Bari, September 25th 2025

Department/Institution: Institute of the Science of Foods Production (CNR-ISPA)
Reviewer: Angela Cardinali
Academic Title: Dr.

Review of Doctoral Thesis

Candidate: Sanling Zuo, MSc

Title: *Metabolic signatures of probiotic and prebiotic functions in the intestine cells*

INTRODUCTION

The maintenance of gut health is central to overall host health, influencing immunity, nutrient absorption, and metabolic homeostasis. Probiotics and prebiotics have emerged as key bioactive agents capable of modulating gut microbiota composition and function, thereby improving intestinal barrier integrity and immune regulation. Advances in metabolomics now provide powerful tools to investigate the molecular signatures of these interactions, enabling the identification of functional biomarkers and pathways of action. This research explores both human and avian intestinal cell models—specifically the well-established Caco-2 and the novel Chick8E11 lines—in combination with GC-MS metabolomic profiling. The study's integrative design, including both *in vitro* and *in ovo* approaches, addresses an important gap in translating cellular-level findings into predictive models for animal and potentially human health.

1. *ASSESSMENT OF DISSERTATION STRUCTURE AND COMPLIANCE WITH TITLE*

The dissertation submitted for review showed a classical structure of a PhD thesis and comprises 183 pages including appendices. The thesis structure is clear and logical with all the chapters appropriately named and proper proportions between individual sections of the thesis. The studies presented are well-organized, moving logically from background and literature review to clearly formulated aims, detailed methodology, and comprehensive results. The research aligns fully with the stated title and

scope, effectively addressing both metabolic signatures and functional aspects of probiotics and prebiotics in intestinal models.

The reported bibliography comprises over 200 references appropriately selected for the thesis content. The majority consist of publications from the last 5–7 years (2018–2025), ensuring the review is up to date. Furthermore, the cited references are sourced from high-impact international journals in the fields of probiotics, prebiotics, microbiology, intestinal health, metabolomics, animal nutrition, and systems biology. Some old references are present in the list and included selectively to provide essential background or methodological foundations (e.g., qPCR methods, early metabolomics approaches). The references generally follow a consistent style, but a few inconsistencies were noted in punctuation, full titles or standardized abbreviations and the DOI format. I suggest to add indentation, or hanging format in order to improve the bibliography readability.

2. ***ASSESSMENT OF METHODOLOGY***:

The Materials & Methods section is methodologically robust and well aligned with the stated objectives, integrating *in vitro* (Caco-2 and Chick8E11), co-culture, and *in ovo* models with advanced metabolomic profiling (GC-MS) to capture probiotic and prebiotic interactions. The literature review preceding the methods is adequate in framing intestinal models and metabolomics, though a more critical comparison with existing methodologies would further justify choices. Study design and implementation are comprehensive, with careful optimization of culture conditions, adhesion assays, qPCR, and immunofluorescence to validate functional outcomes. Statistical analyses are appropriate and sophisticated, combining univariate (t-tests, ANOVA, Wilcoxon) with multivariate (PCA, PLS-DA, OPLS-DA) approaches, supplemented by correlation and pathway enrichment analyses; however, explicit mention of multiple comparison correction (e.g., FDR) would strengthen the rigor. Overall, the section demonstrates strong methodological quality, with only minor refinements recommended to enhance critical integration and statistical transparency.

- **Literature Review Quality:** In Material & Methods chapter relevant and recent references covering both the biological context and the metabolomics analytical framework are cited. The reported references are important for supporting the rationale for using Caco-2, Chick8E11, and *in ovo* chicken embryo models.

- **Research Objectives and Methodology:** Objectives are clearly formulated in the Aims and Hypotheses section, the methodology is well aligned with objectives and describe the study of metabolic interactions of probiotics and prebiotics using GC-MS, intestinal co-culture models, and in ovo assays. In particular, the three-tier design (*in vitro*, co-culture, *in ovo*) increases robustness and translational relevance of the results.
- The **Study Design and Implementation** is comprehensive, technically rigorous, and using well-validated analytical platforms. Replicates (≥3) and controls are included, enhancing reliability**.** Appropriated Research Tools as analytical and imaging techniques, validated cell culture protocols, and optimized GC-MS parameters are used.
- **Statistical Analysis:** Suitable application of both univariate and multivariate statistics; appropriate pathway enrichment analysis; careful interpretation of supervised classification models. Strong statistical design is showed, however explicit mention of multiple comparison correction (e.g., FDR) would strengthen the rigor.

3. *ASSESSMENT OF RESEARCH RESULTS*

The most significant results demonstrated that prebiotic supplementation (fish protein hydrolysate and seaweed extract) significantly altered the metabolic footprint of *Bifidobacterium lactis*, with clear changes in organic acids such as lactic, citric, acetic, and phosphoric acid ($p < 0.05$). Moreover, amino acid metabolism, energy metabolism, and two-component signal transduction systems were activated, indicating functional modulation of probiotic metabolism. In the intestinal cell co-culture model, *B. lactis* enhanced tight junction integrity in both Caco-2 and Chic-8E11 cells, with statistically significant upregulation of markers such as ZO-1, Occludin, Villin, and CK18 ($p < 0.05$), supporting its role in strengthening the epithelial barrier. Additionally, Bacillus strain showed strong adhesion to intestinal cells and biological surfaces, facilitating colonization and potentially supporting beneficial microbiota stability. In addition, probiotic supernatants (postbiotics) also elicited gene expression changes, indicating that metabolites themselves contribute to host modulation. In *in ovo* experiments, probiotics and symbiotic (probiotic + prebiotic) significantly influenced the metabolomic profile of chicken intestinal contents, further validating the translational potential of *in vitro* results to an animal model.

***RESULTS PRESENTATION*:**

The research results were presented in 9 tables and 74 figures. The data presented in this manner are clear and easy to interpret, as tables effectively summarize methodological parameters and enrichment analyses, while figures provide strong visual support for complex metabolomic and cellular results. The result descriptions are substantively correct from a methodological perspective, with appropriate application of statistical tests (e.g., PCA, OPLS-DA, ANOVA, KEGG enrichment). Overall, the presentation is coherent and facilitates the reader's understanding of the findings.

4. ASSESSMENT OF DISCUSSION

The discussion section is comprehensive and well-structured, demonstrating a clear connection between the research aims, hypotheses, and the obtained results. The candidate effectively integrates findings from metabolomics, intestinal co-culture models, and *in ovo* experiments with relevant literature, providing a sound scientific context. The interpretations are consistent with the presented data and show an ability to critically analyze both expected and unexpected outcomes.

The study's limitations are acknowledged, particularly regarding the translational potential of in vitro and *in ovo* models to *in vivo* systems, as well as the relatively narrow scope of probiotic and prebiotic strains tested. Nonetheless, these limitations are discussed appropriately, without undermining the validity of the conclusions.

The conclusions are clearly formulated, substantiated by the results, and provide a logical summary of the work. They emphasize the predictive value of in vitro and *in ovo* metabolomics models in evaluating probiotic and prebiotic functions, while also outlining potential applications in poultry and human health. Importantly, the discussion highlights directions for future research, such as expanding strain diversity and refining metabolomic pathway analysis.

Overall, the discussion demonstrates critical thinking, methodological awareness, and scientific maturity, making it a strong component of the dissertation.

SUMMARY

The doctoral dissertation here presented is a significant and original contribution to the field of gut microbiology and metabolomics. The work is characterized by methodological rigor, innovative use of dual intestinal cell models, and robust analytical techniques. The findings provide valuable insight

into the mechanistic actions of probiotics and prebiotics and their potential translation from laboratory models to *in vivo* systems.

Finally, based on the scientific quality, originality, and execution of the research, I recommend that the doctoral thesis by **Sanling Zuo, MSc, be accepted with minor revisions**. In addition, I would like to know whether scientific papers related to the thesis topics are already in preparation or have been published, and whether the Author could clearly indicate this in the summary of her thesis.

The dissertation meets and, in several respects, exceeds the requirements for the award of the degree of Doctor of Philosophy in Health Sciences. Therefore, it fulfills the conditions specified in Article 187, Sections 1–4, of the Act of July 20, 2018, on Higher Education and Science (consolidated text, Journal of Laws of 2018, item 1668).

RECOMMENDATION: In connection with the above, I submit to the esteemed Disciplinary Council of Health Sciences at Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, a motion to admit Dr Sanling Zuo, to the further stages of doctoral proceedings.

Sincerely,

Dr. Angela Cardinali
Institute of the Science of Foods Production
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