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REVIEW

Doctoral dissertation of Katarzyna Pacyga-Prus, MSc

„ Biological activity of *Bifidobacterium animalis* ssp. *animalis* CCDM 218 and *Bifidobacterium adolescentis* CCDM 368 surface antigens in complex interactions with the host organism - prevention/treatmentof allergy diseases"

The prevalence of allergic diseases has become increasing rapidly worldwide over the last few decades, becoming one of a significant healthcare problem. Among the pivotal hallmarks of allergic diseases is the excessive activation of T helper 2 cells (Th2) that coordinate the activity of other cells involved in allergic inflammation. As a result of these cellular interactions the production of allergen-specific IgE, mast cell degranulation and accumulation of eosinophils in the affected tissues and/or in peripheral blood occurs leading to symptoms of allergy. The Th2 pathway dominates during fetal life to protect the fetus from rejection. We are therefore born with a dominance of the proallergic Th2 pathway. In early life, this dominance is then balanced by the development of a microbe-induced, antagonistic Th1 pathway and, above all, the development of proper immune tolerance mechanisms.

An upward trend in allergic conditions is forecast as a consequence of genetic predisposition, increased environmental pollution, industrialization, urbanization and rising temperatures accompanying climate changes. Limiting the inappropriate medical or agricultural use of antibiotics, implementing of balanced diet supplemented with prebiotics, probiotics and postbiotics, reducing greenhouse gas emissions and preventing exacerbation of existing allergy disease is therefore one of the pressing and global challenges we must address if we want to realistically face the problem of allergic diseases.

In addition to allergy prevention, an equally important challenge in the fight against allergy is the development of effective therapies based both on new drugs and on augmenting the host's natural defenses while inhibiting inappropriate immune response. It highlights the need for

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continuous searching for new or exploring the existing immunomodulators with anti-allergic properties. MSc K. Pacyga-Prus's research work is part of both trends and is focused on the search for natural immunomodulators of allergic reactions among antigens from bacteria of the genus *Bifidobacterium* namely *Bifidobacterium adolescentis* CCDM 368 and *Bifidobacterium animalis* ssp. *animalis* CCDM 218 belonging to healthy microbiota of our organism.
The dissertation presented for review, as the basis for applying for the degree of PhD, was carried out in the Laboratory of Microbiome Immunobiology at the Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences under the supervision of PhD Sabina Górska, Associate Professor at Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. The work is part of the current research conducted by the promoter, who has been successfully working on the immunomodulatory role of probiotic and postbiotic as well. Thus, the evaluated work was carried out in a team under the supervision of a promoter with the great scientific achievements in this subject. The research was carried out under two projects including the National Science Centre of Poland grant (SONATA BIS 7, UMO-2017/26/E/NZ7/01202) grant entitled "The structure and biological role of *Bifidobacterium* components in allergy disease development" and the Polish National Agency for Academic Exchange grant (MOBILITY, PPN/BIL/2018/1/00005) „New strategy for alleviating allergic response: Effect of the surface bacterial antigens in the prevention and treatment of allergic inflammation in mouse model".

From the formal point of view, the dissertation of MSc Katarzyna Pacyga-Prus is a collection of three scientific original articles, two of which have been published in reputable scientific journal - Carbohydrate Polymers in 2023 and 2024 year (IF 10.7, 140 points of Ministry of Science and Higher Education) and one is a not published yet bioRxiv preprint with DOI number. The collection of publications is preceded by an abstract in Polish and English, a list of abbreviations and a short introduction. The introduction introduces the reader to the brief historical outline along with the changing view of the causes of the development of allergic disorders, detrimental role of dysbiosis in allergy, the limited information about influence of postbiotics on allergy as well as the motivations which guided the doctoral candidate to design the presented studies. The introduction, therefore, clearly indicates the goal of the study, which was to identify the surface antigens responsible for the bacteria's ability to alleviate allergy

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symptoms. Each of the attached manuscripts is preceded by a foreword containing rationale of research objectives, methodology, results and conclusions. The dissertation ends summary of research and perspectives, along with declarations signed by the legally required number of co-authors of the publications included in the achievement and an impressive list of other scientific achievements.

When the dissertation presented for evaluation is based on a collection of multi-authored publications, it is of great importance to assess the PhD student's own contribution. Thus, evaluating the accomplishment in terms of author's own contribution, I conclude that in all 3 manuscripts constituting a series of thematically related works the author's contribution is unquestionably leading. This is indicated by her position as the first and corresponding co-author in all of them, and is confirmed by statements of author's contribution included in publications as well as by the signed statements of most of the co-authors.

The extensive methodological skills of PhD student are noteworthy, including a variety of experimental methods and techniques, both in vitro and in vivo. Starting with microbiological, biochemical and immunochemical techniques, we find microscopic methods, instrumental methods such as chromatography-mass spectrometry (GLC-MS), fast protein liquid chromatography (FPLC), nuclear magnetic resonance (NMR) spectroscopy. and statistical or bioinformatic analyses. The study also used relevant murine models such as ovalbumin-induced allergic inflammation model using intranasal and peritoneal immunization and cell line models (Caco-2, HT-29) as well as isolated immune cells (splenocytes, bone marrow dendritic cells-BMDCs). The description of the methods is clear and unobjectionable.

In summary, the work presented for review formally does not raise any objections.

The main objective of the study was to determine the bacterial surface antigens responsible for the ability to alleviate allergic reactions. The main objective was achieved through the

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following specific objectives of the study:

O1. Isolation and purification of *Bifidobacterium* surface antigens and determination of their cytokine profile in OVA-induced mice splenocytes and DCs.

O2. Structure determination of antigens with the most promising abilities to alleviate OVA-induced mouse sensitization.

O3. Analysis of selected surface antigen's impact on the development of the naïve immune system of GF mice when administered intranasally.

O4: Investigation of the anti-allergic potential of selected surface antigens in the mouse OVA-induced allergy model when administered intranasally.

Assuming that the order of the presented manuscripts was adjusted to the chronology of their appearance, I believe, due to the content described and the fact that the publication 3rd as bioRxiv preprint is a continuation of publication 1st, that the following order would be more appropriate: publication 1st, publication 3rd and publication 2nd. Therefore, this is the order I will let myself keep when I go on to short summary of the results of individual publications. Interestingly, the publications were described in this order in the abstract by the doctoral student herself.

In the first of the experimental research papers (Publication 1th) using murine model of OVA allergy and appropriate immunological, cell biology and instrumental methods, the PhD student tested several potential candidates among the structurally different surface antigens of probiotic *Bifidobacterium adolescentis* CCDM 368. Three PS antigens, one LTA antigen, and one PG antigen that could exhibit potential anti-allergic activity, were used to finally select one of them, the polysaccharide antigen BAP1, which acted most efficiently. This action exerted on splenocytes and naive BMDCs included both inhibiting of proallergic Th2 pathway reflected by a decrease in the release of IL-13 and IL-5, as well as enhancing the anti-allergic Th1 pathway reflected by increase in IFN-γ. In addition, the BAP1 caused the induction of immunosuppressive IL-10 release from OVA-induced splenocytes and DCs. Then effective BAP1 transfer from epithelial cells to dendritic cells was shown suggesting the further efficient antigen presentation. Finally, the chemical structure of the BAP1 antigen as linear

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hexasaccharide with a unique structure consisting of glucose, galactose, and rhamnose residues, and linkages, high molecular weight and lack of charge was determined by NMR spectroscopy.

In the second of experimental research papers (Publication 3rd) PhD student and co-authors documented the ability of BAP1 to reduce allergic inflammation using OVA-specific murine model of allergy. They proved also the neutral effect of BAP1on the naïve immune system of germ free mice documenting increased IgA in bronchoalveolar lavage fluid and serum, decreased CCL2, and eotaxin release, and higher *Rorc* gene expression in the lung. The anti-allergic therapeutic potential of the BAP1 polysaccharide has been confirmed in OVA-sensitized mice both locally by reducing lung inflammation through reduced infiltration of eosinophils and IL-5, IL-4, and IL-13 levels, and systemically by reducing of OVA-specific serum IgE and inhibiting Th2-related cytokines released by splenocytes. In this study, and quite surprisingly, a local and systemic decrease in IL-10 was observed.

Question 1. To the best of my knowledge, human IL-10-secreting Treg, Breg lymphocytes and other leukocytes such as monocytes or dendritic cells exert the allergen-specific suppressive properties facilitating immunological tolerance. For example, it has been documented that based on the induction of the aforementioned tolerance, allergen-specific immunotherapy leads to a reduction of allergic symptoms in certain diseases such as allergic rhinitis, asthma or even venom allergy. In the light of these data, which indicate that the underlying cause of allergy is not only a Th1/Th2 imbalance but rather a failure to develop immune tolerance to the allergen, how does the doctoral student view the results of her own research on the inhibition of IL-10 production by the BAP1 antigen tested?

In the third of experimental research papers (Publication 2nd) the PhD student performed structural and chemical analysis of polysaccharide B.PAT and its dephoshorylated counterpart B.MAT isolated from *Bifidobacterium animalis* ssp. *animalis* CCDM 218 and proved that dephosphorylated B.MAT has mor potent immunostimulatory properties than phosphorylated B.PAT. It was expressed by decrease in TNF-α,IL-6, IL-10, and IL-12 cytokine release from BALB/c murine BMDCs cultivated alone or in the presence of a known immunostimulant *Lacticaseibacillus rhamnosus* GG. They then documented that dephosphorylated B.MAT

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reduced proinflammatory cytokine IL-8 release more potently than B.PAT, as demonstrated using human colorectal adenocarcinoma Caco-2 and colon cancer HT-29 epithelial cell lines and an IL-1β-induced inflammatory model, thus indicating the potential of B.MAT in preventing inflammation.

Question 2. Reading this publication, the following question occurred to me: According to my knowledge and my own experience, Caco-2 cells usually reach confluence 3 days after seeding and then undergo spontaneous differentiation into enterocyte-like cells about 18±20 days post confluence. Therefore, why during the experiments on the Caco-2 cell line commonly used as a model of the intestinal epithelial barrier, the line was cultured for only about 3 days prior to the collection of the supernatant?

In conclusion, I evaluate the whole dissertation very high, and among the most important results of the research conducted by MSc Katarzyna Pacyga-Prus are:

1. Comprehensive chemical and biological characterization along with indication of structure-function relationships of the polysaccharide BAP1 from *Bifidobacterium adolescentis* CCDM 368 and both phosphorylated and dephosphorylated polysaccharide B.PAT from *Bifidobacterium animalis* ssp. *animalis* CCDM 218 as a potential postbiotics with preventive anti-allergic/anti-inflammatory properties
2. Proving that the immunomodulatory and anti-inflammatory potential of B. PAT is phosphorylation-dependent and stronger in dephosphorylated form.
3. Demonstration of comprehensive therapeutic effect of BAP1 antigen in inhibiting of local and systemic allergic reaction primarily by Th2 pathway suppression in mice suffering from OVA-dependent allergy.

The publications included in the dissertation (two of them) have already been verified by independent reviewers, so my comments on them are merely an expression of my scientific curiosity. At this point, I would like to ask the doctoral student to share her knowledge on the following issues and related questions that came to my mind while reading the dissertation:

Question 3. Microbiota imbalance has been linked with different disease states such as irritable bowel syndrome (IBS), Crohn's disease, obesity, cancer and even neurological disorders. What

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is the PhD student's opinion on the anti-allergic therapies based on interference with the gut microbiome in the context of the existence of the gut-brain axis linking the enteric and central nervous system? Can microbiota manipulation directly affect human health with potential adverse effects on mood / psyche, affecting mental disorders including anxiety, depression, etc. of those treated? Accordingly, can we say that postbiotic-based therapies would be better and safer than probiotic-based supplementation strategies?

Question 4. What hope does the PhD student see in synthetic biology, related, for example, to the engineering of sugars or the design and engineer commensal / probiotic lactic acid bacteria or *Bifidobacteria* as biotic therapeutics that can improve human health? Are examples of such applications already known? The sugars identified by the PhD student are involved in inhibiting allergic pathways. Could they be modified to also enhance the tolerogenic arm of immunity?

Conclusions

The publications included in the scientific achievement are an original and important contribution to the knowledge of new bacterial antigens capable of exerting anti-allergic properties. The research methods applied are evidence of well mastered technical skills. In my opinion, the results presented by the Author, being a scientific novelty and an original solution to the research problems, present a very high cognitive value. They also have a great application potential, which, given the increase in the prevalence of allergic diseases, opens and even forces a broad research perspectives for the future. All of this shows that the PhD Student is very well prepared for further scientific work.

I would like to emphasize that in addition to the results, included in the scientific achievement within the doctoral thesis, Ms. Katarzyna Pacyga-Prus is the co-author of 9 additional publications, 10 conference posters or oral presentation, is contractor of 7 grants / scientific projects, and participant of 4 national and international research internships.

In my opinion, the doctoral dissertation of Ms. Katarzyna Pacyga-Prus, M.Sc., presented for evaluation, meets the requirements for doctoral dissertations, according to the Act - Law on Higher Education and Science, Article 187 of July 20, 2018, paragraph 1-4 on scientific degrees

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and academic title and on degrees and title in art (Journal of Laws; Dz.U. 2023 item.742, as amended),

In view of the above, I would like to request the High Council of the Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wroclaw to admit the PhD student to further stages of the doctoral dissertation. At the same time, due to the very high scientific value and application potential of the dissertation, I apply for an award.