Review of: "Meta-Omics Analyses of Organic and Conventional Fermented Vegetables Reveal Differences in Health-Boosting Potential"

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Potential competing interests: No potential competing interests to declare.

This manuscript describes a series of tests that were carried out on some samples of fermented vegetables. The experimental methods are clearly described, and the data that were recorded are also clearly presented.

However, the design does not include true replications, and the tested factor (organic versus conventional production method) is confounded with several other factors. Unfortunately, the presentation of the results (as in, what the recorded data mean) does not take these two serious limitations sufficiently into account, and therefore the results are interpreted in a way that is not adequately supported by the data.

Some of the problems can be solved by improving the information in the manuscript as outlined below, and the authors are strongly encouraged to do this.

The main problem with the study is the choice of samples that were initially collected for this research. Presumably, it is not feasible to collect more samples and extend the research in that way, with more replications; otherwise, this would have been the best solution.

It is possible to revise the manuscript to still obtain a good research paper, which can contribute to science, although the contribution is smaller than outlined in the abstract of the present version of the manuscript. This requires that the authors understand and describe the limitations of their research design and point out how their experiences can usefully guide other researchers, or future research by the authors, to eventually achieve what they intended with the research described here: to ‘shed light on how regenerative and conventional farming practices impact the abundance of potentially probiotic microorganisms and metabolites of importance for human nutrition and health maintenance’.

In order to test how two different farming practices impact something, the experimental design must address all the other factors that influence the measured outcome to ensure that the measured effect is not caused by something else (confounding). Examples of relevant factors are: genotype (variety); geographical location (climate); soil type; previous crop on the field (or fallow); organic vs. conventional farming practice; sowing date; distance between plants in the field; harvest date; time and conditions from harvest to processing; size distribution of vegetables; position of the vegetable on the plant (South vs. North, top branch vs. bottom branch); other random differences between individual vegetable items, like the effect of pressure on a vegetable positioned at the bottom of a bag during transport); due to processing conditions;

storage duration after processing; and so on.

There are two ways to address a potentially confounding factor: 1. To prevent the effects of a factor that can be controlled directly by selecting samples that are affected equally by this factor (such as vegetables grown in the same soil type and climate); 2. To collect and separately test replicate samples to capture the variation among factors which cannot be controlled as standard deviation and control it statistically by using an appropriately large sample size when testing for significance (such as preparing more than one jar of fermented product from each bag of vegetables to control for random variation among the vegetables in this bag).

The problem with the present manuscript is that several of the likely relevant factors are not controlled at all, and this limitation is not sufficiently well explained. Every possible confounding factor must be addressed in order to conclude that 'the microbiome and metabolome of the fermented vegetables under each growing system is unique'. The results showed consistent patterns of differences between vegetables collected fresh from one farm compared with the same species of vegetables collected from a farmers market, which were grown at a different farm, and presumably recently harvested. It could be argued that the variety choice and effects of previous crops should be considered an element of the farming system. However, if this is how it is seen, this point should be clearly explained (with references), since not everyone agrees with this view.

As an additional important detail, please specify who certifies the organic farm; different certifying organisations may have slightly different requirements, so this is more accurate than just 'certified'. If the conventional farm is certified, for example, using an 'integrated' approach, then please also specify this.

Another problem with having only one sample from each system is that it is not possible to test for interactions. In other words, the farming system may have a large effect on product quality on one soil type, but little or no effect on the same crop if grown on a different soil type (for example, carrots can be grown in either sandy soil (low organic matter content), which requires high fertiliser input, where the farming system has a large effect on composition, or on peaty soil (high organic matter content), where little or no fertiliser is required for carrots (in either farming system), and there is little difference in the quality. By testing only one soil type, it is impossible to know if the measured effect is also relevant for other soil types. Similar arguments apply to climate, sowing dates, temperature during growth, and so on. This is the reason why most journals publishing papers on agronomy require that a study should include data from 3 years of crop production, just to accommodate the effect of weather variation from year to year.

Including 3 species in the study gives some justification to the argument that if a trend is similar across all 3 species, then it is likely to be due to the difference between the farms. Note that to make this argument, the statistics should be done across all three species, using a two-factor ANOVA; it doesn't actually matter if an effect is significant for each species individually (so in this context, some of the results may be **more** significant than how they are described until now).

The microbiome data used the 6 jars per sample as replications to determine which differences were significant. However, in relation to the farming system, these are 'pseudo-replications'.

However, even if the effect of 'farm' is highly significant, it is not possible to determine if the key relevant difference

between the farms is the farming system, or if it is primarily due to the vegetable washing and packaging procedure after harvest, such as the extent of drying out before processing; since it is known that these were not the same, but it is not known how different they are. Considering the bacterial data, it would be very useful to ask each farmer if they use any detergent or disinfectant for the washing, including chlorinated tap water, since if one of them does and not the other, then this could be a major effect. While the use of disinfectants for washing could also be considered part of the farming system (see above), any difference at this step is likely to affect the microbiome much more than other elements of the farming system, such as the use of pesticides or synthetic fertilisers, which is not how it is presented in this version of the manuscript.

For the reasons explained above, the manuscript should be improved by checking at each step that the limitations of this step are appropriately identified, and it is explained how those limitations affect what can be concluded from this research.

The conclusion already states that ‘The reasons behind this observation need to be further investigated with greater sample sizes’; however, it could be substantially improved by adding a section in the discussion explaining which other types of samples would be required and estimating how many are needed to achieve appropriate statistical power (using relevant literature data to supplement the study’s results where necessary). Including this information in the paper will increase the probability that someone will do this additional research and also cite the paper.

Once the rest of the paper has been revised to adequately reflect the limitations to the research and specify the opportunities it opens, then the title and abstract should be adjusted to match. For example, the title could be changed to: ‘Meta-Omics Analyses of Organic and Conventional Fermented Vegetables Suggest Avenues to Study Differences Which May Affect Health-Boosting Potential’.

A separate issue, not directly related to the above, relates to the use of the terms ‘fermentation’ and ‘autoclaved’ and the interpretation of data from autoclaved jars.

It is explained in the Materials and Methods section that some of the jars were autoclaved, but there are no details about this (equipment type & manufacturer, target temperature, and duration at this temperature in the middle of a jar). Also, no details of the subsequent sample collection, in particular whether it was done aseptically (maintaining a sterile environment inside the jars). Specify the effect of the autoclaving on the microbial community also in absolute numbers (CFU) at the first sampling; presently, it is only shown as diversity, and the lack of effect on the diversity is very surprising if the autoclaving was successful (to eliminate all pre-existing microbes).

If the autoclaving was successful (met standard requirements for time*temperature and reduced CFU (microbe numbers) by log 12), then discuss other sources of microbes, such as contamination from air, sampling equipment, or other sources (which might include researchers’ fingers), to explain the rapid colonisation after autoclaving. Another possible explanation is that the DNA that was analysed in the autoclaved jars when they were first opened after the sterilisation was all from dead microbes, so represented the ‘time 0 days’ value; in this case, any changes after day 1 were caused by contamination.

If the time*temperature was not recorded during the study, then if possible, repeat this process using the same equipment and the same number of jars as in the study. The measured jar must have vegetables in it in the same amount as in the study (since their presence will affect convection); the species of vegetables is not important, and the other jars filling up the autoclave could contain only water (but not just air).

If the initial CFU was not measured during the study, then try to repeat this with a jar that is autoclaved and one that is not. Many companies will do the CFU measurement as a routine test for a small fee.

If the autoclaving was not successful (did not meet standard requirements but still reduced the CFU by approx. log 6), then change the term to ‘pasteurisation’ throughout.

If the autoclaving did not even meet the definition above of pasteurisation, then change the term to ‘heat treated’ throughout.

In several places throughout the manuscript, the non-autoclaved vegetables are described as ‘fermented’, implying that the autoclaved vegetables were not fermented. This gives the impression that maybe the autoclaved jars were not substantially contaminated with microbes. In this case, the measured changes in diversity and such presumably reflect the stability of the DNA in the dead microbes rather than the growth of new microbes. In this case, the drop in alpha diversity in some autoclaved treatments, but not others, might reflect the proliferation of a few contaminant bacterial species.

Please provide the missing information about sterility or lack thereof in the autoclaved jars, and revise the text as relevant, taking this into account.

Overall, the manuscript includes a lot of interesting information that could be of value to the scientific community if presented appropriately and credibly. However, the present title and abstract preposterously exaggerate the importance of the content, which risks confirming a common perception that research on organic farming is unserious, and at the same time, putting off most researchers from even reading the content.