

## Author's response to reviews

**Title:** Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers

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### Author's response to reviews:

Thank you for valuable comments and the work you have done. With most of your comments we really agree and they are taken into account of the revised version. The changes made in text are underlined.

#### Reviewer 1

1. No assessment of the diet before and after the studies (24-hour interview).

Sorry, to try to save article space our information in this aspect remains too limited. Although we did not use a special 24-hour interview, all participants were precisely informed and they did not use anything (vitamins, antioxidants, other probiotics or functional food) that could have affected investigated phenomena. At the same time, the participants of the trials were just asked to follow their usual everyday diet as any positive effect of probiotics on heterogeneous background has a stronger and more realistic every day outcome than in the case of very limited conditions.

We have added the comment in Discussion section.

Line 268-269: "Our aim was to evaluate the functional efficacy of the antimicrobial and antioxidative probiotic *L. fermentum* ME-3 in normal population with variable food intake"

2. There was no exclusion criterion based on the use of antioxidant vitamins or non-steroidal anti-inflammatory (analgesic) drugs.

The part concerning the exclusion criteria as restriction of the use of vitamins and drugs was really missing in the manuscript though it was present in our Study protocol. As summarized it was present under the phrase...

"The subjects with a history of GIT disease, food allergy and acute infection, use of any antimicrobial agent within the last month or use of any regular concomitant medication were excluded."

In the revised version we expanded the sentence and have added the paragraph.

Line 127-128 "The subjects with a history of GIT disease, food allergy and acute infection, use of any antimicrobial agent within the last month or use of any regular concomitant medication including antioxidant vitamins and anti-inflammatory non steroidal drugs that could have affected the evidence-based information were excluded."

3. No assessment was made of the effect of the probiotics studied on the blood cell count, arterial pressure, lipid levels.

As usually the biochemical-clinical indices (including BCC etc) of healthy adult participants' are in normal range, no special assessment of the effect of the probiotic studied on the blood cell count, arterial pressure and lipid level was carried out during trials with healthy persons. This will be the objective of our future trial with sick persons like patients with brain stroke (this trial is just finishing).

4. In the discussion, the authors do not refer to the papers indicating that anti-inflammatory and antioxidant actions of probiotics may result from the increase in the level of short-chain fatty acids, i.e. propionic acid and butyric acid formed as a result of decomposition of fiber by probiotic bacteria, in the circulation.

Propionic acid and butyric acid can lower the level of oxidative stress at the cellular level through their effect on NF-KB.

The strain *L. fermentum* ME-3 does not produce propionic and butyric acids.

Reviewer 2.

1. Abstract; the protocol for the human study is difficult to understand. It should be clearly described that the two groups in OPC received goat milk with or without ME-3 and the other two groups in DBRP received the capsules with or without ME-3.

The description of both human volunteer trials has been rephrased in the abstract of the revised version. Line 28-32:

"Two 3-week healthy volunteer trials were performed. Open placebo controlled (OPC) study participants (n=21) consumed either goat milk or by *L. fermentum* ME-3 fermented goat milk (daily dose 11.8 log CFU (Colony Forming Units). Double blind randomized placebo controlled (DBRP) study participants (n=24) received either capsules with *L. fermentum* ME-3 (daily of dose 9.2 CFU) or placebo capsules."

2. Line 37. What are the "both formulations" The reader may confuse whether they include the control goat milk or not.

You are right; this sentence in the abstract is confusing. Under the term "both formulations" we meant fermented goat milk and capsules. We have replaced the confusing term with "...the significant improvement of blood TAA and TAS indices was seen ...both in case of fermented goat milk and capsules"

What is the meaning of increase in TAS and TAA in goat milk group of goat milk trial?

We have included the explanation for antioxidative effect of goat milk into Discussion. Line 325-330:

"Both fermented goat milk and goat milk elevated the values of TAA and TAS The goat milk contains different biomolecules (e.g. casomorphins, lactorphins, casokinins, etc) having certain antioxidative properties, which can contribute to consumer's plasma antioxidative capacity (Nakagawa et al. 1999). However, the elevation of TAA and TAS was remarkably more expressed in the fermented goat milk group, thus the goat milk fermentation with *L. fermentum* ME-3 results in additive increase of total antioxidativity compared with usual goat milk."

3. Background the second paragraph is too long. The description about oxidation is better to be shortened.

We have shortened the text.

3. Line 239-240. What is meaning of the sentence "Additional increase"? You mention in line 236 that ME-3 capsule also increased lactobacilli count.

Sorry, this sentence was really of no use, as changes in total faecal lactobacilli counts of participants of both study groups have been already described in two previous sentences. The sentence has been removed.

4. Line 243.profiles (Fig. 2); The PCR profiles similar to ME-3 cannot be shown in patients 15 and 17.

The figure has been replaced wit clearer photo of the AP-PCR profiles from the healthy volunteer trial with fermented goat milk.

5. Line 244-245. The meaning of this sentence cannot be understood.

We have rephrased the sentence for more clear understanding. Lines 249-250 in the revised manuscript "However, in different trials the administration of ME-3 strain did not lead to the predominance of *Lactobacillus fermentum* species."

6. Line 249-254. This part should be re-organized for better readability.

The paragraph has been rephrased in the revised manuscript. Lines 252-256:

"The positive effect on the blood ox stress markers as TAA and TAS was seen in the case of both formulations (Fig.3). Particularly, the additive increase in goat milk group was 6% and 9%, respectively, as

compared to control group; however only 4% for TAA and 2.5% for TAS in probiotic capsule study group as compared to placebo."

7. Line 257. This figure number is 3.

Sorry for the mistake. The figure number has been changed to the right one.

8. Line 260. Why do you cite the reference of Kullisaar in this sentence?

Sorry, the citation of the reference of Kullisaar on this sentence was in a wrong place. It has been removed.

9. Line 239, 251, 261, 263 you are frequently using expression "the additional effect". Sometimes it becomes simple repetition of the previous sentence, and sometimes it seems to represent the different meaning. We recommend you to remove these representations.

Representations containing the expression "additional effect" have been removed from the revised manuscript

10. Line 265. Urinary 8-isoprostane should be expressed as the excretion rate per day or minute because the concentration is dependent upon urinary volume. The values for controls are lacking.

We used the first morning urine specimen, which has been traditionally recommended as the standard specimen for urinalysis (for example Kouri T, Fogazzi G, Gant V, Hallander H, Hofmann W, Guder WG. European Urinalysis Guidelines. Scand J Clin Lab Invest Suppl 2000;60(231): 96 and ECLM - European Urinalysis Guidelines). Thus, we used the first morning mid-stream urine is the specimen voided immediately after an overnight bed rest before breakfast and other activities. This is also called early morning urine. We recommended that it be voided after an 8-hour period of bed rest, fast and after at least 4 hours storage time in the urinary bladder (even if the bladder was emptied earlier at night). It has been traditionally recommended as the standard specimen for urinalysis, because it is more concentrated than the day urine.

11. Discussion: the results demonstrated that TAA and TAS were significantly increased by the intake of fresh goat milk. The increase was comparable to that by the fermented goat milk, and larger than the ME-3 capsule group. These issues should be carefully discussed in the revised manuscript.

The issue of changes of TAA and TAS after both goat milk and ME-3 fermented goat milk and probiotic capsule intake have been discussed in the manuscript in the following way. Lines 311-331 in the revised manuscript.

"Thus, our study shows that there is a good association between the mode of formulation of probiotic and expression of its functional properties inside the healthy host. The antioxidative potential of the food supplement containing ME-3 was excellent, as reisolates of the strain from capsule expressed significantly higher TAA in comparison with the base values of the strain in vitro (data not shown). Unexpectedly, the shifts in the antioxidativity markers in blood serum of participants of the probiotic capsule trial and reduction in 8-isoprostanes in urine were less pronounced in comparison with ME-3 fermented goat milk.

Particularly, the explanation for more expressed positive shifts in oxidative stress markers of volunteers of the fermented goat milk trial could be due to the synergistic effect of the probiotic and the substrate. Milk is not just a carrier for the probiotic *Lactobacillus* strain, but contains natural "lactogenic" factors like lactose, minerals, vitamins and other components that enhance the metabolic activity of ingested probiotic strain in GIT. Both fermented goat milk and goat milk elevated the values of TAA and TAS. The goat milk contains different biomolecules (e.g. casomorphins, lactorphins, casokinins, etc) having certain antioxidative properties, which can contribute to consumers' plasma antioxidative capacity [30-36]. This was proved by some antioxidative effect also in persons consuming non-fermented goat milk. However, the elevation these indices were remarkably more expressed in the fermented goat milk group, thus the goat milk fermentation with *L. fermentum* ME-3 results in additive increase of total antioxidativity.

Therefore, the provisional FAO regulations [37] suggesting the need for health claims by specified formulations of probiotic seem to be of the utmost importance."

12. Line 301 the word "reduced" is wrong.

In the revised manuscript (Line 298) the word "reduced" has been changed into correct expression "improved"

"Among the measured blood sera markers both the TAA and TAS values were also improved in the two different study groups."

13. Line 303-305. This sentence is meaningless.

This suggested sentence has been removed from the revised manuscript

14. Line 343 why is there any difference between blood and urine?

TAA and TAS are indices for generalized expression of power of oxidative stress but isoprostanes are oxidative stress markers involve only lipid peroxidation-driven part of events (Halliwell B and Gutteridge JMC: Free radicals in biology and medicine. New York: Oxford University Press; 1999). Thus, the latter parameter alterations occur more easily compared with TAA and TAS. Thus, in the case of healthy persons some differences are possible and realistic.

15. Figure 2. The negative control is required to show what is the specific profile for ME-3. The some lane numbers showed in the legend are wrong.

The molecular fingerprints of type strains of *L. fermentum* ATCC 14931, *L. reuteri* DSM 20016, *L. brevis* ATCC 14869 and *L. buchneri* ATCC 4005 have been added to show what is the specific profile for ME-3. (Figure 2, (a))

The line numbers in figure legend are have been corrected in the revised manuscript. Lines 540-541: "From the left: M - molecular weight marker, Line 1 - ME-3, Line 2...17 - ME-3 like profiles from feces of goa milk trial study group participants."

16. There are many grammatical and typographic errors. The native English speaker should review the manuscript.

Sorry for this, the English language has been revised

17. The abbreviations should be explained by the full-spelled words when firstly appeared.

All the abbreviations throughout the revised manuscript have now been explained full-spelled when firstly appeared.

Line 28-29: Open placebo controlled (OPC) trial

Line 30: Double blind randomised placebo controlled (DBRP) study

Line 30: CFU (Colony Forming Units)

Line 38: TAA (Total Antioxidative Activity) and TAS (Total Antioxidative Status)

Line 54-55: ILSI (International Life Science Institute) and FUFOSSE (The European Commission Concerted Action on Functional Food Science in Europe)

Line 81: gastrointestinal tract (GIT)

Line 92: World Intellectual Property Organization (WIPO)

Line 101: de Man-Rogosa-Sharpe (MRS)

Line 175: arbitrarily primed polymerase chain reaction (AP-PCR).

Line 201: glutathione red/ox ratio (oxidized glutathione and reduced glutathione, GSSG/GSH).

Line 297: low density lipoprotein (LDL)