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The effects of milk matrices on the transit and digestion of secretory immunoglobulin A in the gut

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Abstract:

Bovine secretory immunoglobulin A (BSIgA) has the potential to provide protective effects to the gastrointestinal tract (GIT) when consumed orally. Oral administration of immunoglobulin (Ig) preparations has been explored and proven satisfactory in defence of a variety of enteric microbial infections in humans. Currently Ig preparations focus on bovine colostrum or whole milk. The effects of different milk matrices and how the overall composition may impact BSIgA transit and digestion have not been explored. In this study, an in vivo experiment was used to demonstrate the transit and digestion of BSIgA in two different milk matrices through the GIT of mice. The milk matrices of interest were whey protein concentrate (WPC) and skim milk powder (SMP). Mice were gavaged with 200 µL of each treatment and groups were culled at four time points; 7 minutes, 20 minutes, 1 hour, and 4 hours. The GIT was dissected into four pieces; stomach, small intestine, large intestine, and caecum. These were flushed with phosphate buffered saline (PBS) and the amount of BSIgA in the washouts was measured on an ELISA. Bovine SIgA survived digestive processes in the GIT of mice in SMP and WPC, as it was detected at all time points. Intestinal washouts from mice that were fed SMP measured at 7 minutes, 20 minutes, 1 hour, and 4 hours detected 66.3%, 22.4%, 0.45%, and 0.97%, of BSIgA respectively. The corresponding values for mice that were fed WPC were 43.8%, 10.2%, 0.12%, and 0.14%, respectively. Overall, the results supported the hypothesis that the milk matrix affected transit and digestion of BSIgA through the GIT of mice. BSIgA was digested 10 fold faster in a WPC matrix than SMP matrix. The BSIgA in SMP appeared more protected from digestion than that in WPC. This is the first study to highlight different milk matrices affecting the transit and digestion of BSIgA. It gives an insight into manufacturing BSIgA into a commercial product and the potential benefits it may provide to the consumer.

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Table of Contents

Abstract: ... *i*

Acknowledgements: ... *ii*

Table of Contents ... *i*

Lists of Figures ... *iv*

Lists of Tables ... *v*

Abbreviations used ... *vi*

Chapter 1 Introduction and literature review ... 1

1.1 General introduction ... 2

1.2 Milk ... 3

1.3 Immunoglobulins ... 3

1.3.1 Immunoglobulin development ... 3

1.3.2 Basic immunoglobulin structure and function ... 4

1.3.3 Immunoglobulin in serum and milk ... 5

1.4 Structure and function of Secretory Immunoglobulin A ... 6

1.4.1 Glycosylation of SIgA ... 7

1.4.2 Function of SIgA ... 8

1.5 Importance of SIgA in milk ... 9

1.6 Relevance of bovine SIgA to humans ... 10

1.7 Endogenous SIgA ... 10

1.8 The Digestive System ... 11

1.9 Physical digestion of proteins in the stomach ... 12

1.9.1 Enzymatic digestion in the stomach ... 13

1.9.2 Gastric digestion of IgA and milk proteins ... 13

1.10 Protein Digestion in the small intestine ... 15

1.10.1 Physical digestion of proteins in the intestines ... 15

1.10.2 Pancreatic enzymatic digestion ... 17

1.10.3 Bile.. 18

1.10.4 Brush border enzymes 18

1.10.5 Intestinal IgA and milk protein digestion 19

1.11 Large Intestine.. 20

1.11.1 Physical digestion 20

1.11.2 Microorganisms in the colon 21

1.11.3 SIgA in the large intestine 21

1.12 Summary of Immunoglobulin A survival through the gastrointestinal tract 22

1.12.1 The need for an *in vivo* experiment 25

1.13 Bovine Milk 25

1.13.1 The significance of Bovine milk for human consumption..................................... 26

1.14 Milk Processing 27

1.14.1 Skim milk powder composition and processing 28

1.14.2 Whey Composition and Processing 29

1.15 Conclusions.. 30

1.16 The objectives of this research 31

Chapter 2 Materials and Methods.. 32

2.1 Subjects... 33

2.2 Experimental design and treatments 33

2.3 Preparation of SMP and WPC 35

2.3.1 Treatment preparation and composition 35

2.3.2 Buffer capacity of SMP and WPC matrix.. 36

2.3.3 Animal Management... 36

2.4. Experimental procedure 37

2.4.1 Oral gavage 37

2.4.2 Dissection... 37

2.5 Sample processing and Assays... 38

3.5.1 Preparation of stomach, small intestine, large intestine, and caecum: 38

2.5.2 Preparation of faecal extract: ... 39
2.5.3 Preparation of test samples for Bovine SIgA analysis ... 39
2.5.4 Preparation of test samples for Murine SIgA analysis ... 39
2.6 Enzyme-linked immunoassay ... 39
2.6.1 ELISA for total bovine SIgA ... 39
2.6.2 ELISA for total murine SIgA ... 40
2.7.1 Statistical design ... 41
2.7.2 Statistical analysis ... 41
Chapter 3 Results ... 42
3.1 Non-specific bovine SIgA detection in the water samples ... 43
3.2 Digestion of BSIgA in SMP ... 43
3.3 Digestion of BSIgA in WPC ... 46
3.4 Comparison of bovine SIgA digestion in SMP and WPC ... 48
3.5 Buffer capacity of SMP and WPC matrix ... 51
3.6 Effects of bovine SIgA to endogenous murine SIgA ... 52
Chapter 4 Discussion ... 55
4.1 Digestion of SIgA in SMP ... 56
4.2 Digestion of SIgA in WPC ... 56
4.3 Comparison of BSIgA digestion in SMP and WPC ... 57
4.4 Impact of bovine SIgA on endogenous murine SIgA ... 62
4.5 Limitations ... 62
4.6 Future research ... 64
4.7 Conclusions ... 65
Chapter 5 Bibliography ... 66
Chapter 6 Appendix ... 73
A Methods ... 74
A.1 Gavage procedure ... 74
A.2 SIgA Wash out recovery ... 74

B Results: ... 75

B.1 Non-specific bovine SIgA detection in the water samples ... 75

B.2 Gender effect ... 76

B.3 End weight recovery ... 76

Lists of Figures

Figure 1 Structure of an immunoglobulin (Ig) molecule. ... 5

Figure 2The structure of SIgA showing the heavy and light chains, the antigen binding sites (Fab), effector (Fc) regions, the hinge region, and glycosylation sites (glycans). ... 7

Figure 3 Stomach geometry. ... 12

Figure 4 Paths of tracer particles in the stomach ... 13

Figure 5 Segmentation contractions moving distal of the small intestine ... 16

Figure 6 Pendular contraction from longitudinal muscles of the small intestine ... 16

Figure 7 Peristalsis movement in the small intestine moving the chyme distally. ... 16

Figure 8 Preferential specificities of pepsin, trypsin, and chymotrypsin for peptide linkages. ... 17

Figure 9 Gastrointestinal tract of mice showing the sample collection sites ... 34

Figure 10 Mouse gavage ... 37

Figure 11 Images showing the dissection and collection of GIT components. ... 37

Figure 12 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with SMP at 7 minutes, 20 minutes, 1 hour, and 4 hours post gavage.. ... 45

Figure 13 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with WPC at 7 minutes, 20 minutes, 1 hour, and 4 hours post gavage.. ... 47

Figure 14 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with SMP or WPC at A) 7 minutes B) 20 minutes, C) 1 hour, and D) 4 hours post gavage.. ... 49

Figure 15 Buffering capacity of SMP and WPC. ... 52

Figure 16 Concentration of Murine Secretory IgA through the GIT of mice 20 minutes, 1 hour, and 4 hours post gavage.. ... 54

Figure 17 Wash out recovery for total Murine SIgA with 500uL PBS + protease inhibitor either once, twice, or three times. ... 75

Lists of Tables

Table 1 Concentration (mg/mL) and percentage (%) of immunoglobulin G and SIgA in bovine and human colostrum and milk. Adapted from Butler (1973) and Haneberg, (1974). 6

Table 2 Summary of immunoglobulin A preparations through the digestive tract *in vitro* and *in vivo* 23

Table 3 Main differences in the composition of cow's and human milk casein and whey fractions. 27

Table 4 Composition of reconstituted WPC to 3.7% w/v total solids and SMP to 10% w/v total solids used in this study. 30

Table 5 Experimental procedure showing sex, treatment, gavage time, and cull time. 34

Table 6 Composition of reconstituted WPC and SMP. 36

Table 7 Concentration of murine SIgA through the GIT of mice gavaged with either water, SMP, or WPC at 20 minutes, 1 hour, and 4 hours post gavage.. 52

Table 8 Maximum log values of BSIgA detected in each GIT for 20 minutes, 1 hour and 4 hours for each GIT compartment in water mice. 75

Table 9 Transit and digestion of bovine SIgA through the GIT of male and female mice 76

Table 10. Mean values ± standard error of the difference (SED) (g) for end weights of recovered sample of SMP and WPC at 20 minutes, 1 hour, and 4 hours. 76

Abbreviations used

AA: Amino acids

ANOVA: Analysis of variation

BSA: Bovine serum albumin

BSIgA: Bovine Secretory Immunoglobulin A

ELISA: Enzyme linked immunosorbent assay

GIT: Gastrointestinal tract

HCL: Hydrochloric acid

IgA: Immunoglobulin A

IgG: Immunoglobulin G

MSIgA: Murine Immunoglobulin A

PBS: Phosphate buffered saline

pIgR: Polymeric immunoglobulin receptor

PP: Peyer's patch

P: Probability

SC: Secretory component

SEM: Standard error of the mean

sed: Standard error of the difference

SIgA: Secretory IgA

SMP: Skim milk powder

TBS: Tris-HCL buffered saline

TBST: 1% Tween®20 in TBS

w/v: weight per volume

WPC: Whey protein concentrate

Chapter 1

Introduction and literature review

1.1 General introduction

Manufacturing bovine milk generates a wide variety of nutritional products ranging from infant formula to everyday use products such as butter, yoghurt and cheese. The processing of milk results in the production of different milk matrices. The milk matrix comprises of proteins, carbohydrates, fats, and minerals; the ratio of these is adjusted and altered dependant on the product. The matrix also provides a range of substances that contribute to the biological function of milk, such as immunoglobulins (Ig). The manufacturing processes expose milk proteins to temperature and pH change resulting in different amounts of functional Ig in milk preparations. Secretory immunoglobulin A (SIgA) is the primary class of Ig in human colostrum and milk and is well recognised for its protective function in the gastrointestinal tract (GIT). The efficacy of SIgA function depends upon the Ig survival and transit past the stomach into the small and large intestine (Mantis *et al.*, 2011). The resistance of SIgA to digestion has been acknowledged in literature (Wilson & Williams Jr, 1969; Shuster, 1971; Steward, 1971; Lee *et al.*, 2012). However, there is a lack of information about the digestion of SIgA in milk and, furthermore, the effect of different milk matrices has not been explored. This study investigated the differential milk matrices effects of Whey Protein Concentrate (WPC) and Skim Milk Powder (SMP) on bovine secretory IgA (BSIgA) transit and digestion through the mouse GIT. The transit and digestion of milk proteins through the GIT is of great interest in order to extend knowledge on the nutritional and health benefits they can provide. This study will give insight into determining whether SIgA from cow's milk can be beneficial for commercial immune milk products.

The literature review gives a background on the immune system and the importance of immunoglobulins. Focus is on SIgA structure, function, and digestion. Literature on the digestive system, with specific examples to milk protein digestion and SIgA, is described. Finally, processing and composition of SMP and WPC are presented. This literature review familiarises concepts and existing theories regarding transit and digestion of whey milk protein SIgA. The purpose is to gather sufficient information to support the hypothesis - that the milk matrix affects transit and digestion of SIgA through the GIT of mice.

1.2 Milk

Milk is an essential complete food to all newborn mammals. It provides a rich source of nutrients containing proteins, carbohydrates, fats, minerals, and vitamins unique to the species (Haug *et al.*, 2007). The milk composition between mammals differs depending on the environment and nutrition available to the mothers and growth patterns of the infant (Haug, *et al.*, 2007). The domestication of cows and production of milk has economic and nutritional benefits which has been established since the 18th century (Evershed *et al.*, 2008). To this day we have, and continue to exploit, the benefits cow milk can provide to humans. Breast milk is the best nutritional choice for infants; however, it is not possible for all women. Infant formula, commonly manufactured from bovine milk, provides a necessary alternative to breastfeeding. Due to composition differences between bovine and human milk, there is interest in investigating ways of 'humanising' bovine milk. This includes modifying bovine milk's composition to maximise nutritional and immunological benefits for human health following digestion.

Milk proteins are a major dietary protein source for humans contributing to growth, development, cell repair, and energy (Whitcomb & Lowe, 2007). Some proteins and their peptides have activities beyond nutrition that are associated with benefits to human health. These proteins are termed milk bioactives and include the Igs, cytokines, hormones, nucleotides, peptides, enzymes, and growth factors (Keenan & Patton, 1995). Their functions include antibacterial, antihypertensive, and provision of immunity (O'Riordan *et al.*, 2014). The research on milk bioactives currently focuses on the biological properties demonstrating their role *in vitro* and *in vivo*. There is increasing attention to the Igs in milk and how they can be manufactured into functional foods.

1.3 Immunoglobulins

1.3.1 Immunoglobulin development

Immunoglobulins are proteins produced by B cells as part of the adaptive immune system (Zhao *et al.*, 2010). B cells are generated in the bone marrow. During development B cells that have encountered self-antigen are subjected to negative selection to eliminate autoreactive cells from the immune repertoire (Sandel & Monroe, 1999). The immature B cells that don't engage with self-antigen are positively selected and develop into fully immunocompetent mature B cells and are exported from the bone marrow into the lymphatic circulation. Once in the circulation B cells undergo further development steps (activation, proliferation and

differentiation) that require antigen and lead to antigen driven B cell activation and clonal selection (Ig production). B cells produce Ig in a range of classes; IgG, IgM, IgA, IgE and IgD that are distinguishable by the heavy chain they contain (Butler, 1969; Mantis*, et al.*, 2011).

1.3.2 Basic immunoglobulin structure and function

The basic Ig structure consists of four polypeptide chains comprising of two heavy chains (~50 kDa) and two light chains (25 kDa) forming a Y-shaped glycoprotein that has a total molecular weight of ~150 kDa (Butler, 1969; Riera *et al.*, 2008). The two chains are linked together through disulphide bonds (Corthesy, 2013b). The end of a heavy and light chain region, 100 to 130 AA long, forms a variable region (V). The variable region is responsible for binding specificity to a variety of antigens. There are also carboxyl-terminal sequences located on the light and heavy chains also known as the constant region (C) (Riera*, et al.*, 2008). Light chains contain one variable domain and one constant domain, heavy chains contain one variable domain and up to four constant domains, depending on the antibody class (Figure 1). The structure of an Ig can be broken down by enzyme cleavage into two fragments termed fragment antigen binding (Fab) and fragment constant (Fc) (Hurley & Theil, 2011). These fragments have different activities; the Fab region is the specific antigen binding site responsible for interacting with the target antigen. The Fc region is comprised of constant amino acids and carries out effector functions once Ig has bound to the antigen; the Fc region has no antigen binding ability (Riera*, et al.*, 2008).

There are a variety of effector functions which include; inhibiting antigen from binding to host cellular receptors and promoting their removal from the blood by releasing pro-inflammatory mediators to activate the complement system (Riera*, et al.*, 2008). Also, an important mechanism is antibody dependent cell mediated toxicity (ADCC). This is a mechanism of cell-mediated immune defence whereby an effector cell of the immune system actively lyses a target cell, whose membrane-surface antigens have been bound by specific antibodies. The Fc regions promote opsonisation of the antigenic particles by phagocytic cells such as macrophages, neutrophils, and eosinophils (Riera*, et al.*, 2008).

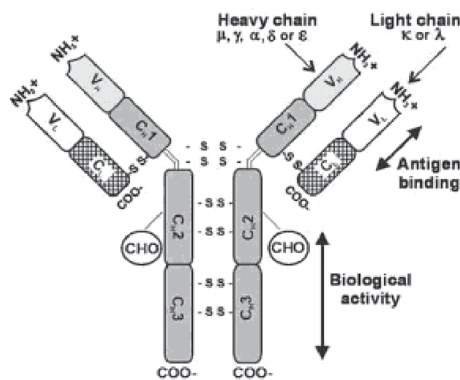

Figure 1 Structure of an immunoglobulin (Ig) molecule.

The Ig molecules are composed of two different polypeptide chains joined by disulphide bridges. The amino-terminal domains of both chains are variable in sequence and bind the antigen. The remaining domains of both chains are constant and are involved in the biological activity. The CH region is further divided into three distinct domains CH1, CH2, and CH3. Diagram from Riera et al., (2008).

1.3.3 Immunoglobulin in serum and milk

In the blood and body fluid of humans, IgG comprises 75-80% of total antibodies. IgG is the principal antibody that mediates protection against bacterial and viral infections (reviewed in Kaur et al., 2012). IgA comprises 10-15% of total antibodies in blood and is predominantly in mucosal surfaces such as the urogenital tract, eyes, respiratory tract, and digestive tract (Woof & Kerr, 2004; Kaur *et al.*, 2012). IgM, IgD, and IgE are also present; however, they will not be discussed in detail as they are not the antibodies of interest in the thesis. Immunoglobulin classes IgA, IgG, IgE, and IgM have all been identified in milk, entering from blood serum (Butler, 1969). SIgA and IgG are of particular interest due to the relative concentrations present in the cow and human milk. As seen in Table 1, human milk has a SIgA concentration of 1.00 mg/mL which corresponds to 87% of the total Igs. Whereas bovine milk has a lower SIgA concentration of 0.14 mg/mL which corresponds to only 18% of the total Igs (Butler, 1969; Haneberg, 1974). In contrast, the main Ig in bovine milk is IgG, at approximately 0.61 mg/mL, corresponding to 75% of the total Igs. In human milk IgG levels are much lower at 0.04 mg/mL corresponding to only 3% of total Igs (Butler, 1969; Haneberg, 1974). Therefore, immune composition in bovine milk is currently not suited to the immunological needs of human

infants. Due to the abundance of IgG in bovine milk, this antibody has been studied extensively in terms of structure, composition, functions, milk processing, digestion, and relevance to humans. In comparison, BSIgA has been overlooked. Human mucosal sub-epithelium cells intensively produce SIgA reflecting the critical requirement for immune protection of mucosal cells. The mucosal surfaces also represent a large surface area and point of contact between the immune system and the possible exposure to invading pathogens (Brandtzaeg & Johansen, 2007). Manipulating the concentration of bovine milk to suit human consumption could lead to great mucosal health benefits which will be explored in further detail, in this chapter.

Table 1 Concentration (mg/mL) and percentage (%) of immunoglobulin G and SIgA in bovine and human colostrum and milk. Adapted from Butler (1973) and Haneberg, (1974).

		Concentration, mg/mL		**% of total immunoglobulins**	
Species	Immunoglobulin	Colostrum	Milk	Colostrum	Milk
Bovine	IgG_1	47.60	0.59	81.0	73.0
	IgG_2	2.90	0.02	5.0	2.5
	SIgA	3.90	0.14	7.0	18.0
Human	IgG	0.43	0.04	2	3
	SIgA	17.35	1.00	90	87

1.4 Structure and function of Secretory Immunoglobulin A

There are several forms of IgA; monomeric, dimeric, and SIgA (Della Corte & Parkhouse, 1973). The IgA in human serum is predominately monomeric (Woof & Russell, 2011). IgA in tissues is a dimer consisting of two Ig units connected tail to tail at the heavy chain regions (Fc) and covalently bound by a small glycoprotein known as the J chain, a 15 kDa polypeptide (Johansen *et al.*, 2000). Dimeric IgA is transported into mucosal secretions by a receptor, called Polymeric immunoglobulin receptor (pIgR), that is expressed on the basolateral surface of epithelial cells (Cakebread *et al.*, 2015). At the apical membrane, IgA is released into the mucosal lumen along, with a portion of pIgR, termed secretory component (SC) bound to the Fc portion of the IgA molecule (Figure 2); this complex is SIgA. The J chain is essential for SIgA formation as it is a requirement for polymerisation of IgA and promotes IgA's affinity for pIgR (Johansen *et al.*, 2007).

Polymeric immunoglobulin receptor expression is regulated by a variety of host and microbial factors. The production of pIgR is crucial for SIgA mediated mucosal defence thus, pro-inflammatory cytokines, as well as recognition of microbials such as LPS, E.coli, and retrovirus, upregulate pIgR production. (Kaetzel & Bruno, 2007).

Figure 2 The structure of SIgA showing the heavy and light chains, the antigen binding sites (Fab), effector (Fc) regions, the hinge region, and glycosylation sites (glycans). Secretory component binds the dimerized SIgA molecule, composed of two monomeric IgA molecules that are joined by the J chain. Diagram from (Cakebread*, et al.*, 2015).

1.4.1 Glycosylation of SIgA

There are two types of protein glycosylation commonly found on milk proteins; O-linked, where the glycan chain is covalently attached to the hydroxyl oxygen of a threonine or serine residue; and N-linked, where the glycan chain is covalently linked via an N-acetyl glucosamine molecule to the amide side chain of an asparagine residue (O'Riordan *et al.*, 2014). In Igs, glycans are in the constant region of the heavy chain, these regions allow interactions of the Fc region. Glycans are also present on the Fab regions of SIgA (refer to Figure 2) (Yoo & Morrison, 2005). N-linked carbohydrates make up 6-7% of total mass of SIgA.

The SC stabilises the quaternary structure of the molecule and acts to protect SIgA against proteolytic degradation (Cakebread*, et al.*, 2015). The SC of SIgA contains between 15 and 24% of glycans. The glycans protect the Ig from digestion by proteolytic enzymes trypsin and pepsin, by increasing the structures stability or through unspecific steric hindrance protecting some bonds from enzyme cleavage which in the absence of SC are sensitive to proteolysis (Boutrou *et al.*, 2013; Zhang *et al.*, 2013). Therefore, SC allows a partially or completely

intact Ig to reach the intestinal tract which is needed in order to obtain mucosal benefits (Section 1.4.2) (Lindh, 1975; Mantis*, et al.*, 2011).

In addition to stability, the carbohydrate components mediated adherence to host and pathogen which is important for the biological function of the pIgR/SC complex. Mutation of the N-glycan chain of the pIgR/SC reduces the complex's binding ability to pathogens (Kaetzel & Bruno, 2007) as demonstrated by the interaction to Gram-positive bacteria, where removal of the glycans resulted in a dramatic drop of interaction with the bacteria (Mantis*, et al.*, 2011). This binding capacity is independent of the antibody variable region. The specific composition of the N-glycans influences the binding of SC and SIgA to lectins (Mantis*, et al.*, 2011). These observations emphasise the importance of the sugar-mediated non-specific component of SIgA. There are few studies focusing on the glycosylation of bovine Igs in relation to their digestion, or to their bioactivity (O'Riordan*, et al.*, 2014). To gain a better understanding of BSIgA in milk, scientists should look at whether digestion of SIgA impacts glycosylation activities.

1.4.2 Function of SIgA

Secretory IgA is the form of IgA that is found in human colostrum and milk (Butler, 1969). The intestinal immune system must protect the GIT by preventing invasion of pathogens but also be able to recognise harmless commensal bacteria. SIgA protects the GIT against enteric toxins and pathogenic microorganisms through a process called immune exclusion (Mantis*, et al.*, 2011). SIgA anchors to the mucosal lining of the epithelium, thereby, decreasing the bacterial binding access (Cakebread*, et al.*, 2015). SIgA prevents pathogenic microorganisms from entering the intestinal lumen by blocking their access to epithelial receptors and trapping them in mucus to facilitate their expulsion through peristalsis in the GIT. SIgA has the ability to extinguish bacterial virulence factors, for example, by binding to O- antigens of *V. cholerae*, *S. Typhimurium* and *S. flexneri* (Mantis*, et al.*, 2011). This is seen in human milk SIgA where the concentration is high enough to inhibit the binding of *clostridium difficile* toxin membrane receptors and, therefore, prevents infection (Mantis*, et al.*, 2011).

SIgA is a non-inflammatory antibody and is important in down regulating proinflammatory responses by binding to pathogenic bacteria and allergenic antigens that are normally associated with proinflammatory responses (Mantis*, et al.*, 2011). Immune tolerance of microorganisms that are coated with SIgA results in down regulation of proinflammatory NF-kb cytokine production but maintains secretion of regulatory IL-10. This promotes normal function of the intestinal barrier and has anti-inflammatory effects (Yoo & Morrison, 2005).

SIgA is also effective in neutralising antigens such as influenza virus (Kikuchi *et al.*, 2014). Specific IgA responses also lead to upregulation of costimulatory molecules of T cells, preferential class switching to IgA, and stimulate long-lived protective immunity (Mantis*, et al.*, 2011).

SIgA influences the composition of intestinal microbiota: the Fab region of SIgA binds to epitopes on microbial antigens competitively inhibiting pathogens preventing them from binding to the host cell. There is evidence to suggest that SIgA is involved in colonisation and selection of tolerance of new born mucosal immune system towards antigens associated with microbial symbiotic partners (Hanson et al., 2005). When the neonate is exposed to commensal microorganism, maternal SIgA has the capacity to bind to them and promote their uptake through mast cells. This shapes the SIgA in the gut with limited affinity and abundant epitopes in the gut (Mantis*, et al.*, 2011). In SIgA knock out mice there is reduced protection against infection with influenza A and B compared to wild type mice (Brandtzaeg & Johansen, 2007). A deficiency in SIgA leads to a higher frequency of gastrointestinal diseases such as inflammatory bowel disease, allergy, and celiac disease, further supporting SIgA value in preventing many intestinal infections. This emphasises the importance SIgA has in the protection against infection or diseases and homeostasis in the GIT of a neonate, infant, or adult (Cunningham-Rundles, 2001; Brandtzaeg & Johansen, 2007; Geuking *et al.*, 2012). Thus, SIgA is important for mucosal homeostasis.

1.5 Importance of SIgA in milk

In humans, IgA is important for the transfer of passive immunity from the mother to the neonate through the consumption of milk (Mehra *et al.*, 2006). SIgA is produced locally within the mammary tissue by plasma cells (Macpherson *et al.*, 2001). Igs are transported through mammary epithelia into the milk by receptor mediated processes, (Macpherson*, et al.*, 2001), then out of the mammary gland to neonate via suckling. The GIT comprises the stomach and intestines which are responsible for transporting, digesting foodstuffs, absorbing nutrients, and expelling waste. The GIT is a high risk area for invasion of pathogenic bacteria. Human neonates have an immature immune system. SIgA from milk enters the GIT to provide a protective benefit against pathogenic bacteria to the offspring as mentioned in Section 1.4.2 (Hurley & Theil, 2011). Purified SIgA from human milk is effective to stimulate mucosal protection against invasion by M6 streptococci and poliovirus (Takahashí *et al.*, 1998). Thus, SIgA in milk helps to ensure passive immunity to the offspring.

1.6 Relevance of bovine SIgA to humans

Bovine SIgA can successfully provide immune protection to mucosal surfaces through the GIT of humans (Cunningham-Rundles, 2001; Brandtzaeg & Johansen, 2007). SIgA can be targeted to specific diseases (Hurley & Theil, 2011) such as rotavirus to protect against inflammation of the intestine (Hilpert *et al.*, 1987; Pacyna *et al.*, 2001). Recent information reveals that bovine SIgA and human SIgA interact with bacteria commonly found in the human GIT in a comparable way. Thereby, suggesting that SIgA bacterial binding is not species specific and that bovine SIgA has potential benefit to providing immune exclusion in the GIT of humans (Hodgkinson *et al.*, 2017). There is great interest in therapeutic benefits of bovine milk particularly investigating the function of SIgA (Wheeler *et al.*, 2007).

1.7 Endogenous SIgA

The information presented in Section 1.4 helps to highlight the vital role exogenous SIgA could have within the immune system. The levels of SIgA at mucosal effector sites in a host can be increased by two approaches, including the induction of bacteria such as lactic acid bacteria and the provision of exogenous SIgA (Takahashí*, et al.*, 1998). As mentioned in Section 1.6, exogenous SIgA can protect against intestinal infection. Due to the potential immune response SIgA could elicit, it is important to investigate the digestion of exogenous SIgA and impacts it has to the endogenous immune production or degradation of SIgA. A preliminary study of this research looked at the survival of BSIgA through the GIT of mice by feeding bovine skim milk and milk with high-IgA and low-IgA content over a period of 21 days. The levels of exogenous BSIgA and endogenous MSIgA were measured in the faecal pellets from the mice. Bovine SIgA was detected in the faecal pellets that reflected the levels of BSIgA in the feeding regimes. Conversely, the highest amount of MSIgA was observed in the water-fed group and the lowest in the high-IgA fed group. However, this does not form conclusive evidence that BSIgA impacts the levels of endogenous MSIgA as it is only one study, using one mouse breed. There is limited information examining how bovine SIgA impacts the levels of endogenous SIgA as the primary research focus has been on the interaction of SIgA to antigens. Therefore, the results from this research will help to determine if exogenous bovine SIgA does impact endogenous SIgA production or degradation.

1.8 The Digestive System

The GIT breaks down ingested food and liquids by biochemical and mechanical processes, to a form that allows nutrients to be absorbed. The processes of digestion can be broken down into four main parts (Boland, 2016):

1) Oral processing
2) Gastric processing
3) Intestinal processing
4) Fermentation

Proteins, carbohydrates, and lipids are the precept macronutrients essential for nutritional homeostasis in humans (Erickson & Kim, 1990). All three macronutrients are subject to a complex interplay of degradation processes that are promoted by enzymatic and physical processes in the GIT (Erickson & Kim, 1990). Proteins are digested by specific proteolytic enzymes present in gastric juice, pancreatic secretions, and intestinal brush border enzymes. Physical processes that contribute to mixing and movement of the digesta, complement enzymatic actions, allowing greater surface area access. Without the physical and enzymatic breakdown of intact protein, the absorption would not be possible (Lentle & Janssen, 2008). The acidic fluid which passes from the stomach to the small intestine (chyme), consists of gastric juices and partly digested food. Enzymes in chyme break down proteins into amino acids (AA) and oligopeptides of up to 2-6 AA residues (Silk *et al.*, 1985).

In the native conformation, proteins are relatively resistant to the action of proteases. This is due to their secondary and tertiary structures being stabilised by covalent bonds; such as disulphide bridges and non-covalent forces; such as hydrogen bonds, ionic interactions, and Van Der Waals forces. For protein digestion to be efficient, peptide bonds need to be accessible. Proteins with complex structures, such as SIgA, to some extent can resist protein digestion. To understand the transit and digestion of SIgA, it is important to understand the digestive processes SIgA is subjected to.

This section will explain the physical and enzymatic processes of protein digestion in the stomach, small intestine, caecum and colon of the mature mammalian gut. Absorption is an important part of digestion, but it is not relevant to the transit and digestion work of SIgA in this study, thus, it will not be reviewed. This section will also give a general overview of the digestion of milk proteins with particular emphasis on SIgA, as this is the subject of the research.

1.9 Physical digestion of proteins in the stomach

The role of the stomach is to store, mix and digest food and to transport it into the small intestine (Lentle & Janssen, 2011c). The stomach consists of the fundus, corpus, and antrum (Figure 3A). The fundus and proximal corpus act as a reservoir, whereas mixing and emptying occurs in the antrum and distal corpus. Peristaltic contractions begin at the corpus and propagate to the pylorus (Figure 3B). Retropulsive flow is generated as the peristaltic contraction wave height increases near the pylorus, decreasing the space between the contraction wave and the pyloric valve. The maximum velocity also increases when the contraction reaches the pylorus and is sustained until the flow reaches the proximal region of the antrum. Thus, the chyme inside the decreasing space is forced into the proximal part of the stomach in a jet like movement, resulting in local mixing efficiency peaking near the pylorus (Miyagawa *et al.*, 2016). Particles moving from the fundus towards the antrum undergo negligible mixing (Pal *et al.*, 2004).

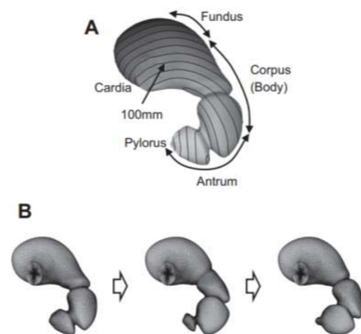

Figure 3 Stomach geometry. The contour lines in A) show the distance from the pylorus at 10mm intervals). B) Shows a systematic image of peristaltic contractions. Retrieved from Imai et al., (2013).

Antral recirculation (a jet-like retropulsive flow) expands the region responsible for gastric mixing and the content is moved out by the forward flow. Retropulsive flow near the pylorus then mixes the content longitudinally, and the antral recirculation transports the content laterally toward the antral wall, creating an instantaneous mixing well (Figure 4). Images C and D in Figure 4 show turbulent flow (Miyagawa*, et al.*, 2016). Retropulsive flow contributes to the dispersion of oil droplets and other solid particles (Lentle & Janssen, 2011b).

Figure 4 Paths of tracer particles in the stomach. Images A and B show antral recirculation at Reynolds number (Re) = 0.1 and images C and D show turbulent flow at Re = 30. Images C and D show greater force due to an increased Re. Retrieved from Miyagawa et al., (2016).

1.9.1 Enzymatic digestion in the stomach

Bits of ingested foods, moistened in the mouth, are swallowed and become mixed with gastric juice in the stomach (Adibi & Mercer, 1973). The components of gastric juice are pepsins, gastric lipase, mucus, urea, intrinsic factor (glycoprotein), H^{+}, Cl^{-}, Mg^{2+}, Na^{-}, K^{+}, H20 and HPO_4^{2-}. Proteolysis in the stomach is initiated by pepsin and hydrochloric (HCL) acid secreted by specialised cells in the stomach (Erickson & Kim, 1990).

There are two types of enzymes: 1) endopeptidases which attack specific peptide bonds within the molecule and 2) exopeptidases which attack certain peptide bonds at the carboxyl terminal end of the molecule. Pepsin is an endopeptidase which attacks proteins preferentially at the peptide linkages formed between L-dicarboxylic acids and L-aromatic amino acids, hydrophobic residues. The acidic condition of the stomach varies before, during, and after meals. The pH rises from a range of 2.0-2.5 (optimum pH of pepsin) before meals (when the stomach is empty), to 4.5-5.8 during and immediately after consumption of a meal. After consumption of milk, the pH increases to greater than 6, thus limiting the activity of pepsin (Ye et al., 2016). As digestive time increases, the pH reverts back to 2.0-2.5. The acidic conditions (pH 2) assists in denaturing native proteins and altering their conformation as mentioned earlier (Adibi & Mercer, 1973; Silk*, et al.*, 1985). Thus, matrices with differential pH buffering capacity may have the ability to withhold the acidic conditions of the stomach and pepsin hydrolysis. Hence, more BSIgA can survive gastric digestion.

1.9.2 Gastric digestion of IgA and milk proteins

Studies have shown, SIgA molecules to be relatively resistant to pepsin digestion (Shuster, 1971; Newby & Bourne, 1976), more so than other IgA forms and Ig subclasses (Newby & Bourne, 1976; Stelwagen *et al.*, 2009). *In vitro* pepsin incubation of human colostrum SIgA at a

pH of 4.5 reveals that SIgA is more resistant to digestion than monoclonal IgA (Wilson & Williams Jr, 1969; Shuster, 1971). Furthermore, SIgA resists pepsin digestion for an extended time; after 1 hour of digestion, 80% of SIgA was detected after incubation and remained relatively constant dropping to around 70% of SIgA after 4 hours of incubation, and 60% of SIgA after 24 hours of incubation (Wilson & Williams Jr, 1969; Shuster, 1971). Elution peaks from chromatography graphs after 3 hours of pepsin incubation revealed that SIgA only eluted 2 peaks. One peak corresponding to the native protein peak and the second to a low molecular weight peptide, indicating that pepsin does degrade SIgA into peptides (Shuster, 1971).

It has been reported that SIgA antibody is relatively sensitive to pepsin at lower pH levels of 2.5 (McClelland *et al.*, 1972). Furthermore, the binding activity of SIgA remains after gastric digestion (Steward, 1971). Equally, McClelland et al., (1972) stated that the biological activity of SIgA is retained in the neonatal GIT due to the high pH but is not detectable in the normal acidic stomach at pH 2.5, despite undigested SIgA reaching the small intestine (McClelland*, et al.*, 1972). In order for SIgA to have a function beyond gastric digestion, adequate intra-gastric neutralisation is needed to increase the pH thus limit the activity of pepsin (Wilson & Williams Jr, 1969; McClelland *et al.*, 1971; McClelland*, et al.*, 1972). This data leads to the supposition that if different milk matrices have differential or long lasting pH effects in the stomach, this will not only affect the survival rate of BSIgA but also the biological activity further in the GIT (Corthesy, 2013a).

Human studies show that the flow of digesta changes depending on its physical state. Liquids are rapidly dispersed through the stomach and can be retained for as short as one minute (Lentle & Janssen, 2011c). In comparison, solid food can be retained in the mammalian stomach for as long as 95 minutes (Lentle & Janssen, 2011c). However, hormonal and osmotic effects of lipids, carbohydrates, and proteins also have an effect on the gastric emptying rate. Therefore, gastric half-emptying time of liquid phase is longer for milk than water. Whey proteins remain soluble in the stomach and empty rapidly, thus, there is less time for whey proteins to be subjected to proteolysis by pepsin and, therefore, enter the duodenum partially intact with the liquid phase (Mahe *et al.*, 1992). In comparison, casein coagulates and forms structured clots at low pH causing delayed gastric emptying with most of the proteins in the form of degraded products (Mahe*, et al.*, 1992). The structured clot formation is due to pepsin digesting kappa-casein (k-casein), thereby, destroying the protective effect k-casein has on the casein micelle (Ye *et al.*, 2011). When intact, micelles structures have a protective effect to whey proteins, helping to stabilise their structure (Ye*, et al.*, 2011).

The stomach is a potentially harsh environment for proteins as acidic conditions and activity of enzyme pepsin leads to their digestion. Therefore, SIgA unique structural stability due to a high degree of glycosylation and hydrophobic amino acids being buried inside the hydrophobic core in conjunction with delivery in an alkaline solution helps to protect SIgA from gastric digestion (Boutrou*, et al.*, 2013). Thus, SIgA can transit into the small intestine intact. Further protective effects may be obtained by the milk matrix and this is yet to be explored.

1.10 Protein Digestion in the small intestine

The small intestine can be subdivided into the duodenum, jejunum, and ileum. The partially digested food (chyme) arrives in the duodenum at acidic pH from 4-5. The pH rises rapidly to 7-8 due to pancreatic and duodenal bicarbonate secretions from the pancreas (Adibi & Mercer, 1973; Roos *et al.*, 1995). Pancreatic secretion also contains bile and four types of proteolytic zymogens; trypsinogen, procarboxypeptidase, chymotrypsinogen, and proelastase (refer to Section 1.10.2).

1.10.1 Physical digestion of proteins in the intestines

The contractile activity in the small intestine consists of phasic and tonic that either act alone or simultaneously to produce three types of contractions; segmentation, pendular, and peristalsis contractions (Lentle & Janssen, 2011a). These contractile activities cause fundamental mobility responsible for the mixing of chyme with digestive enzymes and propel contents distally (Lentle & Janssen, 2011a). Contractions in the GIT differ in the fasted and fed state being rhythmic in the fed state due to the influence of hormones and dietary components (Lentle & Janssen, 2011a). Some peptides such as opioid are known to reduce the contractile activity and transit rate through the GIT (Daniel *et al.*, 1990). For example, caseins which releases opioid peptides during digestion transits through the GIT of rats slower than whey proteins (Daniel*, et al.*, 1990). Thus differences in milk matrices may have a direct effect on contractions in the intestine and hence affect physical digestion and transit rate through the GIT.

Segmentation is the predominant contraction when the bolus enters the small intestine. It consists of rhythmic stationary contraction of the circular muscle layer which alternates in segments along the long axis of the small intestine (Lentle & Janssen, 2011a). Segmentation induces symmetrical vertical flow where after displacement the digesta does not return to the ordinal place, it mixes with the adjacent material (Figure 5) (Thuneberg & Peters, 2001).

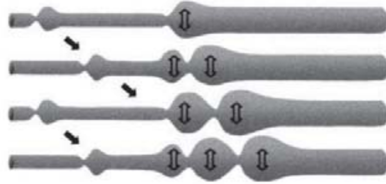

Figure 5 Segmentation contractions moving distal of the small intestine. Diagram from intestinal contents semi-solid. The arrows show symmetrical vertical flow where the chyme is mixed with the adjacent material. Retrieved from Thuneberg and Peters, (2001).

Pendular contraction differs to segmentation where it is brought about by longitudinal contraction providing asymmetrical mixing creating to and fro movements of the digesta (Figure 6) (Lentle & Janssen, 2011a).

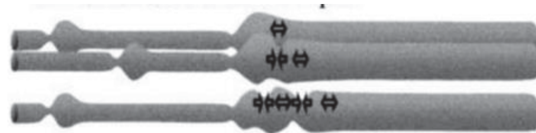

Figure 6 Pendular contraction from longitudinal muscles of the small intestine. Diagram from intestinal contents semi-solid. The movements create backwards and forward movement of the chyme assisting with the mixing of digesta. Retrieved from Thuneberg and Peters, (2001).

Peristalsis is the most commonly described contraction, a high amplitude propulsion contraction in the fed state (Figure 7) (Lentle & Janssen, 2011a). Peristaltic contractions involve both circular and longitudinal smooth muscle and can occlude the lumen which includes flow as high pressure is formed in front of the digesta and low pressure in the rear of contraction. The contractions progress longitudinally causing asymmetric movement as the leading shoulder is at a different rate to the trailing shoulder (Lentle & Janssen, 2011a). Pressure from peristalsis prevents retrograde motion ensuring distal movement of the chyme (Lentle & Janssen, 2011a).

Figure 7 Peristalsis movement in the small intestine moving the chyme distally. Retrieved from Thuneberg and Peters, (2001).

1.10.2 Pancreatic enzymatic digestion

One of the functions of the pancreas is to produce and deliver digestive enzymes to the small intestine for the hydrolysis of complex nutrients (Whitcomb & Lowe, 2007). Nearly 80% of pancreatic enzymes are proteases, needed for the digestion/hydrolysis of the complexity of dietary proteins (Whitcomb & Lowe, 2007). These enzymes, trypsin, elastase, chymotrypsin, and carboxypeptidases A and B enter the duodenum in their inactive precursors (zymogens) and are activated in the GIT (Erickson & Kim, 1990).

Trypsin, elastase, and chymotrypsin are endopeptidases from the serine protease family that hydrolyse nonterminal AA peptide bonds (CO-NH) and release oligopeptides (Figure 8). Trypsin hydrolyses peptide bonds on the carboxyl side at the site of basic AA, lysine and arginine only. Elastase hydrolyses the protein backbone at bonds that have uncharged small AA (i.e. alanine, glycine, valine, leucine, isoleucine and serine). Chymotrypsin hydrolyses peptide bonds on the carboxyl side of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan (Figure 8) (Whitcomb & Lowe, 2007).

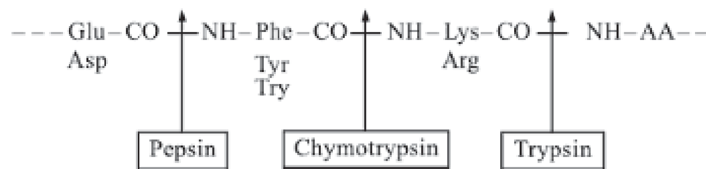

Figure 8 Preferential specificities of pepsin, trypsin, and chymotrypsin for peptide linkages. Retrieved from Whitecomb and Lowe, (2007).

In contrast, exopeptidase enzymes carboxypeptidases A and B hydrolyse peptide bonds between AA at the carboxy terminal end to release single AA. Carboxypeptidase A cleaves the neutral and acidic aliphatic and aromatic AA (phenylalanine, tyrosine, and tryptophan) from the carboxyl terminal and carboxypeptidase B is similar except it cleaves the basic AA (arginine and lysine) from the carboxyl terminal. The combination of these enzymes have the ability to break down different protein molecules allowing for a wide range of dietary proteins to be digested into AA, and oligopeptides if digested from endopeptidase (Whitcomb & Lowe, 2007). The tri and dipeptides are further hydrolysed by brush border (Section 1.10.4) enzymes before being absorbed as AA, dipeptides, and tripeptides through the mucosal wall. When SIgA is in its native form it is capable of resisting digestion of these enzymes as the SC provides steric hindrance inhibiting the enzymes access to their required AA sequences (Steward, 1971; Lindh, 1975; Mantis*, et al.*, 2011). Conformational changes occur during unfavourable processing or digestive conditions such as high temperatures or acidic pH changes which assist with

enzymatic digestion (Li-Chan *et al.*, 1995). Therefore, if SIgA peptide bonds can be protected in a matrix to block SIgA enzymatic digestive sites, then it may help SIgA resist digestion.

1.10.3 Bile

Bile is known to aid digestion of fats and solubilise dietary lipids into mixed micelles to promote their absorption (Gass *et al.*, 2007). Pancreatic and brush border enzymes need to be in physical contact with proteins in order to break them down. Recently it has been found that in the absence of fatty acids and monoglycerides, bile enhances the proteolysis of several dietary proteins by displacing proteins from oil-water interfaces in emulsions. Some of these proteins are bovine serum albumin, myoglobin, β-lactoglobulin, and a commercially available dietary protein supplement (Gass*, et al.*, 2007). To date, there is no known data on as above SIgA and whether the delivery of SIgA in different milk matrices such as full fat milk or skim milk would impact the activity of bile. On the contrary, studies demonstrate the participation of the liver in the secretion of endogenous polymeric IgA and SIgA in the mice, rat, and human (Nagura *et al.*, 1981). The hepatic bile transports circulating SIgA into the upper GIT. Furthermore, it is suggested that in the rat and human bile, SIgA might contribute to immune defences of the gut and biliary tract (Nagura*, et al.*, 1981). Thus, it can be assumed that bile does not assist with the digestion of SIgA and will not be discussed further.

1.10.4 Brush border enzymes

The proteins are broken down by a variety of brush border digestive enzymes (Adibi & Mercer, 1973). The enterocytes of the small intestine produce brush border enzymes comprising of aminopeptidases, endopeptidases, exopeptidases, carboxypeptidases, and di-, tri- and tetra peptidases (Whitcomb & Lowe, 2007). These enzymes are the last essential stage of protein digestion before absorption and are most effective in digesting peptides after initial hydrolysis by gastric and pancreatic enzymes (Whitcomb & Lowe, 2007).

The microvilli are known to increase the surface area of the small intestine, but recent evidence has discovered that another function is as a launching pad for brush border digestive enzymes (Hooton *et al.*, 2015). The cytoskeleton of the microvillus has a motor element displacing the apical membrane towards the apex of the microvillus. At this point, it forms a vesicle and launches into the periapical space. During this process the brush border enzymes remain incorporated in the membranes of these vesicles, therefore brush border digestion has moved from the surface of enterocytes to the periapical space. They can then, bound to brush border membrane vesicles transit to all parts of the lumen either in this form or be released by the action of biliopancreatic secretions (Hooton*, et al.*, 2015). The overall process results in

significant nutrient hydrolysis adjacent to the membrane in a pre-absorptive step (Hooton*, et al.*, 2015). Protein digestion may also be carried out by enzymes synthesised by gut microorganisms, microsomal enzymes (Hooton*, et al.*, 2015). However, as there is a low amount of bacteria in the small intestine compared to the large intestine and because bile acts as a detergent in the duodenum and jejunum, it is likely that microbial enzyme digestion is small (Hooton*, et al.*, 2015). Overall brush border enzymes complement gastric and pancreatic enzymatic actions to reduce a large variety of macro-nutrient oligomers to monomers (Hooton*, et al.*, 2015).

1.10.5 Intestinal IgA and milk protein digestion

As established, the small intestine is a protease rich environment equipped with a combination of physical and chemical digestive factors to maximise protein digestion. Bovine SIgA has the structural capability to enter the small intestine as an intact protein and further transit the GIT. BSIgA is more resistant to digestion than other milk proteins (Yvon *et al.*, 1993; Roos*, et al.*, 1995). Secretory IgA show considerably greater resistance to proteolytic enzymes, trypsin and chymotrypsin, digestion than the monomeric and dimeric IgA isoforms which are found in the blood and do not contain secretory components (Lindh, 1975; Hilpert*, et al.*, 1987). *In vitro* incubation of bovine Igs in pronase for 2 hours resulted in 100% recovery of SIgA compared to 73% of IgG_2 and 86% of IgG_2 (Newby & Bourne, 1976). The resistance of SIgA to proteolytic degradation is correlated to the amount of SC. Removal of the SC results in a decreased resistance of SIgA to proteolytic degradation (Lindh, 1975; Crottet & Corthésy, 1998). *In vitro* studies are also supported by *in vivo* studies where SIgA has been identified in the faeces of humans, indicating an intact structure post digestion (Haneberg, 1974).

In vitro digestion of dimeric IgA and western blot analysis revealed that the α-chain is split into smaller 40 kDa fragments and that digestion begins in the J chain region (Crottet & Corthésy, 1998). For SIgA, however, this degradation is delayed and the k light chain is not digested at all. This suggests that the Fab binding region is left intact. Analysis of SC alone shows that it is not resistant to digestion (Crottet & Corthésy, 1998). SC maintains the integrity of the dimeric structure by delaying the cleavage in the hinge/Fc region, thereby, increasing the resistance of the α-chain to enzyme digestion (Newby & Bourne, 1976; Crottet & Corthésy, 1998). Therefore, it is proposed that SC attached to SIgA supports its survival through digestive processes by increasing the stability of the backbone through an increased resistance to both trypsin and pepsin (Lindh, 1975) and stabilising the quaternary structure (Ben Mkaddem *et al.*, 2013). There is strong evidence demonstrating that SC is involved in protecting the IgA molecule from digestion. Although, the increased resistance may be from unspecific steric

hindrance which protects some peptide bonds that without SC would be sensitive to proteolysis (Lindh, 1975).

It is important to note that SIgA is also resistant to sialidase digestion, therefore, the glycoproteins remain intact and non-specific binding to antigens may then remain after digestion (Crottet & Corthésy, 1998). Also, when digestion of SIgA is achieved, a portion of Ig variable and constant regions remain assembled. More importantly, the antigen-binding activity and agglutinating activity of SIgA remains after digestion (Steward, 1971; McClelland, *et al.*, 1972; Crottet & Corthésy, 1998).

There are numerous *in vitro* digestive studies of Igs, however these simulated conditions do not fully represent the environment of the digestive tract. Furthermore, *in vitro* duodenal digestion methods of milk proteins have resulted in two different peptide patterns (Picariello *et al.*, 2015). Thus, simulated digestion conditions affect peptide patterns and *in vivo* models may be more informative.

1.11 Large Intestine

The large intestine comprises the colon and caecum (Williams *et al.*, 2001). Despite the small intestine being very effective at nutrient digestion and absorption, there is a constant supply of undigested dietary components to the large intestine. The most commonly known function of the large intestine is to absorb water and electrolytes. Although recently, the diverse population of commensal bacteria has been shown to play an important role in health of an individual as well as contributing to the fermentation of carbohydrates and proteins (Williams, *et al.*, 2001). The physical digestion and micro-organism within the colon in relation to SIgA will be discussed further (Section 1.11.3).

1.11.1 Physical digestion

The large intestine is haustrated, meaning there are small pouches due to teniae coli causing sac formation, so consequently the colon has a segmented appearance. The viscosity of digesta in the distal colon is greater than the small intestine (Lentle & Janssen, 2011c). There are four types of contractile activity in the colon, two for moving the contents and two for mixing the contents; mass peristalsis, haustral progression, fast phasic contractions, and ripples, respectively. These contractions facilitate the extraction of water, nutrients, and electrolytes (Lueamsaisuk *et al.*, 2015).

Movement in the colon progresses by mass peristalsis that causes high pressure in the colonic lumen and occurs irregularly (Lentle & Janssen, 2011c). Mass peristalsis in the colon has a

longer duration and occurs more slowly compared to peristalsis in the small intestine (Lentle & Janssen, 2011c). Thus, the transit of the bolus is slow in the large intestine.

In the absence of the mixing haustral contractions, ripples are coordinated movements that may cause movement of watery fluid towards the mouth. However, in the presence of haustra, ripples propagate at varying rates and are uncoordinated across intertaenial domains. This movement mixes the contents of the lumen along the length of the colon (Lueamsaisuk*, et al.*, 2015). In the presence of watery probes, ripples cause the contents to be propelled into the diametrically opposite intertaenial domain of a haustrum (Lueamsaisuk*, et al.*, 2015). Thus, mixing the contents and moving them into contact with microorganisms and the luminal wall for absorption.

1.11.2 Microorganisms in the colon

The principal area of microorganisms colonisation is in the lower ileum and large intestine (Cummings & Macfarlane, 1991). These microorganisms are responsible for fermentation of carbohydrates and proteins that have resisted gastric, pancreatic, and small intestine digestion (Cummings & Macfarlane, 1991). Micro-organism can use both O-linked and N-linked glycans found in host mucous secretions or shed epithelial cells as substrates (Cummings & Macfarlane, 1991). Which also means they have the potential to use the glycans on BSIgA as substrate. Bacterial enzymes have proteolytic activity and can modify nitrogenous compounds by deamination and decarboxylation of AA (Rowan, 1989). Microorganisms can use products of the large intestine for the synthesis of their own proteins and cellular components (Cummings & Macfarlane, 1991).

There is a delicate balance between beneficial commensals bacteria and pathogenic bacteria in the GIT. The commensal bacteria population in a mature GIT is relatively constant. Although, other substances including dietary components can stimulate the microorganisms in the large intestine and impact the immune system which in turn can help to inhibit potential pathogenic species (Williams*, et al.*, 2001).

1.11.3 SIgA in the large intestine

Studies focusing on SIgA digestion in the large intestine are lacking in the literature. Instead, the focus has been on overall SIgA recovery in faecal samples. Both endogenous and exogenous SIgA has an important function to maintain the symbiotic commensal bacteria in the large intestine. It has been demonstrated that the commensal bacteria in the large intestine induce endogenous SIgA production (Yanagibashi *et al.*, 2013). The absence of microorganisms in germ-free mice reveals that SIgA production in the small intestine is

minimal and there is no detection of SIgA in the large intestine (Yanagibashi*, et al.*, 2013). Therefore, there is a symbiotic relationship between SIgA and commensal bacteria. Furthermore, SIgA binds to a large proportion of bacteria in the GIT (Van Der Waaij *et al.*, 1996).

The other important function of SIgA is to provide defence against pathogenic bacteria (Brandtzaeg & Johansen, 2007). Potentially harmful microorganism the GIT include rotavirus, *Escherichia coli*, *Salmonella spp*., *Clostridium perfringens* and *Campylobacter sputorium*. (Williams*, et al.*, 2001). Numerous studies have demonstrated the capability of SIgA in providing passive immunity against the potentially harmful microorganisms (Hurley & Theil, 2011). SIgA has two mechanisms to bind bacteria in the large intestine; either through specific antigenic sites, or non-specifically through the attached glycans (Hodgkinson*, et al.*, 2017). The glycans of SIgA provide competitive binding to mucosal receptors preventing pathogenic bacteria from binding and hence entering the body's system (Mathias & Corthésy, 2011).

The phenomena of SIgA binding to bacteria is not species specific. The binding capability of human and bovine SIgA to bacteria is comparable ranging from 30 to 90% depending on the bacterial species and strains (Hodgkinson*, et al.*, 2017). Thus, the consumption of bovine SIgA may have an impact to the human GIT and provide potential benefits.

1.12 Summary of Immunoglobulin A survival through the gastrointestinal tract

Through the consumption of milk, SIgA enters the stomach where it is fairly resistant to proteolysis and can provide a protective benefit to the offspring in the small intestine (Hurley & Theil, 2011). Bovine SIgA survives transit through the GIT at variable rates which is expected as each study uses a difference source of SIgA (Yvon*, et al.*, 1993; Roos*, et al.*, 1995). Studies have portrayed 10- 86% of the ingested BSIgs to past the stomach, through the lower GIT and be identified in the faeces (Table 2) (Hilpert*, et al.*, 1987; Roos*, et al.*, 1995; Pacyna*, et al.*, 2001). This could be a concentration effect as increasing the dose of Ig results in higher amounts of intact protein in the faeces. It could also be due to the milk matrix and surrounding solution influencing the digestion of SIgA. Further investigation on digestion and absorption of SIgA needs to occur as the exact processes to how SIgA digestion differs in different milk treatments remain unclear (Mahe*, et al.*, 1992; Hurley & Theil, 2011; Cakebread*, et al.*, 2015).

Table 2 Summary of immunoglobulin A preparations through the digestive tract *in vitro* and *in vivo*

Population (n), health status	Donor species	Material	Digestion	Recovery	Recovered immunological activity	Reference
In vitro	Human	Colostrum SIgA bound to radio-labelled diphtheria toxoid	Proteolytic digestion with trypsin and/or chymotrypsin	85-90%	Retained antigen binding sites and ability to bind to antitoxin	Brown et al., (1970)
In vitro	Human	Secretory IgA	Proteolytic digestion with trypsin and pepsin	~60% (tryptic) ~15% (peptic)	NR	Lindh, (1975)
In vitro	Human	Secretory IgA	Pepsin pH 4.5 Incubation 3 hours	~70%	NR	Shuster, (1971)
In vitro	Human	Colostrum Secretory IgA	Pepsin pH 4.1 8 hours	~65%	NR	Wilson and Williams Jr, (1969)

			24 hours	~44%		
In vivo **Adult (7), healthy**	Bovine	Powder IgA 1g/L IgG 51g/L	Intestinal collection	IgG (84%) IgA (0%)	~19%	Roos et al., (1995)
In vivo **Children (105), healthy**	Hyperimmunised Bovine	Liquid Ig	Fecal collection	5%	86% rotavirus which correlated with initial dose	Pacyna et al., (2001)(Pacyna*, et al.*, 2001)
In vivo **Children (164) rotaviral**	Hyperimmunised Bovine	Liquid Ig Concentrate	Feacal collection	10%	43% rotavirus neutralizing activity	Hilpert et al., (1987)

NR= not reported

1.12.1 The need for an *in vivo* experiment

Mimicking the complex system of digestive processes *in vitro* has limitations (Picariello *et al.*, 2010). The digestive conditions are difficult to mimic in terms of substrate ratio, pH, reaction time, and enzymes, in conjunction with physical processes (Boutrou*, et al.*, 2013). *In vivo* digestion is a dynamic phenomenon in terms of continuous delivery of gastric effluents to the small intestine, and at the same time absorption occurring in the intestines (Boutrou*, et al.*, 2013). There is an extensive amount of *in vitro* studies on milk protein digestion; however, *in vivo* models are needed to make clear conclusions. *In vivo* functions of SIgA are poorly understood.

The preliminary findings of this research suggested that exogenous BSIgA remained intact through digestive processes and could offer added protection to pathogenic gut microbes, warranting the digestion of BSIgA of further investigation. However, only testing BSIgA levels in the faecal pellet limits the information on BSIgA digestion and does not portray a picture to the changes of BSIgA levels throughout the digestive tract of mice. The methodology of this study aims to determine the digestion of BSIgA throughout the GIT of mice; therefore it is necessary to check the levels of BSIgA in the stomach, small intestine, large intestine, and caecum individually.

One approach to *in vivo* methods is to fluorescently label the protein of interest to determine movement through the GIT (Nagakura, *et al*., 1996; Carai *et al.,* 2006). This approach is a non-invasive method and shows the distribution of the marker at a more defined location. However, fluorescently labelling proteins is a costly approach. The method in this research cuts the GIT into compartments and uses PBS to flush out the contents and measure the amount of BSIgA in the washouts by an ELISA. This is favourable as a pilot trial revealed that SIgA can successfully be collected by washing out GIT compartments with PBS buffer (Section 4.4). Other merits to this particular *in vivo* approach are the ease of measuring the amount of BSIgA retained by ELISA, the accessibility of mice, and the financial viability of being able to repeat the experiment on a large scale. However, the method of this research is invasive to the animal and only reveals the amount of BSIgA in the entire GIT compartment. But it is sufficient in portraying the transit and digestion of BSIgA through the GIT of mice. Overall, understanding physiological and metabolic consequences requires *in vivo* investigation.

1.13 Bovine Milk

1.13.1 The significance of bovine milk for human consumption

Producing a product that is perceived as valuable is important for future progression of the dairy industry (Boland *et al.*, 2001). Milk is naturally an oil-in-water emulsion that contains milk fat globules suspended in an aqueous phase that consist of proteins, phospholipids, salts, and lactose (Ye*, et al.*, 2011). There are two main types of proteins in milk; soluble whey protein, and micellular casein.

Whey proteins are characterised by disulphide bridges sustaining the portions folding and their ability to bind to fat, specifically β-lactoglobulin. while caseins are open (limited hydrophobic core) and have a flexible conformation (Picariello*, et al.*, 2015). Whey proteins such as β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and the Igs are an important source of bioactive nutrients (Morr & Ha, 1993). Bioactive nutrients are valuable to humans, providing a source of energy and also contribute to normal body functions (Boland*, et al.*, 2001). As established in Table 1**,** there are immune compositional differences between bovine and human colostrum and milk. This is also true for the variation of whey and casein proteins. The differences in protein composition between cow's milk and human milk are shown in Table 3. The differing levels emphasise the nutritional target differences of the two species and how each are relevant to the growth rate of their offspring (Wells, 1996). The most important nutritional target difference is reflected in the slow growth and development of the infant. Compared to bovine milk, human milk has less energy and protein concentration (Wells, 1996). Thus, developing a product that targets human health is important, especially in neonates that rely solely on milk (Boland*, et al.*, 2001). There are many elements that are involved in altering the composition of milk proteins; nutrition, natural selection, and manufacturing processes are a few examples (Boland*, et al.*, 2001). This review will review how manufacturing processes affect bovine protein concentration and how two different matrices can affect the bioactive function of SIgA (see milk processing Section 1.14).

Table 3 Main differences in the composition of cow's and human milk casein and whey fractions. Retrieved from Wells, (1996)

	Cow's milk	Human milk
Whey: casein ratio	20: 80	Changes throughout lactation - Early lactation 90: 10 - Mature milk 60: 40 - Late lactation 50: 50
Whey	Mainly β-lactoglobulin, IgG, and α-lactalbumin	Mainly α-lactalbumin, SIgA, and lactoferrin. No β-lactoglobulin
Casein	Mixture of β-, k-, α_{s1}- and α_{s2}-casein	Mainly β- and k- casein

1.14 Milk Processing

There are various types of milk processing that can be used to increase the shelf life of milk, maintain a year round supply of milk, concentrate proteins, or enhance the digestibility of milk in the GIT. Milk processing can have an adverse effect on the composition of milk, for example, exposing Igs to changes in conditions such as temperature, pressure, and pH, can affect their structure and function (Mehra*, et al.*, 2006; Hurley & Theil, 2011). The initial pasteurisation step, heating milk to 72˚C for 15 seconds, retains between 25-75% of SIgA and IgG compared to that found in raw (unprocessed) milk (Li-Chan*, et al.*, 1995). However, with careful low heat treatment it is possible to retain SIgA activity (Li-Chan*, et al.*, 1995; Mehra*, et al.*, 2006). For example, the levels of antibodies in pasteurised milk was sufficient to neutralise the *in vitro* replication of rotavirus and protect mice against rotavirus infection (*in vivo*) (Yolken *et al.*, 1985).

Ig's are thermolabile, as they are deactivated by heat and are undetectable when heated for long periods at temperatures greater than 75˚C (Dominguez *et al.*, 1997). Also, SIgA is the most thermolabile Ig, losing its ability to bind antigens when heated at 80˚C for 20 minutes (Ustunol & Sypien, 1997). High temperatures result in denaturation or unfolding of molecules which in

turn causes loss of antigenicity (Li-Chan*, et al.*, 1995). A reason for decreased protein resistance in processed milks may be due to denaturation of whey and casein proteins during heating treatment meaning proteins that are usually inaccessible due to the hydrophobic core can be accessed by enzymes (Farnfield *et al.*, 2009). SMP and WPC processing and possible effect on SIgA content will now be discussed in further detail (Section 1.14.1 and 1.14.2).

1.14.1 Skim milk powder composition and processing

Milk can be further processed to create a skim milk powder by skimming pasteurised milk, concentrating the skim milk solids to 45-50% of the total solids by evaporation, heating the skim milk concentrate and then spray drying the milk concentrate to produce a powder (Oldfield *et al.*, 2005). SMP is a widely used as an ingredient in many formulated foods providing functional properties including stimulation of immune functions, enhancing calcium uptake, and opioid activity (Boland*, et al.*, 2001; Oldfield*, et al.*, 2005). Foods that include SMP are soups, evaporated milk, sauces, bakery production, confectionery products, and infant formulae. The bulk composition of SMP is proteins (casein and whey), lactose, and 0-1% fat (Oldfield*, et al.*, 2005). Due to the low fat composition and previous negative views on large consumption of fat, SMP is viewed as a healthy product by the consumer.

The manufacturing processes of SMP has the potential to cause a number of changes to the composition of milk. These include: destruction of bacteria, inactivation of enzymes, whey protein denaturation, transfer of soluble calcium and phosphate to the colloidal phase, denaturation of whey proteins with the casein micelle (Oldfield*, et al.*, 2005). These changes could result in a decreased nutritional and immunological value of SMP. The irreversible denaturation of whey proteins in skim milk occurs mainly in the preheating period (Oldfield*, et al.*, 2005). Careful preheat treatment at 70°C for 52s allows the highest concentration of the whey and casein proteins to remain ensuring high quality SMP (Oldfield*, et al.*, 2005). The evaporation step does effect the milk matrix as it causes tighter packing of casein micelles and increases the concentrations of lactose, and whey proteins (Singh & Creamer, 1991). Further, the casein micelles form intermolecular complexes with whey proteins, such as α-lactalbumin, by hydrophobic interactions and covalent linkage through thiol-disulphide bonds (Singh & Creamer, 1991).

Temperature changes and pressure effects while producing SMP can manipulate the immune composition. Spray drying methods can be altered to increase the retention of Igs, in particular SIgA (Castro-Albarrán *et al.*, 2016). Standard spray drying procedures of human milk powders at 160-180°C with 2% humidity resulted in >88% IgG, 70% IgM, but only 38% SIgA retention

(Castro-Albarrán*, et al.*, 2016). Retention of SIgA is best achieved from freeze drying with a decreased heating plate temperature to 40̊C resulting in 55% SIgA retention. However, the most efficient retention, 75% of SIgA was achieved at 30̊C heating plate temperature (Castro-Albarrán*, et al.*, 2016). Furthermore, heating plate temperature rather than process time is the critical factor in retaining SIgA in milk powder (Castro-Albarrán*, et al.*, 2016). Digestive studies and Ig survival through the GIT focus primarily on IgG due to the higher relative concentrations present in bovine milk (Jasion & Burnett, 2015). Due to the low SIgA starting concentration in processed milk, this protein's survival through the GIT has been overlooked. To my knowledge, no studies have investigated the transit and digestion of SIgA in milk products after manufacturing processes.

1.14.2 Whey Composition and Processing

Whey is the solution remaining after removal of milk fat and casein (Morr & Ha, 1993). Whey proteins remain in the milk serum after coagulation of casein proteins at pH 4.6 and 20°C (Morr & Ha, 1993). WPC is whey protein that has been concentrated by removal of other whey constituents. The whey is filtered and disk-stacked centrifuged to removal all casein curd and concentrate the whey. Ultrafiltration removes lactose, minerals, and low- molecular- weight components less than 10 Kd (Morr & Ha, 1993). Native whey proteins remain stable at 70̊C but the level decreases as the temperature is increased to 120̊C (Oldfield*, et al.*, 2005). Whey proteins are complex globular proteins ranging from 14-1000 Kda, but share a key property, an abundance of sulfhydryl AA residues allowing intermolecular covalent bonds (Morr & Ha, 1993). This property is important during high-temperature processing allowing the protein structures to remain intact (Morr & Ha, 1993). Individual whey proteins have different heat resistance capability which follows the order : Immunoglobulins < bovine serum albumin < β-lactoglobulin < α-lactalbumin (Singh & Creamer, 1991). In comparison to the preheating stage, evaporation and drying has minor changes to the level of native whey proteins (Oldfield*, et al.*, 2005). Therefore, manufacturing processes can optimise heat treatments to ensure maximum immunoglobulin concentration. There are two types of WPC; made from sweet or acid whey. Sweet whey is manufactured by inoculating milk with lactic acid bacteria culture to acidify it to pH 6.2 to 6.4 , and adding the coagulating enzyme rennet (Morr & Ha, 1993). In contrast, acid whey is produced by adjusting the pH of skim milk to 4.6 by adding acid, lactic acid bacteria culture, or glucono delta lactone, and draining the resulting whey (Morr & Ha, 1993). The manufacturing processes result in differential milk matrices (Ando *et al.*, 2005). Due to the lower pH in acid whey, the antibody activity is lower compared to rennet (sweet) whey (Ando*,*

et al., 2005). The WPC used in this study uses acid treatment processes and successfully achieves high SIgA concentration as shown in Table 4.

Table 4 Composition of reconstituted WPC to 3.7% w/v total solids and SMP to 10% w/v total solids used in this study.

Composition	SMP	WPC
Protein %	3.2%	3.2%
SIgA mg/mL	0.19	1.43
IgG mg/mL	0.58	2.36
Lactoferrin mg/mL	0.10	0.45
Fat %	n.d	0.07%
Lactose	4.4	n.d

In conclusion, the SMP matrix provides both whey and casein proteins that may interact causing an effect on the transit and digestion of SIgA through the GIT. Whereas, WPC lacks casein, but has an increased concentration of SIgA per g protein, relative to SMP. The effects a milk matrix has on the transit and digestion of SIgA will be investigated in this research.

1.15 Conclusions

To conclude, there is extensive information regarding the function of SIgA, however, the literature is lacking in information about the implication of milk matrices to BSIgA transit and digestion. Studies compare the digestive of SIgA with other milk proteins but there are no data focusing on SIgA transit and digestion. Finally, there is a requirement for *in vivo* experiments to present the true potential of SIgA through the GIT.

1.16 The objectives of this research

- To compare whether the different matrices of WPC and SMP effect digestion of BSIgA within the product.
- To compare the transit time of SIgA in bovine SMP and WPC through the GIT of mice.
- To identify the impact exogenous BSIgA has on endogenous MSIgA.

Questions to be addressed are:

1. Does the survival rate of BSIgA (as measured by ELISA) change according to the delivery matrix?
2. How quickly is BSIgA digested in these two matrices and how much BSIgA transits the gut relative to the starting amount?

The hypotheses of this research are:

1. The milk matrix affects transit and digestion of BSIgA through the GIT of mice.
2. Ingested BSIgA impacts the level of endogenous MSIgA.

The findings from this study will help give a better understanding as to how protein digestion changes depending on the original solution consumed. The results will add to the current knowledge on digestion process of protein and its effects on health. It will also confirm previous studies and determine what level of SIgA needs to be present in the milk matrix for the survival of SIgA and, therefore, possible benefits. This information may be informative for the development of products designed to deliver bioactive products. In the long term it will give a detailed understanding of the effect of different formula matrixes and how they impact bioactive integrity. Consequently, researchers will have a greater understanding of SIgA bioavailability for future product developments. This knowledge may also be important for the farming community as it could lead to an extra premium for natural high SIgA milk supply.

Chapter 2

Materials and Methods

2.1 Subjects

Approval for animal use and experimental protocols for this study was granted by the Ruakura Animal Ethics Committee (AEC#13986). A total of 120 mixed sex adult (12-13 weeks old) BALB/c mice weighing 19.4-29.5 g (average 23.43 g) that were bred and raised in the Small Animal Facility located at AgResearch Ruakura, Hamilton were used in the study. Groups were housed in single sex groups of up to five per cage throughout the study. The mice were offered a dairy-free mouse chow (Teklad, Research Diets, New Brunswick, USA) prior to treatment and water *ad libitum*.

2.2 Experimental design and treatments

The aim of the experiment was to investigate the digestion and transit of BSIgA in mice from different milk matrices. To do this, mice were gavaged with different treatments. They were then culled at four time points. Two treatment groups and a control group were used to measure the transit and digestion of BSIgA in mice:

1) High SIgA skim milk powder (SMP)
2) High SIgA whey protein concentrate (WPC)
3) Water (control)

Ten mice, comprising of five males and five females, were assigned to each experimental treatment for four different experimental end points as follows:

1) 7 minutes post gavage
2) 20 minutes post gavage
3) 1 hour post gavage
4) 4 hours post gavage

The time points represent the culling time after gavage. Each experimental time point was conducted on separate days.

Sample sites collected from each mouse were as follows:

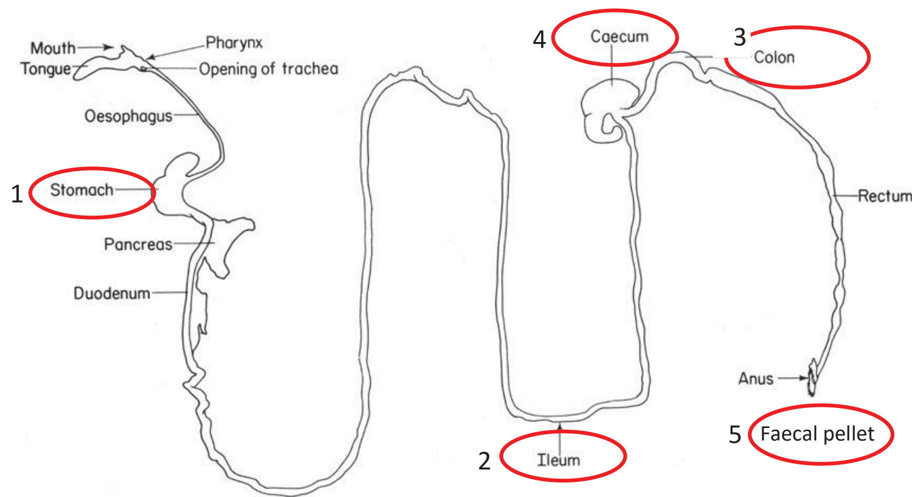

Figure 9 Gastrointestinal tract of mice showing the sample collection sites

1) Stomach
2) Small intestine (SI) (duodenum and ileum)
3) Large intestine (LI) (colon)
4) Caecum
5) Faecal pellet (FP) (only for the 4 hour period)

To dissect a mouse and collect the necessary samples, 10 minutes is required. Therefore, one mouse was gavaged every 10 minutes to stagger their cull times (experimental end points). The sex of the mouse was alternated for each treatment. The treatment order was also alternated after each gavage in the following order; water, SMP, and WPC. Table 5 shows a brief example of the procedure for the 20 minute time point; the full procedure can be seen in the Appendix A.1. Due to time constraints only 15 mice were treated on each experiment day. Thus a total of 8 experimental days was required.

Table 5 Experimental procedure showing sex, treatment, gavage time, and cull time.

Sex	ID	Treatment	Gavage time	Cull time
F	Red	Water	10.00am	10.20am
M	Red	SMP	10.10am	10.30am
F	Blue	WPC	10.20am	10.40am
M	Blue	Water	10.30am	10.50am
F	Yellow	SMP	10.40am	11.00am
M	Yellow	WPC	10.50am	11.10am

2.3 Preparation of SMP and WPC

Cows from a pasture fed Jersey-Friesian commercial milking herd based in Waikato, N.Z, were individually screened and selected for high SIgA milk concentrations. Milk was collected from cows identified as having high SIgA concentration during mid-lactation. The collected milk was pasteurised using a standard protocol (75°C for 15 seconds) and processed into SMP and WPC using in house methodology as follows:

- To produce SMP the pasteurised milk was skimmed, followed by evaporation to 40-45% solids then spray drying using low heat.
- To produce WPC the pasteurised milk was skimmed. It was then transferred to a low heat, and continuously stirred at 40 degrees. Hydrochloric acid (HCL) was added to reach and maintain pH levels of 4.6-4.8. The whey was decanted off the casein curd. The whey was then filtered and disk-stacked centrifuged to remove all the casein curd and concentrate the whey. The pH of the final concentrated whey was adjusted to 6.6 using sodium hydroxide (NaOH) and then spray dried using low heat.

Both SMP and WPC powders were stored in a sealed light-proof bag at ambient temperature.

2.3.1 Treatment preparation and composition

The amount of SMP and WPC required for all experiments was weighed and allocated into individual pots and stored at room temperature prior to the experiments. The treatments were prepared the evening prior to an experimental day and were stored overnight in a fridge.

Preparation for each treatment was as follows:

1) SMP

10 g of SMP was added to 90 mL of Milli Q (Barnstead) purified water (MQ-H_20) water, mixed together by shaking gently until the powder dissolved.

2) WPC

3.75 g of WPC was added to 100 mL of MQ-H2 water. As WPC was difficult to mix, it was mixed in a blender for two seconds then shaken gently until the rest of the powder had dissolved.

3) Water

100 mL MQ-H2 water.

The sample preparation reconstituted SMP at 10% w/v total solids and WPC reconstituted at 3.7 % w/v total solids. This normalised the protein levels in WPC and SMP to 3.2%. The reconstituted SMP and WPC had the following composition, presented in Table 6.

Table 6 Composition of reconstituted WPC and SMP.

Composition	SMP	WPC
Protein %	3.2%	3.2%
SIgA mg/mL	0.19	1.43
IgG mg/mL	0.58	2.36
Lactoferrin mg/mL	0.10	0.45
Fat %	nd	0.07%
Lactose	4.4	nd

nd= not detected.

Levels of SIgA, IgG, and lactoferrin were determined by an enzyme linked immunosorbent assay (ELISA) method using a commercially supplied kits (Bethyl Laboratories, USA.). Procedures were carried out according to the manufacturer's protocol. Protein, fat, and lactose percentage was determined using the Milkoscan FT1.

2.3.2 Buffer capacity of SMP and WPC matrix

To measure the pH buffering capacity of SMP and WPC matrix, 50 µL 2M HCL was added in increments to 40mL of the milk matrix. The solution was mixed after each addition of acid and the pH measured using a Lab 850 pH reader (Schott instruments, probe – SI analytic, 3 mol/l KCL). The results were recorded until the pH of the milk matrix reached 2.15.

2.3.3 Animal Management

At approx 4.30 pm the evening prior to experimental days, the mice were weighed and the food was removed to clear the upper gut of solid matter, enabling easy transit of the treatment gavage. The bedding in the cages was also cleaned to limit the consumption of faecal pellets. During the fasting period, the animal had *ad libitum* drinking water. During the experiment one mouse died from a pre-existing lung/heart condition (diffuse pulmonary oedema) before gavaging and was therefore excluded from the experiment and replaced with a healthy counter.

On experimental days, animals were gavaged with either water, SMP, or WPC. Post gavage, animals were observed for signs of discomfort. During the first experimental trial (1 hour period) post gavage mice were placed into cages in groups of three from mixed treatments.

However, behaviour observations showed the mice licked the mouths of their cage-mates and this risked contaminates between treatments. Thereafter, the mice were separated into treatment-specific groups for the 20 minute and 4 hour time-point experiments. For the 4 hour time-point, mice were moved into individual containers 1 hour before cull time to allow easy collection of faecal samples.

2.4. Experimental procedure

2.4.1 Oral gavage

Oral gavaging was carried out using probe-ended stainless-steel gastric tubes (25 x 1.2 mm, length x outer diameter). All mice were gavaged a total volume of 200 µL of their corresponding treatment. The gavaging was carried out by a trained SAC staff member. To clarify the gavage procedure refer to Figure 10. The mouse was restrained by holding the back skin with the body freely hanging. The animal's head was gently extended back. The tube was placed gently down the oesophagus and the mouse was dosed slowly, put into a secure cage, and monitored for five minutes.

Figure 10 Mouse gavage

2.4.2 Dissection

At the allocated time-point, mice were euthanised in a CO_2 chamber followed by cervical dislocation by a trained staff member. The mice were sprayed with 70% ethanol to restrict fur contamination. A mouse was placed on a dissection tray face up and the paws pinned down to secure the mouse (Figure 11, A). The abdomen was opened by mid-line laparotomy (Figure 11, B). The GIT was cut into segments (stomach, small intestine, large intestine and caecum) to enable sample sites to be collected (Figure 11, C, D, and E). The segments were then placed into separate weigh boats. The stomach and caecum were sliced open to allow easy access to contents. The small intestine was chopped into two due to its long length. The contents were squeezed out, using tweezers if necessary. A previous experiment had showed that the first wash yielded most of the murine SIgA, with a second wash maximising the SIgA yield (see

Appendix A.2). Therefore, segments were flushed out twice using a total of either 0.75 mL (7 minute, 20 minute and 4 hour time-point) or 1 mL (1 hour time-point) of Phosphate-buffered saline (PBS) containing a protease inhibitor (cOmplete Mini 11836153001, Roche Diagnostics, Germany) (Figure 11, F). The wash-out fluid and contents were collected and put into labelled pre weighed Eppendorf tubes.

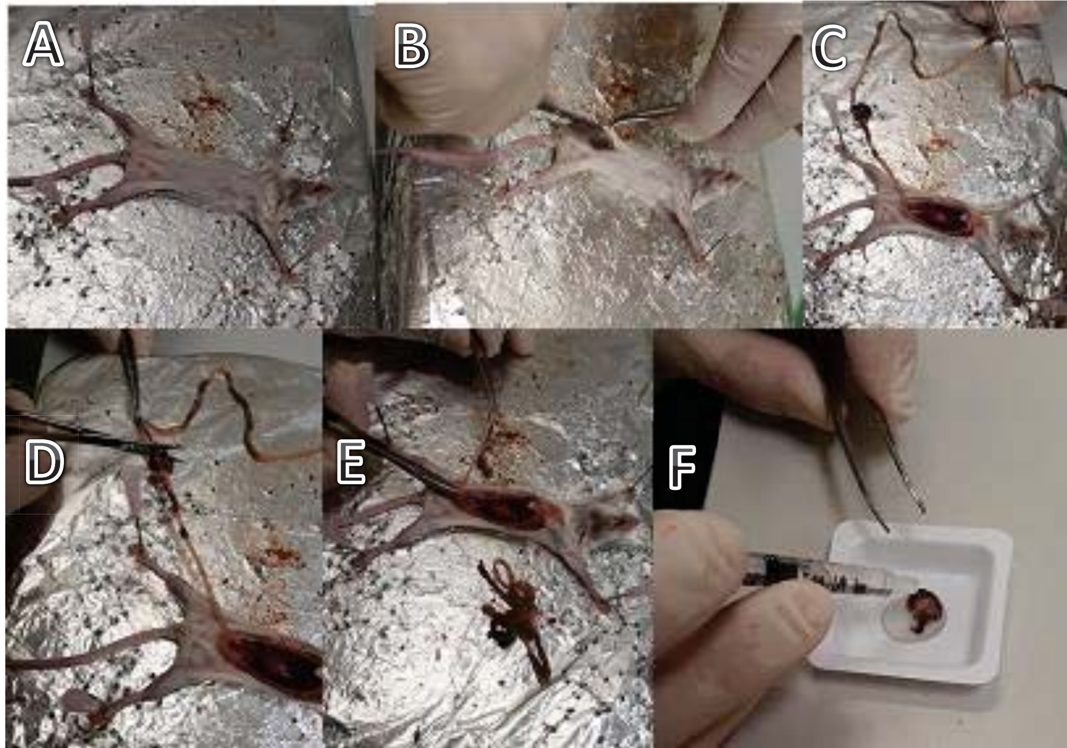

Figure 11 Images showing the dissection and collection of GIT components. A) Mouse pinned face up B) Abdomen open by a mid-line cut C) removal of the GIT from the mouse and excision of the stomach D) excision of the small intestine and caecum E) excision of the large intestine F) example of the caecum being flushed out.

2.5 Sample processing and Assays

3.5.1 Preparation of stomach, small intestine, large intestine, and caecum:

Weights for each sample was recorded. Samples that were viscous, due to digesta contents, were mashed using the end of a microbiology loop until a slurry texture was produced. All samples were vortexed briefly to ensure homogenisation and then centrifuged (13,000 x g for 10 minutes). The supernatant was transferred into a new Eppendorf tube and re-centrifuged (13,000 x g for 10 minutes). The supernatant was then recovered into a new Eppendorf tube and stored in a -20°C freezer for later analysis.

2.5.2 Preparation of faecal extract:

The four hour post gavage time point was the only experiment that had faecal pellets. Sample preparation from faecal pellets was similar to the intestinal contents preparation. However, the volume of PBS containing protease inhibitor added to faecal pellets was based on their weight (weight in mg X 10, e.g. 8 mg -> 80 µL). The samples were mashed using the end of a microbiology loop until a slurry texture was produced. All samples were vortexed briefly to ensure homogenisation and then centrifuged (13,000 x g for 10 minutes). The supernatant was transferred into a new Eppendorf tube and re-centrifuged (13,000 x g for 10 minutes). The supernatant was then recovered into a new Eppendorf tube and stored in a -20°C freezer for later analysis.

2.5.3 Preparation of test samples for Bovine SIgA analysis

Test samples were diluted using Tris-buffered saline and tween (0.5%) (TBST) and the following dilutions were prepared: the 1 hour and 4 hours post gavage samples were not diluted. The 20 minute experimental samples were first measured undiluted, however, samples that were above or below the standard concentrations were repeated at dilution 1:50. The gavage material was also measured as a start point; WPC was diluted 1:2000, 1:4000; and SMP was diluted 1:200 and 1:500.

2.5.4 Preparation of test samples for Murine SIgA analysis

Test samples were diluted using TBST-containing 1% bovine serum albumin (BSA). All samples were diluted as follows: stomach 1:10; small intestine 1:1000; large intestine, caecum, and faecal pellet 1:200. Samples with concentrations that were above or below the standard curve were repeated with different dilutions as follows; stomach 1:10 and 1:40; small intestine 1:2000 and 1:4000; large intestine, caecum, and faecal pellet 1:50 and 1:500.

2.6 Enzyme-linked immunoassay

2.6.1 ELISA for total bovine SIgA

Total bovine SIgA in the samples was measured using a non-competitive sandwich ELISA method using commercial bovine SIgA kit (Bethyl Laboratories, USA. Bovine Cat. No E10-131). Procedures were carried out according to the manufacturer’s protocol. All washes were carried out by an automatic microplate washer (5165010, Thermo Scientific) using TBST, and all samples and reagents were diluted with TBST. At each incubation step, the ELISA plates (ThermoFisher) were wrapped in tinfoil to prevent light contamination.

ELISA plates were coated with 100 µL of affinity purified Sheep Bovine SIgA coating antibody (A10-131A-1) diluted to 1:100 with coating buffer, 0.05M Carbonate-bicarbonate (pH 9.5). Plates were left to incubate in the fridge (4°C) overnight. The following day, the plates were washed three times and non-specific binding sites were blocked by adding 250 µL of TBST to each well and left to incubate at room temperature for 1-2 hours. After washing the plates three times, 100 µL of the standard (1000, 500, 250, 125, 62.5, 31.25, 15.625 ng/mL, and blank), test samples (Section 2.5.3) and a quality control sample (dilution 1:500) were loaded into the wells in duplicate. The plate was left to incubate at room temperature for 1 hour. Plates were then washed three times and 100 µL of HRP conjugated Sheep anti-Bovine SIgA detection antibody (A10-131P-1) diluted to 1:100,000 was added to each well. The plates were washed three times and 100 µL of ELISA substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB) added to each well. The plates were then left to incubate on an automatic shaker for 15 minutes. 50 µL of ELISA stop solution (2 mol/L sulphuric acid, H_2SO4) was added and the optical density measured at 450 nm by an automated plate reader (versa max, Molecular Devices). Absorbance were read on Softmax Pro software.

An 'in-house' total SIgA macro, built in Excel 2010, plotted the standard curve absorbance's against their corresponding known SIgA concentration to create a standard curve. This was used to determine unknown total SIgA concentration in each sample. The macro calculated the mean SIgA concentration from duplicate samples absorbance and standard deviations. The samples and quality control samples measured on an ELISA had good agreement between duplicates (less than 5% difference of the mean values).

2.6.2 ELISA for total murine SIgA

Total murine SIgA in the samples were measured using a commercial murine SIgA kit (Bethyl Laboratories, USA. Murine Cat. No. E90-103) which used the same methodology as for the total bovine SIgA. Procedures were carried out according to manufacturer's instructions and as described above. Differences between the two kits were as follows. ELISA plates were coated with 100 µL of affinity purified Goat anti-Mouse SIgA coating Antibody (A90-103A-30) diluted to 1:100 with coating buffer. The buffer used to block the plates and dilute test samples was TBST-BSA. The murine quality control test sample was diluted 1:200. Refer to 2.4.4 for the diluted test samples plated. HRP conjugated Goat anti-Mouse SIgA detection antibody (A90-103-34) was diluted to 1:40,000 and the plates were left on the automatic shaker with conjugate for only 10 minutes as the colour development was faster.

2.7.1 Statistical design

The 20 minute and 4 hour bovine SIgA levels were scaled up by 0.25 to adjust for the different sample wash volume from 0.75mL to 1 mL to allow all time points to be compared. The actual values for bovine SIgA in SMP and WPC individually were log transformed to normalise the variance. The values are presented as back transformed mean of the logs. The relative values for bovine SIgA comparing the treatments were a percentage of the original SIgA amount in each treatment. The murine SIgA values are presented as total murine SIgA detected (mg/mL). In order to test the consistency of the volume of samples collected, the sample end weights were calculated by taking the difference of tube 3 and tube 1 (tube 3 – tube 1)(Section2.5). Also mean values of BSIgA found in the GIT of male and female mice were calculated to determine if there was a gender effect.

2.7.2 Statistical analysis

An analysis of variance (ANOVA) test was used to determine the significance between treatment, time, and GIT. The significance between each time point was also tested using an ANOVA. Bovine SIgA concentration values from the GIT of male and female mice were analysed by ANOVA to determine if there was a gender effect on BSIgA digestion. Also, end weight recovery was analysed by ANOVA to determine if there was consistency in sample collection. As there was no difference in MSIgA concentration in each GIT compartment for the different treatment types, the averages of the three treatments were calculated and analysed over the post gavage time points using ANOVA. Statistical analysis was performed using Genstat (GenStat for Windows 18th Edition VSN International, Hemel Hempstead, UK). Data are presented as the mean and error bars illustrate the pooled standard error of differences of means (sed). Bovine SIgA values were log10 adjusted to stabilise variation. Significance level $P<0.05$.

Chapter 3

Results

3.1 Non-specific bovine SIgA detection in the water samples

There was apparent BSIgA detection in some of the water control samples (Appendix B.1). As no product containing BSIgA was gavaged to the water control mice and they were separate from the milk gavaged mice, there could be no contamination. We could not see any correlation between animals with high endogenous murine SIgA levels and those with apparent detection of BSIgA. A cross reactivity test of murine IgA protein on a bovine IgA ELISA ruled out the possibility of cross reactivity between murine and bovine SIgA. It was revealed that there was SIgA detected in the dilution buffer control (containing BSA) reading at 163 ng/mL (corresponding to geometric log value of 9.14 ng/mL). We noted that the samples where BSIgA was detected did not dilute out, as would be expected with a true reading. We therefore conclude that the low values detected in the water samples were non-specific binding, or were from something else in the samples that could impact the ELISA assay. We therefore assigned 9.14 ng/mL as the baseline value below which BSIgA values were assumed to be 'background noise'.

3.2 Digestion of BSIgA in SMP

Total bovine SIgA throughout the GIT of mice was measured at 7 minutes, 20 minutes, 1 hour, and 4 hours following gavage of 200 µL of SMP (Figure 12). Values are presented as geometric mean BSIgA amount (ng/mL). BSIgA did survive digestive processes as it was detected at all time points and transits through the GIT. Overall, there was a reduction in BSIgA amount detected in washouts from 7 minutes, 20 minutes, 1 hour, and 4 hours of post gavage ($P<0.05$).

At 7 minutes post gavage BSIgA was detected in both the stomach and small intestine washouts, measured at 6311 ng/mL and 15,063 ng/mL respectively ($P>0.05$). A minimal amount of BSIgA was detected in the large intestine and caecum washouts, measured at 13.60 ng/mL and 9.68 ng/mL respectively ($P>0.05$). The BSIgA measured in the caecum was below the level considered a true BSIgA value.

At 20 minutes post gavage, BSIgA was detected in the washouts from the stomach and small intestine. The BSIgA levels were higher in the stomach and small intestine washouts at 7 minutes compared to the 20 minute post gavage time point ($P<0.05$). Contents of the stomach contained 265 ng/mL BSIgA, but a much higher BSIgA amount was detected in washouts from the small intestine (2465 ng/mL, $P<0.05$). The minimal levels of BSIgA detected in the large

intestine and caecum washouts at 20 minutes were below the level of confidence for believing these were true BSIgA values.

At 1 hour post gavage, the highest amount of BSIgA was detected in the lower GIT segments. At 20 minutes the level of BSIgA detected in the washouts from the stomach was 265 ng/mL but at the 1 hour time point, 6.30 ng/mL of BSIgA was detected in the stomach ($P<0.05$). The highest amount of BSIgA at 1 hour was measured in the washouts from the small intestine at 77.48 ng/mL. There was no difference to the amount of BSIgA detected in the large intestine washouts from the 20 minute to 1 hour period (1.40 vs 1.58 ng/mL, respectively) ($P>0.05$). At this time point, BSIgA was detected in the caecum washouts at 3.90 ng/mL. The BSIgA detected in the stomach, large intestine, and caecum washouts were below the level of confidence for believing these were true BSIgA values.

By 4 hours post gavage little BSIgA could be detected in the washouts from the stomach or the small intestine (1 ng/m and 7.92 ng/mL, respectively). There was 12.55 ng/mL detected in the washouts from the large intestine but the highest amount of BSIgA was located in the washouts from the caecum at 159.17 ng/mL. Figure 12 illustrates BSIgA levels detected in the washouts at four time points. There was a decreased amount of BSIgA in the stomach and small intestine washouts at the longer post gavage time points ($P<0.05$). BSIgA was not confidently detected in the large intestine and caecum washouts until the 4 hour post gavage time point. There was no significant difference in the amount of BSIgA detected in the large intestine washouts at 7 minutes and 4 hours post gavage ($P<0.05$).

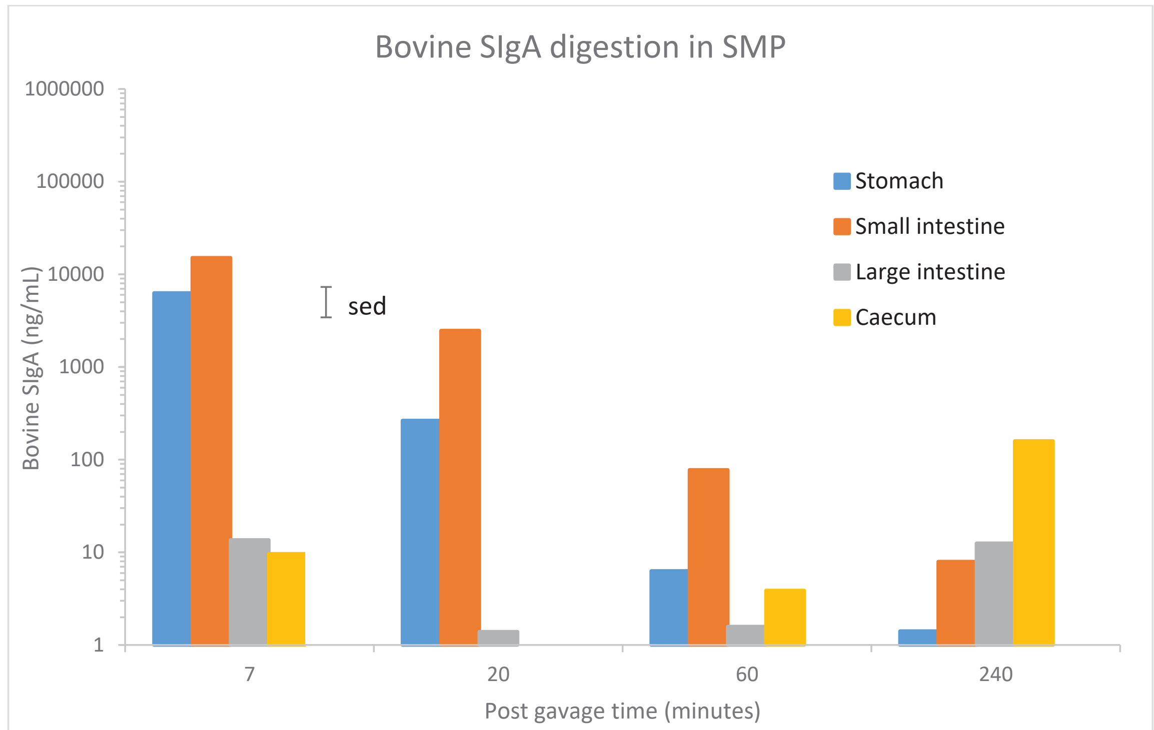

Figure 12 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with SMP at 7 minutes, 20 minutes, 1 hour, and 4 hours post gavage.

Analysed on a log scale, bovine SIgA (ng/mL) at each location is presented. Standard error of the difference for all values is presented as an error bar (sed). The level of confidence for true BSIgA values was 9.14 ng/mL.

3.3 Digestion of BSIgA in WPC

Total bovine SIgA throughout the GIT of mice was measured at 7 minutes, 20 minutes, 1 hour, and 4 hours following gavage of 200 µL of WPC. Figure 13 shows the amount of BSIgA detected in the washouts from the stomach, small intestine, large intestine, and caecum at four time points following gavage with WPC. Values are presented as geometric mean BSIgA amount (ng/mL). The level of confidence for believing values were true BSIgA was 9.14 ng/mL. The amount of BSIgA at each GIT compartment was influenced by the post gavage time.

At 7 minutes post gavage with WPC, BSIgA was detected in all GIT compartments. In the stomach washouts BSIgA was detected at 9,509 ng/mL, but a higher amount was detected in the small intestine washouts at 109,098 ng/mL ($P<0.05$). This is the opposite to the SMP fed mice who had higher levels of BSIgA in the stomach washouts at 7 minutes compared to the small intestine washouts. There was little BSIgA detected in the large intestine and caecum washouts at 27.11 ng/mL and 31.81 ng/mL respectively ($P>0.05$). The amount detected in the caecum washouts at 7 minutes was no different to the amount detected at 4 hours ($P<0.05$).

At 20 minutes post gavage, the highest amount of BSIgA was measured in the washouts from the small intestine at 11,271 ng/mL but there was some BSIgA detected in the stomach at 167 ng/mL ($P<0.05$). The amount of BSIgA detected in the large intestine and caecum washouts was 6.17 ng/mL and 1.43 ng/mL respectively ($P>0.05$), but these values were below the level of confidence for believing these were true BSIgA values.

At 1 hour post gavage, the amount of BSIgA detected in the GIT washouts had decreased significantly in the stomach (11.25 ng/mL), small intestine (208.51 ng/mL) and large intestine (2.20 ng/mL) ($P<0.05$). Minimal levels of BSIgA were detected in caecum (2.29 ng/mL). Levels in the caecum, and large intestine washouts were below the level of confidence in believing these were true BSIgA values. There was no difference in BSIgA amount between 20 minutes to 1 hour in the caecum washouts ($P>0.05$).

At 4 hours post gavage, BSIgA was not detected in the stomach washouts. The BSIgA levels in the small intestine washouts were below the level of confidence, 9.03 ng/mL ($P<0.05$). In contrast, BSIgA levels had increased in the large intestine and caecum washouts (97.51 ng/mL and 149.91 ng/mL, respectively ($P>0.05$)). At 4 hours post gavage Figure 13 shows that the highest amount of BSIgA was detected in the lower GIT, in the large intestine and caecum washouts. Figure 13 displays differing levels of BSIgA detected in the GIT washouts for the different post gavage time points. There were decreased BSIgA levels in the stomach and small

intestine washouts with increased post gavage time (P<0.05). BSIgA was detected in the large intestine and caecum washouts at 7 minutes and 4 hours post gavage only.

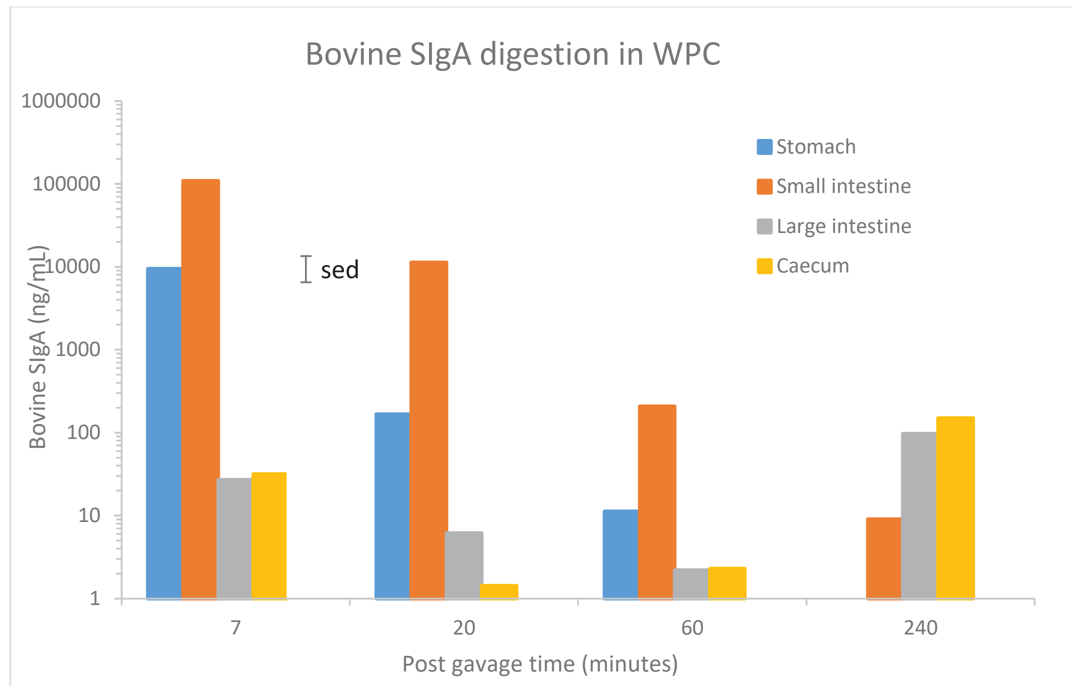

Figure 13 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with WPC at 7 minutes, 20 minutes, 1 hour, and 4 hours post gavage. Analysed on a log scale, bovine SIgA (ng/mL) at each location is presented. Standard error of the difference for all values is presented as an error bar (sed). The level of confidence for tur BSIgA values was 9.14 ng/mL.

3.4 Comparison of bovine SIgA digestion in SMP and WPC

The aim of the experiment was to determine if there was a difference in BSIgA digestion and transit from two different milk matrices; SMP and WPC. The BSIgA levels detected in the washouts are presented as a percentage of the starting concentration of the gavaged milk product preparations. Thus the WPC and SMP BSIgA values in Figure 14 are directly comparable to each other. The values detected in the water samples were irrelevant when comparing WPC and SMP to each other, therefore are not included. The SMP and WPC preparations contained BSIgA at 0.18 mg/mL and 1.43 mg/mL, respectively. This equated to each mouse receiving 37,750 ng/200µL of BSIgA in the SMP and 286,000 ng/200µL of BSIgA in the WPC. All gavages were 200µL volume.

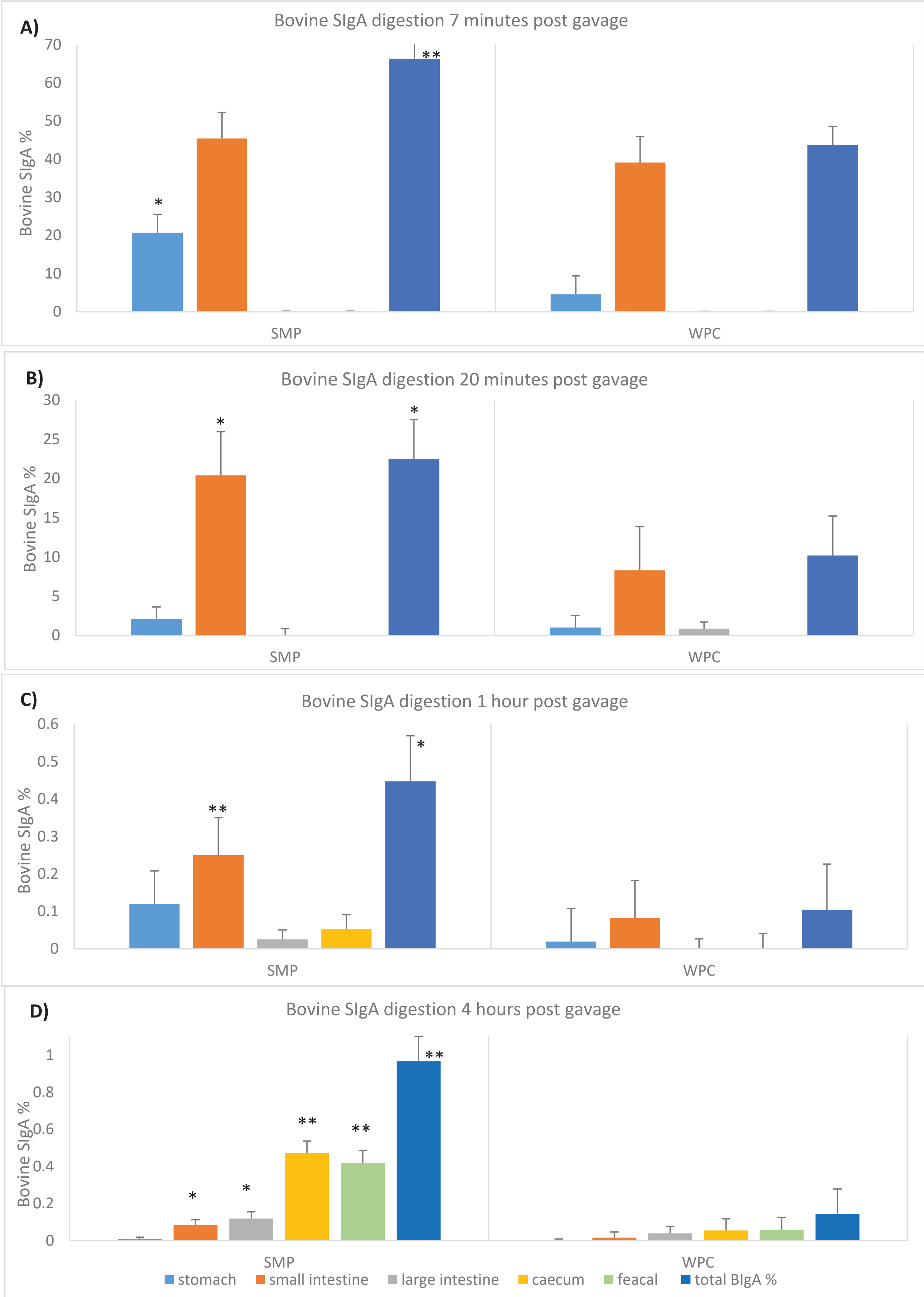
A)
Bovine SIgA digestion 7 minutes post gavage
Bovine SIgA %
70
60
50
40
30
20
10
0
*
**
SMP
WPC
B)
Bovine SIgA digestion 20 minutes post gavage
Bovine SIgA %
30
25
20
15
10
5
0
*
*
SMP
WPC
C)
Bovine SIgA digestion 1 hour post gavage
Bovine SIgA %
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
SMP
WPC
D)
Bovine SIgA digestion 4 hours post gavage
Bovine SIgA %
1
0.8
0.6
0.4
0.2
0
*
*
**
**
**
SMP
WPC
stomach
small intestine
large intestine
caecum
feacal
total BIgA %

Figure 14 Detection of Bovine SIgA in the intestinal washouts from mice gavaged with SMP or WPC at A) 7 minutes B) 20 minutes, C) 1 hour, and D) 4 hours post gavage.

Data is presented as percentage of BSIgA relative to the starting SIgA concentration in each of the products. Error bars illustrate standard errors of differences (sed). Significant differences among the GIT compartments with a probability scores less than 0.05 are indicated by an asterisk * and less than 0.01 indicated by **. The SED and asterisk can only be compared to each segment compartment blue-stomach orange-small intestine grey-large intestine yellow-caecum green-feacal pellet dark blue-total BSIgA %. Note the different axis for each time point.

At 7 minutes post gavage, there was a higher total percentage of BSIgA detected in the intestinal washouts from SMP gavaged mice compared with the intestinal washouts from WPC gavaged mice (66.34 vs 43.79 ± 6.9% respectively) ($P<0.001$)(Figure 14A). Similarly there was a higher percentage of BSIgA located in the stomach washouts from mice that were gavaged with SMP compared with WPC (20.66 vs 4.61 ± 5.0% respectively) ($P<0.001$). This was also true for the small intestine washouts (45.4 vs 39.1 ± 5.6 % respectively) ($P=0.038$). Comparing BSIgA levels in the washouts from the large intestine and caecum, there was no difference in the percentage of BSIgA recovered from SMP and WPC gavaged mice ($P>0.05$). Less than 0.5% of BSIgA was detected in the washouts from the large intestine and caecum for both SMP and WPC gavaged mice.

At 20 minutes post gavage, there was a higher total percentage of BSIgA detected from SMP gavaged mice relative to starting BSIgA amount, compared with WPC gavaged mice (22.40 vs 10.20 ± 5.0% respectively) ($P=0.025$) (Figure 14B). Similarly there was a higher percentage of BSIgA located in the small intestine washouts from mice that were gavaged with SMP compared with WPC (20.30 vs 8.30 ± 5.6 % respectively) ($P=0.045$). Comparing BSIgA levels in the washouts from the stomach, large intestine and caecum, there was no difference in the percentage of BSIgA recovered from SMP and WPC gavaged mice ($P>0.05$). Less than 2.5% of BSIgA was detected in the washouts from the stomach, less than 1% of BSIgA was detected in the large intestine, and less than 0.05% of BSIgA was detected in the caecum for both SMP and WPC gavaged mice.

Despite the small BSIgA percentages detected at 1 hour post gavage, there were still differences in BSIgA levels between the intestinal washouts and digestion of BSIgA in SMP and WPC (Figure 14C). At 1 hour post gavage, there was only 0.45 vs 0.10 ± 0.12% total BSIgA for SMP and WPC respectively ($P=0.013$). Once again there was a higher percentage of BSIgA located in the small intestine from mice that were gavaged with SMP compared with WPC

(0.25 vs 0.08 ± 0.05 % respectively) ($P=0.005$). Although not significant, there was a trend that more BSIgA was detected in the washouts from the stomach, large intestine, and caecum from SMP gavaged mice compared with WPC gavaged mice ($P>0.05$). On average there was less than 0.15% BSIgA recovered from the stomach washouts and less than 0.10% BSIgA recovered from the large intestine, and caecum washouts for both SMP and WPC gavaged mice.

At 4 hours post gavage, the difference in BSIgA levels from SMP and WPC gavaged mice was noticeable (Figure 14D). There was a higher percentage of overall total BSIgA recovered from mice that were gavaged with SMP compared with WPC (0.97 vs 0.14 ± 0.13% respectively) ($P<.001$). Percentage of BSIgA were also higher in SMP gavaged mice compared with WPC gavaged mice in the washouts from the small intestine (0.08 vs 0.02 ± 0.03% respectively) ($P=0.034$), large intestine (0.12 vs 0.04 ± 0.04% respectively) ($P=0.048$), caecum (0.47 vs 0.05 ± 0.07% respectively) ($P=<.001$), and faecal pellet (0.41 vs 0.06 ± 0.07% respectively) ($P=<.001$). The highest percentage of BSIgA was measured in the washouts from the caecum (0.47 ± 0.07%) and faecal pellet (0.42 ± 0.07%) from the SMP gavaged mice. The faecal pellet was only collected at 4 hours. Less than 0.50% BSIgA was measured in the stomach washouts and there was no difference in the amount of BSIgA measured from WPC or SMP gavaged mice ($P>0.05$).

Overall, BSIgA was detected in the washouts from all the GIT compartments and was detected at higher levels in the lower GIT compartments as post gavage digestive time increased ($P<0.05$). Throughout the GIT washouts, BSIgA was detected at higher levels in mice that were gavaged with SMP compared with WPC ($P<0.05$) (Figure 14A, B, C, D).

3.5 Buffer capacity of SMP and WPC matrix

The starting pH measured was 6.51 and 6.68 for WPC and SMP matrix, respectively. A total of 850 µL 2M HCl was added to WPC for the pH to drop to 2.15 whereas, more than double, 2100 µL 2M HCl was added to SMP for the pH to drop to 2.17 (Figure 15). The decline in pH was

rapid in the WPC matrix compared to SMP matrix.

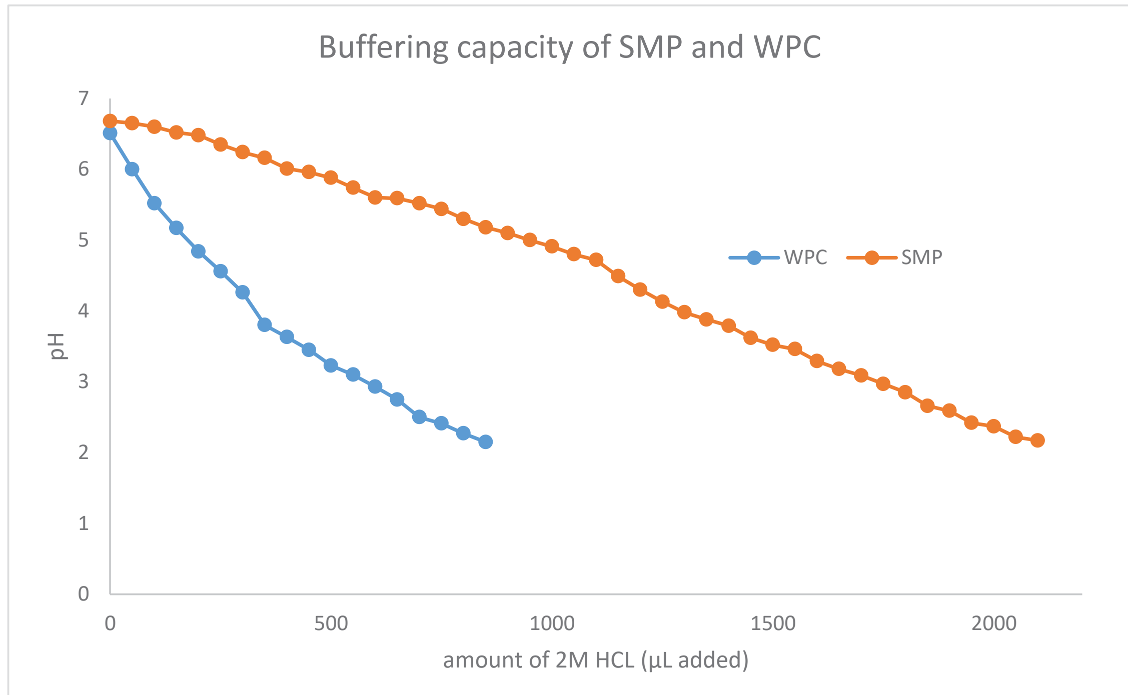

Figure 15 Buffering capacity of SMP and WPC.

Starting pH was 6.51 and 6.68 for WPC and SMP matrix respectively. 50 µL 2M HCL was added in increments and pH measured.

3.6 Effects of bovine SIgA to endogenous murine SIgA

To determine if the consumption of exogenous bovine SIgA had an impact on the amount of endogenous murine SIgA (MSIgA) detected, the murine SIgA levels in the washouts of each GIT compartment was measured. Treatment type and time data are presented in Table 7 as mean amount ± SED (mg/mL). Table 7 shows that there was no difference in the amount of MSIgA between treatment groups at 20 minutes, 1 hour, or 4 hours post gavage ($P<0.05$). There was a difference in the MSIgA levels between GIT compartments at each time point ($P>0.05$). The highest level of MSIgA was located in the small intestine washouts ranging from 0.78 to 1.82 ± 0.18 mg/mL and in comparison only small levels of less than 0.15 mg/mL MSIgA was located in the washouts from the large intestine, caecum, and faecal pellet. The amount of MSIgA detected in the washouts from the large intestine, caecum, and faecal pellet was similar to one another ($P>0.05$). MSIgA was detected in the stomach washouts; however, the amount was minuscule compared to the other GIT areas (> 0.01 ± 0.002 mg/mL).

Table 7 Amount of murine SIgA through the GIT of mice gavaged with either water, SMP, or WPC at 20 minutes, 1 hour, and 4 hours post gavage. Mean values of murine SIgA are presented as mg/mL. The standard error of the difference (sed) and F-probability are also presented.

	Water 20 min	SMP 20 min	WPC 20 min	Water 1hr	SMP 1hr	WPC 1hr	Water 4hr	SMP 4hr	WPC 4hr	SED	Fprob
Stomach	0	0.01	0	0	0	0	0	0	0	0.002	0.7
SI	1.7	1.8	1.5	0.6	0.7	0.8	0.9	0.9	1.0	0.19	0.5
LI	0.06	0.07	0.09	0.04	0.05	0.03	0.12	0.11	0.13	0.03	0.7
Cae	0.06	0.05	0.08	0.04	0.08	0.04	0.07	0.08	0.05	0.03	0.4
total	1.9	2.0	1.6	0.7	0.8	0.9	1.2	1.2	1.2	0.19	0.2
Faecal							0.13	0.08	0.10	0.02	0.2

Although these three treatments did not affect the amount of MSIgA detected, the post gavage time did impact MSIgA amount (Figure 16). The averages of the three treatments over the post gavage time points are presented in Figure 16. The post gavage time impacts the amount of MSIgA in each GIT compartment ($P<0.05$). MSIgA levels were the highest in each GIT compartment at the 20 minutes post gavage time point ($P<0.05$). From 20 minutes to 1 hour post gavage the amount of MSIgA decreased in each GIT compartment washouts ($P<0.05$). From 1 hour to 4 hours post gavage the amount of MSIgA increased ($P<0.05$) but, it did not return to the original levels detected at the 20 minute post gavage time.

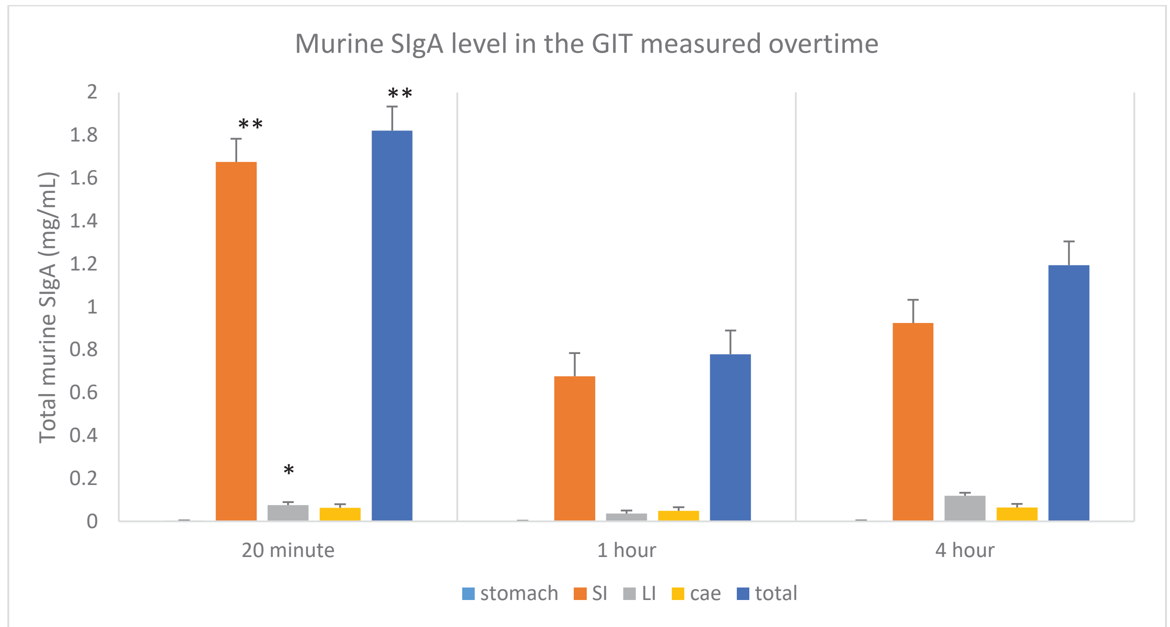

Figure 16 The amount of Murine Secretory IgA through the GIT of mice at 20 minutes, 1 hour, and 4 hours post gavage.

Pooled mean values of murine SIgA from mice that consumed SMP, WPC, and water are presented as mg/mL. Error bars illustrate standard errors of differences (sed). Significant differences among the GIT compartments with a probability scores less than 0.05 are indicated by asterisk * and less than 0.01 indicated by **. The SED and asterisk can only be compared to each segment compartment blue-stomach orange-small intestine grey-large intestine yellow-caecum dark blue-total MSIgA.

Chapter 4

Discussion

The purpose of this thesis was to test the central hypothesis that the milk matrix affects transit and digestion of BSIgA through the GIT of mice. The three aims of this study were:

- To compare whether the matrices of WPC and SMP effect digestion of the BSIgA within the product.
- To determine the transit time of BSIgA in SMP and WPC through the GIT of mice.
- To identify the impact exogenous bovine SIgA has on endogenous murine SIgA.

Data from the *In vivo* digestion of BSIgA through the GIT of mice revealed differences in BSIgA survival and transit rate depending on the milk matrix the SIgA was delivered in. Furthermore, exogenous BSIgA consumption did not impact MSIgA production.

4.1 Digestion of SIgA in SMP

From the experiments, we found BSIgA in SMP was digested, but, a proportion of BSIgA survived digestive processes as demonstrated by detection of BSIgA at all time points throughout the GIT of mice. The transit times of BSIgA and other bioactives will determine the location of the compounds for a timely release in the GIT (Nagakura *et al.*, 1996). This information can then be used to optimise oral administration of Immunoglobulins. Initially, transit times used in this study were based on published results of previous studies. As an example, when examining the transit time of orally administered carmine in mice it was found that a non-absorbable marker travelled approximately 50% along the small intestine after 20 minutes of administration and that the whole gut transit time ranged from 135 to 200 minutes (Nagakura*, et al.*, 1996; Carai *et al.*, 2006). Therefore, in the study reported here it was unexpected that after 7 minutes the highest amount of BSIgA was located equally in the stomach and small intestine washouts and at 20 minutes post gavage the highest amount was located in the small intestine washouts. These results indicate that the passage of BSIgA within SMP and WPC out of the stomach was rapid. After 1 hour post gavage, some BSIgA remained in the stomach, demonstrating the capability BSIgA has in surviving gastric digestion. Bovine secretory IgA is stabilised by the SC (Hurley & Theil, 2011; Corthesy, 2013a) and also resists digestion more efficiently than non SIgA compounds when transiting through enzymatic areas in the GIT (Stelwagen*, et al.*, 2009; Hurley & Theil, 2011). This may be one reason we were able to detect BSIgA throughout the GIT and in the faecal pellets.

Backer et al, (2008) used fluorescein labelled dextran (70kDa, FD70) to measure transit time in mice and similarly found that the marker was detected in the terminal ileum and caecum after

1 hour 30 minutes (Backer *et al.*, 2008). Thus, it was unexpected to observe that BSIgA was detected and remained distributed through the GIT, and was located in the caecum and large intestine after 4 hours post gavage. Reasons for BSIgA retention through the GIT may be attributable to IgA binding properties. SIgA binds to bacterial antigens in the large intestine by both specific binding through the Fab region and non-specific binding from the glycan's (Mathias & Corthésy, 2011; Hodgkinson*, et al.*, 2017). Furthermore, SIgA is retained in the intestinal mucus layer (Elson *et al.*, 1984).

There was some non-specific binding detected in the BSIgA ELISAs. This was confirmed by testing for cross reactivity with murine IgA and assaying a 'blank' (assay diluent containing BSA) sample. Based on these results, a baseline level of 9.14 ng/mL was set. Any values below 9.14 ng/mL were deemed non-specific background noise (refer to section 4.5). Therefore, in mice gavaged with SMP, BSIgA is not detected at meaningful levels in the large intestine or caecum washouts until 4 hours post-gavage. This is consistent with a published study where contrast media containing IgG reached the stomach at 10 mins and was detected in the small intestine at 20 minutes, 30 minutes and 3.5 hours after administration (Lee*, et al.*, 2012). The contrast media was detected in the caecum 5 hours post digestion (Lee*, et al.*, 2012).

4.2 Digestion of SIgA in WPC

From the experiments, we found the digestion and transit of BSIgA in WPC followed a similar trend to that of BSIgA in SMP mice (Figure 13). However, at 20 minutes post gavage, the highest amount of BSIgA in mice gavaged with WPC was observed in the small intestine washouts with lower amounts in the stomach washouts. This was in contrast to mice gavaged with SMP and indicates that movement out of the stomach is more rapid for BSIgA in WPC. Interestingly, at 7 minutes post gavage, in WPC gavaged mice BSIgA was detected in the caecum washouts at a value above the baseline level set for non-specific background noise. This suggests the transit of BSIgA was quicker when in WPC matrix compared to SMP matrix. It has previously been shown that whey proteins remain soluble and pass rapidly through the stomach (Boirie et al., 1997), and that WPC moves out of the stomach at a faster rate than SMP (Ye *et al.*, 2016). A recent *in vitro* study showed a reduction in gut motility when rats were fed WPC compared to a whey protein hydrolysate preparation. (Dalziel *et al.*, 2016). Our study looked at BSIgA transit rather than contractile frequency, however, it appears that the transit of SMP was slower than the WPC in our model, suggesting SMP may cause a reduction in gut motility compared to WPC. This is an area for further research.

After giving WPC, the highest amount of BSIgA was found in the washouts from the small intestine with some remaining in the stomach from both the 20 minutes and 1 hour post gavage period. This suggests BSIgA in WPC survives gastric digestion to some degree. The BSIgA measured in the washouts from the large intestine and caecum at 7 minutes post gavage was minimal but detectable at levels above the baseline value suggesting these values were real. It appeared that BSIgA in a WPC matrix rushed through the GIT quickly. This is possible as fluid components are rapidly dispersed through the stomach and can be retained for as little as one minute (Lentle & Janssen, 2011). Further, WPC does not have coagulating properties allowing rapid exit from the stomach (Boirie *et al.*, 1997) and movement of BSIgA from the stomach into the large intestine and caecum. This may be why BSIgA in a WPC matrix was detected in the lower GIT compartments at earlier post gavage time points than when in a SMP matrix. Using the same cut off criteria as above, no BSIgA was detected in the washouts from the large intestine and caecum at 20 minutes and 1 hour of digestion. There are multiple binding sites for BSIgA in the large intestine, including commensal bacteria and pathogenic bacteria (Macpherson*, et al.*, 2001). Therefore, BSIgA antigen binding sites may be saturated which would prevent ELISA detection. Alternatively, BSIgA could simply have been digested to the point where it could not recognised by the ELISA antibodies. The increasing levels of BSIgA from 1 hour to 4 hours of digestion may be because all GIT BSIgA binding sites were saturated, so more BSIgA was available for detection by ELISA. Also as time increases, BSIgA moved through the GIT and accumulated in the large intestine and caecum as mentioned in Section 4.1.

These reasons help to explain the different transit and detection levels of BSIgA for WPC and SMP gavaged mice. To determine which reason is more likely, microbes in the large intestine and caecum should be investigated further to determine if BSIgA antigen has bound to them. This has been observed by Mathias & Corthesy (2011), SIgA was demonstrated to bind to both gram positive and gram negative bacteria (Mathias & Corthésy, 2011).

In contrast to SMP gavaged mice, BSIgA from WPC gavaged mice is not detected in the small intestine washouts at 4 hours post gavage. Thus again supporting the evidence that BSIgA transit was faster in WPC gavaged mice compared with SMP gavaged mice. The amount of BSIgA detected in the washouts from the large intestine and caecum did not differ. Thus, BSIgA was distributed throughout the lower GIT at 4 hours.

4.3 Comparison of BSIgA digestion in SMP and WPC

Protecting SIgA from the unfavourable environments in the GIT is critical for passive immunity and for providing protection from pathogens in the small intestine via oral administration of BSIgA (Lee*, et al.*, 2012). BSIgA had an increased survival rate when consumed within SMP compared to WPC. The results in this study have shown that the detection of BSIgA through the GIT is not commensurate with the amount of BSIgA delivered. Instead, it is the matrix BSIgA was delivered in that effects the digestion and possibly the function of BSIgA in the GIT.

The gastric juices and acidic environment of the stomach decreased the percentage of BSIgA measured in the washouts by 15% at 7 minutes post gavage in WPC gavaged mice compared to SMP gavaged mice. The partial resistance to gastric digestion in the SMP matrix may be explained by the acidic conditions; stimulated gastric digestion revealed that when skim milk first enters the stomach the pH increased to greater than 6, therefore, pepsin activity was low (Ye et al., 2016). Over time the pH decreased and dropped to pH 2 (Ye et al., 2016). There was indication that BSIgA was digested at a quicker rate in WPC gavaged mice than SMP gavaged mice shown by the differences in total BSIgA recovery after 7 minutes. Movement of BSIgA out of the stomach was rapid, shown by higher amount of BSIgA detected in the small intestine washouts at 7 minutes post gavage for WPC gavaged mice and 20 minutes post gavage for the SMP gavaged mice. The rapid transit from the stomach to small intestine may reduce time available for BSIgA gastric digestion. However, BSIgA from WPC gavaged mice exited the stomach quicker than SMP gavaged mice. Thus, an increased time in the stomach does not mean BSIgA was being hydrolysed quicker as the SMP matrix may be harder to digest. We examined the buffering capability of the SMP and WPC products and observed differential acid buffering capacity which could impact *in vivo* digestion. The pH of WPC dropped from pH 6.51 to 2.15 after adding 850 µL 2M HCl to WPC matrix. In comparison the pH dropped from 6.68 to 2.17 after adding 2100 µL 2M HCl to SMP matrix. The data from this study shows that that a SMP matrix has an increased buffering capability as seen by slower decrease in pH compared with the WPC matrix. This may be another factor that is contributing to the different digestion rates of BSIgA in the two matrices. In order for SIgA to have a function beyond gastric digestion, adequate intra-gastric neutralisation is needed to increase the pH thus limit the activity of pepsin (Wilson & Williams Jr, 1969; McClelland*, et al.*, 1971; McClelland*, et al.*, 1972). As the fasted state of a mouse stomach is pH 4.0 and the fed state pH 3.0 (McConnell *et al.*, 2008), the acid buffering capacity shows that SMP will be able to maintain a more basic pH in the stomach, causing less pepsin activity (Ye et al., 2016).

Our observation that BSIgA was resisting digestion more efficiently in a SMP matrix compared to WPC matrix may be due to the differential milk components. As established earlier, the difference between SMP and WPC is that SMP contains caseins proteins. The caseins micelles present in SMP may be protecting BSIgA from gastric digestion allowing a higher percentage to enter the small intestine as an intact protein. Casein proteins; in conjunction with minerals, form micelle structures which can act as chaperones to help stabilise whey proteins (Holt *et al.*, 2013). This mechanism helps to stabilise and prevent precipitation of whey proteins due to pH and heat (Holt*, et al.*, 2013). Caseins micelles coagulate in the stomach due to both pepsin and low pH (Miranda & Pelissier, 1981; Ye*, et al.*, 2016). Commercial SMP forms a dense casein clot and has a slower rate of casein hydrolysis compared to heated commercial SMP (Ye*, et al.*, 2016). With denser casein clots proteolysis is slower as it occurs at the surface of the clot, so has a large surface area compared with loose clots where there is a faster diffusion of pepsin as the surface area is smaller (Ye*, et al.*, 2016). The SMP used in this study is manufactured with lower heat temperatures than commercial SMP, therefore, it can be assumed that the proteolysis of caseins is at the same or a reduced rate compared with commercial SMP. This information supports our findings that BSIgA was being retained for a longer period in the stomach within an SMP matrix compared with the WPC matrix. It also helps to explain why a higher percentage of BSIgA survived digestive processes more efficiently in SMP gavaged mice compared with WPC gavaged mice. Therefore, a SMP matrix has both casein protective effect and buffering capacity to limit the rate of pepsin digestion of BSIgA in the stomach.

Once BSIgA reaches the small intestine the protein complex in SMP and WPC is relatively resistant to digestion (Hurley & Theil, 2011). BSIgA survived 20 minutes post gavage more efficiently in SMP gavaged mice compared with WPC gavaged mice. The highest percentage of BSIgA was detected in the small intestine. Similar to the results of this study, Roos et al found BSIgA to be partially resistant to human gastric digestion with 20% SIgA located in the ileal effluents (Roos*, et al.*, 1995). Proteolysis in the gastric and intestinal compartments reduced the quantities of intact proteins. This study showed that at 1 hour post gavage nearly all BSIgA in the washouts was digested and less than 1% of BSIgA survived digestive processes. However, at all time points a higher percentage of BSIgA survived digestion in the SMP compared to the WPC. The main difference in BSIgA recovery from SMP compared with WPC was seen in the small intestine, at all time points. This is also the most important difference due to the biological function of BSIgA. In the intestinal lumen, IgA has an antimicrobial function and directly interacts with pathogens. SIgA blocks pathogenic microorganisms from entering the intestinal lumen by blocking their access to epithelial receptors and trapping them

in mucus to facilitate their expulsion through peristalsis in the GIT (Mantis*, et al.*, 2011). These functions require at least partial retention of non-denatured protein conformation (Hurley & Theil, 2011).

From measurement, the amount of BSIgA recovered from the GIT washouts at 4 hours post gavage was similar to the amount of BSIgA recovered from the GIT at 1 hour post gavage (ranging from 0.10-0.90%). The main observational difference between the 1 hour and 4 hour post gavage time points was the higher BSIgA levels seen in the lower GIT, caecum and faecal pellet washouts at the 4 hour post gavage time point (Figure 5). This suggests that after a certain period post gavage (1 hour), BSIgA continued to move through the GIT without further digestion. Although not significant, there was a trend for more BSIgA to be detected in the washouts from the stomach, large intestine, and caecum from SMP compared with WPC ($P>0.05$). Therefore, BSIgA was digested more rapidly when contained in WPC than SMP.

Despite the low BSIgA recoveries measured throughout the GIT, this amount may still be sufficient to have biological significance. The different survival rates of BSIgA in WPC and SMP may help to explain BSIgA differential efficiency to modulating infection. A study by Cakebread et al. (2017) (manuscript in preparation) investigated the effect of BSIgA in SMP and WPC on mice that were infected with Citrobacter rodentium (*C. rodentium*). Cakebread et al (2017) used the same WPC as used in this study and a similarly processed SMP, along with commercial WPC. Their findings showed that mice infected with *C. rodentium* and fed SMP had reduced faecal *C. rodentium* counts compared to their counters that were fed water. In contrast the WPC treatment did not impact *C. rodentium* infection. Their findings suggest that BSIgA in SMP, but not WPC, can suppress pathogens in the GIT. In a mouse model, the differential milk matrix ability to supress pathogens may be due to the level of BSIgA available in the GIT. It is now evident that SIgA survives digestive processes more effectively when consumed in a SMP compared to WPC. Therefore, more BSIgA is available in the GIT to exert biological functions and to supress pathogens.

WPC can be manufactured by either rennet or acid treatments and commercial production results in reduced antigen specific antibody activities of SIgA, IgG and IgM (Ando*, et al.*, 2005). The WPC in this study was manufactured using acid treatment, however, antibody activity is reported to be higher from whey produced from rennet treatment (Ando*, et al.*, 2005). Further, these two products contain different specific vitamin profiles and specific amino acids (Ando*, et al.*, 2005). Therefore, the manufacturing processes of a product are important for

bioavailability of its components. It has also been suggested previously that the loss of antibody activity may be due to the involvement of the Igs in casein precipitate.

Additional work after the main trails of this study investigated SMP processed differently to the first SMP used. The results show similar BSIgA digestive behaviour between the two SMP matrices. Therefore, the results are reproducible and confirm that SMP has a protective effect and increases BSIgA survival in the GIT. Overall, it can be concluded that the food matrix influences the biological activity of foods.

4.4 Impact of bovine SIgA on endogenous murine SIgA

The results from this study indicate that BSIgA did not impact the level of endogenous MSIgA after 20 minutes, 1 hour, or 4 hours post gavage. However, it should be noted that change to endogenous murine production or degradation after 4 hours of treatment is unexpected. In an earlier study, results suggested that BSIgA impacted the level of endogenous MSIgA as the highest MSIgA level was measured in the faecal pellets of mice in the water-fed group and the lowest MSIgA level measured from mice in the high-IgA fed group. The main difference between these two studies was that the preliminary study measured MSIgA from mice that had consumed treatment *ad lithium* for two weeks. Whereas, the mice in this study was gavaged once with the treatment and culled at a significantly shorter time point. Therefore, it is expected that there are differences in the two studies results due to the differing levels of milk consumed by the mice and the time period measured.

Instead, the results of this study did reveal that the level of MSIgA was impacted by gavaging. Interestingly, MSIgA was detected in the stomach washouts, however, the amount was minuscule, at less than 0.01 ± 0.002 mg/mL compared to the other GIT areas. As the stomach does not produce SIgA, the small amounts detected here are likely to be from salivary IgA entering the stomach (Brinkworth & Buckley, 2003). This information supports SIgA capability of surviving gastric digestion. Since SIgA is produced and transported into the small intestine by plasma cells in the lamina propria, it is expected that the highest level of MSIgA would be located in the small intestine which was the case in this study (Macpherson*, et al.*, 2001; Corthesy, 2013a).

Murine SIgA production or degradation did not vary in mice between treatment groups but varied between individual mice. However, this variation was not high enough to effect significant differences between samples. Conversely Elson and colleagues reported total SIgA recovered to be highly variable, even in the same group of mice sampled on multiple occasions

(Elson*, et al.*, 1984). This may be due to differences in technique used; Elson and colleagues used a wash-out technique over a period of 20 minutes. Therefore, their method doesn't account for continuous MSIgA production, degradation and MSIgA already located in the lower GIT (large intestine and caecum) (Elson*, et al.*, 1984). They suggested their variation was due to SIgA being retained by the intestinal mucus layer. The method in this study was based on a pilot study which showed that flushing the intestine twice with a total of 0.75-1 mL PBS is sufficient in obtaining an estimated 80% of MSIgA from the intestine. Therefore, the method in this study is more consistent and this is shown by the results being less varied.

A recent study by Kikuchi, *et al*., (2014) observed that intestinal IgA production increased in response to the oral administration of the virus *L plantarum* (Kikuchi, Kunitoh-Asari *et al.* 2014). It was also evident that starvation impaired both IgA production and transport due to reduced levels of T regulatory cells, important for producing cytokines necessary for IgA production (Fukatsu & Kudsk, 2011). Furthermore, nutrients such as butyric acid and glutamine stimulate IgA production (Fukatsu & Kudsk, 2011).This leads to speculation, that starvation of the mice overnight in this study either reduced MSIgA production or increased MSIgA degradation and the physical administration of oral gavage, or receipt of nutrient into the stomach then stimulated the MSIgA production or decreased MSIgA degradation. Overall, it was concluded that oral administration of BSIgA did not impact the amount of endogenous MSIgA detected.

4.5 Limitations

An ELISA is an analytical technique that has been shown to measure the Ig concentration through fluorescently labelled antibodies with a high level of sensitivity and specificity (Voller *et al.*, 1978). However, through cross reactivity tests from the BSIgA and MSIgA ELISA kits it was discovered that an unknown protein in the MSIgA kit binds non-specifically to the BSIgA standard. The BSA buffer and all MSIgA standard concentrations measured 9.14 ng/mL. The water control test samples also had non-specific and inconsistent binding. These results were accounted for as BSIgA levels below the top BSIgA value detected in the water group for each GIT was the baseline point and anything below these values could not be assumed to be real BSIgA values. To account for the samples measured on different ELISA plates, a quality control sample was run with each assay to monitor inter-assay variation. When comparing BSIgA values relative to their starting concentration for WPC and SMP there is no need to take into account the water values. This is because we can assume that both the SMP and WPC gavaged mice detect the same background amounts so are consistent with each other and are directly

comparable. The end weight flushing recovery for each GIT nor the sex of the mouse did not affect the amount of BSIgA obtained (Appendix B.3 and B.4).

The ELISA used in this study detects the secretory component of the molecule, a small part of the antibody. Thus the detection of total SIgA is only as good as the antibody provided in the kit. Also measuring the change in total amount of SIgA present may be misleading to assess the digestive effects on SIgA functionality. It is possible that some of the SIgA detected may have lost its antigen binding ability. Conversely, fragments of IgA no longer detectable by the ELISA antibody but with glycans still attached could still have activity. This is something that needs further study.

Bovine SIgA had no impact to the level of detectable endogenous MSIgA. Therefore one methodological limitation is that the experiment addresses the effects of BSIgA on endogenous MSIgA at 20 minutes, and 1 hour post gavage. These times differences are too short to expect to observe a measurable difference in endogenous MSIgA levels. The level of MSIgA was measured at the three time points due to convenience. This study wanted to see if BSIgA could impact endogenous murine IgA production or degradation in a short period. As this was discovered to be false it would be beneficial to look at the impact at a longer time point with continuous BSIgA consumption, similar to that of the preliminary study.

4.6 Future research

There are future opportunities for the development of a high BSIgA milk product that can be used for treatment or prevention of gastrointestinal infections by pathogens. For this to occur, it's important to design oral dosage forms of BSIgA with maximum resistance to proteolysis. To my knowledge, this study is the first to report that BSIgA digestion differs depending on the milk matrix it is embedded in. Thus, this study demonstrates how a product can be designed to enhance the amount of bioactive (BSIgA) reaching the small intestine and large intestine.

Examining the different peptides present in the digesta at each stage to compare the movement of other proteins in SMP and WPC could be investigated. Further, investigation on how different amino acid profiles and overall milk composition potentially results in different functionalities is an area of interest. To determine why there may be digestive difference of BSIgA between a SMP and WPC matrix, analysing the GIT samples on using SDS-PAGE would be beneficial. This will help reveal, compositional changes at each GIT site over time and whether or not it is the casein proteins that are providing a protective effect in the stomach. Having a molecular weight marker in a SDS-PAGE gel will help to identify which proteins have been

digested. A western blot or mass spectrometry could also be used for protein/peptide identification. Also measuring the functionality of digested BSIgA may be useful to determine if the digested fragments have a protective function in the GIT.

The possible implications of these experimental results could be advantageous to the farming industry. The increase in consumers wanting natural remedies, rather than antibiotics, may increase the demand for milk products with high levels of SIgA, therefore, increasing its commercial value. Finally, further investigation should be carried out to develop products that protect the bioactive compounds from digestion and deliver the bioactive to the required location.

4.7 Conclusions

It has been established that substances within SMP have a considerable influence on the BSIgA molecule resisting digestive processes. BSIgA within WPC digests at a much faster rate compared with BSIgA within SMP. The results of this research demonstrated that BSIgA was predominantly digested in the stomach. The highest amount of BSIgA was detected in the washouts of the small intestine and as digestion time increases the BSIgA transits through the GIT. After a single gavage of SMP, BSIgA remains in the GIT at levels above baseline for the maximum post digestive time measured, 4 hours. It is suggested that the increased protection of BSIgA from a SMP matrix is due to the caseins present and their increased acid buffering capacity. In conclusion, SMP provides a suitable matrix to assist with BSIgA delivery.

Chapter 5
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Chapter 6
Appendix

A Methods

A.1 Gavage procedure

Sex	Animal #	ID	Treatment	Gavage freq/min	Cull freq/min
F	31	red	Water	10.00am	10.20am
M	41	red	SMP	10.10am	10.30am
F	51	blue	WPC	10.20am	10.40am
M	32	blue	Water	10.30am	10.50am
F	42	yellow	SMP	10.40am	11.00am
M	52	yellow	WPC	10.50am	11.10am
F	33	black	Water	11.00am	11.20am
M	43	black	SMP	11.10am	11.30am
F	53	orange	WPC	11.20am	11.40am
M	34	orange	Water	11.50pm	12.10pm
F	44	green	SMP	12.00pm	12.20pm
M	54	green	WPC	12.10pm	12.30pm
F	35	white	Water	12.20pm	12.40pm
M	45	white	SMP	12.30pm	12.50pm
F	55	green head	WPC	12.40pm	1.00pm

A.2 SIgA Wash out recovery

An experiment was conducted to determine the volume required to recover murine SIgA in the small intestine of mice. Three mice were used to wash out small intestine contents with 500µL PBS + protease inhibitor either once, twice, or three times. The 2.4.2 dissection and 2.5 sample processing methods were used. Figure 17 shows that the first wash out yields most SIgA and that there is not a significant difference between wash two and three. Therefore it was concluded that two washes with a total of 1mL is sufficient to recover ~80% SIgA through the GIT segments.

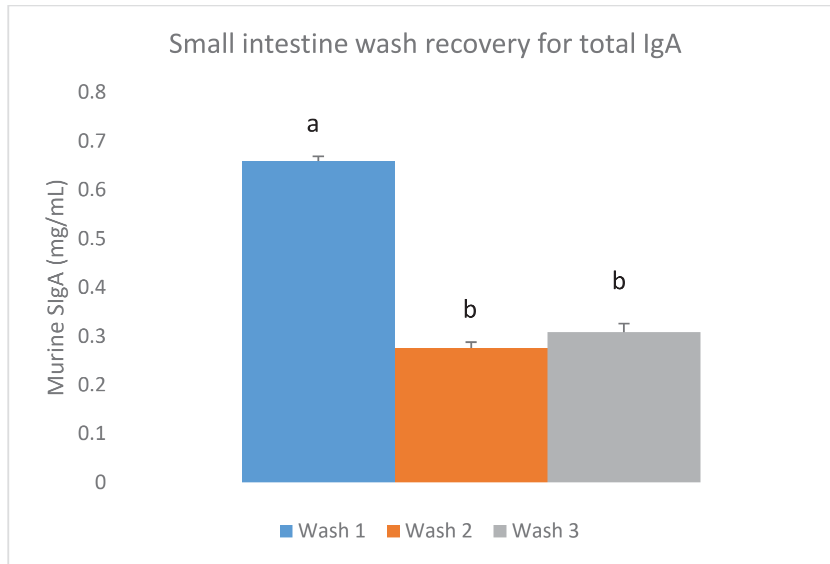

Figure 17 Wash out recovery for total Murine SIgA with 500uL PBS + protease inhibitor either once, twice, or three times.

B Results:

B.1 Non-specific bovine SIgA detection in the water samples

Table 8 Maximum log values of BSIgA detected in each GIT for 20 minutes, 1 hour and 4 hours for each GIT compartment in water mice. Overall maximum geometric log value detected, average geometric log values detected, and percentage of detection (%) for each GIT compartment in water mice. Note water samples were not measured at 7 minutes.

GIT compartment	20 minute maximum	1 hour maximum	4 hour maximum	Overall maximum	Average	% of detection
Stomach	3.87	2.81	5.29	5.29	1.96	23.33
Small intestine	8.77	11.41	0	11.41	4.65	50
Large intestine	3.69	0	0	3.69	1.07	6.67
Caecum	4.86	3.89	5.5	4.86	2.00	16.67

B.2 Gender effect

There was no gender or gender treatment effect on BSIgA values (P>0.05) (Table 9).

Table 9 Transit and digestion of bovine SIgA through the GIT of male and female mice. Log Mean Bovine SIgA percentage ± SED through the GIT of male and female mice. Significant differences are reported as Fprob. Significance P<0.05.

LOG Bovine SIgA %	Female	Male	gender SED	gender Fprob
Stomach	0.25	0.18	0.11	0.48
Small intestine	0.72	0.88	0.18	0.39
large intestine	0.11	0.03	0.08	0.32
Caecum	0.06	0.10	0.02	0.10
Total SIgA	1.05	1.10	0.12	0.69

B.3 End weight recovery

There was no difference in the end weight recovery of volume of PBS + protease inhibitor flushing for each GIT compartment over all time points (P>0.05) (Table 10).

Table 10 Mean values ± standard error of the difference (SED) (g) for end weights of recovered sample of SMP and WPC at 20 minutes, 1 hour, and 4 hours.

End weight recovery (g)	SMP 20min	SMP 1hr	SMP 4hr	WPC 20min	WPC 1hr	WPC 4hr	Interaction sed	Interaction Fprob
Stomach	0.82	0.88	0.78	0.82	0.86	0.77	0.03	0.98
Small Intestine	0.73	0.69	0.75	0.70	0.66	0.73	0.05	0.96
Large Intestine	0.70	0.78	0.61	0.66	0.74	0.59	0.04	0.91
Caecum	0.87	0.76	0.85	0.87	0.75	0.85	0.04	0.96