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

Food Institutional Research Measure

Final Report

Novel food ingredients for the elderly consumer

DAFM Project Reference No: 11 F 053

Start date: 01/07/2013

End Date: 30/06/2018

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Collaborating Research Institutions and Researchers: School of Microbiology and APC Microbiome Ireland; Moorepark Teagasc; Prof. Paul O'Toole, Prof. Catherine Stanton, Prof. Paul Ross, Alexandra Ntemiri, Fodhla Ni Chonchuir, Laure Gonod, Caitriona Cadogan

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	4	5 X	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**

Key words: Diet, microbiome, GMP, milk

1. Rationale for Undertaking the Research

The aim of this project was to improve the intestinal health of older consumers by using selected dairy products or dairy ingredients, and by exploiting the ability of these ingredients to change the population of bacteria that reside in the intestines. These bacteria impact on health by helping to convert components of the diet into smaller compounds that can be used by the body, which in the elderly can mean the difference between a person being frail or not. These gut bacteria can also control the level of non-specific immune activity or "inflammation" which can lead to the person being unable to protect themselves from winter infections, or respond properly to vaccinations. Importantly, milk is a widely accessible nutrient-rich product that can provide important nutrients to the ageing population. However, older people avoid milk consumption due to misconceptions on milk fat-content and lactose intolerance. Avoiding milk (and dairy) can deprive the ageing population from important nutrients such as calcium and phosphorous. With this research we aimed at offering more scientific evidence (gut microbiota -related) on the importance of milk in human diet.

2. Research Approach

Whey, sourced on site, was selected as a starting point for generating GMP and bench scale trials have been performed using a selection of ultrafiltration units. A protocol for HPLC analysis of the GMP content has also been developed. In continuation of these preliminary tests, whey protein isolate (WPI) has been sourced through Kerry Group and used a starting point for the production of a GMP enrichment for *in vitro* trials.

In brief, the method used to produce GMP enrichments intended for *in vitro* trials involved: pH adjustment of a 30% Voac WPI solution to pH3.9; subjecting the resulting protein solution through a membrane of a 30kDa MWCO at a 2.5 bar transmembrane pressure, resulting in a GMP-enriched permeate depleted of a depleted α-lac and β-lg content relative to the initial feedstream; readjustment of the resulting permeate to pH7.0, following 3X concentration of retentate; subjecting the pH-adjusted permeate through a membrane of a 10kDa MWCO, resulting in a 5X concentrated retentate depleted of water, lactose and other minerals relative to the permeate emerging for the 30kDa MWCO membrane. Freeze-drying the final retentate obtained following processing with the 10kDa MWCO membrane. A panel of ten lactobacilli and ten bifidobacteria previously isolated as part of the ELDERMET project has been selected for use in the assessment of GMP in terms of its ability of GMP to stimulate the growth of beneficial bacteria.

Strict anaerobic techniques were used in order to prepare faecal microbiota inocula for our *in vitro* colon model from faecal samples from consented older people. Through this work we standardised the preparation of anaerobic inocula that can be stored and used for future multiple testing of food substrates in *in vitro* colon models. We optimised and standardised our laboratory batch fermentation system that has enabled the development of a continuous fermentation system. With the use of both in vitro colon models developed in our lab we have successfully tested the effect on the gut microbiota of a variety of food substrates, and we have explored the in vitro potential of improving the "dysbiotic" microbiota of older people by various supplementation regimes. Importantly, by using "dysbiotic" faecal microbiota from frail older people we improved our understanding of the "dysbiotic" microbiota responsiveness to intervention and we demonstrated limitations and potentials in the use of such type microbiota in *in vitro* studies. After exploring the in vitro effect on the gut microbiota of the GMP and lactose we employed a mouse model in order to test the effect of the selected dairy products in vivo. We successfully used a mouse model previously used in our lab and we established its validity in testing the effect of dietary compounds on different types of microbiota. We successfully eradicated the indigenous murine microbiota with antibiotic treatment and consecutively, we transferred to the mouse gut two types of human microbiota, i.e. "healthy" and "dysbiotic". By using 16S rRNA gene amplicon sequencing we demonstrated that the distinct profiles were maintained throughout the trial. The human microbiota inocula were prepared from faecal samples under strict anaerobic conditions.

3. Research Achievements/Results

GMP was purified in-house and compared to commercially available product for its ability to support the growth of commensal gut bacteria. Detailed laboratory analysis showed how the product was broken down and metabolized.

Using an *in vitro* colon model we showed the effect of glycomacropeptide (GMP) on the gut microbiota of older people. We reported that 24 h GMP supplementation of the culture medium resulted in an increased relative abundance of health relevant taxa in the gut microbiota of older people compared to glucose or lactose supplementation. The GMP supplementation resulted in increased short chain fatty acids production. These results indicated the potential of GMP to be further explored *in vivo* for its prebiotic potential. We have also applied an in-house process for a small scale production of GMP that was compared to the commercial GMP for its purity and effect on the gut microbiota. We demonstrated that the differential lactose content in the two GMP preparates resulted in differential effect on the gut microbiota. The lactose effect may be relevant especially for lactose malabsorbers and lactase non-persistent people. Importantly, in these *in vitro* tests we used faecal microbiota from frail older people that compared to microbiota from healthy older people, it is characterised by reduced diversity and reduced relative abundance of health-relevant taxa. Faecal microbiota from frail older people has not been extensively used in *in vitro* tests in research. Therefore, with our experiments we demonstrated the potential of using a "dysbiotic" microbiota in *in vitro* tests that can facilitate future research aiming at the improvement of dysbiosis of the gut microbiota of older people related to frailty.

We used an *in vivo* colon model in order to investigate the in vivo effect of GMP on the gut microbiota and the effect of two types of milk, i.e. whole milk and lactose-free milk. With these experiments we successfully established a colonisation mouse model by successfully transferring and maintaining "healthy" (community) and "dysbiotic" (long-stay) microbiota from older people in mice previously treated with antibiotics in order to clear off the native murine microbiota. Mice received normal feed, or feed supplemented with 20% GMP, 20% whole milk or 20% lactose-free milk. The effect of milk and GMP supplemented diets on the gut microbiota of mice was compared with soy protein supplemented diet. Other studies have reported that soy-protein supplemented diet had a prebiotic effect on the mice microbiota and sustained higher murine faecal microbiota diversity compared to milk protein supplemented diet. In our study, in mice humanised with "healthy" microbiota, lactose free milk supplemented diet sustained a higher microbiota diversity comparable to soy protein supplemented diet (no significant differences between the groups); whole milk supplemented diet resulted in similar results but did not sustain the species richness as efficient as lactose-free milk. In the microbiota of mice humanised with "dysbiotic" microbiota from a frail older person, lactose-free milk was the most efficient in sustaining the gut microbiota diversity compared to all diets. Importantly, milk supplemented diets resulted in increased relative abundance of health-relevant taxa in the faecal microbiota of the mice "humanised" with either community or long-stay type microbiota. In contrast to our hypothesis, however, the faecal microbiota of mice fed GMP supplemented diet had the lowest alpha diversity scores compared to feeding with the other diets at the end of the trial. In the faecal microbiota of mice fed GMP supplemented diet, the phylum Bacteroidetes had the highest increase in relative abundance compared to feeding with the other diets. It has been reported in other studies in mouse models that GMP promoted the growth of Bacteroidetes taxa. Importantly, we observed an effect of the milk diets on the family Desulfovibrionaceae and Proteobacteria potentially due to the sialic acid of the milk diets. This observation may be important when designing clinical trials with sialic-acid containing milk substrates.

This Task has consolidated our ability to perform nutritional interventions in pre-clinical models including in mice that have been "humanized" with different microbiota types. Importantly, we have successfully transferred and maintained the compositional profile of the two types of human microbiota used for "humanisation", i.e. healthy COM and frail LS. Furthermore, the successful design of the murine test diets allowed for good palatability for the mice and adequate supplementation of test substrates for the detection of significant microbiota changes.

4. Impact of the Research

The data generated by work conducted in Teagasc indicate that certain groups of bacteria connected with health can be promoted by presence of GMP in the medium; however further research is required to completely understand side-effects on other bacteria. The end-game will be the refinement of GMP that can be considered by industry as an additive to functional foods to promote gut health in older consumers.

Our establishment of the colon model has allowed us to test food ingredients for their effect on the microbiota. This is a valuable resource for the food industry. Working outside of DAFM-provided resources, and in collaboration with USDA-funded labs, we have tested polyphenol ingredients in the artificial colon model established by the Elderfood project. The results are very encouraging. We have established contact with a major player in the Irish horticulture industry and shared our preliminary findings with them. We have arranged to meet them in 2019 to share the results of ongoing work we are doing in this area, not funded by DAFM, but exploiting capability developed on this project.

The results of our pre-clinical trial i.e. animal model, were very surprizing. They suggest that lactose free milk, is capable of maintaining gut microbiota diversity, whereas both whole and lactose free milk promoted the growth of health-relevant taxa. Both these findings would be very valuable for the milk industry. Our results indicate towards the potential of the carbohydrate component of milk (oligosaccharides, lactose) to modulate the gut microbiota. However, it is important to stress that this is data from an animal model only which may not extrapolate directly to humans. For example, mice may not tolerate lactose as efficient as a lactase persistent person and therefore, the observed result of reduced alpha diversity after whole milk feeding compared to lactose free milk, may be a result of the microbiota consuming the simple disaccharide lactose. The lack of lactose in the dietary regime of mice may have made the milk oligosaccharides more accessible to the gut microbiota. These oligosaccharides are complex in structure (similar to the human milk oligosaccharides that have a prebiotic action on Bifidobacteria) and may qualify for prebiotics.
Our observation that the lactose-free milk supports gut microbiota diversity was a major finding of the Elderfood project. Lack of microbiota diversity is linked to several human conditions including pre-diabetes and obesity. We were unable to complete a scheduled Task that comprised a human intervention within the original project timeframe, and a (second) NCE was not granted. We will work with our Teagasc partners to explore and exploit the value to the Irish food industry of these findings on milk benefit, limited though they are by being derived from animal studies, not human. We will actively explore ways to conduct a milk-microbiome trial in humans as soon as possible.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

We consolidated a collaboration with Kerry in Listowel to source whey ingredient for the in-house GMP purification. We interacted with Arla in Sweden, and Valio in Sweden, for sourcing and testing commercial-grade GMP in *in vitro* and pre-clinical models, but we did not pursue a long-term collaboration with them.

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

We developed an in-house GMP purification protocol in Teagasc Moorepark and MTL is well positioned for advising Irish food companies on purifying GMP in the future. The process is not patented or patentable.

(iii) Outcomes with economic potential

We showed that lactose free milk is capable of maintaining gut microbiota diversity, whereas both whole and lactose free milk promoted the growth of health-relevant taxa. Both these findings would be very valuable for the milk industry as a marketing angle, but they first need to be substantiated in human subjects.

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

We believe that further research in this area can prove the value of milk products for improving gut health by maintaining gut microbiota diversity. This can be particularly important for certain consumers such as the elderly, those being treated with antibiotics, or those whose microbiota is depleted due to long term consumption of a restricted diet.

4 (b) Summary of Research Outputs

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

1. Ntemiri, A., Ni Chonchuir, F., O'Callaghan, T. F., Stanton, C., Ross, R. P., O'Toole, P. W. (**2017**). Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota. *J. Agric. Food Chem.*, *65*(8), 1836–1846.
2. Ntemiri, A., Celine Ribiere, Stanton, C., Ross, R. P., O'Connor, E., O'Toole, P. (**2019**). Retention of microbiota diversity by lactose-free milk-in a mouse model of elderly gut microbiota. *J. Agric. Food Chem*., 67(7), 2098-2112.

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

1. **IHMC-International Human Microbiome Consortium, Killarney, Ireland, 2018:**
    - Retention of microbiota diversity by lactose free milk-supplemented diet was comparable to soy protein diet in a mouse model of healthy and frail gut microbiota Ntemiri, A., Stanton, C., Ross, P., O'Toole, P.
2. **ISAPP – SFA Annual Meeting 2017, Chicago, USA (ISAPP Poster Award)**,
3. **SGM Annual Meeting 2017, Edinburgh, UK (SGM Travel Grant)** and
4. **S*f*AM 2017: The Microbiome and Human Health 2017, London, UK:**
    - Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota. Alexandra Ntemiri, Fodhla NíChonchúir, Tom O'Callaghan, Catherine Stanton, R. Paul Ross, Paul W. O'Toole.
5. **SGM Focused Meeting Irish Division 2016, Cork, Ireland:**
    - *In vitro* fermentation of intact glycomacropeptide and lactose by faecal microbiota from healthy and frail older donors. Alexandra Ntemiri**,** Fodhla NíChonchúir, Tom O'Callaghan, Catherine Stanton, R. Paul Ross and Paul W. O'Toole.

(iii) National Report -N/A

(iv) Workshops/seminars at which results were presented

1. EMBL-EBI Bioinformatics Workshop 2016, Hinxton, UK and

- *In vitro* fermentation of intact glycomacropeptide and lactose by faecal microbiota from healthy and frail older donors. Alexandra Ntemiri**,** Fodhla NíChonchúir, Tom O'Callaghan, Catherine Stanton, R. Paul Ross and Paul W. O'Toole.

(v) Intellectual Property applications/licences/patents
n/a

(vi) Other
n/a

5. Scientists trained by Project

Total Number of PhD theses: 1

Effect of dairy based food ingredients on the gut microbiota of older consumers
Ntemiri, Alexandra
School of Microbiology, University College Cork; thesis submitted January 2019.
https://cora.ucc.ie/handle/10468/7423

Total Number of Masters theses: 3

1. Fodhla Ni NíChonchúir, Master by research, Teagasc: Evaluating the effect of glycomacropeptide and lactose on the growth of lactobacilli and bifidobacteria from the intestinal microbiota of elderly subjects
2. Laure Gonod, Master of Science by research, Teagasc: Production of kappa-Casein Glycomacropeptide and its use as a Functional Food
3. Caitriona Cadogan, Master of Science, taught, UCC. Thesis title: Identify and investigate key determinants of acceptance of functional food products, in this case containing the ingredient GMP (Glycomacropeptide), among an elderly cohort

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
UCC	5	2
Teagasc	2	0.16
Total	**7**	**2.16**

7. Researchers Funded by FIRM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates		
Contract Researchers		
PhD postgraduates/ Masters	4	8.4167
Research Assistant	1	0.8333
Total	**5**	**9.2500**

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:	€411,540
Total Award by DAFM:	€500,098
Other sources of funding including benefit in kind and/or cash contribution(specify):	-

Breakdown of Total Expenditure

Category of expenditure	UCC	MFRC	Total Project
	€	€	€
Contract Staff	25,233	-	25,233
Students	93,804	88,000	181,804
Consumables	69,264	32,355	101,619
Travel and subsistence	2,438	1,549	3,987
Sub-total	190,739	121,904	312,643
Durable equipment (**)	-		-
Other (please specify):	276	5,008	5,284
Sub-Contracting Costs			-
Overheads (***)	57,222	36,391	93,613
Total Expenditure	**248,237**	**163,304**	**411,540**

10. Leveraging

The project received approximately 5 kg high grade GMP from Arla Foods for lab analyses.
Teagasc received ca. 20 kg sweet whey powder from Kerry (Listowel) for pilot scale GMP production.

11. Future Strategies

The project team plan to integrate the research findings of ELDERFOOD into future Horizons 2020 applications.

12. Consent to Publish Final Report on the DAFM Website and/or Through Other Dissemination channels

I consent to this report being made available to the public, through the Department's website and other dissemination channels.

Yes X No ☐

13. Declaration

I declare that the information contained in this final report is complete and true to the best of my knowledge and belief.

Signed: ____PWOT_____ Project Coordinator

Date: 19/03/2019