Summary Report: Healthier Sheep Milk with Probiotics (AgMardt Grant 826)

Project objectives (from AgMardt Proposal)

Waituhi Kuratau Trust (WKT) has identified competency in R&D as critical to the success of it future businesses aspirations. It sees its commitment to R&D as fostering innovation and clearly establishes the Trust as a knowledge driven organisation creating wealth not only from the natural resources it manages but also the intellectual capabilities of its partners and the people within its own organisation. This trial marks the beginning of a potentially multi year partnership that initially sees the Trust take existing lab research through to marketable product but eventually having the capability to identify, research and develop its own products and services.

In-line with the Trust's plan to take to market in 2008 a New Zealand sheep yogurt using bacterial cultures identified and researched as being beneficial to gut health, the current proposal has been designed to provide valuable *in vivo* evidence based data to support some of the anecdotal health benefits of sheep milk. Specifically it will provide data required for marketing on:

- Gut health and anti-inflammatory benefits of sheep milk
- Gut health and anti-inflammatory benefits of novel (& patentable) probiotics
- Gut health and anti-inflammatory benefits of a combination of sheep milk and probiotics

The data will be used to secure patents, publish a paper in a peer reviewed scientific journal, produce marketing materials and is a prerequisite to human clinical trials. The patents will form the basis for the development of knowledge-embedded novel sheep milk-based products that add value because they have scientifically-supported efficacy claims for improved gut function.

Milestones

Milestone 1 & 7

Description: File Provisional Patents on Bacterial Strains. Provide data for patent exemplification.

The finding by AgResearch that the WKT bacterial strains improve the integrity of the intestinal barrier is considered inventive and considered by IP experts as potentially patentable. However, without any scientific evidence at the beginning of this project on the gut health/anti-inflammatory effects of sheep milk used alone or in combination with the novel probiotics it was mutually agreed that filing of a provisional patent at the start of the project was premature. It was further acknowledged that AgResearch would file (and pay associated costs) on completion of the AgMardt project. Having discussed the findings of the trial with representatives from WKT on October 8th 2008 and having gained a clearer understanding of their commercial intentions for the strains as well as having had a good discussion on the advantages/disadvantages of patenting, we are now seeking recommendations from the AgResearch IP team and their patent attorneys

on how best to protect (or format a patent application to include) the findings of the three WKT Probiotic strains alone and in combination with sheep milk and in a yoghurt format. AgResearch will also seek clarification regarding potential patentability of the novel addition of the WKT strains in a cows milk based yoghurt product.

Milestone 2 & 3.
Description: Complete tolerance and adherence tests for the three experimental probiotic strains and commercial strain.

The assessment of probiotics to survive passage through the gastrointestinal tract to the colon and to adhere to colon epithelial cells is crucial for the identification of strains that will provide the desired beneficial effects at the required site (the colon).

Of the bacterial strains, WKT3 was the best at tolerating the conditions that mimicked the gastrointestinal tract and at adhering to epithelial cells. It was also better at surviving in acidic conditions and in the presence of bile than the benchmark commercial probiotic strain (*Lactobacillus rhamnosus* HN001).

WKT1 and WKT2 were able to adhere to epithelial cells and tolerate some gastrointestinal conditions; however, both strains did not tolerate high concentrations of bile as well as *L. rhamnosus* HN001 and WKT1 did not tolerate acidic conditions (pH 2) as well as *L. rhamnosus* HN001. This casts doubt on the ability of these strains to deliver their beneficial effects to the colon, but these results are not definitive. It is known that bacteria are better protected from the harsh gastrointestinal conditions when delivered in a milk solution than when delivered alone. It is possible that the less robust strains, particularly WKT2, may be able to survive passage to the colon when delivered in a suitable matrix such as sheep milk.

AgResearch recommended that at least WKT2 and WKT3 be tested in a rodent model (*in vivo*) of intestinal inflammation (Milestone 4). Excluding WKT1 from the *in vivo* trial, allowed more doses of sheep milk and/or WKT2 and WKT3 to be tested.

Milestone 4 & 5.
Description: Complete 3 trials testing the efficacy of sheep's milk solids and three experimental Probiotic strains using a rat model of acute colitis.

1. Effect of sheep milk powder and probiotic administration technique on colitis.
2. The palatability of increasing doses of sheep milk powder and the effect of frequency of probiotic administration on colitis.
3. Effect of sheep milk powder (highest palatable dose) and probiotics on colitis.

Provide a report demonstrating the efficacy of sheep milk or the combination of sheep milk and probiotics in reducing gut inflammation.

Results

A chemically-induced rat model of colitis was used to test the *in vivo* effects of sheep milk powder and/or probiotics on colon inflammation. Acute colitis was induced in rats by dextran sodium sulphate (DSS) in the drinking water. This is a recognised model of intestinal barrier dysfunction and is commonly used to test food components for their effect on colon inflammation.

Two preliminary experiments were carried out to determine the best administration technique and frequency for the probiotics, and the palatability of feeding rats high concentrations of sheep milk powder in the diet. From these results it was decided that in the main experiment the probiotics should be administered in a sugary milk solution via pipette twice a day with and without 51% sheep milk powder (20% sheep milk fat) in the diet.

Rat model of inflammation

In all three experiments, colon inflammation developed in the DSS rat model as expected. Compared to the rats receiving no DSS (healthy controls), the rats receiving DSS had:

1. Increased colon weight to length ratio (indicator of colon inflammation).
2. Increased colon histological parameters (indicator of colon damage and inflammation).
3. Increased colon myeloperoxidase (MPO) activity (indicator of colon inflammatory cell infiltration).

Conclusion: The DSS-induced rat model of colon inflammation was suitable for looking at effects of diet on gut health.

Sheep milk powder effect

In Experiment 1, when the DSS rats were fed 11% sheep milk powder, there was a reduction in colon total histology score and an increase in caecal concentration of short chain fatty acids (SCFA) compared to similar rats fed the control diet. In Experiment 3, the DSS rats fed the diet containing 51% sheep milk powder (compared to DSS rats fed the control diet) also:

1. Increased caecal concentrations of some SCFA.
2. Increased caecal concentrations of beneficial bacteria (bifidobacteria and clostridia).

But:

3. Increased colon total histology score (above that of the DSS control rats).

Conclusion: Overall sheep milk powder largely had a beneficial effect on gut health across all experiments but there was evidence to suggest that the level of sheep milk powder in the diet was important; as that level increased the beneficial effects on gut health declined. The optimal level of inclusion appears to be somewhere between 11 and 51%. This should be investigated further.

Probiotic effect

In Experiment 1, there was some indication that DSS rats fed the control diet (5% fat) and administered the WKT cocktail (combination of WKT1, 2 and 3) had reduced colon inflammation (surface enterocyte disruption and *muscularis* external thickening) compared to the DSS rats fed the same control diet. In Experiment 2, this effect of WKT

cocktail was not observed. However, in Experiment 3, the probiotic treatments (WKT strain alone or cocktail) had both beneficial and detrimental effects in the DSS rats compared to the placebo administration in the DSS rats (all rats fed the control diet containing 20% of fat):

1. WKT2 alone had no effects.
2. WKT3 alone increased caecal concentrations of some SCFA (indicates presence of beneficial bacteria) but also increased the colon total histology score (indicates an increase in colon inflammation).
3. WKT cocktail did not have an effect on any parameters except it increased colon MPO activity (indicates an increase in colon inflammatory cell infiltration).

Conclusion: All the probiotics including the commercial strain we used as a control, either alone or in combination (cocktail) did not have consistent effects on improving gut health.

Sheep milk powder and probiotic effect

In Experiment 3, the combination of the probiotics and 51% sheep milk powder resulted in conflicting effects on several colon parameters in DSS rats compared to DSS rats fed a control diet:

1. WKT2 in combination with 51% sheep milk powder increased colon total histology score, increased colon MPO activity and decreased colon GSH concentration (all indicate an increase in colon inflammation).
2. WKT3 in combination with 51% sheep milk powder increased the caecal concentration of beneficial bacteria and decreased the caecal concentration of undesirable bacteria, but also increased colon total histology score and MPO activity.
3. WKT cocktail in combination with 51% sheep milk powder increased the caecal concentration of beneficial bacteria and decreased the caecal concentration of undesirable bacteria, but also increased colon MPO activity.

Conclusion: The positive effects of sheep milk powder alone on gut health were still present when given in combination with the WKT probiotic cocktail or WKT3 alone.

This information was presented in written report format two weeks following the contracted completion date as agreed by WKT.

Milestone 6

Description: Present outcomes of the research to Trustees. Look to disseminate research outcomes to key sheep milk dairying industry players.

Result: A presentation and discussion was had with William Konui (Chair of Waituhi Kuratau Trust), Graeme Everton (Trustee and Project Manager) and Rex Webby (Consultant to Waituhi Kuratau Trust) on October 8 at Ruakura. Present from AgResearch were Drs Warren McNabb and Rachel Anderson, and Lisa Marcroft. The presentation provided the background to the methodology and trial designs, the research findings and some suggestions followed by discussion on what the results imply and identification of the future steps required to get four new yoghurt probiotic products ready

for a market launch in early 2009. The Powerpoint presentation has been provided to WKT for their use.

Timeframes:
All milestones were completed by the expected completion dates as per Agreement between Waituhi Kuratau Trust and AgResearch.

The future:
As a result of this research project, WKT are planning on developing four new yoghurt products containing the novel WKT3 probiotic and launching these early in 2009. Initially the NZ market (market size $141m) will be targeted but once robust production, marketing and distribution systems have been established in this market they will either look to launch the locally produced products internationally or to license the IP developed by this project and future projects/experience internationally. (This will be dependent on variables such as ability to build sufficient volumes of sheep milk).

Prior to launching the new Probiotic product some further analysis and product development work will be required by a team including Matatoki yoghurt personnel, AgResearch probiotic team and a yet to be identified expert in regulatory and new product development. This team will co-ordinate:

- Formulation work including studying the effect of adding WKT3 on acceptability of taste and texture.
- Safety trials of new formulation
- Shelf life of yoghurt including survivability of probiotics
- Nutritional composition of yoghurt Products for Nutrition information Panel
- Protocol for optimising fermentation of WKT3 so this can be outsourced if necessary.
- Trial production and market sampling

Following the completion of this work, it is envisaged that the data from the AgResearch studies, AgMardt study and the data from the above activities will be patented and then published in a medium-high impact scientific journal. Interestingly, the Journal of Digestive Disease and Science published an original paper in April 2008 conduced by the Women's and Children's Hospital in Adelaide, Australia that provides evidence for the first time that sheep milk Probiotic yoghurt "may be a useful natural prophylactic treatment to minimize the deleterious effects of chemotherapy-induced intestinal mucositits". This study indicates that the efforts by WKT and AgMardt to enter the gut health domain using a combination of probiotics and sheep milk are well founded. As both studies highlight, further work is requires to ascertain the optimal concentration of sheep milk in the diet and optimal combination of probiotics when used in a sheep milk product.

In addition to the above there is the opportunity for WKT to provide samples of sheep milk products for inclusion in the FRST research collaboration, Nutrigenomics New Zealand. The work of NuNZ will further improve the understanding and knowledge of sheep milk for both the scientific and sheep milking industries and will not require direct financial input from WKT. One such study will provide a comparison of sheep milk and milk from other species on gut health and inflammation. Information from this

programme will help WKT build their knowledge about the benefits of their specific products and may guide them in the future with regard to providing direction for the research required to add value to their products.

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