Effects of orally administered ovine serum immunoglobulin in the normal and *Salmonella enteritidis* – challenged growing rat.

A thesis presented in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Nutrition at Massey University,
Palmerston North, New Zealand

Prabhu Balan

2011

MASSEY UNIVERSITY

I am dedicating my thesis to my guru, his holiness arulthiru BANGARU ADIGALAR

Abstract

Immunoglobulins (Ig) are the primary anti-infective component of plasma, colostrum and breast milk. They are the specialized glycoproteins that protect the body from harmful bacteria, viruses and other environmental pathogens by either binding to them or by forming an encapsulating barrier. The development of antimicrobial and immunomodulatory products from natural sources for dietary supplementation in both animals and humans is an active area of research. Purified Ig from sheep plasma (ovine serum Ig) is one such candidate product.

Based on the results of the numerous background growth studies of others, the objectives of this study were to determine whether orally administered ovine serum Ig affected growth performance, digestive organ weights, gut morphology, immunity, the gut microbiota, goblet cell numbers, mucin gene expression and digesta mucin protein contents in the growing rat. The study also sought to understand whether orally administered ovine serum Ig prevented or lessened the negative effects of *Salmonella enteritidis* ATCC 13076 (a pathogen) in the *S. enteritidis*−challenged growing rat. The presence of ingested intact Ig in different parts of the digestive tract was also determined. Investigations were undertaken in normal and *S. enteritidis*−challenged Sprague-Dawley male growing rats. Diets were iso-caloric and had similar protein and amino acid contents. The diets were fed for 21 days (for non-challenged rats) and for 18 days (for the challenged rats).

An ovine Ig fraction improved food conversion efficiency, the weights of several digestive organs and gut histology. Compared with spray-drying, a freeze-drying procedure preserved a higher degree of immunological activity.

In immunity studies, an ovine Ig fraction selectively enhanced ($P < 0.05$) various indices of immune function such as phagocytic acitivity, lymphocyte proliferation and gut and plasma antibodies. In microbiolgical studies, the number of lactobacilli in the gut were increased ($P < 0.05$) by feeding the ovine Ig. Ovine Ig also influenced the transcription and translation of gut mucin protein as evidenced by increased ($P < 0.05$) mucin gene expression and digesta mucin protein concentrations as well as an increased goblet cell count.

After gavaging with *S. enteritidis*, the rats fed the IOI (inactivated ovine Ig) and BD (basal diet) diets grew considerably more slowly (growth declined)

than the challenged rats fed the FDOI (freeze-dried ovine Ig) diet and the latter rats showed no sign of infection. The villus length, crypt depth, villus:crypt ratio and villus surface area (VSA) of the duodenum and jejunum were generally greater ($P < 0.05$) in rats challenged with *S. enteritidis* and receiving the FDOI diet compared to either the unchallenged rats fed the BD diet (except duodenal and jejunal VSA) or the challenged rats fed the BD or IOI diets. Several measures of immune modulation were affected as was the bacterial composition of the gut microflora. The ileal and colonic digesta for the FDOI-fed rats had higher ($P < 0.05$) numbers of goblet cells and higher ($P < 0.05$) digestive luminal mucin protein concentrations than the challenged rats fed either the BD- or IOI-supplemented diets.

Intact ovine Ig were detected in the luminal contents from the stomach through to the colon in the growing rat fed orally with ovine Ig fraction. The amounts (percentages of digesta dry matter) of intact ovine Ig for rats fed the FDOI diet were 2.17%, 3.12%, 5.31%, 2.03% and 5.76% for stomach chyme, duodenal, jejunal, ileal and colonic digesta respectively. Overall, the accumulated amount was 18.4%, which indicates the presence of a high level of active material throughout the digestive tract.

In conclusion, purified ovine Ig improves growth of healthy rats and protects against enteric infection by immunomodulation, mucin protein and/or modification of commensal microbial composition. The results contribute to knowledge of how orally administered ovine Ig can modulate and enhance key indicators of gut function and overall growth performance in the growing rat.

ACKNOWLEDGEMENTS

PhD is not just a degree, it's a discovery journey. I didn't do this journey alone. So, there are many kind hearted well-wishers I need to acknowledge and thank.

First and foremost my chief supervisor Distinguished Professor Paul J Moughan for his kind support, motivation, immense patience, deep scientific insights, dedication and willingness to let me explore unusual avenues in order to solve research problems and satisfy my curiosity. Without his scholarly insight, substantial corrective comments, prompt and high-quality feedback, my PhD thesis would not have appeared in its present form.

I am thankful to my second supervisor Professor Harjinder Singh for his kind encouragement, guidance and motivation for my PhD journey. I also take this opportunity to especially thank my third supervisor, Dr Kyoung-Sik Han; without his continuous help, supervision and advice I would not have completed my PhD journey. I would like to thank Dr Shane M Rutherfurd for his invaluable support, scientific advice and guidance for my research work. Without his reference letter, I would not have received a hardship bursary.

I express my deep sense of gratitude to Dr Kay Rutherfurd-Markwick for her immense support in completing the immunology research works and publications. I should also like to thank Dr Mike Boland (Riddet Institute), Prof Gerald Tannock and Dr Blair Lawley (Department of Microbiology and Immunology, University of Otago) and Dr Nicole Roy (Food Nutrition and Genomics, AgResearch Ltd) for their valuable comments in the preparation of the thesis chapter and manuscript describing the DGGE work.

I am thankful to Dr Venkata Sayoji Rao Dukkipati (Institute of Veterinary, Animal and Biomedical Sciences, Massey University) for his kind assistance in performing Western Blot studies. I also thank Dr Ganesalingam (Institute of Fundamental Sciences, Massey University) for his valuable advice on the statistical analysis.

I am deeply grateful to Professor Ravi Ravindran and Dr Rana Ravindran for their valuable support during PhD.

I am truly thankful to the Riddet Institute for providing me PhD Riddet Scholarship, Research Assistant position during my 4th year of PhD and now Research Officer Position. I am also thankful to Massey University for providing me with a Bailey Bequest Bursary.

I am indeed thankful to Ms Willi Twight, Ms Felicia Stibbards, Ms Ansley Te Hiwi, Ms Paula McCool, Mr John Henley-king, Mr Mark Ward and Mr Andy Lim and Ms Terri Palmer for their immense cooperation, administrative assistance and invaluable support.

I am very grateful to Mr Jack Cui for his kind support in preparing the sheep immunoglobulin fraction.

I am extremely thankful to Mr Joe Crenshaw and Mr Scott Dorr (American Protein Corporation, APC, USA) for providing me with a free sample of spray dried porcine plasma - AP920 for my research work.

I appreciate everyone at the Riddet Institute who played the role of counsellors as well as well-wishers. Especially I would like to thank Mr Shantanu Das, Ms Sharon Henare, Ms Janiene Gilliand, Mr Carlos Montoya, Mr Mallesh Peram, Mr Anant Dev, Ms Lovedeep Kaur, Mr Arup Nag, Ms Anwesha Sarkar, Ms Maggie Zou and Mr Guillaume Brisson. I am also thankful to all based at the Institute of Food Nutrition and Human Health for their support. A special thanks to Ms Kelly OFlaherty and Ms Shay Rutherfurd for their kind support during my animal trials. Special thanks to Mr Mathew Levin for his IT support.

I take this opportunity to say a big thanks to Ms Michelle McGrath, Ms Anne Broomfield and Ms Shampa de (Institute of Food Nutrition and Human Health) for their full hearted support in the immune work and DGGE study.

I am deeply grateful to Ms Debbie Chesterfield (Manager, Small Animal Plant Unit, Massey University, Palmerston North) for her enormous contribution during animal studies.

I would not have completed my animal trials without approval from the Animal

Ethics Committee, Massey University, thank you. I would like to extend my heartfelt thanks to Ms Juliet Cayzer (Estendart, Palmerston North) for her guidance during my *S. enteritidis* − challenged rat trial.

I wish to sincerely thank Mr. Aidan Wood, IT Assistant, Massey University, for his valuable support in making my table of contents in PhD thesis.

The blessings of my family have given me the strength to complete this PhD research study to the best of my effort. There are no words to explain the affection and love that I have for my family. I am not here, without my parents Mr Sambasivam Balan Mudliyar and Ms Punithavathi Balan (late) and Ms Selvi Balan and I thank them for their constant love, encouragement and guidance. I am extremely thankful to my eldest sister Ms Saraswathi Shankar, my second eldest sister Malleshwari Suresh and younger sweet and lovely sister Ms Yamini Balan for their constant prayers, motivation and love. I am thankful to my brothers-in-law, Mr. Shankar, Mr. Suresh, Mr Karthick. Last but not least, my final thanks and undying gratitude go to my lovely wife and kids, Ms Suchitra Prabhu, Master Sakthi Bala Prabhu and Master Kavin Sakthi Prabhu. Susi, Yamini, Sakthi, and kutty Sakthi without your love, endurance, strength, prayer and patience, I would not have completed this PhD journey. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA – Amino acid

ADCC – Antibody dependent cell-mediated cytotoxicity

ADFI – Average daily feed intake

ADG – Average daily gain

AMI – Antibody-mediated immunity

AP – Animal plasma

BD – Basal diet

BSA – Bovine serum albumin

C3b – complement component 3

cAMP – Cyclic adenosine monophosphate

cGMP – Cyclic guanosine monophosphate

CD – Crohn's disease

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

cDNA – complementary DNA

CDR – complementarity determining regions

CFU – Colony forming unit

C_H – Constant heavy chain

C_L – Constant light chain

CLR – C-type lectin receptor

Con A – Concanvallin A

CPM – Counts per minute

CT – cholera toxin

CXC R1 – chemokine receptor 1

d – day

DC – Dendritic cells

DGGE – Denaturing Gradient Gel Electrophoresis

DNA – Deoxyribonucleic acid

dsRNA – double-stranded RNA viruses

EAggEC – enteroaggregative E. coli

EDTA –Ethylenediaminetetraacetic acid

EHEC– enterohemorrhagic *E. coli*

ELISA – Enzyme Linked Immunosorbent Assay

ELLA – Enzyme-Linked Lectin Assay

EPEC – enteropathogenic *E. coli*

ETEC – enterotoxigenic *E. coli*

Fab – “Fragment, antigen-binding”

FACS – Fluorescence-activated cell sorter

FAE – Follicle-Associated Epithelium

Fc – “Fragment, crystallisable”

FcR – Fc receptor

FCγbp – Fc-gamma binding protein

FDOI – Freeze dried ovine immunoglobulin

FOS – fructo-oligosaccharides

GALT – Gut associated lymphoid tissue

G:F – Gain:feed ratio

GIT – Gastrointestinal tract

GOS – galacto-oligosaccharides

H_2O_2 – hydrogen peroxide

HD – human defensins

HIP / PAP – hepatocarcinoma-intestine-pancreas / pancreatic-associated protein

HRP – Horseradish peroxidase

HSP – Heat shock protein

HVR – Hyper variable region

IBD – Inflammatory bowel disease

IEC – intestinal epithelial cells

IFNγ – Interferon gamma

Ig – Immunoglobulin/ Immunoglobulins

IgA – Immunoglobulin A

IgD – Immunoglobulin D

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IgY – Egg yolk antibodies

IL-1– Interleukin -1

IL-1R – Interleukin -1 receptor

IMO – isomalto-oligosaccharide

iNOS – inducible nitric oxide synthase

IOI – Inactivated ovine immunolglobulin

IPS-1 – Interferon promoter stimulator-1

JAM – junctional adhesion protein

kDa – kiloDalton

LEAPs – liver-expressed antimicrobial peptides

LPS – lipopolysaccharide

LT – Heat Labile Toxin

LTB4 – leucotrien B 4

M-SAA3 – mammary-associated serum amyloid A isoform 3

MALT – mucosal-associated lymphoid tissue

M-cell – Microfold cells

MDA5 – Melanoma differentiation associated gene 5

MHC – Major histocompatibility complex

mRNA – messenger ribonucleic acid

MUC – Mucin genes (human)

Muc – Mucin genes (rat)

NA – Natural antibodies

NF-κB – Nuclear factor kappa B

NLR – Nucleotide-binding domain leucine-rich repeat

NK cells – Natural killer cells

NO – Nitric oxide

NOD – Nucleotide-binding domain

NOS – nitric oxide synthase

$O^{\cdot}{}_2^-$ – Superoxide anion

OIC – Ovine serum immunoglobulin concentrate

OPD – o-phenylenediamine dihydrochloride

PAMPs – Pathogen-associated molecular patterns

PBL – Peripheral blood leukocytes

PBS-T – Phosphate buffer saline–tween 20

PCR – Polymerase chain reaction

PHA – Phytphaemagglutinin

pIgR – Polymeric immunolglobulin receptor

PLA_2 – Phospholipase A_2

PP – Peyer's patch

PRR – pattern recognition receptors

qRT-PCR – Quantitative Real Time –Polymerase Chain Reaction

RELMβ – resistin-like molecule beta

RLR – Rretinoic-acid-inducible protein 1–like receptors

RNA – Ribonucleic acid

s – Seconds (time)

SAP130 – Sin3-associated polypeptide p130

SDAP / SDBP – Spray dried animal plasma / Spray dried bovine plasma

SDOI – Spray dried ovine immunoglobulin

SDP – Spray dried plasma

SDPP – Spray dried porcine plasma

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEB – *Staphyloccocus aureus* enterotoxin B

SED – subepithelial dome

SEM – Standard error of mean

SIgA – Secretory immunoglobulin A

SIgM – Secretory immunoglobulin G

ST – Stable enterotoxins

TER – transepithelial electric resistance

TFP – trefoil factor peptides

TGFβ – Transforming growth factor

T_H1 – T helper cells 1

T_H2 – T helper cells 2

T_H17 – T helper cells 17

TLR – Toll like receptor

TNFα – Tumour necrosis factor alpha

UC – Ulcerative colitis

VR – Variable region

VSR – Villous surface area

WGA – Wheat germ agglutinin

Wk – Week

ZO – *zonula occluden*

Zot – *Zonula occludens* toxin

LIST OF PUBLICATIONS

Peer-reviewed articles published or in progress

1. **P Balan**, KS Han, K Rutherfurd-Markwick, H Singh and PJ Moughan. (2011). Immunomodulatory effects of ovine serum immunoglobulin in growing rats gavaged with *Salmonella enteritidis* J Nutr. 141, 950-956.
2. **P Balan**, Kyoung-Sik Han, H Singh and PJ Moughan. (2011). Dietary supplementation with ovine serum immunoglobulin is associated with increased gut mucin secretion in the growing rat. (Animal). DOI:10.1017/S1751731111001108.
3. **P Balan**, KS Han, SM Rutherfurd, H Singh and PJ Moughan. (2011). Dietary supplementation with ovine serum immunoglobulin attenuates acute effects on growth, organ weights, gut morphology and intestinal mucin production in the growing rat challenged with *Salmonella enteritidis*. Animal. 5, 1570-1578
4. KS Han, **P Balan**, F Gasa and M Boland. (2011). Green kiwifruit modulates the colonic microbiota in growing pigs. Lett Appl Microbiol. 52, 379-385.
5. **P Balan**, KS Han, K Rutherfurd-Markwick, H Singh and PJ Moughan. (2010) Immunomodulatory effects of ovine serum immunoglobulin in the growing rat. Animal. 4, 1702-1708.
6. **P Balan**, KS Han, SM Rutherfurd, H Singh and PJ Moughan. (2009) Orally administered ovine serum immunoglobulins influence growth performance, organ weights, and gut morphology in growing rats. J Nutr.139, 244-9.

Confidential reports

7. SJ Henare, SM Rutherfurd, M Zou, KS Han, **P Balan**, N Strobinger, S Saigeman, T Olson, C Sawatdeenaruenat, A Purba, MJ Boland and PJ Moughan. (2011). Gastrointestinal interactions of kiwifruit: Effects on mucin production in piglets
8. SM Rutherfurd, TK Chung**,** DV Thomas, ML Zou, KS Han, **P Balan**, E Maier and PJ Moughan (2011). Effect of microbial phytase on broiler performance, AME, toe ash, bone mineral density, ileal and colonic microbial population, gut mucin production and the digestibility of phytase P, minerals and amino acids.

Manuscripts ready for submission

9. **P Balan**, KS Han, B Lawley, H Singh and PJ Moughan. Orally administered ovine serum immunoglobulins modulate the levels of *Lactobacillus* and *Enterobacteria* in the growing rat.
10. **P Balan**, KS Han and PJ Moughan. Recovery of intact immunoglobulin in the digesta of the growing rat following ingestion of an ovine serum immunoglobulin.

Manuscripts in preparation

11. **P Balan** and PJ Moughan. Immunoglobulins – Review
12. **P Balan** and PJ Moughan. Ovine serum immunoglobulin supplements prevent the release of mucosal proinflammatory mediators in the growing rat challenged with *Salmonella enteritidis*.
13. **P Balan** and PJ Moughan. Stimulatory effect of ovine serum Ig on multiplication of lactic acid bacteria under *in vitro* condition.
14. **P Balan**, SM Rutherfurd and PJ Moughan. Effects of ovine serum immunoglobulin on dental health and immunomodulation in the cat.
15. **P Balan**, G Mal, S Das and PJ Moughan. Synergistic antimicrobial activity of curcumin, manuka honey and whey protein isolate.
16. KS Han, **P Balan**, A Purba and PJ Moughan. Effect of Korean ginsengs on gut microbiota and mucin secretion in growing rats.
17. KS Han, **P Balan**, A Purba and PJ Moughan. Effect of Korean traditional foods-derived polysaccharides on lymphocyte proliferation of Peyer's patch, cytokine and immunoglobulin production in the ileum of growing rats.
18. SM Rutherfurd, TK Chung, **P Balan**, KS Han, E Maier and PJ Moughan. Effect of three microbial phytases on mucin output and gut microbiota in broilers fed low-phosphorus corn-soybean diets.
19. SJ Henare, SM Rutherfurd, M Zou, **P Balan**, KS Han, N Strobinger, MJ Boland and PJ Moughan. Gastrointestinal interactions of kiwifruit: Effects on mucin production in piglets.
20. SM Rutherfurd, SJ Henare, RK Richardson, ML Zou, **P Balan**, C Sawatdeenaruenat and PJ Moughan. The effect of dietary protein content on endogenous ileal tryptophan flow in the growing rat.

Patent application

21. **P Balan**, Shane M Rutherfurd, H Singh and PJ Moughan. Ovine serum immunoglobulins positively modulate dental health and immunity in the cat.

Abstract, conference and other presentations

22. **P Balan**. Ovine serum Immunoglobulins – *In vitro* and *In vivo* studies. Oral presentation at the Research day, Riddet Institute, Massey University, Palmerston North, New Zealand, August 2011.
23. **P Balan** and PJ Moughan. Potential application of ovine serum immunoglobulins during total parenteral nutrition. Poster presentation at the International Symposium: Dietary Protein for Human Health Auckland 1142, New Zealand, March 2011.
24. **P Balan**, KS Han, H Singh, and PJ Moughan. Orally administered ovine serum immunolgobulins modulates the immunity and gut function in the growing rat. Oral presentation at the CORE meeting, Palmerston North, New Zealand, June 2010.
25. **P Balan**, KS Han, H Singh and PJ Moughan. Gut microbial modulation and immunomodulation by ovine serum immunolgobulins in the growing rat. Oral presentation at the USA/IRELAND functional food conference, Cork, Ireland, March 2010.
26. **P Balan**, KS Han, H Singh and PJ Moughan. Gut microbial modulation and immunomodulation by ovine serum immunolgobulins in the growing rat. Oral and poster presentation at the PhD student Colloquium, Palmerston North, New Zealand, October 2009.
27. **P Balan**, KS Han, H Singh and PJ Moughan. Immunomodulation of ovine serum immunolgobulins in the growing rat. Poster presentation at the Functional food conference, Riddet Institute, Palmerston North, New Zealand, February 2009.
28. KS Han, R Sengupta, **P Balan**, A Deglaire, H Singh and PJ Moughan. Effect of bioactive protein on mucin gene expression in rat small intestine. Poster presentation at the 17th Queenstown Molecular Biology Meeting. Queenstwon, New Zealand. 2007.