Catania, September 4th 2025

Department/Institution: Agriculture, Food and Environment, University of Catania, Italy

Reviewer: prof. Cinzia Lucia Randazzo

Academic Title: full professor of Food Microbiology

REVIEW OF DOCTORAL THESIS

Candidate: Sanling Zuo, MSc

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

Supervisor: Dr hab. Katarzyna Stadnicka

2nd Supervisor: Dr hab. Przemysław Kosobucki

Field of Study: Health Sciences

1. INTRODUCTION

This thesis explores the beneficial role of probiotics, alone and in combination with prebiotics, in enhancing intestinal barrier function, cellular metabolism, and microbiota balance, thereby contributing to intestinal health. The aim was to detect key metabolic signatures and pathways involved in energy production, amino acid metabolism, and antioxidant defense, demonstrating synergistic effects between specific probiotics and prebiotics, through in vitro and in ovo models. The findings provide new perspectives for the development of targeted strategies for probiotic screening, validation, and the design of personalized probiotic formulations for therapeutic and preventive purposes. Overall, the introduction provides a solid and detailed background on metabolomics and the gut microbiota, highlighting their importance in host health. However, it would benefit from a more concise metabolite section and an expanded focus on probiotics and prebiotics. Improving clarity and explicitly stating research gaps and study aims would strengthen its impact and relevance within the broader field. Regarding the current state of knowledge the thesis shows metabolomics and microbiota as well-established tools for biomarkers and mechanistic studies; however molecular mechanisms of probiotics and prebiotics remain unclear and require further investigation. It is clearly established the value of metabolomics for understanding host-microbiota interactions, but we need clearer articulation of the study's objectives and clinical relevance.

2. ASSESSMENT OF DISSERTATION STRUCTURE AND COMPLIANCE WITH TITLE,

The dissertation submitted for review follows a classical and well-organized structure, spanning 183 pages including appendices. The layout is clear and logical, with chapters appropriately titled and balanced in length, ensuring a coherent flow throughout the thesis. It begins with a comprehensive table of contents and a detailed list of abbreviations, facilitating reader navigation.

- **Structural components**: The thesis includes all essential sections expected in academic research, such as introduction, materials and methods, results, discussion, and conclusions, which are well proportioned and logically arranged.
- **Bibliography:** The reference list contains 161 citations carefully selected to support the thesis content. Most references are recent (within the last 15 years) and drawn from reputable international journals. The literature predominantly focuses on probiotics and prebiotics, cell culture models, and metabolomics, reflecting the multidisciplinary nature of the research.
- **Editorial Observations and areas for editorial improvement**: Only a few oversights and typographical errors are present in the dissertation.

3. ASSESSMENT OF METHODOLOGY

Overall comment: The methodology is generally well-structured and appropriate for the research objectives. The literature review provides a solid background, and the study design is coherent and systematic. Minor weaknesses include limited critical analysis of literature gaps, incomplete reporting of certain methodological details, and a lack of deeper discussion on statistical power and confounding factors. Overall, the methodological framework is robust and suitable to support the research outcomes. Below the main criteria, assessments and overall rating considered.

Criterion	Assessment	Overall rating
Literature review quality	Up-to-date and relevant, covering key research in the field	Good
Research objectives and methodology	Objectives are clearly stated and well aligned with the literature. Methodology is coherent with the research aims.	Very good
Study design and implementation and applied research tools	Well-structured design with adequate description of samples, operational phases, and applied tools.	Good
Statistical analysis	Statistical tests are appropriate for the type of data	Good

4. ASSESSMENT OF RESEARCH RESULTS

Remarks: Overall, I would like to commend the PhD candidate for the large amount of data presented and subjected to statistical analysis. Results description are correct from a methodological perspective and are reported in Sections 1.1 to 1.5 and are summarized in Figures 5 to 74 and in Table 5. The data presented in this manner are clear and easy to interpret. The results highlight that the methodological approach employed, supported by statistical analysis, is correct and has allowed us to highpoint the combined effect of the best symbiotic formulation. Below the main key findings of each paragraph.

Key findings of 1.1. Influence of protein hydrolysate and algal extract on Bifidobacterium lactis metabolism

- Protein hydrolysate and algal extract strongly affect *B. lactis* metabolism.
- Algal extract promotes a broader diversity of metabolites and activates energy-related pathways (oxidative phosphorylation).
- Protein hydrolysate enhances specific metabolites (lactate, citrate, phosphate)
- Metabolite profiles clearly reflect the type of available substrate, showing differential prebiotic effects

Key findings of 1.2. Characterization of the intestinal co-culture model

- By comparing Chic-8E11 and Caco-2 cells under both monolayer and spheroid conditions, distinct morphological and growth features was confirmed, reflecting their potential to mimic different aspects of intestinal physiology.
- Monolayers allow the study of barrier integrity and cell–cell interactions.
- Spheroids offer a more physiologically relevant 3D architecture resembling early organoid stages.

The model is applicable for investigating nutrient absorption, metabolite production, host–microbiota interactions, and the impact of bioactive compounds on intestinal function.

Key findings of 1.3. Direct interaction of probiotics (Bifidobacterium lactis and Bacillus strain) with intestinal cells

- *Bifidobacterium lactis* adheres strongly to both Caco-2 and Chic-8E11 (stronger to Chic-8E11)
- Chic-8E11*: Bifidobacterium* up-regulates villin, cytokeratin 18, occludin; *Bacillus* down-regulates ZO-1
- Caco-2*: Bifidobacterium* up-regulates E-cadherin, tight junction genes, IL-18; *Bacillus* induces mixed effects
- Caco-2: Occludin enhanced by both probiotics; E-cadherin and claudin-1 affected differently depending on probiotic and supernatant

Key findings of 1.4. Metabolic footprint and fingerprint of probiotic activity in intestinal cells

- Co-culture with *Bifidobacterium* increases bioactive metabolites and a metabolic shift (Volcano plots & Heatmap, Figures 6–7)

- *Bacillus* and *Bifidobacterium* modulate intracellular metabolism; *Bifidobacterium* favors energy metabolism, intestinal barrier, and lipid metabolism as showed in the KEGG plots (figures 10-11)

Heatmaps show clear metabolite shifts, highlighting significantly enriched metabolic pathways.

Key findings of 1.5. Metabolomic analysis of chicken intestinal contents after in ovo injection of candidate probiotics, prebiotics and synbiotics

- *Bifidobacterium lactis* NCC2818: improves intestinal barrier, increases tight junction gene expression, enriches bioactive metabolites.
- *Bacillus* strain: selective effects; strong adhesion to Chic-8E11, variable influence on genes and metabolites.
- Combined data highlight a functional interaction between probiotics and intestinal cells, confirmed at both morphological and metabolic levels.

5. ASSESSMENT OF DISCUSSION

The discussion section is clear, well-structured, and effectively compares the results of the study with existing literature. The findings on the role of probiotics in strengthening intestinal barrier function and the comparative evaluation of Caco-2 and Chic-8E11 cells are well-argued and highlight the potential of the Chic-8E11 3D model. The integration of advanced metabolomics analyses adds value and provides a systemic interpretation of the data.

Some limitations remain, mainly the reliance on in vitro models, which cannot fully reflect in vivo complexity, and the need for confirmation of enriched metabolic pathways in animal or clinical studies.

Overall, the discussion is strong and well-written, though it would benefit from a brief concluding section (currently included only in the Summary) to reinforce the key message.

SUMMARY:

The doctoral dissertation under review provides an in-depth investigation of gut microbiota–host interactions, with particular emphasis on metabolomics approaches to study probiotics, prebiotics, and their symbiotic effects. The work is presented in a well-structured and rigorous manner, supported by an appropriate methodology, comprehensive statistical analyses, and a thorough comparison with relevant literature. While the discussion would benefit from a brief concluding section, the dissertation demonstrates originality, scientific maturity, and a valuable contribution to the field of intestinal health research. Therefore, it meets 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).

I consider the dissertation to be of high scientific quality and therefore suitable for acceptance and public defense. 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 Sanling Zuo, MSc to the further stages of doctoral proceedings.

Cinzia Lucia Randazzo

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