Rapport d'évaluation du mémoire de thèse de Ruth Raspoet

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DOCTORATE IN VETERINARY SCIENCES

Form for the evaluation of the PhD thesis by the members of the Reading Committee

Candidate: Ruth Raspoet

Title of the PhD thesis: Survival strategies of Salmonella Enteritidis to cope with antibacterial factors in the chicken oviduct and in egg white

Name of the member of the Reading Committee: ***Sophie REHAULT-GODBERT***

Acceptability

This PhD thesis is :

x acceptable as such*

❑ acceptable after minor revision*

❑ acceptable after major revision

❑ unacceptable

*Undersigned states that the candidate was successful for the written part of the doctoral examination and may proceed to the second part, the oral defence.

Please use the attached form for argumentation of your decision.

Date : .15-04-2014..... Signature :

.....

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Comments (if necessary, use additional pages)

1. General comments

It was a great pleasure to read this manuscript, which was very interesting for me in terms of new approaches to study *Salmonella* Enteritidis and of very new data leading to a better understanding of the mechanisms of resistance developed by this pathogen in the *Gallus* model.
The introduction is clearly written and brings the social, economic and scientific context leading to questions raised by the PhD project. It reviews the various mechanisms of transmission to the reproductive tract of the hen and to the egg. It focuses first on the transmission and the survival of *Salmonella* within the oviduct and secondly on the interaction between the pathogen and egg components. Since this part is very dense, it might have been useful to give a short conclusion summarizing the major published information on *Salmonella* survival mechanisms in both oviduct and the eggs and the more promising points to be explored. Altogether, the various sections of this introduction give a very good overview of the literature. The objectives of the study are nicely introduced in the next part (Part 2) as it presents the studies performed during the PhD to better appreciate how this specific strain is able to colonize the reproductive tissues of laying hens and recover from the hostile environment of the egg white. Part 3 is related to the four main experimental studies conducted during the thesis. They all start with an abstract and follow the classical "Introduction/Material and Methods/Results, Discussion/ Conclusion" organisation. In this regard, it would have been quite interesting to write a short text between two consecutive articles to better appreciate the link between them and give a more integrated view of the experiments. These experiments are however well included in the last part where Ruth Raspoet performed a nice effort to integrate the experimental data in a general discussion, bringing some new hypotheses and further perspectives for the research team.
The quality of the redaction and discussion combined to the diversity of the techniques used to address the questions are very impressive and underline the capacity of Ruth Raspoet to conduct a research project from the conception and realization of the experiments to the publication and valorisation of the results. Indeed, at present, these PhD data allowed for the publication of two articles as first author and one as the second author. They were presented several times orally or in posters in international meetings and R. Raspoet also participated in the redaction of one book chapter. For all these reasons, I am very pleased to accept this thesis as such.

2. Detailed comments (please refer to the (sub)chapter and page)

Below are detailed some comments that might be discussed during the PhD defence.

Chapter 1.2. It is assumed that the main route for egg contamination is via vertical transmission. The contamination through horizontal transmission is discussed only as it is likely to occur during the passage through an infected cloaca. What about contamination along the entire supply chain from the production to the distribution of eggs and storage by consumers? Eggs are likely to be exposed to variation in temperature during transportation and depending on the production system, which

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might favour bacterial contamination. They can also be contaminated by infected products in the fridge. Do we have any information about the ability of *Salmonella* to infect egg white (after alteration of the eggshell) after a few days of storage? Indeed, egg white undergoes major physicochemical modifications and we can wonder about the consequences of these changes on the activity of egg white antimicrobial molecules but also on the ability of *Salmonella* to grow in such conditions.

In this regard, I have several questions about egg white, particularly in oviduct.
We know that pH of egg white from freshly laid egg is about 7.4 and that the viscosity is high. Storage of eggs results in an increase of pH from 7.4 to about 9.3 and a thinning of egg white. Moreover, fresh egg whites are more favourable to *Salmonella* growth than stored egg whites. What about egg white within the magnum (pH ?)? Viscosity must be high as only 50 % of the albumen water is deposited in the egg during albumen formation. The other half of water content is added in the uterus by the 'plumping' before egg shell formation. Additionally, egg white is composed of thick and thin egg white. Do we know whether protein composition of both parts is different and if *Salmonella* is similarly able to survive in both? One suggestion is that viscosity could imply that antimicrobial molecules are trapped (including lysozyme which is bound to ovomucin, the main responsible for viscosity) and that thinning would allow for the release of active antimicrobials.

Fimbriae (Chapter 1.3.2.) plays an important role in the colonization of the reproductive tract. Is there any comparison available about the structures of fimbriae between *Salmonella* Enteritidis and other *Salmonella* serovars including Gallinarium that could explain the colonisation of the oviduct by this strain as compared with the others?

Article 1.
The experimental approach was designed to study the colonization of the oviduct after intravenous or intra-oviducal injection of the mutants. Can we expect to have similar results after an oral contamination?
It might be interesting to study the expression of uspA and uspB after contact with thin/thick egg whites collected from the magnum (before plumping) and within the uterus (after plumping). Is the expression of uspA and B transient or continuous when in contact with egg white? Does this activation result from some specific egg white proteins or from a combination of all antimicrobial proteins, pH and viscosity ? What are the bacterial intracellular pathways activated by the overexpression of both genes to give this "persistence/survival" phenotype?

Article 2.
In this chapter, the candidate explored the genes involved in the persistence of *Salmonella* inside cells and within the reproductive tract. Several hundreds of genes were shown to be important for oviduct colonization including 81 that were common.
It might be useful to perform a Venn diagram to illustrate the results showing the numbers of genes which are common and those that are specific to the *in vitro* experiment and the *in vivo* experiment. The discussion is made on the common genes but will the other genes further explored and what could we expect from such analysis?

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Article 3.

A hierarchical classification of all the genes shown in Table 2 with the corresponding families would be very informative (like it was performed for the previous articles).

Is there is a relationship between the overexpression of uspA and B genes (article 1) and that of TolC after contact with egg white?

Regarding the results from purification and mass spectrometry analysis, the active fraction was containing ovotransferrin and albumin. It would be useful to have a gel showing the various steps of purification. I guess it is ovalbumin, the major egg white protein and not albumin (albumin also exists but is an egg yolk and plasma protein). Gel filtration is a good separation technique to purify major proteins. Minor proteins including antimicrobial peptides are often discarded as they are retained by the gel filtration and eluted very late as a very small peak. Moreover, visualization of these antimicrobial peptides on classical SDS-PAGE is quite difficult when not concentrated. To be sure that only ovotransferrin and ovalbumin are present it might be important to perform mass spectrometry analysis in solution and not from gel bands. You might identify new antimicrobial candidates. The fact that purified ovotransferrin increases tolC expression is very interesting and this approach might be applied to other purified proteins/peptides from egg white. The present story about ovotransferrin is very complete and relevant.

Is there any selectivity of the MDR pump to export specific molecules? Is it efficient with high molecular weight molecules as well as small ones? What do we know about the mechanisms by which the pump recognizes specifically host-derived antimicrobial proteins to export them out of the periplasmic space?

Article 4

From the results obtained in this last part, one major mechanism (genes involved in LPS synthesis) seems to be involved in the survival of *Salmonella* Enteritidis in egg white. This experiment also allowed for the identification of htrA protease as another actor. Since egg white contains many proteases inhibitors, it might be interesting to explore whether the periplasmic htrA is inhibited by some of them.

From all these studies, which would be the more promising pathway(s) to target if we wanted to develop a new efficient multifunctional antimicrobial agent against *Salmonella* Enteritidis?

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Minor modifications in the manuscript:

The term AvβD is not the usual abbreviation. It is more appropriate to write AvBD (Cf the new nomenclature by Lynn et al. 2007)

Reference to be added: the structure of gallin has been recently published. Hervé V, Meudal H, Labas V, Réhault Godbert S, Gautron J, Berges M, Guyot N, Delmas AF, Nys Y, Landon C. 3D NMR structure of hen egg gallin (chicken ovo-defensin) reveals a new variation of the beta-defensin fold. J Biol Chem. 2014

Chapter 1.4.2.4 Protease inhibitors. 1) Ovalbumin is not considered as an inhibitor. It is structurally related to the serpin family (“serine protease inhibitor family”) but does not exhibit protease inhibitory activity. 2) Replace “the final serine protease ovomucin” by the final “serine protease inhibitor ovomucoid”.

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