Title: Supplemental treatment of rheumatoid arthritis with natural milk antibodies against enteromicrobes and their toxins: results of an open-labeled pilot

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Author's response to reviews: see over
**Supplemental treatment of rheumatoid arthritis with natural milk antibodies against enteromicrobes and their toxins: results of an open-labelled pilot study**

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Reviewer 1: Douglas L Schmucker

**Major compulsory revisions:**

**Comment 1.** As this manuscript describes what amounts to a brief, preliminary study, it seems appropriate that its length should be shortened. Since the differences in responses to the primary variable are barely statistically significant (<.05-.09), the Discussion can be shortened.

**Our response:** We agree with the Reviewer’s comment that the manuscript should be shortened. However, this is a clinical study with many variables resulting in large standard deviations. Therefore, removal of detailed information will make more difficult to understand. But, we shortened the discussion section equivalent to 1 page by eliminating the parts of discussion that do not directly relate to this current study, such as probiotics and its physiological mechanism.

**Comment 2:** The authors should clearly state why they did not measure serum titers of anti-cyclic citrullinated peptide antibodies (CCP) as it is a major rheumatoid factor and an index of the disease. This is particularly important since some of the conclusions are based on clearly subjective clinical assessments by both the subjects and the investigators.

**Our response:** ELISA is an immunoassay system based on the strong hydrophobic binding of proteins on plastic surfaces. Immunoglobulins (Ig) in human sera also bind to the ELISA plate in a dose dependent manner. Importantly, blocking agents, such as BSA, gelatin, diluted heterologous serum or casein hydrolysate are not capable of completely blocking the hydrophobic binding of human Ig on plastic surfaces. The sera especially from patients with RA contain immunoglobulins, which have extremely high affinity to plastic surfaces. Therefore, in order to determine specific antibodies to antigens, it is critical to determine the background values of individual human serum specimens in antigen non-coated wells, and subtract the OD values from the OD values in antigen-coated well (See our manuscript: Fujii et al. An improved enzyme-linked immunosorbent assay of anti-collagen antibodies in human serum. J Immunol Methods. 13:63-70, 1989).
This important concept has not been taken in consideration in any assay systems. The anti-CCP antibody assay system is not an exception. It is highly likely that the results obtained by these current assay systems contains a large part of non-specific reaction values, and mislead and create serious consequences in both research and clinical field. We hope we can improve the current assay system by our efforts, and be able to assay anti-CCP antibodies in our future studies.

**Minor essential revisions:**

**Comment 1:** While this reviewer recognizes that certain deficiencies in this study cannot be corrected at this time, some concerns may be assuaged by addressing them in the text or muting the interpretation or conclusions. For example, the authors chose to measure serum anti-LPS IgA antibodies, yet serum IgA titers are rarely indicative of this particular isotype response at the primary site, e.g., the gastrointestinal tract or other mucosal surfaces. A more precise measurement would have been to monitor the IgA titers in colonic effluent, e.g., GoLytley-induced intestinal lavage (Taylor et al., Immunology 75, 614, 1992)

**Our response:** We are afraid that this concern was raised by both reviewers based on a miss understanding, which may be caused by our poor explanations. These data are pre-clinical data at the starting point of this study, and do not indicate the effect of milk antibody treatment on these marker values. These values were determined for searching the fundamental differences between the responders and non-responders, because the antibodies to dietary or intestinal components will indicate the mucosal permeability of individual patients.

We have revised the paragraph in both the abstract and results sections for clarifying our aims. In addition, we also revised the paragraph in the discussion section to emphasize the importance of these clinical markers for studying the potential pathogenesis of intestinal bacteria and their toxins.

We believe that the colon will be the most important part for endotoxin translocation rather than small intestine, and agree that it will be important to measure the IgA antibody levels in the colonic effluent for studying the physiological roles of IgA, but our experimental purpose in this current study was not for studying the roles of IgA antibodies produced in the intestinal tract.

**Comment 2:** The subjects are all aged 59 years or older. It is fairly well established that the intestinal flora in the elderly is more subject to overgrowth by one or more commensal species of bacterial than is the case in the younger population. This may reflect, in part, the documented intestinal immunosenescence in old animals and geriatric patients. Such shifts in the balance of normal intestinal flora in the elderly may contribute to the exacerbation of RA, yet the authors fail to discuss this issue.

**Our response:** We agree with the reviewer’s opinion. In the discussion section, we addressed the beneficial effects of milk antibodies in elderly who do not respond to immunostimulants to distinguish from the effect of kefir and other probiotics.

**Comment 3:** Although probiotic foods have been shown to alleviate certain gastrointestinal disorders, the mechanism responsible have not been resolved. Interestingly, feeding kefir to young adult rats immunized intraduodenally with cholera
holotoxin resulted in significantly higher serum anti-cholera toxin IgA titers and in vitro anti-cholera toxin IgA secretion by intestinal lamina propria lymphocytes. However, kefir had no apparent effect on either parameter in senescent rats. (Thoreux et al., Communicating Current Research and Educational Topics and Trends in Applied Microbiology, A. Mendez-Vilas (ed), Formatex, Spain, 2007).

Thus, the authors may wish to briefly discuss the possibility that age per se may be a factor in the response to the milk antibodies.

**Our response:** This comment is relating to Comment 3. In the discussion section, we have addressed the difference of possible mechanisms of milk antibodies and probiotics that provide the beneficial effects, especially for elder patients with immunosenescence.

**Comment 4:** There are several typographical error and inappropriate syntax in the cur
**Our response:** The revised manuscript was reviewed by a native English speaker with Ph.D.

**Comment 5:** The authors refer to this as a "quasi-randomized" study (pg. 5). What is meant by this phrase, i.e., how is it not random?
**Our response:** The patients enrolled in this study were chosen by a physician, because their diseases are uncontrollable by authentic anti-rheumatic drugs due to drug resistance, complications and/or risk factors. Since these patients required immediate care and treatment, patients were received a supplemental treatment with milk antibodies in the order of visiting date to the hospital. This is one example of clinical study design, which has been accepted as a randomized study.

**Comment 6:** The description of the modified ACR response criteria and the ad hoc evaluation point is confusing to this Reviewer.
**Our response:** The wording “The modified ACR response criteria” used in the text was our mistake due to our English barrier. We actually did not use the modified ACR response criteria for evaluating patients. Instead, we set up an ad hoc “Evaluation Point” for this particular study using 8 core variables listed in the ACR response criteria. The wording “modified ACR response criteria’ used in the result section was replaced with “evaluation point”.

**Comment 7:** The finding that the serum titers of inflammatory cytokines were not affected by the milk antibodies, yet the titer of anti-collagen antibodies increased dramatically requires some explanation, Could this not reflect an increased autoimmune response against synovial cartilage in the "responder" group?
**Our response:** This comment relates to the comment 1. We are afraid that this concern was raised by both reviewers based on a miss understanding caused by our poor explanations. These data are pre-clinical data at the starting point of this study, and not the results after milk antibody treatment to search for the fundamental differences between the responders and non-responders to milk antibody treatment. We have revised the paragraph in both of abstract and result sections relating to antibodies to type II collagen and LPS. In addition, we also revised the paragraph in discussion section to emphasize the importance of these clinical markers for studying the potential pathogenesis of intestinal bacteria and their toxins.
**Comment 8**: Figures 2 and 3 are very difficult to understand based on the brief figure legends provided. What do the thin lines signify in Figure 3?

**Our response**: We have revised Figure 2 and 3, and then combined all 3 graphs into Figure 2. Accordingly, we have also revised the legends with a note as follows:

Figure 2. Transitional changes of CRP (a), ESR (b) and DAS-ESR (c) before, during and after the repeating treatment with milk antibodies with 4 months resting period

Thin lines with ρ: Patient ID198, □: ID3188, ○: ID3240, ▲: ID3709, □: ID4119, and

Heavy line with ★: Average ± SE, *: P<0.05 (compared to the values at the starting point of milk antibody treatment), Shadow area: Treatment period with milk antibodies.

☆: Constipation worsened during secondary treatment with milk antibody (ID3188).

NOTE: CRP and ESR values of 5 patients who received the second treatment among the 8 responders (Figure 1) were plotted in Figure 2. Due to abnormal CRP and ESR values in one patient (ID4119) at the 3rd month of the second treatment, the average values of both CRP and ESR at the end of the second treatment seem to increase slightly.

**Comment 9**: Also in Figure 2 it appears that the serum C-reactive protein titer actually begins to increase during both the first and second milk treatments. Furthermore, both the C-reactive protein level and the RBC sed rate begin to decline between the first and second treatments. These seemingly incongruent results require some explanation since they suggest changes in the absence of the milk antibodies.

**Our response**: This relates comment 8. We have revised Figure 2 and 3 as described above.
Reviewer 2: Susanna Rokka

Minor Essential Revisions
Comment 1: Check the symbols in figures/figure legends
Our response: We have revised Figure 2 and 3, and then combined all 3 graphs into Figure 2.

Discretionary Revisions
Comment 2: Are the other whey proteins present in concentrate? How can you know that it is the antibodies that are behind the effect? What about lactoferrin, growth factors, enzymes, complement or peptides?
Our response: This whey protein concentrate used for this study contains various kinds of biologically active ingredients, and thus it cannot be denied that other components are involved in beneficial effects of milk antibody treatment in this study. However, it is apparent that none of these components except antibodies have a blocking activity on bacteria growth. Our previous date clearly indicated that native IgG enriched whey protein is capable of reducing the population of certain pathogenic bacteria in feces: See a graph in the additional file attached (Iwatsuki et al: manuscript was submitted). Further more, lactoferrin content in the whey protein concentrates is only 15 mg, which is significantly lower than 300 mg, which is the expected effective dose of lactoferrin (www.brinkzone.com/article/lactferrin-the-bioactive-peptide-that-fights-disease/).

Comment 3: Please, clarify why milk antibodies raise the levels of serum antibodies and what is their effect on immune response.
Our comment: We are afraid that this concern was raised by both reviewers based on a miss understanding caused by our poor explanations. These data are pre-clinical data at the starting point of this study, and do not indicate the effect of milk antibodies on these marker values.
We have revised the paragraph in both the abstract and results sections. In addition, we also revised the paragraph in the discussion section to address the potential difference in the mucosal permeability between responders and non-responders, which might influence the IgA and IgG anti-LPS antibody levels.

Comment 4: The microbial flora varies significantly in different parts of GI tract. The fecal bacteria don’t tell very much about the situation in small intestine.
Our comment: We believe that colon will be the most important part for endotoxin translocation rather than small intestine.
Please see references listed bellow.

Comment 5: What is the connection of probiotics (page 14) to milk antibodies?
Our response: Milk antibodies are not considered as a probitocs, thus we eliminate the paragraph talking about the probiototics in the discussion section (Page 14) to avoid unnecessary confusions. Instead, we added a paragraph to discuss the mechanisms of how milk antibodies provide the beneficial effects for elderly patients with immuno-senescence.

Comment 6: There are a number of studies that show that bovine IgG1 is quite resistant to pepsin cleavage. Refer for instance Petschow 1994, Roos 1995, Warny 1999.
Our response: We added references suggested by the reviewer.