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1 **Nutritional Modulation of Sleep Latency, Duration, and Efficiency: A**
2 **Randomised, Repeated-Measures, Double-Blind Deception Study**

3
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13
14 **Running Title:** NUTRITIONAL BLEND IMPROVES MEASURES OF SLEEP

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24 presented clearly, honestly, without fabrication, falsification, or inappropriate data manipulation.
25 The results of the present study do not constitute endorsement by the American College of Sports
26 Medicine.

27 **ABSTRACT**

28 **Purpose:** To test the hypothesis that a novel nutritional blend comprised of tryptophan, glycine,
29 magnesium, tart cherry powder and L-theanine, enhances subjective and objective measures of
30 sleep during free living conditions. **Methods:** In a randomised, repeated measures crossover and
31 double blind deception design, participants ($n = 9$ male and 7 female; age: 24 ± 3 years; body
32 mass: 69.8 ± 11.6 kg; stature: 170.8 ± 9.1 cm) completed a 3 day familiarisation period, followed
33 by 3 day intervention and placebo trials. Subjective Pittsburgh Quality Sleep Index, Core
34 Consensus Sleep Diary and Karolinska Sleepiness Scale survey tools, alongside objective
35 actigraphy measures of sleep were assessed, with daily nutritional intake, activity and light
36 exposure standardised between trials. Participants provided daily urine samples for assessment of
37 targeted and untargeted metabolomes. **Results:** The intervention trial reduced sleep onset latency
38 (-24 ± 25 mins; $p = 0.002$), increased total sleep time (22 ± 32 mins; $p = 0.01$) and sleep
39 efficiency (2.4 ± 3.9 %; $p = 0.03$), whilst also reducing morning sleepiness ($p = 0.02$).
40 Throughout the study, 75 % of participants remained blinded to sleep assessment as a primary
41 outcome measure, with 56 % subjectively indicating improved sleep during the intervention trial.
42 Metabolomic analysis highlighted several significantly altered metabolomes related to sleep
43 regulation between trials, inclusive of 6-sulfatoxymelatonin, D-serine and L-glutamic acid.
44 **Conclusions:** Data demonstrate that employing the proposed blend of novel nutritional
45 ingredients during free living conditions reduced sleep onset latency, increased total sleep
46 duration and increased sleep efficiency, leading to reduced perceptions of morning sleepiness.
47 These effects may be mediated by the upregulation of key metabolites involved in the
48 neurophysiological modulation of the sleep/wake cycle. **Key Words:** ACTIGRAPHY,
49 NUTRITION, ERGOGENIC DIETARY SUPPLEMENTS, METABOLOMICS

50 INTRODUCTION

51 Sleep is an essential process in aiding the recovery of several psycho-physiological
52 functions, including learning and memory, the central nervous and metabolic cost of daytime
53 activities and the restoration of the immune and endocrine systems (1). In order to maintain
54 optimal health and daytime functioning, it is recommended that adults achieve at least 7 hours of
55 total sleep time with ≥ 85 % sleep efficiency (2, 3). However, a large scale global survey of
56 participants aged 15 to 65 years, has highlighted that 56 % of respondents from the United States
57 of America, alongside 31 % in Western Europe and 23 % in Japan have experienced some form
58 of sleep disturbance, such as poor sleep induction (55-69 %), maintenance (78-63 %) and overall
59 sleep quality (52-31 %) (4). The impact of these sleep disruptions often results in deleterious
60 effects on daytime functioning, therefore causing negative repercussions to both personal and
61 professional activities. Indeed, there is accumulating evidence demonstrating how sleep
62 disturbance may cause reductions in both athletic performance and physical activity levels (5, 6).
63 Moreover, it has been reported that sleep disturbance is a well understood factor in all cause
64 morbidity and mortality across a range of populations (7). To that end, changes in lifestyle
65 factors, improved sleep hygiene and nutrition have all been proposed as potential modulators for
66 sleep disturbances in order to offset these issues (8, 9).

67 The nutritional regulation of sleep has been of consistent research interest for many years,
68 most notably in athletic populations, as has been recently addressed in a number of narrative
69 reviews and an expert consensus statement (1, 10, 11). Several studies have examined various
70 nutritional ingredients with proposed sleep enhancing qualities across a range of participant
71 groups with and without sleep disorders, inclusive of high glycaemic index carbohydrates (12,
72 13), tryptophan (14), tart cherries (15, 16), glycine (17), magnesium (18) and L-theanine (19).

73 Within these investigations there have been numerous suggested regulatory mechanisms, with
74 metabolomic interactions of specific neurotransmitters involved in the sleep/wake cycle,
75 purported to enhance regulation of the circadian rhythm of sleep (20). However, despite
76 promising outcomes in the capacity to improve sleep, many of these studies have been conducted
77 with predominantly male participants and with limited samples sizes in order to make definitive
78 conclusions (21). Furthermore, these ingredients have principally been studied in isolation, with
79 only one investigation to date examining their combined efficacy on various sleep outcome
80 measures (22). On this basis, there is considerable scope to further investigate the combined
81 benefit of these sleep enhancing agents, particularly given their reported individual effectiveness.

82 A recent critical review (23) has suggested that to sufficiently investigate and determine
83 the true efficacy of any intervention treatment on sleep mechanisms, participants should be
84 appropriately blinded to their sleeping patterns being monitored. To examine sleep as a primary
85 variable there are several available methods, with the criterion standard of polysomnography
86 affording an in-depth view across numerous outcome measures, inclusive of sleep stages (24).
87 However, despite the validity of this technique, measurement needs to be conducted within a
88 laboratory setting, leading to indirect disturbances in participants normal sleeping patterns and
89 reducing ecological validity as assessments cannot be conducted during free living conditions
90 (23). Consequently, given the complexity of configuration prior to and during polysomnography
91 utilisation, this makes the capacity to deceive participants difficult, thereby not fulfilling the
92 potential to truly blind experiments. Despite being limited in the capability to assess sleep stages,
93 the use of actigraphy has been proposed as a method that is less invasive than polysomnography
94 and has been suggested as less likely to interrupt participants regular sleeping patterns (11, 25).
95 As such, given actigraphy monitors can be easily administered and also utilised to measure

96 daytime activities, this facilitates the opportunity to blind participants and provide a more
97 accurate overview of sleep assessment in free living conditions (26).

98 With this in mind, the aim of the present study was to test the hypothesis that a novel
99 nutritional blend comprised of tryptophan, glycine, magnesium, tart cherry powder and L-
100 theanine, would improve subjective and objective measures of sleep in male and female
101 participants during free living conditions. To this end, we adopted an experimental design
102 inclusive of an initial familiarisation period for the assessment of habitual sleeping patterns, light
103 exposures, physical activity levels and daily energy intakes, prior to participating in a
104 randomised crossover trial whereby participants ingested either an intervention or placebo
105 treatment. Importantly, the intervention was administered double blind, with participants being
106 deceived as to the true aim of the study.

107

108 **METHODS**

109 **Participants**

110 The study was approved by the Liverpool John Moores University Research Ethics
111 Committee (Protocol code: 21/SPS/003) and all procedures were conducted according to the
112 manual of the Declaration of Helsinki 1964 and its later amendments. Sample size was
113 determined a priori according to an effect size of $d = 1.00$, where a sample size of 16 would
114 provide an α -value of 0.05 and a power of 0.96 (G*Power, version 3.1.9.6). Sixteen participants
115 (9 males and 7 females; age: 24 ± 3 years; body mass: 69.8 ± 11.6 kg; stature: 170.8 ± 9.1 cm)
116 were recruited for the study and prior to commencement, were provided with an information
117 sheet and gave written informed consent. Inclusion criteria stipulated participants must be a.)
118 aged between 18-40 years, b.) non-smokers, c.) not experiencing any medically diagnosed sleep

119 disorders, environmental sleep disturbances and/or taking sleep aid medications, d.) had not
120 travelled across different time zones in the month prior to study, e.) have no history of
121 neurological, cardiovascular, metabolic and/or psychiatric illnesses or diseases and f.) not be
122 taking any medications or supplements, which may contraindicate the study.

123

124 **Study Design**

125 Initially, participants were familiarised to the study measures and procedures, whilst also
126 establishing a baseline for nocturnal sleeping patterns, daily energy/macronutrient intakes and
127 activity profiles, which enabled duplication during the main intervention and placebo trials (see
128 Figure 1a & b). In a randomised, repeated measures crossover and double blind deception
129 design, participants were then assigned to either the intervention or placebo trial determined by
130 an online randomisation system (See Figure 1c & d). Both the familiarisation and the main trial
131 assessments were 3 days in length, all of which commenced on the same days of the week for all
132 participants and with a 4 day period between conditions to minimise any crossover effects. The
133 intervention treatment comprised of 1000 mg of tryptophan, 3000 mg of glycine, 300 mg of
134 magnesium, 220 mg of tart cherry powder and 200 mg of L-theanine, with the placebo treatment
135 containing 4720 mg of cellulose, both of which were administered across all 3 nights of each
136 respective main trial in opaque pill format. Additionally, the participants were also informed that
137 the study aim was to assess the effect of two supplemental treatments on resting fat oxidation
138 measured on the final day of main trial periods (see Figure 1c) and with all subsequent
139 assessments being administered for standardisation between conditions. The intention of this
140 deception blinding, was to make participants unaware of the true aim of the study (27).

141 **Study Measures**

142 *Body Mass and Stature:* Body mass was measured to the nearest 0.01 kg on a calibrated digital
143 scale, with measures of stature established to the nearest 0.1 cm using a free standing stadiometer
144 (Seca 702 & 123; Seca GmbH, Hamburg, Germany).

145

146 *Subjective Sleep Assessments:* To examine subjective assessments of nocturnal sleep patterns,
147 alongside evening and morning alertness and sleepiness, participants recorded responses within
148 electronic versions of the Pittsburgh Quality Sleep Index (PSQI) (28), Core Consensus Sleep
149 Diary (CSD) (29) and Karolinska Sleepiness Scale (KSS) (30). The PSQI is a 9-unit
150 questionnaire, which retrospectively examines sleep across a one month period and is rated on 7
151 components of sleep quality, latency, duration, efficiency, disturbance, medication and day time
152 dysfunction, to generate a global score. The core CSD is a 10-unit survey, assessing a range of
153 information related to sleep patterns, whereas the KSS is a 9-point Likert scale utilised to
154 examine levels of alertness or sleepiness, both prospectively captured at the time of response.
155 Upon bed time, each participant recorded the clock time for the first and second units within the
156 core CSD and the following morning upon getting out of bed, recorded the information for the
157 remainder of the units.

158

159 *Objective Sleep, Activity and Light Assessments:* To objectively assess nocturnal sleep patterns,
160 daytime activity profiles and light luminous intensity (lux) exposure, each participant was
161 equipped with a lightweight, waterproof, wrist worn actigraphy monitor (MotionWatch 8,
162 CamNtech Ltd, Cambridgeshire, United Kingdom). The tri-axial accelerometer within the
163 monitor detects acceleration ranging from 0.01 to 8 G in magnitude, with a frequency of 3-

164 11 Hz. Additionally, the monitor contains a digital human eye response optimized light sensor,
165 capable of capturing 0 to 64,000 lux at a resolution of 0.25 to 16.0 lux in one sample per second.
166 For sleep and activity measurement, the monitor has been assessed for validity and reliability of
167 its algorithms against both polysomnography and indirect calorimetry across a range of
168 demographics and is effective when set above medium sensitivity and epoch lengths of 30
169 seconds (31, 32). Each monitor was configured in these modes and participants were requested to
170 press the marker button during specific morning and night time periods (see *Study Procedures*).
171 The recorded markers from the monitor and the collated information from the core CSD, were
172 then used to determine bed time, sleep onset, sleep offset and get up time, so that nocturnal sleep
173 behaviours could be automatically calculated using the appropriate software (MotionWare
174 version 1.2.5, CamNtech Ltd, Cambridgeshire, United Kingdom). From the analysis, the
175 following sleep characteristics were established: sleep onset latency (SOL), wake after sleep
176 onset (WASO), total sleep time (TST), time in bed (TIB), and sleep efficiency (SE). After
177 excluding sleep measurement periods, day time activity profiles were automatically calculated by
178 the software into vigorous, moderate, low and sedentary phases, with light exposure averaged
179 across each of the night and day time periods.

180

181 *Daily Energy and Macronutrient Intakes:* Within the familiarisation period, all *ad libitum* food
182 and fluid intake were prospectively recorded by participants using both weighed food inventory
183 and the remote food photography method (RFPM) (33). In brief, participants were guided on
184 how to measure all food and fluids on an electronic digital scale (AccuWeight 201, Nanlgood
185 Network Technology Co. Ltd., Shenzhen, China) and record the details within the MyFitnessPal
186 application (Francisco Partners, San Francisco, California, USA). Additionally, participants were

187 also instructed to capture photographs at 45 and 90° angles before and after each eating and/or
188 drinking occasion, which was recorded and timestamped via the WhatsApp application
189 (WhatsApp Inc, Mountain View, California, USA) as previously described (34). These data were
190 then assessed by two accredited sport nutrition practitioners utilising dietary analysis software
191 (Nutritics V5. Nutritics Ltd., Swords, Co. Dublin, Ireland) to establish respective daily energy
192 and macronutrient values (see Table 1), with systematic bias of measurements via independent *t*-
193 tests highlighting no differences between analyses ($p < 0.05$). Within both main intervention and
194 placebo trials, participants were instructed to repeat the eating and drinking patterns at the same
195 times of day as in the familiarisation period, with reminders inclusive of previous recorded
196 images, descriptions and MyFitnessPal data sent by members of the research team via the
197 WhatsApp application. The participants were also instructed to follow the same procedures of
198 weighed food inventory and RFBM as described above, for confirmation of adherence.
199 Compliance to these procedures was 100% across all participants, which ensured daily energy
200 and macronutrient values were duplicated across main trials.

201
202 *Urine Collection, Storage and Metabolomic Analysis:* Urinary voids were collected by
203 participants into sterilised measuring cylinders and immediately stored at -20 °C within a home
204 freezer unit. The samples were then returned to the research team, thawed at room temperature,
205 centrifuged at 12,000 *G* for 1 min and subsequently transferred to a 1.5 mL Eppendorf tubes for
206 immediate storage at -80 °C. A random sub group of $n = 8$ participants samples were then
207 examined for targeted and untargeted metabolomic profiles. For sample preparation, 500 µL of
208 urine was desalted using Zeba™ spin columns (Thermo Scientific, Hemel Hempstead, United
209 Kingdom), flash frozen in liquid nitrogen and lyophilized overnight. The samples were then

210 reconstituted in 100 μ L (90/10 v/v) LC/MS grade water acetonitrile and spin filtered with
211 Costar® Spin-X® 0.22 micron cellulose filters (Corning, Glendale, Arizona, USA) at 2000 g for
212 5 mins. The resulting concentrated urine samples were then transferred to 1.5 mL amber vials
213 with 150 μ L micro inserts. For the targeted analysis of 6-Sulfatoxymelatonin, conventional
214 standard addition methodology was applied, whereby 1.0 mg of chemical standard (Biosynth®
215 Carbosynth Ltd, Berkshire, United Kingdom) was reconstituted in 100 μ L of LC/MS grade water
216 to generate a 30 mM stock and then serial diluted down to a 10 μ M working solution. The RT
217 and accurate mass were confirmed using hydrophilic liquid interaction chromatography (HILIC)
218 and the mass spectrometry signal acquisition was performed and quantified using a single ion
219 monitoring mode with 10 ppm mass channel tolerance. The HILIC profiling of the samples was
220 performed on a Vanquish™ Ultra High Pressure Liquid Chromatography (UHPLC) separation
221 system connected to an IDX high-resolution mass spectrometer system (Thermo Scientific,
222 Hemel Hempstead, United Kingdom).

223 Mass spectrometry data were captured using the AcqvieX acquisition workflow (data
224 dependent analysis methodology) and orbitrap detector operating parameters were MS1 mass
225 resolution 60K, for MS2 30K collisional stepped energy (HCD) 20, 35, 50 in step collision
226 mode. The mass scan range was 100-1000 m/z, RF lens was 35%, AGC target mode custom and
227 normalised AGC target was set to 25 % (100 % = 3e6) with a maximum injection time of 50 ms.
228 The intensity threshold was set to 2e4, with all data acquired in profile mode. A corresponding
229 extraction blank was used to create background exclusion list and a pooled QC were used to
230 create the inclusion list.

231 HILIC separation was achieved using a Waters™ Acquity UPLC BEH amide column
232 (2.1 x 150mm with particle size of 1.7 μ m), operating at 65°C with a flow rate of 200 μ L·min⁻¹

233 (Waters Ltd., Wilmslow, UK). The LC gradient consisted of a binary buffer system, of which
234 buffer A was 95/5 % (LC/MS grade water/ACN) and Buffer B was 90/10 % (ACN/water), with
235 both containing 10 mM ammonium formate additives. Independent buffer systems were used for
236 positive and negative modes, respectively. For positive modes, the pH of buffers was adjusted
237 using 0.1 % formic acid and for negative modes 0.1 % ammonia solution. The LC gradient were
238 the same for both polarities, with 95 % B at T0 hold for 2 min and a linearly decrease to 50 % B
239 at 11 min hold for 4.5 min and return to the starting condition and hold for further 4.5 min
240 (column stabilization). The total run was approximately 21.5 min per injection, with each volume
241 and applied voltage being 3 μ L/3.5 kV and 5 μ L/2.5 kV for positive and negative modes,
242 respectively. The HESI conditions for 200 μ L \cdot min⁻¹ were sheath gas 35, aux gas 7 and sweep gas
243 of 0, with an ion transfer tube temperature of 300°C and vaporizer temperature of 275°C.
244 Positive and negative data sets were processed via Thermo Scientific Compound Discoverer 3.2
245 with untargeted metabolomic workflow, alignment model adaptive curve, minimum intensity
246 1e⁶, S/N threshold 3:1, compound consolidation, mass tolerance 10 ppm and retention time
247 tolerance 0.3 min settings. Database matching was performed using Thermo Scientific m/z
248 CloudTM spectral library for targets with a similar index of >70% MS2 spectra. Those
249 metabolites that could be matched ($n = 134$) and had a relative standard deviation of 30% or less
250 within the quality controls were retained for analysis.

251

252 *Deception Blinding Protocol:* Given the status of COVID-19 at the time of the study assessment
253 period and with governmental restrictions on certain testing procedures in place, the research
254 team were unable to conduct valid assessments of resting fat oxidation via indirect calorimetry.
255 To compensate, a *mock* fat oxidation assessment was conceived, whereby participants were

256 provided with an exetainer collection vial equipped with a bespoke breathing tube. Following the
257 final assessment day of both intervention and placebo main trials and upon waking, participants
258 were instructed to blow into the breathing tube for a period of 60 seconds, so their subsequent
259 breath sample could be assessed for endogenous fat utilisation utilising indirect calorimetry.
260 However once samples were collected, they were discarded, given they were not required for
261 analysis within the primary examinations of the deception design.

262

263 **Study Procedures**

264 *Familiarisation:* On the day prior to the beginning of the familiarisation assessment period,
265 participants were measured for body mass and stature, completed the PSQI and then provided
266 with the subjective and objective sleep measurement apparatus and urine cylinders as described
267 in the *Study Measures*. Participants were then subsequently categorised based on their global
268 PSQI score, with < 5 regarded as *good sleep quality* and > 5 as *poor sleep quality* types (28).
269 During this assessment period, all communication was recorded and timestamped via the
270 WhatsApp application. On the evening prior to the first day of assessment, participants were
271 instructed to wear the actigraphy monitor on their non-dominant wrist, with recording
272 programmed to begin at 6.00am on the following morning. Participants were requested to
273 continuously wear the monitor at all times, other than if there was a need to remove it for safety
274 reasons i.e., contact exercise. On the morning of each assessment day, upon sleep offset and
275 getting out of bed, participants were instructed to press the marker button of the monitor, provide
276 an initial urine sample and then complete the electronic core CSD and KSS survey tools.
277 Throughout the remainder of each assessment day, participants provided weighed food inventory
278 and RFPM for each subsequent individual food and fluid intake occasion (see *Study Measures*),

279 whilst also indicating the details of any exercise bouts, inclusive of modality and load (i.e.,
280 duration, distance, sets, repetitions etc.). A further two urine samples were collected at regular
281 spaced intervals, based on the estimated get up and bed times derived from individual participant
282 PSQI responses. Finally, participants were directed to complete the relevant section within the
283 core CSD and KSS, press the wristwatch actigraphy monitor marker button when getting into
284 bed and again when they intended to go to sleep. The monitor and urine samples were then
285 returned to the research team on the day after the final assessment day (see Figure 1a & b).

286

287 *Main Trials:* Following the familiarisation period, sleep measures were analysed from the
288 actigraphy monitor and if SE was $\geq 85\%$ and TST ≥ 7 hours, then participants were further
289 classified as *normal* and below both of these values as *deficient* sleep types (2, 3). During main
290 trials, participants continued to be contacted via the WhatsApp online application, with
291 collection of measures, morning procedures, energy/macronutrient/fluid intakes, exercise
292 occasions and urine sampling repeated in the same manner and time of day as established in the
293 familiarisation period. The placebo and intervention treatments were administered two hours
294 prior to each participant's respective bed time, which was replicated at the same time within the
295 familiarisation period. The sleep offset and get up times of participants were not standardised, to
296 examine if any of the conditions resulted in an extended time in bed (see Figure 1c & d). Upon
297 completion of the study, all participants were interviewed to assess the efficacy of the deception
298 blinding procedures. Initially, participants were queried as to if they could detect any differences
299 between the two main trials. Participants were then informed that the deception was not the true
300 aim of the study and asked if they could speculate as to the real aim. Finally, participants were

301 informed of the study aim and asked if they could identify the intervention and placebo
302 conditions.

303

304 **Statistical Analyses**

305 Descriptive statistics inclusive of mean \pm *SD*, 95 % confidence intervals (95 % CI) and
306 frequency are provided for all data where appropriate, with analyses performed using SPSS
307 version 26 (PASW, Chicago, Illinois, USA) and the α -level set at $p < 0.05$. Residuals of the data
308 were explored for normality utilising histograms and Shapiro-Wilk tests, with box plots
309 examined for outliers. All data are presented as the mean of each 3 day period for the
310 intervention and placebo trials; therefore, comparisons were assessed via parametric paired
311 samples *t*-tests or non-parametric Wilcoxon tests for normally and non-normally distributed data,
312 respectively. Additionally, effect sizes (ES) were calculated utilising Hedges *g* with the
313 following quantitative criteria to explain the practical significance of the findings: trivial < 0.2 ,
314 small 0.2 – 0.59, moderate 0.6 – 1.19, large 1.20 – 1.99, and very large > 2.0 (35). Given the
315 ordinal nature of the KSS data, Pearson's Chi Squared test was employed to cross tabulate
316 frequency percentages between participant responses. Metabolomics data was auto scaled and
317 cube root transformed using MetaboAnalyst 5.0 software (36) before performing detailed partial
318 least squares discriminant analysis (PLS-DA) between intervention and placebo trial conditions.
319 The relative abundance of the metabolites from the intervention with variable importance in
320 projection (VIP) factor > 1 was then compared with the placebo trial.

321 RESULTS

322 Sleep Classifications and Bed Times

323 Prior to starting the familiarisation assessment period, $n = 8$ participants were
324 subjectively classified as having *poor sleep quality* (50 %) with the remaining $n = 8$ classified as
325 having *good sleep quality* (50 %) based on global PSQI scores. Following the familiarisation
326 period, $n = 14$ participants were objectively classified as *deficient* (87.5 %) and $n = 2$ classified
327 as *normal* (12.5 %) sleep types based on actigraphy monitoring. Mean bed time during the
328 familiarisation assessment period was $11.47 \text{ pm} \pm 26 \text{ mins}$, which was consistently replicated at
329 $11.48 \text{ pm} \pm 26 \text{ mins}$ and $11.50 \text{ pm} \pm 26 \text{ mins}$ in both the intervention and placebo trials,
330 respectively.

331

332 Activity Profiles and Light Exposure

333 There were no differences in mean night time light exposure between intervention ($1.06 \pm$
334 0.91 lux) and placebo ($1.29 \pm 0.95 \text{ lux}$) main trials, with a $-0.24 \pm 0.53 \text{ lux}$ variance of a small
335 effect between conditions ($p = 0.10$; 95 % CI = -0.53 to 0.55 lux ; ES = 0.24). Additionally, there
336 were also no differences in mean day time light exposure between intervention (351.66 ± 289.58
337 lux) and placebo ($352.48 \pm 361.74 \text{ lux}$) main trials, with a $-0.80 \pm 295.19 \text{ lux}$ difference of a
338 trivial effect between conditions ($p = 0.99$; 95 % CI = -158.10 to 156.50 lux ; ES = 0.01).
339 Measurements of mean participant activity are highlighted in Figure 2, demonstrating no
340 differences in vigorous (Figure 2a, 9 ± 1 vs. $8 \pm 11 \text{ mins} \cdot \text{day}^{-1}$, $p = 0.83$; 95 % CI = -4 to 5
341 $\text{mins} \cdot \text{day}^{-1}$; ES = 0.09), moderate (Figure 2b, 25 ± 23 vs. $35 \pm 29 \text{ mins} \cdot \text{day}^{-1}$, $p = 0.22$; 95 % CI
342 = -25 to $6 \text{ mins} \cdot \text{day}^{-1}$; ES = 0.37), low (Figure 2c, 473 ± 93 vs. $453 \pm 80 \text{ mins} \cdot \text{day}^{-1}$, $p = 0.15$; 95
343 % CI = -7 to $47 \text{ mins} \cdot \text{day}^{-1}$; ES = 0.22) or sedentary (Figure 2d, 360 ± 117 vs. 378 ± 109

344 mins·day⁻¹, $p = 0.13$; 95 % CI = -40 to 5 mins·day⁻¹; ES = 0.16) profiles, resulting in trivial to
345 small effects between the intervention and placebo main trials, respectively.

346

347 **Actigraphy Sleep Assessments**

348 For measures of SOL, there were differences of a large effect between the intervention
349 (16 ± 6 mins) and placebo (40 ± 25 mins) main trials, with a mean reduction of -24 ± 25 mins (p
350 $= 0.002$; 95 % CI = -38 to -10 mins; ES = 1.29) between conditions (see Figure 3a). There were
351 no differences in WASO between the intervention (49 ± 15 mins) and placebo (51 ± 17 mins)
352 main trials, resulting in a trivial effect based on the mean difference of -2 ± 11 mins ($p = 0.36$; 95
353 % CI = -8 to 3 mins; ES = 0.12) as highlighted in Figure 3b. For measures of TST there were
354 differences of a small effect between the intervention (422 ± 34 mins) and placebo (400 ± 44
355 mins) main trials, due to a mean increase of 22 ± 32 mins ($p = 0.01$; 95 % CI = 6 to 39 mins; ES
356 $= 0.55$) between conditions (see Figure 3c). There were no differences for overall TIB between
357 the intervention (506 ± 38 mins) and placebo (495 ± 42 mins) main trials, with a small effect
358 given the mean difference of 11 ± 26 mins ($p = 0.13$; 95 % CI = -4 to 25 mins; ES = 0.27) as
359 shown in Figure 3d. Finally, there were differences of a small effect in measures of SE between
360 the intervention (83.3 ± 3.5 %) and placebo (80.9 ± 5.9 %) main trials, based on an increase in
361 efficiency of 2.4 ± 3.9 % ($p = 0.03$; 95 % CI = 0.3 to 4.5 %; ES = 0.48) between conditions (see
362 Figure 3e).

363

364 **Subjective Sleep Assessments & Deception Blinding**

365 There were no differences evident in subjective scores of evening sleepiness between the
366 intervention and placebo main trials (see Figure 4a). However, there were differences in morning

367 sleepiness (see Figure 4b), whereby in the intervention trial participants reported a lower level of
368 some signs of sleepiness when compared to the placebo trial ($p = 0.02$). During post testing exit
369 interviews, $n = 12$ (75 %) participants were unable to identify that the true outcome of the study
370 was to assess sleep, with the remaining $n = 4$ (25 %) being aware. Of the $n = 16$ participants, $n =$
371 9 (56 %) identified the intervention trial as the assessment period where they perceived they had
372 their best sleep, $n = 1$ (6 %) stated the placebo trial and $n = 6$ (38 %) were unable to identify any
373 difference between conditions.

374

375 **Targeted and Untargeted Urinary Metabolomic Analysis**

376 Results of the PLS-DA visualization for both treatments is presented in Figure 5,
377 demonstrating a distinct phenotypic change in the urinary metabolome between the intervention
378 and placebo treatments. Twenty five database matched metabolites were highlighted to be
379 statistically different between the intervention and placebo trials based on VIP score factor > 1 .
380 Figure 6 highlights those metabolites identified as proxy markers of sleep regulation inclusive of
381 the targeted metabolite 6-sulfatoxymelatonin (a.), alongside untargeted amino acids D-serine (b.)
382 and L-glutamic acid (c.), both of which were up and downregulated between the intervention and
383 placebo trials, respectively.

384

385 **DISCUSSION**

386 In a randomised, repeated measures crossover and double blind deception design, the aim
387 of the present study was to test the hypothesis that a novel nutritional blend comprised of
388 tryptophan, glycine, magnesium, tart cherry extract and L-theanine, would improve subjective
389 and objective measures of sleep in male and female participants during free living conditions.

390 The data from this study demonstrates that a blend of sleep enhancing agents, shortened time to
391 fall asleep and extended time asleep, therefore resulting in greater sleep efficiency and leading to
392 reduced feelings of morning sleepiness. Evaluation of the urinary metabolome also suggests that
393 such alterations to sleeping patterns may be mediated by the upregulation of 6-
394 sulfatoxymelatonin and glycine co-agonist D-serine, in parallel to a downregulation of the
395 excitatory neurotransmitter L-glutamic acid, which are modulators of the sleep/wake cycle,
396 respectively. Taken together, these results indicate the efficacy of this novel blend of nutritional
397 ingredients to improve outcome measures of sleep during free living conditions, in both male and
398 females without medically diagnosed sleep disorders.

399 A key strength of this investigation was in deception blinding participants to sleep
400 assessment as the true aim of the study. On this basis, 75 % of participants were unaware that
401 sleep was being assessed as a primary outcome measure and over 50 % identified they had a
402 perceived sense of improved sleep during the intervention trial. Whilst this method has been
403 utilised in other nutritional investigations (27), to the authors' knowledge this study represents
404 the first application of this approach when examining the potential for nutritional modulation of
405 subjective and objective sleep outcomes and therefore enhances the applicability of the findings
406 (23). Additionally, participants were also exposed to an initial assessment period with the dual
407 objective of familiarisation to outcome measures, whilst also establishing a baseline for
408 standardisation of bed times, daily nutritional intake, activity profiles and light exposure within
409 the main intervention and placebo trials. This has been previously highlighted as an important
410 consideration in deciphering the translational potential of nutrition intervention studies (37) and
411 serves to further establish the ecological validity of the outcome results.

412 To date, only one other study by Halson and colleagues (22) has examined the effect of a
413 combined blend of sleep active ingredients, inclusive of high glycaemic carbohydrates, tart
414 cherry, tryptophan (in the form of α -lactalbumin), adenosine-5-monophosphate, valerian and L-
415 theanine in a double blind, placebo controlled crossover design. Whilst utilising
416 polysomnography in contrast to the actigraphy measures within this study, Halson et al.
417 highlighted a 9 minute reduction in SOL between the intervention and placebo treatments, yet
418 contrary to our findings, found no other differences in subjective or objective measures of sleep
419 outcomes between conditions. It should be noted that a limitation within the present study, is the
420 inability of a reductionist approach to understand the individual or combined effects of the agents
421 within the intervention treatment, on the capacity to improve the subjective and objective
422 measures of sleep. However, given the strength of the study design, the nutritional blend did
423 indeed impact some of these measures and to that end, the subsequent sections will serve to
424 discuss the potential sleep enhancing mechanisms of each ingredient contained within the
425 intervention treatment.

426 Dietary tryptophan (Trp) is a well-established sleep active ingredient, which has over
427 four decades of research within this area (14). Trp crosses the blood brain barrier by active
428 transport, therefore acting to release the monoamine neurotransmitter serotonin, a precursor of
429 melatonin (38). However, given other large neutral amino acids (LNAA) also compete to cross
430 the blood brain active barrier system, it is the addition of an adequate dose of dietary Trp (1000-
431 3000 mg), alongside an optimal Trp:LNAA ratio which increases brain bioavailability and can
432 subsequently lead to enhanced sleep through the upregulation of melatonin (14, 39). In
433 comparison to our study, Hartmann and Spinweber (40) found that a dose of 1000 mg of
434 tryptophan resulted in significantly reducing subjective assessments of SOL in those with mild

435 insomnia (over 30 minutes SOL). Additionally, Chauffard-Alboucq and colleagues (41) also
436 highlighted that a dose of 1000 mg of tryptophan was effective in quadrupling the Trp:LNAA
437 ratio, whilst also leading to enhanced feelings of sleepiness after 90 minutes of ingestion. Based
438 on these investigations, it could be postulated that the dosage of tryptophan included in the
439 presented study increased brain Trp levels, thereby upregulating serotonin and melatonin
440 pathways. Despite not directly assessing the Trp:LNAA ratio, this notion is supported by an
441 enhanced metabolomic abundance of urinary 6-sulfatoxymelatonin (see Figure 6a), which could
442 further explain the exhibited improvements in sleep during the intervention trial. In parallel to
443 Trp, tart cherries are known to contain endogenous melatonin (42), which can also directly
444 regulate sleep mechanisms via the aforementioned pathways. Howatson and colleagues (15)
445 demonstrated that a 7 day administration of tart cherry juice resulted in significant improvements
446 in sleep time and quality. Indeed, whilst the provision of the intervention treatment within the
447 present study was for a 3 day period, this resulted in similar favourable increases in both TST (22
448 vs. 34 mins) and enhancements in SE (2.4 vs. 5.5 %). Furthermore, Howatson et al. also
449 highlighted significant increases in urinary melatonin during tart cherry supplementation, also
450 corresponding to the amplified metabolomic abundance of urinary 6-sulfatoxymelatonin within
451 this study.

452 Glycine and L-theanine are amino acids, which are both endogenously synthesised/stored
453 and can be ingested through diet. In a similar manner to Trp, glycine is also able to cross the
454 blood brain barrier, which alongside co-agonist D-serine, targets the *N*-methyl-D-aspartate
455 (NMDA) receptors within the suprachiasmatic nucleus, further acting to upregulate whole body
456 vasodilation and therefore reduce core temperature leading to subsequent sleep enhancement
457 (17). Additionally, L-theanine also crosses the blood brain barrier and acts on the ionotropic

458 glutamate receptors and partially on the NMDA receptors to upregulate both serotonin and
459 glycine (19). A study by Yamadera and colleagues (43) examined the effects of glycine on
460 subjective sleep parameters and objective sleep via polysomnography. Whilst utilising different
461 measures compared to those within this study, a comparable dose of 3000 mg of glycine
462 improved objective assessments of SOL and subjective assessments of sleep quality and daytime
463 sleepiness. Although no assessments of core temperature were conducted within the present
464 study, given the known effects of core temperature reduction on upregulating sleep (44), it is
465 tempting to speculate that the glycine modulation of this mechanism may have also contributed
466 to the improvements in sleep outcome measures, particularly given the increased metabolomic
467 abundance of D-serine (see Figure 6b). However, to date this has only been conducted in animal
468 model studies, with more conclusive evidence needed within human populations (45). In a
469 similar manner to the present study, Ozeki and colleagues also examined the efficacy of L-
470 theanine on sleep via both subjective sleep scores and objective measurements of actigraphy
471 (19). Utilising a 200 mg dose and across a 3 day measurement period, the intervention
472 significantly improved subjective assessments of sleep, alongside an actigraphy derived 2.8 %
473 improvement in sleep efficiency when compared to a placebo condition, in direct parallel to the
474 results of this study.

475 An additional interesting finding within this investigation was a downregulated
476 metabolomic abundance of L-glutamic acid within the intervention compared to the placebo
477 trials (see Figure 6c). L-glutamic acid has been highlighted as a potent excitatory
478 neurotransmitter, resulting in stimulation of the orexinergic neurons, which in turn promotes
479 arousal and inhibition of both non-rapid eye and rapid eye movement sleep stages (46).
480 Conversely, both endogenous melatonin and D-serine have been demonstrated to attenuate the

481 excitatory neurotransmitter action of L-glutamic acid via inhibition of specific NMDA receptor
482 binding sites (47, 48). To that end, it is tempting to speculate that the upregulated metabolomic
483 abundance of urinary 6-sulfatoxymelatonin and D-serine in the intervention trial may have
484 resulted in the downregulated metabolomic abundance of L-glutamic acid, therefore leading to
485 greater subjective and objective sleep enhancement in comparison to the placebo trial. However,
486 much of the former evidence in this area has principally been conducted in rodent models and
487 more research is needed in human trials to substantiate this hypothesis.

488 Finally, magnesium is a key micronutrient, which also acts to regulate the conductivity of
489 the NMDA receptors, whilst aiding the binding of monoamines such as serotonin to their
490 respective sites (49), presenting the assumption that magnesium deficiency may lead to
491 disturbances in sleep, which can be enhanced with exogenous supplementation (50). A recent
492 systematic review and meta-analysis highlighted that administration of 320-729 mg·day⁻¹
493 magnesium, resulted in improvements in subjective and objective sleep, that included 17 minute
494 reductions in SOL and 16 minute increases in TST when compared to placebo conditions (50).
495 However, it should be noted that these treatments were delivered across various dosing
496 protocols, in predominantly elderly populations with magnesium deficiencies and across a range
497 of sleep disturbances and disorders. Consequently, given magnesium status was not assessed in
498 the present study participant cohort, it is difficult to consider the interaction of this ingredient
499 within the intervention treatment and with more research on the efficacy of magnesium
500 supplementation in diverse populations needed.

501 Finally, the present data may be considered of practical relevance for those individuals
502 who partake in regular exercise and physical activities. Indeed, in athletic populations, it has
503 been established that sleep disturbances can result in negative consequences on physical

504 performance, exercise recovery and exercise induced injuries/diseases, with these outcomes
505 being mediated by an enhanced perception of exertion via increased psycho-physiological strain
506 (5). Furthermore, reductions in sleep duration and quality are likely to reduce participation in
507 physical activity, which contributes to a wide range of health related outcomes within general
508 populations (6). Accordingly, improvements in sleep have been shown to enhance exercise
509 performance and increase the likelihood of participation of physical activities in athletic and
510 general populations, respectively (5, 6). On this basis, further investigation implementing the
511 nutritional blend proposed within this study and the potential effects of ameliorated sleep
512 outcomes on exercise performance and physical activity is certainly warranted.

513

514 **CONCLUSIONS**

515 In conclusion, this study highlights the efficacy of a novel blend of sleep enhancing
516 nutritional agents on subjective and objective measurements of sleep in both male and female
517 participants without medically diagnosed sleep disorders and during free living conditions.
518 Additionally, a key strength of this investigation included 75 % of participants being unaware
519 that sleep was being measured as a primary outcome, therefore enhancing the ecological validity
520 and applicability of the findings. The combination of ingredients contained within the blend, led
521 to significant decreases in sleep onset latency and increases in total sleep time and sleep
522 efficiency, alongside subjective reductions in morning sleepiness, as has been demonstrated
523 within the previous research literature. Furthermore, potential mechanisms for these effects may
524 have been as the result of metabolomes related to the sleep/wake cycle. The replication of this
525 study to examine potential efficacy across longer time periods and in those with medically
526 diagnosed sleep disorders is also warranted.

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532

533 **Conflicts of Interest**

534 James P. Morton is a consultant for SiS plc. The other authors disclose no conflicts of interest. SiS plc
535 had no role in the design, execution, interpretation, or writing of the study. The results of the study are
536 presented clearly, honestly, without fabrication, falsification, or inappropriate data manipulation. The
537 results of the present study do not constitute endorsement by the American College of Sports Medicine.

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- 659

660 **FIGURE LEGENDS**

661

662 **Figure 1** Overview of study design, inclusive of (a.) familiarisation assessment period, (b.)
663 familiarisation assessment methods and procedures, (c.) intervention and placebo assessment
664 periods and (d.) intervention and placebo assessment methods and procedures

665

666 **Figure 2** Comparison of mean and individual responses for (a.) vigorous, (b.) moderate, (c.) low
667 and (d.) sedentary activity profiles, between the intervention (grey bars) and placebo (white bars)
668 trials

669

670 **Figure 3** Comparison of mean and individual responses for (a.) sleep onset latency, (b.) wake
671 after sleep onset, (c.) total sleep time, (d.) time in bed and (E.) sleep efficiency outcomes,
672 between the intervention (grey bars) and placebo (white bars) trials. *denotes significant
673 difference between conditions ($p < 0.05$)

674

675 **Figure 4** The frequency of subjective Karolinska Sleepiness Scale scores across (a.) evening and
676 (b.) morning measurement periods between intervention and placebo trials. *denotes significant
677 difference between equitable scoring measure ($p < 0.05$)

678

679 **Figure 5** Partial least squares discriminant analysis of key identified urinary metabolomes
680 between the intervention (red) and placebo (green) trials

681

682 **Figure 6** Original and normalised concentration of key urinary metabolites (a.) 6-
683 sulfatoxymelatonin, (b.) D-serine and (c.) L-glutamic acid between the intervention (red bars)
684 and placebo (green bars) trials

Table 1 Estimated daily energy and macronutrient intakes assessed during the familiarisation period, which were duplicated during main intervention and placebo trials

	Energy	CHO	Protein	Fat
INTAKE	(kcal·day⁻¹)	g·day⁻¹ <i>(g·kg⁻¹)</i>	g·day⁻¹ <i>(g·kg⁻¹)</i>	g·day⁻¹ <i>(g·kg⁻¹)</i>
Day 1	2010 ± 567	257 ± 79 <i>(3.5 ± 1.0)</i>	101 ± 38 <i>(1.6 ± 0.5)</i>	62 ± 27 <i>(0.9 ± 0.3)</i>
Day 2	2114 ± 769	273 ± 90 <i>(3.7 ± 1.1)</i>	104 ± 49 <i>(1.6 ± 0.8)</i>	65 ± 39 <i>(1.0 ± 0.5)</i>
Day 3	2036 ± 782	264 ± 110 <i>(3.8 ± 1.2)</i>	100 ± 50 <i>(1.6 ± 0.7)</i>	63 ± 35 <i>(0.9 ± 0.4)</i>
TOTAL	2053 ± 640	265 ± 82 <i>(3.7 ± 1.0)</i>	101 ± 39 <i>(1.6 ± 0.6)</i>	63 ± 28 <i>(0.9 ± 0.3)</i>

Relative values are presented in italics within parenthesis. CHO = carbohydrate











