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REVIEW

**Doctoral dissertation by Mr Haihao Huang
titled: 'Effect of dietary Paulownia leaves on ruminal
methanogenesis and biohydrogenation in dairy cows'**

**Ph.D. supervisor: prof. dr hab. Adam Cieślak
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The legal and formal basis

Mr. Haihao Huang received his Master of Engineering degree on 13.03.2018 at the Faculty of Veterinary Medicine and Animal Sciences, Poznan University of Life Sciences.

Haihao Huang, M.Sc., was employed in 2019–2020 in the OPUS project funded by the National Science Centre (Poland) entitled: 'The role of biologically active substances of Paulownia CLON IN VITRO 112 in the regulation of methanogenesis and biohydrogenation in ruminants' (Grant No. 2016/23/B/NZ9/03427). The candidate has not previously applied for a doctoral degree.

The formal and legal basis for the review is the Resolution of the Scientific Council of Discipline: Animal Science and Fisheries, Poznan University of Live Sciences (official letter: WWZ-4000-03/2023, 13.07.2023) appointing me as a reviewer in the doctoral proceedings for Haihao Huang, M.Sc. The dissertation was reviewed in accordance with the requirements specified in Article 187 of the Law Higher Education and Science of 20.07.2018 (Journal of Laws of 2023, item 742, as amended).

The materials for the review were prepared reliably. The results of the research were presented in a legible manner, which enabled their proper and comprehensive evaluation.

Research problem and significance of the study

The world's population continues to grow and so does the demand for food. Climate changes observed in recent years have negative impact on the global agricultural sector. Water scarcity, heatwaves (agricultural drought), heavy rainfall, and other extreme weather and climate conditions are expected to negatively impact crop and livestock productivity.

Animal production aimed at providing humans with products of animal origin necessary for rational nutrition has an undoubted impact on the climate changes taking place on Earth, e.g. through the emission of greenhouse gases. Ruminants are responsible for 80% of total emissions from the livestock sector, or about 56% of total greenhouse gas emissions from agriculture. During 24 hours, an adult cow can produce 250–400 l of methane, which is 30–40% of all rumen gases. It is estimated that cattle account for as much as 40% of the total production of this gas.

The reduction of methane production can be achieved by changing the nutrition of ruminants, by reducing roughage in the diet in favour of concentrates, and thus changing the fermentation profile in the rumen – the portion of acetate fermentation decreases and the portion of propionate fermentation increases.

Rumen functions can be modulated by biologically active compounds of plant origin. Supplementation with plant material containing bioactive substances such as saponins, tannins, alkaloids, flavonoids, phenolic acids and volatile oils can affect the rumen metabolism and may decrease ruminant methane production. By using the appropriate components of the ration for ruminant animals, which are the source of substrates of microorganisms colonizing the rumen, their population can be modulated, and thus the fermentation processes, aiming, for example, at the production of compounds characterized by biological activity, e.g. of fatty origin. These compounds, being an integral component of milk and meat fat, may have a beneficial effect on the human body. This is particularly important due to the growing awareness of consumers and the demand for health-promoting products of animal origin. This seems to be particularly important considering the growing awareness of consumers and the demand for health-promoting products of animal origin.

Tree of the *Paulownia* genus is characterized by extremely fast growth – in 6 years it can reach 16 meters. With such rapid development, it absorbs huge amounts of carbon dioxide, and

thus – it also produces a lot of oxygen (10 times more than other deciduous trees). Its leaves are rich in protein - hence can be used as an alternative source of forage for ruminants and contain a high concentration of bioactive compounds such as phenolic acids and flavonoids as well as fatty acids.

Bearing the above in mind, it is important to undertake research aimed at developing new systems and methods of feeding and keeping animals – methods that are safe for the environment, limiting the production of greenhouse gases, and at the same time conducive to obtaining high-quality products of animal origin. In my opinion, the research problem addressed by Haihao Huang, M.Sc., fits very well in the current trend of searching for a possibility of an effective use of plants with high content of bioactive substances in cattle production, and the issue addressed in the doctoral dissertation is relevant and important for the Discipline: Animal Science and Fisheries.

Description and review of the study

The doctoral dissertation submitted by Haihao Huang, M.Sc. is written in English, as a series of publications consisting of two original research studies published in peer-reviewed scientific journals indexed by the Journal Citation Report with Impact Factor (IF) values of 3.313 and 7.000 (IF in the year of publication).

The dissertation under the collective title: ‘Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows’ consists of the following publications:

1. **Haihao Huang**, 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.

Impact Factor (2021) = 3.313*; Impact Factor 5 years = 3.914*; MEiN = 200 points*

2. **Haihao Huang**, Dorota Lechniak, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Martyna Kozłowska, Pawel Kolodziejski, 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.

Impact Factor (2022) = 7.000*; Impact Factor 5 years = 7.300*; MEiN = 200 points*

* *Current bibliometric data corrected by reviewer*

The series of publications constitute a coherent set of valuable research papers. In both publications, Haihao Huang M.Sc. is the first author with a percentage contribution of 55% in the creation of the publication. This was confirmed in the statements attached to the doctoral dissertation, in which the co-authors declare that the doctoral student's tasks included introducing the idea of the manuscript, participating in the creation of the research hypothesis, participation in the implementation of research (conducting the *in vitro* and *in vivo* experiments), devising ideas for discussion of the results, writing the original draft of the manuscript, and being responsible for responses to the journal reviewers in the first round of evaluation. This indicates the extensive involvement of the Ph.D. candidate in the entire process of the creation and submission of the publications and that the Ph.D. student who was the main person in charge of the work resulting in the attached publications.

The research described in both publications included in the doctoral dissertation was supported by a grant from the National Science Centre, Poland (Grant No. 2016/23/B/NZ9/03427), under the supervision of the doctoral dissertation supervisor prof. dr hab. Adam Cieślak. The publishing process was supported by the framework of the Ministry of Science and Higher Education programme 'Regional Initiative Excellence' in 2021, project number: 005/RID/2018/19, task no. 13, Animal Science and Fisheries 3/2021. These papers have been published in recognized international journals and have received positive opinions from independent experts related to the given issues, which proves their reliability and high scientific value. The bibliometric data of both publications is total IF = 10.313 and parametric assessment scores of the Ministry of Education and Science (MEiN) = 400 points. Moreover, both articles are cited (with self-citations) in total 19 and 3 times (respectively; according to Web of Science). The small number of the second paper citations may be due to the relatively recent publication date.

The dissertation presented for the review is a 62-page study with the following sections characteristic for this type of work: Title Page, Abstract in English and Polish, Abbreviations, Introduction, Hypothesis and Aim, Material and Methods, Results, Discussion with Summary, References, and Supplementary Data (statements provided by the co-authors and photocopies of both publications (Manuscript No.1 and Manuscript No.2)). Additionally, it includes the list of seven scientific articles published during Ph.D. studies - in which Haihao Huang M.Sc. is the co-author.

The doctoral dissertation is about the impact of Paulownia leaves on *in vitro* and *in vivo* rumen fermentation characteristics, *in situ* nutrient degradation, ruminal methane production,

microbial population, milk production, and thereof composition. The doctoral dissertation begins with an Abstract (in English and Polish). After the abstract, the Ph.D. student presents a list of the most important symbols and abbreviations, followed by a theoretical introduction, based on the well-fitted literature. In the **Introduction** chapter the Ph.D. candidate describes the research problem comprehensively on 7 pages and provides the reason for undertaking the research. He describes the research in detail with diverse aspects ranging from animal husbandry production and its environmental impact (climate changes) through ruminant metabolism (ruminal fermentation and methanogenesis) to biologically active compounds in plants (their effect on microbial populations and rumen metabolism), particularly in Paulownia leaves, up to use theirs as a potential dietary component in the daily ruminant nutrition.

Next, the Ph.D. student posted **the Hypothesis and Aim** of the work. The main goal of the research is clearly defined and includes determining the possibility of using Paulownia leaves as a dietary component in the nutrition of dairy cows, improving milk production and their composition as well as rumen fermentation, and modulating the microbial population to reduce methane. The formulation of the research hypothesis common to all 5 experiments emphasizes the coherence of the scientific concept presented in the doctoral dissertation.

In the chapter **Material and Methods**, the candidate presents detailed methodological assumptions of the doctoral dissertation. The scope of the experiments presented in the reviewed dissertation is very extensive and impressive. The study was divided into five experiments:

1. Batch culture (Exp. 1 – *in vitro*) to examine effects of Paulownia leaves and their silage on rumen fermentation methane production and microbial populations;
2. RUSITEC (Exp. 2 – *in vitro*) to examine effects of Paulownia silage on rumen fermentation, methane production, and digestibility;
3. The *in-sacco* (Exp. 3 – *in vivo*) used for estimating nutrient degradation (present in alfalfa silage, Paulownia leaves and Paulownia leaves silage) in the rumen of cows;
4. The cannulated cows (Exp. 4 – *in vivo*) to examine the effect Paulownia leaves silage on microbial population, ruminal fermentation, fatty acid proportion, and methane production as well as the total-tract nutrient degradability;
5. The commercial experiment (Exp. 5 - *in vivo*) to investigate the effects of Paulownia leaves silage on milk composition (performance indices), and ruminal methane production in dairy cows.

All experimental procedures were performed according to the National Ethical Commission for Animal Research guidelines.

Furthermore, it should be emphasized, that the Ph.D. student carried out numerous chemical analyses and measurements in a very wide range. The analytical methods used are relevant to the adopted standards and have contributed to the achievement of the assumed goal.

In the next chapter of the dissertation titled **Results**, the candidate presents the results of his research. The amount of data obtained and processed is impressive. While reading this part of the dissertation and the works of Haihao Huang, M.Sc., one can realize the enormity of the work done, as well as the innovative nature of the results obtained and the advancement of methods that the doctoral student freely uses. The research work is illustrated with 1 figure and contains 13 tables that are legible and significantly help the reader to get acquainted with the large number of results obtained. In my opinion, the most important results presented in the Ph.D. thesis are: Paulownia leaves are rich in CP with a high proportion of essential AA and contain large amounts of phenolic substances and that the use of fresh and silage Paulownia leaves reduced methanogenesis, beneficially modulate ruminal fermentation and biohydrogenation processes without a negative impact on milk production performance of lactating dairy cows.

Mr. Haihao Huang showed good substantive preparation for conducting research by preparing the **Discussion** chapter. To facilitate the interpretation of the obtained results, he used the same arrangement of subsections in this chapter as in the case of Material and Methods and Results. This 10-page part of the work is based on a deep knowledge of the research topic, supported by a properly selected and carefully compiled bibliography with a total of 113 literature items related to the topic and scope of the work, of which almost 72% come from the last 10 years. This confirms that the presented dissertation fits well into the area of modern research. The discussion is followed by a one-page summary.

The doctoral dissertation submitted for review was carefully prepared, but such an extensive, multi-faceted study contains some minor inaccuracies, shortcomings, and so-called typos that appeared in the text, and which did not have a significant impact on my positive opinion about the dissertation. Below are some of them:

- 1) There is a mistake in the Polish version of the title of the doctoral dissertation. English version of the title is: 'Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows', whereas the Polish version – 'Liści Paulowni w regulacji

metanogenezy i biouwodorowania u krów mlecznych'. The proposed version of the Polish version of the title is: 'Wpływ liści Paulowni na metanogenezę i biouwodorowanie żywca u krów mlecznych'.

2) Both publications included in the doctorate dissertation are multi-authored. In the Manuscript no. 1 – there are 9 authors, while in the Manuscript no. 2 – 10 authors. In both publications, the listed co-authors (Statement no. 1 and Statement no. 2) declare a 55% share of Mr. Haihao Huang. Therefore, I understand that the contribution of the other co-authors (not mentioned in the declaration) to the creation of the papers was marginal? – Please comment briefly.

3) In the doctoral dissertation, the Author uses the term 'Paulownia leaves', which were obtained from *Paulownia tomentosa* × *Paulownia fortunei* hybrid trees. In my opinion, it would be good to have some information about: Why these hybrid trees were chosen? What guided this choice? Are there any others with similar properties?

4) The doctoral dissertation lacks clearly defined conclusions. The author presents a one-page summary in which he informs us about the benefits of using Paulownia leaves in the nutrition of dairy cows. However, it does not provide specific, practical application conclusions that could constitute a recommendation for agricultural practice. So, I have a question – would you advise farmers to use silage from Paulownia leaves or would you rather advise against it? Please, explain briefly.

5) Based on the obtained results, the Author showed that the use of Paulownia leaves silage in the feeding of dairy cows had a positive effect, among other things, on the FA profile of milk. So, the question arises: did the use of this silage affect the taste of the milk?

Minor items:

- Abstract: The abundance of bacteria was increased, whereas the abundance of protozoa and archaea was decreased by PLS.; Methane production decreased by 11% (*delete 14%*) in PLS-fed cows;
- Abbreviations: LT: longissimus thoracis – there is no information regarding muscles in the dissertation;
- Table of Contents: 'Hypothesis and aims', while on page 8 it is singular form 'aim';
- Introduction: '*Entodiniomorpha* protozoa have a hard surface membrane and cilia located mostly (*delete 'only'*) near the 'mouth', capable of digesting particulate matter' – see page 3 (3.2.1 Batch culture);
- Material and Methods: 'Alfalfa silage was used as a control to compare with tested feeds (PL and PLS) because of its similarity to PL and PLS (*delete 'AS'*) nutritional value' – see page 10 (3.2.1 Batch culture);

- Results: Table 6 – the correct name of bacteria is *Fibrobacter succinogenes* – see page 22 (4.3.1 Batch culture); Table 8 – Footnotes contain lowercase letters ‘a’, ‘b’, ‘c’, while in the table are capital letters ‘A’, ‘B’, ‘C’ – see page 26 (4.4.1 *In sacco*); Table 10 – Footnotes contain explain abbreviation OM; EE; NDF, which are not in the table – see page 29 (4.4.2 The cannulated cows); Table 13 – incorrect markings in footnotes – see page 35 (4.4.3 Commercial dairy cows);
- Discussion: ‘In this study, observed a reduction protein, and lactose yield ($P < 0.05$), however, without reduction the milk production’ (*delete ‘we observed a reduction in milk production’*); ‘Higher ammonia levels in the ruminal fluid may interact with milk parameters which results in higher urea content ($P < 0.05$) in milk when the diet was supplemented with PLS (*delete ‘PLE’*) (Nousiainen et al., 2004)’ – see page 43 (5.6 Effects of Paulownia silage on commercial dairy cows).

Formal evaluation of the dissertation

A doctoral dissertation submitted for evaluation entitled: ‘Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows’ is a compact study prepared based on two monothematic, mutually complementary publications, constituting an original solution to a scientific problem. It is an extensive research study on 62 pages, including 13 tables, 1 figure, and 113 items of references. The structure of the presentation of the contents follows the rules adopted in such theses and does not raise any objections. The dissertation is written logically and clearly, and the subsequent parts form a coherent whole. The use of well-thought-out methodological solutions allowed the candidate to fully complete the intended goal. Carrying out such ambitious and multi-faceted research required good theoretical preparation and extensive manual and analytical skills. Certainly, the research results determined by the Ph.D. student have significant cognitive and application value and the dissertation has a pronounced contribution to the development of the Discipline of Animal Science and Fisheries.

In summary, I declare that the reviewed work fully meets the requirements for doctoral dissertations specified in Article 187 of the Law Higher Education and Science of 20.07.2018 (Journal of Laws of 2023, item 742, as amended). Therefore, I recommend the Scientific Council of the Discipline: Animal Science and Fisheries, Poznan University of Life Sciences to admit Haihao Huang, M.Sc. for the subsequent stages of the doctoral proceedings.

Furthermore, considering the degree of difficulty of the thesis, the high substantive value of the dissertation, a comprehensive approach to the problem, the amount of work put into the research and the practical aspect of the results of the investigation, I recommend the Scientific Council of the Discipline: Animal Science and Fisheries, Poznan University of Life Sciences to award the doctoral dissertation of Haihao Huang, M.Sc.

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