Author's response to reviews

Title: Fermented milk improves glucose metabolism in exercise-induced muscle damage in young healthy men

Authors:

Masayo Iwasa (masayo.iwasa@taiwa.ac.jp)
Wataru Aoi (waoi@kpu.ac.jp)
Keitaro Mune (kanshakansha11@yahoo.co.jp)
Haruka Yamauchi (y_haru_0504@yahoo.co.jp)
Kaori Furuta (fk126116@zeus.eonet.ne.jp)
Shota Sasaki (nutrition13@gmail.com)
Kazuya Takeda (k.tetsu.t15@gmail.com)
Kiyomi Harada (s810731010@kpu.ac.jp)
Sayori Wada (poisson@kpu.ac.jp)
Yasushi Nakamura (yas@kpu.ac.jp)
Kenji Sato (k_sato@kpu.ac.jp)
Akane Higashi (higashi@kpu.ac.jp)

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Author's response to reviews: see over
April 5, 2013

Nagaraj Nagathihalli
Managing Editor
Nutrition Journal

Dear Dr. Nagathihalli,

I, along with my co-authors, hereby submit a revised version of the article “Fermented milk improves glucose metabolism in exercise-induced muscle damage in young healthy men” for consideration for publication in Nutrition Journal.

We thank the reviewers for their comments and suggestions, which we believe have contributed to improve the quality of our manuscript. The revised version of the article addresses all of the concerns raised by the reviewers; the corrections are highlighted in red. We believe that the present article provides valuable contribution to the literature.

Along with the revised article, we hereby attach point-by-point responses to each of the reviewers’ comments.

We thank you for your consideration and look forward to hearing from you.

Sincerely,

Dr. Wataru Aoi
Laboratory of Health Science
Graduate School of Life and Environmental Sciences
Kyoto Prefectural University
1-5 Hangi-cho Shimogamo
Sakyo-ku, Kyoto 606-8522, Japan
waoi@kpu.ac.jp
<Response to Reviewers>
Reviewer: Thomas Love

Reviewer's report:
Method
Study Design
The authors use a repeated-measures design to determine the effects of fermented milk on muscle damage and acknowledge the existence of a repeated bout effect. Whilst trials were administered in a random order please could they clarify if this was 1) a counter-balanced design, 2) report trial order effects for responses (soreness, CK) and 3) further explain the reason for using a 6 week intervening period given the data presented by Nosaka et al (1991) which suggests a protective effect might still be evident. This study had a counter-balanced design to avoid practice bias. We have specified this in the text.

The levels of serum CPK and muscle soreness did not change significantly between the first and second exercise trials as shown in the below table, which suggests that the muscle damage response were not affected by trial order.

<table>
<thead>
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As you suggested, a 6-week intervention period may have been too short to avoid the effect of exercise in the first exercise trial on the muscle damage response in the second exercise trial. However, in this study, the level of muscle damage estimated from the CPK and soreness values was moderate; therefore, we believe that the initial trial minimally affected the next trial. Indeed, the muscle damage parameters did not differ between trials as mentioned above. The use of more intervals may lead to other problems such as seasonal variations of parameters.

Examination Beverage
It is unclear what the authors mean by “adjusted contents of protein...”. It would perhaps be more informative to report protein, fat, carbohydrate and pH values of both drinks to allow comparison with other guidelines such as the effects of carbohydrate and/or protein on muscle damage to be determined.

Both fermented milk and placebo beverages contained the following: energy, 34 kcal; protein, 2.2 g; fat, 0.0 g; carbohydrate, 7.2 g/200 mL. Each was adjusted to pH 3.75. Because the subjects took each beverage 3 times before and after exercise, they ingested 3-fold amounts of nutrients. The information regarding this point has been revised in the text. The carbohydrate and protein contents did not differ
between the beverages, which suggested that the nutrient amount did not influence the results.

Experimental Schedule

Diet – The authors state that food and fluid intake was recorded for the day before the trial, but there is not data/attempt to standardise the diet on day 1 of each trial. Consequently the ability to infer that the alteration in CK at 24hr and indices of oxidation were due to the drinks that were consumed is not possible.

**Food and fluid intake before the trial is important to ensure metabolic and damage parameter accuracy as you suggested. In this study, we did not adjust meal contents in any of the subjects and trials, although food intake was recorded on the day before the measurement (Day 1 in the initial trial); thereafter, the diet was repeated in same contents through all 3 trials, which would lead to accurate examinations in individual trials.**

Diet – The lack of dietary control on day 1 has implications for the metabolic data collected on day 2. Recently Stevenson and colleagues have reported the influence of the GI index of an evening meal on subsequent responses to a high GI meal the following morning. Whilst this did not appear to influence carbohydrate or fat oxidation it did have a significant effect on blood glucose and insulin responses. Can the authors report what dietary standardisation was performed on day 1 of each trial?

As mentioned above, each subject took the same meal on the day 1 in all 3 trials. None of the subjects consumed extremely large or small intakes of energy, fat, and carbohydrate. As a result, they consumed the following; energy, 1997±94 kcal/day; protein, 14.6±0.4%; fat, 26.3±1.7%; and carbohydrate, 59.4±1.7% (mean ± standard error), which corresponds to the recommended dietary allowance.

Urine Collection – Urine collections and blood samples (analysed for CRP, TNF-α, carbonyl protein) were obtained only on the morning of day 2. Given the 6 week intervening period between trials, could these values represent a day-day change rather than attributable to the intervention?

As you pointed out, a day-day or seasonal variation would influence urine and blood parameters, which is a limitation of this study. However, we confirmed from the interview that the subjects kept a sedentary life, normal diet, and had no illnesses before each trial; therefore, we believe that the effect of variation was suppressed as much as possible.

Visual Analogue Scale – It is currently unclear what a muscle soreness value of 4.8 means. Please can the authors provide further evidence of the scale used. A total soreness value has been reported by simply adding the soreness values from the 3 muscle groups together. If whole body muscle soreness was a variable of interest this would have been best determined by a specific question rather than later inferred from data. The absence of muscle soreness data from the Control trial is disappointing as this would have been informative and enabled a more guided assessment of the efficacy of drinks to have been made. Was this data collected and if so, why was it not included?

The visual analogue scale (VAS) was used to examine the level of muscle pain. Subjects were asked to indicate the intensity of perceived pain for each muscle part.
on a 100-mm horizontal line. The left side stated “having no pain,” while the right side stated “having max pain”. The total soreness value was calculated by adding the soreness values of 3 muscle parts, while the soreness scores in the rest of the trials were expressed as not detected (N.D.). The related description has been added to the methods section.

Statistical Analysis – The data gathered, (3 trials using the same participants) would suggest that a repeated-measures ANOVA would be the most appropriate statistical test (if data was normally distributed) rather than a one-way ANOVA that has been used. Statistical Analysis – For some variables (eg. lactate, glucose) the authors report 2-3 collection points per trial. This data should therefore be analysed by a two-way repeated measures ANOVA rather than a one-way ANOVA. Please can the authors clarify the reasons for the statistical tests run.

We agree with your suggestion. Repeated-measures ANOVA was used to compare the data of the 3 trials. In the index of 2–3 collection points per trial, such as blood glucose and lactate, a 2-way repeated-measures ANOVA was used.

Discussion
It is surprising that the recent work by Cockburn et al (2012) and other studies from the growing literature on milk and exercise was not included in the study. This data indicate that just 500ml of milk will confer a benefit on muscle damage. This volume of milk is similar to that used in the current study (400ml). If indeed an improvement was found with fermented milk, then the authors might find it beneficial and indeed a strength of the study to report this due to the comparison of fermented milk to a PLA (milk) which is already considered to be beneficial.

Consumption of milk (unfermented) partially attenuates the muscle damage as shown by Cockburn et al.; therefore, the placebo trial, which used unfermented milk, may have also suppressed muscle damage to some extent. Indeed, the elevation of serum CPK in the placebo trial was not too large. However, our results showed that fermented milk is more effective than milk. The related discussion has been described in the Discussion section.

Conclusion
The authors conclude “we found that fermented milk prevents glucose metabolic impairment and muscle damage induced....”. But, from the data that was presented, there was no difference in muscle damage between trials.

We agree with this point. The description “muscle damage” has been changed to “muscle soreness” because the level of CPK, a muscle damage parameter, was not significantly different between the placebo and fermented milk trials.

Impaired Glucose Metabolism – The data presented indicates no differences in blood glucose responses to a glucose load and in the absence of insulin concentrations, I believe the statement “These observations suggest that dietary fermented milk reduces impairment of glucose metabolism....” is difficult to justify.

Our recent paper (Aoi W et al. Physiol Res 2012) using mice showed that damaged muscle impairs insulin-induced glucose uptake and reduces IRS/PI3K/Akt signals and carbohydrate oxidation, although blood glucose and plasma insulin levels were
not changed. While we could not examine metabolic performance in muscle tissue in
the present study, inflammation and metabolic impairment are thought to have
occurred in the local muscle but did not spread to the blood or urine parameters in
the consideration of earlier reports.

Athletes – This study used “healthy young males who were not habituated to a regular
exercise regimen. It is therefore difficult to justify the comment suggesting athletes may
benefit.

The description regarding application of our results to athletes may be an
overexplanation, as you suggested. Here, the outcome has applied to only healthy
persons who performed recreation sports, and the benefit to athletes needs to be
determined in a future study. We have added this information to the Discussion.

Reviewer: Koichi Nakazato

Reviewer's report:
The authors investigated the effect of fermented milk supplementation on muscle damage
after one bout of strength exercise. They observed significant changes in muscle soreness
and RQ in the fermented milk group compared to the placebo group. The authors
concluded that intake of fermented milk supplementation improves glucose metabolism
and alleviates muscle damage. The reviewer thinks that the aim of this study is clear, but
there are several concerns especially in the Methods and Discussion sections. Since the
experimental setup seemed unsuitable for the purpose of the study, the obtained results
were rather weak to support their hypothesis.

Background
The authors previously reported that Lactobacillus helveticus-fermented milk prevents
exercise-induced muscle damage in animal models. They also showed that
insulin-dependent glucose uptake was affected in damaged muscle, and such impairment
was rescued by fermented milk supplementation in animals. In this report, they raised the
question of whether this favorable effect of fermented milk supplementation is also
effective in humans. The reviewer thinks that the question is original and clearly defined.
It is also obvious that prevention of muscle damage is important, for example, for
weekend athletes.

Method
1 Repeat bout effect
The authors classified 3 groups as control, placebo, and fermented milk groups, and all
subjects participated in all 3 trials in a random order. It is well known that the repeat-bout
effect exists in exercise-induced injury. The reviewer recommends that the authors
explain why they thought that 6 weeks was enough to diminish the repeat-bout effects.
A 6-week intervention period may have been too short to avoid the effect of previous exercise on the muscle damage response. However, in this study, the level of muscle damage estimated from the CPK and soreness values was moderate; therefore, we believe that the initial trial minimally affected the next trial. Actually, as shown in the below table, the serum CPK and muscle soreness levels did not change significantly between the first and second exercise trials. In contrast, the use of more intervals may lead to other problems such as seasonal variations of parameters.

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2 Exercise protocol
The reviewer is not sure why the authors selected the “usual” exercise protocol. In their animal model, they employed eccentric contraction exercise, which induces muscle damage. In this human trial, eccentric exercise is suitable for replicating the author’s previous animal studies. The reviewer also wonders why the strength of the exercise was as broad as 70–100%. The strength and volume is critical for inducing muscle damage. Please provide details for the exercise protocol employed.

**Heavy resistance exercise at the intensity of 10–12 RM has been often used as for inducing muscle damage in earlier studies. All subjects performed 10 repetitions at the load of 100% 12 RM in 1–3 sets and then the load of 70% 12 RM in 4–5 sets. Detailed information has been added to the Methods section.**

3 RQ
It is obvious that indirect metabolic performance is affected by the experimental setup. Please clarify how you measured the respiratory parameters. The cited manuscript is inappropriate, because it refers to an animal-model study.

**Thank you for your suggestion. We have revised the cited manuscript to refer to a human study.**

4 Experimental schedules
It is easier for readers to understand schematic illustrations of the experimental schedule rather than text. Please consider making this change.

**We agree with the suggestion to include a schematic illustration of the experimental schedule. The following figure has been added.**
VAS and blood-profiling parameters change over time. The reviewer thinks that only 1 time point is insufficient for precise evaluation. Why did the authors select the next day of exercise as the only measuring time point?

A number of studies have shown that muscular damage peaks at 24–48 h after exercise. We set the point of measurement at 24 h rather than 48 h because it is easy to control the subjects’ lives in shorter periods after exercise. However, we would also like to examine the time-course changes, as you suggested, which will be a theme of a future study.

5 VAS
Why did the authors evaluate muscle pain during butterfly and squat movements? It is easier to evaluate the degree of pain by palpation and specify the muscle group involved. If the authors have referenced previous studies, please indicate so.

Pain was induced by leg press and chest press in the pectoralis major, quadriceps, and gluteus maximus muscles. Therefore, subjective muscle pain of the 3 parts was evaluated by both palpation and movement during the butterfly and squat positions. The butterfly position is widely known to contract the pectoralis major, while the squat is known to contract the quadriceps and gluteus maximus muscles. Subjects who exercise regularly can easily check for muscle pain even without an exercise machine. However, since it would be more accurate to evaluate by palpation as suggested by reviewer, we have shown the results in the table.

Results and Discussion
1 Effect of fermented milk for muscle damage
The significant differences between the fermented milk and the placebo were only seen in muscle soreness and respiratory parameters. Further, evaluation of muscle soreness was conducted at only 1 time point, and significance was observed only in the pectoralis major muscle. The pain during butterfly motion is suspected to have radiated from other muscle groups. With regard to RQ, the difference of averaged values was rather small (0.88 and 0.84).
Although the reviewer agrees that fermented milk might have beneficial effects, the experimental setup and obtained data were too weak to support their hypothesis. The present human study is based on our previous animal studies. First, exercise-induced muscle damage impairs glucose metabolism in the damaged muscle (Physiol Res 2012). Second, the intake of fermented milk suppresses muscle damage (J Nutr Biochem 2007). Thus, the purpose of this study was to examine the effect of fermented milk on metabolic impairment with muscle damage in humans. We believed that the present study could demonstrate the beneficial effect of fermented milk, although it was not examined in muscle tissue to avoid excessive invasion, and larger variations were seen in the human study versus the animal study.

Indirect metabolic examination using respiratory analysis reflects metabolism in the entire body, including the brain, heart, liver, and adipose tissue. With regard to skeletal muscle, because the damage occurred primarily in the pectoralis major, quadriceps, and gluteus maximus but not in the other muscles, almost all of the parts retained normal function. Therefore, we believe that the observed magnitude in the reduction of RQ is reasonable.

2 Serum glucose, lipid profile, inflammatory factors, and oxidant stress markers The tendency of averaged values seemed to support the author’s hypothesis, but there was no significance. The reviewer thinks that the strength of the selected exercise protocol might be weak for this experiment. In the present study, the response of muscle damage was not so large and other blood markers were not significantly changed. It has been reported that consumption of unfermented milk partially attenuates muscle damage (Cockburn et al. Eur J Appl Physiol 2012); therefore, a placebo trial may already suppress muscle damage to some extent. However, our results have shown that fermented milk is more effective than milk. In addition, inflammatory responses are found in local muscle tissues but not in circulation as shown in our animal study (Physiol Res, 2012).
The related discussion has been described in the Discussion section.

Reviewer: Anne Pihlanto

Reviewer’s report:
The manuscript by Iwasa et al describes fermented milk on glucose metabolism on young health men. The manuscript is interesting and should be published. The structure of the manuscript is good as well as the language. The methods are described sufficiently and are suitable to the study.