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Review of the PhD thesis entitled „Host cell response to polymicrobial biofilms: implications in aspiration pneumonia" prepared by Ms. Miriam Gonzalez Gonzalez

The PhD thesis was completed in the Institute of Zoology and Biomedical Research (Faculty of Biology) and in the Department of Comparative Biochemistry and Bioanalytics (Faculty of Biochemistry, Biophysics and Biotechnology) of the Jagiellonian University under the supervision of Professor Maria Rąpała-Kozik. This research group is highly experienced in characterization of biofilm formation and its influence on host response in diseases caused by *Candida albicans* and *Porphyromonas gingivalis*, and the content of the thesis extends the knowledge in this research area.

The thesis (206 printed pages) is dived into four main chapters, describing particular stages of the theoretical and experimental work. The Introduction chapter contains overview of the literature, which clearly introduces the subject studied. Significant part of the introduction contains description of aspiration pneumonia and its association with periodontal diseases and virulence factors produced by the main etiological agent of periodontitis, *Porphyromonas gingivalis*. Also, correlation between *C. albicans* and virulence factors produced by this microorganism and their implication in aspiration pneumonia is presented. Particular attention is paid to biofilm formation by both pathogens and its relation to the host response. The introductory part of the thesis is very useful, especially for readers not familiar with this subject, and may serve as a good review.

Several subsequent pages contain broad and detailed description of chemicals, materials and a variety of methods and techniques used. This chapter confirms broad and multidisciplinary experience of Ms. Miriam Gonzalez Gonzalez in methods used to culture bacteria, fungi and host cells, experience in molecular biology techniques, and experience in analytical, biophysical, microscopic, and biochemical methods. Materials and Methods section is written well, with many details, and may serve as a good laboratory guide for students.

Further sections of the thesis contain description of results. Each chapter is focused on separate experiments, contains short introduction, details of the experiments performed, obtained results and short conclusions. In general, all experiments were designed and performed properly, were repeated very often several times and some of the results obtained were also confirmed by alternative methods. All results are described in very details in the text, and shown in tables and figures. Figures were prepared well, clearly demonstrate results, and contain sufficient statistical analysis. However, although in many cases legends are described properly, often with many details, some figure legends are incomplete [for example: page 74, Figure 15: substrate used in this experiment is not indicated; page 87, Figure 24: what was measured and then shown as % Metabolic activity (A450)"]. In some figures (for example pages 77 and 78, Figures 17 and 18) OY axis is labeled "Optical density (A600)" instead of "Optical density (OD600) or labeling is confusing (for example "Ratio mAbs/millions of W83"; page 68, Figure 10B). In addition, very often legends to figures are placed on separate pages than figures (double page printing would be helpful in case of large figures and long legends) or even figure is divided and shown on two separate pages (pages: 121 and 122, 127 and 128, 143 and 144). Although the results are interesting, their huge number (the reviewer is really impressed by the work done) and not a very perfect style of writing, makes it a little difficult to understand and

formulate conclusions. In order to make easier to understand so many results, the reviewer suggests improving the style of writing, because the work, despite the fact that it is very interesting and important, is quite difficult to read and follow all findings. The reviewer is not an English native speaker and will not comment on the language, but it must be improved before publication.

In the Discussion section, a reference to published data, indication of some limitations, and future perspectives are presented. The style of the Discussion section presentation in the form of separate groups of three types of experiments is very useful to resume the results obtained. The entire thesis is supported by ~400 references, in general cited properly, which support the current knowledge and discussion regarding the subject presented in the thesis. However, a few examples of insufficient citing (on page 26 papers Kosno et al. 2022 and Olczak et al. 2015 are cited – review Smalley and Olczak 2017 would be more useful, because it shows better, in a wider context, the problem discussed on this page) or the lack of important reference (Guo et al. 2020 – analysis of dual *P. gingivalis-C. albicans* biofilm) have been found.

Besides minor critical comments indicted above, the aims of the PhD thesis are clearly defined and the data obtained fully answer questions asked. The influence of mutual interaction between *P. gingivalis* and *C. albicans*, as well as between these potential pathogens and the host immune system (comprising both direct and indirect contact) adds to the knowledge of bases of processes, which could occur during aspiration pneumonia, but have been not taken into consideration before.

According to the reviewer's opinion, the most important general findings presented in this thesis include:

1. demonstration that *P. gingivalis* co-cultured with *C. albicans* in the form of heterotypic biofilm under aerobic conditions, due to the supportive role of *C. albicans*, increases gingipain activity and causes that *P. gingivalis* replicates with higher efficiency,
2. characterization of the pro-inflammatory and profibrotic responses of lung fibroblasts stimulated with the pathogens using fibroblasts on both 2D and 3D surfaces,
3. characterization of pro-inflammatory response of macrophage-like cells (THP-1-derived macrophages) towards agents produced by members of heterotypic biofilm and present in supernatants.

In general, Ms. Miriam Gonzalez Gonzalez demonstrates many interesting, original, and novel data, forming important contribution to the research area studied. Moreover, data gained in this work suggest a direction for future studies, also considering therapeutic strategies.

To clarify some achievements presented in the thesis and to broaden the discussion, the reviewer would like to ask for a few additional explanations.

1. The cell viability of *C. albicans* and *P. gingivalis* in heterotypic biofilms demonstrated on page 83 was examined by CFU analysis. How colonies of *P. gingivalis* were differentiated from colonies formed by *C. albicans* ?
2. The paper published by Guo et al. in 2020 (Frontiers in Microbiology, 11:596459, "Heme competition triggers an increase in the pathogenic potential of *Porphyromonas gingivalis* in *Porphyromonas gingivalis-Candida albicans* mixed biofilm"), and not cited in the thesis, showed increased virulence potential of *P. gingivalis* when *P. gingivalis* was grown in a biofilm together with *C. albicans* (including increased gingipain expression among other findings). There are some differences in experiments performed in this PhD thesis and in experiments demonstrated in the paper - whether it is possible to compare the results obtained for different culture conditions in the case of these microorganisms and correlate results presented in the paper with results presented in this thesis ?

3. In the PhD thesis, more virulent *P. gingivalis* W83 strain was used. How conclusions drawn from the results gained in this study using laboratory strains could be correlated with those obtained for clinical *P. gingivalis* isolates ?
4. Is there any possibility that detached from the biofilm structure and then co-aggregating *P. gingivalis* and *C. albicans* cells, but taking advantage also from compounds secreted by microorganisms forming biofilm structures, would be more virulent in comparison to the cells being trapped in biofilm in lungs ?
5. *P. gingivalis* releases outer membrane vesicles, with important virulence factors as their cargo. Whether it is possible that coexistence of *P. gingivalis* and *C. albicans* in biofilm structures or in a co-aggregated form, in contrast to mono-cultures, may cause increased ability of outer membrane vesicles production by *P. gingivalis* ?

Finally, it is worth noting that part of the data presented in the PhD thesis has been published in one research paper, with Ms. Miriam Gonzalez Gonzalez as the second author (Front Cell Infect Microbiol, 2022). It is certain that also the remaining results will be published soon. It is also worth mentioning that Ms. Miriam Gonzalez Gonzalez is the co-author of 5 additional papers, focused on other research areas than the PhD thesis content.

In conclusion, the reviewer's overall opinion is very good. This PhD thesis meets the conditions set out in Article 187 of the Act of July 20th 2018 on Higher Education and Science (Artykuł 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce; Dz.U. z 2018 r. poz. 1668 z późn. zm.). The overall content and presentation of this thesis is satisfactory. Therefore, I recommend to the Biological Sciences Discipline Council of Jagiellonian University (Rada Dyscypliny Nauki biologiczne Uniwersytetu Jagiellońskiego) Ms. Miriam Gonzalez Gonzalez for admission to further stages of the procedure for conferring a doctoral degree in the field of exact science and life sciences in the discipline of biological sciences (dziedzina nauk ścisłych i przyrodniczych, dyscyplina nauki biologiczne).