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Opinion on the doctoral dissertation

“Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation  
in dairy cows”

(title in Polish: Liści Paulowni w regulacji metanogenezy i biouwodorowania u krów  
mlecznych

by Mr. Haihao Huang

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Growing consumer awareness and the need to feed a growing population and protect the natural environment are the driving forces behind the search for alternative feed components that do not compete with human foods, and are an ally in climate protection. These include alternative dietary components used in cattle nutrition and waste management. Ecologists focus on implementing those solutions in high-tech and urbanized countries, where such adverse impacts are evident. Agriculture is an inherent source of greenhouse gases, which is assumed to be responsible for 10–12% of total green-house gases caused by human activity, of which half of those emissions is produced by ruminant livestock production. Moreover, with the increasing demand for animal products, ruminants could contribute more to global methane emissions unless methane mitigation measures are not adopted. Different nutritional strategies are implemented in livestock production to mitigate methane emissions. Recently, many biologically active substances and tree leaves have been used as a source of nutrients and plant bioactive compounds to decrease methane production and improve ruminal fermentation. An

example could be a Paulownia tree. The leaves of Paulownia could be used as an alternative feed ingredient for different animals because of their varied biochemical properties. Paulownia leaves are rich in minerals such as calcium (2.1%), phosphorus (0.6%), zinc (0.9%), and iron (0.6%), contain 15.1% cellulose and 8.8% crude protein. Paulownia dried leaves or silage positively affected the rumen fermentation parameters by changing acetate to a propionate ratio without any adverse effect on nutrient digestion, which could be exploited in the nutrition of ruminants. It has also been shown that phenolic compounds, including phenolic acids and flavonoids, are the most biologically active substances in Paulownia leaves. These compounds can influence the rumen fermentation processes, including methanogenesis.

The PhD student carried out a series of interesting studies, the task of which was to determine the chemical and phytochemical composition of Paulownia leaves and to investigate the effects of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production, microbial population, milk production, and their composition.

This dissertation is written in English, consists of 2 published articles.

**Huang Haihao**, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Sylwester Ślusarczyk, Dorota Lechniak, Mina Vazirigohar, Zora Varadyova, Martyna Kozłowska, and Adam Cieślak. "Chemical and phytochemical composition, *in vitro* ruminal fermentation, methane production, and nutrient degradability of fresh and ensiled Paulownia hybrid leaves." *Animal Feed Science and Technology* 279 (2021): 115038.

**Huang Haihao**, Dorota Lechniak, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Martyna Kozłowska, Pawel Kolodziejcki, Min Gao, Sylwester Ślusarczyk, Daniel Petrič, and Adam Cieslak. "The effect of ensiled Paulownia leaves in a high-forage diet on ruminal fermentation, methane production, fatty acid composition, and milk production performance of dairy cows." *Journal of Animal Science and Biotechnology* 13, no. 1 (2022): 1-19.



The doctoral dissertation begins with an abstract (in English and Polish). After the abstract, the Mr. Haihao Huang presents a list of the most important symbols and abbreviations (for the reader it would be more convenient to list abbreviations than like continuous text), followed by a theoretical introduction, presenting the issues: animal husbandry production and environmental impact, ruminal fermentation and methanogenesis, effect of plant bioactive substances on ruminal fermentation, bioactive substances in Paulownia leaves and finally their leaves as a dietary ingredient in ruminant nutrition.

Next, the PhD student posted the hypothesis and aims of the work, a description of materials and methods, and then the results. The discussion takes 9 pages of printout. The discussion is followed by a one page of the summary.

The dissertation ends with a references, contained in 16 pages and covering 113 items of literature related to the topic and scope of the work, of which almost 72% comes from the last 10 years. Unfortunately the work is poorly illustrated with charts, diagrams, figures etc. but contains tables that are legible and significantly facilitate the reader to get acquainted with the large number of results obtained.

The materials have been prepared in a transparent manner and in a way that allows for proper and complete assessment and familiarization with the essence of the scientific achievement.

The issues discussed in the doctoral dissertation are very typical. The research topic taken is original, innovative and important, both for basic research, and has practical significance in relation to the effects of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production, microbial population, milk production, and their composition.

The doctoral thesis has been presented as a cycle of publications, comprising two original and creative papers published in renowned scientific journals Journal of Animal Science and Biotechnology and Animal Feed Science and Technology indexed by the Journal Citation Report. The combined impact factor (IF) of these journals is 9.422, and the

number of points granted by the Polish Ministry of Science and Higher Education (MEiN) is 340. The collective title of the doctoral thesis is "Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows." These papers have been published in recognized international journals and have already received positive feedback from independent experts in the respective field, attesting to their reliability and high scientific value.

When assessing the series of publications, it should be stated that they constitute a coherent, homogeneous cycle of innovative and valuable research works. In both publications, the PhD student is the first author. The other co-authors of the papers provided statements, which show that the involvement of his in the preparation of these articles is 55 for each of them. I believe that this is a significant contribution and shows that it was the PhD student who was the main person in charge of the work contained in the attached publications.

The PhD thesis of Mr. Haihao Huang is thematically homogeneous and begins with the presentation of the current state of knowledge related usage appropriate nutrition to reduce methane emissions by ruminant. This dissertation provides important information on the impact of livestock nutrition on the environment. The introduction of a new feeding strategy with Paulownia leaves is a promising perspective that may contribute to the improvement of current climatic conditions but requires further research on effectiveness and animal welfare. It is worth praising the fact that in the description of the current state of knowledge, the PhD student cites many already published articles, the list of which fits well the subject of this thesis.

#### **General comments**

The presented doctoral thesis has been prepared in a clear manner, allowing for proper and comprehensive assessment and understanding of the essence of the scientific achievement. The topics addressed in the thesis are highly relevant, and the chosen research subject is original and innovative. It holds significance for both fundamental research and practical applications, especially concerning the utilization of Paulownia



leaves in the nutrition of dairy cows. The research focuses on the influence of Paulownia leaves on rumen fermentation, nutrient degradation, ruminal methane production, microbial population, milk production, and its composition. The title is fine and informative. Polish title should be Liście not Liści. Structure of thesis is not the one of a classical PhD document, since the main part of the original research is reported in the journal articles, but it is fine in general.

As the main goal of the doctoral thesis, the PhD student identified the chemical and phytochemical composition of Paulownia leaves and to investigate the effects of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production, microbial population, milk production, and their composition. PhD candidate hypothesized that Paulownia leaves with high concentrations of crude protein and bioactive components modulate ruminal fermentation and nutrient degradation, and thus can be considered as a valuable dietary component for dairy cows. The research hypothesis was verified in five experiments (both *in vitro* and *in vivo*).

A detailed analysis of the methodologies reveals that the research was conducted using modern and well-selected methods. The reliability and credibility of scientific research results are key aspects that influence the quality and value of scientific discoveries.

During the determination of phenolic acids, flavonoids, and saponins in raw Paulownia leaves and silages prepared from them, the PhD student applied analytical procedures based on Petrič et al. (2020) and Szumacher-Strabel et al. (2019), utilizing solid-phase extraction and liquid chromatography with mass spectrometric detection. Were the research results presented by the PhD student validated and could the PhD student provide the recovery and repeatability values for the analytes determined in Paulownia leaves and in the silage prepared from them. Additionally, it is inquired whether the PhD student investigated the matrix effects on analyte signals (an important parameter in mass spectrometry) and observed any differences in their values between fresh leaves and

silages. It should be noted that the research by the authors, based on which the analytical procedures were adopted, was conducted on different matrices: Petrič et al. - wormwood, chamomile, fumitory, and mallow; Szumacher-Strabel et al. - alfalfa.

Scope and quality of results are appreciable for a PhD work. The chapter "Conclusion" is a logical effect of the results obtained in the research. They prove the author's great ability to synthetically analyze the obtained results against the background of previous research.

Concerning the questions related to the research work itself, some are formulated comments, mainly as an invitation for the Candidate to look beyond and as a support for discussion at the PhD defence.

The problem is clearly of actuality and originality, the tasks for the PhD research have been correctly identified and solved. The originality of research has been confirmed through publications in scientific journals of international reputation.

#### Other comments:

- At the beginning of the work, the author puts the abbreviations used in the work in the form of solid text. In my opinion, it should be written in the form of a table or list, then it would be more transparent for the reader.
- The numbering of the work is unclear, namely, on the initial pages of the work, the notation "i-xii" was used, which is not the correct Roman numeration, while the rest of the work uses Arabic numbering.
- The author on page "xi" has included a "Table of contents", in which he has included all the chapters of the work. Unfortunately, an error broke in, namely, the Abstract of the work is on p. "viii" and not on "vii" as it is stated in the "Table of contents".
- page 4 the year is missing when citing Bahromovich and Becker et al.
- page 6, the author noted that the use of excessive amounts of paulownia leaves can adversely affect the body of ruminants, due to the bioactive substances they contain. On the other hand, he did not explain what negative effects in the body of



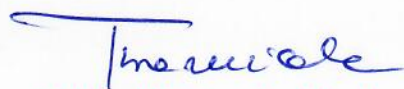
ruminants can be caused by high concentrations of bioactive compounds contained in paulownia leaves.

- In the theoretical part, the author describes the genus Paulownia from the *Paulowniaceae* family, while only in subsection 3.1 he indicates what forms were taken for analysis. There are at least 7 species in this genus. In my opinion, the inclusion in the theoretical part of the two forms used in the research will significantly enrich this part.
- In subsection 3.1 the author writes: "Before the in vitro (batch culture) and in situ (rumen in sacco) experiments, all the Paulownia samples were pooled and thoroughly mixed (separately for PL and PLS). One representative of a homogeneous sample was prepared for each plantation. Finally, 4 samples of PL and 4 samples of PLS were prepared. One sample represented one plantation." Please explain how representative samples for individual plantations were prepared, since earlier, as described by the author, all Paulownia samples were mixed together. Was it about mixing a sample from individual plantations?
- In subsection 3.3.1 the author writes: "Bags were incubated in the rumen of each cow for 0, 2, 4, 8, 12, 24, 48, and 72h. The zero-hour bags were not placed in the rumen but were treated with distilled water at 39°C for 15min.". These two sentences are mutually exclusive.
- On page 19, in table 2, in the legend, there are explanations of min and max abbreviations. However, these values are not included in the table.
- On page 19, the author of the paper writes: "The content of amino acids increased by 22.5% ( $P < 0.003$ ) for essential AAs and by 23% ( $P < 0.005$ ) for non-essential AAs (Table 3). ". However, in Table 3 there are mean, SD, min, max. From what data were the percentages calculated?
- page 20, in tab 4, again in the legend, there are abbreviations of values not included in the table.
- Bibliography: there are items that are not in the literature list, namely:
  - page 43 – Khorsandi et al. (2019)

- page 45 – Garnsworthy et al. (2010)
- page 5, no year when cited Becker

### Final conclusion

The doctoral dissertation presented by Mr. Haihao Huang provides a proof of his good knowledge of investigate the effects of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production, microbial population, milk production, and their composition. The Candidate has demonstrated his capabilities to critically scrutinize the bibliography of the subject as well as various variants of computational approaches to turbulent flows. The thesis contains original analyses and novel findings beyond the state of the art. Judging by the PhD document, the Candidate has proven his good knowledge of the subject area, the professional skills, as well as the ability to think and work creatively. Given all the above, my final conclusion about Mr. Haihao Huang being a doctoral candidate is positive and I recommend that he orally defends the PhD dissertation with no reserve at all. Moreover, given (i) the degree of difficulty of the thesis subject, (ii) the quality findings reported in the PhD work, listed in this review and published in renowned research journals I propose that the PhD thesis of Mr. Haihao Huang be awarded distinction (*summa cum laude*).



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