



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

Food Institutional Research Measure

Final Report

'Novel strategy for exploitation of milk glycoproteins in infant formula (Milk Glycoproteome)'

DAFM Project Reference No: 10/RD/Milk Glycoproteome/NUIG/707

Start date: 1 April 2012

End Date: 30 June 2016

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

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	3 X	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)	H Food for Health
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Key words: (max 4)

Infant formula, Milk glycoproteins, Commensals, Anti-infectives

1. Rationale for Undertaking the Research

The goal of the infant formula industry is to mimic the composition of human milk and thereby ensure optimal nutrition and development of the human infant. Human milk oligosaccharides (HMOs) are the third largest component of human milk and comprise 12–14 g/L, higher than the total protein content. The functions of the HMOs include prebiotic activity to promote commensal growth, protecting the gut epithelium from pathogenic invasion, and stimulating development of the normal immune system. The oligosaccharide content of cow's milk is less than 5% that of human milk, although both have some similar structures. To increase oligosaccharide levels, infant formulae are currently being supplemented with chemically or enzymatically-produced fructooligosaccharides (FOS) or galactooligosaccharides (GOS). A natural source of human milk-like oligosaccharides would be highly desirable to supplement infant formulae.

Much is known about the types and roles of free oligosaccharides in bovine milk, but the glycans of milk glycoproteins have been largely ignored. Yet, they could represent an important source of biologically active structures that might mimic and/or complement the activities of HMOs. Some of the bioactivities ascribed to specific milk proteins have been associated with their glycan components, but to date these proteins have not been specifically exploited for their glycan components. Protein glycosylation is a complex process and not template-driven. Hence, glycoproteins tend to be heterogeneous with respect to their glycan composition. Instead of isolating individual glycoproteins, we proposed to fractionate milk glycoproteins on the basis of their terminal glycan moieties. The terminal structures of oligosaccharide branches interact with cognate biological receptors and are responsible for biological activity.

This project proposed to develop convenient fractionation methods for soluble bovine milk glycoproteins from different processing streams and to survey the biological activities of each fraction, in particular for their activity with probiotics and pathogens and influence on host immune system. This will result in the identification of milk glycoprotein fractions enriched with specific biological activities, which can be exploited as novel infant formula ingredients, giving a strategic advantage over existing formulations. This is consistent with FIRM goals to pursue research that will underpin the development of the Irish infant formula industry.

2. Research Approach

It was proposed to generate milk glycoprotein fractions which were enriched for certain terminal carbohydrate structures and to assess these enriched glycoproteins for biological activities. Using readily available demineralised whey protein (DWP) as the initial starting material, a range of lectin affinity matrices were assessed to prepare various glycoprotein isolates with differing terminal glycan motifs. This approach resulted in poor yield, so a number of other chromatographic approaches, including reverse phase, activated charcoal and anion exchange chromatographic methods, were assessed for separation of the glycoproteins based on polarity, hydrophobicity and charge of the DWP glycoproteins to yield a similar outcome of differentially glycosylated DWP fractions. Each isolate was fully characterised in terms of its terminal glycan and protein composition by analysis on lectin microarrays, electrophoresis and western blotting. Additionally ammonium sulfate precipitation was employed on the WPI before AEC due to issues with build up on the column. The anion exchange chromatography (AEC) method was successful in achieving differentially glycosylated WPI fractions as assessed by lectin microarray and SDS-PAGE. AEC is also compatible with the food industry and was optimised on lab scale to generate quantities sufficient for biological assays.

A panel of bioassays were used to assess the bioactivity of the final isolates and milk glycoproteins that are commercially available to identify fractions and glycoproteins with potentially useful activities.

Initially, *in vitro* bioassays aimed at identifying pro- and anti-microbial activities, and modulatory effects on mammalian cell lines that are representative of gut, immune and neural tissues were planned. However, assays were revised to concentrate on expanding the assessment of the microbial, gut and immune responses as these are more interconnected and have proven direct impacts on one another.

AEC fractions were also protease-treated to simulate their form in the gastrointestinal tract. Both AEC fractions and hydrolysed fractions were assessed in mammalian cell viability, proliferation and death assays using gut epithelial cell lines. Immune activity was assessed by macrophage activation assays for *E. coli* and zymosan (i.e. pathogenic bacteria and fungi or yeast). These assays were optimised for AEC fractions using RAW cells. Prebiotic activity of the AEC fractions and their hydrolysates were assessed using a number of probiotic strains important to infant and adult gut health. A microtitre plate assay was developed for this prebiotic assay to enable the assessment of limited sample quantities. The prebiotic activity of milk fractions buttermilk hydrolysate, peptone, WPC and peptone hydrolysate was assessed in comparison to GOS. In addition, the supernatant of the bacteria cultured in the prebiotic assays were also harvested for short chain fatty acid (SCFA) analysis which are known to influence gut epithelial cell behavior and health. The supernatants were derivatised and analysed by high pressure liquid chromatography (HPLC). Anti-infective activity of glycomacropeptide (GMP) was assessed in anti-adherence studies and lactoferrin was assessed for its ability to enhance the antimicrobial effect of penicillin G on *Staphylococcus aureus*. The effect of GMP the transcriptome of colonic epithelial (HT-29) cells was also assessed. Finally, methods for the large scale production of hydrolysed PPF fractions which demonstrated bioactivity in the anti-adherence assays and GMP enriched powder containing 72% GMP on a dry matter basis were optimised.

3. Research Achievements/Results

The AEC method was successful in achieving differentially glycosylated WPI fractions as assessed by lectin microarray and SDS-PAGE. AEC is also compatible with the food industry and was optimised on lab scale to generate quantities sufficient for biological assays. The prebiotic activity of the AEC fractions from was shown to be dependent on the particular commensal species assessed and thus could be promising for modulating the bacterial population *in vivo*. All fractions increased the growth of *Faecalibacterium prausnitzii* and fraction F2 increased the growth of *Bifidobacterium bifidum*. Fractions did not have any effect on the growth of *B. longum* and *Lactobacillus reuteri*.

Whole IgG glycoproteins from milk from days 1, 2, 3 and 10 of lactation was profiled on lectin microarrays and no major differences in glycosylation were found between pooled samples but sialic acid content was found to be greatest in day 1 IgG.

It was also shown that GMP significantly reduced pathogen adhesion, albeit with a high degree of species specificity toward enteropathogenic *E. coli* (EPEC) strains O125:H32 and O111:H2 and enterohemorrhagic *E. coli* (EHEC) strain 12900 O157:H7. The anti-adhesive effect resulted from the interaction of GMP with the *E. coli* cells and was also dependent on GMP concentration. Pre-incubation of intestinal Caco-2 cells with GMP reduced pathogen translocation as represented by a decrease in transepithelial electrical resistance (TEER).

Hydrolysed WPI reduced the population of necrotic and apoptotic HT29 cells and increased the proportion of healthy cells in the population. This observation may be promising for employing WPI as a functional food ingredient for promoting gut health.

Certain hydrolysed WPI fractions and the resulting bacterial metabolites after treatment with the hydrolysed fractions increased phagocytosis of *E. coli* by RAW cells. This finding could be similarly promising for individuals with weak or compromised immune responses. In addition, GMP was found to

have a positive immunomodulatory effect on intestinal cells while mature skimmed milk also induced a balanced inflammatory response *in vitro*.

Methods for the large scale production of hydrolysed PPF fractions which demonstrated bioactivity in the anti-adherence assays and GMP enriched powder containing 72% GMP on a dry matter basis produced gram quantities of the hydrolysed PPF fractions and 10 kg of GMP enriched powder are available for future projects.

Overall, these results are promising given that in particular skimmed mature milk is readily available in the dairy industry.

4. Impact of the Research

4(a) Summary of Research Outcomes

Collaborative links were made with 1 academic labs and 1 industry during the course of this project. There were a number of important findings during this project which may translate into new functional food products. However, it is too early to see the commercial, economic, policy and social impacts as the outputs have not been published yet.

(i) Collaborative links developed during this research

Anti-GMP antibody was gifted from Dr. Eva Salinas Miralles of the Department of Microbiology, Autonomous University of Aguascalientes, Aguascalientes, Mexico as it is not commercially available.

GMP was kindly gifted to Teagasc by Agropur Ingredients. After contact was made with Peggy Ponce, Senior Scientist, Sales and Marketing, interest was expressed in collaborating further. Additional glycoprotein samples have been offered to the Teagasc group and Agropur are very interested in having the results of the anti-infective work on GMP published. Agropur reviewed this manuscript before submission to Foods and were very positive in relation to the quality of the research undertaken.

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

It is too early to see impacts such as these yet.

(iii) Outcomes with economic potential

Certain AEC fractions favoured one probiote over the other, so this could translate as a valuable infant formula component to favour the growth of certain probiotes that are known to be beneficial to the developing infant. The positive effect of hydrolysed WPI on gut epithelial cells means that it may be a promising and valuable functional food component for promoting gut health. As certain hydrolysed WPI fractions and glycoproteins had positive immune effects (increased phagocytosis and balanced inflammatory responses), these products could be promising functional food or therapeutic components for individuals with weak or compromised immune responses.

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

Not applicable as it is too early to see impacts such as these yet.

4 (b) Summary of Research Outputs

This project has resulted in 6 presentations at national and 3 presentations at international conferences to date. There are two PhD theses in preparation currently with planned submission dates in Spring 2018. Currently, 3 papers are currently being prepared for submission to internationally peer-reviewed

journals by December 2017, with a further 2 papers planned for submission after submission of the PhD theses.

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

Papers submitted

Feeney S., Ryan J.T., Kilcoyne M., Joshi L. and Hickey R.M.: Glycomacropptide Reduces Intestinal Epithelial Cell Barrier Dysfunction and Infection of Enterohemorrhagic and Enteropathogenic *Escherichia coli* *In Vitro*. Submitted to a special issue of Foods (September 2017).

Papers in preparation

Feeney S. and Hickey R.M. invited to write booked chapter on “Dairy glycoproteins: extraction, characterisation and potential bioactivities” in the following book Novel Proteins for Food, Pharmaceuticals and Agriculture: Sources, Applications and Advances. Manuscript in preparation (anticipated submission December 2017).

Feeney S., Gerlach, J.Q., Hickey R.M., Kilcoyne M. and Joshi L. Temporal changes in the glycosylation of IgG over the course of lactation. Manuscript in preparation (anticipated submission December 2017).

Asadi, A.S., Le Costouec, T., Kilcoyne, M., Joshi, L. Extraction, purification, characterisation and biological activity of *Ascophyllum nodosum* poly- and oligo-saccharides. Manuscript in preparation (anticipated submission late Spring 2018).

Asadi, A.S., Hickey, R., Kilcoyne, M., Joshi, L. The effect of whey protein glycosylation on immunomodulation and probiotic growth. Manuscript in preparation (anticipated submission late Spring 2018).

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

Ms Akram Asadi Shehni made an oral presentation of her work at the Theresis biomedical science and engineering, NUI Galway, on 1st September 2014.

(iii) **National Report**

Not applicable.

(iv) **Workshops/seminars at which results were presented**

Ms Akram Asadi Shehni presented a poster at the 13th European Training Course on Carbohydrates, The Netherlands. The poster was entitled ‘Extraction, purification, characterisation and biological activity of the brown seaweed *Ascophyllum nodosum* poly- and oligo-saccharides’. The conference was 1 - 4, April 2014.

Ms Akram Asadi Shehni presented a poster entitled ‘Biological evaluation of poly- and oligo-saccharides extracted from seaweed’ at a scientific showcase at NUI Galway on 1st of July 2014.

Ms Akram Asadi Shehni presented the poster entitled ‘Bioactivity of poly- and oligo-saccharides from brown seaweed *Ascophyllum nodosum*’ at the 1st Matrix Biology Ireland Conference at NUI Galway, 19th -21st November 2014.

Ms Akram Asadi Shehni presented the poster entitled ‘Bioactivity of poly- and oligo-saccharides from brown seaweed *Ascophyllum nodosum*’ at the Young Life Symposium, UCD, 12th November 2014

Ms Akram Asadi Shehni's submission entitled 'The effect of whey protein glycosylation on immunomodulation and probiotic growth' was invited for an oral presentation at EuroCarb18 in Moscow, Russia on 2nd-6th of August, 2015.

Poster presentation: Feeney S., Ryan J. T., Kilcoyne M., Joshi L. and **Hickey R. M.** (2014) Glycomacropeptide reduces infection of enterohemorrhagic and enteropathogenic *Escherichia coli in vitro* (Poster) 43rd Annual Food Research Conference (9-11 December, University College Dublin, Dublin, IRELAND.

Mr Shane Feeney presented a poster at the 44th Food Research Conference, TFRC, Moorepark, Fermoy, Co. Cork on 14th December 2015.

Mr Shane Feeney presented a poster entitled 'Glycomacropeptide reduces intestinal epithelial cell barrier dysfunction and infection of enterohemorrhagic and enteropathogenic *Escherichia coli in vitro*' at the 8th Probiotics, Prebiotics & New Foods for microbiota and human health, Rome, Italy, on the 13th – 15th of September 2015.

The Proceedings from this conference were also published and this presentation has the reference Journal of Clinical Gastroenterology, 50, S224-S224, 2016.

Mr Shane Feeney presented a poster entitled 'Glycomacropeptide from bovine milk reduces intestinal E. coli colonisation and associated barrier dysfunction *in vitro*', Walsh Fellow Seminar, Royal Dublin Society, Dublin On the 12th November 2015.

(v) Intellectual Property applications/licences/patents

Not applicable at this time.

(vi) Other

Both Prof. Joshi and Dr. Hickey are members of the Food for Health Ireland (FHI) 2 research consortium funded by Enterprise Ireland which includes research labs and industrial partners. Dr. Hickey has made regular reports to FHI2 which have included updates of other funded projects including this project. Relevant stakeholders who attend these meetings include the dairy and infant formula industries (Glanbia, Kerry Group, Ornu, Dairygold and Carbery), infant nutrition and dairy research communities (UCC, UCD, NUIM, UL and DCU) and the state agency Enterprise Ireland. FHI2 ran until July 2017.

5. Scientists trained by Project

Total Number of PhD theses: 2 (pending)

Ms Akram Asadi Shehni, The effect of whey protein glycosylation on immunomodulation and probiotic growth

Mr Shane Feeney, Structural and functional characteristics of glycoproteins from whey
Anticipated submission Spring 2018 for both theses.

Total Number of Masters theses: 0

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
NUI Galway	2	0.38
Teagasc Moorepark	2	0.926
Total	4	1.306

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers	3	1.538
PhD students	2	5.645
Masters students		
Temporary researchers		
Other		
Total	5	7.183

8. Involvement in Agri Food Graduate Development Programme

Name of Postgraduate / contract researcher	Names and Dates of modules attended
N/A	Did not attend

9. Project Expenditure

Total expenditure of the project:	€346,436.22
Total Award by DAFM:	€348,285.60
Other sources of funding including benefit in kind and/or cash contribution(specify):	€

Breakdown of Total Expenditure

Category	NUI Galway	Teagasc Moorepark	Name Institution 3	Name Institution 4	Total
Contract staff (includes Postdocs)	35,406.19	23,849.95			59,256.14
Temporary staff					
Post graduates	56,133.47	63,000			119,133.47
Consumables	46,084.21	37,538.21			83,622.42
Travel and subsistence	2,477.37	2,000			4,477.37
Sub total					266,489.40
Durable equipment					
Other					
Overheads	42,030.37	37,916.45			79,946.82
Total					346,436.22

10. Leveraging

Not applicable to date. However, findings from this project are being incorporated into funding applications being prepared and submitted currently.

11. Future Strategies

The work on GMP is being continued at Teagasc and a paper has been recently submitted to Biomed Research International on "Bovine glycomacropeptide promotes the growth of *Bifidobacterium longum* subsp. *infantis* and modulates its gene expression".