

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

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15 **Conflict of Interest Statement**

16 All authors declare that there are no conflicts of interest.

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23 **ABSTRACT**

24 Bovine colostrum (COL) has been advocated as a nutritional countermeasure to
25 exercise-induced immune dysfunction and increased risk of upper respiratory illness
26 (URI) in athletic populations, however, the mechanisms remain unclear. During winter
27 months, under double-blind procedures, 53 males (mean training load \pm SD, 50.5 \pm
28 28.9 MET-h \cdot week⁻¹) were randomized to daily supplementation of 20 g of COL (N =
29 25) or an isoenergetic/isomacronutrient placebo (PLA) (N = 28) for 12 weeks. Venous
30 blood was collected at baseline and at 12 weeks and unstimulated saliva samples at
31 4-week intervals. There was a significantly lower proportion of URI days and number
32 of URI episodes with COL compared to PLA over the 12 weeks ($p < 0.05$). There was
33 no effect of COL on *in vitro* neutrophil oxidative burst, salivary secretory IgA or salivary
34 antimicrobial peptides ($p > 0.05$), which does not support previously suggested
35 mechanisms. In a subset of participants (COL = 14, PLA = 17), real-time quantitative
36 PCR, targeting the 16S rRNA gene showed there was an increase in salivary bacterial
37 load over the 12-week period with PLA ($p < 0.05$) which was not as evident with COL.
38 Discriminant function analysis of outputs received from serum metabolomics showed
39 changes across time but not between groups. This is the first study to demonstrate
40 that COL limits the increased salivary bacterial load in physically active males during
41 the winter months which may provide a novel mechanism of immune-modulation with
42 COL and a relevant marker of *in vivo* (innate) immunity and risk of URI.

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

45

46 1. Introduction

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

58 Bovine colostrum (COL) may be effective at alleviating recurrent URI in situations of
59 immune deficiency (Patel and Rana, 2006). Previous evidence has shown that 8-10
60 weeks of COL supplementation can reduce the incidence of URI in physically active
61 populations but the mechanism(s) behind such effects remains unclear (Brinkworth
62 and Buckley, 2003; Crooks et al., 2010). Animal and *in-vitro* culture studies
63 demonstrate that COL has a mediating effect on cell-mediated immunity by influencing
64 the production of cytokines (Biswas et al., 2007; Boudry et al., 2007; Shing et al.,
65 2009). Increasing concentrations of COL, *in-vitro*, has been shown to modulate
66 cytokine production in peripheral blood mononuclear cells from resting, healthy
67 individuals, to promote a Th1 profile (cell-mediated immunity) (Shing et al., 2009),
68 which may suppress the binding of pathogens (e.g. rhinovirus) (Sethi et al., 1997).
69 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·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.,

95 2002). To date, the majority of both longitudinal and cross-sectional exercise training
96 studies have focused on changes in salivary sIgA (Walsh et al., 2011). Although the
97 importance of other salivary antimicrobial peptides (AMPs) (e.g. lysozyme, lactoferrin)
98 for host defense have been recognized, they have received limited attention (West et
99 al., 2006).

100 In addition to the presence of inducible factors such as AMPs at mucosal surfaces,
101 protection from invading microorganisms is also provided by the diverse community of
102 commensal microbes which colonize the upper respiratory tract (Blaser and Falkow,
103 2009; Bosch et al., 2013). Subsequently, disturbance of this respiratory microbial
104 community can contribute to acquisition of new pathogens which may result in
105 respiratory illness, particularly if host immunity is compromised (Murphy et al., 2009).
106 However, the effects of exercise and nutritional interventions on changes in the
107 salivary microbiome have not previously been investigated.

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

116 **2. Methods**

117 **2.1. Participants**

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

126 **2.2. Supplementation**

127 All 57 participants were randomly allocated into COL or placebo (PLA) groups with
128 stratification by age and type of exercise training only. In a double blind manner,
129 participants were asked to consume 20 g·day⁻¹ (10 g prior to morning and evening
130 meal) of COL (Neovite UK, London) or an isoenergetic/isomacronutrient PLA (as used
131 in Davison and Diment, 2010) for 12 weeks. Four participants (COL = 3, PLA = 1) were
132 lost due to lack of compliance with the study protocol (e.g. lack of training or
133 supplement consumption due to injury, family bereavement or air travel). All
134 participants who successfully completed the study (COL group, n = 25, age: 30.5 ±
135 13.8 years, height: 179.9 ± 6.4 cm, body mass: 77.2 ± 8.9 kg); PLA group, n = 28, age:
136 31.5 ± 13.2 years, height 178.4 ± 6.6 cm, body mass 74.5 ± 8.7 kg) commenced the
137 study between September and December.

138 **2.3. Monitoring of upper respiratory illness and training volume**

139 Participants completed a health questionnaire (Gleeson et al., 2011, 2012) on a daily
140 basis. This involved participants indicating if they were suffering from any of the illness
141 symptoms listed on the questionnaire: sore throat, catarrh in the throat, runny nose,
142 cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains,
143 weakness, headache, and loss of sleep. Upon reporting of any of the above
144 symptoms, participants were asked to provide a subjective rating of the severity of
145 symptoms (light, moderate, severe). As used previously (Fricker et al., 2005; Gleeson
146 et al., 2011, 2012), these ratings of light, moderate and severe were given numerical
147 scores of 1, 2 and 3 respectively for data analysis. At any given point during the 12
148 weeks, a total symptom score of ≥ 12 was used to indicate that an URI was present.
149 Each week, participants were asked to complete a standard short-form International
150 Physical Activity Questionnaire (<http://www.ipaq.ki.se/downloads.htm>) to provide
151 quantitative data of training loads in metabolic-equivalent (MET)-h \cdot week $^{-1}$ (Craig et al.,
152 2003). Participants were allowed unrestricted use of medication during episodes of
153 URI but were asked to report such use and report how their training was affected by
154 the URI (1 – training maintained, 2- training reduced, 3 – training discontinued).

155 **2.4. Blood sampling**

156 Blood samples were drawn from an antecubital vein into 4 ml K₃EDTA (BD, Oxford,
157 UK) and 6 ml plain (BD, Oxford, UK) vacutainers at baseline and 12 weeks following
158 COL or PLA supplementation. All participants avoided strenuous exercise for 24 h
159 prior to each visit and arrived at the laboratory following an overnight fast of at least
160 10 h. Blood collected in the K₃EDTA vacutainer was used for determination of total
161 and differential leukocyte counts (Pentra 60C+, Horiba, Montpellier, France) and

162 neutrophil function. Blood collected in the 6 mL plain vacutainers was allowed to clot
163 at room temperature for 1 h 20 min. Following centrifugation (1300 g for 10 min at
164 4°C), serum was stored at -80°C for later metabolomic analysis.

165 **2.5. Neutrophil function**

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

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

186 2.6. Serum metabolomics

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

198
199 Analysis was carried out using Direct Injection Electrospray Ionisation Mass-
200 Spectrometry (DI-ESI-MS) on a Micromass LCT mass-spectrometer
201 (Micromass/Waters Ltd., UK) in negative ionization mode where metabolites are singly
202 ionized by the loss of H^+ . The polar extracts were reconstituted in 0.25 mL 30% [v/v]
203 methanol: H_2O and 50 μL added to 200 μL inserts in 2 mL (Waters Ltd. UK) and
204 introduced by direct-infusion (DI) at a flow rate of 0.05 mL min^{-1} in 30% [v/v] methanol:
205 H_2O running solvent. Data were acquired over the m/z range 100-1400 Th and were
206 imported into MATLAB, binned to unit mass and then normalized to percentage total
207 ion as stated in Johnson et al. (2007). Tentative identification of metabolites of interest
208 was achieved through interrogation of the HMDB Serum Metabolome database
209 (Psychogios et al., 2011).

210 **2.7. Saliva sampling**

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

230 **2.8. Blood contamination**

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

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

238 **2.9. Saliva sIgA**

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

243 **2.10. Saliva antimicrobial peptides**

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

248 **2.11. Extraction of microbial DNA**

249 Microbial DNA was extracted from 200 μL of the salivary pellet at baseline and 12
250 weeks of 31 participants (COL = 14, PLA = 17) who all commenced the study late
251 September/early October and completed the study late December/early January.
252 Extraction was performed using a FastDNA SPIN Kit (MP Biomedical, Santa Ana,
253 USA) following the manufacturer's guidelines, except that bead beating was carried
254 out using a FastPrep24 (MP Biomedical) machine with three cycles at speed setting
255 6.0 for 30 s, with cooling on ice for 60 s between each cycle. Extracted DNA was
256 quantified using Epoch (BioTek, Winooski, USA) spectrophotometry. All extractions

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

259 **2.12. 16S rRNA gene terminal restriction fragment length polymorphisms (T-** 260 **RFLP)**

261 Amplification of the 16S rRNA gene was accomplished through PCR using primers 27f
262 (5'-AGA GTT TGA TCC TGG CTC AG-3' with FAM labelled on 5' end) and 1389r (5'-
263 ACG GGC GGT GTG TAC AAG-3') as described by Huws et al. (2011). All PCR
264 products were verified using a 1.0% agarose gel. Triplicate reactions for each sample
265 were pooled and purified using a QIAquick PCR purification kit (Qiagen, West Sussex,
266 UK) following the manufacturer's guidelines. 25 ng of purified PCR product for each
267 sample was digested for 5 h at 37°C with restriction enzymes *HaeIII* and *MspI*
268 (Promega, Madison, USA), in separate reactions. Restriction products were separated
269 through size using an ABI PRISM1 377 Automated DNA Sequencer (Applied
270 Biosystems, Warrington, UK). As there was an inability to sequence the 16S rRNA
271 genes at one of the timepoints for 2 participants, peak profiles and Shannon Diversity
272 Indexes were determined on 29 participants only (COL= 12, PLA= 17). Peak profiles
273 were examined using Genemapper software (Version 3.7, Applied Biosystems).
274 Those peaks with an estimated fragment size below 50 nucleotides were removed and
275 the remaining data were modelled using FingerPrinting II software (BioRad, Hercules,
276 USA). Shannon Diversity Indexes were determined using Fingerprint Analysis with
277 Missing Data software (Version 1.2) (Schluter and Harris, 2006).

278 **2.13. 16S rRNA Gene Quantitative PCR**

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

281 1 μ l of each sample in a PCR reaction using 27f and 1389r primers, as detailed above,
282 except that the 27f primer did not have FAM on the 5' end, to amplify the gene. The
283 resulting PCR product was purified and quantified, as previously detailed, to estimate
284 the total number of 16S rRNA gene copies and serial dilutions made to a 10^{-10} level.
285 Serial dilutions of 10^{-0} , 10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , and 10^{-10} were used in subsequent qPCR
286 reactions using a C100 thermal cycler (BioRad) and CFX96 optical detector (BioRad),
287 with data captured using CFX Manager software (BioRad). qPCR reactions were
288 completed in 25 μ l volumes consisting of 1X SYBR Green Mastermix (Applied
289 Biosystems), 400 nM of each of the EubF forward (5'-GTG STG CAY GGT TGT CGT
290 CA-3') and EubR reverse (5'-ACG TCR TCC MCA CCT TCC TC-3') primers, as
291 detailed by Kim et al. (2008) and 3 μ l of neat DNA. The final volume was made up with
292 PCR grade water (Roche, Hertfordshire, UK).

293 **2.14. Microbial Growth Curve Analysis**

294 To ascertain the antimicrobial properties of each supplement, 10 μ l of a 50 g·L⁻¹ (w/v)
295 solution of COL or PLA (made using autoclaved ultrapure water), were cultured with
296 200 μ l of artificial saliva medium and 10 μ l of a salivary microbial culture as previously
297 described by McBain et al. (2003). In addition, 10 μ l of the COL and PLA solutions
298 were incubated with 200 μ l of artificial saliva medium, without the addition of the
299 salivary microbial culture, to determine the level of microbial load for each solution.
300 These cultures, alongside appropriate positive and negative growth controls, were
301 incubated in a CellStar tissue culture 96 well plate with flat bottom and lid (Greiner Bio-
302 one, Nürtingen, Germany) in a BioTek ELx808 microplate reader (BioTek Instruments,
303 Winooski, USA) set at 37°C for 72 h. An optical density reading was taken every 20
304 min at a 630 nm wavelength, before which, the plate was shaken for 5 s.

305

306 Kinetic read data was exported from the Gen5 software package (BioTek Instruments)
307 and corrected to a baseline (the first reading taken for each of the 96 wells). To allow
308 for log transformation of the raw data, all data points, after baseline correction, were
309 added to 10. The Log₁₀ value for each data point was then calculated. After 3 replicate
310 96 well plates were completed, the mean and standard deviation for each growth
311 condition, across all 3 plates, was calculated.

312 **2.15. Statistical analyses**

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

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

344 **3. Results**

345 **3.1. Training load**

346 Analysis of IPAQ questionnaires showed no significant differences between COL (53.1
347 \pm 6.0 MET-h-week⁻¹) and PLA (48.2 \pm 5.4 MET-h-week⁻¹) groups for weekly training
348 volume at moderate-vigorous intensity ($t(51) = -0.61, p = .546$).

349 **3.2. Upper respiratory illness**

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

352 $p < .001$). Further chi-squared analyses of URI at 4 weeks intervals showed a
353 significantly lower proportion of days with URI in the COL group at 1-4 weeks (COL =
354 6 %, PLA = 8%; $\chi^2 (1) = 4.56, p = .021$) and 5-8 weeks (COL = 2 %, PLA = 13% ; χ^2
355 (1) = 64.12, $p < .001$) but not 9-12 weeks (COL = 6%, PLA =7%; $\chi^2 (1) = 0.29, p =$
356 $.348$) (Figure 1). Independent t -test showed a significantly lower mean number of URI
357 episodes in the COL group compared to PLA group over the 12 weeks (COL, $0.4 \pm$
358 0.7 ; PLA, 0.8 ± 0.7 ; $t (51) = 1.88, p = .033$). The proportion of participants who reported
359 URI during the study period (all 12 weeks combined) was not lower in the COL group
360 (40%) than PLA group (64%) ($\chi^2 (1) = 3.13, p = .067$). There were, however, a
361 significantly lower proportion of participants who reported URI in the COL group (12%)
362 compared to PLA (36%) at 5-8 weeks ($\chi^2 (1) = 0.29, p = .044$) which was not evident
363 at 1-4 weeks (COL = 16 %, PLA = 25%; $\chi^2 (1) = 0.65, p = .322$) or 9-12 weeks (COL
364 = 20 %, PLA =18%; $\chi^2 (1) = 0.04, p = .559$) (Figure1). When URI episodes were
365 reported by participants, the severity (COL, 35.3 ± 26.9 ; PLA, 42.0 ± 27.4 ; $t (32) =$
366 $0.68, p = .250$) and duration of symptoms (COL, 7.9 ± 4.4 days; PLA, 9.9 ± 5.6 ; $t (32)$
367 $= 1.02, p = .159$) were similar between groups. During episodes of URI, there were no
368 differences between groups for the proportion of participants who used medication
369 (COL = 50%, PLA =78%; $\chi^2 (1) = 2.27, p = .139$) or had training negatively affected
370 (i.e. reduced or prevented) (COL = 60%, PLA =54%; $\chi^2 (1) = 1.15, p = .249$). All of the
371 above patterns/effects were similar in the subset of participants used for microbiome
372 analysis (see section 2.11).

373 **3.3. Cell counts and neutrophil function**

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

377 **3.4. Salivary sIgA and antimicrobial peptides**

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

385 **3.5. Salivary bacterial load and diversity**

386 The ANOVA to analyze log of estimated copy number of 16S rRNA gene revealed a
387 significant time ($F(1,29) = 15.38, p < .001$) and interaction effect ($F(1,29) = 4.90, p =$
388 0.035) but no group effect ($F(1,29) = 0.28, p = .602$) (Figure 2). Post-hoc analysis of
389 interaction revealed a significant increase in bacterial load over the 12 weeks in the
390 PLA group ($t(16) = -6.64, p < .001$) that was not present in COL group ($t(16) = -0.90,$
391 $p = 0.386$) (Figure 2). ANOVA on total TRFLP peak number obtained from the
392 restriction enzyme *MspI* revealed a main time effect ($F(1,27) = 21.80, p < .001$), with
393 decreased bacterial diversity at 12 weeks, but no interaction ($F(1,27) = 0.43, p = .515$)
394 or group effect ($F(1,27) = 0.01, p = .917$). However, analysis of total TRFLP peak
395 number using *HaeIII* 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 *MspI* 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 *HaeIII*. 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.

419 **4. Discussion**

420 This study aimed to determine the effects of COL on innate and mucosal markers of
421 immunity and the subsequent incidence of URI in regularly exercising males.
422 Compared to the PLA group, there was a significantly lower number of URI episodes
423 and subsequently less proportion of URI days in the COL group over the 12 weeks.
424 This supports previous evidence in studies of both active and immune-deficient
425 populations (Brinkworth and Buckley, 2003; Crooks et al., 2010; Patel and Rana,
426 2006). Beneficial effects of COL were most evident during periods of greater
427 prevalence of URI within the study population by reducing the proportion of
428 participants who report URI (hence a greater scope for intervention). As not all
429 participants commenced the study at the same time of year, it is important to recognize
430 that these findings reflect the effects of COL within the timeframe and the incidence of
431 illness in participants of the current study rather than proposing a specific seasonal
432 window where COL affects URI. There was, however, no effect of COL on severity
433 and duration of URI episodes, which supports some previous studies (Brinkworth and
434 Buckley, 2003; Crooks et al., 2006, 2010) but not the beneficial effects reported by
435 Papiroglu and Kondolot (2011).

436 In contrast to the effect of COL on URI, there was no significant effect of COL on
437 salivary sIgA and AMPs or fMLP-stimulated blood neutrophil oxidative burst. The lack
438 of effect on immune parameters despite differences in URI is in accordance with
439 previous evidence (Crooks et al., 2006; Shing et al., 2007). Although previous studies
440 have found beneficial effects in mucosal protection (sIgA/AMPs) following COL
441 supplementation, either a blunting of the exercise-induced immune dysfunction
442 (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

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

485 Of particular note was the lack of simultaneous increase in bacterial diversity within
486 the PLA group during the study. This suggests that it was not the acquisition of new
487 bacteria that caused the significant increase in bacterial load in the PLA group but
488 rather amplification of bacteria resident at baseline. The oral cavity is exposed to a
489 constant array of exogenous and endogenous factors, thus measurement via saliva
490 has been recognized to provide a 'fingerprint' of the whole oral microbiota (Dewhirst
491 et al., 2010; Fabian et al., 2008; Li et al., 2005; Boutaga et al., 2007). We propose that,
492 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

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

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

564

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573

574 **References**

- 575 Abbasi, A., Fehrenbach, E., Hauth, M., Walter, M., Hudemann, J., Wank, V., Niess,
576 A.M., Northoff, H., 2013. *Exerc Immunol Rev* 19, 8-28.
- 577 Appukutty, M., Radhakrishnan, A., Ramasamy, K., Majeed, A.B.A., Chinna, K., Noor,
578 I.M., Safii, N. K., Koon, P. B., 2010. *Br. J. Sports Med.* 44, 145.
- 579 Bals, R., 2000. Epithelial antimicrobial peptides in host defense against infection.
580 *Respir. Res.* 1, 141-150.
- 581 Bals, R., Wang, X., Zasloff, M., Wilson, J.M., 1998. The peptide antibiotic LL-
582 37/hCAP-18 is expressed in epithelia of the human lung where it has broad
583 antimicrobial activity at the airway surface. *Proc. Natl. Acad. Sci. U. S. A* 95, 9541-
584 9546.
- 585 Bals, R., Weiner, D.J., Moscioni, A.D., Meegalla, R.L., Wilson, J.M., 1999.
586 Augmentation of innate host defense by expression of a cathelicidin antimicrobial
587 peptide. *Infect. Immun.* 67, 6084-6089.
- 588 Biswas, P., Vecchi, A., Mantegani, P., Mantelli, B., Fortis, C., Lazzarin, A., 2007.
589 Immunomodulatory effects of bovine colostrum in human peripheral blood
590 mononuclear cells. *New. Microbiol.* 30, 447-454.
- 591 Blaser, M.J., Falkow, S., 2009. What are the consequences of the disappearing
592 human microbiota? *Nat. Rev. Microbiol.* 7, 887-894.
- 593 Bosch, A.A., Biesbroek, G., Trzcinski, K., Sanders, E.A., Bogaert, D., 2013. Viral and
594 bacterial interactions in the upper respiratory tract. *PLoS Pathog* . 9, e1003057.
- 595 Boudry, C., Buldgen, A., Portetelle, D., Gianello, P., Thewis, A., Leterme, P.,
596 Dehoux, J.P., 2007. Effect of bovine colostrum supplementation on cytokine mRNA
597 expression in weaned piglets. *Livest. Sci.* 108, 295-298.

598 Boutaga, K., Savelkoul, P.H., Winkel, E.G., van Winkelhoff, A.J., 2007. Comparison
599 of subgingival bacterial sampling with oral lavage for detection and quantification of
600 periodontal pathogens by real-time polymerase chain reaction. *J. Periodontol.* 78,
601 79-86.

602 Brinkworth, G.D., Buckley, J.D., 2003. Concentrated bovine colostrum protein
603 supplementation reduces the incidence of self-reported symptoms of upper
604 respiratory tract infection in adult males. *Eur. J. Nutr.* 42, 228-232.

605 Chicharro, J.L., Lucia, A., Perez, M., Vaquero, A.F., Urena, R., 1998. Saliva
606 composition and exercise. *Sports Med.* 26, 17-27.

607 Craig, C.L., Marshall, A.L., Sjostrom, M., Bauman, A.E., Booth, M.L., Ainsworth,
608 B.E., Pratt, M., Ekelund, U., Yngve, A., Sallis, J.F., Oja, P., 2003. International
609 physical activity questionnaire: 12-country reliability and validity. *Med. Sci. Sports*
610 *Exerc.* 35, 1381-1395.

611 Crooks, C., Cross, M.L., Wall, C., Ali, A., 2010. Effect of bovine colostrum
612 supplementation on respiratory tract mucosal defenses in swimmers. *Int. J. Sport*
613 *Nutr. Exerc. Metab.* 20, 224-235.

614 Crooks, C.V., Wall, C.R., Cross, M.L., Rutherford-Markwick, K.J., 2006. The effect of
615 bovine colostrum supplementation on salivary IgA in distance runners. *Int. J. Sport*
616 *Nutr. Exerc. Metab.* 16, 47-64.

617 Daele, J., Zicot, A.F., 2000. Humoral immunodeficiency in recurrent upper
618 respiratory tract infections. Some basic, clinical and therapeutic features. *Acta*
619 *Otorhinolaryngol. Belg.* 54, 373-390.

620 Davison, G., 2013. Bovine colostrum and immune function after exercise. In:
621 Lamprecht, M. (Ed.), *Acute topics in sports nutrition*. Karger, Basel, pp. 62-69.

622 Davison, G., Allgrove, J., Gleeson, M., 2009. Salivary antimicrobial peptides (LL-37
623 and alpha-defensins HNP1-3), antimicrobial and IgA responses to prolonged
624 exercise. *Eur. J. Appl. Physiol.* 106, 277-284.

625 Davison, G., Diment, B.C., 2010. Bovine colostrum supplementation attenuates the
626 decrease of salivary lysozyme and enhances the recovery of neutrophil function after
627 prolonged exercise. *Br. J. Nutr.* 103, 1425-1432.

628 Dewhirst, F.E., Chen, T., Izard, J., Paster, B.J., Tanner, A.C., Yu, W.H.,
629 Lakshmanan, A., Wade, W.G., 2010. The human oral microbiome. *J. Bacteriol.* 192,
630 5002-5017.

631 Fabian, T.K., Fejerdy, P., Csermely, P., 2008. Salivary Genomics, Transcriptomics
632 and Proteomics: The Emerging Concept of the Oral Ecosystem and their Use in the
633 Early Diagnosis of Cancer and other Diseases. *Curr. Genomics* 9, 11-21.

634 Fricker, P.A., Pyne, D.B., Saunders, P.U., Cox, A.J., Gleeson, M., Telford, R.D.,
635 2005. Influence of training loads on patterns of illness in elite distance runners. *Clin.*
636 *J. Sport Med.* 15, 246-252.

637 Gleeson, M., 2007. Immune function in sport and exercise. *J. Appl. Physiol.* 103,
638 693-699.

639 Gleeson, M., Bishop, N.C., Oliveira, M., McCauley, T., Tauler, P., Lawrence, C.,
640 2012. Effects of a *Lactobacillus salivarius* probiotic intervention on infection, cold
641 symptom duration and severity, and mucosal immunity in endurance athletes. *Int. J.*
642 *Sport Nutr. Exerc. Metab.* 22, 235-242.

643 Gleeson, M., Bishop, N.C., Oliveira, M., Tauler, P., 2011. Daily probiotic's
644 (*Lactobacillus casei* Shirota) reduction of infection incidence in athletes. *Int. J. Sport*
645 *Nutr. Exerc. Metab.* 21, 55-64.

646 Goldman, M.J., Anderson, G.M., Stolzenberg, E.D., Kari, U.P., Zasloff, M., Wilson,
647 J.M., 1997. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is
648 inactivated in cystic fibrosis. *Cell* 88, 553-560.

649 Holmes, E., Wilson, I.D., Nicholson, J.K., 2008. Metabolic phenotyping in health and
650 disease. *Cell* 134, 714-717.

651 Huws, S.A., Kim, E.J., Lee, M.R., Scott, M.B., Tweed, J.K., Pinloche, E., Wallace,
652 R.J., Scollan, N.D., 2011. As yet uncultured bacteria phylogenetically classified as
653 *Prevotella*, *Lachnospiraceae* incertae sedis and unclassified *Bacteroidales*,
654 *Clostridiales* and *Ruminococcaceae* may play a predominant role in ruminal
655 biohydrogenation. *Environ. Microbiol.* 13, 1500-1512.

656 Jarvis, R.M., Broadhurst, D., Johnson, H., O'Boyle, N.M., Goodacre, R., 2006.
657 PYCHEM: a multivariate analysis package for python. *Bioinformatics* 22, 2565-2566.

658 Johnson, H.E., Lloyd, A.J., Mur, L.A.J., Smith, A.R., Causton, D.R., 2007. The
659 application of MANOVA to analyse *Arabidopsis thaliana* metabolomic data from
660 factorially designed experiments. *Metabolomics* 3, 517-530.

661 Kim, E.J., Huws, S.A., Lee, M.R.F., Wood, J.D., Muetzel, S.M., Wallace, R.J.,
662 Scollan, N.D., 2008. Fish oil increases the duodenal flow of long chain
663 polyunsaturated fatty acids and trans-11 18:1 and decreases 18:0 in steers via
664 changes in the rumen bacterial community. *J Nutr.* 138, 889-896.

665 Leicht, C.A., Bishop, N.C., Goosey-Tolfrey, V.L., 2011. Mucosal Immune Responses
666 to Treadmill Exercise in Elite Wheelchair Athletes. *Med. Sci. Sport Exer.* 43, 1414-
667 1421.

668 Li, Y., Ku, C.Y., Xu, J., Saxena, D., Caufield, P.W., 2005. Survey of oral microbial
669 diversity using PCR-based denaturing gradient gel electrophoresis. *J. Dent. Res.* 84,
670 559-564.

671 Marchbank, T., Davison, G., Oakes, J.R., Ghatei, M.A., Patterson, M., Moyer, M.P.,
672 Playford, R.J., 2011. The nutraceutical bovine colostrum truncates the increase in gut
673 permeability caused by heavy exercise in athletes. *Am. J. Physiol. Gastrointest.*
674 *Liver. Physiol.* 300, 477-484.

675 McBain, A.J., Bartolo, R.G., Catrenich, C.E., Charbonneau, D., Ledder, R.G., Gilbert,
676 P., 2003. Effects of a chlorhexidine gluconate-containing mouthwash on the vitality
677 and antimicrobial susceptibility of in vitro oral bacterial ecosystems. *Appl. Environ.*
678 *Microbiol.* 69, 4770-4776.

679 Mero, A., Kahkonen, J., Nykanen, T., Parviainen, T., Jokinen, I., Takala, T., Nikula,
680 T., Rasi, S., Leppaluoto, J., 2002. IGF-I, IgA, and IgG responses to bovine colostrum
681 supplementation during training. *J. Appl. Physiol.* 93, 732-739.

682 Morozov, V.I., Pryatkin, S.A., Kalinski, M.I., Rogozkin, V.A., 2003. Effect of exercise
683 to exhaustion on myeloperoxidase and lysozyme release from blood neutrophils. *Eur*
684 *J. Appl. Physiol.* 89, 257-262.

685 Murphy, T.F., Bakaletz, L.O., Smeesters, P.R., 2009. Microbial interactions in the
686 respiratory tract. *Pediatr. Infect. Dis. J* 28, S121-126.

687 Nieman, D.C., 2000. Special feature for the Olympics: effects of exercise on the
688 immune system: exercise effects on systemic immunity. *Immunol. Cell Biol.* 78, 496-
689 501.

690 Nieman, D.C., 2007. Marathon training and immune function. *Sports Med.* 37, 412-
691 415.

692 Nieman, D.C., Miller, A.R., Henson, D.A., Warren, B.J., Gusewitch, G., Johnson,
693 R.L., Davis, J.M., Butterworth, D.E., Herring, J.L., Nehlsencannarella, S.L., 1994.
694 Effect of High-Intensity Versus Moderate-Intensity Exercise on Lymphocyte
695 Subpopulations and Proliferative Response. *Int. J. Sports Med.* 15, 199-206.

696 Patel, K., Rana, R., 2006. Pedimune in recurrent respiratory infection and diarrhoea--
697 the Indian experience--the pride study. *Indian J. Pediatr.* 73, 585-591.

698 Patiroglu, T., Kondolot, M., 2013. The effect of bovine colostrum on viral upper
699 respiratory tract infections in children with immunoglobulin A deficiency. *Clin. Respir.*
700 *J.* 7, 21-26.

701 Pedersen, B.K., Bruunsgaard, H., 1995. How Physical Exercise Influences the
702 Establishment of Infections. *Sports Med.* 19, 393-400.

703 Psychogios, N., Hau, D.D., Peng, J., Guo, A.C., Mandal, R., Bouatra, S., Sinelnikov,
704 I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E.,
705 Huang, P., Hollander, Z., Pedersen, T.L., Smith, S.R., Bamforth, F., Greiner, R.,
706 McManus, B., Newman, J.W., Goodfriend, T., Wishart, D.S., 2011. The human
707 serum metabolome. *PLoS One* 16, e16957.

708 Sansone, S.A., Fan, T., Goodacre, R., Griffin, J.L., Hardy, N.W., Kaddurah-Daouk,
709 R., Kristal, B.S., Lindon, J., Mendes, P., Morrison, N., Nikolau, B., Robertson, D.,
710 Sumner, L.W., Taylor, C., van der Werf, M., van Ommen, B., Fiehn, O., 2007. The
711 metabolomics standards initiative. *Nat. Biotechnol.* 25, 846-848.

712 Schluter, P.M., Harris, S.A., 2006. Analysis of multilocus fingerprinting data sets
713 containing missing data. *Mol. Ecol. Notes* 6, 569-572.

714 Sethi, S.K., Bianco, A., Allen, J.T., Knight, R.A., Spiteri, M.A., 1997. Interferon-
715 gamma (IFN-gamma) down-regulates the rhinovirus-induced expression of
716 intercellular adhesion molecule-1 (ICAM-1) on human airway epithelial cells. *Clin.*
717 *Exp. Immunol.* 110, 362-369.

718 Shing, C.M., Peake, J.M., Suzuki, K., Jenkins, D.G., Coombes, J.S., 2009. Bovine
719 colostrum modulates cytokine production in human peripheral blood mononuclear

720 cells stimulated with lipopolysaccharide and phytohemagglutinin. *J. Interferon*
721 *Cytokine Res.* 29, 37-44.

722 Shing, C.M., Peake, J., Suzuki, K., Okutsu, M., Pereira, R., Stevenson, L., Jenkins,
723 D.G., Coombes, J.S., 2007. Effects of bovine colostrum supplementation on immune
724 variables in highly trained cyclists. *J. Appl. Physiol.* 102, 1113-1122.

725 Smith, J.J., Travis, S.M., Greenberg, E.P., Welsh, M.J., 1996. Cystic fibrosis airway
726 epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell* 85, 229-
727 236.

728 Spence, L., Brown, W.J., Pyne, D.B., Nissen, M.D., Sloots, T.P., McCormack, J.G.,
729 Locke, A.S., Fricker, P.A., 2007. Incidence, etiology, and symptomatology of upper
730 respiratory illness in elite athletes. *Med. Sci. Sports Exerc.* 39, 577-586.

731 Sugisawa, H., Itou, T., Ichimura, Y., Sakai, T., 2002. Bovine milk enhances the
732 oxidative burst activity of polymorphonuclear leukocytes in low concentrations. *J.*
733 *Vet. Med. Sci.* 64, 1113-1116.

734 Sugisawa, H., Itou, T., Saito, M., Moritomo, T., Miura, Y., Sakai, T., 2003. A low-
735 molecular-weight fraction of bovine colostrum and milk enhances the oxidative burst
736 activity of polymorphonuclear leukocytes. *Vet. Res. Commun.* 27, 453-461.

737 Sugisawa, H., Itou, T., Sakai, T., 2001. Promoting effect of colostrum on the
738 phagocytic activity of bovine polymorphonuclear leukocytes in vitro. *Biol. Neonate*
739 79, 140-144.

740 Sumner, L.W., Mendes, P., Dixon, R.A., 2003. Plant metabolomics: large-scale
741 phytochemistry in the functional genomics era. *Phytochemistry* 62, 817-836.

742 Walsh, N.P., Gleeson, M., Shephard, R.J., Woods, J.A., Bishop, N.C., Fleshner, M.,
743 Green, C., Pedersen, B.K., Hoffman-Goetz, L., Rogers, C.J., Northoff, H., Abbasi, A.,

744 Simon, P., 2011. Position statement. Part one: Immune function and exercise. *Exerc.*
745 *Immunol. Rev.* 17, 6-63.

746 Wedzicha, J.A., Donaldson, G.C., 2003. Exacerbations of chronic obstructive
747 pulmonary disease. *Respir Care* 48, 1204-1213; discussion 1213-1205.

748 West, N.P., Pyne, D.B., Renshaw, G., Cripps, A.W., 2006. Antimicrobial peptides
749 and proteins, exercise and innate mucosal immunity. *FEMS Immunol. Med.*
750 *Microbiol.* 48, 293-304.

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752

753

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

760

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

764

765 Fig. 3. Discriminant Function analysis (DFA) plot of metabolomic profiles before and
766 at end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation.

767 ● - PLA group at baseline; ■ - COL group at baseline; ● - PLA group at 12 weeks; ■ -
768 COL group at 12 weeks. A satisfactory separation was obtained between time points
769 but not groups. Rings on figure display 95% confidence intervals for DFA separation.

770

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

774

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

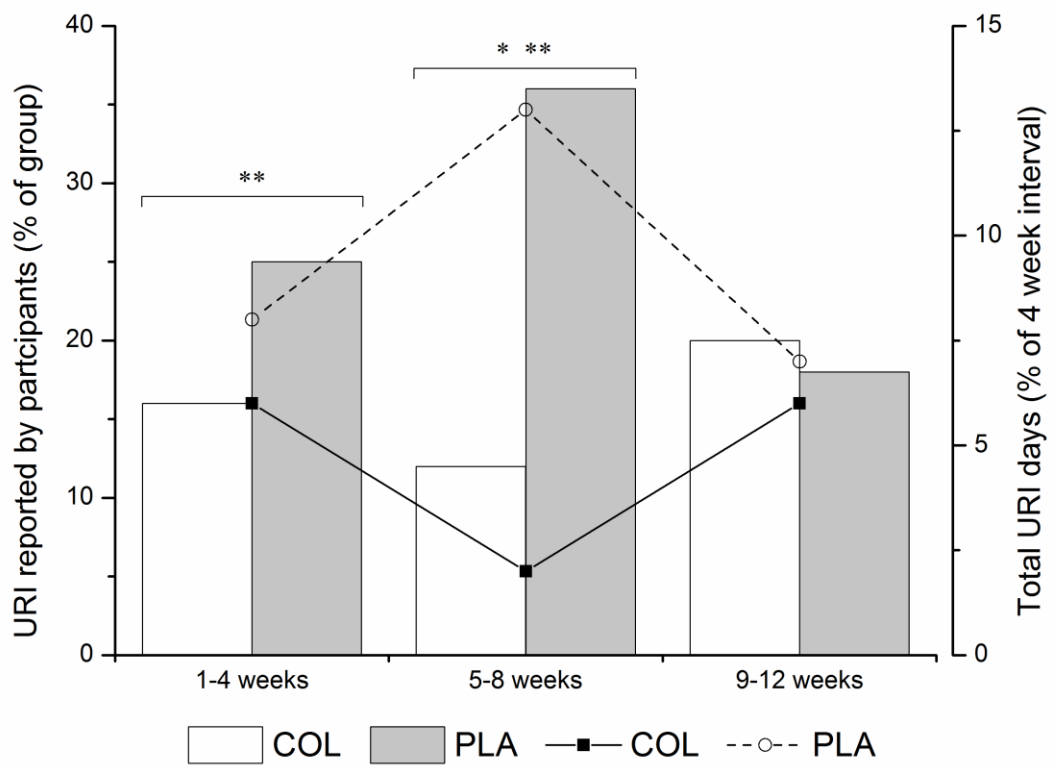
777

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 \pm 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 \pm 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



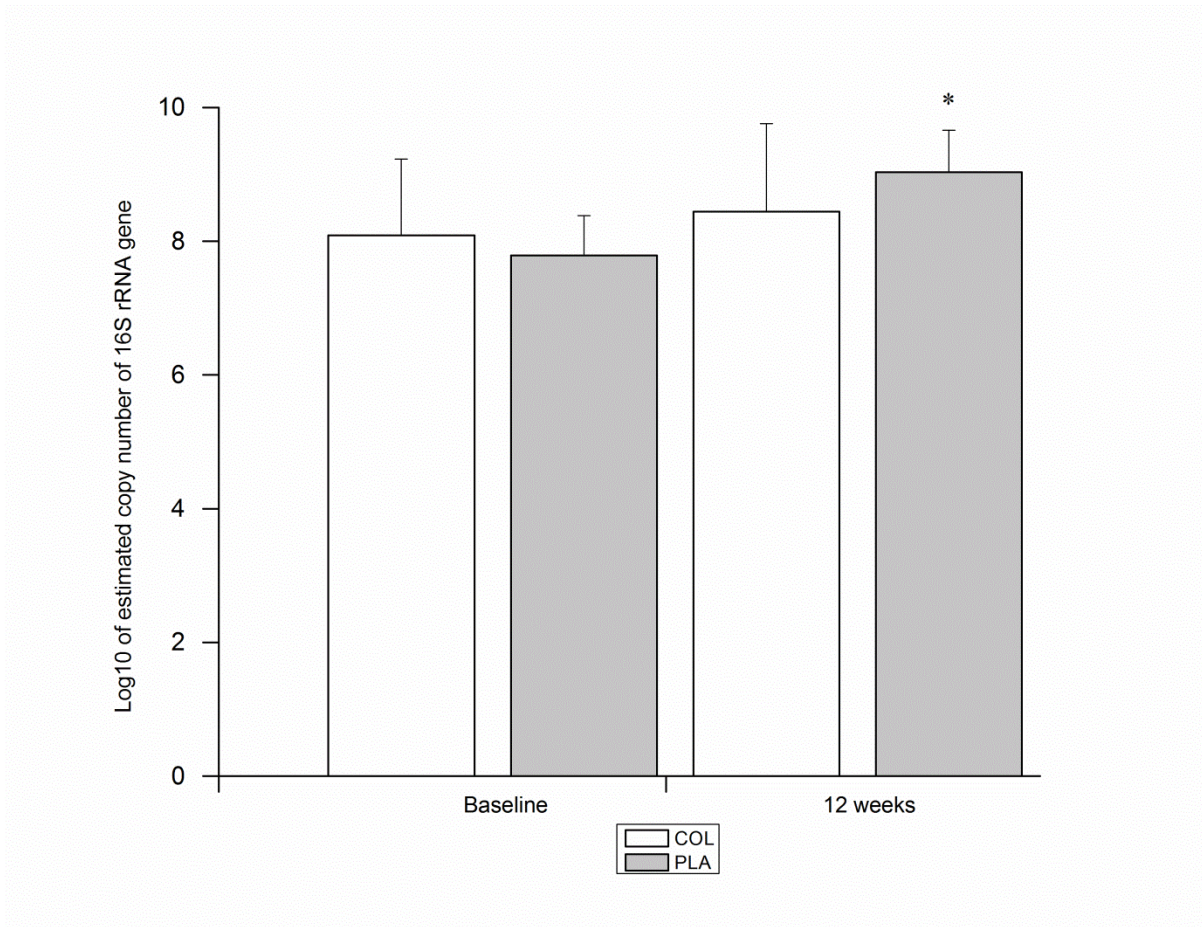
791

792 **Fig.1**

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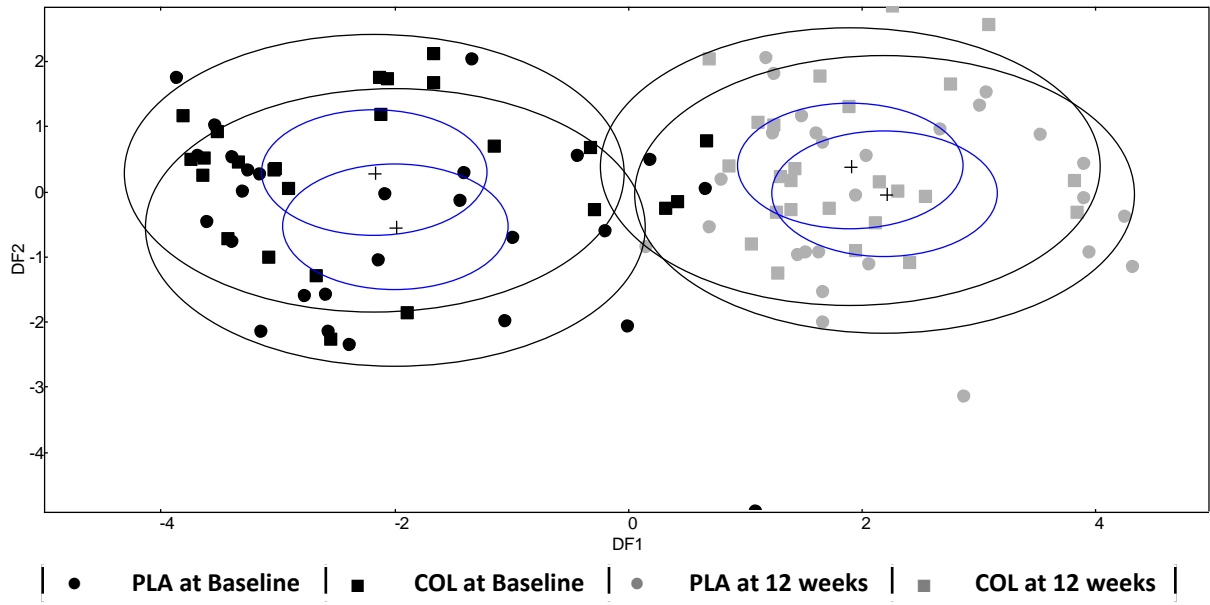
796

797 **Fig.2**

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802 **Fig.3**

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Immune measure	Baseline	12 weeks	<i>F</i> (df) <i>P</i> time
			<i>F</i> (df) <i>P</i> trial
			<i>F</i> (df) <i>P</i> interaction
Total leukocytes (cells ×10 ⁹ ·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 ×10 ⁹ ·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 ×10 ⁹ ·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 ×10 ⁹ ·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 ×10 ⁹ ·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 ×10 ⁹ ·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

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

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

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

822

823 Table 3. sLac and sLys before, during and at end of 12 weeks of bovine colostrum (COL)/placebo (PLA) supplementation. Main effect
824 of time indicated by * $p < .05$. Post hoc analysis could not reveal any significant differences between timepoints for sLac concentration.
825 There was a significant increase in sLac secretion rate (at 8 and 12 weeks) and sLys secretion rate (at 8 weeks) from baseline (p
826 $< .05$). Values are mean \pm SD.

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Immune measure	Group	Baseline	4 weeks	8 weeks	12 weeks	<i>F</i> (df) <i>P</i> time		<i>F</i> (df) <i>P</i> trial	
sLac concentration (mg·L ⁻¹)	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
	PLA	3.8 ± 1.2	3.8 ± 1.0	4.0 ± 1.0	3.8 ± 1.0				
sLac secretion rate (µg·min ⁻¹)	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
	PLA	1.9 ± 1.0	2.1 ± 1.2	2.3 ± 1.4	2.3 ± 1.3				
sLac:osmolality (mg·mOsmol ⁻¹)	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
	PLA	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02				
sLys concentration (mg·L ⁻¹)	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
	PLA	22.0 ± 14.6	20.1 ± 12.1	21.3 ± 11.5	21.1 ± 12.1				
sLys secretion rate (µg·min ⁻¹)	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
	PLA	10.8 ± 8.8	10.7 ± 8.4	11.7 ± 7.4	11.6 ± 8.8				
sLys:osmolality (mg·mOsmol ⁻¹)	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
	PLA	0.33 ± 0.22	0.31 ± 0.20	0.33 ± 0.19	0.33 ± 0.19				

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840

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	<i>F</i> (df) <i>P</i>
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-Acetylnithine 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

