participants in the COL group who report URI at 5-8 weeks ($p<.05$).

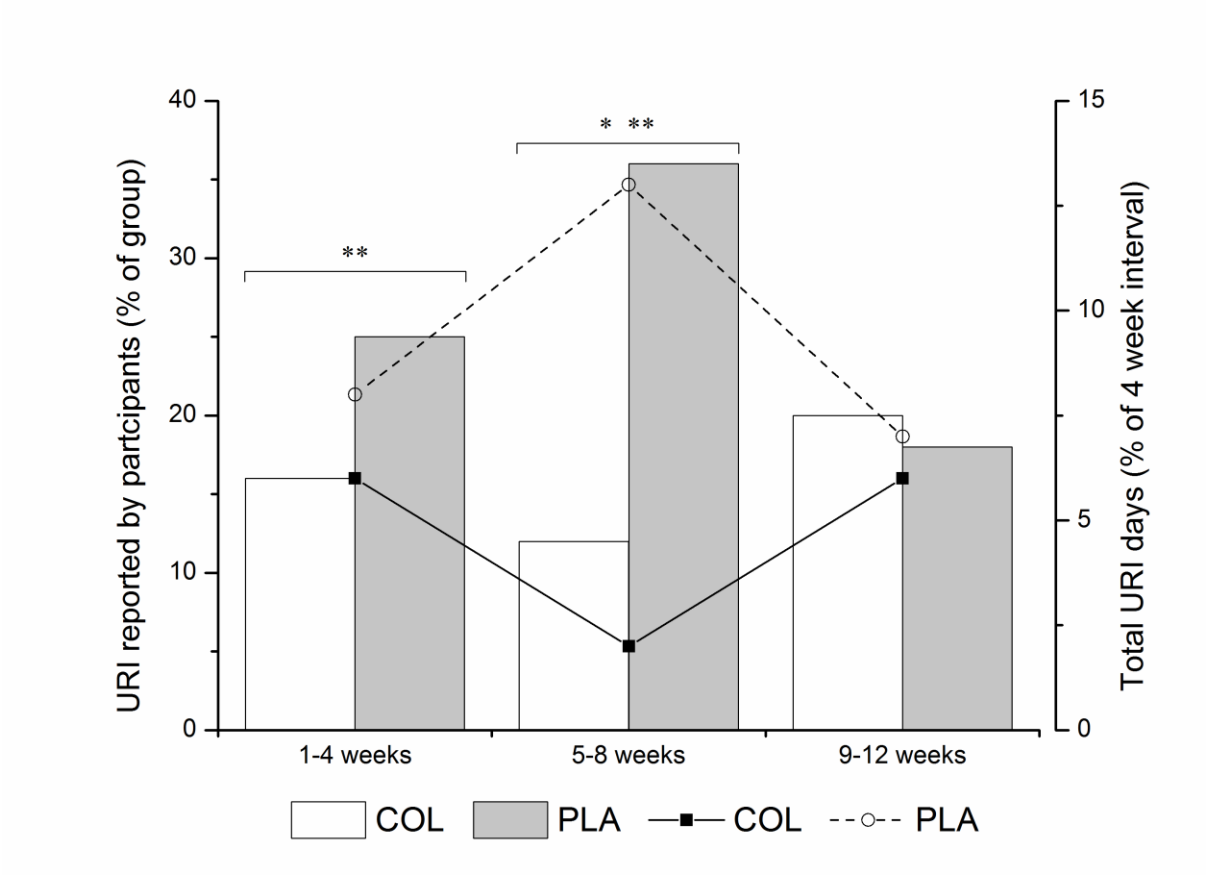
Fig. 2. Salivary bacterial load in bovine colostrum (COL)/ placebo (PLA) groups. Standard deviation as error bars. *Significant increase in salivary bacterial load from baseline to 12 weeks in PLA group ($p<.001$).

Fig. 3. Discriminant Function analysis (DFA) plot of metabolomic profiles before and at end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation. ● - PLA group at baseline; ■ - COL group at baseline; ● - PLA group at 12 weeks; ■ - COL group at 12 weeks. A satisfactory separation was obtained between time points but not groups. Rings on figure display 95% confidence intervals for DFA separation.

Table 1. Blood leukocytes and neutrophil function in bovine colostrum (COL)/placebo (PLA) groups. Statistically significant difference between baseline and 12 week measures (main effect of time) indicated by * $p <.05$. Values are mean ± SD.

Table 2. sIgA before, during and at the end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation. Values are mean ± SD.

778 Table 3. sLac and sLys before, during and at end of 12 weeks of bovine colostrum
779 (COL)/placebo (PLA) supplementation. Values are mean ± SD. Main effect of time
780 indicated by * p <.05. Post hoc analysis could not reveal any significant differences
781 between timepoints for sLac concentration. There was a significant increase in sLac
782 secretion rate (at 8 and 12 weeks) and sLys secretion rate (at 8 weeks) from baseline
783 (p <.05). Values are mean ± SD.
784
785 Table 4. Tentative identifications of metabolites which may be responsible for the
786 separation between the 2 timepoints visible in DF1 of Figure 3. For some of the whole
787 number molecular weight bins there are multiple tentative IDs which may or may not
788 contribute to the DF1 loading. Change from the baseline and subsequent ANOVA *P*
789 values are detailed alongside the tentative IDs.
790



791

792 **Fig.1**

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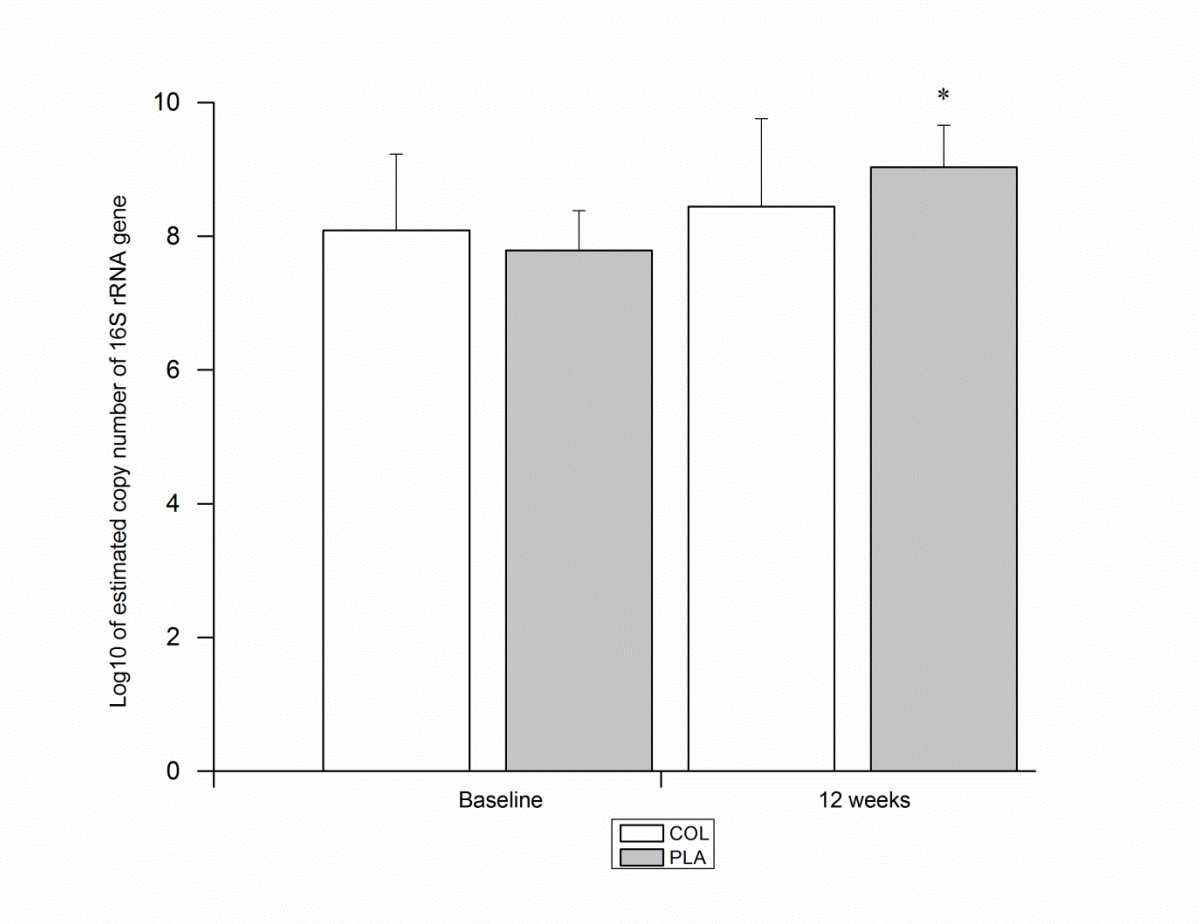

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797 **Fig.2**

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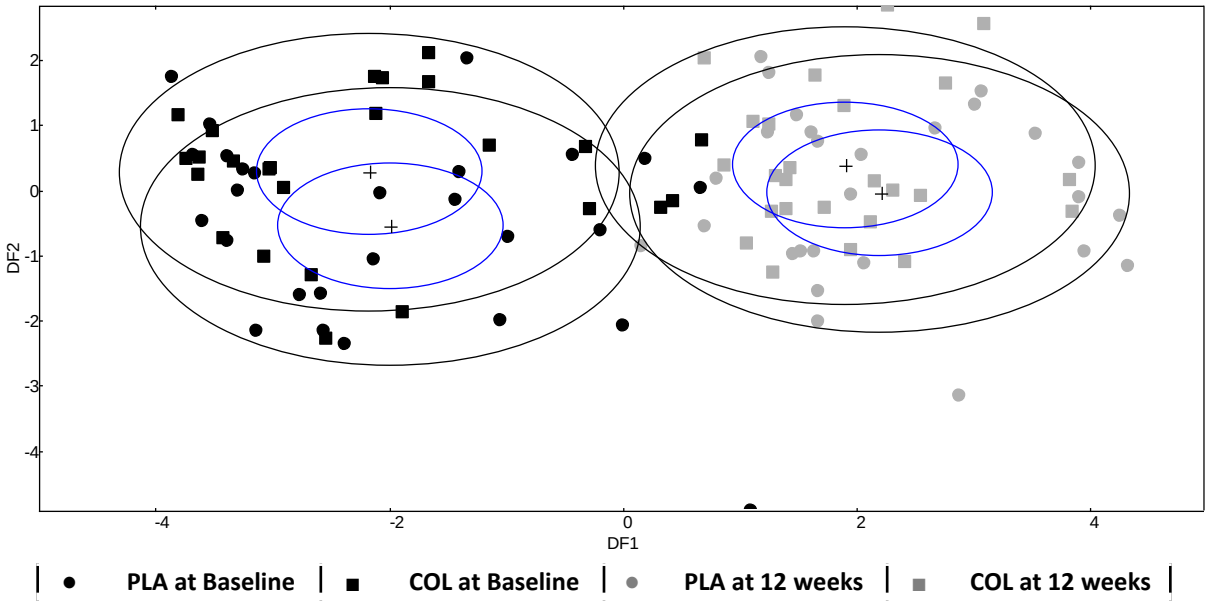

801

802 **Fig.3**

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Immune measure	Baseline	12 weeks	*F* (df) *P* time *F* (df) *P* trial *F* (df) *P* interaction
Total leukocytes (cells $\times 10^{9} \cdot L^{-1}$)			1.74 (1,51) .193
COL	4.74 ± 1.02	4.90 ± 1.01	0.78 (1,51) .382
PLA	5.20 ± 1.68	5.27 ± 1.63	0.17 (1,51) .687
Neutrophils (cells $\times 10^{9} \cdot L^{-1}$)			0.86 (1,51) .359
COL	2.31 ± 0.61	2.31 ± 0.59	0.51 (1,51) .268
PLA	2.58 ± 1.18	2.74 ± 1.28	1.26 (1,51) .480
Monocytes (cells $\times 10^{9} \cdot L^{-1}$)			0.33 (1,51) .567
COL	0..46 ± 0.11	0.46 ± 0.12	0.09 (1,51) .766
PLA	0.46 ± 0.13	0.47 ± 0.20	0.63 (1,51) .431
Total lymphocytes (cells $\times 10^{9} \cdot L^{-1}$)			0.26 (1,51) .619
COL	1.77 ± 0.50	1.91 ± 0.67	<0.01 (1,51) .970
PLA	1.90 ± 0.61	1.79 ± 0.47	3.21 (1,51) .079
Atypical lymphocytes (cells $\times 10^{9} \cdot L^{-1}$)			2.92 (1,51) .093
COL	0.04 ± 0.01	0.04 ± 0.02	0.56 (1,51) .458
PLA	0.05 ± 0.02	0.05 ± 0.02	2.44 (1,51) .124
Large immature cells (cells $\times 10^{9} \cdot L^{-1}$)			0.05 (1,51) .832
COL	0.03 ± 0.01	0.03 ± 0.02	1.08 (1,51) .305
PLA	0.05 ± 0.04	0.04 ± 0.03	0.27 (1,51) .605

fMLP-stimulated CL per neutrophil (RLU per s^{-1} per $cell^{-1}$)			5.31 (1,51) .025*
COL	43.65 ± 27.44	53.99 ± 26.51	0.68 (1,51) .413
PLA	42.79 ± 25.58	44.52 ± 24.91	0.84 (1,51) .365

Table 1. Blood leukocytes and neutrophil function in bovine colostrum (COL)/placebo (PLA) groups. Statistically significant difference between baseline and 12 week measures (main effect of time) indicated by * $p < .05$. Values are mean ± SD.

Table 2. sIgA before, during and at the end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation. Values are mean ± SD.

Immune measure	Baseline	4 weeks	8 weeks	12 weeks	*F (df) P* time F (df) P trial F (df) P interaction
sIgA concentration ($mg \cdot L^{-1}$)					0.71 (1,50) .404
COL	267.0 ± 126.5	252.9 ± 119.7	256.0 ± 142.8	272.2 ± 152.5	1.09 (1,50) .301
PLA	259.9 ± 202.9	217.1 ± 124.4	230.4 ± 132.3	215.0 ± 98.5	0.37 (1,50) .546
sIgA secretion rate ($\mu g \cdot min^{-1}$)					2.66 (1,50) .065
COL	116.5 ± 89.3	125.6 ± 105.6	129.5 ± 89.6	143.9 ± 96.8	1.65 (1,50) .733
PLA	121.1 ± 125.9	104.7 ± 51.5	114.3 ± 63.0	108.1 ± 57.3	0.12 (1,50) .180
sIgA:osmolality ($mg \cdot mOsmol^{-1}$)					0.97 (1,50) .409
COL	3.8 ± 1.5	3.6 ± 1.4	3.5 ± 1.4	3.8 ± 1.8	1.19 (1,50) .280
PLA	3.6 ± 2.0	3.2 ± 1.4	3.4 ± 1.5	3.1 ± 1.2	0.75 (1,50) .525

Table 3. sLac and sLys before, during and at end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation. Main effect of time indicated by * $p < .05$. Post hoc analysis could not reveal any significant differences between timepoints for sLac concentration. There was a significant increase in sLac secretion rate (at 8 and 12 weeks) and sLys secretion rate (at 8 weeks) from baseline ($p < .05$). Values are mean ± SD.

Immune measure	Group	Baseline	4 weeks	8 weeks	12 weeks	*F* (df) *P* time	*F* (df) *P* trial
						F (df) *P* interaction	
sLac concentration	COL	3.3 ± 0.8	3.7 ± 0.8	3.7 ± 0.8	3.8 ± 0.8	3.02 (1,44) .032*	0.63 (1,44) .431
($mg \cdot L^{-1)}$	PLA	3.8 ± 1.2	3.8 ± 1.0	4.0 ± 1.0	3.8 ± 1.0	1.58 (1,44) .198	
sLac secretion rate	COL	1.6 ± 1.0	1.9 ± 1.2	2.0 ± 0.9	2.1 ± 0.8	6.29 (1,44) .001*	0.84 (1,44) .365
($\mu g \cdot min^{-1}$)	PLA	1.9 ± 1.0	2.1 ± 1.2	2.3 ± 1.4	2.3 ± 1.3	0.29 (1,44) .829	
sLac:osmolality	COL	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	2.04 (1,44) .111	0.36 (1,44) .549
($mg \cdot mOsmol^{-1}$)	PLA	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	1.29 (1,44) .282	
sLys concentration	COL	21.5 ± 13.3	23.0 ± 13.5	22.3 ± 12.6	20.1 ± 12.8	0.57 (1,44) .636	0.04 (1,44) .837
($mg \cdot L^{-1}$)	PLA	22.0 ± 14.6	20.1 ± 12.1	21.3 ± 11.5	21.1 ± 12.1	1.25 (1,44) .295	
sLys secretion rate	COL	9.9 ± 8.3	10.5 ± 6.5	11.4 ± 8.4	10.0 ± 5.1	2.91 (1,44) .037*	0.09 (1,44) .764
($\mu g \cdot min^{-1}$)	PLA	10.8 ± 8.8	10.7 ± 8.4	11.7 ± 7.4	11.6 ± 8.8	0.58 (1,44) .627	
sLys:osmolality	COL	0.34 ± 0.23	0.36 ± 0.25	0.33 ± 0.17	0.30 ± 0.18	0.59 (1,44) .622	0.01 (1,44) .941
($mg \cdot mOsmol^{-1}$)	PLA	0.33 ± 0.22	0.31 ± 0.20	0.33 ± 0.19	0.33 ± 0.19	0.95 (1,44) .420	

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841 Table 4. Tentative identifications of metabolites which may be responsible for the
842 separation between the 2 timepoints visible in DF1 of Figure 3. For some of the whole
843 number molecular weight bins there are multiple tentative IDs which may or may not
844 contribute to the DF1 loading. Change from the baseline and subsequent ANOVA *P*
845 values are detailed alongside the tentative IDs.

MW	Tentative ID	Change from Baseline	*F* (df) *P*
108	Cresol	Decrease	5.31 (3,100) .002
154	Gentisic acid 2-Pyrocatechuic acid Protocatechuic acid Hydroxytyrosol	Decrease	84.70 (3,100) < .001
155	L-Histidine	Increase	94.18 (3,100) <.001
157	Unknown	Increase	53.65 (3,100) <.001
168	Uric acid Homogentisic acid 3-Hydroxymandelic acid 3,4-Dihydroxybenzeneacetic acid Pyridoxamine	Decrease	16.11 (3,100) <.001
169	Norepinephrine Pyridoxine 3-Methylhistidine D-Glyceraldehyde 3-phosphate Dihydroxyacetone phosphate	Increase	11.13 (3,100) <.001
170	Gallic acid 3,4-Dihydroxyphenylglycol cis-4-Decenoic acid	Increase	36.68 (3,100) <.001
172	Glycerol 3-phosphate Glycylproline	Decrease	6.57 (3,100) <.001
173	2-Oxoarginine Pyrophosphate	Increase	12.43 (3,100) <.001
174	Dehydroascorbic acid Suberic acid N-Acetylornithine L-Arginine	Increase	68.56 (3,100) <.001
186	2-Phosphoglyceric acid 3-Phosphoglyceric acid	Decrease	17.48 (3,100) <.001
285	Unknown	Increase	6.19 (3,100) <.001
325	10-Nitrolinoleic acid N-Oleoylethanolamine	Decrease	11.86 (3,100) <.001

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