Centrum Zaawansowanych
Materiałów i Technologii
CEZAMAT
Politechnika Warszawska

Warsaw, 20 February 2023

Associate Professor Monika Staniszewska
Centre for Advanced Materials and Technologies
(CEZAMAT), Warsaw University of Technology

**Review of the Doctoral Dissertation**

**Candidate:** MSc Miriam Gonzáles Gonzáles

**Title of the doctoral thesis/dissertation** Host Cell Response to Polymicrobial Biofilms: Implications in Aspiration Pneumonia

**Study Programme Tutor:** Professor Maria Rąpała-Kozik

Jagiellonian University in Krakow, Institute of Zoology and Biomedical Research Faculty of Biology, Department of Comparative Biochemistry and Bioanalytics, Faculty of Biochemistry, Biophysics and Biotechnology.

**Reviewer's e-mail:** monika.staniszewska@pw.edu.pl

**Topicality of the doctoral dissertation theme:**

The microbial (fungal and bacterial) community implications in aspiration pneumonia were analysed in the dissertation. It is the continuation of the thorough studies conducted by Prof. Rąpała-Kozik's team; the scientific background was described in the dissertatioin's introduction. Thus, the PhD student undertook the phenotypic, molecular, and biochemical characterization of the main etiological agent of periodontitis: anaerobic gram-negative *Porphyromonas gingivalis* bacteria cooperating with the fungal commensal *Candida albicans.* This approach is justified due to a correlation between periodontal diseases and the development of aspiration pneumonia. Both pathogens co-exist in the oral cavity, and display many virulence factors responsible for periodontitis progression which is the cause of secondary infections such as aspiration pneumonia. As enumerated by the PhD student, the following: dysphagia, mental state (dental plaque), and nosocomial infections (invasive procedures) promote aspiration pneumonia. In the dissertation the studies on *C. albicans* virulence are fully justified due to the commensal of the human oral cavity and the 'silent fellow' responsible for life-threatening infections named candidiasis or candidaemia. **On the other hand, the protective role of *C. albicans* against pathogenic bacteria is still under investigation. These two microorganisms are responsible for aspiration pneumonia, while their role and behaviour in this pathology are still not understood. Especially the mutual interplay of the oral cavity inhabitant on lung fibroblasts. The studies are of particular importance in the proper therapy that influences public health issues.**

For these reasons the dissertation of Ms Miriam Gonzáles Gonzáles is an interesting proposal and responds to current research challenges of biology and biomedical research. The ideas formulated and developed in this dissertation as well as the results presented are a perfect response to the current needs of the scientific community.

**Fulfilment of the doctoral dissertation's objectives**

The goals are related to the three general research fields of the team, thus Ms Miriam Gonzáles Gonzáles undertook the continuation of the studies, e.g.:

1. Characterization of heterotypic biofilms formed by indicated pathogens in aerobic conditions, considering the anaerobic behaviour of bacteria.
2. Characterization of the interactions between lung fibroblasts in 2D and 3D cell culture with homo- and heterotypic biofilms of these pathogens.
3. Characterization of the interactions between macrophage-like cells stimulated with homo- or heterotypic biofilms of these pathogens.

Based on the above, the specific goals were stated as follows: briefly, to characterize gingipain production in heterotypic biofilms; to find out the biofilm behaviour in the presence and absence of gingipain (null mutant was tested); to assess fungal morphology in heterotypic biofilm; to optimize 3D infectious cell culture model; to study the interplay between fibroblasts and microbial biofilm in 2D and 3D models; to characterize macrophage-like THP-1 cell gene expression levels after stimulation with biofilms. In order to accomplish these supernatants and coculture of microbes were studied.

**Regarding Materials and Methods:** The Author accurately (1) selected WT strains and mutants to prove the hypothesis; (2) performed the culturing of human cell lines and microorganisms as well as single and dual cell culturing to produce biofilm; (3) assessed the metabolic activity and proliferation of microbes (yeasts) and human cells. In detail, CFU/mL of single and dual-species biofilm assessment and quantification of gingipain activity were described. The Author precisely described the *in vitro* culturing methods, e.g. THP-1 macrophage-like differentiation, colonization (singular or dual) with microbes in 2D or 3D model, and production of recombinant aspartic proteases; most of the described methods were established and published by the scientific team which Ms Miriam Gonzáles Gonzáles worked with. All molecular techniques: from immunocytochemistry to quantitative RT-PCR (paragraph 9) were used to understand the mechanism of infection with polymicrobial biofilm and its implication in pneumonia. **Thus the procedures to study the host cell response to polymicrobial biofilms meaning implications in pneumonia can be treated as successfully met goals.**

**Results of the doctoral dissertation, the Author's concrete achievements**

The Author first assessed the production of gingipains correlated with bacterial survival in mixed biofilm; furthermore, optimization of the enzyme production was achieved. Secondly, the Author showed that fungal-mediated biofilm in the presence of FBS, but in an unfavourable aerobic environment, boosted the bacterial cell activity (production of Rgp and Kgp). The above results decided that the proposed microbiological system (mixed-biofilm) can be further tested to fulfil other goals.

Using the *C. albicans* morphogenesis mutants the Author proved that these yeasts promote *P. gingivalis* growth under aerobic conditions. It was shown that the fungal component is pivotal to bacteria survival in aerobic conditions. The Author showed that an increase in optical density in heterotypic biofilms (including attachment) and microbial coexistence is governed by gingipains produced by the W83 strain.

Relative gene expression profiles were assessed in 2D followed by 3D cell culturing, where the direct contact of fibroblasts with microbes or soluble components was examined; the Author observed an up and down expression of the tested genes e.g.: proinflammatory cytokines, fibrotic genes; finally, the accurate conclusions regarding the comparison of these two conditions were included. The same tendency for 2D and 3D in the case of proinflammatory and fibroblastic genes was observed. The differences were noted for ACTA2 considering 2D and 3D models. The analysis of fibroblast migration considered accurate microbial supernatants, and the Author marked the influence of hyphal secretome and gingipains on fibroblast migration.

The Author was critical of the obtained results concerning the 3D model of embedded fibroblasts in the collagen matrix. Evident shortcomings of the used assessment can be seen. Thus, the thesis does not confirm the hypothesis that fibroblasts are more susceptible to undergoing myofibroblast transition after stimulation with biofilm supernatant. Moreover, the Author demonstrated shortages of the resazurin-based reagent (named PrestoBlue) in the viability quantification of fibroblasts.

The Author displayed that rSap6 does not trigger proinflammatory and fibrotic effects on lung fibroblasts based on the alpha-SMA fluorescence intensity gene relative expression assay followed by protein production. Characterization of the viability and relative gene expression patterns of macrophage-like cells stimulated with biofilm supernatant (homo and heterotypic) showed that gingipains play a pivotal role in initiating and perpetuating proinflammatory states of macrophages.

**Regarding the results, the Author presented novelties as follows: (1) evidence that hyphal secretome and gingipains direct cell migration; (2) characterization of the fibrotic potential of the *C. albicans* secretome; (3) wound healing assays with MRC-5 stimulated with rSap6; (4) rSap6 on alpha-SMA protein production in lung fibroblasts.**

**In the Discussion,** based on her own achievements in the presented dissertation, the Author skilfully discussed the world literature on the action of the two pathogens mutually supporting each other in occupying the host niches. In the dissertation the Author indicated the new studies undertaken in response to the needs of the literature. The Author emphasized the role of gingipain in the detachment of heterotypic biofilm-favoured variable cell dispersion involved in pathogenesis. The Author presented findings of high importance for the practice and development within a branch of science.

The Author discussed the overall novelty of the dissertation, namely the findings on the molecular potential implication of these two inhabitants of the oral cavity in aspiration pneumonia. Briefly, the PhD student showed the role of fungal filamentation as crucial for the colonization process, with no remarkable meaning for SAP deficiency in this. The Author first undertook the attempt to characterize the gene expression profile of (1) fibrotic markers in 2D and 3D models, and (2) THP-1 macrophage-like cells. For the first time the Author showed the involvement of Saps in promoting pro-myofibroblast. The Author outlined further areas of future research that are a continuation of her research, e.g. the transcriptomic profile of fungal biofilm regulation using the W38 strain and gingipain-null mutant (KRAB). The Author discussed the limitations of her studies versus literature achievements.

**However, some issues should be raised:**

1. Considering the methodology: Methods, p. 54; it is not clear why biofilm activity was assessed with XTT after 10 min of incubation.
2. The Author presented high standard deviations between replicates of Cal (WT and mutants) adhesion to fibroblasts or myofibroblasts, yeast staining with CFW and SYBR green was used. The Author considered further verification using another technique so why was not the CFU method (adopted in other experiments) included in paragraph 2.1.1?
   I propose to consider the quantification of *Candida* morphotypes attached to fibroblasts/ myofibroblasts to be confirmed using CFU counting, briefly, applying cell washing (to remove untouched cells), lysing the fibroblasts with water to recover fungal morphotypes, and finally culturing the attached cells. Continuing the above in the results:
3. The fibroblast viability under rSap6 influence was quantified using PrestoBlue (a resazurin-based reagent); it needs to be verified with a more sensitive method the Author is expected to propose.

**Editorial mistakes to be corrected:**

4. For *alpha* smooth actin's (abbreviated as ACTA2) another acronym of αSMA (often used in the text, e.g., p. 145) ought to be included in the table named 'List of abbreviations and symbols', p. 9.
5. Goal 5 on p. 47 is difficult to understand.
6. P. 46 editorial mistake: 'dot' at the beginning of the second sentence (line 2, p. 46).
7. Double space in the middle of the sentence of line 13 p. 46.
8. Double space in line 13 p. 52.
9. Bracket ought to be removed in line 16 p. 53.
10. Double space in line 12 p. 86.
11. In vitro - italic is needed in line 6 p. 96.
12. Put 150,000 instead of 150000 in line 19 p. 55.
13. The GAPDH housekeeping protein instead of the gene ought to be given in line 19 p. 128.
14. P. 132 figure 45: the legend description is not properly organized: it extends to the margin area.
15. P. 138 line 3: 24 h ought to be presented in the same line without separating them.
16. Whatever instead of whatsoever in line 9 p. 152.
17. Put *F. nucleatum* instead of F. nucleaturn in line 10 p. 162.
18. Lack of KCN explanation in the List of abbreviations and symbols on pp. 9-10.
19. Lack of KYT-1 explanation in the List of abbreviations and symbols on pp. 9-10.
20. Lack of OMV explanation in the List of abbreviations and symbols on pp. 9-10.
21. Lack of MMP explanation in the List of abbreviations and symbols on pp. 9-10.

The Author adopted the formal layout of a doctoral dissertation which is typical and obligatory for this procedure. The quality of the language is accurate.

**Final assessment of the doctoral dissertation**

In conclusion, I would like to state that all the remarks contained in this review do not affect the quality and validity of the conducted research. I assess this doctoral dissertation very highly, especially emphasizing its substantiveness, transparency, and importance for medical application. Master Miriam Gonzáles Gonzáles showed very good theoretical preparation, mastery of modern research techniques with well-chosen biomolecular tools containing elements of innovation, and the

ability to critically analyse the results obtained. The work contains original elements as indicated above in the review. It is worth emphasizing that the achievements of the PhD student are of cognitive importance supplementing the knowledge in the field of aspiration pneumonia pathogenicity.

The dissertation is a significant achievement of the PhD student. I am fully convinced that the doctoral dissertation submitted for evaluation entitled *Host Cell Response to Polymicrobial Biofilms: Implications in Aspiration Pneumonia* is a valuable contribution to the field of natural sciences in the discipline of biological sciences and meets the criteria set out in Art. 187 of the Act of July 20, 2018 Law on higher education and science (Journal of Laws 2018, item 1668 with later amendments). Based on the above opinion I am applying to the Scientific Council of the Jagiellonian University in Krakow to admit MSc Miriam Gonzáles Gonzáles to the further stages of the doctoral dissertation degree programme and to distinguish her doctoral dissertation.

PL: Z pełnym przekonaniem uważam, że przedstawiona do oceny praca doktorska pod tytułem" Host cell response to polymicrobial biofilms: implications in aspiration pneumonia" stanowi wartościowy wkład dla dziedziny nauk ścisłych i przyrodniczych w dyscyplinie nauki biologiczne i spełnia warunki określone art. 187 Ustawy z dnia 20 lipca 2018 roku Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.). W oparciu o powyższą opinię wnioskuję do Rady Dyscypliny Nauki biologiczne, o dopuszczenie mgr Miriam Gonzáles Gonzáles do dalszych etapów przewodu doktorskiego oraz wyróżnienie rozprawy doktorskiej.

Date: 20 February 2023

Reviewer's signature: Monika Staniszewska