Author's response to reviews

Title: Dietary milk fat globule membrane supplementation combined with regular exercise improves skeletal muscle strength in healthy adults: a randomized double-blind, placebo-controlled, crossover trial

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Author's response to reviews: see over
Dear Dr. Kumagai:

Thank you for your e-mail dated May 11, 2015 regarding our manuscript (MS: 1196492366158306 - Habitual exercise with dietary milk fat globule membrane supplementation improves skeletal muscle performance in healthy adults: a randomized double-blind, placebo-controlled, crossover trial) together with the comments made by the reviewers.

We have read the comments very carefully. We thank you and the reviewers for your helpful comments, which have guided the revision of our work. In response, we have made extensive corrections that we hope will meet your approval. We believe that the revised manuscript is a great improvement on the original, providing a clearer and more balanced presentation of our data. We hope that the revised manuscript is satisfactory and that it will be considered acceptable for publication in Nutrition Journal.

Sincerely,

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AUTHORS’ RESPONSES TO THE REVIEWERS’ COMMENTS:

The revised text is highlighted in red font in the manuscript.

Reviewer 1:

We sincerely thank the reviewer for the insightful comments about our paper, which we feel have helped us substantially improve the paper.

Discretionary points

1. The authors may want to consider broadened the scope of application of this work, this may include its potential usefulness for athletes and post training recovery and also possibly neuromuscular diseases/atrophy other than ageing. This could help to boost the intro which feels a little vague at the moment.

Response: We agree with the reviewer’s comments and regret that we described the background information poorly in the original manuscript.

In response to the reviewer’s comments, we extensively revised the Background section. Concomitantly, we changed the title of the manuscript.

Following the reviewer’s suggestion, we added the sentences that describe the potential usefulness of MFGM supplementation for athletes and post-training recovery in the Discussion section.

(Title)

Dietary milk fat globule membrane supplementation combined with regular exercise improves skeletal muscle strength in healthy adults: a randomized double-blind, placebo-controlled, crossover trial

(Background, pages 4-5)

Muscle strength is an excellent indicator of general health. Optimal muscle function is important in relation to rehabilitation and quality of life in many musculoskeletal diseases [1-4]. However, muscle strength in healthy men decreases in a linear fashion from the age of 25 years to between 54% and 89% at the age of 75 years [5]. Therefore, the decline of skeletal muscle strength in young adults should be prevented to maintain higher quality of life in their older age.

Resistance training is well known to increase muscle mass and strength [6]. However, high-intensity and long-term resistance training is necessary to achieve satisfactory improvement of muscle mass and strength. Accordingly, more efficient strategies that boost the exercise-induced improvement of skeletal muscles are required to maintain general health for non-athletes. Nutritional
supplementation may facilitate more efficient muscle improvement for untrained people, even combined with low-intensity and low-frequency exercise. Recent studies demonstrated that dietary supplementation with amino acids [7] or tea catechins [8], when combined with regular exercise of low intensity, significantly improved muscle mass and strength in the elderly.

Our recent studies in mice have demonstrated that dietary supplementation with milk fat globule membrane (MFGM), when combined with habitual exercise, significantly improved muscle mass and strength [9], as well as swimming endurance capacity [10]. MFGM is the structural membrane covering a triglyceride globule that is dispersed as emulsified bodies in milk [11]. Our previous study revealed that the beneficial effects of dietary MFGM on skeletal muscles was associated with stimulation of neuromuscular junction (NMJ) development [9], which is a critical structure of a motor unit (a single motor neuron and all of the muscle fibers that it innervates). MFGM supplementation in the diet (1%) combined with voluntary exercise (wheel-running) significantly increased muscle strength and expression of NMJ-related molecules in adult mice [9]; however, whether nutritional supplementation with MFGM may facilitate neuromuscular improvement by regular exercise in humans has yet to be investigated.

The present study aimed to demonstrate the ability of dietary MFGM combined with regular exercise to increase skeletal muscle strength and neuromuscular function in healthy young adults.

(Discussion, page 14, line 243)

The application of this study may include not only maintenance of general health of non-athletes, but also improvement of physical performance and post-training recovery of athletes, as well as treatment of neuromuscular diseases/atrophy.

2. Review the key words: I don't think that motor unit is a key search term for this paper.

Response: We agree with the reviewer’s comments and regret that we improperly inserted “motor unit” as a key word.

Surface EMG comprises the sum of the electrical contributions made by the active motor units, and the amplitude of the surface EMG is related to the net motor unit activity (Farina et al. 2004, Semmler 2014). Because the change from the baseline for either leg extension strength (Table 4) or RMS observed during surface EMG (Table 5) was significantly greater in the MFGM group than in the Placebo group, we inferred that MFGM supplementation improved the muscle strength by increasing motor unit activity. Since this is one of the key findings in the present study, we would like to use “motor unit activity” as a key word in the revised manuscript. If the reviewer and the
editor feel otherwise, we shall abide by their decision.

In response to the reviewer’s comment, we changed the key word “motor unit” to “motor unit activity,” and revised the Abstract, Methods, and the Discussion sections as follows:

(Keywords, page 3, line 45)

motor unit activity

(Abstract, page 2, line 36)

Surface EMG showed that the MFGM group had a significantly higher root mean square amplitude than the placebo group, which indicated that the MFGM group had higher motor unit activity.

(Methods, page 8, line 140)

Motor unit activity during the exertion of muscle force was evaluated using surface electromyography (EMG) [14, 15].

(Methods, page 9, line 146)

the root mean square (RMS) amplitude, an indicator of motor unit activity [16, 17], was calculated using MegaWin 3.0 software (Mega Electronics Ltd., Kuopio, Finland).

(Discussion, page 12, line 189)

Another finding was that dietary MFGM supplementation plus regular exercise also increased the RMS of surface EMG, indicating that dietary MFGM increased motor unit activity during muscle contraction.

Surface EMG comprises the sum of the electrical contributions made by the active motor units, and the amplitude of the surface EMG is related to the net motor unit activity (i.e., the recruitment and/or the discharge rates of the active motor units) [16, 17].

3. Consider that thigh circumference is a very insensitive measure of muscle mass; did the BIA give a predicted muscle mass value? Not sure it would be much more sensitive but reporting both measures might give this assumption of no muscle increase more weight.

Response: We agree with the reviewer’s comments regarding the measurement of muscle mass, and appreciate the suggestion to evaluate muscle mass using BIA. BIA
can predict muscle mass and also supports the assumption that there was no muscle mass increase during the experimental period.

In response to the reviewer’s comments, we included muscle mass data obtained using BIA in Table 2 in the revised manuscript, and revised the Methods, Results and the Discussion sections as follows:

(Methods, page 8, line 124)

Body weight, body fat ratio, and muscle mass were measured using a bioimpedance body fat analyzer (BC-621, Tanita, Co., Tokyo, Japan).

(Results, page 10, line 163)

No overall changes in body weight, body fat ratio, whole body and leg muscle mass, or thigh circumference were observed during the intervention period (Table 2).

(Discussion, page 12, line 187)

The first was that daily intake of 1 g MFGM combined with regular, twice weekly exercise improved skeletal muscle strength (leg extension) in young adults, despite a lack of change in muscle mass.

(Discussion, page 12, line 197)

Because the leg muscle mass did not change after MFGM ingestion in the present study, 4. The increased Hb is intriguing and warrants greater consideration.

Response: We agree with the reviewer’s comment and appreciate the suggestion. Interestingly, dietary supplementation with MFGM also increased hemoglobin levels. However, the reason for the increased hemoglobin after MFGM ingestion was unclear. MFGM is abundant in phospholipids that are critical components of the plasma membrane. Decreased phospholipid content in the cell membrane of red blood cells (RBCs) has been shown to attenuate the vulnerability of the cells, and may result in loss of hemoglobin from the cells. Accordingly, one possible explanation is that absorbed phospholipids from MFGM might be incorporated into and stabilize the cell membrane of RBCs.

In response to the reviewer’s comment, we revised the Discussion as follows:

(Discussion, pages 13, line 217)
We also observed increased hemoglobin levels after dietary supplementation with MFGM. This result is also consistent with our previous findings in mice [9]. The reason for the increased hemoglobin after MFGM ingestion was unclear. MFGM is abundant in phospholipids that are critical components of plasma membrane (Table 1). Decreased phospholipid content in the cell membrane of RBCs has been shown to attenuate the vulnerability of the cells [22], which may result in loss of hemoglobin from the cells. One possible explanation is that absorbed phospholipids from MFGM might be incorporated into and stabilize the cell membrane of RBCs. In fact, the number of RBCs tended to increase after MFGM ingestion for 4 weeks (Table 3). Because increased hemoglobin improves oxygen transport and exercise performance [23], dietary supplementation with MFGM might also improve endurance capacity in humans. However, further study is needed to confirm the increase in hemoglobin by dietary MFGM and clarify its underlying mechanism.

5. Considering that carbohydrate levels and the concomitant effect on insulin are key in muscle uptake of nutrients the paper would be enhanced with some report of what was consumed around the time that the supplement was taken.

Response: We agree with the reviewer’s comments that insulin plays a key role in muscular nutrient uptake. In this study, the subjects were instructed to take the test tablet at their favorite time during their daily physical activity. On the exercise training enforcement days, they were instructed to take the tablet within an hour before the training program. One gram of MFGM contains 0.1 g of carbohydrate, which did not increase blood glucose and insulin levels in a preliminary study. Accordingly, the role of insulin signaling in MFGM uptake in the skeletal muscles does not seem to be significant.

In the response to the reviewer’s comments, we revised the Methods section, adding the following sentence to describe the timing of MFGM ingestion more clearly:

(Methods, page 7, line 107)

On the other days, the subjects were instructed to consume the tablet at their favorite time during their daily physical activity.

6. Reporting the degrees of freedom, F and t values would make the statistical analysis more through. This is a small group to have sufficient power for a repeated measures ANOVA, not that this takes away from those that are significant.

Response: We agree with the reviewer’s suggestion and appreciate it.
Following the reviewer’s suggestion, we added the df, F and t values in Tables 3–5 and Results section (pages 10-11) in the revised manuscript.
Reviewer 2:

We sincerely thank the reviewer for the insightful comments about our paper, which we feel have helped us substantially improve the paper.

Major issues
1. The background section is unfocused and needs to be rewritten. The authors highlight problems with muscle wasting and myelination loss in elderly populations despite the fact that the participants in this study were healthy adults under the age of 50. Moreover, the section describing the effects of milk ingestion and resistance training is also not relevant to the current study as concentrations of MFGM are very low in milk.

Response: We agree with the reviewer’s criticisms and regret that we described the background information poorly in the original manuscript.

In response to the reviewer’s comments, we extensively revised the Background section.

(Background, pages 4-5)

Muscle strength is an excellent indicator of general health. Optimal muscle function is important in relation to rehabilitation and quality of life in many musculoskeletal diseases [1-4]. However, muscle strength in healthy men decreases in a linear fashion from the age of 25 years to between 54% and 89% at the age of 75 years [5]. Therefore, the decline of skeletal muscle strength in young adults should be prevented to maintain higher quality of life in their older age.

Resistance training is well known to increase muscle mass and strength [6]. However, high-intensity and long-term resistance training is necessary to achieve satisfactory improvement of muscle mass and strength. Accordingly, more efficient strategies that boost the exercise-induced improvement of skeletal muscles are required to maintain general health for non-athletes. Nutritional supplementation may facilitate more efficient muscle improvement for untrained people, even combined with low-intensity and low-frequency exercise. Recent studies demonstrated that dietary supplementation with amino acids [7] or tea catechins [8], when combined with regular exercise of low intensity, significantly improved muscle mass and strength in the elderly.

Our recent studies in mice have demonstrated that dietary supplementation with milk fat globule membrane (MFGM), when combined with habitual exercise, significantly improved muscle mass and strength [9], as well as swimming endurance capacity [10]. MFGM is the structural membrane covering a triglyceride globule that is dispersed as emulsified bodies in milk [11]. Our previous study revealed that the beneficial effects of dietary MFGM on skeletal muscles was
associated with stimulation of neuromuscular junction (NMJ) development [9], which is a critical structure of a motor unit (a single motor neuron and all of the muscle fibers that it innervates). MFGM supplementation in the diet (1%) combined with voluntary exercise (wheel-running) significantly increased muscle strength and expression of NMJ-related molecules in adult mice [9]; however, whether nutritional supplementation with MFGM may facilitate neuromuscular improvement by regular exercise in humans has yet to be investigated.

The present study aimed to demonstrate the ability of dietary MFGM combined with regular exercise to increase skeletal muscle strength and neuromuscular function in healthy young adults.

2. The authors need to provide a rationale why 1g of MFGM was chosen for the supplemental dose.

Response: We agree with the reviewer’s comment and regret that the reason for the dose of MFGM was poorly described in the original manuscript.

In our previous studies, dietary MFGM significantly improved muscle function (Haramizu et al. 2014a) and endurance capacity (Haramizu et al. 2014b) at the dose of 1000 mg/kg body weight in mice. To convert by calculation based upon body surface area, the dose was estimated to be 4000 mg/60 kg body weight for humans (equivalent to 2400 mL of milk). This exceeds the range of the eating experience of whole milk for Japanese in the long term. Because dietary MFGM supplementation tended to improve the endurance capacity at the dose of 250 mg/kg body weight in mice (Haramizu et al. 2014b), we arrived at the dose of 1000 mg (equivalent to 600 mL of milk), which warrants the nutritional safety for the subjects in the present study.

In response to the reviewer’s comments, we revised the Method section as follows:

(Methods, pages 7, line 103)

The daily dose of MFGM (1 g; equivalent to 600 mL of whole milk) was chosen based on what is known about its nutritional safety and efficacy, after converting the minimal effective dose in mice [10] to a human equivalent, taking into account the relative body surface areas.

3. The MFGM and milk powder composition description would be more appropriate as a table.

Response: We agree with the reviewer’s comments.

Following the reviewer’s suggestion, we added the composition of MFGM and milk
powder in Table 1 of the revised manuscript, and revised the Methods section as follows:

(Methods, page 7, line 111)

The composition of the MFGM and whole milk powder are shown in Table 1.

4. Can the authors provide a rationale or a reference for the leg extension strength and surface electromyography tests? Are these validated protocols?

Response: Both the leg extension strength and surface EMG results have been used to evaluate the muscle strength and neuromuscular function in this research field. We adopted the standard and relevant protocols for the measurements.

In response to the reviewer’s requests, we revised the Method section, adding references (Abe et al. 2003, Ito et al. 2007, Burnley et al. 2012, Roatta & Farina 2011) regarding the measurement of leg extension strength and surface EMG results.

(Methods, page 8, line 135)

The isokinetic extension strength of each leg was the primary outcome and was measured using the StrengthErgo 240 (Mitsubishi Electric Co., Tokyo, Japan) [12, 13].

(Methods, page 8, line 140)

Motor unit activity during the exertion of muscle force was evaluated using surface electromyography (EMG) [14, 15].

5. The authors need to report if there was a difference in baseline between the experimental periods. If the subjects were previously untrained they may have had higher baseline muscle strength in the final experimental period because of the training effect from the first period. Even though the study is a crossover, it would be important to know if there is an experimental period effect.

Response: We agree with the reviewer’s comments and appreciate the valuable suggestion.

There was a significant difference in the baseline value of muscle strength between the first and the second experimental periods. The difference seemed to be caused by the training effect in the untrained subjects.

Following the reviewer’s requests, we stated that there was a training effect on the
muscle strength from the first period in the Results section of the revised manuscript as follows:

(Results, page 10, line 178)

There was a significant difference in the baseline value of muscle strength between the first and the second experimental periods, suggesting that the training influenced muscle strength in the untrained subjects. Nevertheless, the muscle strength at baseline did not differ significantly between the groups in this crossover design study.

6. Much like the background, much of the discussion centers around mechanisms unique to the elderly. The study population was not elderly so the discussion should be framed towards mechanisms relevant to the study population.

    Response: We agree with the reviewer’s criticism and regret that the Discussion was improperly constructed in the original manuscript. In response to the reviewer’s comments, we extensively revised the Discussion section. Following the suggestion from Reviewer 1, we added sentences that describe the potential usefulness of MFGM supplementation for athletes and post-training recovery in the Discussion section. Concomitantly, we revised the Abstract as follows:

    (Abstract, page 2, line 40)

    Dietary MFGM supplementation combined with regular exercise improves skeletal muscle strength, which may be due to increased motor unit recruitment in healthy Japanese young adults.

    (Discussion, pages 12-13)

    This study had two major findings. The first was that daily intake of 1 g MFGM combined with regular, twice weekly exercise improved skeletal muscle strength (leg extension) in young adults, despite a lack of change in muscle mass. Another finding was that dietary MFGM supplementation plus regular exercise also increased the RMS of surface EMG, indicating that dietary MFGM increased motor unit activity during muscle contraction.

    Surface EMG comprises the sum of the electrical contributions made by the active motor units, and the amplitude of the surface EMG is related to the net motor unit activity (i.e., the recruitment and/or the discharge rates of the active motor units) [16, 17]. Improved neurological adaptation in skeletal muscle has been recognized to improve muscle strength earlier than muscle hypertrophy [18]. This muscle reinforcement is accompanied by increased RMS that indicates increased motor unit recruitment, as the involvement of neurological adaptation. Because the leg
muscle mass did not change after MFGM ingestion in the present study, increased muscle strength by dietary MFGM supplementation seems to be due to the increase in motor unit recruitment.

Pathway analysis after transcriptomic measurement in our previous study [9] revealed that dietary MFGM combined with regular exercise improved muscle strength in adult mice primarily by stimulating the pathway involving “neuro-muscular system development” in the skeletal muscle. This pathway includes the functional annotations such as formation of synapse, growth of neuritis, or development of NMJ. Dietary MFGM combined with exercise increased skeletal muscle expression of docking protein-7 (Dok-7) and muscle-specific receptor tyrosine kinase in mice [9], both of which play a critical role in NMJ formation [19, 20]. Defects in NMJ function causes muscle weakness in neuromuscular disorders, and Dok-7 gene therapy improves NMJ formation and rescues the motor activity [21]. The results in the present study are consistent with the previous findings and indicate that dietary MFGM plus exercise increases motor unit recruitment and enhances muscle strength, probably due to the neuromuscular mechanism.

Minor issues
1. Line 34: Awkwardly written sentence

   Response: We changed the sentence as follows:

   (Abstract, page 2, line 35)

   The MFGM group had significantly greater leg extension strength than the placebo group after the 4-week study period.

2. Line 80: Awkwardly written sentence

   Response: We changed the sentence as follows:

   (Methods, page 6, line 79)

   Fourteen male subjects (aged 31 to 48 years) were enrolled in the present study.

3. Line 124: Change 'comprised' to 'consisted of'

   Response: Following the reviewer’s request, we changed “comprised” to “consisted of” in the revised manuscript (page 7, line 120).
4. Line 166- Awkwardly written sentence

Response: We changed the sentence as follows:

(Results, page 10, line 162)

All subjects completed the intervention protocol, and there were no adverse side effects from the ingestion of the test tablets.

5. Line 197- Can the authors explain “nervous system development”

Response: Following the reviewer’s request, we added the following sentences to explain “nervous system development:”

(Discussion, page 12, line 201)

Pathway analysis after transcriptomic measurement in our previous study [9] revealed that dietary MFGM combined with regular exercise improved muscle strength in adult mice primarily by stimulating the pathway involving “nervous system development” in the skeletal muscle. This pathway includes the functional annotations such as formation of synapse, growth of neuritis, or development of NMJ. Dietary MFGM combined with exercise increased skeletal muscle expression of docking protein-7 (Dok-7) and muscle-specific receptor tyrosine kinase in mice [9], both of which play a critical role in NMJ formation [19, 20]. Defects in NMJ function causes muscle weakness in neuromuscular disorders, and Dok-7 gene therapy improves NMJ formation and rescues the motor activity [21]. The results in the present study are consistent with the previous findings and indicate that dietary MFGM plus exercise increases motor unit recruitment and enhances muscle strength, probably due to the neuromuscular mechanism.

6. Table legends need to be more descriptive and should stand alone without looking elsewhere in the manuscript.

Response: In response to the reviewer’s requests, we revised the legends of Tables 2–5 in the revised manuscript.