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REVIEW

**Doctoral dissertation by Haihao Huang, MSc.
titled “Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation
in dairy cows”**

**performed under the guidance of Prof. dr hab. Adam Cieślak,
in the Department of Animal Nutrition, Faculty of Veterinary Medicine
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Legal and formal basis

The formal and legal basis for the review is the Resolution of the Scientific Council of the Discipline: Animal Science and Fisheries (specific task contract No. WWZ 9/2/2023 of 13.07.2023), Poznań University of Life Sciences of June 13.07.2022 appointing me as a reviewer in the doctoral proceedings for Haihao Huang, MSc. The dissertation was reviewed in accordance with the requirements specified in Article 187 of the Law on Higher Education and Science of 20.07.2018 (Journal of Laws 2021, item 478, 16.03.2021). The materials to be reviewed were prepared accurately, clearly presenting the research outcomes, which allowed adequate and complete evaluation thereof.

Research problem and significance of the study

Livestock production is an important part of global food production chains. Simultaneously, it has a significant impact on the climate, natural environment, economic development, income

resources, and sustainable food security. Animal husbandry is one of the main sources of global greenhouse gas emissions, especially methane and carbon dioxide. It is estimated that animal husbandry contributes to approximately 14.5% of global anthropogenic greenhouse gas emissions. In the rumen metabolism in ruminants, microorganisms are used in the biotransformation process for decomposition and fermentation of complex carbohydrates to produce short-chain fatty acids and gases, e.g. methane. This process provides energy and nutrients for ruminants but concurrently exerts an impact on the natural environmental. Appropriate nutrition provided to ruminants can substantially reduce the production of greenhouse gases. Feed materials containing bioactive substances are especially important in this respect. They include e.g. flavonoids, saponins, or alkaloids, which are able to inhibit the growth of methanogens in the rumen, thereby reducing methane production. Concurrently, the literature in this field confirms that the use of natural bioactive substances in animal nutrition facilitates dietary modification of animal products, such as milk.

Paulownia trees are rich in bioactive phytochemical substances. Its leaves contain a large amount of nitrogen glycosides such as adenosine, guanosine, and cytidine, which contribute to regulating immune function, lowering blood pressure, and improving blood circulation. Polysaccharides present in Paulownia leaves, such as arabinose, xylose, galactose, etc., can resist oxidation; they lower blood glucose and have anti-tumor properties. The leaves contain a variety of flavonoids, such as rhamnoside and rutin, which have antioxidant, anti-inflammatory, and blood pressure-lowering effects. Cellulose-rich Paulownia leaves can promote intestinal peristalsis and increase satiety in ruminants. The presence of these specific substances in the diet for dairy cows not only contributes to the maintenance of a good health status of animals, but also results in a high level of production of milk with a favorable chemical and dietary composition.

The research problem addressed by the Author 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. Therefore, the issue addressed in the doctoral dissertation by Haihao Huang, MSc. is relevant, up-to-date, and important for this area of science.

Description and review of the study

The doctoral dissertation submitted for the review by Haihao Huang MSc. is 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 3,247 and 6,175



(IF in the year of publication) under the collective title “Effect of dietary Paulownia leaves on ruminal methanogenesis and biohydrogenation in dairy cows”.

The series consists of the following publications:

1. **Huang Haihao**, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Sylwester Ślusarczyk, Dorota Lechniak, Mina Vazirigohar, Zora Varadyova, Martyna Kozłowska, and Adam Cieślak (2021). "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: 115038.
Impact Factor (2021) = 3.247; MEiN = 200 pts
2. **Huang Haihao**, Dorota Lechniak, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Martyna Kozłowska, Pawel Kolodziejski, Min Gao, Sylwester Ślusarczyk, Daniel Petrič, and Adam Cieslak (2022). "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, 1: 1-19.
Impact factor (2022) = 6.175; MEiN = 140 pts

The series of publications constitutes a coherent homogeneous set of innovative and valuable research papers. In both publications, Huang Haihao MSc. is the first author with a percentage contribution of 55%. This has been confirmed in the statements attached to the doctoral dissertation, in which the co-authors declare that the tasks performed by the doctoral student included introducing the idea of the manuscript, collecting, preparing, and ensiling Paulownia leaves, conducting the *in vitro*, *in sacco*, and *in vivo* experiment, performing the basic chemical analysis, determining ruminal pH, concentration of ruminal ammonia, and volatile fatty acids, ruminal total gas production, and ruminal methane concentrate analysis, determining the number of ruminal protozoa, analysis of the fatty acid profile in milk and ruminal samples; contributing to formulation of ideas for discussing the results, writing the original draft, and replying to the journal reviewer’s concerns in round 1. This indicates the extensive involvement on the part of the PhD candidate in the entire process of the creation and submission of the publications.

The doctoral dissertation, i.e. the two publications presented by the PhD student, has the following bibliometric indicators: total **IF = 10.055** and parametric assessment scores of the



Ministry of Education and Science (MEiN) = 340. The bibliometric data of the publications are measurable and objective and clearly evidence the high quality and importance of the research results presented in the doctoral dissertation.

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 Polish and English, Abbreviations, Introduction, Hypotheses and aims, Materials and methods, Results, Discussion, Summary, and References. In the dissertation, the role of the standard Conclusion section is taken over by the Summary, in which conclusions from two publications are presented. Additionally, it includes the publications and statements provided by the co-authors.

The form of the doctoral dissertation, i.e. articles published in international journals and subjected to detailed assessment by independent experts during the publishing process, facilitates the evaluation thereof by the reviewer. It is also a proof of the substantive correctness and relevance of the scientific issues discussed therein.

The dissertation also includes a list of seven other scientific publications co-authored by the PhD candidate. They were all published in journals indexed by the Journal Citation Report with Impact Factor (IF). The total IF of these publications is 20.154, and the MEiN score is 950. Such high publication achievements of the doctoral candidate prove his great commitment to the research work of the team and thus confirm his excellent abilities to meet scientific challenges as part of his doctoral work.

Based on the well-selected literature, the PhD candidate describes the research problem comprehensively on 7 pages and provides the rationale for undertaking the research. The background of the research has been described in detail with its multifarious aspects ranging from animal husbandry production and environmental impact, through ruminant metabolism (rumen fermentation) to the effect of plant bioactive substances on ruminal ferment, bioactive substances in Paulownia leaves, and Paulownia leaves in ruminant nutrition. This proves Author's excellent preparation for research work and commitment to studying literature in great detail. As many as 112 publications, adequately corresponding to the subject of the study and originating from the last 15 years in approximately 83%, were used in the dissertation. This confirms that the presented dissertation fits well in the scope of contemporary research.

Research Objective and Hypotheses

The aim of the study is presented clearly and precisely. An important element of the doctoral dissertation is the formulation of research hypotheses common for the entire series of the 5



experiments. This underlines the consistency of the scientific concept implemented in the extensive experiments.

Research methodology

In the “Material and Methods” section, the Author presents detailed methodological assumptions of the doctoral dissertation. The scope of the experiments presented in the reviewed dissertation is very extensive. The study was designed into five consecutive experiments:

1. Batch culture;
2. RUSITEC test;
3. *in sacco* test;
4. with cannulated cows,
5. with commercial dairy cows.

In experiment 1, the rumen inoculum was obtained from three ruminal cannulated Polish Holstein-Friesian dairy cows (body weight 625 ± 25 kg, second month of lactation) before the morning feeding. The rumen inoculum donors were fed 24 kg dry matter (DM) of a total mixed ration (TMR). Alfalfa silage (AS) was used as a control to compare with the tested feeds: Paulownia leaves (PL) and Paulownia silage (PLS). The experiment was repeated for three consecutive days. In rumen fluid, after incubation, the basic rumen fermentation, methane production, and microbial populations were determined.

In experiment 2, an *in vitro* experiment was carried out using the Rumen Simulation Technique (RUSITEC). Ruminal fluid and solid digesta for the *in vitro* experiment were collected 3 h before the morning feeding from four rumen-cannulated multiparous Polish Holstein–Friesian dairy cows (630 ± 25 kg body weight) at their 3rd month of lactation. The following diets were tested: a control diet (CON) and three PLS diets. The PLS diets contained Paulownia silage that replaced alfalfa silage at 25%, 50%, and 75%. They corresponded to the PLS content in the diet at the level of 20, 40, and 60 g/kg DM. The collected fluid samples were analyzed for pH, volatile fatty acids (VFA), ammonia concentration, protozoa, bacteria and methanogen counts.

In experiment 3, three lactating multiparous Polish Holstein Friesian cows (625 ± 25 kg body weight) fitted with rumen cannulas were used. The cows were fed twice daily at 06:00 and 18:00 h, with the TMR consisting of 400 g/kg of alfalfa forage, 300 g/kg of grass forage, and 300 g/kg of concentrate (wheat grain, extracted rapeseed meal, extracted soybean meal, and mineral and vitamin premix). Clean water was available *ad libitum*. PL and PLS were tested and



AS was used as a control to compare the tested feeds for their degradability. Bags were incubated in the rumen of each cow for 0, 2, 4, 8, 12, 24, 48, and 72 h. For each incubated feed (AS, PL, or PLS), three cows were used, and the procedure was repeated twice.

In experiment 4, four multiparous cannulated Polish Holstein–Friesian dairy cows (625 ± 20 kg body weight; 4–5th month of lactation) were assigned to two dietary treatments (CON vs. PLS60) with two cows in each treatment in a replicated 2×2 crossover design. The CON and PLS diets containing the higher level of PLS, i.e., 60 g/kg DM (PLS60), were implemented. The cows were fed two times a day. Each period lasted for 36 d, with a 21-d adaptation and a 15-d sampling period (5 d of rumen fluid collection and 10 d for gas collection). The ruminal fluid was collected from each cannulated cow. Rumen samples (about 400 g/animal) were analyzed for the pH value, ammonia and VFA concentrations, and the FA profile. Quantification of total bacteria and methanogens was carried out only in rumen fluid sampled 3 h after morning feeding. Feed intake, feed residue, and amount of feces were recorded daily (d 27 to 36) from individual cows kept in respiratory chambers.

In experiment 5, 16 multiparous lactating Polish Holstein–Friesian dairy cows were assigned to two dietary treatments: CON vs. PLS60 (600 ± 30.4 kg body weight, 2.4 ± 0.45 parity, 160 ± 32 d in milk, and 33 ± 2.1 kg/d milk production). Each period consisted of a 21-d adaptation period followed by a 5-d sampling period with a total of 26 d. The two groups (CON and PLS60) had separated controlled access to a computer-controlled feeder station, where concentrate was served. The rest of the diet was offered twice a day (at 06:00 and 18:00 h) as a partial mixed ration (PMR) in individual feeding boxes located on the feeding table. The control PMR contained (g/kg of DM) corn silage (441), alfalfa silage (93), meadow grass silage (103), beet pulp (118), brewer's grain (108), rapeseed meal (122), and mineral and vitamin premix (16), whereas the experimental PMR contained (g/kg of DM) corn silage (438), alfalfa silage (29), Paulownia leaf silage (68), meadow grass silage (102), beet pulp (117), brewer's grain (108), rapeseed meal (122), and mineral and vitamin premix (16). The cows had access to clean water *ad libitum*. The dry matter intake was measured daily for the last 5 d (d 22 to 26) of the experiment. Feces were individually collected from each cow directly after defecation and the floor was kept clean. The cows were milked twice a day at 5:30 and 17:30 h. Milk samples were collected from all cows at each milking during the sampling period (d 22 to 26) for milk basic constituents, FA analysis, and gene expression analysis.

It should be emphasized that the Author carried out numerous observations, measurements, and



chemical analyses in an exceptionally wide range, e.g.:

- chemical analyses of the quality of the feed components and feed mixes, taking into account their basic chemical composition, fiber fraction, amino acid composition, and fatty acid profile, and the content of starch, ammonia, flavonoids, saponins, and phenolic and organic acids,
- *in vitro* crude protein, dry and organic matter degradation,
- analyses of the ruminal fermentation profile, CH₄ production, and FA composition in the rumen,
- analyses of protozoa, methanogens, and bacterial populations,
- analyses of such parameters as pH, ammonia content, and the volatile fatty acid (VFA) level,
- analyses of mRNA expression in milk samples
- measurements of milk yield and composition (dry matter, fat, protein, lactose, urea, methane)

The design of the experiments, the number of observations, and the analytical methods used do not differ from the adopted standards and have contributed to the achievement of the assumed goals. Adequate statistical methods were used in the study. In the doctoral dissertation, it would be worth providing information whether the results obtained in the research were checked for normal distribution.

Assessment of the results and discussion

In the dissertation, the doctoral student discusses the research results in an extremely detailed and substantive way with reference to the well-chosen literature. The study results have been analyzed with great care and scientific maturity. In experiments 1 and 3 (*Huang Haihao, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Sylwester Ślusarczyk, Dorota Lechniak, Mina Vazirigohar, Zora Varadyova, Martyna Kozłowska, and Adam Cieślak (2021). "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: 115038*), the candidate observed that the use of PL and PLS mitigates methane production by reducing methanogen counts and improves rumen fermentation characteristics. Paulownia leaves are rich in crude protein with a high proportion of essential amino acids and



contain large amounts of phenolic substances. The nutritionally valuable profiles of amino acids and fatty acids along with the highly effective degradability and fractional degradation rates of PL and PLS suggest that their inclusion in ruminant diets would increase nutrient and energy supply into the rumen and improve the fatty acid profile of milk. The *in vitro* results were verified by the PhD candidate in *in vivo* experiments.

In experiments 2, 3, and 4 (**Huang Haihao**, Dorota Lechniak, Malgorzata Szumacher-Strabel, Amlan Kumar Patra, Martyna Kozłowska, Pawel Kolodziejski, Min Gao, Sylwester Ślusarczyk, Daniel Petrič, and Adam Cieslak (2022). "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, 1: 1-19), the Author confirmed that PLS (60 g/kg DM) reduced methanogenesis and beneficially modulated ruminal fermentation and biohydrogenation processes without a negative impact on milk production performance of lactating dairy cows. The PLS used in dairy cow nutrition improved the milk FA profile, including greater proportions of total UFA, PUFA, conjugated linoleic acid, and C18:1 trans-11 along with reduction of the n6/n3 ratio. A negative effect of the use of PLS was also observed, as an increased ruminal ammonia concentration affecting the milk urea content was reported. The author points out that the energy and protein synergy in PLS-containing diets should be improved.

The "Summary" section presents logical conclusions of the research results. They were adequately formulated, which proves the Author's great capability of synthetic analysis of the investigation findings. The results of all 5 experiments have great practical importance and indicate a fairly simple dietary way to increase the production performance of dairy cows. They also show the possibility of maintenance of the optimal level of rumen bacteria and animal health status, production of milk with improved dietary values, and minimization of the problem of environmental pollution through reduction of the amounts of gases produced by animals. The study formulated an application aspect with a recommendation for breeding practice, which nevertheless should be regarded as a suggestion, as the author himself mentions the need to improve it.

During the evaluation of the doctoral thesis, I had a few questions to the doctoral student:

1. What toxic substances are contained in Paulownia leaves? What is the safe level of administration in a ration for cattle?



2. Can Paulownia flowers and fruits, likewise leaves, be used in animal nutrition? What bioactive substances do they contain and what properties do they have?
3. Which feeds and why can increase methane production in dairy cows?
4. How can the energy and protein synergy in dairy cows fed diets with Paulownia leaves silage be improved?

The doctoral dissertation submitted for the review is performed with extreme care, although this extensive study does have some inevitable minor inconsistencies and shortcomings. Here are some of these points:

- page 4: the year at the “Bahromovich” reference is not specified,
- page 5: the year at the “Becker et al.” reference is not specified,
- page 39: there is a citation of “Patra’sPatra (2012)”, which is not included in the References section or in the publications
- page 44: there is a citation of “Khorsandi et al. (2019)”, which is not included in the References section or in the publications
- page 44: there is a citation of “Garnsworthy et al., 2010”, which is not included in the References section or in the publications

Formal evaluation of the dissertation


The reviewed work is a reliable, up-to-date, and multi-faceted source of knowledge of the possibility of using plant sources of bioactive substances in the ruminant husbandry. It is a comprehensive and original approach to the research problem and a proof of the Author’s extensive and profound knowledge. It is characterized by a very high substantive level. **The dissertation is a significant contribution to the development of the Discipline: Animal Science and Fisheries and has great application-related importance.** The research required considerable commitment, work, and cooperation as well as expertise in research techniques and methods.

To sum, I declare that the reviewed work fully meets the requirements for doctoral dissertations specified in Article 187 of the Act of July 20, 2018: Law on Higher Education and Science (Journal of Laws of 2021, item 478, 16.03.2021). Therefore, I recommend that the Scientific Council of the Discipline: Animal Science and Fisheries, Poznań University of Life Sciences, should admit Huang Haihao, MSc. for the subsequent stages of the doctoral proceedings.



Given the high substantive value of the dissertation, the comprehensive approach to the problem, the huge amount of work devoted to the research, and the practical aspect of the research, I recommend that the PhD candidate should be awarded by the Scientific Council of the Discipline: Animal Science and Fisheries, Poznań University of Life Sciences.

Lublin, 7.08.2023



Prof. dr hab. Bożena Kiczorowska

