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1 **Whey protein with potassium bicarbonate supplement attenuates the reduction in**
2 **muscle oxidative capacity during 19 days bed rest**

3

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14

15 **Running head:** Whey protein sustains muscle oxidative capacity in bed rest.

16

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20 **Abstract**

21 The effectiveness of whey protein plus potassium bicarbonate enriched-diet
22 (WP+KHCO₃) to mitigate disuse-induced changes in muscle fibre oxidative capacity and
23 capillarization was investigated in a 21-day crossover design bed rest study. Ten healthy
24 men (31±6 years) once received WP+KHCO₃ and once received a standardized isocaloric
25 diet. Muscle biopsies were taken two days before and during the 19th day of bed rest (BR)
26 from the soleus (SOL) and vastus lateralis (VL) muscle. Whole body aerobic power
27 (VO_{2max}), muscle fatigue and isometric strength of knee extensor and plantar flexor
28 muscles were monitored. Muscle fiber types and capillaries were identified by
29 immunohistochemistry. Fiber oxidative capacity was determined as the optical density
30 (OD) at 660 nm of succinate dehydrogenase (SDH)-stained sections. The product of fiber
31 cross-sectional area and SDH-OD (integrated SDH) indicated the maximal oxygen
32 consumption of that fiber. The maximal oxygen consumption supported by a capillary was
33 calculated as the integrated SDH in its supply area. BR reduced isometric strength of knee
34 extensor muscles ($P<0.05$), and the fiber oxidative capacity ($P<0.001$) and VO_{2max}
35 ($P=0.042$), but had no significant impact on muscle capillarization or fatigue resistance of
36 thigh muscles. The maximal oxygen consumption supported by a capillary was reduced by
37 24% in SOL and 16% in VL ($P<0.001$). WP+KHCO₃ attenuated the disuse-induced
38 reduction in fiber oxidative capacity in both muscles ($P<0.01$). In conclusion, following 19
39 days bed rest, the decrement in fiber oxidative capacity is proportionally larger than the
40 loss of capillaries. WP+KHCO₃ appears to attenuate disuse-induced reductions in fiber
41 oxidative capacity.

42

43 **Key words:** bed rest, oxidative capacity, capillarization, whey protein, muscle atrophy,
44 microgravity, KHCO₃, maximal voluntary contraction, muscle fatigue.

45

46

47 **New and noteworthy:** Reduced muscle oxidative capacity and capillary rarefaction may
48 be critical factors in disuse-induced muscle weakness in space flight or bed-rest. Here we
49 show that 19 days bed rest induced a reduction in the fiber oxidative capacity, irrespective
50 of muscle (soleus and vastus lateralis muscle) or fiber type, without significant capillary
51 loss, that was in part attenuated by a whey protein plus potassium bicarbonate enriched
52 diet.

53

54 **Abbreviations.** BDC, before bed rest; BR, bed rest; BSA, bovine serum albumin; C:F,
55 capillary to fiber ratio; CD, capillary density; CFD, capillary fiber density; DLR, Deutsches
56 Zentrum für Luft- und Raumfahrt; ECG, electrocardiography; ESA, European Space
57 Agency; FCSA, fiber cross-sectional area; HDT, head-down-tilt; HRP, horseradish
58 peroxidase; KHCO_3 , potassium bicarbonate; LCFR, local capillary to fiber ratio; $\log_{10} \text{SD}$,
59 standard deviation of the logarithm of domain areas; LTBR, long-term bed rest; MatLab,
60 Matrix Laboratory; $\text{MO}_{2\text{max}}$, maximal oxygen consumption supported by a capillary;
61 MTBR/MEP, Medium-Term Bed Rest Whey protein; MyHC, myosin heavy chain; MVC,
62 maximal voluntary contraction; NOS3, nitric oxide synthase 3; ns, not statistically
63 significant; O.C.T., optimum cutting temperature; OD, optical density; PBS, phosphate
64 buffered saline; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1-
65 alpha; SDH, succinate dehydrogenase; SOL, soleus; VL, vastus lateralis; $\text{VO}_{2\text{max}}$, maximal
66 oxygen uptake.

67

68 **Introduction**

69

70 Skeletal muscle disuse, such as occurs during prolonged immobilization, bed rest
71 and spaceflight, is associated with muscle wasting, weakness and reduced fatigue
72 resistance (17). As muscular forces are important to maintain bone density, the reduction
73 in muscle mechanical forces may lead to an increased risk of falls and bone injury (24). In
74 astronauts, microgravity-induced changes in the musculoskeletal system may lead to
75 muscle or bone injury during activity and may limit their ability to perform their mission and
76 daily tasks, and presents a potential risk to their safety and health (1). There is therefore
77 considerable interest to develop effective nutritional and exercise interventions to
78 attenuate the muscle wasting following prolonged space missions.

79

80 Besides muscle atrophy, disuse also causes arterial structural remodeling and
81 reductions of blood flow to the active muscles (54). Mechanical signals and endothelial cell
82 shear stress are crucial for capillary maintenance and angiogenesis (31), and a reduced
83 blood flow during muscle disuse may result in capillary rarefaction. An adequate capillary
84 supply is crucial not only for delivery of oxygen but also for the delivery of nutrients and
85 removal of heat and waste products and hence for tissue remodeling and repair. As fatigue
86 resistance correlates positively with capillarization and fiber oxidative capacity of the
87 muscle (20), capillary rarefaction in disuse or in microgravity, combined with a reduced
88 oxidative capacity, may lead to a lower muscle fatigue resistance (17), or even exacerbate
89 tissue damage.

90

91 Although it has been well documented that gravitational unloading during short-term
92 spaceflight is associated with muscle atrophy and a reduced oxidative capacity in humans
93 (4,23) and rodents (4,50), there are still limited data on changes in muscle capillarization
94 and fiber oxidative capacity during prolonged microgravity in humans. Microgravity-
95 induced muscle weakness, reduced fiber cross-sectional area and a slow-to-fast fiber type
96 transition (4) are more pronounced and occur earlier in oxidative and antigravity muscles
97 (such as the soleus) than in non-postural mixed muscles (i.e. the vastus lateralis) (17).
98 One might therefore expect that also the reduction in oxidative capacity and loss of
99 capillaries are more pronounced in the more oxidative weight-bearing muscles, but this
100 has hitherto not been investigated systematically.

101

102 It has been shown that high protein intake and essential amino acid supplements
103 have anti-catabolic, anti-inflammatory and anti-oxidant effects, where in particular whey
104 protein (WP) appears effective in overcoming protein wasting during short-term bed rest
105 (3,52). Although WP has been reported to enhance the gain in lower body strength and
106 VO_{2max} and mitochondrial enzyme activities in combination with resistance- (39) or
107 aerobic- (see review: 42) training, we (8) and others (in review: 52) did not see any
108 significant effect on muscle fiber size of a WP-enriched diet during prolonged period of bed
109 rest (8,52). It remains to be seen, however, whether WP could attenuate any disuse-
110 induced reductions in muscle fiber oxidative capacity in bed rest.

111

112 A potential limitation of a daily high protein intake is the introduction of an acid-
113 load, caused by the endogenous oxidation of cationic and sulfur-containing amino acids,
114 which during bed rest will add to the acidogenic load resulting from the amino acids
115 derived from broken down muscle proteins. If the acidogenic effect of high protein intake is
116 not compensated by an alkaline agent a chronic low-grade metabolic acidosis may cause
117 further activation of [muscle proteolysis](#) (57), bone demineralization (24) and potentially
118 also inhibit aerobic energy metabolism, resulting in an earlier onset of muscle fatigue (36).
119 The supplementation of alkaline mineral salts, such as potassium bicarbonate ($KHCO_3$),
120 has been shown to effectively reduce muscle wasting in the setting of acidogenic or high
121 vitamin D diets and in chronic metabolic acidosis in human (10) and animal models (14). It
122 was therefore expected that the addition of the alkaline salt $KHCO_3$ supports the action of
123 whey protein and helps to sustain muscle fiber aerobic capacity during prolonged periods
124 of disuse.

125

126 In the present study, we investigated the potential of whey protein
127 supplementation plus $KHCO_3$ to counteract the effects of 19 days 6° head down-tilt bed
128 rest (21 days of medium-term bed rest, MTBR/MEP study) on muscle fiber capillarization
129 and oxidative capacity. Our principal hypothesis was that bed rest-induced reductions in
130 fiber oxidative capacity and capillary rarefaction are more pronounced in the soleus than
131 the vastus lateralis muscle, which can all be prevented by alkaline whey protein enriched
132 diet.

133

134 **Materials and Methods**

135

136 ***Bed rest study***

137 The 21-day 6° head-down-tilt (HDT) **Medium-Term Bed Rest** Whey protein
138 (MTBR/MEP) study was performed at the German Aerospace Center (DLR) in Cologne,
139 Germany, in accordance with the European Space Agency (ESA) bed rest standardization
140 plan. The design of the study was described previously (11). Briefly, the study was a
141 controlled randomized crossover design performed in two campaigns, separated by a 125-
142 day wash-out period. Each campaign comprised a 7-day adaptation, a 21-day bed rest
143 (intervention period) and a 6-day recovery phase. The caloric intake was controlled
144 throughout the study and was during the 7-day adaptation and 6-day recovery phases
145 around 2700 kCal·d⁻¹ and reduced to around 2030 kCal·d⁻¹ during bed rest (for details see
146 11). For the first campaign (September and October 2011), five healthy participants were
147 randomly assigned to a bed rest-only (BR), and another five healthy participants to a bed
148 rest plus whey protein + KHCO₃ intervention (NUTR). For the second campaign (February
149 and March 2012), the participants were assigned the other way around (Fig.1). The
150 crossover design minimized any potential bias from carry-over and seasonal effects
151 (possible differences in the habitual activity levels during the summer and mid-winter) on
152 the structure and function of skeletal muscle. Table 1 shows the participant characteristics.

153

154 The recruited subjects (ten healthy men aged between 23 to 43 years, an age
155 typical for astronauts) successfully completed all medical, physical and psychological
156 screenings (11). Exclusion criteria included presence of muscle/cartilage/joint diseases,
157 herniated disc, chronic back pain, chronic hypertension, diabetes, obesity, arthritis,
158 hyperlipidemia, any infectious and hepatic disease, disorders of calcium or bone
159 metabolism, history of orthostatic intolerance or vestibular disorders (11). Negative results
160 of a thrombophilia screening panel (Antithrombin III, Protein C and S, Factor-V-Leiden,
161 Pro- thrombin muteins, Lupus- Partial Thromboplastin Time) were mandatory for final
162 inclusion in the study (11).

163

164 The study was conducted in compliance with the protocol (and its subsequent
165 amendments) for the MEP bed rest study, as approved by the independent ethics
166 committee of the Ärztekammer Nordrhein, Düsseldorf, Germany. During the study the
167 rights, safety and well-being of subjects were protected according to the Declaration of

168 Helsinki. All subjects participated after providing signed informed consent. More detailed
169 data on exclusion criteria, anthropometric characteristic, energy intake and baseline data
170 of the MTBR/MEP study are reported in (www.clinical.trials.gov, Identifier: NCT01655979;
171 [8,11](#)).

172

173 **Nutritional intervention**

174 The nutritional intervention (NUTR) was a combination of whey protein (0.6 g whey
175 protein·kg body mass⁻¹·day⁻¹; Diaprotein®, Dr. Steudle Inc, Krueger GmbH) plus
176 potassium bicarbonate (90 mmol KHCO₃·day⁻¹), that isocalorically replaced fat and
177 carbohydrates in the daily diet in a 1:1 ratio ([11](#)). During the control bed rest condition (BR)
178 the participants received a basic protein diet of 1.2 g protein·kg⁻¹·day⁻¹. This intake was
179 higher than the current recommended daily intake (0.8 g protein·kg⁻¹·day⁻¹) and was
180 moderately acidifying (potential renal acid load of the diet: 13±1 mEq·day⁻¹). During the
181 NUTR condition, the alkaline urine content confirmed an alkali over acid production,
182 suggesting that there was no acidification in this group. More detailed data are reported in
183 ([11](#)).

184

185 **Maximal Oxygen Uptake (VO_{2max})**

186

187 Maximal oxygen uptake was assessed using a graded exercise protocol on an
188 electronically-braked cycle ergometer (Model Excalibur Sport, LODE B.V, The
189 Netherlands). The oxygen uptake throughout the test was measured with a Metalyzer
190 (Spirometer: Cortex Metalyzer, CORTEX Biophysik GmbH, Germany), before (BCD-7) and
191 post (R+1) bed rest. Heart rate, ECG and blood pressure were monitored continuously
192 during the test (Finometer, TNO, The Netherlands, Biopac systems inc. USA).
193 Participants were considered to have reached VO_{2max} if they fulfilled at least two of the
194 following three criteria: they could not maintain the cadence of 60 revolutions per minute
195 due to voluntary exhaustion, reached the predicted maximal heart and/or had a respiratory
196 exchange ratio > 1.1.

197

198 **Isometric maximal voluntary contraction (MVC)**

199 The torque during maximal voluntary isometric contractions (MVC) was determined
200 for the knee extensors and the plantar flexors before (BCD-7) and post (BR+0) bed rest,
201 using a dynamometer (Biodex Medical Systems, Inc., Shirley, NY) as described previously

202 (38). The highest torque (Nm) was considered the subject's maximum. If a subject
203 continued to improve at the third trial contraction, testing was continued until no further
204 improvement was observed.

205

206 **Muscle fatigue resistance**

207 Muscle fatigue resistance was determined before (BCD-3) and post (BR+0) bed
208 rest, in the knee extensors. Muscle fatigue resistance was given as the time to failure
209 during a sustained contraction at 50% of the actual MVC (38).

210

211 ***Muscle Biopsies***

212 Muscle biopsies were obtained two days before bed rest (BDC-2) and during the
213 19th day of bed rest (BR+19) from the vastus lateralis (VL) and soleus (SOL) muscles of
214 the right leg. Biopsies of the vastus lateralis were taken at 40% of the length between the
215 knee joint cleft (0% being the knee joint cleft) and the anterior superior iliac spine. Soleus
216 biopsies were obtained via a lateral approach, at least 2 cm below the distal end of the
217 lateral gastrocnemius muscle. In both muscles, sequential biopsies were at least 2 cm
218 apart. To minimize any bias due to regional differences in muscle morphology, sequences
219 (distal vs. proximal) of biopsy localization were permuted between subjects. In the
220 second campaign, two of the subjects provided no biopsies (one for medical reasons and
221 one withdrew from the study for personal reasons during the second campaign and did not
222 provide a post-bed rest biopsy). There were no adverse events or side effects in the MEP
223 study, associated with neither the bed rest nor the biopsies. However, one subject
224 developed petechiae during the orthostatic tests that were performed after bed rest in both
225 campaigns, as previously reported (25). The samples were subdivided into a piece for
226 histological analysis and other tissue pieces (approx. 20 mg each) for biochemical and
227 molecular analysis, as described (8). The histology piece was embedded in a 3-mm
228 silicone tube filled with Optimum Cutting Temperature (O.C.T.) compound (Scigen®
229 Gardena) to facilitate cross-sectional orientation. All samples were immediately frozen in
230 liquid nitrogen and stored at -80°C until analysis.

231

232 ***Histological staining for muscle capillarization and fiber typing***

233 Muscle cross-sections were prepared as previously described (8). Briefly, from all
234 biopsies, serial 8- μ m cross-sections were cut in a cryotome at -20°C (CM 1860, LEICA
235 Microsystems). The sections were mounted on polarized glass slides (SuperFrost® Plus,
236 631-0108, VWR International) and stored at -80°C until use. Capillaries and type I fibers
237 were stained in the same section using a combined immunostaining (Fig. 2). The cross-
238 sections were dried at room temperature for 30 min and then fixed for 15 min in ice-cold
239 acetone (100%). The sections were then washed twice for 5 min in phosphate buffered
240 saline (PBS) at pH 7.6 and blocked for 1 h in 0.1% bovine serum albumin (BSA) in PBS.
241 The sections were then washed twice in PBS for 5 min and the endogenous peroxidases
242 blocked by incubation in 3% H₂O₂ and 10% Triton X-100 in PBS for 30 min at room
243 temperature. The anti-mouse myosin heavy chain type I (MyHC I, 1:100; Novocastra,
244 Leica Biosystems, UK) and biotinylated *Ulex europaeus* agglutinin I (50 μ L·mL⁻¹ in 1%
245 BSA in HEPES; Vector Laboratories, USA) were used to visualize type I fibers and
246 capillaries, respectively. Unlike previously reported (8), further sub-classification of type II
247 fibers and of hybrid fibers (co-expressing both MyHC types I/II) was not performed here.
248 The effect of bed rest and the WP-enriched diet on fiber cross-sectional area (FCSA) and
249 myosin heavy chain composition have been published previously (8). After two 5-min
250 washes in PBS the sections were incubated with the VECTASTAIN® Elite ABC System
251 (Vector Laboratories, USA), as described by the manufacturer. After a further 2x5-min
252 washes the sections were incubated 30 min with a secondary goat anti-mouse horseradish
253 peroxidase (HRP) labelled antibody (1:200; Dako, UK) and then stained using the
254 Vector® VIP HRP substrate kit (Vector Laboratories, USA), as described by the
255 manufacturer. After the staining, the sections were washed in distilled water, mounted in
256 glycerol-gelatin and stored at 4°C.

257 258 ***Analysis of muscle capillarization and fiber type composition***

259 The capillarization of a muscle has traditionally been described by the overall
260 indices of capillary density (CD) and capillary to fiber ratio (C:F). Here, in addition to
261 conventional measures of muscle capillarization, we used the method of capillary domains,
262 as described previously (9), where the capillary domain is the area around a capillary
263 delineated by equidistant boundaries from adjacent capillaries. The capillary domain
264 provides an estimation of the capillary supply area (2). The capillary domain method also
265 gives information about the distribution of capillaries within the tissue, considers fibers that

266 lack direct contact with a capillary and allows the analysis of the capillary supply to
267 individual fibers (9).

268

269 The data processing was performed on photomicrographs of stained muscle cross-
270 sections containing at least 70 complete fibers. The coordinates of the outlines of the
271 fibers and capillary coordinates were collected using a digitizing tablet (Model MMII 1201,
272 Summagraphics Digitizers, Austin, Texas, USA). These data were then fed into a
273 computer program (AnaTis, BaLoH Software, <http://www.baloh.nl>) that calculates capillary
274 domains (9) and parameters related to muscle fiber size and composition (). For each
275 muscle biopsy, the fiber cross-sectional area (FCSA) and the numerical and areal fiber
276 type composition were calculated (55). In addition, the % connective tissue was given as
277 the % area of the region of interest not covered by contractile material. The number of
278 capillaries supplying a fiber, or the local capillary to fiber ratio (LCFR) for a given fiber, was
279 determined by the sum of the domain fractions overlapping that fiber (9). Note that the
280 LCFR of a fiber takes into account remote capillaries, thus allowing the determination of
281 the capillary supply to a fiber even when it lacks direct capillary contacts. The capillary
282 fiber density (CFD) was calculated as the LCFR divided by the fiber cross-sectional area
283 and was expressed as the number of capillaries per mm². To get information about the
284 capillary contacts per fiber, reflecting the oxygen exchange area per fiber (28), the LCFR
285 per fiber perimeter (LCFR/perimeter) was also calculated. Finally, the standard deviation of
286 log transformed domain areas (log_DSD) was used as an index for the heterogeneity of
287 capillary spacing.

288

289 ***Succinate Dehydrogenase and maximal oxygen consumption***

290 The succinate dehydrogenase (SDH) activity in individual muscle cells was
291 determined in histological sections (Fig, 2B), as described previously (9,55). Briefly, a
292 section adjacent to the capillary-stained section was incubated at 37°C in the dark for 20
293 min in 37 mM sodium phosphate buffer pH 7.6 with 74 mM sodium succinate and 0.4 mM
294 tetra-nitroblue-tetrazolium. After 20 min of incubation, the reaction was stopped with 0.01
295 N HCl (5 s) and after washing with water mounted in glycerol gelatin (9,55).
296 Photomicrographs of stained cross-sections were then captured and the SDH optical
297 density (OD) of a fiber was determined by measuring the absorbance of the final reaction
298 product using an interference filter at 660 nm (9,55). Absorbance was converted to the rate

299 of staining quantified by a calibration curve specific for each individual section created with
300 a set of filters with known OD (ImageJ software) to minimize bias related to differences in
301 lighting. The OD of the SDH stain was determined in fibers also identified in the serial
302 section stained for myosin type I and capillaries (Fig. 2B). The OD of the SDH stain is a
303 measure of the mass-specific fiber maximal oxygen consumption. For each of those fibers
304 the product of FCSA and OD SDH gives the integrated SDH, a reflection of the maximal
305 oxygen consumption of that fiber when oxygen is not rate limiting (55). The maximal
306 oxygen consumption supported by a given capillary was calculated as the sum of the
307 overlap areas times the SDH OD of that overlap area of a given domain (9), using Matrix
308 Laboratory (MatLab).

309 **Statistics**

310 All analyses were done on the data of individual fibers. During the design of the
311 study we hoped that all participants completed both trials, and thereby make full use of the
312 power of such a design allowing paired observations (and hence no 'between-factor'
313 analysis). However, not all participants completed both campaigns and to be able to
314 include all data nevertheless, we decided to treat all observations as non-paired
315 observations. Appropriateness of the wash-out period in the MEP/MTBR crossover-
316 designed study has been reported previously (11). Here we tested for possible differences
317 between the baseline data for each of the analyzed factors between the campaigns, with a
318 3-way ANOVA, with as factors muscle, fiber type and campaign, and as random variable
319 subject. This showed that baseline data did not differ significantly between the two
320 campaigns. To assess the effects of the intervention, the baseline data were pooled and a
321 3-way ANOVA performed with as factors condition (baseline, BR and NUTR), muscle (VL
322 and SOL) and fiber type (I vs II), with subjects again as random factor. Three way
323 interactions and interactions with subject were excluded. The differences between
324 baseline data, 19 days bed rest (BR) and 19 days bed rest plus diet (NUTR) on %CT,
325 numerical and areal fiber type composition, domain area, domain radius, C:F, CD, and
326 \log_{10} SD were tested with a repeated-measures ANOVA, with muscle as within-factor and
327 condition (BL, BR, NUTR) as between-factor. Regression analysis (SPSSX 19.0) of
328 individual data was performed to analyze relationships between selected variables.
329 Differences and relationships were considered significant at $P < 0.05$. All P-values were
330 Bonferroni corrected to adjust for multiple comparisons.

331

332

333 Results

334 **Maximal voluntary force (MVC), fatigue resistance and fiber type composition**

336 Knee extensor MVC was significantly reduced after BR ($P = 0.021$; Fig. 3A), but no
337 significant changes were seen in plantar flexor MVC. There were no significant differences
338 between NUTR and BR for either knee extensor or plantar flexor MVC (Fig. 3A), or muscle
339 fatigue resistance of thigh muscles (Fig. 3B). The impact of BR or NUTR on myosin heavy
340 chain composition and fiber size (FCSA) has been presented previously (8). Here, we
341 show that the % connective tissue did not differ significantly between the SOL and VL and
342 was not significantly affected by BR or NUTR (Table 2). The SOL contained a larger
343 number % and areal % of type I fibers than the VL, irrespective of condition (Table 2; $P <$
344 0.001). Neither BR nor NUTR induced a significant change in the fiber type proportions.

346 **Oxidative capacity**

347 To investigate whether the BR and whey protein + KHCO_3 intervention (NUTR) may
348 affect fiber oxidative capacity, we quantified the succinate dehydrogenase (SDH) activity of
349 muscle fibers (Fig. 4). The specific SDH activity (reflected by the OD) was higher in type I
350 than type II fibers (Fig. 4A; $P < 0.001$) in both SOL and VL. In addition, the integrated
351 SDH, reflecting the maximal oxygen consumption of a fiber, was higher in fibers of the
352 SOL than the VL ($P = 0.046$). BR did result in a reduced fiber oxidative capacity in type I
353 and type II fibers in both muscles, both in terms of specific SDH activity (Fig. 4A) and
354 integrated SDH activity (Fig. 4B; $P < 0.01$). WP + KHCO_3 attenuated the BR-induced
355 reduction in specific SDH activity in both VL and SOL, as reflected by higher SDH activities
356 in the NUTR than the BR condition (Fig. 4A; $P < 0.01$). This was also reflected by an
357 attenuated reduction in integrated SDH in the SOL ($P < 0.01$), but not in the VL, of the
358 NUTR than the BR condition (Fig. 4B). These changes in integrated SDH activity in the VL
359 were mirrored by the bed rest-induced reductions in whole body $\text{VO}_{2\text{max}}$ ($P = 0.042$) that
360 was not attenuated by the nutritional intervention (Fig. 3C).

362 **Overall capillarization**

363 The CD (Table 2) and C:F (Table 2) were higher in the SOL than the VL ($P < 0.01$).
364 The capillary domain area was smaller in the SOL than the VL (Table 2; $P < 0.001$), but
365 there was no significant difference in the heterogeneity of capillary spacing (Los_DSD)
366 between muscles (Table 2). Neither BR nor NUTR did significantly affect the CD, C:F,

367 Los_DSD or domain area ([Table 2](#)). Noteworthy, not only the maximal fiber oxygen
368 consumption, indicated by a reduced integrated SDH in fibers of both muscles after BR
369 (Fig. 4B), but also the maximal oxygen consumption supported by a capillary (MO_{2max}),
370 (Table 2, BR vs BL; $P < 0.001$), was attenuated by NUTR intervention ([Table 2](#); $P <$
371 0.001). There was a non-significant trend ($P=0.057$) for a difference between the MO_{2max} at
372 baseline between the two campaigns, suggesting a possible carry-over effect of bed rest
373 or nutritional intervention, or a seasonal effect on MO_{2max}.

374

375 **Fiber specific capillary supply**

376 The local capillary to fiber ratio (LCFR; Fig. [5A](#)) and the capillary fiber density (CFD;
377 Fig. [5B](#)), were higher in SOL than in the VL ($P < 0.01$). The LCFR of type II was higher
378 than that of type I fibers in both muscles ($P < 0.001$), while type I fibers had a higher CFD
379 than type II fibers ($P < 0.001$). The LCFR/perimeter ratio was larger in type I than type II
380 fibers ($P = 0.012$), and it was larger for fibers in the SOL than the VL ($P < 0.001$).
381 Irrespective of fiber type, NUTR, but not BR, was associated with a reduction in LCFR in
382 the SOL muscle ($P < 0.001$; [Fig. 5A](#)). BR did induce an increase in CFD in both muscles
383 ($P < 0.001$). We found that the fibers became less circular during BR, as indicated by an
384 increased perimeter:FCSA ratio ([Fig. 6](#); $P < 0.001$) and this was even more pronounced in
385 the SOL, but not in the VL after NUTR ([Fig. 6](#); $P < 0.001$). The LCFR/perimeter ratio was
386 lower in BL than in BR and NUTR ($P < 0.001$; [Fig. 5C](#)).

387

388 **Discussion**

389 The main observations of the present study are that 19 days of bed rest significantly
390 reduced the fiber oxidative capacity, irrespective of fiber type, in both the soleus and
391 vastus lateralis muscle. This was associated with a reduction in the whole body maximal
392 oxygen uptake (VO_{2max}). There was no significant loss of capillaries, resulting in a denser
393 capillary network than expected for the fiber size and fiber oxidative capacity, suggesting a
394 superfluous capillarization. The reduction in fiber oxidative capacity was to some extent
395 prevented by a WP + KHCO₃-enriched diet.

396

397 Bed rest has been widely used as a model to mimic the effects of microgravity and
398 unloading, and to test the efficacy of exercise, nutritional and pharmacological
399 interventions to prevent or attenuate unloading-induced muscle wasting and weakness

400 (43). Previously, our group showed that after 19 days of bed rest there was no marked
401 atrophy in either the SOL or VL muscle nor a significant change in myosin heavy chain
402 composition (8), corresponding with the absence of significant changes in fiber type
403 composition observed here (Table 2). The reduction in maximal voluntary isometric force
404 (MVC) of the knee extensor muscles we observed (Fig.3) can thus not be attributable to
405 atrophy after 19 days, but may be mainly due, as suggested by others, to a decreased
406 ability to activate motor units (7, 33) and/or to a disproportionate loss of thin filaments (46).

407

408 ***The effect of bed rest on skeletal muscle morphology***

409

410 Capillarization

411 During unloading and bed rest, there is little contractile activity and few, if any,
412 periods of elevated muscle blood flow. Since both mechanical strains and shear stress are
413 important for angiogenesis and the maintenance of the capillary bed (31), and there is
414 reportedly, a close correlation between the fiber oxidative capacity of a fiber and its
415 capillary supply (5), one might expect that bed rest is associated with capillary rarefaction.
416 In line with this, it has been observed that the capillary to fiber ratio, was reduced in the
417 human soleus, but not in the vastus lateralis muscle, after 90 days bed rest and was
418 maintained by exercise during bed rest (47). We, however, did not observe reductions in
419 the number of capillaries per fiber (Table 2) or capillary density (Table 2) after 19 days bed
420 rest in the soleus or vastus lateralis muscle. Others also found no atrophy or changes in
421 capillary density in the vastus lateralis muscle after 5 weeks bed rest (34). In another study
422 with 6 weeks bed rest, the decrease in FCSA in the VL was associated with a maintained
423 capillary density (22), suggesting that in the long-term capillary loss may occur during bed
424 rest that is proportional to the decrease in fiber size. Importantly, in our study, bed rest did
425 not significantly affect the capillary spacing within the muscle (Table 2), a factor that can
426 have a significant impact on local tissue oxygenation (18,26).

427

428 *Oxidative capacity*

429 The bed rest-induced reduction in the oxidative capacity of the fibers, indicative for
430 a decreased mitochondrial volume density, was independent of muscle or fiber type (Fig.
431 4) and was accompanied by a reduction in whole body VO_{2max} . A reduction in
432 mitochondrial volume density and mitochondrial enzyme activities has also been observed
433 in the vastus lateralis muscle after 37 days bed rest (22), indicating that even after 37 days

434 the loss of mitochondria is proportionally larger than the atrophy. In denervated rat soleus
435 muscles something similar was observed, where initially the loss of mitochondria was
436 disproportionately more than fiber atrophy (19). Our observations were also consistent with
437 an earlier report on the effects of 4 weeks unilateral lower limb suspension (7), where
438 unloading did reduce work and oxidative capacity of skeletal muscle without changes in
439 capillary to fiber ratio, fiber type composition or FCSA of the vastus lateralis muscle. Part
440 of the impairment of peripheral gas exchange (O_2 transfer and/or utilization) and maximal
441 oxygen consumption (VO_{2max}) after medium- and long-term bed rest may thus not only be
442 attributable to cardiovascular "deconditioning" and muscle atrophy (13,22), but also to a
443 reduced capacity for oxidative metabolism of the disused muscles (32).

444
445 Because of the unaltered morphology of the capillary network and the reduction of
446 the fiber oxidative capacity, the maximal oxygen consumption supported by a capillary
447 (Table 2) was significantly reduced after bed rest. Thus, in terms of oxidative capacity, the
448 muscle has an 'excessive' capillary supply; something also observed in old rat muscles
449 without significant fiber atrophy (27) and in atrophied denervated muscles (19). A similar
450 situation occurs after cessation of a training program where the decrease in muscle
451 oxidative capacity develops faster than the decrease in muscle capillarization and whole-
452 body VO_{2max} (28). These observations suggest that reductions in mitochondrial volume
453 may precede capillary rarefaction and thus might represent one of the early hallmarks of
454 muscle adaptation to disuse.

455
456 Previously we suggested that the increased ability of older people to sustain a 50%
457 MVC (37) is more a reflection of their slower contractile properties or fiber type
458 composition than changes in oxidative capacity, where more economical type I fibers (53)
459 are better able to sustain a prolonged isometric contraction than type II fibers. Similarly,
460 the absence of a significant change in fatigue resistance observed in our study in the face
461 of reductions in fiber oxidative capacity, could thus be explicable by the absence of
462 significant changes in fiber type composition.

463
464 It remains unclear how unloading would result in a reduction in mitochondrial
465 content. It is possible that a disuse-induced increase in the generation of reactive oxygen
466 species (ROS) contributes to impaired mitochondrial homeostasis and biogenesis (45). In
467 spaceflight or bed rest, the transition from the standing weight-bearing position to

468 microgravity or a supine position may affect the cell tensegrity, as several *in vitro* and *in*
469 *vivo* (murine) studies indicated that gravitational changes caused cytoskeleton
470 disarrangement (15) that in turn may be responsible for aberrant mitochondrial distribution
471 and impair respiratory function (41). This has been confirmed in other models of disuse-
472 induced muscle atrophy, such as denervation-induced atrophy, where changes in inter-
473 myofibrillar mitochondrial content or in mitochondrial distribution are paralleled by
474 increased generation of ROS during active respiration, altered fiber metabolism and
475 impaired muscle cell survival (6). Disarrangement of the cytoskeleton may also contribute
476 to the increase in the 'perimeter:FCSA' ratio, as we observed in bed rest (Fig. 6), indicating
477 that the fibers became more angular. The changes in cytoskeletal components, such as
478 microtubules, may therefore explain the effects of the lack of weight-bearing on the
479 distribution of mitochondria, shape of the fiber and other cellular functions (56).

480 481 ***The effects of whey protein and KHCO₃ on oxidative capacity***

482 Dietary amino-acids and protein supplements have been suggested to attenuate the
483 loss of muscle mass after space flight, aging and bed rest, possibly by stimulating anabolic
484 signaling pathways and reducing proteolysis (3,52). To date, there is little information on
485 the effectiveness of alkaline whey protein-enriched diet to attenuate the bed rest-induced
486 reduction in muscle oxidative capacity. Here we found that a whey protein +KHCO₃-
487 enriched diet attenuated the bed rest-induced reduction in fiber oxidative capacity (Fig. 4),
488 irrespective of muscle or fiber type.

489 It has been reported that whey protein supplementation improved mitochondrial
490 activity in mouse brain and liver by reducing oxidative stress and stimulating mitochondrial
491 biogenesis *via* transcriptional activation of the peroxisome proliferator-activated receptor
492 gamma coactivator 1-alpha (PGC-1 α) (51). A similar action of whey proteins on
493 mitochondria may occur in muscle, as a reduced expression of PGC-1 α plays a major role
494 in disuse atrophy, while its overexpression prevents activation of catabolic systems and
495 disuse atrophy (12). It is likely that the attenuated bed rest-induced reduction in muscle
496 fiber oxidative capacity by alkaline whey protein was due to an increased expression of
497 PGC-1 α or other proteins involved in mitochondrial biogenesis.

498
499 While, the whey protein-enriched diet attenuated the bed rest-induced reduction in
500 fiber oxidative capacity (in terms of oxidative capacity per gram of muscle), it did not result
501 in an attenuated reduction of whole body VO_{2max} (Fig. 3B). Something similar was also

502 found in a 60-day bed rest study in women (35,48), where the protein-intervention without
503 exercise proved ineffective to attenuate the bed rest-induced reduction in VO_{2max} (48). The
504 discrepancy between the attenuated reduction in fiber oxidative capacity and no such
505 effect of whey protein-enriched diet on whole body VO_{2max} may be explained by the fact
506 that VO_{2max} is primarily determined by the cardiovascular system rather than by the
507 oxidative capacity of the working muscles (22, 49).

508
509 We cannot exclude that $KHCO_3$ itself may have contributed to the attenuated loss of
510 fiber oxidative capacity during bed rest. Bicarbonate salts have been demonstrated to
511 improve muscle strength and endurance, primarily by increasing the buffering capacity of
512 the extracellular fluid and hydrogen ion efflux from muscle cells (16). Extracellular acidosis
513 slows down proton efflux from mitochondria, which may affect fiber oxidative capacity
514 (16,30). Thus, one would expect that by removing intracellular proton excess, $KHCO_3$ may
515 have contributed to improved fiber oxidative capacity during bed rest. However, we are
516 lacking specific information whether oral whey protein and $KHCO_3$ intake do change proton
517 concentrations in muscle tissue. Finally, it is important to consider that during bed rest the
518 moderate acidogenic dietary load may have acted synergistically with disuse to negatively
519 impact on mitochondrial function and content, as observed in the kidney (40).

521 *Perspective*

522 There is a large interest to develop nutritional interventions to attenuate bed rest-
523 induced muscle wasting and reduction in muscle oxidative capacity in the clinical setting.
524 This is particularly relevant for older adults or sarcopenic individuals as they may have
525 slower recovery to the pre-inactivity muscle condition than young adults (44). Our data
526 suggest that a whey protein plus $KHCO_3$ -enriched diet attenuates the decrements in
527 muscle oxidative capacity and may well enhance the benefits of integrated physical
528 therapy to counteract the loss of muscle oxidative capacity during hospitalization not only
529 in the young (35,48), but also in the older (21) patient.

531 **Conclusion**

532 In conclusion, medium-term bed rest, even without overt muscle fiber atrophy,
533 induces a reduction in the fiber oxidative capacity of the soleus and vastus lateralis
534 muscle. As the capillary bed was not significantly affected, there was an excessive

535 capillary supply to the muscle during bed rest. Part of the reduction in bed rest-induced
536 oxidative capacity was prevented by supplementation with whey protein plus KHCO_3 .

537

538

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540 Metropolitan University, Manchester, UK. Preparations of muscle cryosections were done
541 at the Charité Center of Space Medicine Berlin (ZWMB), Berlin, Germany. MTBR/MEP
542 bed rest study was performed at the Institute of Aerospace Medicine, German Aerospace
543 Center DLR, Cologne, Germany. H.D.: conceived and designed the experiments. A.B.
544 performed the experiments. H.D. and A.B. analyzed and interpreted the data. A.B. wrote
545 the first draft of the manuscript. M.S. and D.B. prepared the muscle cryosections and
546 helped in muscle sampling. J.B. conducted the organization of the MTBR/MEP study. E.M.
547 collected the torque data, the body $\text{VO}_{2\text{max}}$ and its related parameters. J.R. and B.G. took
548 the muscle biopsies and over-saw the medical care of the volunteers. M.H.Y. set MatLab
549 programming. A.B. and H.D. wrote the final version of the manuscript. All authors
550 discussed the results, gave input to writing of manuscript, revising it critically and approved
551 the final version of the manuscript.

552

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557

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560

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562

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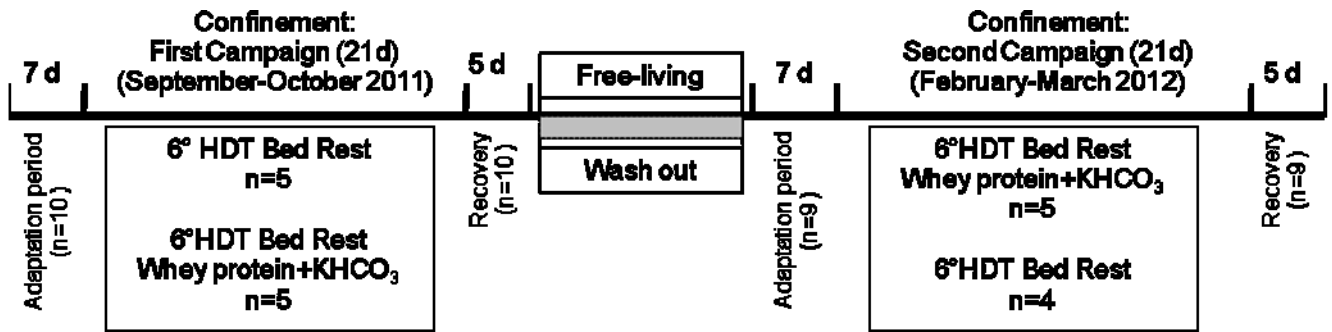
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739 **Figures and Figure legends:**

740 **Fig. 1**

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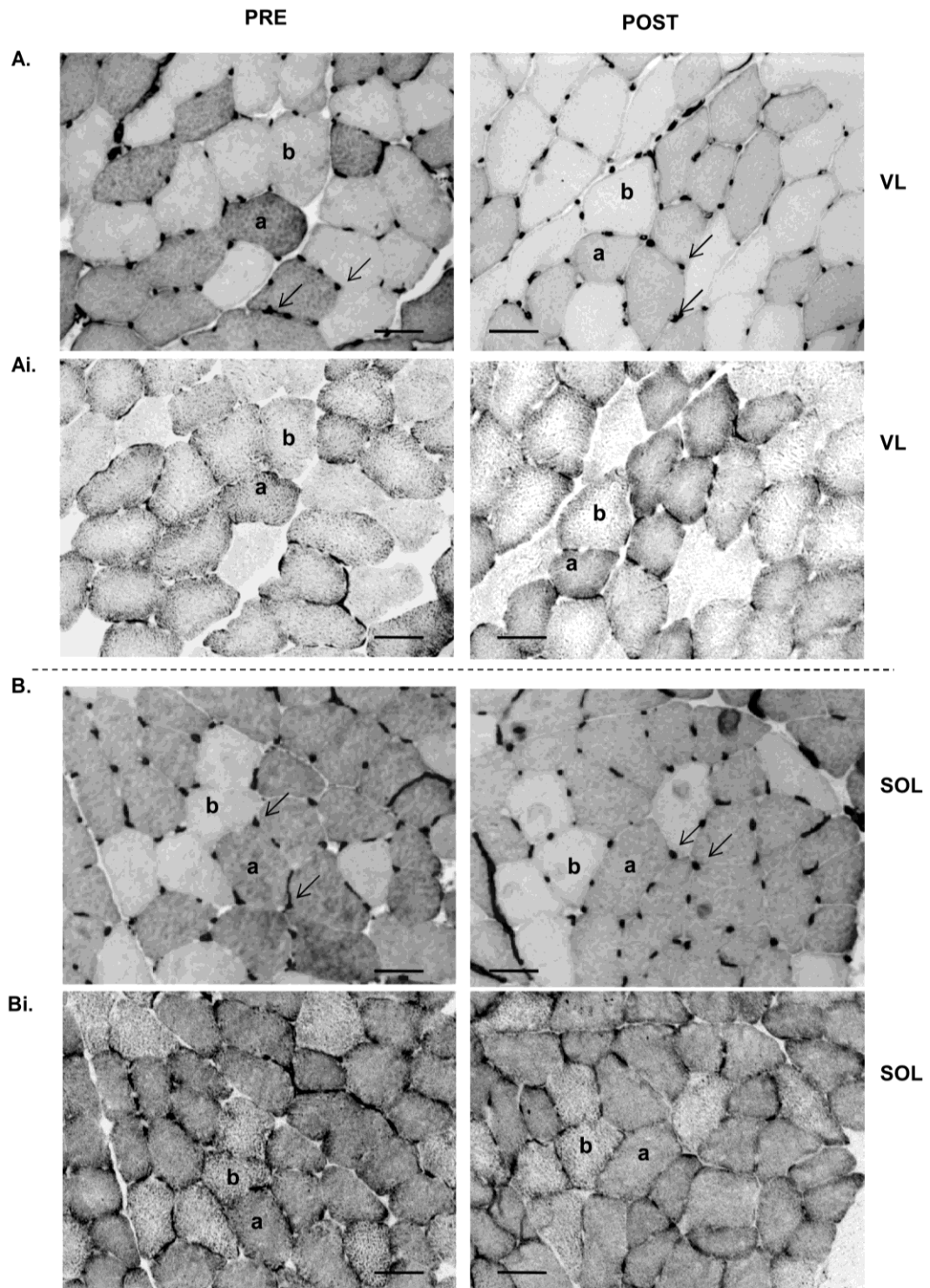
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744 **Fig.1** Schematic diagram showing the crossover study design of the bed rest study. HDT,
745 head down tilt bed rest.

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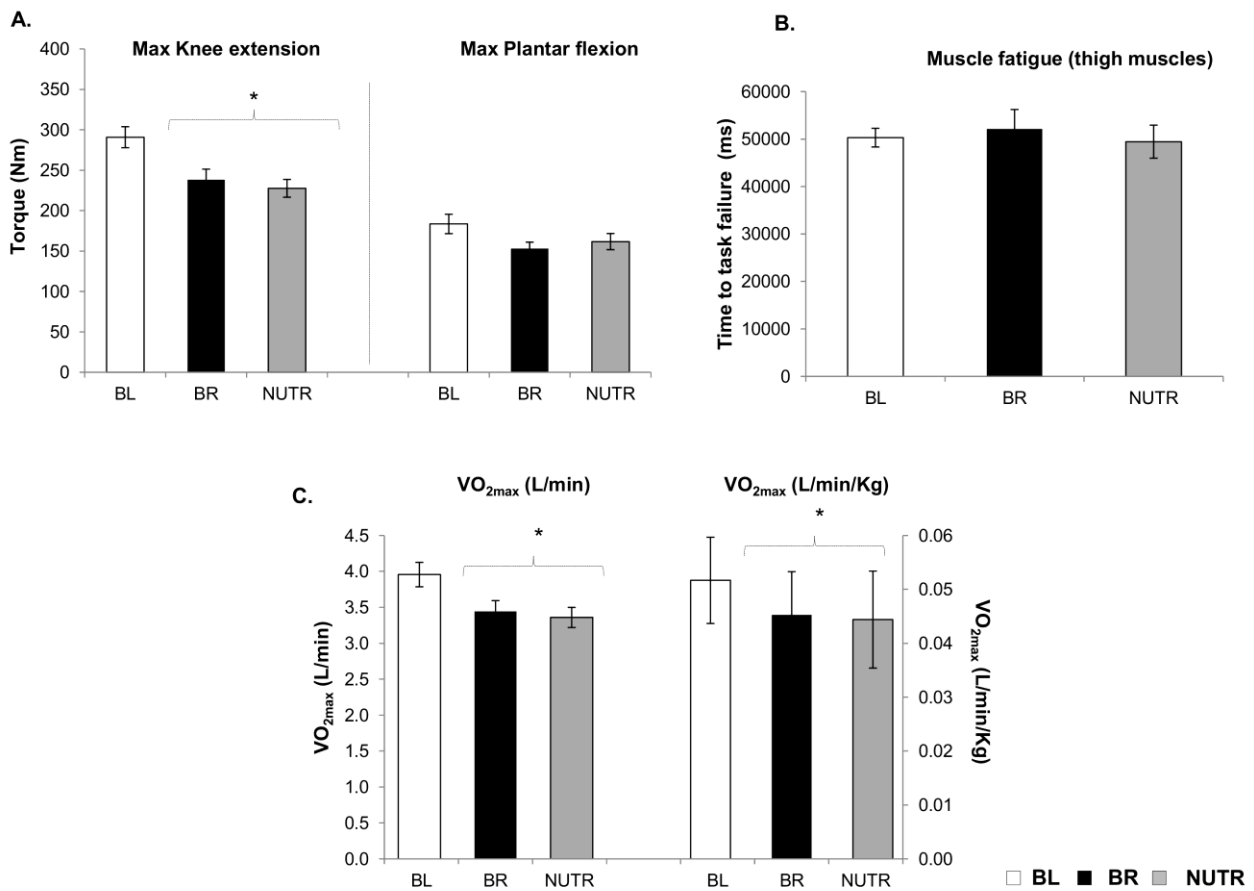
749 **Fig. 2.** Representative micrographs showing immunohistochemical co-staining with anti-
 750 myosin type I and lectin to identify type I (darker stained; example indicated by **a**) and type
 751 II fibers (indicated by **b**) and to visualize capillaries (some indicated by arrows) in frozen

752 muscle cross-sections of vastus lateralis (VL; **A**) and soleus (SOL; **B**) muscles, before
753 (PRE) and after (POST) 19 days of bed rest. **Ai** and **Bi**: Representative micrographs
754 showing enzyme histochemical staining for succinate dehydrogenase (SDH) activity in the
755 VL (**Ai**) and in SOL (**Bi**) of the same participants before (PRE) and after (POST) 19 days of
756 bed rest. Scale Bar, 50 μ m.

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758

759 **Fig. 3.**



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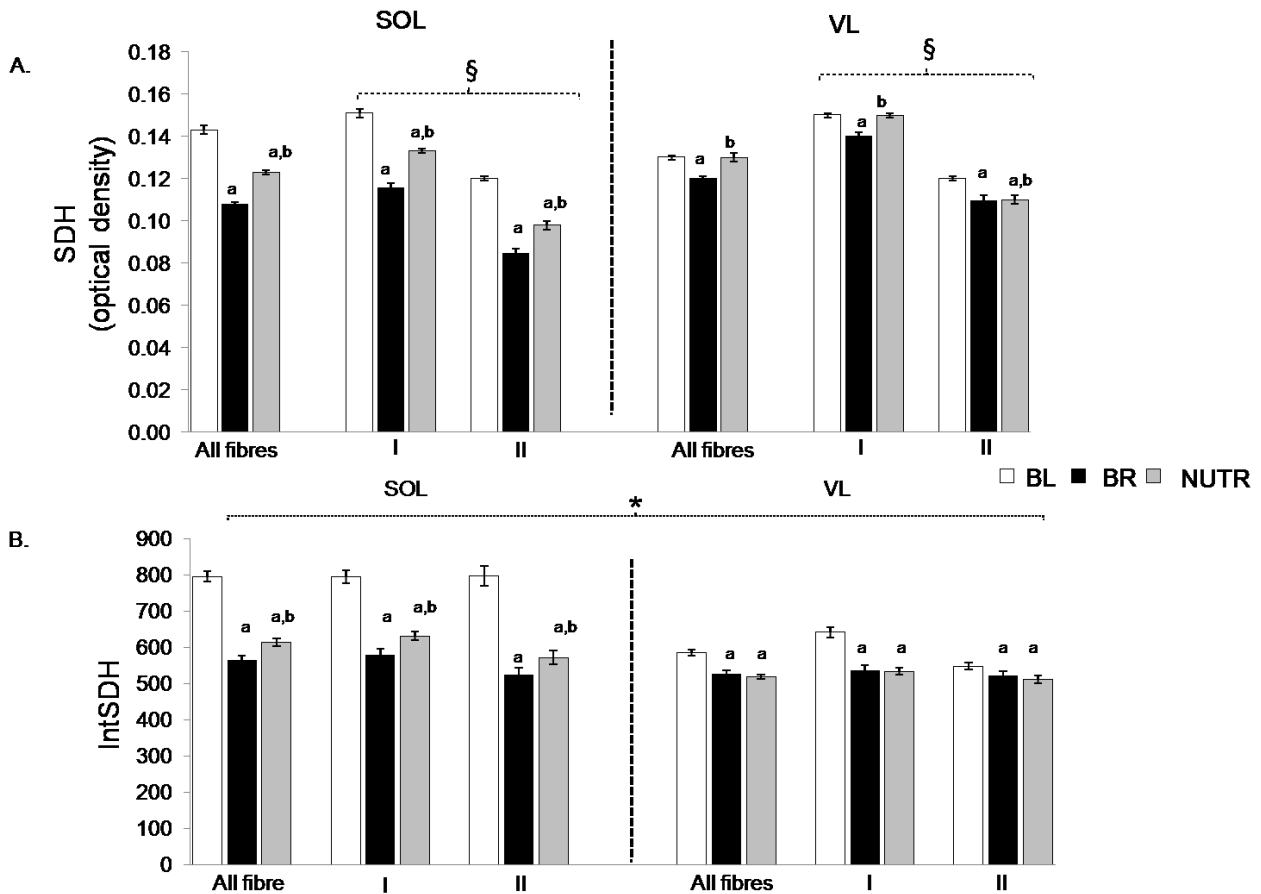
761 **Fig. 3.** The effect of 19 days of bed rest with or without WP+KHCO₃ supplementation on
762 (A) maximal voluntary contraction of knee extensors and plantar flexors of the left leg, (B)
763 muscle fatigue of thigh muscles and (C) whole body peak oxygen uptake (VO_{2max}). In C,
764 secondary axis: peak oxygen uptake normalized per body mass. BL: baseline; BR: bed-
765 rest plus standardized diet; NUTR: bed-rest plus WP+KHCO₃-enriched diet. Data are
766 expressed as mean ± SEM.

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768 In A.: *Significantly different from the corresponding value before bed rest ($P = 0.021$); in
C.: *Significantly different from the corresponding value before bed rest ($P = 0.042$).

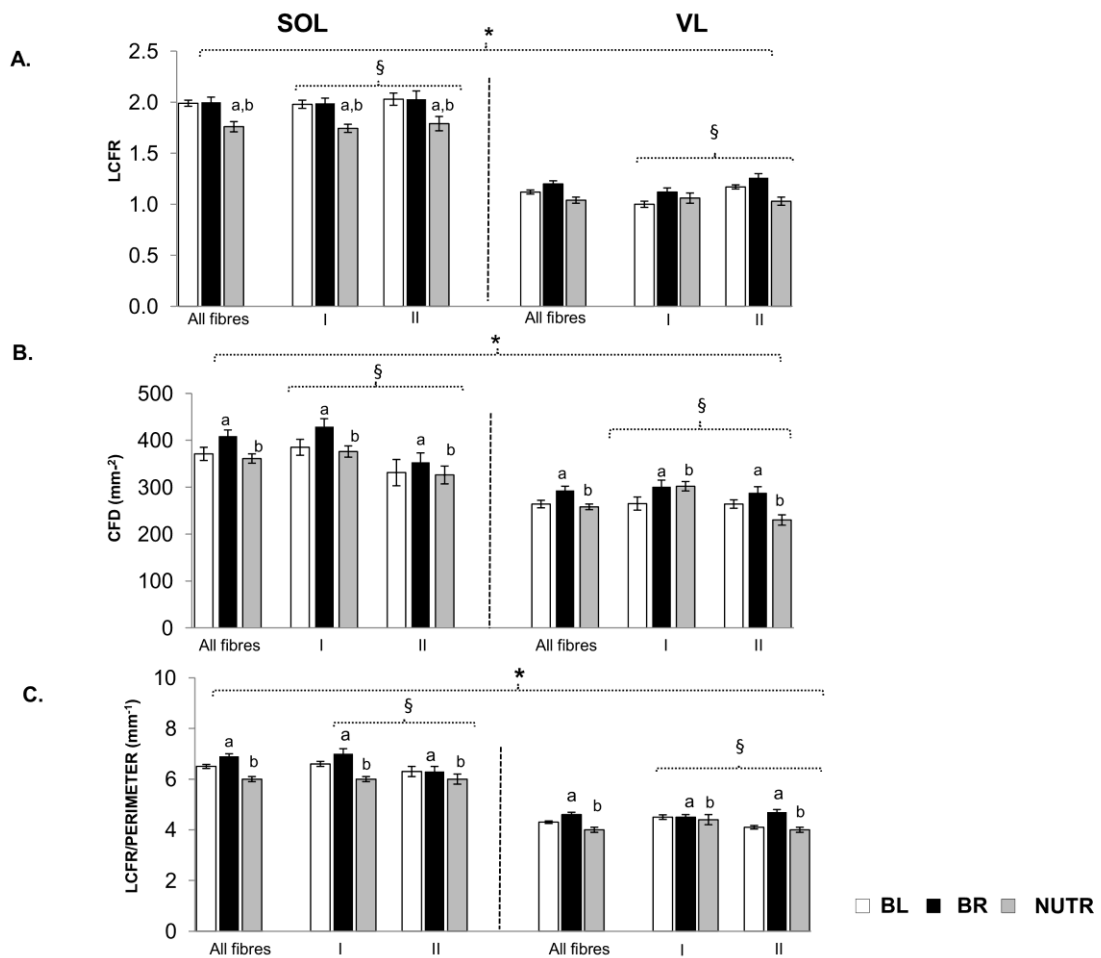
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Fig. 4.



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Fig. 4. The effect of 19 days of bed rest with or without WP+KHCO₃ supplementation on (A) specific succinate dehydrogenase (SDH) and (B) integrated SDH activity in the soleus (SOL) and vastus lateralis (VL) muscle. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed-rest plus WP+KHCO₃ supplementation. Data are expressed as mean \pm SEM. *: significant difference between muscles at $P = 0.046$; §: significant difference between fiber types at $P < 0.001$. a: different from BL; b: different from BR at $P < 0.01$.



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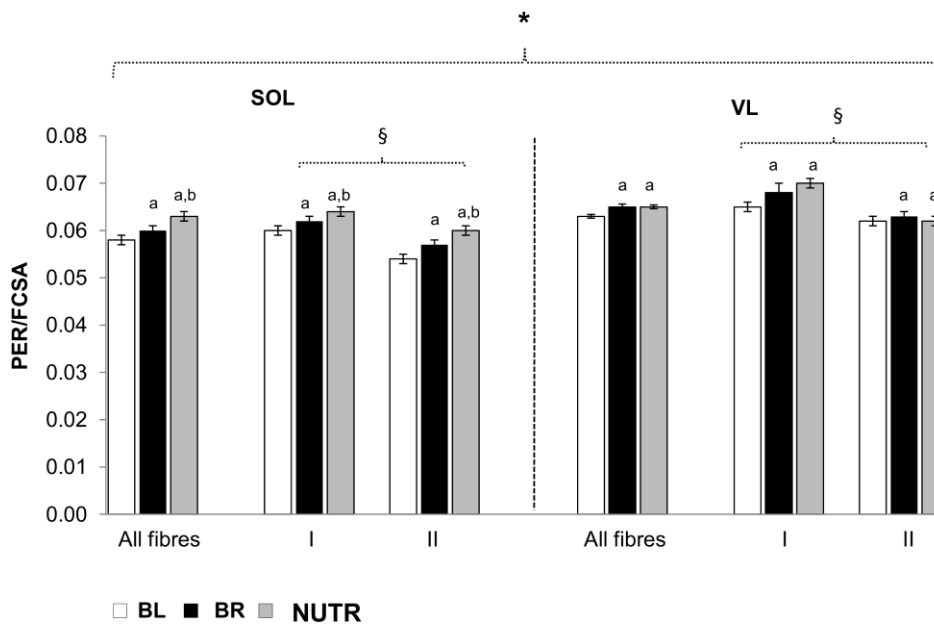
786

787 **Fig. 5.** The effect of 19 days of bed rest with or without WP+KHCO₃ supplementation on
 788 the (A) local capillary to fiber ratio (LCFR; sum of domain fractions overlapping a fiber); (B)
 789 capillary fiber density (CFD) and (C) LCFR/perimeter ratio in the soleus (SOL and vastus
 790 lateralis (VL) muscle. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed rest
 791 plus WP+KHCO₃. In A and B: *: significant difference between muscles at $P < 0.001$; §:
 792 significant difference between fiber types at $P < 0.001$. In C: *: significant difference
 793 between muscles at $P < 0.001$; §: significant difference between fiber types at $P = 0.012$.
 794 In all panels: a: different from BL at $P < 0.001$. b: different from BR at $P < 0.001$. There
 795 were no significant interactions. Data are expressed as mean \pm SEM.

796

797

798 **Fig. 6.**



799

800 **Fig. 6.** The effect of 19 days of bed rest with or without WP+KHCO₃ supplementation on
801 the perimeter:FCSA ratio. BL: baseline; BR: bed-rest plus standardized diet; NUTR: bed
802 rest plus WP+KHCO₃. *: significant difference between the two muscles at $P < 0.001$; §:
803 significant difference between fiber types at $P < 0.001$; a: different from BL at $P < 0.001$. b:
804 different from BR at $P < 0.001$. Data are expressed as mean ± SEM.

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807 **Tables:**

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Table 1. Anthropometric characteristics of participants

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Participants	1st campaign n = 10	2nd campaign n = 9
Age (years)	31.6 ± 6.2	31.5 ± 6.2
Height (m)	1.80 ± 0.05	1.80 ± 0.06
Mass (kg)	76.1 ± 5.4	77.7 ± 4.8
BMI (kg·m⁻²)	23.4 ± 1.6	24.0 ± 1.5

810

811 Cross-over design: BMI: Body Mass Index; more details see www.clinicaltrials.gov
812 Identifier NCT01655979 (See also 10,13).

813 **Table 2: Skeletal muscle morphometric parameters and capillary oxygen supply areas.**

		% CT	% n. type I	% n. type II	% Area type I	% Area type II	Capillary Domain Area (μm^2)	Capillary Domain Radius (μm)	CD (mm^{-2})	Log _D SD	C:F	MO _{2max} ($\mu\text{L}\cdot\text{mm}^{-1}\cdot\text{min}^{-1}$)
SOL	BL	7.4±0.6	75±4	25±4	71±5	29±5	2912±166	30±1	352±21	0.187±0.007	2.50±0.23	213±3
	BR	6.4±0.7	75±6	25±6	71±7	29±7	2603±204	29±1	378±27	0.175±0.007	2.25±0.19	163±3
	NUTR	9.1±1.1	70±5	30±5	71±6	29±6	2859±146	30±1	349±18	0.194±0.009	2.13±0.19	192±3
VL	BL	10.8±1.5	36±4	64±4	31±4	69±4	3818±178	35±1	261±13	0.188±0.009	1.18±0.12	273±4
	BR	11.8±1.0	40±3	60±3	37±4	63±4	3655±212	34±1	264±18	0.195±0.013	1.26±0.14	237±6
	NUTR	11.2±1.6	35±3	63±3	28±5	72±5	4271±323	36±1	236±19	0.215±0.014	1.10±0.19	293±6
Muscle		ns	P < 0.001	P < 0.001	ns	ns	P < 0.001	P < 0.001	P < 0.01	ns	P < 0.01	P < 0.01
Condition		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	P < 0.001
Interaction		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	P < 0.001

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815 **Table 2: Skeletal muscle morphometric parameters and global capillarisation parameters.** The table shows the numerical (%n)
816 and areal (%Area) fiber type composition, connective tissue content (CT%), oxygen supply area (capillary domain area and capillary
817 domain radius), the numerical capillary density (CD), capillary to fiber ratio (C:F), the heterogeneity of capillary spacing (Log_DSD;
818 logarithmic standard deviation of the domain area) and the maximal oxygen consumption supported by a capillary (MO_{2max}) in the
819 soleus (SOL) and vastus lateralis (VL) muscles, at baseline (BL) and after 19 days bed rest without (BR) or with (NUTR) WP+KHCO₃
820 enriched diet. Data are expressed as mean ± SEM.