Author's response to reviews

**Title:** Vitamin D3 and Gargling for the Prevention of Upper Respiratory Tract Infections: A Randomized Controlled Trial

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Dear Mr. Nazareno,

Thank you for reviewing our manuscript titled “Vitamin D3 and Gargling for the Prevention of Upper Respiratory Tract Infections: A Randomized Controlled Trial” (MS 2300874421067077). We appreciate the thoughtful comments and questions raised by the reviewers. We have addressed these issues and enclose detailed responses below to each comment along with a revised manuscript.

Thank you for the invitation to resubmit our manuscript. We appreciate the ongoing consideration of our work for publication in BMC ID.

Sincerely,

Emma Goodall, on behalf of the authors
Reviewer 1 Major Revisions:

1. Background. Very short background, should be expanded and also included some of the more recent RCTs on vitamin D and RTI. In particular, the studies by Bergman et al (BMJ Open, 2012) and by Rees et al (CID, 2013) should also be discussed here. In fact, REFs 11-18 are not correctly commented upon. The sentence “many of these trials were post-hoc analyses // other were limited by small sample size // and relatively low dose” it not correct. The studies referred to are very heterogeneous also with regard to study population, length of the study and dosing schedule. This could be clarified better. Moreover, it is not clear to me what the authors actually mean when they state that “more rigorously designed trials” are needed? Please, explain what kind of studies that are warranted and expand this para.

Thank you for your constructive comments and suggestions to strengthen our manuscript. We welcome the opportunity to incorporate the results from the study by Rees et al., which was not published before we submitted this manuscript for review. We have revised our background section on page 3 to include all currently published randomized controlled trials investigating vitamin D for the prevention of URTI and to acknowledge the substantial heterogeneity among these trials. We have also clarified our concluding sentence to recognize the need for large, rigorously designed clinical trials to investigate the effect of vitamin D₃ on URTI in a healthy population. The revision on page 3 is as follows, with new text underlined:

“Results from several trials of vitamin D₃ supplementation report reduced risk of infection, but only three studies have reported statistically significant findings.[12-21] The lack of consensus among these studies may reflect substantial heterogeneity across trials resulting from variation in participant populations, degree of vitamin D deficiency, the dose and duration of vitamin D supplementation, and definitions of URTI.[12-21] Additionally, several trials were limited by retrospective data collection and post-hoc analyses of trials with non-respiratory outcomes.[12-14] Consequently, there remains a need for rigorously-designed, large clinical trials to investigate the effect of vitamin D₃ on URTI in a healthy population.”

Additionally, we have added new information about the studies by Rees et al., and Bergman et al. in the discussion on page 12. The revision on is as follows, with new text underlined:
“Rees et al. performed a substudy of 759 participants in a trial of colorectal adenoma chemoprevention. Participants were randomized to receive 1000 IU vitamin D3, calcium carbonate, both or placebo daily and were followed for an average of 13 months which covered two winter seasons. The authors reported that supplementation did not significantly reduce the incidence of URTI.[21] Bergman et al. followed 140 adults with increased susceptibility to respiratory infections at an immunodeficiency clinic. After one year of daily supplementation with either 4000 IU vitamin D$_3$ or placebo, the authors reported a significantly lower infection score in the vitamin D$_3$ group.[20]”

2. Methods/Study design: How did the study team deal with consent from legal guardians from participants below the legal age in Ontario? (18 or 21 for clinical study consent in Ontario?).

In Canada, the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada and the Social Sciences and Humanities Research Council of Canada jointly authored the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS). This document, now in its second edition, is the government’s official human research ethics policy and these guiding principles are upheld by individual research ethics board. TCPS 2 does not rely strictly on “age of majority” to determine whether an individual can consent to participate in research. Rather, the critical factor is whether or not the individual has the capacity to understand the significance of the research and the potential risks, benefits and implications associated with their participation, as well having the capacity ability to make this decision on their own behalf. In the context of this study, university aged students were deemed capable of making this decision. This study, including the informed consent process, was approved by the Hamilton Integrated Research Ethics Board.

3. Methods/Study design: were only those on vitamin D above 1000 IU/day excluded? In this case, how many were taking vitamin D and which doses were used? This could be of great importance for the interpretation of the study. Rees et al (CID, 2013) had the same acceptance for vitamin D supplements and encountered some problems (also null effect). Please, refer to Rees et al and expand this very important point in the discussion. In fact, if the placebo-group were diluted with vitamin D takers, it could very well explain the null effect of the current study.
We very much appreciate your comment about the potential for vitamin users in the placebo group to dilute the effect of the vitamin D intervention and contribute to the non-significant effect observed in this study. The process of randomization resulted in slightly more baseline vitamin users in the vitamin D intervention arm compared to the placebo arm (23% and 18% respectively). As such, we don’t believe that baseline vitamin use contributed to the non-significant effect observed in this study.

We sought to conduct a pragmatic trial which would reflect the way this intervention might be used, as an addition to current practice, by the general public. We therefore chose to include individuals who were taking vitamin D either in combination with any other vitamin or mineral, or in isolation so long as the daily dose was less than or equal to 1000 IU. At the time of enrollment, prospective participants were screened for this criterion however, exact details of their vitamin use were not recorded. Similarly, although the baseline survey asked participants about their daily vitamin use the details about dose were not recorded. The majority of participants who reported vitamin use reported taking a multivitamin. In Ontario, most multivitamins provide 600-800 IU vitamin D3 per dose, and are recommended to be taken once daily.

4. Discussion, page 11: “further meta-analysis // should be conducted”. Please, explain how additional meta-analyses could move this field forward. In fact, there is a strong need for additional interventional studies, just like the current study by Goodall et al.

We agree with the reviewer that there is a need for more interventional studies to investigate the effect of vitamin D supplementation on respiratory outcomes. However, as we discuss in our concluding paragraph we also feel that a meta-analysis of all current studies would be useful to inform the design of additional interventional studies. An individual patient data meta-analysis would be particularly beneficial for the evaluation of potential interacting variables such baseline serum vitamin D levels, dosing regimens and age. We have modified this sentence on page 13 to read “Although these results support the use of vitamin D₃ supplementation, further meta-analysis, particularly an individual patient data meta-analysis, including the results from more recent trials and an exploration of heterogeneity between trials should be conducted.”

5. Discussion. It is somewhat odd to draw conclusions and to reason along lines based on non-significant results, such as “the vitamin D group appeared to experience more severe symptoms”. I would not focus
on this non-significant finding although it is an interesting topic if it could be shown to hold true (remove?).

Thank you for your thoughtful comment. We thought this result, despite being non-significant, may be of interest to readers and we chose to provide some hypotheses which could explain the observation and, potentially, inspire future investigations. However, we appreciate your perspective and recognize that this might distract the reader from the true merit of the study and we have decided to remove this topic.

6. The study does not evaluate the vitamin D levels, not at baseline, not during the study or not at the end of the study. This is a major flaw of the study, since it is becoming increasingly clear that healthy individuals with a sufficient vitamin D status does not benefit from extra vitamin D. This is particularly well described in the study by Murdoch et al. In contrast, the studies by Camargo et al and Bergman et al, where there is vitamin D deficiency and a non-healthy population, show small (23-50%) but statistically significant effects. It is quite possible that vitamin D-supplementation could be even more beneficial in a VitD-deficient subgroup in the current study. However, since no such information is available we cannot know. Please, comment this further in the discussion section.

We agree that not measuring serum vitamin D levels is a limitation to our study and we have acknowledged this in our discussion. However, we chose not to include blood tests since we sought to conduct a pragmatic study that reflected the way vitamin D supplementation is used by the Canadian general public. Serum vitamin D testing in healthy individuals is not routinely available as part of universal health care and if vitamin D supplementation had the potential to be a new public health campaign, individuals would not have blood tests before initiating use. Although it certainly would have been interesting to have this data, particularly to be able to evaluate the potential interaction between baseline serum vitamin D levels and supplementation, we felt it was a reasonable limitation rather than a major fault. We have expanded our paragraph about study limitations on page 14 to include the following sentence “An additional limitation was the lack of serum vitamin D testing without which we were unable to investigate whether individuals with lower baseline vitamin D levels benefitted incrementally compared to individuals with sufficient baseline vitamin D levels.”

7. Discussion. Page 12. The discussion on dosing and that “larger and less frequent doses may be an effective alternative” is not fully correct and updated with regard to RTIs. It could well be true for other
indications apart from infections, such as bone health. However, Bergman et al discuss this point in detail (Bergman et al, PLoS One, 2013) and argues along the opposite line, i.e.that a daily dosing schedule is preferable to a bolus schedule. Please comment.

Thank you for sharing your thoughts on this important topic. Currently, there is no consensus about the optimum dosing regimen for vitamin D supplementation for respiratory health and we believe that this remains one of the most important topics for future investigation. As Bergman et al (PLoS One, 2013) acknowledged, there is a tradeoff between frequency of dosing and potential compliance. As the authors wrote, weekly supplementation might be a successful dosing regimen: “However, a bolus scheme could be preferred when compliance is expected to be poor. For example, dosing schemes once a week may be a good compromise to improve effect compared to bolus doses while still facilitating compliance.” In a student population, we anticipated that compliance would be a challenge and consequently we chose to use the weekly dosing strategy. As we acknowledge in our concluding paragraph, more trials are needed to investigate optimal dosing strategies.

8. Conclusion. I do not agree on the final sentence in this manuscript. In fact, additional meta-analyses would not be informative. The field is in great need of larger interventional studies in different study populations.

We agree with the reviewer that large interventional studies are needed to better understand the relationship between vitamin D supplementation and respiratory health. However, we also believe that an updated meta-analysis which includes all new available data, and particularly an individual patient data meta-analysis, would help inform the design of future clinical trials. We have modified our concluding sentence on page 15 to read “A rigorous systematic review and meta-analysis of current studies, particularly an individual patient data meta-analysis which would facilitate the evaluation of potential variable interactions, would be instrumental to the design of future trials in this field which may need to include a larger sample size, longer period of follow-up or different dosing regimens, and targeted populations who may benefit disproportionately from vitamin D supplementation.”

9. Table 1: Between 14-26.7% was taking vitamin D according to this table. What doses? How did this affect the study outcome and why was this allowed in the study?

We sought to conduct a pragmatic trial which would reflect that way this intervention might be used, as an addition to current practice, by the general public. We therefore chose to include individuals who
were taking vitamin D either in combination with any other vitamin or mineral, or in isolation so long as the daily dose was less than or equal to 1000 IU. At the time of enrollment, prospective participants were screened for this criterion however, exact details of their vitamin use were not recorded. Similarly, although the baseline survey asked participants about their daily vitamin use the details about dose were not recorded. As noted by the reviewer, the proportion of participants who reported using any kind of vitamin ranged from 14% to 26.7% across the four trial arms. The majority of participants who reported vitamin use reported taking a multivitamin, and in Ontario, most multivitamins provide 600-800 IU vitamin D3 per dose, and are recommended to be taken once daily.

We don’t believe that allowing these individuals to participate in our study had a negative impact on our study. Rather, we believe that including these individuals was consistent with our pragmatic study design and facilitated participation since individuals did not have to stop practicing their current personal health routines. It is possible that if the placebo arm included a high number of baseline vitamin users the effect of the interventional vitamin D supplementation would have been diluted and this could have contributed to a null effect. We do not believe that this risk was present in our study as the process of randomization resulted in slightly more baseline vitamin users in the vitamin D intervention arm compared to the placebo arm (23% and 18% respectively).

**Reviewer 1 Minor Revisions:**

1. Abstract. The intervention should be mentioned here, ie the dose and time of vitamin D.

Thank you for identifying this omission, the abstract has been revised to include the dosing regimen. The revision is as follows, with new text underlined:

“We undertook a 2X2 factorial, randomized controlled trial (RCT) to assess whether vitamin D$_3$ supplementation (10,000 international units per week) versus placebo and gargling versus no gargling could prevent viral, clinical upper respiratory tract infection (URTI) in university students.”


Thank you for this comment which will improve the clarity of our abstract. We have revised the abstract to identify the primary and secondary outcomes of interest. The revision is as follows, with new text underlined:
“We randomized 600 students into 4 treatment arms: 1) vitamin D₃ and gargling, 2) placebo and gargling, 3) vitamin D₃ and no gargling, and 4) placebo and no gargling. Students completed weekly electronic surveys and submitted self-collected mid-turbinate nasal flocked swabs during September and October in 2010 or 2011. Symptomatic students also completed an electronic symptom diary. The primary and secondary outcomes were the occurrence of symptomatic clinical URTI and laboratory confirmed URTI respectively.”

3. Abstract. It should be defined that URTI only includes viral infections and that bacterial infections is not included in the definition.

Thank you for this constructive comment, we have adjusted the abstract to clarify that this study was interested in the effect of vitamin D supplementation for the prevention of “…viral, clinical upper respiratory tract infection…” We have also revised the “Outcome Measures” section on page 7 to clarify that we did not test for bacterial agents. The revision is as follows, with new text underlined:

“Laboratory confirmed illness was determined by testing the Day 1 nasal swabs using an in-house enterovirus/rhinovirus polymerase chain reaction (PCR) and, if negative, a commercial multiplex PCR able to detect 16 respiratory viruses and viral subtypes (xTAG RVP FAST, Luminex, Austin TX). No testing was performed to identify bacterial agents.”

4. Please explain the rationale for performing a study where gargling and vitamin D is combined.

From the literature, both vitamin D supplementation and gargling with tap water appeared to be promising interventions for the prevention of URTI. Both interventions are safe, inexpensive and easy to carry out and both represent potential interventions that could benefit public health in Canada. Conducting a factorial randomized controlled trial allowed us to investigate both interventions simultaneously and maximize the efficiency of running a clinical trial rather than conducting two separate trials. With these interventions, we felt it was reasonable to accept the assumption in our analysis that the effect of vitamin D would not differ depending on whether or not a participant was also gargling. To be rigorous, we tested for these interactions and none were identified.

5. Methods/Study design: Did the participants ever pass by a hospital outpatient clinic? Were there any contacts with doctors involved prior to inclusion? Please specify.
Our study did not include a consultation with any health care provider as part of the standard procedures. The study did not limit the participants’ ability to seek medical care at any point during the study. The definition of our primary outcome, clinical URTI, was a self-reported outcome dependent on the presence of symptoms and did not require consultation with or validation by a health care provider.

6. Method/intervention: please describe where the vitamin D and placebo came from, which company, country? Were there any financial connections between the study team and the company? Please specify and explain.

The study team purchased the vitamin D in the form of Euro D 10,000 IU tablet manufactured by Euro-Pharm International Canada Inc. Each tablet was over-encapsulated with a gelatin capsule and packed with calcium carbonate to prevent the tablet from moving within the capsule. The same gelatin capsules were filled with calcium carbonate to make aesthetically matched placebo capsules. The over-encapsulation process and development of the placebo pills was carried out by a research pharmacist at McMaster University. There were no financial connections between the study team and Europharm. The information about the placebo contents has been added to the “Interventions” section of the manuscript on page 6. The revision is as follows, with new text underlined:

“The vitamin D was purchased from Euro-Pharm International Canada Inc. and the placebo contained only calcium carbonate. The pills were made aesthetically identical through use of a gelatin capsule.”

7. Method/assessment: please describe the survey, what questions were asked?

The weekly survey contained 11 questions which screened for symptoms of URTI and asked the participant if (s)he had been sick in the past seven days. The participant was also asked if (s)he had taken the study pill and if any housemates had been sick during the previous week. The remaining questions inquired about health and lifestyle factors such as the frequency of hand washing, quantity of exercise, quantity of sleep, changes in health, and any absences from school. A single question about frequency of gargling was inserted among the health and lifestyle questions and all participants received the same set of questions.

8. Since seven consecutive swabs were collected, the results from this interesting material would be relevant to disclose. How was the kinetics here? Which criteria were used to define a positive swab, one positive day / 7 or 7/7. Please expand.
To establish confirmation of a viral pathogen, our study protocol dictated that only the first swab collected during an episode of URTI would undergo laboratory testing. A swab that was submitted from a symptomatic individual was first tested using an in-house polymerase chain reaction (PCR) to detect rhinovirus/enterovirus. If the swab was negative after the in-house PCR, it was tested using a commercial multiplex PCR able to detect 16 respiratory viruses and subtypes (xTAG RVP FAST, Luminex, Austin TX). If a swab was negative after both tests, no additional swabs were tested. Routine testing of each series of swabs was not feasible within our financial and human resources. To clarify this issue, the sentence on page 7, has been modified to read “Laboratory confirmed illness was determined by testing the Day 1 nasal swabs using an in-house enterovirus/rhinovirus polymerase chain reaction (PCR) and, if negative, a commercial multiplex PCR able to detect 16 respiratory viruses and viral subtypes (xTAG RVP FAST, Luminex, Austin TX).”

The collection of serial swabs was intended to facilitate the investigation of the persistence and rate of viral shedding, however this is beyond the scope of the current manuscript. A small sample of serial swabs which have been tested by PCR has revealed that the same viral pathogen can be detected up to seven days from the onset of symptoms, but not longer than fourteen days. However, no data is available to address the question of what proportion of episodes which were negative based on testing the first swab, may have been positive if subsequent swabs were tested. This presents an interesting question to be considered for future analyses, however it is beyond the scope of this paper and the manuscript has not been modified.


9. Method/assessment: it would be extremely interesting to report also the viral findings from those not having symptoms. It is well known that the presence of a virus does not always connect to clinical symptoms. A ‘Devil’s Advocate’ could argue that vitamin D affects the general well-being in yet undescribed ways and that symptoms and the presence of viruses are not connected. The design of 7 consecutive samples from symptomatic individuals could thus inflate vitaminD-mediated effects on the general health. A thorough description of viral findings also from asymptomatic persons could help to clarify these matters. Likewise, it is interesting to note that effect on symptoms (1’ endpoint) was not
significant but viral findings (2’ endpoint) and viral load (which are based on symptoms, a priori) were highly significant. How do the authors explain this? This could be discussed more in detail.

We share your interest in better understanding asymptomatic infections and intend to conduct some analyses using the swabs collected in this study, however that analysis will form a separate manuscript. In a separate study of a subset of asymptomatic participants, which is currently under review, we demonstrated that asymptomatic infections were common in the university student population. These asymptomatic infections were associated with statistically significantly lower viral loads, and viral load was significantly correlated with symptom presence. The collection of serial weekly nasal swabs from each participant was intended to facilitate the investigation of asymptomatic infection through future analyses and manuscripts. Because only one swab was tested for each event, the risk of inflating vitamin-D mediated effects with serial swabs is not a concern in this study.

Although the effect on the primary outcome, clinical URTI, was not statistically significant the point estimate was in favour of the vitamin D intervention which is consistent with the statistically significant effect observed on laboratory confirmed illness. As we hypothesized in the discussion, it is possible that our definition of a clinical URTI was too broad and allowed for some misclassification of reports which contributed to a non-statistically significant result.

10. Methods/statistics: please describe which statistical analyses were predefined and those that were chosen after the study was completed.

We a priori planned regression analysis of the primary and secondary outcomes to account for randomization strata and interventions a priori. We also a priori planned the analysis of the duration and severity of symptoms. After the first year of the trial, and before any analysis was conducted, we revised the analysis plan to include the analysis of viral load. We have revised our description of the statistical analysis on page 8 to clarify this issue. The revision is as follows, with new text underlined:

“An identical complete-case analysis was conducted to assess the secondary outcome of laboratory confirmed infections. Symptom severity and viral load (log viral copies/mL) were compared by t-test. Cox regression was used to assess time to symptom resolution adjusted for the variables listed above. All analyses were planned a priori.”

11. Methods/statistics: How was the result from the ‘per-protocol’ analysis?
Throughout the manuscript, we presented results from both an intention-to-treat analysis and a complete case analysis. The complete case analysis supported the same interpretation as the intention-to-treat analysis and the point estimates of effect were consistently very similar.

12. Discussion: page 10. The sentence “previous trials in adult populations have not yielded statistically significant results” is not correct, since the study by Bergman et al provided data on modest, but statistically significant effects. If the sentence above include the word “healthy” it is correct, since the study by Bergman et al included patients with primary immune deficiency and frequent RTIs. Please comment and correct.

Thank you for your detailed review and comments. We agree that this sentence does not accurately reflect the current published literature and we have revised the sentence on page 11 so that it clearly acknowledges that statistically significant results have not yet been observed in healthy adults. Furthermore, we have revised the preceding sentence to acknowledge the results of Bergman at al., 2012. The revision is as follows, with new text underlined:

“Two placebo controlled RCTs in pediatric populations and one trial in adults with primary immune suppression have demonstrated that vitamin D₃ significantly reduced the risk of clinical and lab-confirmed URTI and improved annual infection scores, respectively.[17, 18, 20] However, previous trials in healthy adult populations have not yielded statistically significant results.”

13. Likewise, the sentence “consistent with previous studies in adults...” is erroneous in line with the statement above.

As above we have revised the sentence to reflect that our results are consistent with previous studies in healthy adults. The revision is as follows, with new text underlined:

“Consistent with previous studies in healthy adults, our primary analysis did not show that vitamin D₃ significantly reduced the risk of clinical URTI;...”

14. Discussion/Result: it would be informative to have information on other viruses, apart from rhino/entero-viruses.
We agree that it would be interesting to investigate the potential effect of vitamin D on infections caused by viruses other than rhinovirus and enterovirus. Due to the timing of our study, rhinovirus and enterovirus were the predominant pathogens in circulation. Our testing protocol was designed to identify other pathogens, and did identify one case of coronavirus NL63, however our study did not capture infections caused by a variety of viral pathogens.

15. Again, I miss information on the viral findings.

As described in the results section, laboratory testing identified viral pathogens in 70 infections, with rhinovirus being the predominant viral pathogen (61 cases), six cases of enterovirus and one case of coronavirus NL63. This study was conducted during the months of September and October when rhinovirus and enterovirus were the dominant pathogens, consequently there was a lack of infections caused by other viruses.

Reviewer 2 Major Revisions:
1. Almost one-third of the URTI events (44 out of 150) were adjudicated – please explain the adjudication process. Did the two clinician’s contact the subjects? How did the clinicians decide whether the subject had URTI or not?

Participants were asked to report the onset of URTI symptoms directly to study staff. In addition, each week participants received a short online survey with screening questions for URTI. If a participant replied that (s)he was unsure if (s)he was sick, and did not report an episode of URTI during the subsequent week, the record went through the adjudication process. Two independent clinicians reviewed the records and considered the reported symptoms. A record was considered an adjudicated event if the following criteria were met: 1) a minimum of two symptoms, one of which was nasal congestion, sneezing, cough, sore throat, and wheezing, were reported, and 2) there was an absence of additional comments that attributed the symptoms to another cause. The participants were not contacted by any study staff. The independent clinicians who performed the adjudication process had perfect agreement. To clarify this process, the following sentence has been added on page 6: “These reports were deemed adjudicated events if 1) at least two symptoms were reported and included one of nasal congestion, sneezing, cough, sore throat, and wheezing, and 2) no additional information was provided that attributed the symptoms to another cause.”
2. Please explain which nasal swab was cultured – was it from day 1 of 7 consecutive daily nasal swabs from symptom onset?

To establish confirmation of a viral pathogen, our study protocol dictated that only the first swab collected during an episode of URTI would undergo laboratory testing. A swab that was submitted from a symptomatic individual was first tested using an in-house polymerase chain reaction (PCR) to detect rhinovirus/enterovirus. If the swab was negative after the in-house PCR, it was tested using a commercial multiplex PCR able to detect 16 respiratory viruses and subtypes (xTAG RVP FAST, Luminex, Austin TX). If a swab was negative after both tests, no additional swabs were tested. Routine testing of each series of swabs was not feasible within our financial and human resources. To clarify this issue, the sentence on page 7 has been modified to read “Laboratory confirmed illness was determined by testing the Day 1 nasal swabs using an in-house enterovirus/rhinovirus polymerase chain reaction (PCR) and, if negative, a commercial multiplex PCR able to detect 16 respiratory viruses and viral subtypes (xTAG RVP FAST, Luminex, Austin TX).”

3. When did the URTIs occur? The events were recorded 1 week after randomization – was the frequency of URTIs the same throughout the 8 weeks? Were there less infections after more doses of vitamin D in the vitamin D group? Would you expect vitamin D supplementation to work this quickly and in such a short duration (8 weeks)?

The URTIs occurred across the study period with varying frequency however the total number of self-reported and laboratory confirmed infections peaked during calendar weeks 39, 40, and 41. Although our study was not designed to compare the weekly frequencies of infections according to the treatment arms, the proportion of URTIs reported in the vitamin D group relative to the total number of URTIs tended to decrease across the study period. This may have been related to increased exposure to vitamin D, however our study was not designed to statistically evaluate this hypothesis.

As presented in the Discussion section on page 13, we initiated our study in early September which allowed us to both capture peak rhinovirus activity and to initiate vitamin D supplementation before the natural decline in serum vitamin D levels could occur following seasonal summer highs. Consequently, the vitamin D supplementation was not intended to overcome depleted vitamin D levels, which may have required a longer period of time than our study provided. Our study was designed to maintain, or even enhance vitamin D levels, however in the absence of blood tests this cannot be proven.
Reviewer 2 Minor Revisions:

1. Under “Background” 1st paragraph, I would mention that URTIs are caused by a number of viruses. I would also mention that the peak of rhinovirus infection is in September. This would explain why the study was done in September to October.

Thank you for this thoughtful suggestion. We have added the following sentence on page 4: “Multiple respiratory viruses are known to cause episodes of URTI, however rhinovirus has consistently been identified as the most common cause of the common cold.” The natural peak in rhinovirus was addressed in the discussion and consequently we did not add this information to the Background section.

2. Under “Discussion” 4th paragraph, if vitamin D supplementation reduced rhinovirus load, why did the vitamin D group experience more severe symptoms? How could your explanation of vitamin D preventing “milder infections” be valid if you are referring to rhinovirus infection? This leads to another question: does severity of URTI symptoms correlate with viral load? One thing to take note of is that though the common cold is caused by viruses, the symptoms are caused by the body’s immune response to the virus.

It would be interesting to know the cultures from the asymptomatic subjects. I am sure that if their nasal swabs were cultured we would find positive cultures. We may view these positive cultures as contaminants or the asymptomatic subjects may not have had an inflammatory response to the viruses.

More research is needed to understand whether or not vitamin D has any impact on symptom severity. Despite identifying a statistically significantly lower viral load in participants randomized to vitamin D compared to placebo, we also observed greater symptom severity scores among those randomized to vitamin D compared to placebo, although this difference was not statistically significant. We hypothesized that vitamin D may have completely prevented milder infections, perhaps by preventing asymptomatic infections from becoming symptomatic, and that this effect would not depend on the viral agent. Alternatively, we hypothesized that vitamin D supplementation may have enhanced intracellular killing and the associated inflammatory responses which could have resulted in increased symptom severity and lower viral load. Much more research is needed to investigate this possible effect of vitamin D on symptom severity. We thought this result, despite being non-significant, may be of interest to readers and we chose to provide some hypotheses which could explain the observation and,
potentially, inspire future investigations. However, we now feel that this topic might distract the reader from the true merit of the study and we have decided to remove this topic.

We share your interest in better understanding asymptomatic infections. In a separate study which is currently under review, we demonstrated that asymptomatic infections were common in the university student population. These asymptomatic infections were associated with statistically significantly lower viral loads, and viral load was significantly correlated with symptom presence. The collection of serial weekly nasal swabs from each participant was intended to facilitate the investigation of asymptomatic infection through future analyses and manuscripts.

Reviewer 2 Discretionary Revisions:

Why is there an asterisk (*) next to Vitamin Use in Table 1? I do not see a comment for the asterisk.

Thank you for identifying this typo, we have removed this unnecessary asterisk.

Reviewer 3 Major Revisions:

1. [Page 4 line 18] Open permuted-block randomization of 1:1:1:1 adopted in this study might allow the participants or the investigator to anticipate the next participant’s allocation of gargling. Did the authors take measures to prevent a violation of allocation concealment?

To maintain allocation concealment, the randomization scheme was only known by study personnel who were not involved in the enrollment and randomization of participants. Allocation was concealed in opaque, sealed, serially numbered envelopes that corresponded to serially numbered pill containers, as developed by the study pharmacist. The size of the blocks used during the randomization process was not known by the participants nor by the study personnel conducting the enrollment and randomization. Only two study personnel could access the envelopes, and neither was ever alone with the envelopes. These precautions ensured that there was no opportunity for a violation of the allocation concealment. To clarify these methods, we have added the following sentences on page 5: “The allocation was concealed using opaque, sealed, serially numbered envelopes. To prevent any violations of the allocation concealment, the envelopes could only be accessed by two study personnel who were not involved in their preparation. The envelopes were only accessed when both personnel were present, and the size of the randomization blocks was not known.”
2. [Page 5 line 3-5] How did the investigators direct the participants to carry out gargling in a community where gargling is uncommon? Did the participants lift ones' chin and swirl water deep in the throat making some embarrassing noise as Japanese people do? Did they perform a single gargling per one procedure? A 30-second continuous gargling seems rather hard to practice.

At the time of randomization, each participant received an opaque envelope which contained study instructions, general health advice, and, half of the participants also received printed instructions on how to gargle. The process was broken down into the following six steps:

1. Take a small mouthful of plain tap water (about 1 ounce)

2. Tilt your head back (not beyond your personal comfort) and let the water settle at the back of your throat.

3. Close your throat so you don’t swallow!

4. Breathe out slowly and let the air mix with the water so that you are generating bubbles. This might tickle and you will probably make a funny noise

5. Gargle like this for 30 seconds

6. Spit out the water

We were unable to monitor the quality of gargling or whether participants performed a continuous 30 second gargle or several shorter, serial gargles and we have acknowledged this limitation in our discussion on page 15:

“Gargling did not appear to reduce the risk of URTI in our study population, in contrast to previous reports.[22] Although our collection period captured peak rhinovirus activity, gargling may be more effective for pathogens which predominantly colonize the oropharynx. Additionally, we were unable to observe whether, or how often, gargling was practiced by participants; gargling may need to be carried out more frequently than twice daily to be beneficial.”
3. [Page 5 line 5-6] When did the participants receive general lifestyle and health advice: before the randomization or after the randomization? If advice was given after the randomization, some bias (a kind of allocation-dependent co-intervention) might occur.

The general lifestyle and health advice was provided to each participant at the moment of randomization. Each participant received a serially numbered pill container which contained either vitamin D or placebo. At the same time, each participant also received an opaque, sealed envelope which contained a copy of the study instructions and a copy of the general lifestyle and health advice. Half of these envelopes also contained a separate, fluorescent sheet of gargling paper. Participants were instructed to open this envelope in private and to keep the contents of their envelope to themselves. We feel that this approach minimized the risk of any allocation-dependent bias since neither study staff nor participants knew who received the gargling information in addition to the standard health advice. To clarify this point, we have modified the following sentence on page 5 so that it now reads: “All participants received general lifestyle and health advice about the benefits of appropriate sleep, nutrition, hand hygiene, and exercise at the time of randomization.”

4. [Page 5 Assessment] Didn't the online survey system include the practice of gargling and other hygienic actions? Didn't the authors collect the unused medicines to infer the compliance?

The weekly survey did include questions about the consumption of study pills, as well as questions about health and lifestyle factors such as the frequency of hand washing, quantity of exercise, quantity of sleep, changes in health, and any absences from school. A single question about frequency of gargling was also inserted among the health and lifestyle questions and all participants received the same set of questions. Every effort was made to collect all pill containers and unused pills at the end of the study and 75% of the containers were recovered. Based on the pill counts from these containers, 88% of the participants consumed all the doses, 10% of the participants missed one dose, and 2% of participants missed two or more doses. Compliance estimated via pill counts was strongly correlated (R=0.76) to the reports of pill consumption collected via the weekly survey.

5. [Page 7 line 1] Poisson regression is used for an analysis of count data. When this type of analysis is used in a follow-up study, make-up would be needed for the censored cases. When some participants were censored, logistic regression would be better, where the incidence rate, i.e., the number of colds per person-day (or person-month), is applied. However, the reviewer agrees with the authors' opinion
that the logistic regression model is not well fitted when the outcome events is so frequent. The Cox proportional hazard model is another option, where the multiple outcome occurrences in a person cannot be treated.

Thank you for your thoughtful comment. We selected Poisson regression because we felt it was better aligned with the primary outcome of interest which was whether or not the participant had a URTI over the entire course of the study. We used multiple imputation to handle the missing data and performed a complete case analysis as a sensitivity analyses. To overcome an assumption of a Poisson distribution, we used a semi-parametric model set-up. We felt that this analysis was more appropriate than either logistic regression or time-to-event analysis for the question of interest.

6. [Page 7 line 14-16] Cox regression would not be appropriate for such an inevitable outcome (recovery from cod). The duration of illness (ill days) should be used as an algebraic manner and was compared with multiple regression.

We encouraged participants to complete a daily symptom diary for seven consecutive days however we anticipated, and observed, incomplete reporting in these diaries. We felt that Cox regression was the most suitable type of analysis to accommodate this censoring and also address our question of whether or not the time to symptom resolution was different among individuals taking vitamin D compared to those taking placebo. Cox regression is frequently used in the analysis of inevitable outcomes, such as death in oncology studies, and the inevitable nature of symptom resolution was not a concern.

7. [Page 8 line 11-14] How well did the participant's perception of a cold and microbiological cold agree?

We a priori predicted that the participant’s perception of a cold and a laboratory confirmed infection would not agree perfectly and so we designated these as separate outcomes. As presented in the results section, laboratory testing identified viral pathogens in 70 of the 150 (~47%) reported URTI. To establish confirmation of a viral pathogen, our study protocol dictated that only the first swab collected during an episode of URTI would undergo laboratory testing. A swab that was submitted from a symptomatic individual was first tested using an in-house polymerase chain reaction (PCR) to detect rhinovirus/enterovirus. If the swab was negative after the in-house PCR, it was tested using a commercial multiplex PCR able to detect 16 respiratory viruses and subtypes (xTAG RVP FAST, Luminex, Austin TX). If a swab was negative after both tests, no additional swabs were tested. It is possible that
we would have detected more viral infections if we had tested multiple swabs per URTI episode, and this is a topic of interest for future studies.

8. [Discussion section] Describe some limitations of this study other than its insufficient power for vitamin D3.

We designed a pragmatic clinical trial of two potentially effective interventions for the prevention of URTI. By design, we did not take blood samples from our participants. However, as mentioned in the discussion, this limited our ability to assess whether or not our vitamin D supplementation regimen did in fact prevent the seasonal decline in serum vitamin D levels that occur during the fall. Without these blood samples, we were also unable to investigate whether individuals with the insufficient serum vitamin D levels at baseline benefitted more from the supplementation compare to individuals with sufficient baseline serum vitamin D levels. We’ve expanded our discussion on page 14 to reflect this limitation.

Although we were able to capture peak rhinovirus activity, we did not identify many URTI caused by other viruses and thus, we’re unable to investigate whether the effect of vitamin D differs among infections caused by other viruses. Finally, although we provided detailed gargling instructions to our participants, we were unable to monitor the quality of gargling that occurred and we had to rely on self-reported data to assess the frequency of gargling.

The revision of this section is as follows, with new text underlined:

“However, in the absence of blood tests this cannot be proven. Additional differences include the frequency and quantity of vitamin D₃ supplementation. Our study used a relatively high dose of 10,000 IU of vitamin D₃ per week ingested as a single dose, an average of approximately 1400 IU/day. Although the optimal dosing regimen remains uncertain, it has been acknowledged that adherence with daily supplements is often suboptimal and larger, less frequent doses may be an effective alternative.[36, 37]

A final methodological difference was our use of self-collected nasal swabs and laboratory confirmation of URTI. Our definition of clinical URTI may have been excessively broad and insufficiently specific which may have led to incorrectly classified events. It is possible that this definition captured episodes attributable to allergies or other causes, creating error in the statistical model and pushing the results towards a null effect.
Ultimately, our study was underpowered since the observed event rate was lower than predicted. This would contribute to larger variance in the estimates and uncertainty surrounding our results. This study was conducted over a relatively short period of time and while it captured peak rhinovirus activity, it was unable to capture URTI caused by other viruses. Consequently, it is uncertain whether vitamin D₃ supplementation is beneficial for the prevention of non-rhinovirus URTI. An additional limitation was the lack of serum vitamin D testing without which we were unable to investigate whether individuals with lower baseline vitamin D levels benefitted incrementally compared to individuals with sufficient baseline vitamin D levels.

Gargling did not appear to reduce the risk of URTI in our study population, in contrast to previous reports.[22] Although our collection period captured peak rhinovirus activity, gargling may be more effective for pathogens which predominantly colonize the oropharynx. Additionally, we were unable to observe whether, or how often, gargling was practiced by participants; gargling may need to be carried out more frequently than twice daily to be beneficial.”

Reviewer 3 Minor Revisions:

1. [Abstract and Tables] Log viral load should be described with the unit (log viral copies/mL).

Thank you for your detailed review, we have described the unit (log viral copies / mL) in the abstract and tables.

2. [Methods section] It would be desirable to describe what ingredients did the placebo capsules contain, and where, how and by whom were they manufactured.

The study team purchased the vitamin D in the form of Euro D 10,000 IU tablets manufactured by Euro-Pharm International Canada Inc. Each tablet was over-encapsulated with a gelatin capsule and packed with calcium carbonate to prevent the tablet from moving within the capsule. The same gelatin capsules were filled with calcium carbonate to make aesthetically matched placebo capsules. The over-encapsulation process and development of the placebo pills was carried out by a research pharmacist at McMaster University. There were no financial connections between the study team and Europharm. The information about the placebo contents has been added to the “Interventions” section of the manuscript on page 6. The revision is as follows, with new text underlined:
“The vitamin D was purchased from Euro-Pharm International Canada Inc. and the placebo contained only calcium carbonate. The pills were made aesthetically identical through use of a gelatin capsule.”

3. [Page 9 line 1-2] Clarify the criteria of the adjudication by two clinicians?

Participants were asked to report the onset of URTI symptoms directly to study staff. In addition, each week participants received a short online survey with screening questions for URTI. If a participant replied that (s)he was unsure if (s)he was sick, and did not report an episode of URTI during the subsequent week, the record went through the adjudication process. Two independent clinicians reviewed the records and considered the reported symptoms. A record was considered an adjudicated event if the following criteria were met: 1) a minimum of two symptoms, one of which was nasal congestion, sneezing, cough, sore throat, and wheezing, were reported, and 2) there was an absence of additional comments that attributed the symptoms to another cause. The participants were not contacted by any study staff. The independent clinicians who performed the adjudication process had perfect agreement. To clarify this process, the following sentence has been added on page 6: “These reports were deemed adjudicated events if 1) at least two symptoms were reported and included one of nasal congestion, sneezing, cough, sore throat, and wheezing, and 2) no additional information was provided that attributed the symptoms to another cause.”