Department of Agriculture, Food and the Marine
An Roinn Talmhaíochta, Bia agus Mara

Research Stimulus Fund

Final Report

'Effect of chicken mucin on Campylobacter jejuni global gene expression and colonization of poultry

DAFM Project Reference No: 13S434

Start date: 01/03/2014

End Date: 29/02/2016

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Collaborating Research Institutions and Researchers: Dr. Rita Hickey, Teagasc, Moorepark, Fermoy, Co. Cork.

Please place *one* "x" below in the appropriate area on the research continuum where you feel this project fits

Basic/Fundamental →			Applied →			Pre Commercial
1	2	3X	4	5	6	7

Please specify priority area(s) of research this project relates to from the National Prioritisation Research Exercise* (NRPE) report;

Priority Area (s)	**I Sustainable Food Production and processing**

Key words: *(max 4)*
Campylobacter jejuni, poultry processing, oligosaccharides, chicken intestinal mucin

1. Rationale for Undertaking the Research

This section should outline the rationale for carrying out the research and identify the need / problem to be addressed

C. jejuni contamination of chicken carcasses means that chickens are a source of infection for humans. Ireland has the highest rate of Campylobacter colonization in chickens in Europe with 98% of birds at the end of slaughter contaminated.,In 2013 2,388 cases of campylobacteriosis were reported to the Health Protection Surveillance Centre which represents an annual incidence rate of 52 cases per 100,000 of the population, nearly 8 times the incidence of salmonellosis (7 cases per 100,000). As many cases go unreported to health authorities, the actual or real incidence is likely to be much higher. In the EU 9.2 million cases occur annually, and costs €2.4 billion. Infection with *C. jejuni* can lead to serious sequelae such as development of Guillan-Barre syndrome and irritable bowel syndrome (Marshall *et al.,*2006). The host cell environment has been shown to modulate bacterial virulence.. *C. jejuni* lives in the supramucosal layer of the chicken intestinal tract and strikingly it does not penetrate this layer and interact with the underlying epithelium. In contrast when the organism infects the human intestinal tract it penetrates the mucosal barrier and invades intestinal epithelial cells resulting in disease. We have published that chicken intestinal mucin prevents *C. jejuni* from invading epithelial cells and that *C. jejuni* exhibits a tropism for chicken intestinal mucin compared to mucin from other animal species and binds avidly to it. In this study we hypothesized that chicken mucin modulates *C. jejuni* gene expression and commensal behavior in chickens. We aimed to examine *C. jejuni* global gene expression in the presence of chicken mucin and identify bacterial adhesins involved in binding in order to design strategies to reduce the burden of Campylobacter colonisation in chickens and thus prevent or limit infection of humans. The research conducted as part of this project was necessary as an understanding of how *C. jejuni* behaves as a commensal in chickens is essential for the development of novel strategies to reduce the burden of Campylobacter in chickens.

2. Research Approach

Specify the research methodologies employed, emphasising novel techniques and also outline any modifications from the original approved project proposal

WP1. Optimization of growth of *C. jejuni* in the presence of mucin and RNA isolation.

Bacteria were grown in the presence and absence of mucin. Growth was monitored by measuring the OD600 of the culture and bacterial RNA was isolated using the Isolate II RNA Mini kit (Bioline). Virulence gene expression was examined using Reverse transcriptase polymerase chain reaction (RTPCR) following co-culture of *C. jejuni* strain 81-176 with 10, 30 and 50 µg/ml purified chicken caecum mucin for 4 hours or with 10 µg/ml chicken small intestine,or large intestine mucin.

WP2. RNA-seq of *C. jejuni* grown in the presence and absence of chicken mucin

RNA-sequencing (RNA-seq) was used to determine the global transcriptional profile of *C. jejuni* grown in the presence of mucin from the chicken intestinal tract. Originally it was proposed that we would use Microarrays to assess the transcriptional response of

C. jejuni to mucin. However as the cost of RNA-seq has reduced and expertise in RNA-seq was developed in the laboratory, RNA-seq which is ideal for discovery-based experiments was used.

WP3. Affinity Purification of mucin binding proteins using Millipore Magnetic Avidin-coated beads and identification.

Surface plasmon resonance was used to identify bacterial fractions which interacted with chicken intestinal mucin immobilised on a Biacore chip. This method identified the culture supernate as the fraction that bound best to mucin.

Magnetic avidin beads were coated with biotinylated chicken mucin and incubated with concentrated *C. jejuni* culture supernatant. SDS PAGE was used to assess proteins that bound to the mucin beads.

Specificity of affinity purified secreted proteins for chicken mucin was confirmed by mucin blot (probing of protein blotted onto a membrane with biotinylated mucin). Mass spectrometry was used to identify proteins that bound to chicken mucin. Recombinant proteins expressed in *E. coli* or *C. jejuni* strains mutated in specific genes for proteins of interest were tested for binding to mucin

WP4. Assessment of the effect of bovine colostrum oligosaccharides on Campylobacter proteins interacting with mucins.

Overlay of membrane containing Campylobacter proteins with bovine colostrum oligosaccharides or with camel milk oligosaccharides prior to probing with biotinylated chicken mucin was used to assess the effect of oiligosaccharides and milk on *C. jejuni* binding to mucin.

3. Research Achievements/Results

Outline main results achieved

Global gene expression analysis of bacteria grown in the presence of chicken intestinal mucus revealed a number of cell surface carbohydrate modifications as well as down-regulation of a number of stress-related genes. Notably the secreted protease HtrA, which has recently been shown to promote *C. jejuni* invasion, was one of the most significantly down regulated genes. These results suggest that chicken mucin can modulate gene expression in *C. jejuni* and go some way to explaining the commensal nature of *C. jejuni* in chickens.

The capsular polysaccharide KpsD was identified as playing a role in modulating adherence of *C. jejuni* to mucin. A mutant strain lacking KpsD demonstrated reduced binding to mucin and interestingly enhanced binding to epithelial cells. This suggests that the capsular polysaccharide of *C. jejuni* may shield ligands on the bacterial surface which mediate binding to epithelial cells.

Bovine colostrum oligosaccharides (BCOs) inhibited the binding of *C. jejuni* proteins to mucin suggesting that BCOs bind to the same adhesin on *C. jejuni* that chicken mucin targets.

4. **Impact of the Research**

A summary of the tangible impact of the research project should be provided under the outcomes' and 'outputs' heading below. In addition, please provide a short narrative synopsis of the benefits / improvements the research has made to the area under investigation particularly as regards end users, e.g. industry, consumers, regulatory authorities, policymakers, the scientific community, etc

In this project *C. jejuni* genes have been identified that can be modulated by mucin of chickens, an avian species that *C. jejuni* colonises naturally and which is a problem for poultry producers as the organism is an important cause of gastroenteritis in humans. Identification of genes which are affected by the presence of chicken mucin is useful knowledge for members of the scientific community working to develop strategies to prevent the burden of infection in chickens and hence improve the quality of poultry produced for human consumption. This work allows for the formation of hypotheses on how chicken mucin influences the lifestyle of Campylobacter and the design of experiments for the testing of those hypotheses. It also serves as a valuable reference system for the scientific community currently assessing the bidirectional cross talk between bacteria and the host and the role of mucins in modulating that cross talk, a keen area of interest at the moment.

Identification of mucin binding ligands in *C. jejuni* such as KpsD, allows for the use of that ligand to probe glycan arrays for interaction with neoglycoconjugates and oligosaccharides which it may interact with on the mucins. This could lead to the identification of oligosaccharides that could be used to block colonization in chickens.

Bovine colostrum oligosaccharides were shown to inhibit KpsD, from interacting with mucin. The blocking was specific for bovine colostrum oligosaccharides as camel milk oligosaccharides used at the same concentration had no effect. This suggests that colostrum oligosaccharides could be mined for inhibitors of bacterial infection.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

This research has strengthened the links between the two applicants funded ie Teagasc researcher Dr. Rita Hickey and UCD researcher Dr. Marguerite Clyne.

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

N/A

(iii) Outcomes with economic potential

We have provided preliminary evidence which suggest that it may be feasible to use milk sugars as a potential anti infective ingredient to limit or prevent *C.*

jejuni in chickens. These results may be of great relevance to the end users as they suggest that the use of material that can be easily isolated from natural sources could be included in feed to reduce the burden of Campylobacter in the chicken and hence the spread of infection to humans. Another advantage of using bovine colostrum oligosaccharides as an inhibitor is that it is much easier to isolate oligosaccharides from colostrum or milk than it is from mucin.

(iv) Outcomes with national/ policy/social/environmental potential

A primary focus of improving the health of our population is prevention and early intervention. In 2013, the Government approved Healthy Ireland - A Framework for Improved Health and Wellbeing 2013-2025. The vision of Healthy Ireland is to improve health and wellbeing and the quality of people's lives. This project aimed to focus on generating data that would lead to strategies to prevent infection with a bacteria that is a common source of food poisoning in Ireland and the rest of Europe. By understanding how bacteria colonise the gut of chickens we can develop strategies and promote products that will reduce or prevent colonisation and subsequent infection of humans.

4. Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

Acceptable Format: ***Walsh, D.R., Murphy, O., Cosgrave, J. (2008). Echinococcosis - an international public health issue. Research in Veterinary Science 774, 891-902.***

Naughton J, Duggan G, Bourke B, Clyne M. 2014 Interaction of microbes with mucus and mucins: recent developments. Gut Microbes 5(1):48-52.

Clyne M, Duggan G, Naughton J, Bourke B. 2016. Methods to Assess the Direct Interaction of *C. jejuni* with Mucins. Methods Molecular Biology, Vol. 1512, James Butcher and Alain Stintzi (Eds): Campylobacter jejuni, 978-1-4939-6534-2, 330282_1_En, (12) (In Press)

Clyne, M., Duggan, G., Dunne, C., Dolan, B., Alvarez, L., Bourke, B. Assays to Study the Interaction of *Campylobacter jejuni* with the Mucosal Surface. Methods Molecular Biology, Vol. 1512, James Butcher and Alain Stintzi (Eds): Campylobacter jejuni, 978-1-4939-6534-2, 330282_1_En, (12) (In Press).

(ii) Popular non-scientific publications and abstracts including those presented at conferences

(iii) National Report

(iv) Workshops/seminars at which results were presented

Dublin Academy of Pathogenomics and Infection Biology Annual meeting, January

2016. Trinity College Dublin.

(v) Intellectual Property applications/licences/patents

(vi) Other (For noting)
Duggan G, Bourke B, Clyne M. 2016. A multi-omics approach to deciphering the effect of chicken intestinal mucus on Campylobacter jejuni. Manuscript in preparation

Duggan G, Bourke B, Clyne M. 2016. Characterisation of a KpsD mutant of Campylobacter jejuni. Manuscript in preparation.

5. Scientists trained by Project

Total Number of PhD theses: __1__

*Gina Duggan PhD thesis submitted to University College Dublin, October 2016
Title: Mucin and Glycan mediated interactions of the gastrointestinal pathogen Campylobacter jejuni in humans and chickens.

Total Number of Masters theses: ___N/A_

*Although the original proposal funded a masters student for two years, the successful candidate had already completed two years on the MSc register at the time of starting work so she decided to combine the work she had already done with the work she completed for this proposal in order to obtain a PhD.

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
University College Dublin	1	0.2
Teagasc, Moorepark	1	0.01
Total	**2**	**0.21**

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers		
PhD students	1	2 (see note above)
Masters students		
Temporary researchers		
Other		
Total	**1**	**2**

8. Involvement in Agri Food Graduate Development Programme

Name of Postgraduate / contract researcher	Names and Dates of modules attended
N/A	

9. Project Expenditure

Total expenditure of the project:	€92,205
Total Award by DAFM:	€99,580
Other sources of funding including benefit in kind and/or cash contribution(specify):	€N/A

Breakdown of Total Expenditure

Category	Name University College Dublin	Name Teagasc Moorepark Cork	Name Institution 3	Name Institution 4	Total
Contract staff					
Temporary staff					
Post doctorates					
Post graduates	43,953.94				43,953.54
Consumables	26,188.79	314.61			26,503.40
Travel and subsistence		469.56			469.56
Sub total	70,142.73	784.17			70,926.90
Durable equipment					
Other					
Overheads	21,042.82	235.25			21,278.07
Total	**91,185.55**	**1019.42**			**92,204.97**

10. Leveraging

Summarise any additional resources'/funding leveraged by this award from other sources e.g. Additional Staff, National/EU funding secured, EI Commercialisation Fund, etc.

The results of the work from this grant were used as preliminary data in a proposal that was submitted by the applicants in collaboration with Prof Lokesh Joshi and Dr. Michelle Kilcoyne of NUIGalway to the SFI Investigators Programme 2016. This proposal was submitted in Dec 2016. The results of this competition are not yet available.

11. Future Strategies

Outline development plans for the results of the research.

Dr. Marguerite Clyne and Dr. Rita Hickey together with Prof Loksh Joshi in NUIGalway have recently submitted an application to the SFI investigator proposal scheme 2016 (SFI IvP 2016) and in that proposal results acquired from this project was used as preliminary data. We have shown using RNA seq that chicken mucin alters the global transcriptional profile of *C. jejuni* (this project). Key findings are that a number of cell surface carbohydrates are modified upon exposure to chicken mucin and stress-related genes are down-regulated. As a result of these findings we hypothesise that these changes are specific for chicken mucin and that, upon exposure to human mucin, key virulence genes are up-regulated. Using a novel cell culture model designed to mimic the intestinal tract in vivo we aim to assess the effect of human mucus on the interaction

of *C. jejuni* with epithelial cells. We will then test the effect of chicken mucus on *C. jejuni* infection of these cells to assess if using this model chicken mucus can modulate *C. jejuni* virulence as our results suggest. We also aim to assess the effect of bovine colostrum oligosaccharides on prevention of infection using this model to further examine their potential to prevent infection. Thus it may be possible to develop functional foods that play a role in prevention of infection and development of disease.

2 papers for submission to international peer reviewed journals are in progress.

1. Duggan G, Bourke B, Clyne M. 2016. A multi-omics approach to deciphering the effect of chicken intestinal mucus on *Campylobacter jejuni*.

This paper will describe the use of both transcriptomics and proteomics to assess the effect of chicken intestinal mucin on *C. jejuni*. Results indicate that chicken mucus induces a number of cell surface carbohydrate modifications on *C. jejuni* as well as down regulation of stress related genes. Chicken mucus also reduced protein secretion by the organisms. The results of this study suggest that chicken mucus plays a role in promoting the commensal life style of *C. jejuni* in chickens and highlights the diversity exhibited by *C. jejuni* in response to environmental stimuli.

2. Duggan G, Bourke B, Clyne M. 2016. Characterisation of a KpsD mutant of *Campylobacter jejuni*

This paper will describe the identification of the capsular polysaccharide protein KpsD as a potential mucin binding ligand of *C. jejuni* and the construction of a *kpsD* mutant. This is one of the first studies to identify a *C. jejuni* protein that may play an important role in mediating binding to mucin. Elucidation of the mechanism of interaction of *C. jejuni* with mucins may allow for development of strategies to block that interaction and hence prevent colonisation in chickens.