

1
2 **Evaluation of the functional efficacy of an antioxidative probiotic in healthy**
3 **volunteers**

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5 Epp Songisepp^{1†}, Jaak Kals^{2†}, Tiiu Kullisaar^{2†}, Reet Mändar^{1†}, Pirje Hütt^{1†}, Mihkel Zilmer^{2†},
6 Marika Mikelsaar^{1*}

7
8 ¹Department of Microbiology, University of Tartu, 50411 Tartu, Estonia

9 ²Department of Biochemistry, University of Tartu, 50411 Tartu, Estonia

10

11 *Corresponding author

12 †These authors contributed equally to this work

13

14

15 ES: epp.songisepp@ut.ee

16 JK: jaak.kals@ut.ee

17 TK: tiiu.kullisaar@ut.ee

18 RM: reet.mandar@ut.ee

19 PH: pirje.hutt@ut.ee

20 MZ: mihkel.zilmer@ut.ee

21 MM: marika.mikelsaar@ut.ee

22

22 **Abstract**

23 **Background:** In persons without clinical symptom it is difficult to assess an impact of
24 probiotics regarding its effect on health. We evaluated the functional efficacy of the probiotic
25 *Lactobacillus fermentum* ME-3 in healthy volunteers by measuring the influence of two
26 different formulations on intestinal lactoflora, fecal recovery of the probiotic strain and
27 oxidative stress markers of blood and urine after 3 weeks consumption.

28 **Methods:** Two studies with healthy adults were performed; altogether 45 randomly allocated
29 persons consumed either the ME-3 capsules/placebo or fermented goat milk/goat milk in a
30 daily of dose 9.2 to 11.8 log CFU respectively for 3 weeks. The fecal lactoflora composition,
31 fecal ME-3 recovery, effect of the consumption on intestinal lactoflora, and oxidative stress
32 markers of blood (total antioxidative activity; total antioxidative status and glutathione red-ox
33 ratio) and urine (8-isoprostanes) were measured.

34 **Results:** ME-3 was well tolerated and a significant increase in total fecal lactobacilli yet no
35 predominance of ME-3 was detected in all study groups. Fecal recovery was documented by
36 molecular methods only in fermented milk group, however the significant improvement of
37 blood TAA and TAS indices was seen in case of both formulations, yet glutathione re-ox ratio
38 and urine isoprostanes values decreased only in case of fermented by ME-3 goat milk.

39 **Conclusions:** The functional efficacy of both consumed formulations of an antioxidative
40 probiotic *L. fermentum* ME-3 is proved by the increase of the intestinal lactobacilli counts
41 providing putative defense gainst enteral infections and by reduction of the oxidative stress
42 indices of blood and urine of healthy volunteers. In non-diseased host the probiotic health
43 claims can be assessed by improvement of some measurable laboratory indices of well-
44 established physiological functions of host, e.g. markers of antioxidative defense system.

45

45 **Background**

46 Probiotics are defined as live microbial food supplements, which beneficially influence
47 human health [1;2]. Widely accepted probiotics contain different lactic acid producing
48 bacteria of human origin: bifidobacteria, lactobacilli or enterococci. Nowadays the concept of
49 functional foods, incl. probiotic food and dietary supplements implies to their ability to
50 beneficially influence body functions in order to improve the state of well-being and health
51 and reduce the risk of disease [2,3]. The important areas of human physiology that are
52 relevant to functional food science according ILSI and FUFOSE (The European Commission
53 Concerted Action on Functional Food Science in Europe) are besides others, the modulation
54 of basic metabolic processes and defense against high-grade oxidative stress [4, 5].

55

56 Human nutrition is clearly associated with oxidative metabolism, which beside production of
57 energy is involved in a number of vital functions of the host. For example, under
58 physiological conditions the reactive species (including peroxy radicals, nitric oxide radical,
59 superoxide anion) figure a crucial role in primary immune defense of the human body by
60 phagocytic cells against harmful microorganisms [6, 7]. On the other hand, a prolonged
61 excess of reactive species is highly damaging for the host biomolecules and cells, resulting in
62 dysbalance of the functional antioxidative network of the organism and leading to substantial
63 escalation of pathological inflammation. Recently Petrof *et al.* showed *in vitro* that some
64 probiotics protect intestinal epithelial cells against oxidant stress also through inducing heat
65 shock proteins known as cytoprotectors against inflammatory cell-derived oxidants [8].

66 By our knowledge, no systematic studies have been performed to approve the functional
67 efficacy of different formulations of probiotic on the antioxidative defense system of a
68 healthy human. In our previous study *Lactobacillus fermentum* ME-3 (DSM 14241) [9-11],
69 expressed strong antimicrobial activity against Gram-positive and Gram-negative entero- and

70 uropathogens [12, 13]. The cells and cell lysate of *L. fermentum* ME-3 possessed substantial
71 antioxidative potency [14]. In an animal experiment ME-3 suppressed the excessive oxidative
72 stress reaction caused by *Salmonella* infection in intestinal mucosa and thus improved the gut
73 mucosal antioxidative status [15]. The antioxidative effect of *L. fermentum* ME-3 on human
74 body oxidative stress markers was confirmed by our pilot study with fermented goat milk
75 [16].

76

77 The aim present study was to evaluate the functional efficacy of the probiotic strain *L.*
78 *fermentum* ME-3 in the human GIT of healthy volunteers. The fecal recovery, effect of two
79 different formulations on total fecal lactoflora and oxidative stress markers of blood and urine
80 were compared after 3 weeks consumption.

81

82 **Methods**

83 **Formulations**

84 The efficacy of two different formulations (experimental fermented goat milk and probiotic
85 capsules) on the human body oxidative stress markers was evaluated.

86 *Lactobacillus fermentum* ME-3, a probiotic strain of healthy human intestinal origin (17), has
87 been identified by biochemical and molecular methods [9]. The patent application has been
88 submitted to the Estonian Patent Agency (Application No. 0356/01PV) as well as to the
89 International Bureau of WIPO (Application No. WO03002131) [11]. *L. fermentum* ME-3 was
90 used as freeze-dried powder in capsulated form and in fermented milk.

91 *Capsules.* Gelatine coated capsules were manufactured by the Tallinn Pharmaceutical
92 Company. The freshly prepared probiotic capsules contained 9.0 log CFU of *L. fermentum*
93 ME-3 per capsule in addition to 250 mg of saccharose and microcellulose. Identical placebo
94 capsules contained only saccharose and microcellulose. All capsules were stored at +4°C.

95 *Survival of ME-3 in capsule.* Survival of ME-3 in capsule was monitored during 12 months at
96 +4°C. The content of one capsule was dissolved aseptically in 2 ml of 0.9% NaCl solution.
97 The suspension was vortexed, serially diluted and seeded 0.1 ml on MRS agar medium
98 (OXOID, U.K.) and incubated 48 hours at 37°C microaerobically (10% CO₂). The number of
99 colonies was counted and the viable cell count in capsule was calculated.

100 *Experimental fermented milk.* Three different lots of experimental fermented goat milk was
101 prepared for the 3-week trial with healthy volunteers in order to establish the health effects of
102 ME-3 consumption. The study group was supplied with fresh product once a week.

103 Experimental fermented milk was prepared as described previously [16] by combining the
104 probiotic strain with two supportive lactobacilli cultures *L. plantarum* LB-4 and *L. buchneri*
105 S-15. *L. buchneri* strain S1-5 decreased the specific taste of the goat milk. *L. plantarum* LB-4
106 was included as a strong producer of exopolysaccharides, which gives the fermented milk a
107 cream-like consistence and delightful acidity. The goat milk was inoculated with 2% mixture
108 of *Lactobacillus* strains and incubated at 37°C for 24 hours. The product, ready to use, was
109 cooled and stored at 4°C.

110 *Survival of L. fermentum ME-3 in fermented goat milk.* To measure the viable cell count of
111 ME-3 in fermented goat milk, samples were taken at the end of fermentation (before cooling
112 the product), and after 24h, 32h, 48h and 7 days from the preparation, when the product was
113 stored at 4°C. The amount of 0.5 ml of the fermented milk was serially diluted in saline and
114 plated on MRS agar medium and incubated for 48 h at 37°C in microaerobic conditions.

115

116 **Design of human volunteer trials**

117 Two healthy volunteer (n 45) trials, particularly open placebo controlled (OPC) study and
118 double blind randomized placebo controlled (DBRP) study were carried on to evaluate the
119 functional efficacy of *L. fermentum* ME-3 in the human body. The inclusion criteria included

120 the wish to participate, no known health problems, and no medical conditions requiring drug
121 therapy, no other yoghurts or no special diets. The subjects with a history of GIT disease,
122 food allergy and acute infection, use of any antimicrobial agent within the last month or use
123 of any regular concomitant medication were excluded. The members of the trial were daily
124 questioned about their general welfare, intestinal function (general welfare, gut gas
125 production, stool frequency) and putative adverse effects. The withdrawal criteria from the
126 trials included acute infections during the study. Reasons for dropout were the unwillingness
127 to proceed with the study or relocation to new area. The blood samples (6 ml) from the
128 antecubital vein, fecal and urine samples were collected before and at the end of all clinical
129 trials.

130 Participants of all trials gave informed consent to the study protocols approved by the Ethical
131 Committee of Tartu University.

132 *Open placebo controlled fermented goat milk trial.* The study participants were 5 men and 16
133 women, mean age 50 years (range 35-60). During three weeks of the trial the study group (3
134 males and 13 females) consumed daily 150 ml fermented goat milk. The daily dose of
135 probiotic *Lactobacillus* strain was 11.2 to 11.8 log CFU per person.

136 The control group (1 male and 4 females) consumed the same dose of fresh goat milk.

137 *Probiotic capsule trial.* A DBRP study was carried out as follows. The study group consisted
138 of 15 men and 9 women, mean age 52 years (range 40-60) allocated according to their wish to
139 participate and randomly divided by an independent person and computer program for two
140 groups. The study group members (8 males and 4 females) took three probiotic containing
141 capsules (8.4 log CFU per capsule) two times daily (the daily dose 9.2 log CFU) during three
142 weeks. The placebo group (7 males and 5 females) received identical capsules without the
143 probiotic strain.

144 Fecal samples of all participants to assess change in fecal lactoflora and the persistence of the
145 ingested probiotic strain were collected before and at the end of trial. Several laboratory
146 indices of blood and urine were measured before and after the consumption of ME-3. Here we
147 report on changes in human body oxidative stress markers as total antioxidative activity
148 (TAA), total antioxidative status (TAS) and glutathione red-ox ratio (GSH/GSSG) from blood
149 serum and 8-isoprostanes in urine.

150

151 **Microbiological analyses of feces**

152 The total count of lactobacilli and the count of *L. fermentum* were evaluated in fecal samples.

153 The fecal samples were collected at day 0 and 21 in both trials. Samples were kept at -80°C
154 before analyzed. Serial dilutions (10^{-2} - 10^{-9}) of the weighed fecal samples were prepared with
155 phosphate buffer (pH 7.2) and 0.05 ml of aliquots was seeded onto MRS agar medium [17].

156 The plates were incubated at 37°C for 4 days microaerobically in 10% CO₂ environment
157 (incubator IG 150, Jouan, France). The catalase negative colonies were selected on the basis
158 of typical for LAB colony morphology, cells microscopy and Gram staining.

159 The count of *Lactobacillus* species was expressed in log₁₀ colony forming units per gram
160 feces (log₁₀ CFU/g) and percentage (relative share) in the total count of lactobacilli. The
161 detection level of lactobacilli was a 3.0 log CFU/g feces.

162 The relative amount of *L. fermentum*, colonizing the gastrointestinal tract of persons in the
163 study groups was expressed as a proportion of the total count (%), using the Bioquant
164 program [18]). The program gives output data for every microorganism as an absolute count
165 (log₁₀ CFU/g) and their percentage in the total count with its normal values.

166

167 **AP-PCR typing**

168 The putative ME-3 isolates were typed by arbitrarily primed polymerase chain reaction (AP-
169 PCR). Genomic DNA was extracted from 24h old cultures, cultivated on MRS agar
170 microaerobically with the QIAamp DNA Mini Kit 50 (QIAGEN GmbH., Hilden, Germany)
171 according to the manufacturers instructions. AP-PCR typing was done with two primers:
172 ERIC1R (5'-ATGTAAGCTCCT GGGGATTCAC-3') and ERIC2 (5'-
173 AAGTAAGTGAAGTGGGGTGAGCG -3') (DNA Technology A/S, Aarhus, Denmark). A 30
174 µl volume of reaction mixture consisted of 10xPCR buffer (Fermentas, Vilnius, Lithuania),
175 2.5 mM MgCl₂ (Fermentas, Vilnius, Lithuania), 200µM deoxynucleoside triphosphate
176 mixture (dATP, dGTP, dTTP and dCTP, Amersham Pharmacia Biotech, Freiburg, Germany)
177 0,60µg of each primer and 2.5U Taq DNA Polymerase (Fermentas, Vilnius, Lithuania,) and 5
178 µl of extracted DNA according to Matsumiya *et al.* [19]. The PCR mixture was subjected to
179 thermal cycling 35 cycles of denaturation at 95°C for 1 min, annealing at 35°C for 1 min, and
180 extension at 74°C for 2 min, with a final extension at 74°C for 5 min with the PTC-200
181 thermal cycler (Eppendorf AG, Hamburg, Germany). The PCR products were separated by
182 electrophoresis in a horizontal 2% agarose gel containing 0.1 µl/ml ethidium bromide in Tris-
183 acetic acid–EDTA (TAE) buffer (40mM Tris, 20mM boric acid, 1mM EDTA, pH 8.3) (Bio-
184 Rad Laboratories, Hercules, USA) at constant voltage of 120V. A 1kb ladder (GeneRuler,
185 Fermentas, Vilnius, Lithuania) was used as a base pair size marker. The banding patterns of
186 isolates were visualized with UV light and compared with that of *L. fermentum* ME-3 strain.
187

188 **Measurement of human body oxidative stress status**

189 In urine the changes of the oxidative stress marker 8-isoprostanes concentrations (ng/ml) were
190 assessed by a competitive enzyme-linked immunoassay (ELISA) (BIOXYTECH 8-
191 Isoprostane Assay, Cat No 21019) as described previously [16].

192

193 Blood serum was analysed for total antioxidative activity TAA, total antioxidative status TAS
194 and GSSG/GSH. TAA of the serum was assessed by the linolenic acid test (LA-test)
195 described previously [16]. This test evaluates the ability of the sample to inhibit lipid
196 peroxidation. TAS of the serum was measured with a commercially available kit (TAS,
197 Randox Laboratories Ltd. Ardmore, UK) as described elsewhere [16], water-soluble vitamin
198 E (Trolox) serving as a standard. This method is based on the inhibition of the absorbance of
199 the ferrylmyoglobin radicals of 2,2'-azinobis-ethylbenzothiazoline 6-sulfonate (ABTS+)
200 generated by activation of metmyoglobin peroxidase with H₂O₂.

201

202 The cellular oxidative stress markers as total glutathione and oxidized glutathione were
203 measured using the method of Griffith [20] as described elsewhere [16]. The glutathione
204 content was calculated on the basis of a standard curve generated with known concentration
205 of glutathione. Amount of GSH (µg/ml) was calculated as a difference between the total
206 glutathione and GSSG (total glutathione – GSSG). The glutathione red/ox ratio was expressed
207 as GSH/GSSG.

208

209 **Statistical Analysis**

210 The computer program Sigma Stat for Windows 2.0 (Jandel Corporation, USA) was applied.
211 The counts of fecal lactoflora were compared by using Student's t-test and Mann-Whitney
212 rank sum test. Changes in oxidative stress markers of blood sera (TAA, TAS and glutathione
213 red-ox ratio) and urine (8-isoprostanes) were evaluated by Student's t-test, paired t-test and
214 Mann-Whitney rank sum test. The choice of tests was made automatically according to the
215 distribution of the data. Both microbial and biochemical markers were given as mean and
216 standard deviation.

217 One-way ANOVA test was performed to compare the effect of different formulation on TAA,
218 TAS and fecal lactoflora parameters.
219 Differences were considered statistically significant if the value was $p < 0.05$.

220

221 **Results**

222 **Survival of ME-3 in formulations**

223 In capsule after approximately 1-log drop after one week from the production of the capsules,
224 the viable count of the probiotic strain remained stable at the level of 8.4 log CFU per
225 capsule. Additional results have shown at +4°C the stability of the freeze-dried capsulated
226 culture at least 17 months from the production.

227 In fermented goat-milk the cell count of the probiotic strain varied insignificantly from 9.0 to
228 9.7 log CFU/ml from one preparation to the other. The viable count of ME-3 in the fermented
229 goat milk was found to remain stable at least during 7 days of storage at 4°C.

230

231 **Human volunteer trials**

232 No dropouts were registered during volunteer trials, yet one participant was withdrawn from
233 the probiotic capsule trial due to acute respiratory viral infection. Besides, no adverse affects
234 in general welfare or changes in GI functionality were assessed during the trial.

235 *Changes in total LAB count.* The consumption of both ME-3 fermented milk and ME-3
236 capsule significantly increased the total count of lactobacilli in feces as compared to the initial
237 levels (Fig. 1). In opposite, in the group of volunteers consuming non-fermented goat-milk
238 there was even a decrease in total LAB counts during the 3-week trial and no changes were
239 found in capsule placebo group. Additional increase of lactobacilli counts was found only in
240 persons consuming fermented goat milk.

241 *Recovery of the probiotic strain.* In goat milk group *L. fermentum* as a species appeared in
242 fecal samples of all individuals (n=16) after consumption of fermented goat milk (Table 1).
243 The AP-PCR confirmed the recovery of ME-3 in the feces of all study group members (Fig.
244 2). However, in different trials the strain did not perform the predominant *Lactobacillus*
245 species in total lactobacilli count of participants (Table 1), though there was a tendency for
246 increase in *L. fermentum* counts. In the probiotic capsule trial the strain ME-3 was not
247 detectable among *L. fermentum* isolates by AP-PCR.

248 *Antioxidative health effect of ME-3.* The consumption of ME-3 in both formulations had a
249 positive effect on the blood oxidative stress markers as TAA and TAS (Fig. 3). Consumption
250 of goat milk and fermented goat milk enhanced TAA and TAS in the study and control group.
251 There was a significant additional increase (6% and 9% respectively) in both indices in the
252 fermented goat milk group. Significant increase in TAA and TAS values occurred during the
253 consumption of the probiotic strain in capsulated form. No changes were detected in the
254 placebo group. Additional effect of probiotic consumption in capsulated form was 4% for
255 TAA and 2.5 % for TAS.

256 The effect of goat-milk consumption on the TAA and TAS values was significantly higher
257 ($p < 0.001$) than by the consumption of the capsulated probiotic (Fig. 2).

258 The decrease of the glutathione red-ox ratio was significant in both groups: the study group
259 (from 0.15 ± 0.01 to 0.11 ± 0.04 $\mu\text{g/ml}$, $p < 0.01$) and control (from 0.14 ± 0.03 to 0.11 ± 0.02
260 $\mu\text{g/ml}$, $p < 0.01$) in the goat milk trial (Kullisaar *et al.* 2003). The fermented goat milk
261 containing *L. fermentum* ME-3 had no statistically significant additional effect. When the
262 probiotic was consumed in capsulated form, no significant decrease was noticed in the
263 glutathione red-ox ratio. The additional effect of ME-3 fermented goat milk consumption was
264 6%.

265 Compared with the baseline values, the consumption of ME-3 in goat milk reduced urine 8-
266 isoprostanes concentrations from 5.5 ± 0.4 before to 5.0 ± 0.5 ng/ml after the treatment ($p<0.01$)
267 [16]. No changes in urine 8-isoprostanes concentrations were seen between baseline and end
268 of the capsule trial (from 3.59 ± 1.3 to 3.0 ± 1.6 ng/ml).

269

270 **Discussion**

271 We have assessed the functional efficacy of the antimicrobial and antioxidative probiotic *L.*
272 *fermentum* ME-3 for healthy host. First of all the safety of the *L. fermentum* strain ME-3 was
273 confirmed as no adverse side effects were registered in volunteers. Even relatively high ($>10^{11}$
274 CFU) doses of consumed ME-3 had no negative impact on the hosts' general well-being.
275 *Lactobacillus fermentum* as species, used in various food applications, has a well-established
276 history of safe use and is evaluated as GRAS according to the Food and Drug Administration
277 of the USA [21].

278

279 Second, a clear improvement of laboratory indices of antioxidative defense system of a
280 healthy host was documented, using both formulations as fermented by *L. fermentum* ME-3
281 goat-milk and probiotic capsules. This effect was simultaneous with the increase of intestinal
282 lactoflora of healthy volunteers even without necessity for fecal recovery of the strain. In the
283 human population, persons without clinical symptoms have still a quite different health status,
284 including stability, capacity and potency of antioxidative defence to counteract sufficiently to
285 oxidative stress-caused adverse effects [7]. If a probiotic is able to exhibit a positive
286 functionality on oxidative stress-related indices, it helps both to stabilize and promote the
287 potency of the whole body antioxidative defence system in subclinical situations without
288 disease symptoms. That in turn may have an impact for lowering the risk of atherosclerotic

289 damage of blood vessels associated with several cardiovascular and neurodegenerative
290 diseases [22-24].

291
292 In our study of healthy volunteers for validation of the antioxidative functionality of probiotic,
293 four well-known oxidative stress markers of blood and urine were chosen. Urine 8-
294 isoprostanes reflect the whole body oxidative stress-load, normally present at low
295 concentrations in human body fluids [25]. We found that both formulations of ME-3 lowered
296 their concentration as compared to individual baseline values in our clinical settings. This
297 may be understood as indirect evidence for suppression of LDL oxidation [26, 27]. The state
298 of the lipid fraction (including also LDL) in the antioxidative defence system of the blood is
299 evaluated by TAA. TAS on the other hand reflects more the antioxidativity of the water-
300 soluble fraction of the human blood. Among the measured blood sera markers both the TAA
301 and TAS values were also reduced in the two different study groups. However, there was
302 found a significantly lower improvement of TAA and TAS values in cases of capsule than
303 fermented goat-milk where the recovery of the strains was assessed by AP-PCR. The AP-PCR
304 is an easily performed technique very effective for identification of potential ME-3 fecal
305 isolates.

306
307 Similarly, the reduction of the glutathione red-ox ratio was detected after the consumption of
308 fermented by ME-3 goat-milk but not with the capsule. The crucial non-enzymatic cellular
309 antioxidant is GSH [28], present in the millimolar range mainly in the red blood cells, liver,
310 pancreas, kidneys, spleen, eyes, lungs and intestinal cells [29]. The oxidized form of
311 glutathione becomes even at low concentrations toxic, and therefore in the cells the
312 glutathione red-ox ratio is kept as low as possible. In the case of inflammation this balance is
313 shifted towards the oxidized form, indicating non-physiological intracellular oxidative stress.

314 Thus, our study shows that there is a good association between the mode of formulation of
315 probiotic and expression of its functional properties inside the healthy host. Particularly, the
316 explanation for more expressed positive shifts in oxidative stress markers of former
317 volunteers could be due to the synergistic effect of the probiotic and the substrate. Milk is not
318 just a carrier for the probiotic *Lactobacillus* strain, but contains natural “lactogenic” factors
319 like lactose, minerals, vitamins and other components that enhance the metabolic activity of
320 ingested probiotic strain in GIT. Besides, a variety of bioactive peptides (e.g. casomorphins,
321 lactorphins, casokinins, *etc.*) revealed in milk [30-32] may have the antioxidative potency.
322 This was proved by some antioxidative effect also in persons consuming non-fermented goat
323 milk. The composition of goat milk differs from cow milk, containing several biomolecules,
324 which by consumption may also contribute to some additional antioxidative effect, as
325 discussed elsewhere [16]. Therefore, the provisional FAO regulations [33] suggesting the
326 need for health claims by specified formulations of probiotic seem to be of the utmost
327 importance.

328

329 Additionally, in our study with experimental fermented milk the average daily dose of *L.*
330 *fermentum* ME-3 being 11.5 logs CFU was clearly higher than that of capsule (max 9.5 log
331 CFU). It is possible that the dose exceeds the amount of bacteria necessary for interacting
332 with intestinal mucosa and the unattached lactobacilli are excreted with faeces. The finding of
333 Saxelin and colleagues confirmed that the fecal recovery of the probiotic strain started from
334 the consumption of more than 9.0 log CFU daily doses of capsulated LGG [34]. To our
335 surprise, in the present study the similar dose did not result in faecal recovery of the strain.

336

337 It is possible that the ME-3 strain germinated mainly in some upper parts of intestinal tract
338 where the advantageous conditions for survival and metabolic activity of probiotic lactobacilli

339 were present. Using molecular tools, Marteau *et al.* showed that lactobacilli figuring only 7%
340 of fecal microbiota performed up to 30% of microbial communities in human colon [35]. If
341 administered in lower quantities as in case of capsule trial, ME-3 did not reach the detectable
342 level in fecal samples. Yet, its presence in gut was proved by the positive antioxidative health
343 effect in blood but not in urine. Therefore it is understandable that the higher load of
344 metabolically active probiotic bacteria in goat-milk resulted also in their fecal recovery and
345 the highest impact on the oxidative stress indices.

346

347 Moreover, in our study the positive impact of ME-3 consumption on the host lactoflora was
348 proved by the increase of fecal lactobacilli counts in all participants of human volunteer
349 studies. In experimental settings the high counts of intestinal lactobacilli have been shown as
350 an important defensive factor against enteral infections [36, 37]. Though up to now the period
351 of consumption of probiotics has not been defined, the 3-week ingestion of fermented goat-
352 milk and capsule seemed enough for reaching the aims.

353

354 It is important to mention that after consumption of ME-3, a strain with high antagonistic
355 activity, neither the species nor the strain predominated among total lactoflora. This shows a
356 well-granted microbial balance inside the gut, which cannot be disturbed by high load of
357 probiotic bacteria. Apparently, the interconnected advanced metabolism of large gut
358 microbiota keeps the proportions of different species quite stable. Some other investigators
359 have obtained similar results showing the proportional increase of different microbial
360 populations (bifidobacteria, coliforms) after administration of *Lactobacillus* sp. probiotic [38,
361 39].

362

363 Thus, the functional efficacy of different formulations of antiinfectious and antioxidative
364 probiotic *L. fermentum* ME-3 were proved both by the increase of the lactobacilli counts
365 providing putative defense against infectious agents in gut and by reduction of the oxidative
366 stress indices of blood and urine of healthy volunteers. Further, Phase III studies evaluating
367 the efficacy of ME-3 as adjunct to conventional therapy in patients with atherosclerotic
368 damages and a high-grade oxidative stress are ongoing.

369

370 **Conclusions.**

371 In non-diseased host, the probiotic health claims can be assessed by improvement of some
372 measurable laboratory indices of well-established physiological functions of organism. In our
373 case, the possibility for augmentation of the antioxidative defence system by the probiotic *L.*
374 *fermentum* ME-3 in normal population can be proposed.

375

376 **Competing interests.**

377 Marika Mikelsaar, Mihkel Zilmer, Tiiu Kullisaar, Heidi Annuk (Hynes) and Epp Songisepp
378 are sharing the Estonian patent application: no. EE 2001 00356 29.06.01 and International
379 Patent application: no. WO03002131.

380

381 **Authors' contributions**

382 Epp Songisepp, and Pirje Hütt have been in charge of the microbiological analysis. The
383 former has been also in charge of analysing the results and writing of the manuscript. Jaak
384 Kals was responsible for performance and management of the volunteer trials. Tiiu Kullisaar
385 has been in charge of the biochemical analysis and writing the manuscript. Mihkel Zilmer has
386 conducted the biochemical estimations and writing the manuscript. Reet Mändar has been in
387 charge of the molecular analysis and revising the manuscript. Marika Mikelsaar is the main

388 conductor of the *L. fermentum* ME-3 research; for this paper she has been in charge of the
389 clinical trial design and writing the manuscript.

390

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396

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510 **Table 1. Changes in fecal recovery of *L. fermentum* during healthy human volunteer**
 511 **trials**

Groups	<i>L. fermentum</i>					
	* Prevalence (%)		† Count (log ₁₀)		‡ Proportion (%)	
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
Goat milk trial, ME-3 (n=16)	25 (4/16)	100 (16/16)	7.0±0.7	7.3±1.4**	21	13
Control (n=5)	-	20 (1/5)	-	3.6	-	28
Capsule trial, ME-3 (n=11)	16.7(2/12)	33.3 (2/12)	4.3±0.5	5.8±1.6	4	9
Placebo (n=12)	25 (3/12)	16.7 (2/12)	6.3±2.5	8.0±1.6	11	19

512 * Percentage of subjects with fecal *L. fermentum* inside the group

513 ** Significantly different from the pre-treatment values (paired t-test): p<0.001

514 † Median value± SD

515 ‡ Proportion of *L. fermentum* among fecal LAB

Additional files provided with this submission:

Additional file 1: additional.pdf : 37KB

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