



# **Modulation Of Fat Digestion Using Bioactive Alginates**

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## Abstract

Obesity is a fast growing medical issue worldwide and is one of the leading causes of mortality. There is evidence to suggest that various forms of dietary fibre may be used in weight management and for their general health benefits. There is evidence to suggest that the addition of alginate to a food or beverage has the potential to alter the digestion process and potentially reduce the activity of digestive enzymes. Data from this laboratory have demonstrated that alginate possesses the ability to inhibit pancreatic lipase in-vitro. Pancreatic lipase is secreted in the small intestine and hydrolyses fat so that it can be absorbed in the digestive system. If the activity of pancreatic lipase can be reduced then the amount of fat absorbed will be reduced.

A modified Periodic Acid Schiff's (PAS) assay is an effective, repeatable and simple method for quantifying alginate in solution. Furthermore with any potential weight loss treatment the release rate must be determined. The model gut system developed in this laboratory is able to digest the alginate enriched bread and the modified PAS assay system is able to quantify the release rate of the alginate, with between 12-20% released in the gastric phase and 80-88% released in the small intestinal phase. The data here also confirms that the bread is protecting the alginate during the cooking process, and that the alginate retains its inhibition properties despite the cooking and digestion process.

The model gut is an effective model for digesting fat substrates and foods high in fat such as butter and olive oil. Alginate enriched bread has the ability to inhibit fat substrates glyceryl trioleate, trioctanoate, tributyrates, and foods butter and olive oil 62, 39 and 32, 95 and 78% respectively after 180 minutes in the model gut.

Alginate enriched bread is able to modulate fat digestion in ileostomy subjects following ingestion. This was evident by a significant increase in fat content of the effluent fluid. There was an increase in fat content of 81g and 112g when subjects consumed alginate bread when compared with control bread at 240-270-300 minutes and when total fat was combined for all time points respectively. There was also an increase of 292g in wet effluent weight for alginate bread when compared with the control bread. In addition a significant relationship between the wet effluent weight and fat content of the effluent was reported. The alginate enriched bread had no problems with palatability or adverse side effects associated with fat digestion and suggest that alginate enriched bread may be a suitable method for the delivery of alginate into a normal diet. The data in this thesis

suggests that alginate enriched products are able to attenuate the digestion of fat and therefore may be used in weight management and in the treatment of obesity, however further longitudinal clinical research is required in a healthy population before any health claims can be made.

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## List of Abbreviations

AB	Alginate Bread
AOAC	Association of Official Agricultural Chemists
ATP	Adenosine Triphosphate
BMI	Body Mass Index
DAP	Dihydroxyacetone Phosphate
DB	Dextran Blue
DMMB	Methylene Blue Hydrate
CB	Control Bread
CNS	Central Nervous System
CRF	Clinical Research Facility
FA	Fatty acids
FFA	Free fatty acids
G-1-P	Glycerol-1-Phosphate
GI	Gastrointestinal Tract
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
$K_m$	Michaelis Menton Constant
MO	Methyl Orange
MRI	Magnetic Resonance Imaging
NHS	National Health Service
$\eta_{sp}$	Specific Viscosity
$\eta_{rel}$	Relative Viscosity
OD	Optical Density
PAS	Periodic Acid Schiffs Assay

R	Pearsons correlation coefficient
S.E.M	Standard error of the mean
SD	Standard deviation
S-O	Safranin-O
T-B	Toludine Blue
WHO	World Health Organisation
4-AAP	4-aminoantipyrine

## Chapter 1: General Introduction

### 1:1 Obesity

The World Health Organisation (WHO) defines the term “obese” as “abnormal or excessive fat accumulation” which has been strongly linked with ill health and disease. To classify whether an individual is obese a simple technique that uses an index of height and weight, called body mass index (BMI) is used. A BMI of  $25\text{kg/m}^2$  is judged to be overweight, and over  $30\text{kg/m}^2$  is defined as obese, with almost all social science associated with obesity uses BMI, despite its inability to distinguish between fat and fat free mass such as bone and muscle (Burkhauser & Cawley, 2008). Despite the flaws with this technique the WHO reported that in 2005 1.6 billion people were overweight and 400 million were obese, however this is expected to have risen to 2.3 billion and 700 million, respectively by 2015. Furthermore obesity can no longer be considered an issue just for western countries. Similar obesity trends are being experienced in the so called developing 3<sup>rd</sup> world countries, specifically in urban settings (Tucci, Boyland, & Halford, 2010).

The fundamental cause for becoming obese and overweight is an energy imbalance between the number of calories consumed, and the number of calories expended. The current social environment provides frequent opportunities for consuming large amounts of food that are easily accessible and relatively inexpensive (Hill, Peters, & Wyatt, 2007). These easily accessible foods are generally processed and high in fat and sugars, but low in fibre, vitamins and mineral (Hill, 2006). This dependency on processed foods that are high in fat and sugars has been compounded by the low levels of physical activity. Low levels of physical activity are due to the sedentary nature of many jobs, increased urbanisation and a greater reliance on transport as opposed to walking.

Those individuals with a BMI of over  $30\text{kg/m}^2$  have been linked to an increased risk of chronic diseases, such as cardiovascular disease (CVD), which accounts for over 17 million deaths per year. The WHO indicated the number of people who are obese is increasing, as is the number of people with diabetes. Musculoskeletal disorders such as osteoarthritis is also on the rise, due to the excess weight placing extra stress on the weight bearing joints, such as the knees and hips increasing the risk of cartilage damage.

Although not completely understood or the initial cause, certain cancers have been associated with obesity. Cancers such as pancreatic, endometrial, breast and colon have all been linked to obesity. Due to the excessive cost associated with the high level of inactivity and increased risk of disease, a suitable and cost effective treatment for obesity is sought after.

### **1.1.2 Current obesity treatments**

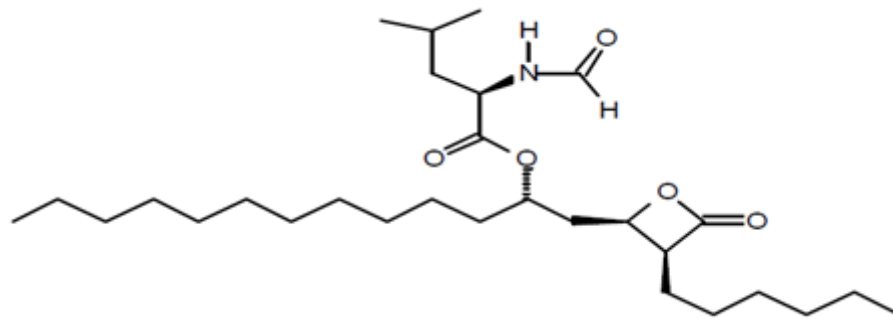
The NHS's attempt at prevention as opposed to treatment is to advise people to eat a more healthy diet and exercise more, thus increasing energy expenditure and decreasing calories consumed. Exercising more is a key factor to weight loss with the NHS advising the most effective types of exercise as 'aerobic' activities that use large muscle groups in an attempt to increase the energy expenditure. Despite the advice of many experts, exercise is rarely maintained, with people preferring to lose weight through a variety of different diets (Miller, 1999). Anderson, Konz, and Jenkins (2000) addressed the issue that the majority of diets are 'fad' diets supporting unsafe practices, which have detrimental health issues attached. Such diets include "Atkins Diet", "Protein Power", "Sugar Busters" and "The Zone". Anderson et al. (2000) echoed the recommendations from the WHO and NHS, and stated that diets should include wholegrain or wholemeal, such as brown rice, pasta and bread, as these can contain large quantities of fibre. A healthy diet or even the fad diets as mentioned previously are rarely maintained, with an overall success rate of just 15% (Ayyad & Andersen, 2000) thus the weight they have lost generally returns within one or two months of the initial weight loss. Due to individual's inability to maintain diet and exercise other obesity treatments have been developed, including pharmaceutical interventions. (Ayyad & Andersen, 2000).

Of those alternative obesity treatments possibly the most common one is bariatric surgery, specifically for those who are morbidly obese. Bariatric surgery includes a wide variety of procedures, with one of the most common reducing the size of the stomach with a medical apparatus which is inserted into the stomach (gastric banding). Another common treatment is the removal of part of the stomach or re-routing the small intestine to a small stomach pouch (gastric bypass) (Santry, Gillen, & Lauderdale, 2005). A review by Buchwald et al. (2004) identified that between 1997 and 2003 patients who underwent gastric banding and gastric bypass had a weight loss of 61.6% and 68.2% respectively. In addition to weight loss a considerable number of patients also had improvement in a number of obese related conditions including hypertension, obstructive sleep apnea and

diabetes. Irrespective of the beneficial effects of bariatric surgery the costs and complications associated with it may outweigh the benefits. Encinosa, Bernard, Chen, and Steiner (2006) conducted a study of insurance claims in the USA and 21.9% of all patients suffered with some form of complication whilst in hospital, and a further 40% in the subsequent six months. Complications included gastric dumping syndrome, leaking at the surgical site, incisional hernia, infections and pneumonia.

An additional treatment is drugs that target the mechanisms underlying the regulation of appetite and energy. There are a number of pharmacological anti-obesity treatments available that target the central nervous system to suppress appetite and therefore decrease food intake. Although these treatments do possess the ability to aid in weight loss, they also have serious detrimental health effects. Amphetamines, Phenylpropanolamine and Rimonabant are all drugs used as appetite suppressant and reduce the Kcal an individual may consume (Fujimiya, Okumiya, & Kuwahara, 1997; Pi-Sunyer, 2006; Rich, Rubin, Walker, Schneeweiss, & Abenhaim, 2000). Although all of these drugs have been associated with weight loss, the adverse side effects including headaches, irregular heartbeat, vision problems, rash, psychological problems and in extreme cases stroke and damage to the valves of the heart has led to the removal of these drugs from the market.

The most frequently used anti-obesity drug is Orlistat, released in 1998 by Roche Pharmaceutical Company. Orlistat (tetrahydrolipstatin) (**Figure 1**) as an alternative treatment to obesity. Unlike the previously mentioned drugs Orlistat acts peripherally at the gastrointestinal tract, with only 1% being absorbed into circulation, therefore reducing the risk of physiological and psychological side effects. Orlistat is a semi synthetic hydrogenated derivative of natural occurring *Streptomyces toxytricini*, which has been shown to inhibit gastric and pancreatic lipase and colipase (Al-Suwailem, Al-Tamimi, Al-Omar, & Al-Suhibani, 2006; Sternby, Hartmann, Borgstrom, & Nilsson, 2002).



**Figure 1.** Chemical structure of tetrahydrolipstatin (Orlistat).

Orlistat has the ability to bind to the active site of pancreatic lipase, resulting in acylation of a hydroxyl group on serine residue at the active site of the enzyme. The acylation of a hydroxyl group is an irreversible reaction, and one that results in its redundancy as a lipase (Hadvary, Lengsfeld, & Wolfer, 1988). In human studies enzyme inhibition up to >90% has been reported, without affecting trypsin, amylase, chymotrypsin and phospholipases, which is surprising especially as trypsin has a serine at the active site of the enzyme (Cudrey, van Tilbeurgh, Gargouri, & Verger, 1993; Sternby et al., 2002).

Sternby et al. (2002) observed the effects of Orlistat mixed into a liquid meal and in capsule form in healthy individuals. The authors reported that adequate mixing of Orlistat (60mg three times a day) and the liquid meal in the gastrointestinal tract was sufficient to inhibit all the major enzymes involved in lipid digestion, such as gastric lipase, colipase dependant lipase and carboxyl ester lipase by up to 90%. Furthermore Cudrey et al. (1993) identified that Orlistat has a powerful inhibition specifically towards pancreatic lipase which is responsible for the breakdown of dietary triglycerides into absorbable free fatty acids and monoglycerides. Therefore by inhibiting pancreatic lipase Orlistat reduces triglyceride digestion and thus its absorption by up to 35% (Al-Suwailem et al., 2006).

In a clinical setting Orlistat has also been used to treat obesity in humans, in an attempt to observe if enzyme inhibition can reduce fat digested, and ultimately fat absorbed and stored as adipose tissue. Prior to the release of Orlistat in the UK, Lucas (1998) conducted a longitudinal study to test the efficacy of the drug as a long term treatment for obesity. The study was conducted over 52 weeks to observe the effects on weight loss, low density lipoproteins (LDL)-cholesterol and blood pressure in 753 obese and overweight individuals with BMI of 28-40 kg/m<sup>2</sup>. The treatment group received 120mg of Orlistat and the second group received 120mg of placebo with both groups consuming 600

Kcal/day less than the normal recommendation for male and females. In the treatment group participants lost 10.25% of their weight, compared to 6% in the placebo group. In addition the treatment group had greater reductions in LDL-cholesterol and diastolic blood pressure in comparison with the placebo group (5.5% vs. 2.2% and 8.2mmHg vs. 6.1mmHg, respectively). The conclusion from this study suggest that long term treatment of obese and overweight individuals using Orlistat in combination with a hypocaloric diet can significantly reduce weight and reduce additional risk factors associated with obesity. Unlike amphetamines and phenlypropanolamine only one serious misuse of Orlistat has been reported. This individual used Orlistat during binge eating which resulted in abnormal bowel movements up to seven times a day, severe fecal urgency, oily rectal spotting and severe flatulence after every binge (Malhotra & McElroy, 2002). Despite this one incident of misuse there have been limited to no physiological or psychological adverse effects. Zhi et al. (1996) provided evidence for the safe use of Orlistat and confirmed this by demonstrating that only 1% was recovered in the urine of participants; with a further 96% recovered in the stool samples over four days post treatment. In addition the authors also analysed plasma samples over 16 days post treatment, reporting that Orlistat was undetectable in plasma samples, supporting the indication that Orlistat has minimal risk of physiological and psychological adverse effects. Although the findings of Zhi et al. (1996) suggest that Orlistat was not present in urine, stool and plasma samples four days post treatment there was no evidence regarding cell content in the gastrointestinal tract. As previously mentioned Orlistat is lipophilic in nature and therefore could be readily incorporated into cellular membranes and may even result in cellular death due to a build-up of Orlistat.

Although there is substantial evidence supporting Orlistat as an obesity treatment, there is also research suggesting that Orlistat has adverse effects associated with it (Finer, James, Kopelman, Lean, & Williams, 2000). Despite the positive results in terms of weight loss of Orlistat participants did suffer with undesirable side effects including over 58% suffering with gastrointestinal events. Such events ranged from flatulence, vomiting, uncontrolled oily discharge, increased defecation, abdominal pain, faecal urgency and loose stools. In addition respiratory problems, headaches and back pain were also experienced, albeit a smaller percentage of participants. These findings were also echoed in a more recent short-term study by Kaya et al. (2004). This study conducted a 12 week randomised study using Orlistat as an anti-obesity treatment on participants with a BMI of 36kg/m<sup>2</sup>. Just as with previous studies weight loss was reported, with mean of 9.35kg



in the treatment group. Nevertheless similar to previous studies (Finer et al., 2000) 48% participants reported adverse side effects such as diarrhoea, flatulence, fatty stools, fecal incontinence, headaches and even forgetfulness in some participants throughout the entire 12 week study. In retrospect the long and short-term benefits of Orlistat as an obesity treatment cannot be denied; nonetheless the adverse side effects can make it an unpleasant treatment for patients, therefore an obesity treatment which may still offer the same benefits as mentioned, without adverse side effects needs to be produced.

## 1.2 Dietary Fibre

The term dietary fibre was believed to be first applied by Hipsley (1953), as a shorthand expression for non-digestible constituents that make up the cell walls of plants. It is essentially a non-starch polysaccharide, which includes components of plants such as arabinoxylans, cellulose, inulin, lignin, pectins and beta-glucans (DeVries, 2003). Dietary fibre is not homogeneous in nature and can vary in its composition, which is generally a mixture of water soluble (pectin, algal polysaccharides and some hemicelluloses) and water insoluble (cellulose, lignin and some hemicelluloses) components (Roehrig, 1988).

Hipsley (1953) first made the observation that populations consuming a diet high in dietary fibre had lower rates of pregnancy toxemia, leading to other health implications. In light of the earlier work numerous studies have been conducted since, suggesting that dietary fibre intake was associated with a range of nutritional and health related benefits (Burkitt, Walker, & Painter, 1972; Trowell, 1973, 1978). Asp (1987) concluded that dietary fibre resisted hydrolysis by human alimentary enzymes and therefore escape digestion and absorption in the gastrointestinal tract. The beneficial effects of fibre are related to gut health where fibres add bulk to faeces and aid passage through the digestive system preventing constipation. Additional advantages of a high fibre diet include reducing harmful microflora (Terada, Hara, & Mitsuoka, 1999), prolonging satiety, increasing transit time, decreasing gastric emptying (Holt, Heading, Carter, Prescott, & Tothill, 1979), reducing break down of carbohydrates thus slowing down absorption of glucose, reducing LDL in blood and increasing cholesterol turnover (Sandberg et al., 1994).

The WHO and the NHS recommend that a healthy diet should include dietary fibre. Howarth, Saltzman, and Roberts (2001) confirmed this in a review and suggested that a diet including up to 30g/d of dietary fibre may reduce the growing number of people becoming obese. Despite the recommendation from the WHO, NHS and the review from

Howarth et al. (2001) regarding the potential health benefits of dietary fibre the recommend quantity of dietary fibre that should be consumed varies between countries. The daily fibre intake recommended today by the America Heart Association is 14g per 1000 calories consumed; therefore the average male consuming 2700 calories should be consuming 38g of fibre per day. Contrastingly the British Heart Foundation suggests only 18g of fibre per day, in the average male consuming 2550 Calories. It is apparent that there are varying recommendations for the levels of fibre intake each day, however it remains an area of debate what the optimal amount of fibre consumed should be. Irrespective of this debate, there is substantial research supporting the idea that dietary fibre may reduce the risk of morbidity and aid the digestive process, through such mechanisms as enzyme inhibition and those previously mentioned.

### 1.3 Enzyme Inhibition

Current evidence suggests that dietary fibres may inhibit digestive enzymes such as lipase, trypsin, pepsin and amylase amongst others (Eastwood, 1992). The research into the use of dietary fibres as enzyme inhibitors stemmed from earlier work (Burkitt et al., 1972; Hipsley, 1953; Trowell, 1973, 1978). Schneeman and Gallaher (1980) fed rats for 10 days on semi-purified diets including either 20% cellulose or no fibre for 10 days. The rats were killed 30 minutes post eating, after which the whole intestine was removed and washed with cold sodium chloride before being frozen at  $-60^{\circ}$ . Prior to analysis of the intestinal contents were defrosted and then homogenised. They were then assayed for the activity of trypsin, chymotrypsin, amylase and lipase. The group of rats who consumed cellulose had lower activity of trypsin, chymotrypsin, amylase and lipase. In a slightly later study, in which Schneeman was involved, Dunaif and Schneeman (1981) provided further evidence for the use of dietary fibres as enzyme inhibitors. They observed the effects of dietary fibre on amylase, lipase, trypsin and chymotrypsin in human pancreatic juice. A sample of human pancreatic juice was collected from a pancreatic fistula from a female participant, before being incubated with one of several dietary fibres ranging from alfalfa, pectin, cellulose, hemicelluloses, wheat bran or oat bran. The cellulose and hemicelluloses samples resulted in a significant reduction in all enzyme activity. Wheat bran and oat bran reduced amylase and chymotrypsin activity and alfalfa reduced the activity of trypsin and chymotrypsin. Interestingly pectin increased the activity of amylase and chymotrypsin. Although these results do provide an interesting insight into *in-vitro* research there was only one participant, therefore making it difficult to generalise the findings. Further supporting evidence was provided by Shah, Mahoney, and Pellett

(1986), who demonstrated that feeding growing rats a diet containing 10% pectin, guar gum and lignin resulted in reduced pepsin activity of 57%, 44% and 20% respectively.

In contrast to the aforementioned studies there is also evidence that suggests dietary fibre has no effect on enzyme activity (Calvert, Schneeman, Satchithanandam, Cassidy, & Vahouny, 1985; El Kossori et al., 2000). Calvert et al. (1985) reported that feeding rats on strict diet including 10% levels of insoluble fibre (cellulose and alfalfa) and 5% levels of viscous fibre derivatives (pectin, guar gum and metamucil) for four weeks had no effect on pancreatic amylase and proteolytic enzymes. In more recent work from El Kossori et al. (2000) they also provided evidence to suggest that dietary fibres locust bean gum, citrus pectin, arabic gum, carrageenan and alginic acid had no inhibitory effect on the activity of pepsin. Despite El Kossori et al. (2000) reporting no decrease in enzyme activity, they do report that the level of casein digested was lower in the dietary fibre group. However the authors do not discuss how they have distinguished between enzyme activity and binding, which makes the findings of this study questionable. Although there is evidence to suggest that dietary fibres may be able to attenuate the activity of digestive enzymes, some of the methods are questionable. For example the lack of controls used make it difficult to compare the dietary fibre group with a control, animal models used and small sample sizes making the findings difficult to generalise.

In contrast to the work of El Kossori et al. (2000) work from this laboratory has demonstrated that dietary fibre possesses the ability to alter digestion in the gastrointestinal tract. Sunderland, Dettmar, and Pearson (2000) demonstrated *in-vitro* pepsin activity could be inhibited by a mean percentage of 52%. This inhibition was observed in 58 various alginate concentrations by using an N-terminal assay. Further work by this group in 2005 observed the inhibitory effect of a wide variety of alginates with various levels of mannuronic (M) and guluronic (G) residues on pepsin activity (Strugala, Kennington, Campbell, Skjak-Braek, & Dettmar, 2005). Strugala et al. (2005) reported that the greatest mean inhibition of alginate was as high as 89% by alginates at a concentration of 5mg/ml. There was a range of 39-89% inhibition of pepsin which was dependant on the structure of the alginate. The authors reported that there was a negative correlation between pepsin inhibition and G residue but a positive correlation of pepsin inhibition and alternating blocks of G and M residues. The authors suggested that the increased flexibility that is observed in alternating M and G residues provides an available interaction with the active site of the pepsin. In addition Smidsrod and Skjak-

Braek (1990) reported a positive correlation between alternating sequences of M and G residues and flexibility. Consequently alginates that are made up of consecutive G residues have poor flexibility and are therefore more resistant to an interaction with the pepsin active site and G residues, which was demonstrated in a significant negative correlation (Strugala et al., 2005). (Wilcox, Brownlee, Richardson, Dettmar, & Pearson, 2014) reported *in-vitro* that alginate was able to inhibit pancreatic lipase by up to 75%, and that this inhibition may be able to aid in weight loss by reducing the amount of fats digested, and therefore absorbed.

Although there is a considerable basis for the use of dietary fibre as an anti-obesity treatment, the earlier inconsistencies may be due to the large range of dietary fibres used. For example as mentioned here there is conflicting evidence for the use of pectin as an enzyme inhibitor, which may be due to the degree of esterification involved (El Kossori et al., 2000). Despite these inconsistencies there is a sizeable body of research reporting that dietary fibre can affect digestion, and may possess enzyme inhibition properties. This evidence along with the beneficial nutritional and health related benefits associated with dietary fibre suggests that alginate may be able to be used as a dietary fibre in the treatment of obesity and aid in weight loss, without the undesirable side effects associated with current pharmacological obesity treatments. Although dietary fibres may not have the side effects associated with the obesity treatments previously mentioned there is not enough clinical evidence to support the claim that dietary fibres may be used to treat obesity.

#### **1.4 Potential Enzyme Inhibition Mechanisms**

The mechanisms by which dietary fibres affect the digestive process is not completely understood, however there are several suggested mechanisms. As previously mentioned Shah et al. (1986) reported that guar gum, pectin and lignin inhibited pepsin activity. The authors suggested that the greater inhibition reported in the pectin and guar gum group compared to lignin may have been due to the viscosity within the stomach. With a greater viscosity the mobility of enzymes and substrates will be slower, therefore slowing digestion. Both pectin and guar gum are highly viscous when dissolved in solutions above pH 2, therefore when consumed these become gels within the gastrointestinal tract thereby slowing mixing and digestion. This sluggish mixing process may slow the access of the enzymes to substrates and ultimately reduce the amount of protein digested. These findings contradict those mentioned earlier by El Kossori et al. (2000), who indicated a possible mechanism responsible for reduced protein digestion may be substrate binding.

Although both of the mechanisms mentioned above may be involved in the reduction in protein digestion neither offer a complete explanation and therefore raise the question as to what other mechanisms are involved.

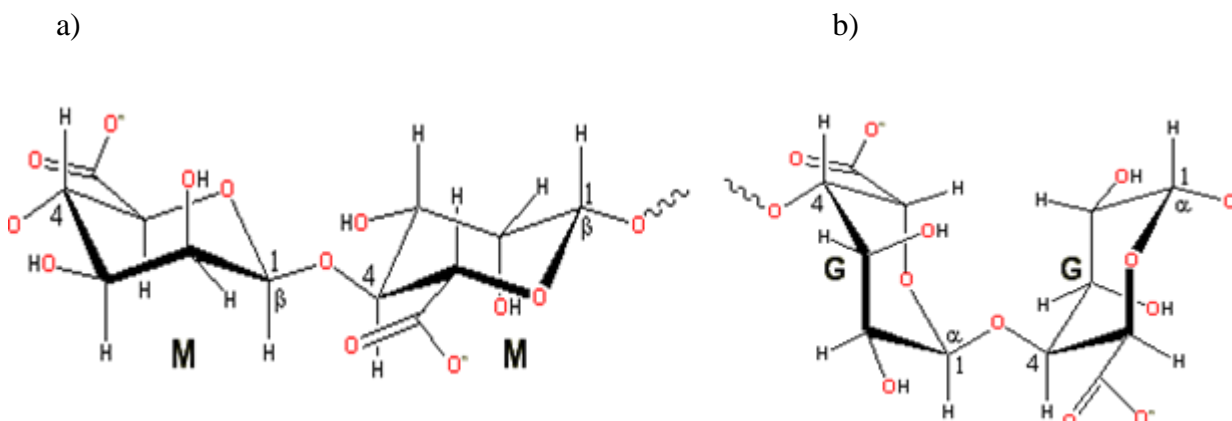
Schneeman and Gallaher (1980) suggested that dilution of intestinal contents may be an additional mechanism that is responsible for a reduction in enzyme activity. The authors observed that enzyme activity in the intestinal contents of rats fed cellulose was significantly different from the controlled diet rats. In the rats who consumed cellulose there was less enzyme activity per milligram of intestinal contents and a reduction in the total amount of enzyme activity. The authors reported that the enzymes and substrates may be diluted due to the addition of non-digestible material (cellulose). This was further supported by the fact that there was less enzyme activity reported in the chyme, resulting in slower digestion. This slower rate of digestion was evident due to the elevated level of protein in intestine of the cellulose group of rats. The authors also suggested that an additional mechanism that may play a role in inhibiting digestive enzymes may be a direct effect by reducing the enzyme substrate binding. Strugala et al. (2005) suggested that alginates may replace the substrate and potentially interact with the active site of pepsin, and therefore pepsin is unable to effectively digest protein. In addition to a potential binding of alginate with digestive enzymes, there is evidence to suggest that alginates may chelate calcium and thus impact upon pancreatic lipase activity. Although the exact role that calcium may play in pancreatic lipase activity remains unknown the enzyme is more active when calcium is present. If alginate chelates calcium then this too may have a role to play in reducing the activity of pancreatic lipase (Smidsrod, 1974). However Wilcox, Brownlee, Richardson, Dettmar, and Pearson (2013) reported that even if the alginate did bind to all of the calcium present, then this may only result in 20% inactivation of the lipase. Therefore this mechanism cannot account for the high levels of inhibition of lipase reported (Wilcox et al., 2013).

It is apparent that dietary fibres possess the ability to govern the activity of the enzymes involved in digestion. By reviewing the current literature it appears that there is no one specific mechanism that is responsible, and that there may be several mechanisms that contribute towards regulating the digestive process. It is also apparent that further research is required as the majority of research thus far investigating the mechanisms involved in regulating digestive enzymes are either in animals, consist of a small sample or are *in-vitro* and thus cannot truly be comparable to *in-vivo* digestion.

## 1.5 Alginate Structure

Alginates are present in brown algae as a matrix polysaccharide in the cell walls, and are by far the most abundant polysaccharide present, making up to 40% of the dry weight. Ertesvåg and Vallaa (1998) suggested that the habitats of various species of the brown algae vary regarding exposure to periodic drying due to the tide and waves. These varying habitats may be responsible for the variability in stiffness, elasticity and water binding capacity between alginates. These properties have been suggested to be accountable for why various species of algae produce different conformations of M and G residues.

The structure of alginate was first fully defined in 1972 by Penman and Sanderson (1972) using proton magnetic resonance (PMR) spectroscopy, however a further technique called C-nuclear magnetic resonance (C-NMR) which allows further definition of the  $\alpha$ -L-guluronic acid (G residue) and  $\beta$ -D-mannuronic acid residues (M residue) in an unbranched chain (Grasdalen, Larsen, & Smidsrod, 1977) (**Figure 2**). These residues can combine to form G rich (G blocks), M rich (M blocks) or a mixture of M and G as seen in **Figure 2**. It is this make up of MM blocks, GG blocks or MG blocks that account for the differences and function of alginates that allows them to be used in nutrition, medicines and the textile industry (Brownlee et al., 2005).



**Figure 2.** Diads of MM (a) and GG (b) residues (Remminghorst & Rehm, 2006).

## 1.6 Gelling of Alginate

As mentioned previously alginate possesses an innate ability of chelating divalent cations, eg. calcium and thus forming a stable gel. This interaction between divalent ions and G residues despite conflicting views is commonly referred to as the “egg box model”

(Morris & Rees, 1978). The ability of the inter chain binding sites has been suggested by several authors to involve the carboxyl and hydroxyl groups and the ring oxygen atoms which are all involved in the binding sites. Rees (1981) indicated that the egg box model can be used to describe the chain packing of M and G residues in alginate, that accommodate the ions of various sizes. Evidence supporting the “egg box model” was provided by Braccini and Perez (2001) who used a molecular modelling program. They used a pairing procedure that analysed all the possible associations of the G and M residue chains bindings to calcium ions to form dimmers. Further evidence for the “egg box model” was provided by Draget, Stokke, Yuguchi, Urakawa, and Kajiwara (2003) who demonstrated that the forming of this ionic gel occurs in two stages. Initially quasicrystalline junction zones composed of three to four lateral chains form; although this is insufficient lateral growth to support continuous chain formation. Therefore during second phase around three to four of the junction zones join together, forming domains of around 50Å and a gel is formed.

The stiffness of alginate gels is generally measured by Young's modulus, which is a measure of the stiffness of an elastic material. Smidsrod (1974) indicated that the mechanical property (stiffness) of the individual polymer chains is one of the main features which determines the viscosity of the gel. Smidsrod (1974) demonstrated that GG blocks had a superior ability of binding to calcium ions in solution, greater hysteresis and signs of heterogeneity with regard to binding sites, compared to MM and MG blocks. This superior ability of GG blocks to bind calcium ions has been suggested to be responsible for higher Young's modulus and thus a stronger and more rigid gel (Draget, Skjak-Braek, & Smidsrod, 1997). Although the G residue content is paramount to the stiffness of the gel, length of the G blocks also plays a role. Whereas M blocks link together via end to end, GG blocks are linked side to side, thus a greater content of GG blocks shortens the length of the interacted chains. The shorter chains in GG blocks increase mechanical stiffness, as opposed to alternating M and G residues which increase the flexibility and reduce stiffness. Despite the literature supporting the “egg box model” for alginates it remains an area of debate as to whether this is the most suitable model for all ionic polysaccharides, such as pectins (Braccini & Perez, 2001).

### 1.6.1 Ionic Gels

In addition to ionic gel formation alginates can also form acid gels in the presence of a low pH. Atkins, Parker, and Smolko (1971) indicated that acid gel formation is dependent on the acid dissociation constant (pKa) of the individual residues that make up alginate. The authors reported that if the pH of the solution containing alginate was reduced below the pKa for mannuronic and guluronic residues (3.38 and 3.65 respectively) an acid gel is formed. It must be emphasised that when the pH is lowered in a controlled manner an acidic gel is formed. In contrast an abrupt reduction in pH below the pKa will result in precipitation of the alginate.

The formation of an acid gel was suggested to be as a result of intermolecular hydrogen bonds that connect the polyuronon chains, however it remains uncertain how the molecular weight and composition of the alginates will affect acid gel formation (Atkins et al., 1971). Since this earlier work Draget, Skjåk Bræka, and Smidsrøda (1994) conducted research using Youngs modulus in an attempt to identify what effect the chemical sequence, molecular weight and composition had on acid gel formation. The authors determined that gel strength was dependent on the chemical sequence and molecular composition. The stronger gels were those rich in G residues and those that consisted of multiple GG blocks, as represented by a significantly larger Youngs modulus when compared to alginates rich in M residues and consisting of MM and MG blocks. Hoad et al. (2004) confirmed these earlier findings when comparing alginates with high and low G residues using magnetic resonance images (MRI) in human. They reported that alginates with a high G content accounted for large gel formation in the stomach as opposed to those alginates with a low G content. In addition the authors indicated that along with chemical composition gel strength was dependant on molecular weight, with the larger apparent Youngs modulus being recorded for the larger molecular weights. A possible explanation for the low gel strength and poor gel formation in MM and MG blocks may be due to a less hospitable environment for the formation of stable intermolecular  $H^+$  bonds (Atkins et al., 1971; Draget et al., 1997; Hoad et al., 2004).

### 1.6.2 Acidic Gels

As mentioned previously alginate can form ionic gels at a range of pHs in the presence of divalent cations such as  $Ca^{2+}$ , (Morris & Rees, 1978), and acidic gels when the pH is reduced below the pKa value of the uronic acid residues, which as mentioned earlier may



be due to intermolecular hydrogen bonds (Atkins et al., 1971). Yang, Chen, and Fang (2009) observed the effect of a range of pH's on the viscosity of alginate in solution. They reported that as the pH was reduced from 6.6 to 5.0 that there were minimal changes in viscosity, suggesting that there was only a small, if any alteration in electrostatic repulsion. However when the pH was reduced further from pH 5.0 to 3.0, the authors reported a significant augmentation in viscosity. The increase in viscosity was suggested to be the result of inhibition of electrostatic repulsion. With reduction in electrostatic repulsion there is the opportunity for an enhancement of intermolecular hydrogen bonds, and possibly entanglements between parallel polymer chains forming structures. The enhancement of intermolecular hydrogen bonds and entanglement between polymers is a result of two interactions.

The first interaction is a reduction in the charge repulsion between dissociated carboxylic groups and the hydrogen bonding formed between carboxylic acid and ionised carboxylate groups (Bu, Kjoniksen, & Nystrom, 2005). In conjunction with a reduction in pH of the alginate solution, the number of dissociated carboxylic acid groups in the M and G residues of the alginate also reduces, thus having a knock on effect on the hydrophilicity of the alginate. Cao et al. (2005) demonstrates that as the pH continues to decrease the carboxylic acid groups in the alginate will become protonated, and as a consequence hydrophobic sections appeared in the alginate chains. In addition the reduction in negative charge of the opposing carboxylic acid groups reduces and therefore encouraging the development of intermolecular hydrogen bonds, and thus increase the viscosity through entanglements of opposing alginate polymer chains (Yang et al., 2009).

## **1.7 Uses of Alginate**

Hipsley (1953) defined dietary fibre as a shorthand expression for non-digestible constituents that make up the cell walls of plants. Haug, Larsen, and Smidsrod (1966) established that alginate is a dietary fibre as it is resistant to hydrolysis in mineral acid. Through the use as a dietary fibre and its ability to form viscous gels, alginate can be used for various applications. These include stabilisers, gel forming, film forming and water binding agents. These application allow alginates to be used in industries ranging from pharmaceutical to the textile industry, however the main uses of alginate are in the food industry (Brownlee et al., 2005). In the pharmaceutical industry alginates are used for dental impressions, control of drug release and have also been used since 1947 as wound

dressing. For example Groves and Lawrence (1986) used alginate in an attempt to reduce blood loss from skin graft sites. There was significant reduction in the amount of blood lost post-surgery when compared to other treatments, with no adverse reactions reported. In the textile industry alginates have been used for various applications ranging from textile printing and manufacturing of ceramics (Ertesvåg & Vallaa, 1998). Despite the various uses of alginates as mentioned there is substantially more income generated from the use of seaweed and seaweed products in human nutrition. Alginates can be made up to various viscosities by altering the level of calcium ( $\text{Ca}^{+2}$ ) and hydrogen ( $\text{H}^{+}$ ) which are stable at temperatures up to  $100^{\circ}\text{C}$  and at a low pH, thus providing an excellent gelling agent in food. Amongst the many applications of alginates in the food industry it can be used in bakery creams (Gibbs, Kermasha, Alli, & Mulligan, 1999) with approximately 0.1-0.3% of the dry weight of the product being added. When alginate is added to the cream of cakes and pastries it increases the stability of the product when freezing and thawing and therefore reduces the separation of the solid and liquid components (Brownlee et al., 2005).

## 1.8 Obesity treatment

Data from previous research suggests that alginate as a dietary fibre may be used as an obesity treatment. However the main obstacle appears to be how to introduce alginate into the everyday diet. The addition of alginate to food vehicles is not a new concept and has been developed since the early 90s. With the addition of alginate to food and drink vehicles resulting in a reduction in glycaemic response (Williams et al., 2004), a reduction in blood glucose, reduced gastric emptying (Torsdottir, Alpsten, Holm, Sandberg, & Tolli, 1991; Wolf et al., 2002), increased fat excretion, decreased bile excretion (Sandberg et al., 1994) and a reduction in Kcal intake (Paxman, Richardson, Dettmar, & Corfe, 2008). Despite these beneficial effects, alginate enriched products are plagued with poor palatability. Ellis, Apling, Leeds, and Bolster (1981) reported that foodstuffs that contain viscous fibres usually exhibit slimy, sticky and gummy characteristics resulting in poor palatability and therefore poor compliance. This was especially evident when alginate is mixed with calcium carbonate, forming a strong rigid gel in the mouth resulting in poor palatability (Paxman et al., 2008).

As previously mentioned there are a number of studies suggesting that diets including dietary fibre possess the ability to lower the risk of morbidity and mortality from various

diseases. However for every study suggesting that dietary fibre may reduce the risk of morbidity, there are a number of studies to the contrary (Brownlee, 2009). These indiscrepancies are generally due to small sample sizes, animal models such as mice and dogs being used or the various amounts and types of dietary fibre that have never been directly compared with each other. Of these dietary fibres alginate has received considerable attention due to its ability to modulate the activity of the enzymes involved in digestion (Strugala et al., 2005; Sunderland et al., 2000; Wilcox et al., 2014). The unique physical property of alginate separates them from other dietary fibres. This is generally because alginate can be taken at low viscosity, which is excellent for palatability and allows for larger doses to be consumed. Additionally alginates are able to form gels in both acidic and calcium rich environments and can be used to reduce the digestion of fats and increase satiety (Brownlee et al., 2005).

Although there is good evidence supporting the use of alginate, the main hurdle appears to be how to introduce alginate into the diet. As mentioned beforehand the overall success rate of diets is only 15% (Ayyad & Andersen, 2000) therefore alginate must be included in foods/drinks that people consume on a regular basis. In addition to ensuring that the alginate is in food/drinks that people regularly consume is the concern of palatability. Previous work that has attempted to include dietary fibre into beverages and crispy bars has resulted in poor palatability and therefore has never been produced into an everyday product. The question remains as to whether the addition of alginate to a food/drink will affect the palatability (Ramirez, 1990).

There have been a number of *in-vivo* studies attempting to use alginate as a dietary fibre, with mixed results. Torsdottir et al. (1991) was one of the first studies that used alginate as a dietary fibre. The authors used a beverage which included 5g of sodium alginate, containing 60% mannuronic and 40% guluronic acid. The authors reported significantly lower postprandial rise in blood glucose and a slower gastric emptying in the treatment group as compared to the control group in diabetic patients. A similar study was conducted by Sandberg et al. (1994) who attempted to observe the effects of a 500g milk shake containing 7.5g of alginate (40% guluronic) per day for two weeks in ileostomy patients. The authors reported an increase in fat excretion and decreased bile excretion in the ileostomy effluent. Despite the positive effects from both of the aforementioned studies, the samples used were small with seven in Torsdottir et al. (1991) and six in Sandberg et al. (1994) making it difficult to interpret the findings. Both studies used alginate with chemical composition of 60% mannuronic acid, and as mentioned

previously G blocks have a greater ability to form stronger more stable viscous gels. Furthermore acid gels made up of M blocks or alternating MG residues have a lower apparent Young's modulus compared to G blocks, thus making them less stable than G blocks in the stomach (Draget et al., 1994; Hoad et al., 2004). Paxman et al. (2008) addressed the molecular make-up of the alginate used in beverages and used an alginate made up of 65-75% guluronate. All participants recorded a seven day food diary of all food and drink consumed. The treatment and control group were given seven 100ml coded plastic bottles with tamper evident caps. The treatment beverage included 1.5g sodium alginate, 0.7g calcium carbonate and flavourings, and the control group consumed a slim fast drink. Both groups were advised to add water to one drink per day to make them up to 100ml and then consume the beverage 30 minutes prior to the breakfast or evening meal once a day for seven days. Upon analysing the seven day food diaries the authors reported that those in the treatment group consumed on average 134.8 Kcal less per day over the seven day treatment compared to the control group. However due to the difference in beverages consumed between the groups the question remains as to whether the difference in Kcal consumed would have been greater if the control group had consumed a beverage similar to the treatment without alginate. Irrespective of the above there is sufficient evidence to suggest that the use of alginate beverages at reducing such variables as energy intake and postprandial blood glucose and increasing satiety. Nevertheless the question still remains over the palatability of the drinks in the mouth and as it travels down the gastrointestinal tract. When alginate is added to solution it can become viscous, even in de-ionised H<sub>2</sub>O (DH<sub>2</sub>O) at relatively low concentrations and exhibit slimy characteristics (Ellis et al., 1981).

Wolf et al. (2002) attempted to tackle the problem of poor palatability of alginate in solution. They indicated that they could produce a liquid that is not viscous at neutral pH, but then becomes viscous in the stomach. They combined alginate with water and potassium citrate, in an attempt to reduce the level of viscosity within the mouth and stomach. The authors suggested that by adding potassium citrate to H<sub>2</sub>O it will bind the calcium. This would mean that when alginate is added the viscosity of the solution should remain low at a neutral pH and therefore not affect palatability. Upon drinking the solution and mixing with gastric juice the pH will reduce to around 1-2 and the alginate will only then form an acidic gel. Through the formation of an acid gel the authors reported attenuation of postprandial glycaemic response, nevertheless the question remains would they have observed even greater reduction if potassium citrate was not used.

Despite the larger sample size used by Wolf et al. (2002) (30 healthy participants) in comparison with those mentioned previously (Sandberg et al., 1994; Torsdottir et al., 1991), the authors did not report what the molecular makeup or concentration of the alginate, thus making it difficult to draw any conclusion based on the alginate added to the beverage. Hoad et al. (2004) indicated that the major flaw with using alginate in a beverage form is that no chewing is required and therefore gastric secretion would initially be low resulting in a low initial dilution of the beverage (Hoad et al., 2004). The lack of gastric juice secretion may result in a poor gel formation and therefore the effects of alginate beverages previously mentioned may not be as effective as food including alginate.

An alternative delivery of alginate which has been considered is the inclusion of alginate into a cereal bar. Williams et al. (2004) added 1.6g of alginate to a crispy bar and observed the effect on postprandial glycaemic response. The treatment group had a significantly attenuated postprandial glycaemic response in comparison with the control cereal bar, with no adverse gastrointestinal effects. The authors attributed this blunting of the postprandial glycaemic response to the cereal bar becoming viscous as the bar is digested and the alginate is released. Although the authors did not measure additional effects such as gastric emptying they suggested that the formation of a gel in the stomach would have increased motility through the upper GI tract and therefore also affect nutrient digestion and absorption. Contrastingly Mattes (2007) reported no significant difference between an alginate cereal bar and the control bar for hunger and dietary intake. Although they didn't specify the molecular composition of the alginate, they did include 4.9g of dietary fibre compared to 0.62g in the control bar. Therefore the dietary fibre used may not have been alginate. Mattes (2007) may have used another dietary fibre that is not soluble and thus there was no gel formation in the stomach and no impact upon hunger. The molecular composition of the alginate may have been the reason why there was no significant difference between groups. Furthermore the taste, pleasantness, sweetness and texture and palatability of the control bar were all significantly higher compared to the treatment bar. The most recent study using alginate as a supplement was a study by Jensen, Kristensen, and Astrup (2012 (a)) who used alginate as a blackcurrant beverage with a calorie restricted diet. The study reported a reduction in weight loss in the treatment group of 6.78 compared to 5.04kg in the control group, however these differences were non-significant. Furthermore the treatment group also reported poorer palatability and other symptoms such as abdominal pain and distension compared to the

control group. Issues with both beverages and cereal bars may be due to a lack of appropriate mixing in the stomach and specifically in beverages the slimy texture of the beverage resulting in poor palatability despite the addition of a calcium source to reduce this. The mixing of the alginate with other ingredients and other meal constituents appears to be necessary to increase the viscosity inside the stomach which appears to be essential in order to experience the physiological benefits of alginate (Torsdottir, Alpsten, Andersson, & Einarsson, 1989).

## 1.9 Bread Rationale

Although there is both *in-vitro* and *in-vivo* evidence supporting the use of alginate as a possible anti-obesity treatment, the vehicle in which to deliver alginate in appears to be a major obstacle. The work from this laboratory to date has only been carried out in test-tubes and has not taken into account the various stages involved in digestion (Strugala et al., 2005; Sunderland et al., 2000; Wilcox et al., 2014). Furthermore the *in-vivo* studies that have added alginate to a food or drink have faced issues such as palatability and a slimy feeling in the mouth.

Bread was chosen as a potential delivery method for alginate as it is regularly eaten on a day to day basis by most people. Furthermore the UK bread market is worth in excess of £3.4 billion per year, which equates to 11 million loaves of bread cooked per day (Federation of bakers). In addition to this the ingredients of bread make is a suitable vehicle for the delivery of alginate. The main constituent of bread is carbohydrates and as the majority of carbohydrates in bread which is generally starch is digested in the small intestine. If the alginate is bound to the ingredients within the bread then the anticipated result would be that the alginate would be released here as the bread is digested as long as alginate does not inhibit  $\alpha$ -amylase in the small intestine. Although the release of the alginate in the small intestine is not crucial, it may have its benefits as it is in the small intestines where between 85-90% of fat is digested (Miled et al., 2000).

## Chapter 2: Alginate Quantification

### 2.1 Introduction

Alginates are present in brown algae as a matrix polysaccharide in the cell walls, and are by far the most abundant polysaccharide present, making up to 40% of their dry weight (Ertesvåg & Vallaa, 1998). Alginates are a co-polymer of 1-4 linked D-mannuronic (M) and L-guluronic (G) acid residues (Stokke et al., 2000). These M and G residues can combine to form MM blocks (mannuronic), GG blocks (guluronic) and MG blocks which have a profound effect on the physiochemical properties of the alginate (Brownlee et al., 2005). The differing physiochemical properties are what makes alginate unique and highlights its applicability for use, ranging from shoe polish to medical use (Uludag, De Vos, & Tresco, 2000; Yamamoto, Kumagai, Sakiyama, Song, & Yano, 1992). Despite the variety of industries alginate is used within, it is its use in the food industry that generates the most revenue with over 27,000 tonnes produced yearly (Jensen, 1993).

Interestingly more and more work is being conducted regarding the use of alginate as an obesity treatment (Strugala et al., 2005), and more specifically the addition of alginate to a food vehicle as an obesity treatment. Cereal bars and drinks have been used previously, with studies reporting increased fat excretion (Williams et al., 2004), increased bile excretion (Sandberg et al., 1994) and a reduced Kcal intake (Paxman et al., 2008). Studies such as these support the use of alginate as a possible treatment for obesity; however the main issue amongst these studies is the poor palatability of the vehicle used for delivery, and therefore people think of these as a medicine as oppose to a dietary supplement. Furthermore the sample sizes used previously are relatively small making it difficult to compare the findings. Previous research into diets has reported an adherence rate of only 15% (Ayyad & Andersen, 2000), therefore any weight lost during this time period is regained quickly afterwards. It therefore seems a reasonable assumption that if alginate can be delivered in a vehicle that is not associated with poor palatability, and that people are already consuming then this may be a useful tool that may contribute towards tackling the obesity epidemic.

If alginate is to be used as an obesity treatment the release rate of alginate from said vehicle needs to be determined. Methods to quantify alginate must be sensitive, accurate, repeatable, high throughput and must be robust enough to remove any interference from un-digested debris. The most accepted techniques used for the quantification of alginate include chromatographically, after acid hydrolysis, colorimetrically subsequent to uronic

acid degradation and UV spectrophotometry following detergent complexation (Usov, 1999). Although these techniques are effective at quantifying alginates, they involve extensive sample preparation and are only sensitive over a narrow pH range. Such methods may not be suitable for samples taken from *in-vivo* digestion, as the pH can range from anywhere between 1-8. Furthermore there are other factors such as bile and pancreatic juice which may also interfere with current techniques for quantifying alginate.

Several alternative methods for the quantification of alginate have been designed which all use varying methods to prepare the alginate, but essentially a colorimetrically technique to stain the alginate and then measure absorbance has been used. Blumenkrantz and Asboe-Hansen (1973) adapted an earlier technique and quantified alginate by heating uronic acids to 100°C in concentrated sulphuric acid/tetraborate treated with meta-hydroxydiphenyl. They then measured the absorbance of samples through the appearance of chromogen. This heating process may not be suitable if the samples concerned require further analysis of digested products, as temperatures in excess of 100°C will denature the enzymes within the samples. Furthermore there is evidence to suggest that alginate is not heat stable at temperatures above 80°C (Leo, McLoughlin, & Malone, 1990). Leo et al. (1990) and Serp, Mueller, Von Stockar, and Marison (2002) reported that once the alginates were exposed to temperatures in excess of 80°C the gel bead strength and viscosity decreased, however bead diameter increased, suggesting the bonds between the alginate molecule are also collapsing. An alternative method for quantifying alginate was designed by Halle, Landry, Fournier, Beaudry, and Leblond (1993), who developed a sensitive assay with the purpose of quantifying the amount of alginate in a solution and microcapsules. This method was based on the metachromatic changes induced when the cationic dye 1,9-dimethyl methylene blue (DMMB) binds to alginate. This assay was sensitive enough to work over a wide range of concentrations of alginate and a wide range of pH ranging from 6.5 to 13. Nonetheless as with the earlier methods this range of pH would not be suitable for *in-vivo* digestion. Richardson, Dettmar, Hampson, and Melia (2004) developed and evaluated this method further to observe its reliability, and to observe if alginate molecular composition and weight affected the complexation of DMMB and alginate. They reported that this method had excellent reproducibility (coefficient of variation (CV) <3%), sensitivity (0.3g/L) and excellent linearity ( $r = 0.99$ ). However the alginate in the study was used in combination with an anti-reflux treatment and was scraped from the mucosal of the oesophagus, which may explain why these results were not repeatable with samples within this laboratory. An alternative assay that



may be suitable is the Periodic acid Schiff's (PAS) assay which was developed by Mantle and Allen (1979). The PAS assay was developed as a technique for the estimation of glycoproteins containing carbohydrates with adjacent hydroxyl groups, which was sensitive enough to detect small amounts of glycoproteins (10-100µg/ml). The PAS assay is dependent on the number of hydroxyl groups in the molecule and the mechanism of how it works can be subdivided into three steps. The initial phase involves preparation of the basic fuchsin with metabisulphate, which decolourises the solution forming sulphonic acid groups. Secondly periodic acid and acetic acid are mixed and then added to the sample, resulting in oxidation. This oxidation splits the rings between adjacent hydroxyl groups converting them to aldehydes. Upon addition of the fuchsin the adjacent sulphonic acid group combines with the adjacent aldehydes, producing a red colour. As the intensity of the red colour is dependent on the number of hydroxyl groups, the larger amounts of alginate should produce a darker red and thus a larger OD value (Mantle & Allen, 1979). Alginate also possesses adjacent hydroxyl groups at C<sup>2</sup> and C<sup>3</sup> suggesting that the PAS may be a suitable method for quantifying alginate within a solution.

In addition to quantify alginate within a solution, any methods must also be able to quantify the release rate of alginate from a given vehicle once said vehicle has been digested. The use of alginate as a food additive is not new concept, however its use as an obesity treatment in a palatable food vehicle consumed during an ordinary day to day diet is. Determining the release rate of alginate from the vehicle and understanding how the alginate acts within foods and how these foods behave both *in-vivo* and *in-vitro* digestion requires further understanding.

## 2.2 Aims

As previously mentioned bread is one of the food types that is consumed on a large basis and is easily accessible to the majority of the population. For these reasons bread was developed in conjunction with Greggs PLC, which contained a powder mix of alginate added to a bread mixture based on 4% by weight, and can be seen in **Figure 3**. If alginate is to be used as a potential obesity treatment the release rate of alginate from the bread during digestion in a synthetic model gut must be determined.

The aims of the current chapter are:

- Assess the ability of cationic dyes DMMB, Safranin-0 and Toluidine Blue to quantify alginate in DH<sub>2</sub>O and in samples taken from the model gut.
- To modify and utilise the PAS assay and determine if it can be used to quantify alginate in DH<sub>2</sub>O and in samples taken from the model gut.
- To assess whether the model gut is able to replicate *in-vivo* digestion and digest the bread and potentially release the alginate into the model gut.
- To determine if the cationic dyes or the PAS assay is able to determine the release rate of alginate from an alginate enriched bread.

## 2.3 Methods

### 2.3.1 Materials

The alginate bread (AB) was similar to an everyday bread and this can be seen in **Figure 3**. Greggs master baker was given a selection of alginates that inhibited pancreatic lipase and selected the alginate that created the most palatable and aesthetically pleasing bread. The only major addition to the AB was sodium alginate and an increase in the fat (**Table 1** and **Table 2**). The alginate used for the bread was MANUCOL DM selected by Greggs PLC master baker as this alginate made the best performing bread. The alginate was a gift from FMC BioPolymer AS, Drammen, Norway and was stored at 4°C in tightly-sealed containers and all alginate weights were corrected for water content.



**Figure 3.** Alginate bread produced by Greggs Plc.

**Table 1.** Greggs Plc control bread ingredients.

<b><u>Regular Bread</u></b>	<b><u>BF0003-5</u></b>	
<b><u>Nutrients per</u></b>		<b><u>100g</u></b>
Energy (kcal)		247
Energy (kJ)		1046
Protein (g)		10.2
Carbohydrates (g)		46.2
	sugars	1.1
	starch	45
Fat (g)		1.7
	saturates	0.5
	monosaturates	0.3
	polyunsaturates	0.6
	trans	0
Dietary Fibre (AOAC) (g)		3
Sodium (g)		0.4 (374mg)
Water (g)		36.8

**Table 2.** Greggs Plc alginate enriched bread ingredients.

<b><u>Alginate Bread</u></b>	<b><u>R110063/0</u></b>	
<b><u>Nutrients per</u></b>		<b><u>100g</u></b>
Energy (kcal)		335
Energy (kJ)		1406
Protein (g)		8.1
Carbohydrates (g)		43.9
	sugars	2.6
	Non milk extrinsic sugars	2.6
	starch	38.4
Fat (g)		14.1
	saturates	6.2
	monosaturates	6.3
	polyunsaturates	0.8
	trans	0.1
Dietary Fibre (AOAC) (g)		3
DM Alginate		4
Sodium (g)		1.5
Water (g)		31.3

Sodium metabisulphate, basic fuchsin, periodic acid at 50%, acetic acid, methanol, Safranin-O (S-O) and Methylene Blue Hydrate (DMMB) were purchased from Sigma-Aldrich Co Ltd, Dorset, UK. Artificial saliva was composed of 62mM sodium hydrogen, 6mM di-potassium hydrogen, 15mM sodium chloride, 6.4mM potassium chloride and 3mM calcium chloride. Artificial gastric juice was composed of 49.6mM sodium chloride, 9.4mM potassium chloride, 2mM monopotassium phosphate and 5mM urea. Artificial pancreatic juice was composed of 110mM sodium bicarbonate, 2.5mM di-potassium hydrogen, 54.9mM sodium chloride, 1mM calcium chloride and 1.67mM urea, all components of artificial solutions were purchased from Sigma-Aldrich Co Ltd, Dorset, UK. Enzymes  $\alpha$ -amylase from hog pancreas (50 U/mg), Pepsin from gastric mucosa ( $\geq$  2500 units/mg protein, P7012-5G) and Pancreatin from porcine pancreas (P7545-500G) were purchased from Sigma and gastric like lipase was purchased from Amano enzyme (Inc AP12) for use in the model gut.

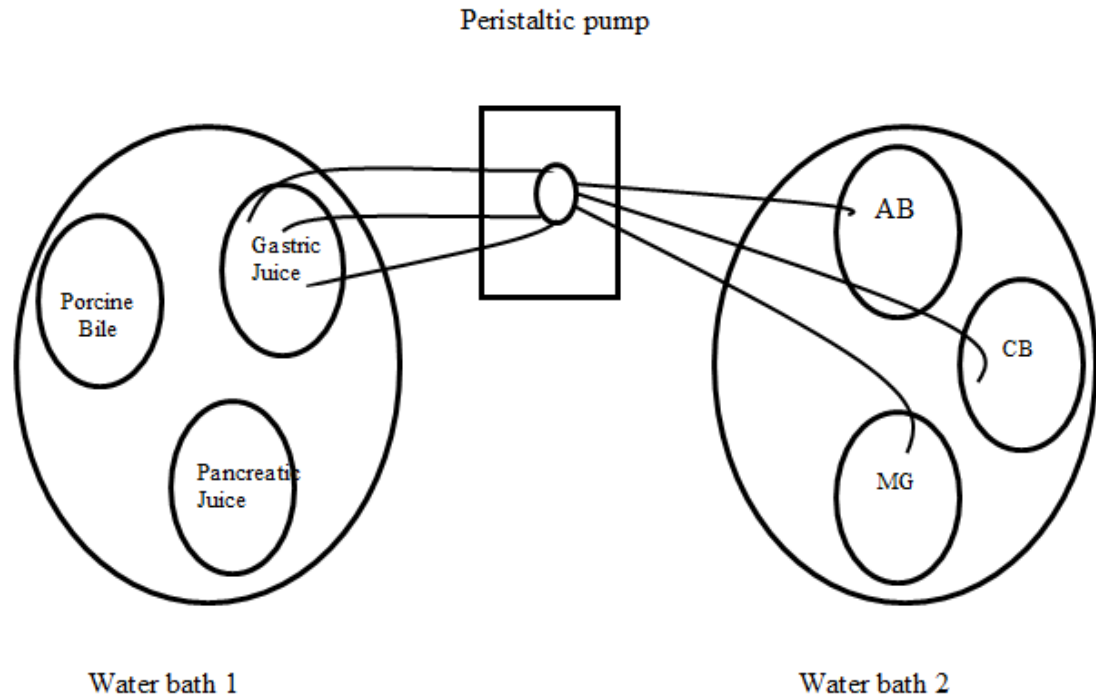
### 2.3.2 Equipment

All assays were carried out using BioTek 96 well plate reader at 550nm (ELx808 BioTek, Bedfordshire, UK). Two Grant GD 100 water baths were used for incubating synthetic model gut solutions and samples within the model gut at 37°C (Fisher Scientific, Loughborough, UK). A Sci-Q 401U/D peristaltic pump was used to pump synthetic solution into samples in the synthetic model gut (Watson-Marlow, Fisher Scientific, Loughborough, UK), three IKA<sup>®</sup> laboratory egg compact mixers were used for mixing in the model gut. A 213 microprocessor pH meter (Hana instruments, Leighton Buzzard, UK) was used to assess pH. A Thermo Jouan BR4i Mu Scientific Centrifuge (Basingstoke, UK) and an Eppendorf Centrifuge 5415 R (Stevenage, UK) were used for samples <4ml and >2ml, respectively.

### 2.3.3 Synthetic Model Gut Procedure

In order to ascertain if alginate is released from the bread vehicle the bread had to be digested. Therefore an open model gut system, as seen **Figure 4**, which replicates digestion in the mouth, stomach and small intestines with three experiments running simultaneously. Each sample initially started at zero minutes with either i) 5.2g AB, ii) 5.2g control bread (CB) or iii) model gut (MG) and all with 5 $\mu$ l of 3mg/ml amylase ( $\alpha$ -amylase from hog pancreas, Sigma 10080-25G c.50 U/mg), 5ml DH<sub>2</sub>O and 5ml synthetic saliva. Samples of bread were taken at random and were homogenous in nature. The alginate and control bread were broken up into crumbs ranging between 2-4cm prior to adding to the model gut to simulate mastication in the mouth. The three samples were

then placed into water bath two and mixed at 75 revolutions (rpm)/min for 30 seconds, which represents mastication in the mouth. 50ml synthetic gastric juice was then added, which included 0.5mg/ml pepsin (Sigma, P7012-5G) and 0.04mg/ml gastric lipase (Amano Enzyme Inc AP12) whilst mixing continued. The remaining synthetic gastric juice (also containing pepsin and gastric lipase) was fed to the three experiments via a peristaltic pump at 0.5ml/min for 60 minutes whilst continually mixing, which represents digestion in the stomach. After 60 minutes 25ml porcine bile, which was homogenised and pre warmed to 37°C (a gift from Thompson abattoir, Bishop Auckland, UK) before being added to each of the three experiments. Synthetic pancreatic juice (which included pancreatin at 7g/100ml) was then pumped into the three experiments at 0.25ml/min for 120 minutes. During the model gut process a one ml sample was taken at 0.5 minutes (after mastication in the mouth), and then every 15 minutes for a total of 180.5 minutes. In addition to confirm the model gut system is replicating the pH of *in-vivo* digestion the pH was monitored after 0.5minutes (mouth), and then every 15 minutes for a total of 180.5 minutes. All enzymes, unless specifically mentioned are added fresh and both water baths were set at 37°C throughout the whole process.



**AB = Alginate Bread**

**CB = Control Bread**

**MG = Model Gut Solution**

1. 0-30 seconds - Samples added to water bath 2 with synthetic saliva
2. 30 seconds – 60 minutes – 50ml synthetic gastric juice added to each sample. Gastric juice then pumped from water bath 2 at 0.5ml/min into each sample.
3. 61-180.5 minutes – 25ml porcine bile added and synthetic pancreatic juice pumped in from water bath 2 at 0.25ml/ml into each sample.

**Figure 4.** Model gut set up for *in-vitro* digestion shown in the gastric phase.



#### 2.3.4 DMMB, S-O, T-B and PAS Assay

DMMB, S-O and T-B were made up at concentrations ranging from 0.1-2.5mM in DH<sub>2</sub>O. Alginate DM was made up in DH<sub>2</sub>O using serial dilutions ranging from 0.1-10mg/ml. 200µl of each concentration of alginate were then added to a 96 well plate in duplicate and 50µl of each dye at varying concentrations was added to each concentration of alginate and incubated at room temperature for 30 minutes, and then the absorbance was measured at wavelengths between 575 and 595nm on the microplate reader. The PAS assay was adapted from Mantle and Allen (1978) to be used on a 96 well plate. 20µl of 50% periodic acid was added to 10ml of acetic acid at 7% in DH<sub>2</sub>O and mixed. 20µl of this mixture was then added to 200µl of sample in a 96 well plate in duplicate. Sodium metabisulphate was added to schiffs fuchsin-sulphite reagent at 16.7mg/ml and mixed. The 96 well plate and schiffs fuchsin-sulphite mixture was then incubated for one hour at 37°C. After incubation 20µl of schiffs fuchsin-sulphite was added to each well and the plate was then left at room temperature for 30 minutes for colour development, before absorbance was measured at 550nm.

#### 2.3.5 Mucin and Alginate Standard Curves

Techniques previously described by Mantle and Allen (1978). Isolated porcine gastric mucin was made up to a concentration of 1mg/ml in DH<sub>2</sub>O. Sodium alginate was dissolved in DH<sub>2</sub>O or model gut solution (MG) from 180 minutes at a concentration of 0.49mg/ml and at 2mg/ml respectively. The mucin and alginate in DH<sub>2</sub>O were then diluted down further in DH<sub>2</sub>O to produce a standard curve, and then 200µl of each concentration was added to a 96 well plate in duplicate. The alginate in the MG was diluted by 50% in methanol and placed in the freezer at -20°C for 30 minutes, before being centrifuged at 4100rpm for 20 minutes at 4°C. The supernatant was then removed, leaving a pellet which was then re-suspended in 4ml DH<sub>2</sub>O. Based on the dilutions from 2mg/ml this left a concentration of 0.25mg/ml which was then diluted in DH<sub>2</sub>O to create a standard curve. The alginate in DH<sub>2</sub>O was used to represent the stomach phase (0.5-60 minutes) and the alginate in MG was used to represent the small intestinal phase (60.5-180 minutes). Once mucin and alginate in DH<sub>2</sub>O and MG had been added to a 96 well plate the PAS assay was run. The absorbance for DH<sub>2</sub>O alone was subtracted from the mucin and alginate absorbance as a control. The control for the alginate in MG was MG alone with the same dilutions used to process the alginate in MG, again to account for any interference.

### 2.3.6 Alginate Isolation

Once the samples have been extracted from the model gut the alginate was isolated. Upon extraction samples taken at from 0.5 – 60.5 minutes were titrated to between pH 6-7 using 1M sodium hydroxide and then centrifuged at 13,500 rpm for 10 minutes to remove any insoluble material. Following centrifugation 500µl of the supernatant is added to 500µl DH<sub>2</sub>O and then vortexed. 200µl of the sample was added to a 96 well plate in duplicate for each time point, and for each of the three samples (MG, AB and CB). The samples taken between 60.5 minutes (after adding bile) and 180.5 minutes were diluted in methanol by 1:1 (vol:vol) and mixed. The samples were then placed in a freezer at -20°C for 30 minutes, before being centrifuged at 4100 rpm for 20 minutes at 4°C. The supernatant from the samples is then removed and 4ml DH<sub>2</sub>O is added and the pellet was re-suspended. 200µl of the sample was added to a 96 well plate in duplicate for each time point for each of the three samples (MG, AB and CB) and the PAS assay was carried out as described earlier. As a control the absorbance for the CB was removed from that of the AB.

## 2.4 Statistical Analysis

Statistical calculations were undertaken using SPSS Statistics 19 (IBM, Predictive Analysis Software, USA). Data presented as mean and standard error of mean ( $\pm$  S.D). A Two-way Repeated ANOVA followed by a Post-Hoc Bonferroni were undertaken at a significant level ( $\alpha$ ) of 0.05 to compare the pH of the AB, CB and MG, and to compare alginate release from the bread vehicle at 0, 60 and all time points between 60.5 and 180 minutes.

## 2.5 Results

### 2.5.1 Cationic Dyes

**Table 3.** Ability of cationic dyes and basic fuchsin (PAS assay) to estimate alginate concentrations. For DMMB \* indicates the range covers the absorbance range 1.2-2.1 derived from absorbance at 595 and 550nm greater than 1. + This range covers absorbance 2.7-3.6 (where absorbance greater than 1 are not reliable). n = 6 measurements for each assay system.

Dye and Concentration	Wavelength	Concentration Range	Pearson's coefficient (r)	Absorbance Range
2.5mM DMMB	Ratio 595/550nm	0-2 mg/ml	0.86	1.2-2.1 (0.7)*
2mM Safranin-0 (S-O)	595nm	0-10 mg/ml	0.98	0.14-0.22 (0.08)
2mM Toluidine Blue (TB)	595nm	0-0.3 mg/ml	0.63	2.7-3.6 (0.9) <sup>+</sup>
PAS Assay on samples from gastric phase of model gut	550nm	0-0.5 mg/ml	0.99	0.02-0.45 (0.43)

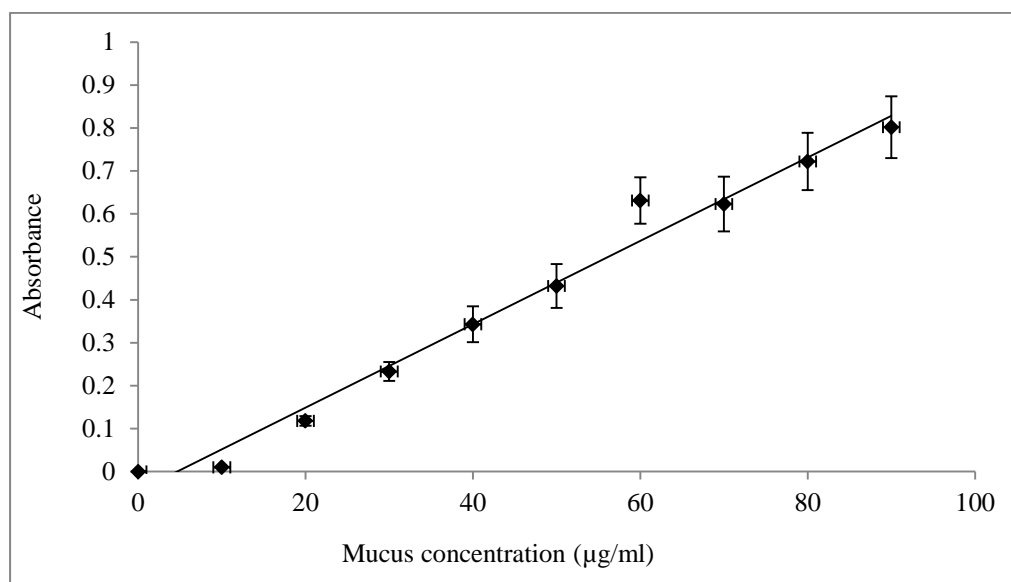
DMMB - **Table 3** shows the results from the addition of DMMB at 2.5mM to sodium alginate at a ratio of 595:550nm, at concentrations ranging from 0-2mg/ml. The Pearson's correlation coefficient (r) was 0.86 with an absorbance range of 1.2-2.1 (0.7) for a 0-2mg/ml.

S-O - DM sodium alginate was added to 2mM S-O at concentrations ranging from 0-10mg/ml and the absorbance was measured at 595nm. The r was 0.98, and the absorbance increased as the alginate concentration increased. However the absorbance range for 0-10mg/ml was 0.14-0.22 (0.08) (**Table 3**).

T-B – The r was 0.63 when 2mM T-B was used, with a decrease in absorbance as the alginate concentration increased. Although the range of absorbance of 2.7-3.6 (0.9) was greater than S-O, the absorbance was beyond the linear scale of 1 (**Table 3**).

### 2.5.2 Mucin Standard Curve

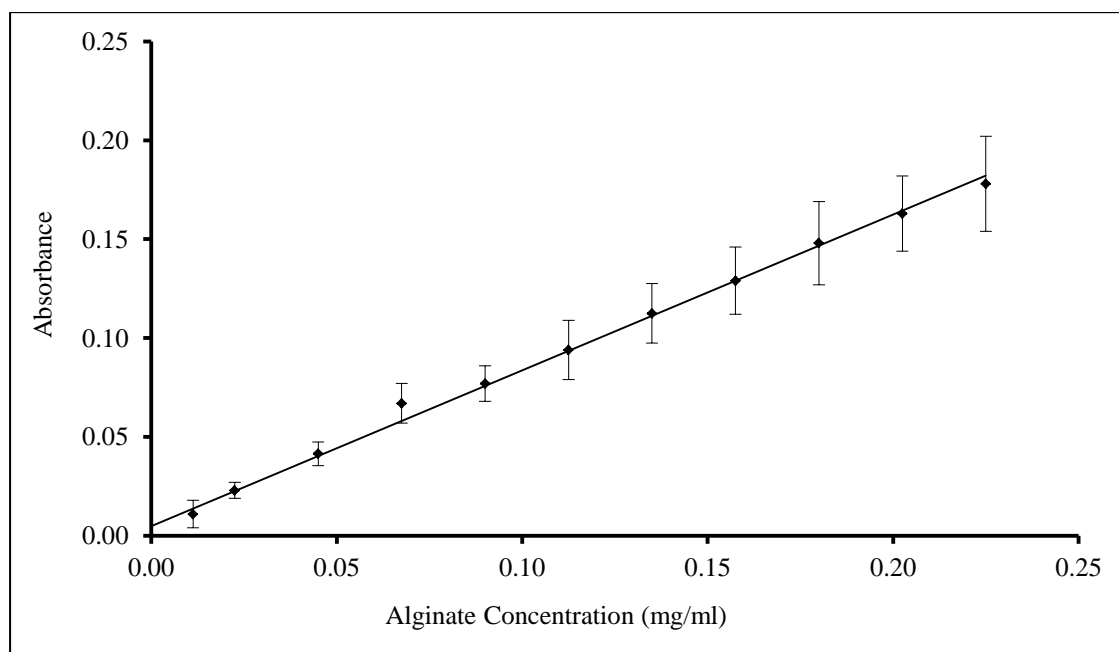
The data in **Figure 5** illustrates the standard curve for mucin in  $\text{DH}_2\text{O}$ , which was used as a positive control and confirms the previous research of Mantle and Allen (Mantle & Allen, 1978). There is excellent linearity and the range of absorbance and it remains on the linear scale between 0-1OD.



**Figure 5.** Mean ( $\pm$  S.D) standard curve of mucin in  $\text{DH}_2\text{O}$  using PAS assay ( $R^2 = 0.98$ ) ( $n=6$ ).

### 2.5.3 Alginate Standard Curve for stomach and small intestinal phase

The data in **Table 3** and **Figure 6** illustrate the absorbance range and standard curve for sodium alginate in gastric juice (30 seconds-60 minutes) and MG (180.5 minutes) respectively. The range of absorbance was 0.02-0.45 and 0.01-0.2 for 0-0.5mg/ml and 0-0.25mg/ml respectively, with excellent linearity for both phases of the model gut.

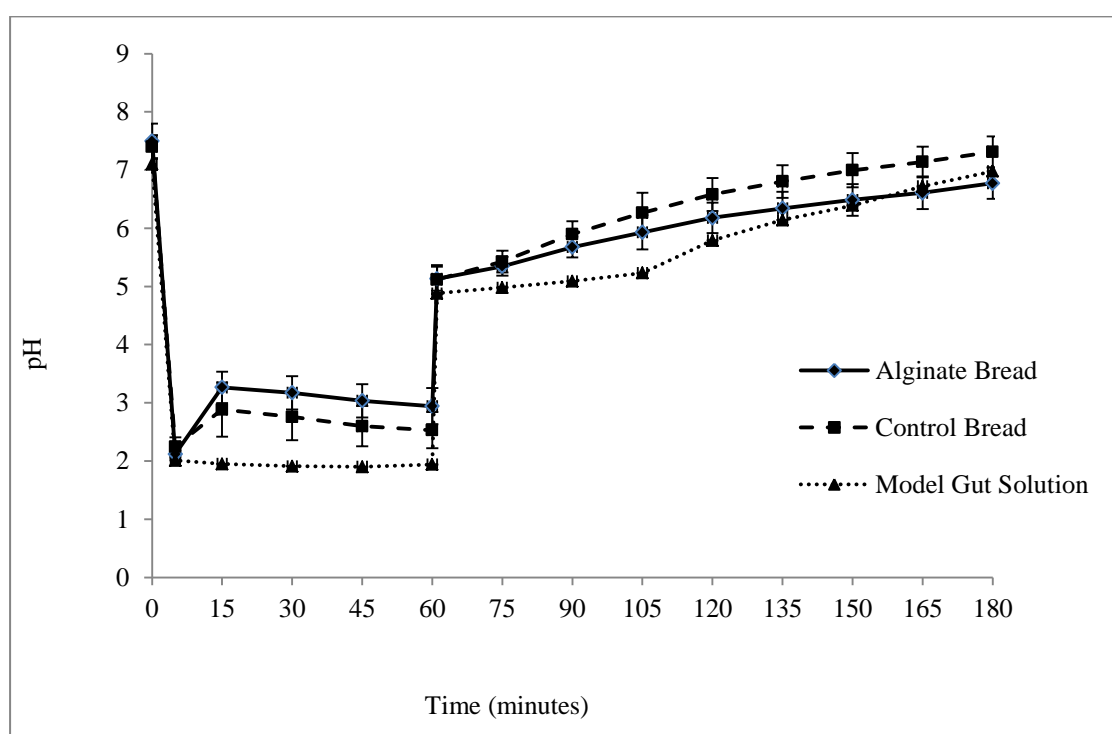


**Figure 6.** Mean ( $\pm$  S.D) standard curve for sodium alginate in model gut solution following alginate isolation, using PAS assay (n=6) (representing the intestinal phase) ( $R^2= 0.99$ ).

The data presented here demonstrates that the modified PAS is sensitive, repeatable, high throughput and is able to overcome interference from the model gut. When comparing dyes DMMB, T-B and S-O at various concentrations with the PAS assay, it is only the PAS assay that has good linearity, range of absorbance, and an increase in absorbance with an increase in concentration for relatively low concentrations of alginate.

## 2.5.4 pH of model gut system

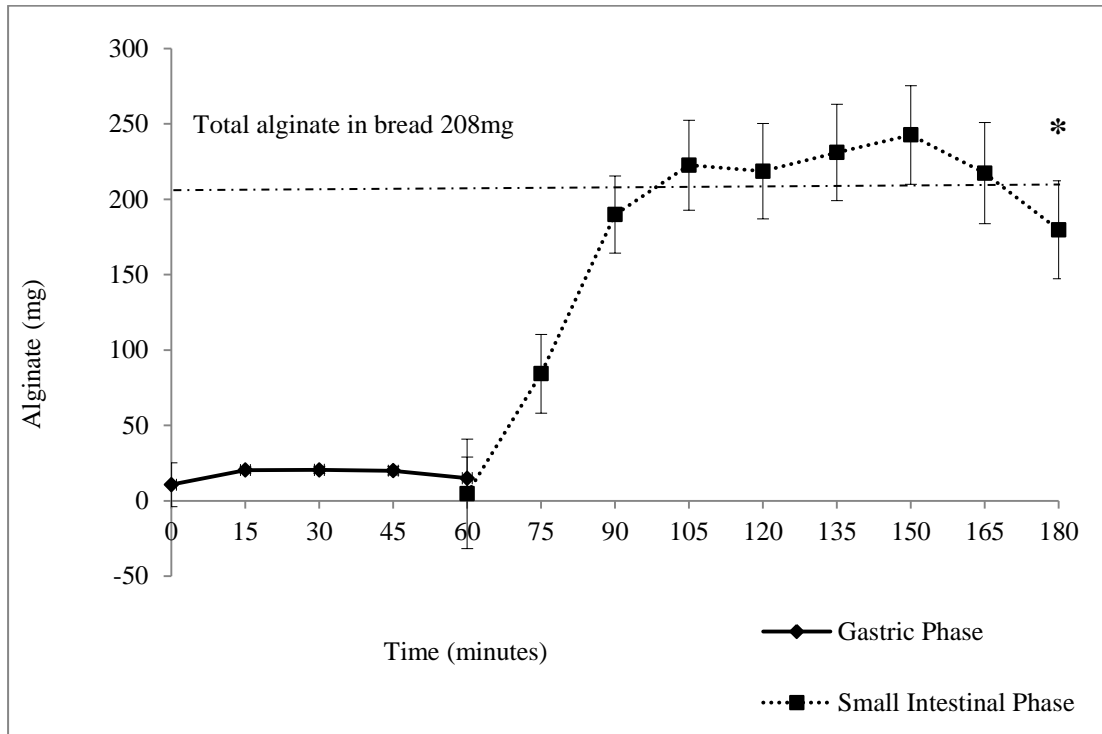
The model gut replicates the pH that would be expected for in-vivo digestion (**Figure 7**). The pH at 0 minutes in the mouth was 7.1 ( $\pm$  0.1), 7.4 ( $\pm$  0.2) and 7.5 ( $\pm$  0.3) for MG, CB and AB respectively. Following the addition of gastric juice the pH was reduced to 2.0 ( $\pm$  0.1), 2.2 ( $\pm$  0.2) and 2.1 ( $\pm$  0.1) for MG, CB and AB respectively. After 60 minutes in the gastric phase the pH had reduced further to 1.9 ( $\pm$  0.1) for MG, but rose for CB and AB to 2.8 ( $\pm$  0.4) and 3.2 ( $\pm$  0.3) respectively. At 180.5 minutes the pH had risen for all three samples to 7.0 ( $\pm$  0.4), 7.3 ( $\pm$  0.3) and 6.8 ( $\pm$  0.3) for MG, CB and AB respectively. There were no significant differences between MG, AB and CB at any time points ( $p > .05$ ).



**Figure 7.** Mean ( $\pm$  S.D) pH of the model gut throughout digestion of model gut solution alone, alginate and control bread (n=14).

### 2.5.5 Alginate release from bread.

The standard curve of the modified PAS assay was used to determine if any alginate had been released from the model gut during digestion. It is evident from **Figure 8** that the majority of alginate is released from the bread in the small intestinal phase of the model gut. Based on 4% by weight and the amount of bread added to the model gut there could be a total of 208mg,  $((5.2 / 100) \times 4 = 208$ , depicted on **Figure 8**) of alginate released if complete digestion occurs in the model gut. There was a significant effect of time on the release of alginate ( $p < .05$ ). Based on the total amount of alginate that is present in the bread there was 5.1 (7.0), 9.8 (1.0) and 86.1 (15.6) percent of the total alginate released at 0.5, 60 and 180.5 minutes respectively. The Post-Hoc Bonferroni reported no significant difference between 0.5 and 60 minutes ( $p > .05$ ). In contrast there was a significant difference in alginate release from the bread vehicle for all time points from 90-180.5 minutes when compared to 0.5 and 60 minutes ( $p < .05$ ) (**Figure 8**).



**Figure 8.** Mean ( $\pm$  S.D) milligrams of alginate released from digested bread in the stomach and small intestinal phase (corrected for interference from control bread) ( $n = 13$ ) (\* indicates significant difference in alginate release between 0 and 180 minutes and 60 and 180 minutes at  $p < .05$ ). The line at 208mg to indicate total alginate content within the alginate bread.



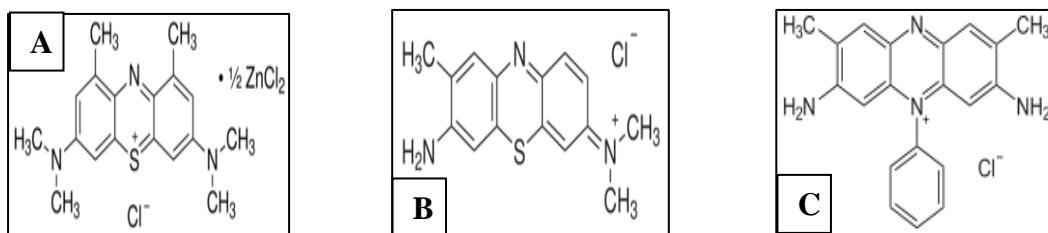
## 2.6 Discussion

The purpose of this chapter was to assess if cationic dyes were able to bind with alginate, and provide a suitable method to quantify alginate in a solution. Furthermore to assess if this method is suitable for determining if alginate is released from a bread vehicle at multiple time points. The data presented here indicates that the PAS assay is a simple and reproducible method for quantifying alginate in a solution. In addition the data here also indicates that this method may be robust enough to withstand the interference from the model gut system and still quantify the release of alginate from a bread vehicle at various time points.

Measurement of alginate is confounded within this system as the model gut has additional factors that may interfere with any techniques. Throughout the model gut there may be undigested bread which would need to be removed in order to ascertain the alginate released from the bread matrix during digestion. In addition the two major phases of the model gut also bring with them interference. The pH in the mouth can range between 5-7, and between 1.5-3.5 in the stomach (Allen, Flemstrom, Garner, & Kivilaakso, 1993) which is confirmed in **Figure 7**. This may pose a problem because once the pH of a solution passes below the acid dissociation constant (pKa) of the alginate gel formation is likely to occur (Atkins et al., 1971), making any binding with a dye difficult. Furthermore with the addition of porcine bile and pancreatic juice the colour of solution goes from transparent to a dark green/brown solution, therefore affecting any colorimetric measurement techniques.

Previous research has suggested several cationic dyes which may bind with alginate such as DMMB, T-B and S-O (Day & Marceau-Day, 1990; Richardson et al., 2004). An alternative method for quantifying alginate was designed by Halle et al. (1993), who developed a sensitive assay with the purpose of quantifying the amount of alginate in a solution and microcapsules. This method was based on the metachromatic changes induced in the cationic dye 1,9-dimethyl methylene blue (DMMB) as it binds to alginate. This assay was sensitive enough to work over a wide range of concentrations of alginate and a wide range of pH ranging from 6.5 to 13. Nonetheless as with the earlier methods this range of pH would not be suitable for *in-vivo* digestion. Richardson et al. (2004) developed and evaluated this method further to observe its reliability, and to detect if alginate molecular composition and weight affected the complexation of DMMB and alginate. Richardson et al. (2004) used alginate to bind with an anti-reflux treatment that was sprayed onto oesophageal mucosa and then scraped off. These scrapings were then

processed and DMMB was added and the absorbance was measured. They reported that this method had excellent reproducibility (coefficient of variation (CV) <3%), sensitivity (0.3g/L) and excellent linearity ( $r = 0.99$ ). However the alginate in the study was used in combination with an anti-reflux treatment and was scraped from the oesophageal mucosa, which may explain why these results were not repeatable with samples within this laboratory. The data presented in **Table 3** illustrates the most repeatable data replicating the methods of Richardson et al. (2004) produced in this laboratory. The  $R^2$  values of 0.86 and 0.63 for DMMB and T-B, respectively indicate a poor relationship between alginate concentration and absorbance. An alternative dye used here was S-O, which like DMMB and T-B is a cationic dye, and thus may complex with alginate through electrostatic forces. Although the data in **Table 3** elucidate an excellent  $R^2$  of 0.98, the range of absorbance is less than 0.1. This is despite the large concentrations of alginate that would suggest a larger absorbance if the dye is binding to the alginate. The anticipated result would be that if there is a larger concentration of alginate then this would be represented by a substantial increase in absorbance. Pal and Mandal (1990) suggest that cationic dyes may possess the ability to bind via electrostatic forces to the uronic acid carboxyl groups within alginate. Based on the work of Pal and Mandal (1990) the assumption would be that DMMB, S-O and T-B should possess the ability to bind with alginate; however the data presented here does not support these suggestions. One possible explanation of why these may not bind are that the research of Richardson et al. (2004) and Day and Marceau-Day (1990) used DMMB and gentian violet respectively, in conjunction with other compounds. For example Richardson et al. (2004) used alginate in combination with an anti-reflux treatment. This was sprayed onto oesophageal mucosa and then scraped off. This may suggest that it may be something within the anti-reflux treatment, mucin or something else from the oesophagus that is binding with the alginate, and in turn binding with the DMMB. Furthermore all three dyes are hydrophilic, with minimum positive charge, whereas alginate molecules are hydrophilic in nature (Smidsrod, 1974). For example DMMB (Taylor & Jeffree, 1969) has one potential binding site, although T-B (Bergeron & Singer, 1958) and S-O (Bandoni, 1979) contain more than one potential binding sites which may explain the results presented here. Furthermore based on the pKa of the dyes there is potential for charge:charge interactions as well as hydrogen bonding which would mean additional binding sites for the alginate with two, three and four potential binding sites for DMMB, T-B and S-O respectively (**Figure 9**). There is no research reporting a simple high throughput method for the easy and rapid quantification of alginate alone in a solution.



**Figure 9.** Structure of cationic dyes, A –1,9-dimethyl methylene blue (Taylor & Jeffree, 1969), B – Toluidine Blue (T-B) (Bergeron & Singer, 1958) and C - Safranin-O (S-O) (Bandoni, 1979).

As an alternative option Mantle and Allen (1978) developed a technique, known as the PAS assay which was designed to quantify glycoproteins. The PAS is dependent on the number of adjacent hydroxyl groups within a molecule, so in essence the larger the number of hydroxyl groups, the larger the absorbance value. As the intensity of the red colour is dependent on the number of adjacent hydroxyl groups, the larger amounts of glycoproteins should produce larger absorbance values. Alginates have similar structures to the carbohydrate side chains of glycoproteins and possess adjacent hydroxyl groups at C<sup>2</sup> and C<sup>3</sup> (Smidsrod, 1974), suggesting the PAS assay may be suitable technique for quantifying alginate in solution. To be able to quantify alginate within the model gut a standard curve must be created, however as previously mentioned there is interference within the model gut which did affect the PAS assay. Sodium alginate was dissolved in DH<sub>2</sub>O and model gut solution that had been run through the entire model gut up to 180.5 minutes, at a concentration of 0.49 and 2mg/ml respectively. The background colour from the model gut solution added to colour of the PAS assay and therefore this needed to be reduced. Using a precipitation and dilution step, as described in the methods, the colour of the samples was reduced. A further correction was used to subtract the absorbance for the model gut solution alone. It is clear that the standard curve for alginate in both DH<sub>2</sub>O and model gut solution has a good range of absorbance and linearity as demonstrated in **Table 3** and **Figure 6**, and the mucin standard curve confirmed the PAS assays effectiveness. A further point of interest is that the absorbance per unit weight was larger for alginate in gastric juice than alginate in the small intestine. At any given alginate concentration the absorbance was larger in the stomach phase when compared with the small intestinal phase. A potential explanation for this may have been that the alginate may be interacting with something within the model gut, such as bile acids (Wang,

Onnagawa, Yoshie, & Suzuki, 2001). If this is the case then this may reduce the ability of the alginate to interact with the PAS assay and account for the lower absorbance.

As previously mentioned the PAS appears to be an effective method for quantifying alginate under the conditions mentioned here. In order for the bread to be digested the model gut must be operating at the optimal pH conditions for digestion (Allen et al., 1993). Previous research has reported the pH in the stomach to range between 1.8-2.5 (Lindahl, Ungell, Knutson, & Lennernas, 1997) however this pH can be buffered with the addition of a food or drink. The pH of the small intestines can begin as low as 2.5 in the duodenum and then raises up to around pH 8 with the addition of bile and pancreatic juice as the digesta pass through the small intestines (Brownlee, Forster, et al., 2010). These changes in pH during *in-vivo* digestion are optimal for digestion of protein, fat and carbohydrates and therefore it is essential that the model gut follows a similar pattern to ensure that digestion of the bread occurs, and that alginate is released from the bread. The data presented here supports the use of the model to replicate *in-vivo* digestion, and has optimal pH conditions for the digestion of bread to occur.

If alginate is to be used as an obesity treatment the release rate of the alginate from the bread during digestion must be achieved. It is apparent from the data presented here that there is only a small amount of alginate released in the gastric phase, however the alginate is predominantly being released from the bread matrix in the small intestine. The main constituents of bread are carbohydrates, specifically starch and it has been well documented that salivary and pancreatic amylase liberates  $\alpha$ -1-4glucose linkages in starch, with the majority occurring in the small intestines (Crapo, Reaven, & Olefsky, 1977; Jenkins et al., 1978; Jenkins et al., 1980). This liberation releases maltose maltotriose,  $\alpha$ -limit dextrins and small portion of glucose. Once liberated the molecules that are released are then hydrolysed at the brush border before being absorbed into the blood stream (Jenkins et al., 1980). If the alginate is thoroughly mixed into the bread matrix then the expectation would be that as the starch is digested the alginate will be released from the bread matrix and this is what the results here show. These data also confirm that the PAS assay is a suitable method for determining the release of alginate from a bread vehicle in an *in-vitro* model gut.

The time point of 180.5 minutes is generally the end of the *in-vitro* model gut, which does replicate *in-vivo* digestion in the small intestines; however *in-vivo* digestion may take longer depending on the contents of the digested meal. 85-90% of fat in the human diet is

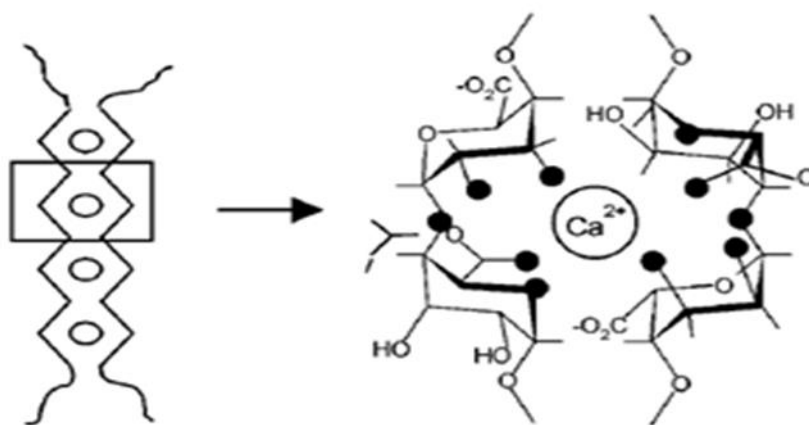
digested in the small intestines and absorbed into the blood stream (Miled et al., 2000). Previously reported data from this laboratory has demonstrated that alginates can attenuate the activity of digestive enzymes (Richardson, Dettmar, Wilcox, Brownlee, & Pearson, 2011; Strugala et al., 2005; Sunderland et al., 2000). The reduction in the activity of digestive enzymes is dependent on the composition of the alginate, with some alginates reducing activity by up to 85% (Richardson et al., 2011). These data and the data presented here suggests that if the alginate is released in the small intestinal phase this may have an inhibitory effect on the activity of pancreatic lipase. If the lipase does not digest the di and triglycerides then they are too large to pass through the cell walls of the small intestines and will therefore pass through the upper gastrointestinal (GI) tract undigested. These data and the data presented here suggests that if the alginate is released in the small intestinal phase this may have an inhibitory effect on the activity of pancreatic lipase. Further work is required to assess if the alginate bread is able to inhibit fat digestion in the model gut system in the development of potential therapeutics treatments against obesity.

## Chapter 3: Biological Activity of Alginate

### 3.1 Introduction

If dietary fibre, but specifically alginate is to be used in the treatment for obesity an appropriate delivery method must be designed. There is evidence that has investigated the use of alginate as an obesity treatment, and particularly using beverages and cereal bars as a delivery vehicle. Despite the numerous physiological benefits reported when adding alginate to a beverage or cereal bar, subjects generally experience poor palatability or other symptoms such as abdominal pain or distension compared to the controls (Jensen et al., 2012 (a); Sandberg et al., 1994; Torsdottir et al., 1991; Williams et al., 2004). The bread vehicle selected in the present study was used to observe an acute effect on triglyceride digestion and acceptability in ileostomy patients (both made by Greggs Plc) (data presented in Chapter 5). There were no significant differences between the two breads suggesting that acceptability of the bread in a clinical trial would not be an issue. In addition to the acceptability study the data presented suggests that as the alginate bread is digested within the model gut the alginate is released from the bread. This being the case then bread may be a suitable delivery method for alginate. Although the digestion of the bread, and release rate of alginate have been determined, further work must be conducted to assess the ability of the AB is able to reduce fat digestion both *in-vitro* and *in-vivo*.

As previously mentioned the ability of alginate to form both ionic and acidic gels has received considerable interest, partially as this may be a possible mechanism for a reduction in the activity of digestive enzyme activity and increased satiety. Rees (1981) provided early evidence to suggest that the carboxyl and hydroxyl groups of the alginate were responsible for the binding sites of alginate and the formation of the “egg box model”, as depicted in **Figure 10**. This was later confirmed by the work of Braccini and Perez (2001) who developed a molecular modelling program which involved a pairing technique that assessed all the possible associations of the polymer chains with calcium ions to form dimers. Although Braccini and Perez (2001) generally discussed calcium, they indicated that the “egg box model” was able to accommodate ions of various sizes. The formation of ionic gels which is likely to occur in beverages that alginate is added to may account for the slimy mouth feel and poor palatability (Jensen et al., 2012 (a); Wolf et al., 2002).



**Figure 10.** Egg-box model (Braccini & Perez, 2001). The dark circles depict oxygen atoms required for the organisation of the calcium ions.

Furthermore, upon consuming the beverage it will pass from the mouth and into the stomach, where the pH can be as low as 1.8. At a pH below the pKa of the guluroinic and mannuronic acid (3.65 and 3.38, respectively) within alginate, an acidic gel is formed (Atkins et al., 1971). If the alginate forms an acidic gel Draget, Skjåk Bræka, and Stokke (2005) suggested that the “egg box model” would not be able to be maintained due to increased intermolecular hydrogen bonds. This formation of an acidic gel was confirmed when alginate was added to a milk based meal replacement beverage in a study by Hoad et al. (2004). This research used MRI scans to observe the characteristics of the beverage within the stomach. They reported that the milk based meal replacement that contained alginate formed large lumps within the stomach and increased satiety, however there was no difference between gastric emptying between the treatment and control groups. The formation of large lumps was said to be due to the formation of an acidic gel within the stomach and Hoad et al. (2004) suggested that this may be enough to cause poor mixing within the stomach between foods and digestive enzymes such as pepsin and gastric lipase. Although the development of an acidic gel may be enough to disrupt the digestion within the stomach it remains to be seen as to whether this acidic gel can withstand the muscular contractions within the stomach and the small intestines. If the gel cannot withstand the muscular contractions within the stomach then the gel will cease to exist at the high pH of the small intestine. Draget et al. (2005) demonstrated that acidic gels were considerably more turbid and had brittle textures compared to ionic gels. Furthermore compression analysis showed that acidic gels failed at a tenth of the load required to disrupt an ionic gel. These data demonstrate that alginates do form acidic gels within the

stomach, nonetheless it remains to be seen as to whether or not this formation is enough to have any impact upon digestive enzymes within the upper GI tract. Work by Seal and Mathers (2001) indicated that the formation of a gel may be responsible for reducing cholesterol and fat digestion in rats fed dry alginate in their food. Within the small intestine, the authors suggested that the alginate may reduce the interaction of bile salts and cholesterol, and thus affect cholesterol and its metabolites (Seal & Mathers, 2001). The data produced by Seal and Mathers (2001) may be due to a re-formation of an ionic gel as the pH will increase within the small intestines, indicating a more robust gel that may withstand the muscular contractions within the small intestines. However the authors failed to confirm this.

The viscous/gel solution that alginates are able to form once combining with gastric juice are also believed to increase satiety and reduce calorie intake (Kristensen & Jensen, 2011). It is by the same mechanism that Shah et al. (1986) suggested that the viscous solutions formed by water soluble dietary fibres was responsible for a reduction in pepsin activity. The authors indicated that the viscous solutions created a sluggish mixing process within the stomach, resulting in poor substrate-enzyme binding and thus causing a reduction in pepsin digestion. The *in-vivo* and *in-vitro* data here implies that viscosity may have a role to play in satiety and reducing digestive enzyme activity. Nevertheless, the question remains as to whether the alginate that is being released during digestion in the model gut increases the viscosity of the solution, as the previous data proposes.

A further point of interest is whether the alginate contained within the bread is affected during the mixing and cooking processes involved in bread baking. Although we know that alginates are able to form ionic and acidic gels at 37°C (Draget et al., 1994; Hoad et al., 2004), there is not a substantial amount of data on the properties of the alginate once it has been heated. McDowell (1977) suggested that when polymers are heated at temperatures above 100°C the alginates structure may depolymerise. McDowell (1977) also indicated that at extreme temperatures in excess of 200°C complete breakdown of the alginate and a rapid evolution of one molecule of CO<sub>2</sub> for every uronic acid group would occur. This would hinder the ability of alginates forming ionic and acidic gels. These temperatures as mentioned above can easily be reached during the cooking process of bread (Hasatani et al., 1991), and therefore raises the questions as to whether the alginate released from the bread during digestion as illustrated in **Figure 8** retains its ability to form a viscous solution in the upper GI tract.



## 3.2 Aims

The data depicted in **Figure 8** implies that the release rate of the alginate can be determined. However the attributes of the alginate released from the vehicle requires further analysis. Specifically how the release of alginate from the bread affects the attributes of the solution during digestion. Hoad et al. (2004) and Seal and Mathers (2001) indicate that alginate enriched products can form an acidic or an ionic gel in the stomach and small intestine, respectively. Although the suggestion is that this gel formation may be a possible mechanism in reducing enzyme activity, or the interaction between substrate and enzyme this remains to be seen. The aims of this chapter are to:

- Utilise a model gut that will digest the bread and allow samples to be taken at multiple time points throughout, including the stomach and small intestinal phase of digestion. This will determine whether the alginate released, as suggested in **Figure 8**, chapter 2 will affect the properties of the solution within the model gut. If the alginate is in-fact being released then the expectation would be for the viscosity in the small intestines to increase as this is where between 80-90% of the alginate is released, and confirm the suggestions of Seal and Mathers (2001). This may provide further evidence as to whether viscosity is a potential mechanism responsible for reducing digestive enzyme activity.
- To determine whether alginate is able to withstand temperatures up to 180°C, and retain its ability to form a viscous solution within the model gut. McDowell (1977) has reported that when polymers are exposed to temperatures in excess of 100°C they can undergo depolymerisation. This may ultimately affect the ability of the alginate to form a gel within the upper GI tract.
- To assess the physiochemical properties of the solution within the model gut following digestion of alginate bread. In addition to the cooking process of the bread, this chapter will assess whether alginate can be isolated from the model gut and determine whether the alginate retain its inhibition properties despite being cooked into the bread, digested in the model gut and isolated. These data will ultimately provide an insight into how robust alginate is and whether viscosity is a mechanism that contributes to attenuation in the activity of digestive enzymes.

### 3.3 Methods

#### 3.3.1 Materials

Sepharose 2B (60-200µm diameter), methyl orange (MO), dextran blue (DB), sodium chloride, sodium azide, Tris, methanol, acetone, colipase, lipase, orlistat (tetrahydrolipstatin) were purchased from Sigma-Aldrich (Poole, UK). Aluminium oxide were purchased from Fisher Scientific (Loughborough, UK), and olive oil was purchased from Co-operatives Foods (Manchester, UK). Bile acids (deoxycholic acid sodium salt and taurodeoxycholic acid sodium salt) were purchased from Fluka (Buchs, Switzerland). The alginate was a gift from FMC BioPolymer AS, Drammen, Norway and was stored at 4°C in tightly-sealed containers and all alginate weights were corrected for water content. Alginate and control bread and materials for PAS assay were those used in chapter 2.

#### 3.3.2 Equipment

All assays were carried out using BioTek 96 well plate reader at 550nm (ELx808 BioTek, Bedfordshire, UK). Two Grant GD 100 water baths were used for incubating synthetic model gut solutions and samples within the model gut at 37°C (Fisher Scientific, Loughborough, UK). A Sci-Q 401U/D peristaltic pump was used to pump synthetic solution into samples in the model gut (Watson-Marlow, Fisher Scientific, Loughborough, UK), three IKA<sup>®</sup> laboratory egg compact mixers. A Thermo Jouan BR4i Mu Scientific Centrifuge (Basingstoke, UK) and an Eppendorf Centrifuge 5415 R (Stevenage, UK) were used for samples <4ml and >2ml, respectively. A Fraction Collector FRAC-100 and a Peristaltic Pump P-1 were purchased from Pharmacia Fine Chemicals (Stockholm, Sweden) to calculate the elution volume from gel filtration. Viscosity measurements were assessed using a Low-Shear-30 viscometer from Contraves (Malacca, Malaysia), Gallenkamp Hot Box Bench Top Laboratory Economy Incubator - with Fan SIZE 2 (Germany). Ultra-Turrax T18 IKA Homogeniser with dispersing tools S 18 N – 10G and 19G from Fischer Scientific (Loughborough, UK) was used to emulsify olive oil substrate and lipase buffer. An Edwards Modulyo EF4-174 Freeze Dryer (London, UK) was used to isolate alginate.

#### 3.3.3 Model Gut Procedure

The model gut was run as per chapter 2 with model gut solution alone run as a control, with the following experiments carried out:

- i) 5.2g Alginate bread (AB) alone

- ii) 5.2g Control bread (CB) alone
- iii) 208mg of DM alginate alone
- iv) 5.2g CB and 208mg DM alginate
- v) Model gut solution from 180 minutes spiked with 208mg DM alginate
- vi) 5.2g CB from 180 minutes spiked with 208mg DM alginate

In the experiments above 50ml of solution was taken from 180 minutes and incubated at 37°C. Model gut solution alone from 180 minutes was used as a control for all samples. In experiments v and vi the solutions were spiked with 208mg DM alginate to replicate the concentration that would be present if the alginate was released from 5.2g of AB. The concentration of alginate in the model gut solution at the end of the model gut can be calculated using **Equation 1**.

$$5.2\text{g AB} = 4\% \text{ by weight of DM alginate powder}$$

$$(5.2/100) \times 4 = 208\text{mg at 180 minutes and 30 seconds}$$

$$208\text{mg}/132\text{ml (total solution at end of model gut)} = 1.57\text{mg/ml}$$

**Equation 1.** Calculation of amount of alginate in 5.2g AB.

### 3.3.4 Viscosity Measurements

The six samples of model gut solutions taken from the end of the model gut were, compared against model gut solution alone. The heated alginates were measured at 2mg/ml in DH<sub>2</sub>O, compared to DH<sub>2</sub>O alone. A 2ml sample from each experiment was added to the measuring cup of the viscometer. The viscometer was set to a range 4 or 5 to ensure that the sample being measured gave less than 100% deflection. The measuring bob was then lowered into the measuring cup and the viscometer records percentage over a range of shear rates. This can be used to calculate specific viscosity ( $\eta_{sp}$ ) using **Equation 2** and **Equation 3**.

$$\eta_{rel} = \frac{\text{Percentage Deflect Sample}}{\text{Percentage Deflect Control}}$$

**Equation 2.** Relative Viscosity ( $\eta_{rel}$ ).

$$\eta_{sp} = \eta_{rel} - 1$$

**Equation 3.** Specific Viscosity ( $\eta_{sp}$ ).

### 3.3.5 Alginate Isolation

Upon completion of the model gut procedure the solutions from 5.2g AB, 5.2g CB and 5.2g CB with 208mg DM alginate (i, ii and iv) were put through an isolation process. 4ml and 8ml samples of 5.2g AB and 5.2g CB runs were prepared and a 4ml sample of the 5.2g CB with 208mg DM alginate. All five samples were diluted by 50% with methanol and then mixed and placed into a freezer at -20°C for 30 minutes. The samples were then centrifuged at 4100 rpm for 20 minutes. The supernatant was removed and 4ml methanol was added and the pellet re-suspended, before being placed in the freezer at -20°C for 30 minutes and then centrifuged at 4100 rpm for 20 minutes. After centrifugation the supernatant was removed and the process was repeated using the pellet. All four samples were then placed in the freeze dryer until the DH<sub>2</sub>O had completely sublimated leaving a dry pellet. The pellets for each sample were then weighed. The predicted weights of the samples can be calculated using **Equation 4**.

Alginate Bread is 4% alginate by weight =  $(5.2\text{g}/100) \times 4\% = 208\text{mg}$

Predicted Weight:  $5.2\text{g AB } 4\text{ml} = (208/132\text{ml}) = 1.57\text{mg/ml} \times 4\text{ml} = 6.3\text{mg}$

$5.2\text{gAB } 8\text{ml} = (208/132) = 1.57\text{mg/ml} \times 8\text{ml} = 12.6\text{mg}$

$5.2\text{g CB and } 208\text{mg DM} = (208/132) = 1.57\text{mg/ml} \times 4 = 6.3\text{mg}$

**Equation 4.** Predicted weight of samples containing alginate from the end of the model gut.

### 3.3.6 Heating of Alginate

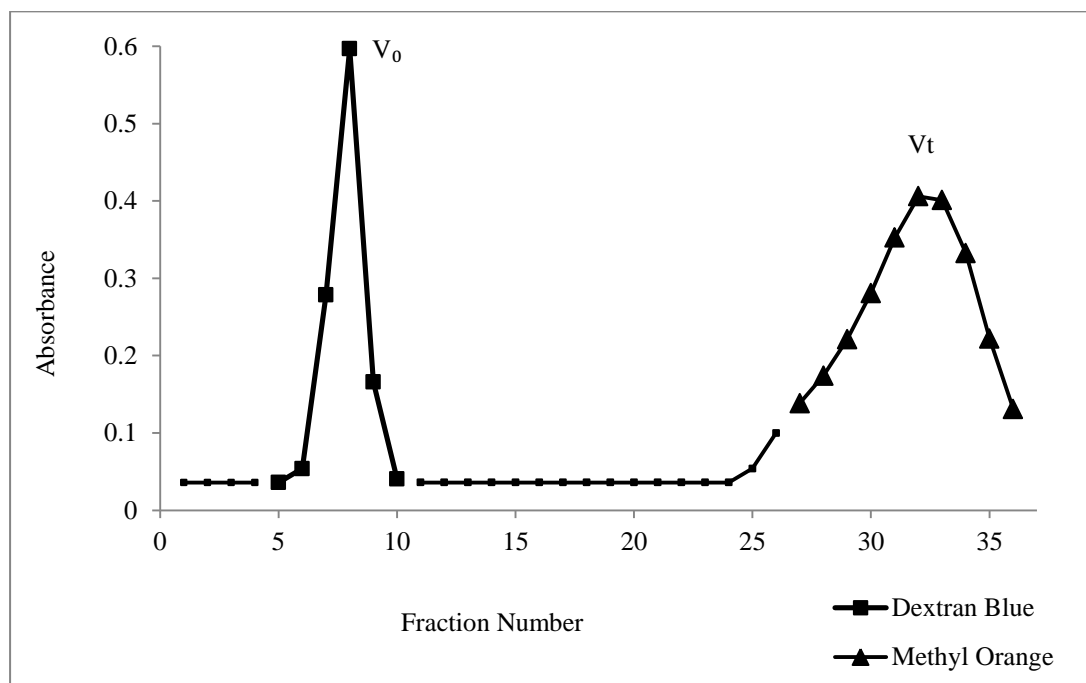
5g of three sodium alginates (LFR 5/60, DM and SF200) with a molecular weight with a range of 40-380 kDa and a mannuronate/guluronate ratio of 0.44-1.38 were added to glass pyrex tubes and then heated at 37, 100 and 200°C. DM was also heated at further temperatures of 50 and 150°C. Each alginate was heated at the temperatures above for 30 minutes before being cooled to room temperature.

### 3.3.7 Gel Filtration

Sepharose 2B was added to approximately 500ml of gel filtration elution buffer consisting of sodium chloride (0.2M), sodium azide (0.003M) and then made up to 1L in DH<sub>2</sub>O and then left over night to ensure complete hydration. The sepharose 2B was then packed into a column measuring 30 cm in length and 1.46 cm in diameter. Once tightly packed the ends of the column were sealed and a tube from the top of the column was placed into the buffer reservoir, and a tube from the bottom of the column was placed into the peristaltic pump. An additional tube ran from the peristaltic pump into the fraction collector, which was set to collect elution volume in 2ml volumes.

### 3.3.7.1 Calibration

Void volume ( $V_o$ ) and Total volume ( $V_t$ ) were determined using dextran blue (DB) and methyl orange (MO) respectively, as seen in **Figure 11**.



**Figure 11.** Calibration for gel filtration using MO and DB.  $V_o = 16\text{ml}$  and  $V_t = 64\text{ml}$  ( $n=6$ ).

### 3.3.7.2 Gel Filtration Samples

Sodium alginates alginate LFR 5/60, DM and SF200, post heating at 37, 100 and 200°C were re-suspended in elution buffer at a concentration of 1.43mg/ml. The freeze dried samples from 5.2g AB, 5.2g CB and 5.2g CB with 208mg DM were also re-suspended in the elution buffer at a concentration of 1.43mg/ml, based on the freeze dried weight. After each of the samples had passed through the column 200 $\mu\text{l}$  of each fraction were added to a 96-well in duplicate plate and the PAS assay was run as per chapter 2.

### 3.3.8 Lipase Inhibition Properties of Heated Samples and Isolated Alginate

Standard olive oil ( $\eta_{sp}$  of 72.5 ( $\pm 10$ )) was filtered by passing it through aluminium oxide (8cm deep in a glass chromatography column) to remove free fatty acids. 10g of the filtered olive oil was made up to 100ml using acetone, giving a 10% solution. This was then diluted down with acetone to a 1% stock solution and stored in the fridge at 4°C ready for use. The 1% solution was then used as the olive oil stock solution for all

experiments. The substrate solution was 0.05M Tris buffer at pH 8 and was stored in the fridge at 4°C. 100ml of the substrate solution was heated up to 70°C and sodium deoxycholate was then added at 0.35%. 4ml of the 1% olive oil stock solution was then added and homogenised for 10 minutes whilst maintaining at 70°C. The solution was then cooled to room temperature and could then be used for the assay for up to 6 hours. The enzymes used were 1.29mg/ml lipase and 18µg/ml colipase in DH<sub>2</sub>O. Orlistat was made up to 0.025mg/ml in DH<sub>2</sub>O at the same concentration as a reagent blank. Heated DM alginate at 37, 100 and 200°C and freeze dried 5.2g AB were added to the substrate solution that contained the olive oil at 3, 2, 1mg/ml.

240µl of either substrate solution alone (as a control) or the substrate containing heated or freeze dried alginate were added to a 96-well plate in duplicate. On a second 96 well plate 10µl of enzyme solution or DH<sub>2</sub>O was added to the wells. Orlistat was included with one row of enzyme solution and one row of DH<sub>2</sub>O. Both plates were incubated at 37°C for 15 minutes. After incubation 200µl of the substrate solution plate were added to the corresponding wells on the 96-well containing the enzymes or DH<sub>2</sub>O. The plate was then placed on the plate reader at 37°C and read every 5 minutes at 405 nm for 35 minutes. In order to calculate the level of lipase inhibition the blanks were subtracted from their corresponding controls, an example can be seen in **Equation 5**.

$$\text{Percent of lipase inhibition} = 1 - \frac{\text{Inhibition Control} - \text{Polymer Sample}}{\text{Inhibition Control} - \text{Lipase Control}} \times 100$$

**Equation 5.** Equation to calculate the percentage of lipase inhibition.

### 3.4 Statistical Analysis

Statistical calculations were undertaken using SPSS Statistics 19 (IBM, Predictive Analysis Software, USA). Data presented as mean and standard error of mean (S.E.M). A Two-way Repeated ANOVA followed by a Post-Hoc Bonferroni were undertaken at a significant level ( $\alpha$ ) of 0.05 to compare the level of pancreatic lipase inhibition of DM alginate heated at 37, 100, 150 and 200°C.

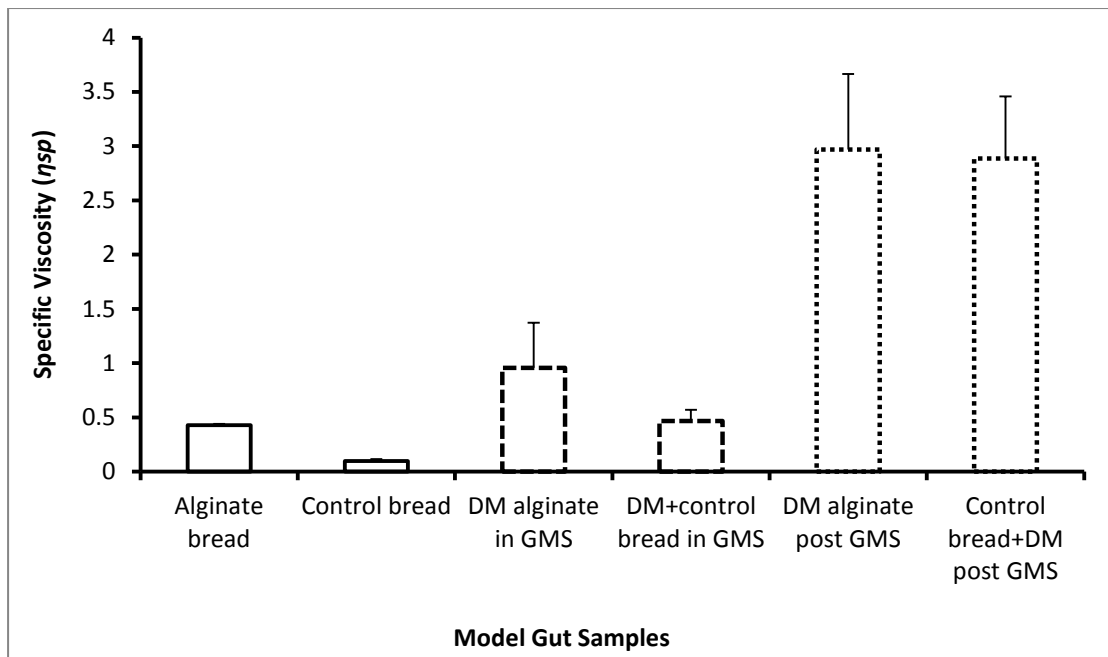
## 3.5 Results

### 3.5.1 Viscosity

#### 3.5.1.1 Model Gut Samples

The viscosities of the samples from the end of the model gut are shown in **Figure 12**. The viscosity for 5.2g AB and CB were relatively low with 0.42 ( $\pm 0.01$ ) and 0.11 ( $\pm 0.01$ ) respectively. DM alginate alone and 5.2g CB and DM alginate that passed through the model gut had a larger specific viscosity of 0.91 ( $\pm 0.41$ ) and 0.46 ( $\pm 0.11$ ) respectively. These data indicate that when bread is present there is a reduction in the ability of alginate to form a viscous solution. The viscosity of the gut model solution and 5.2g CB that were spiked with DM alginate after the model gut had a substantially larger viscosity of 2.96 ( $\pm 0.71$ ) and 2.88 ( $\pm 0.57$ ) respectively than the 5.2g AB sample. The data in **Figure 12** demonstrate that alginate passing through the model, either in a bread vehicle or dry had a lower specific viscosity than when it is added to solution from the model gut once it has finished. A combination of the bread and the model gut appear to be attenuating the ability of the alginate to form a viscous solution.





**Figure 12.** Mean (+ S.E.M) viscosity of samples taken from the end of the model gut (n=6). Alginate Bread (5.2g), Control Bread (5.2g), DM alginate (208mg) and 5.2g CB with DM alginate (208mg) were added at beginning of model gut. DM alginate (208mg) was added to model gut solution and 5.2g CB solution from the end of the mode gut. All viscosity measurements were made at the end of the model gut.

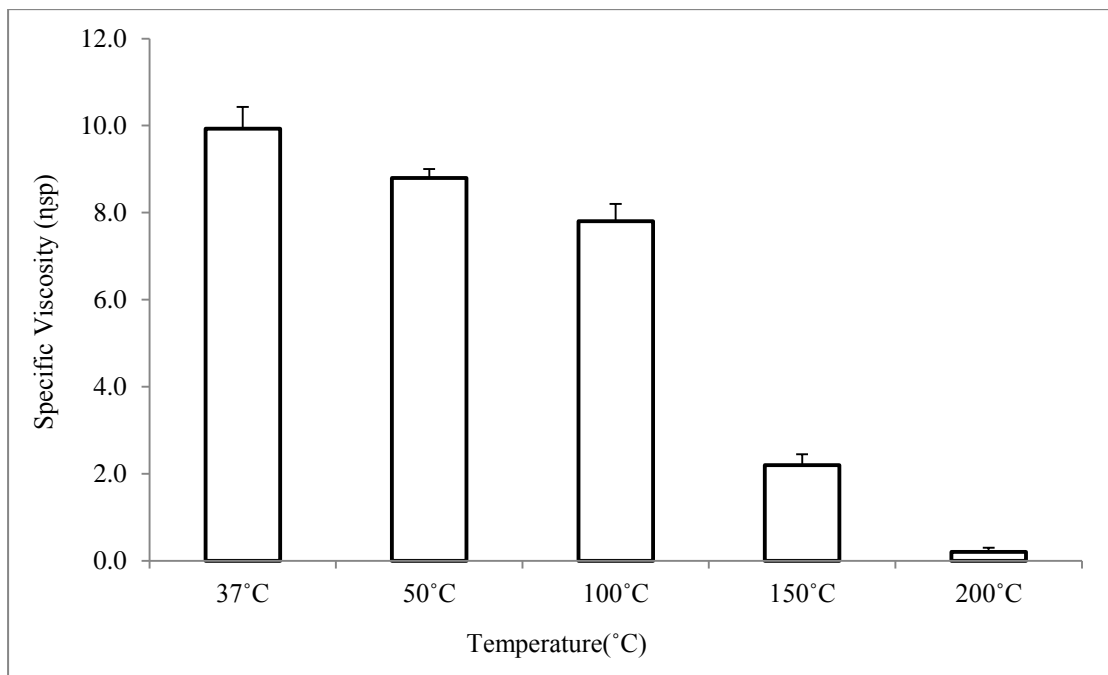
### 3.5.1.2 Alginate Thermal Treatment

The data presented in **Table 4** is for alginates LFR 5/60, DM and SF200 at 37°C and post heating at 200°C. The specific viscosity relates to the molecular weight with SF200 having a specific viscosity of 14, DM alginate 12 and LFR 5/60 2.2. Post heating at 200°C there was a substantial decrease in  $\eta_{sp}$  for all three alginates down to 0.1, 0.2 and 0.2 for DM, LFR 5/60 and SF200 respectively. These data indicate extensive fragmentation of the alginate chains following heating at 200°C.

**Table 4.** Mean  $\eta_{sp}$  of alginates at 2mg/ml in DH<sub>2</sub>O at 37°C and post heating at 200°C for 30 minutes (n=6).

<b>Alginate</b>	<b>Molecular Weight</b>	<b>Pre Heating</b>	<b>Post Heating @ 200°C</b>	<b>% <math>\Delta</math> in <math>\eta_{sp}</math></b>
<b>DM</b>	250,000-320,000	12.0	0.1	99.6
<b>LFR 5/60</b>	40,000	2.2	0.2	90.1
<b>SF200</b>	380,000	14.0	0.2	98.9

The  $\eta_{sp}$  for DM alginate heated at temperatures between 37-200°C is illustrated in **Figure 13**. The  $\eta_{sp}$  remained relatively stable between 37, 50 and 100°C, with only a reduction of 16 and 21% for 50 and 100°C when compared with 37°C. Beyond 100°C there is a large drop in  $\eta_{sp}$  compared to 37°C. The  $\eta_{sp}$  for 150 and 200°C has 2.2 and 0.2 respectively, equating to a reduction of 78 and 99%. The data in **Figure 13** indicate that alginates may not be able to withstand temperatures in excess of 100°C, and that these high temperatures alter the ability of alginate to form viscous solutions.



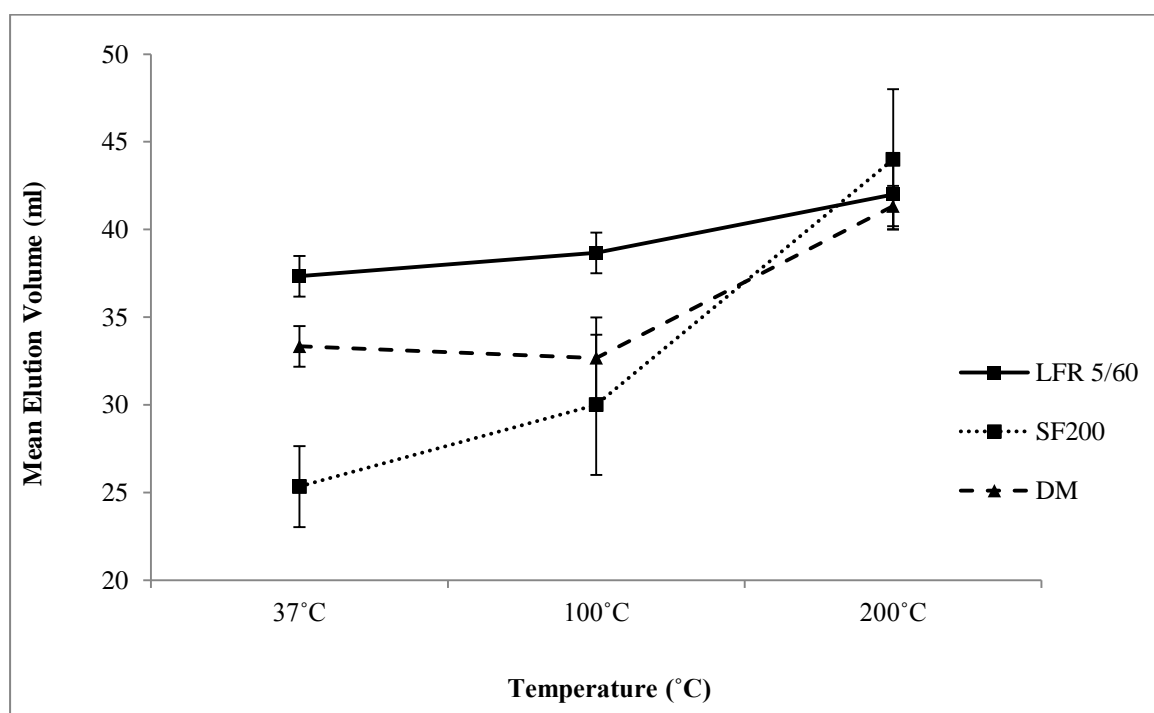
**Figure 13.** Mean (+ S.E.M)  $\eta_{sp}$  of DM alginate after being heated at 37, 50, 100, 150 and 200°C for 30 minutes. Each sample was then allowed to return to room temperature and then re-suspended in DH<sub>2</sub>O at 1.43mg/ml (n=6).

## 3.5.2 Gel Filtration

### 3.5.2.1 Heated Alginates

Following incubation at 37°C the initial elution volumes correspond well with the  $M_r$ . The elution volumes were 25, 33 and 37ml for SF200, DM and LFR 5/60 respectively.

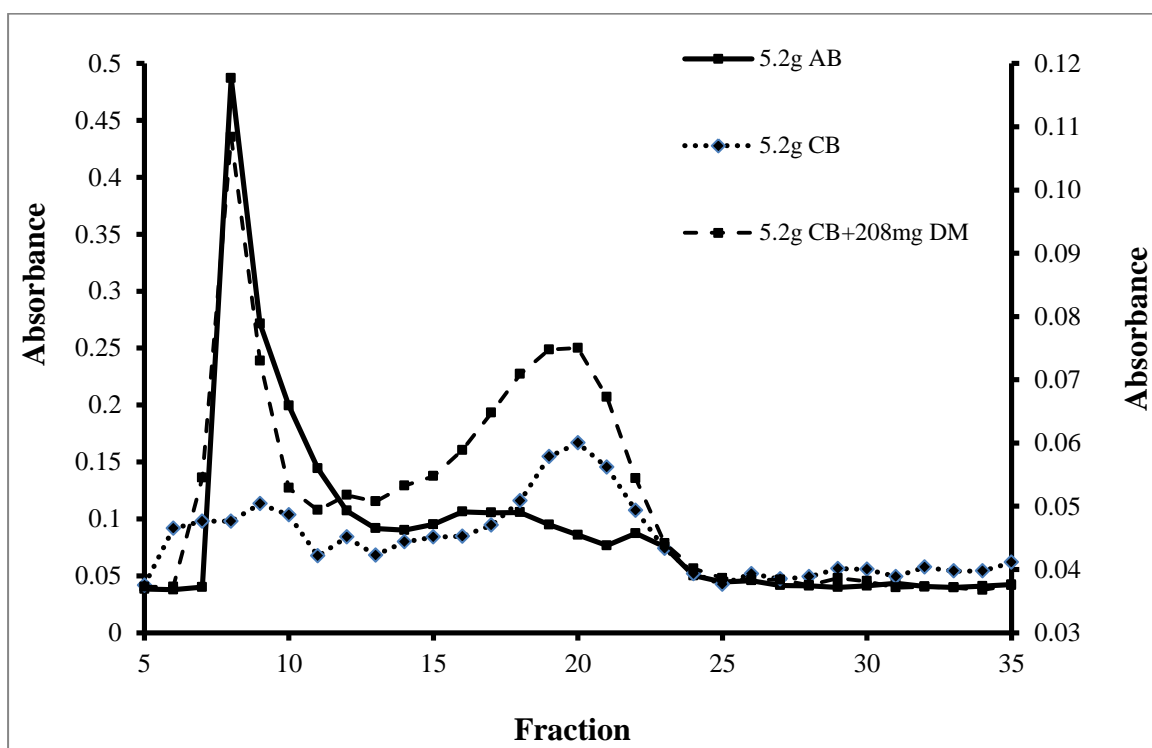
Heating to 100°C resulted in a small increase in elution volume for LFR 5/60 up to 38ml and 30ml for SF200. However there was no change in elution volume for DM following heating at 100°C. Post heating at 200°C all three alginates had an increase in elution volumes, rising to 44, 41 and 42ml respectively for SF200, DM and LFR 5/60, indicating a temperature effect on the alginate **Figure 14**. These data indicate that the increase in elution volume is likely to be as a result of a reduction in  $M_r$ .



**Figure 14.** Mean (+S.E.M) elution volumes for DM, SF200 and LFR 5/60 alginates following 30 minutes heating at 37, 100 and 200°C. All three alginates were then re-suspended in DH<sub>2</sub>O at 1.43mg/ml (n=6).

### 3.5.2.2 Freeze dried samples isolated from the end of the model gut.

The elution profile for freeze dried samples from the end of the model gut following isolation and freeze drying is depicted in **Figure 15**. 5.2g AB is depicted below with a peak elution volume of 18ml which was consistent with the mean elution volume of DB. 5.2g CB and 208mg DM alginate had a mean peak elution volume of 18ml, which was the same as 5.2g AB. In contrast the 5.2g CB alone had a mean elution volume of 42ml, which was 58% larger than both the 5.2g AB and 5.2g CB with 200mg DM alginate. These data indicate that samples that contain alginate elicit a smaller elution volume than those without, and that the alginates  $M_r$  is not being reduced despite the cooking process of the bread, digestion in the model gut or the isolation process. These data indicate that the CB does not contain high molecular weight material. The PAS assay was a suitable method for quantifying samples that contained alginate with absorbance values ranging from 0.48 for AB and 0.12 for CB with DM alginate.



**Figure 15.** Mean (+ S.E.M) ■ = elution profile from freeze dried 5.2g AB, ▲ = 5.2g CB and ● = 5.2g CB and 208mg DM alginate from the end of model gut and isolation process. Freeze dried samples were then re-suspended in  $DH_2O$  at 1.43mg/ml based on the freeze dried weight and processed through the gel filtration. PAS assay was used to quantify alginate in fractions collected from gel filtration. Vertical axis 1 is for AB and vertical axis 2 is for CB alone and CB with DM alginate (n=6).

### 3.5.3 Alginate Recovery at End Point of the Model Gut

The predicted weight for the freeze dried samples can be calculated using

#### Equation 6.

$$\text{Sample weight} = \text{concentration of alginate in sample} \times 4 \text{ (or 8)} = \text{weight in mg}$$

**Equation 6.** Calculation for predicted weight of freeze dried samples.

The predicted, measured and difference between the weights of the freeze dried samples are illustrated in **Table 5**. Although the measured weight was larger than the predicted weight, 4g, 1.8g and 7.7g larger respectively for 4ml CB and 208mg DM alginate, 4ml and 8ml AB, these samples still weighed more than the 4ml and 8ml 5.2g CB samples alone, suggesting that alginate was isolated from the samples.

**Table 5.** Mean weight of freeze dried samples from the end point of the model gut after isolation (n=6). 4ml and 8ml samples were taken for 5.2g AB and CB, and a 4ml sample of 5.2g CB with 208mg DM alginate.

<u>Sample</u>	<u>Predicted wt (mg)</u>	<u>Measured wt (mg)</u>	<u>Difference in wt (mg)</u>
<b>4ml CB and 208mg DM alginate</b>	6.3	15.9	4
<b>AB 4ml</b>	6.3	13.7	1.8
<b>AB 8ml</b>	12.6	31.8	7.7
<b>CB 4ml</b>	-	5.6	-
<b>CB 8ml</b>	-	11.5	-

Predicted weight was calculated using Equation 4.

Measured weight was the mean weight of samples following isolation and freeze drying procedure.

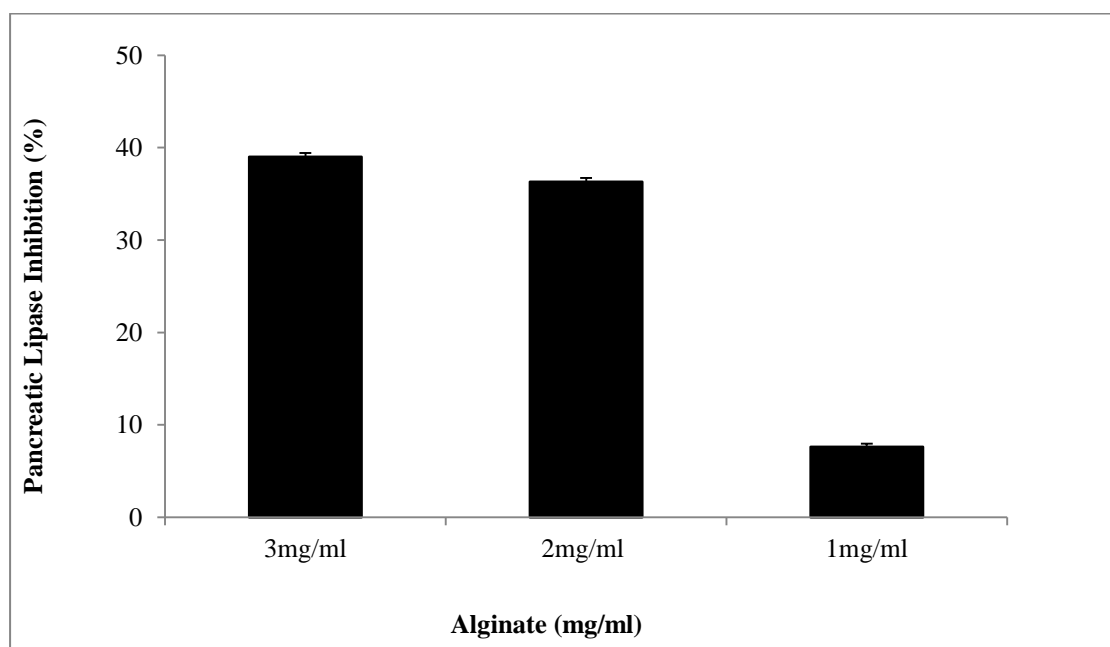
Difference in weight is the predicted weight + control weight

- 4ml CB with 208mg DM 6.3mg + 4ml CB 5.6mg = 11.9g →  $15.9 - 11.9 = 4\text{mg}$
- 4ml AB 6.3mg + 5.6g 4ml CB = 11.9mg  $13.7 - 11.9 = 1.8\text{mg}$
- 8ml AB 12.6mg + 8ml CB 11.5g = 24.1mg  $31.8 - 24.1 = 7.7\text{mg}$

### 3.5.4 Lipase Inhibition by Isolated and Heated Alginate

#### 3.5.4.1 Freeze dried AB and CB extracts

The alginate extract from the end of the model gut was weighed and re-suspended in substrate solution to ascertain whether the alginate is able to retain its inhibition properties post cooking into the bread, digestion and isolation. The freeze dried extracts from 5.2g AB inhibited pancreatic lipase by 39 ( $\pm 0.42$ ), 36 ( $\pm 0.43$ ) and 7% ( $\pm 0.32$ ) at 3, 2 and 1mg/ml respectively. (Figure 16). These data indicate that although the alginate has been cooked into the bread, digested in the model gut and isolated it retains its inhibition properties.

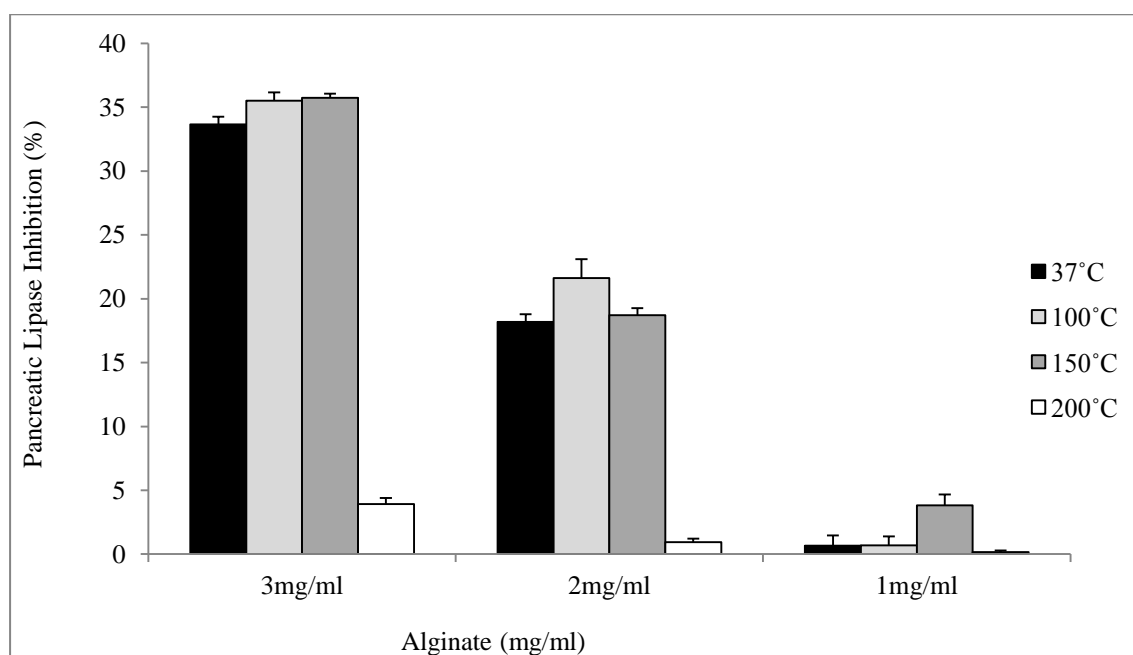


**Figure 16.** Mean (+ S.E.M) pancreatic lipase inhibition with isolated alginate from end of the model gut and following freeze drying. Freeze dried AB were re-suspended in lipase buffer at 3, 2 and 1mg/ml and the olive oil turbidity assay was run (n=9).



### 3.5.4.2 Heated alginate samples

Heated DM alginate at 3, 2 and 1mg/ml inhibit pancreatic lipase. These data indicate that there is a significant effect for concentration and temperature ( $p < 0.05$ ). There was a dose response for 3, 2 and 1mg/ml at temperatures 37, 100 and 150°C which was significantly different ( $p < 0.05$ ). The level of inhibition for 37°C was 33% ( $\pm 0.58$ ), 18% ( $\pm 0.57$ ) and 0.6% ( $\pm 0.81$ ), 100°C was 35 ( $\pm 0.64$ ), 21% ( $\pm 1.46$ ) and 0.6% ( $\pm 0.69$ ) and 150°C was 35% ( $\pm 0.32$ ), 18% ( $\pm 0.55$ ) and 3% ( $\pm 0.84$ ) for 3, 2 and 1mg/ml respectively. There appears also to be a dose response relationship for DM heated at 200°C, however this was not significant ( $p > .05$ ). The level of pancreatic lipase activity reduced from 4 ( $\pm 0.48$ ), 0.9 ( $\pm 0.26$ ) and 0.2 ( $\pm 0.12$ ) for 3, 2 and 1mg/ml respectively when heated at 200°C. There was no significant difference between temperatures 37, 100 and 150°C for matched concentrations at 3, 2 and 1mg/ml ( $p > 0.05$ ). The DM alginate heated at 200°C was significantly different from temperatures 37, 100 and 150°C at concentration 3 and 2mg/ml ( $p < .05$ ) but not at 1mg/ml ( $p > 0.05$ ). The data in **Figure 17** indicate that DM alginate is able to inhibit pancreatic lipase despite being heated at 150°C, and demonstrates its robustness.



**Figure 17.** Mean (+ S.E.M) pancreatic lipase inhibition using DM alginate after it has been heated at 37, 50, 150 and 200°C for 30 minutes. After being left at room temperature the DM alginate was then re-suspended in lipase substrate solution at 3,2 and 1mg/ml and the turbidity assay was performed (n=9).

### 3.6 Discussion

The data in the current chapter demonstrates that alginates are heat stable up to temperatures of 100°C. However beyond 100°C there is a gradual decrease in viscosity as the temperature increases. By contrast the ability of the alginate to inhibit pancreatic lipase appears to be unaffected up to temperatures of 150°C, despite the reduction in viscosity and by implication size. Furthermore, despite being used as an additive to a bread vehicle, cooked, digested in a model gut and isolated the alginate retains its inhibition properties and does not appear to break up.

The ability of alginate to form acidic and ionic gels is well documented (Braccini & Perez, 2001; Draget et al., 2005), which is one of the principal reasons that make alginate unique and lends them to various uses including pharmaceutical and the textile industry, but predominantly the food industry (Brownlee et al., 2005). It is this unique ability of alginate to form gels in both acidic and ionic conditions that has been suggested to be responsible for reduced kcal intake (Paxman et al., 2008) and increased satiety and reduced hunger (Jensen, Kristensen, Belza, Knudsen, & Astrup, 2012 (b)). In addition to human studies Seal and Mathers (2001) reported that rats fed food containing alginate had reduced fat and cholesterol digestion. The precise mechanism responsible for the effects reported above remain unknown, although amongst those suggested viscosity has come under scrutiny. Smidsrod (1974) and Jensen et al. (2012 (b)) indicated that the ability of alginates to form both acidic and ionic gels may be responsible for poor mixing within the stomach and small intestines, and ultimately this will interfere with the ability of digestive enzymes to interact with substrates and attenuate nutrient digestion. Paxman et al. (2008) and Hoad et al. (2004) also added to this and stated that if the alginate forms a viscous solution/gel in the stomach this may cause distension within the stomach and increase satiety, and could therefore reduce the amount of energy absorbed from consumed calories.

Although viscosity may be a mechanism by which digestion is reduced it may also be a problem for alginate. The addition of alginate to a beverage or the coating of a cereal bar in previous studies has resulted in poor palatability when compared with a control beverage or cereal bar (Jensen et al., 2012 (b); Sandberg et al., 1994). If a food or drink vehicle is not pleasant in texture and taste then an individual will be less inclined to consume said food or drink on a regular basis (Ellis et al., 1981), as would be required if alginate is to be used to treat obesity. Amongst others this is one of the reasons why bread was selected as the delivery vehicle for alginate. If the alginate can be mixed in with the

bread during the baking process then this may reduce the ability of the alginate to form a viscous solution in the mouth, yet still possess the potential to increase the viscosity within the stomach and small intestines, and ultimately attenuate digestion.

The data presented in this thesis demonstrates that the majority of the alginate would be released in the small intestine based on the model gut data. Therefore it seems reasonable to assume that if the alginate is in fact being released, then the viscosity of the solution at the end of the model gut should increase. The data in **Figure 12** suggests that there is not a substantial increase in viscosity from the digested AB when comparing it to CB. The  $\eta_{sp}$ 's of AB and CB from the end of the model gut were  $0.42 (\pm .01)$  and  $0.11 (\pm .01)$  respectively, which is a difference of 74%. Nonetheless this is much lower than the viscosity that would be expected for a similar concentration of alginate. For this reason alginate was added at the start of the model gut alone and at the end to see if the model gut had any effect on the ability of alginate to increase the viscosity. The data presented here demonstrates that alginate added to the end of the model gut had a substantially larger  $\eta_{sp}$  than when alginate was added to the start of the model gut. These data suggest that the alginate may be digested or be binding to something within the model gut. If the alginate binds with something in model gut such as bile acids (Wang et al., 2001) this may impact upon the ability of the alginate to increase viscosity. Additionally it may be that the alginate is being digested within the model gut and therefore the alginate is broken down and unable to increase the viscosity, although this would contradict numerous studies including the work of Trowell (1972) and Asp (1987) who demonstrated that dietary fibres evade digestion in the upper GI tract. This may in part explain the lack of viscosity, although an alternative explanation may be that the bread processing and baking may be affecting the ability of the alginate to form a viscous solution. During the cooking process the alginate structure may be altered when the bread is exposed to temperatures up to 200°C. McDowell (1977) reported that if alginates are heated beyond 100°C intra-molecular depolymerisation may occur, which would ultimately affect the ability of the alginate to form a viscous solution. The question therefore raised is whether the material being released from the bread is in fact alginate, and whether this alginate has been affected during the cooking and digestion process.

The isolated weights of the material can be observed in **Table 5**. It is immediately apparent that the samples containing alginate had a larger weight than those that contained no alginate. The weights were marginally larger than the predicted weights, which may be due to binding with the contents of the model gut such as bile acids (Adiotomre,

Eastwood, Edwards, & Brydon, 1990; Wang et al., 2001). Adiotomre et al. (1990) confirmed that a range of dietary fibres were able to bind with bile salts. They used a dialysis sacks in an attempt to quantify whether a range of dietary fibres including gellan, pectin and carboxymethylcellulose were able to retain bile acids. The dialysis sacks simulated bile acid absorption in the small intestines, and anything left in the sacks at the completion of the experiment would be retained through binding with the various forms of dietary fibre. There was an increase in bile acids retention from 33, 14 and 89%, for gellan, pectin and carboxymethylcellulose, respectively. More specifically Wang et al. (2001) looked at soluble and insoluble dietary fibres of seaweeds. The authors reported that both the soluble and insoluble forms of dietary fibre from seaweeds were able to bind with bile acids, although soluble dietary fibres were significantly better at binding them. Although both of the afore mentioned studies indicate the ability of dietary fibres to bind bile salts these were both *in-vitro* and require further research to confirm if this is the case *in-vivo*. For the purpose of the *in-vitro* data presented here suggests that this binding of bile salts may account for a portion of the weight discrepancy here. Although Adiotomre et al. (1990) and Wang et al. (2001) do not suggest how dietary fibres bind bile acid; there are a number of potential mechanisms of how this may occur. The carboxyl group of the alginate may be able to bind with the hydroxyl group of the bile acids. In addition the hydrophilic aspect of the bile acids may also bind with hydrophilic alginate; however neither of these mechanisms have been confirmed, and require further research. Although slightly larger than the predictions the essential theme here is that the samples containing alginate had a larger weight than the control samples, which supports the previously presented data that alginate was being released from the bread. Although this still does not answer the question as to why the AB did not cause an increase in viscosity when the alginate was released in the model gut.

A potential mechanism for the lack of viscosity was suggested by McDowell (1977), that if the alginate is heated beyond 100°C then depolymerisation can occur which would impede gel formation. During the cooking process the bread may be subject to temperatures up to 200°C for 30 minutes. Although the exact temperature of the entire loaf of bread here cannot be determined, Hasatani et al. (1991) observed the effects of various bread recipes and measured the temperature of different parts of the bread. Although this could not be done during the cooking process this gives a good insight into the various temperatures during baking of bread. Hasatani et al. (1991) indicated that the centre of the bread may only reach 70-80°C, however the crust may be exposed to

between 180-200°C. It was for these reasons three alginates SF200, DM and LFR 5/60 were heated at 37°C and 200°C to observe if  $\eta_{sp}$  is affected post heating at 200°C. It was immediately apparent that there is a substantial loss in viscosity ranging between 92-98%. These data indicate that when alginate is exposed to 200°C the alginate is unable to form a viscous solution. As DM alginate was chosen for the bread this alginate was heated at 37, 50, 100, 150 and 200°C in an attempt to ascertain at what point if any depolymerisation may occur. The  $\eta_{sp}$  of DM alginate at 37, 50 and 100°C was not significantly different. There was however a significant reduction in  $\eta_{sp}$  once the temperature exceeded 100°C. These data correspond with the work of McDowell (1977) who demonstrated that as alginates in solution are exposed to temperatures above 100°C a gradual depolymerisation occurs until complete breakdown of the polymer. If depolymerisation occurs, not only will the bonds between adjacent GG, MG and MM blocks be broken, but the size of the alginate molecule will also be reduced which is essential for the formation of ionic or acid gels. This breaking down of the polymer may account for the reduction or complete lack of viscosity as reported here. McDowell (1977) also suggested that exposing alginate to temperatures in excess of 180°C the alginate may completely breakdown causing a rapid evolution of one molecule of CO<sub>2</sub> for every uronic acid group. This would mean that the -COOH would then become H, and thus almost entirely render the alginate incapable of forming an ionic gel.

Leo et al. (1990) indicated that if the glycosidic links that holding polymers together are affected by heat then this may not only affect viscosity but the size of the polymer as well. If alginate is being broken down during the cooking process of the bread then the size of the alginate may also be affected. Larger alginates have a larger Young's modulus (Smidsrod, 1974), meaning they can form more viscous gels. Consequently if the size of the alginate is being reduced during the cooking process then this also may have an impact upon the alginate's ability to form a gel. Gel filtration can be used to separate molecules based on hydrodynamic size. Consequently if alginate is undergoing some degree of depolymerisation then the alginate should elute later from the column. The alginates DM, LFR 5/60 and SF200 from the viscosity experiments when heated at 37°C and eluted based on the  $M_r$ . The elution volume increased for all the alginates post heating at 200°C when compared to 37°C. These data suggest that at higher temperatures the alginate may undergo some degree of depolymerisation and therefore reduce  $M_r$  as indicated with an increase in elution volume, and reduce the ability of the alginate to form a gel. Leo et al. (1990) observed the effects of heat on viscosity of a 3% alginate solution

and calcium alginate beads. They exposed the solution and beads to temperatures ranging from 20°C up to 130°C for 20 minutes. The authors reported that both were unaffected up to 100°C, however beyond this time point there were significant alterations. As the temperature increased beyond 80°C viscosity and gel strength decreased, whilst the bead diameter increased. These studies support the earlier work of McDowell (1977) that as alginates are heated they undergo some form of intra depolymerisation. Although the work of Leo et al. (1990) do lend some support to the work presented here however they report a reduction in viscosity of 53% between 37°C and 110°C, however here there was only a reduction of 13%. This may have been due to the different heating procedures used. Leo et al. (1990) heated the alginate once it was in solution, however here we heated dry alginate, and thus the alginate in the solution may have been exposed to more heat as the alginate would have been mixed throughout. An additional point of interest may have been the different alginates used. Although Leo et al. (1990) do state the G:M ratio they do not state the Mr of the alginate used. If they used an alginate with a larger Mr than the present study then this would be broken down more easily, as demonstrated by a more substantial reduction in viscosity. This was also confirmed by the larger reduction in Mr as demonstrated by SF200 when compared with LFR 5/60 using gel filtration. An additional point of interest may be the molecular composition of the alginate used. There is currently no research comparing the heat stability of a range of alginates varying in GG, MM and MG blocks. It is well known that the properties of GG blocks are different from GM and MM blocks in gel formation. This therefore raises the question as to whether alginates with a larger GG content are more or less heat stable than a combination of MG and MM blocks. This would not only be beneficial for adding alginates to food that requiring cooking but also in drug delivery where alginate gel beads are required to undergo some form of heat sterilisation. Further work is required to confirm these suggestions, although it is clear that alginates undergo some form of intra depolymerisation and suggest that alginate cooked into the bread may not be able to withstand the temperatures involved within the cooking process.

If the alginate is affected by the heating during the cooking process then the isolated alginate from the digested alginate bread would have an elution volume, comparable with DM alginate after being heated at 200°C. The gel filtration data shows that the alginate released from the bread has not been altered in size by the cooking process of the bread. A possible explanation for this may be that the alginate is completely mixed throughout the bread and may only be exposed to temperatures ranging from 70-100°C, however

there may be some alginate that is exposed to higher temperatures. Although it appears that the bread matrix is actually protecting the alginate from the high temperatures. Furthermore the lack of a change in elution volume demonstrates that AB and dry DM alginate had not been digested within the model gut. This demonstrates that alginates are resistant to digestion in the upper GI tract supporting previous reports in the literature (Asp, 1987; FAO/WHO, 2003; Trowell, 1972, 1976). Although these data suggest that the alginate is not being digested in the model gut, the question remains as to exactly what is the cause for the lack of viscosity at the end of the model gut after the alginate bread has been digested. It may be a combination of the alginate binding with something in the bread such as sodium, which would replace calcium in the egg box model and hinder any development in viscosity. The viscosity data from the end of the model gut also suggests that there may be something in the model gut that alginate is binding to, such as bile acids which may reduce the ability of alginate to form viscous solution. Although still unclear it may be a combination of these suggestions that is causing the lack of viscosity, although if the alginate extracts are still able to inhibit pancreatic lipase activity then the lack of viscosity may hold no bearing on fat digestion.

If the alginate cooked into the bread is able to withstand cooking and digestion then it would appear that the bread may indeed be a suitable vehicle to add alginate to an individual's diet. Despite being robust to withstand these processes the question remains as to whether the alginate retains its inhibition properties once cooked into the bread, despite the reported lack of viscosity. Previous *in-vitro* work from this laboratory by Sunderland et al. (2000) and Strugala et al. (2005) have reported the effects alginate have on attenuating the activity of pepsin. More specifically Wilcox et al. (2014) and Richardson et al. (2011) have reported that certain alginates are able to reduce the activity of pancreatic lipase by up to 75%, which is dependent on the structure of the alginate. The data from the present study reveals that DM alginate retains its inhibition properties after being cooked, digested, isolated or heated at 150°C. These data not only support the work of Asp (1987) but also demonstrates the robustness of alginate after being processed in the various methods described here. The data also highlights the ability of alginates to withstand high temperatures, even when not being protected by the bread matrix. There was no significant difference at temperatures 37, 100 and 150°C at matched concentrations for lipase inhibition despite the reduction in viscosity at these temperatures. Upon exposing the alginate to 200°C the alginate appeared to lose 88% of its inhibition properties when matching it for concentration with DM alginate at 37°C.

The ability of alginate to inhibit pancreatic lipase after being exposed to temperatures 37-100°C corresponds well with the  $\eta_{sp}$  data, which again lends support to earlier work (Leo et al., 1990; McDowell, 1977; Serp et al., 2002). The data presented here in accordance with previous work indicates that alginates undergo depolymerisation when exposed to temperatures above 100°C, which has a profound detrimental impact upon gel formation. Interestingly, when exposed to 150°C the DM alginate lost 78% of its  $\eta_{sp}$  when compared with 37°C, however there was no such reduction when observing the effect of heat on alginates ability to inhibit pancreatic lipase activity. Conversely there was a reduction of 98 and 88% for  $\eta_{sp}$  and pancreatic lipase inhibition respectively, when exposed to 200°C. These data suggest that there is substantial alteration in polymer structure at temperatures beyond 150°C which is essential for gel formation (Braccini & Perez, 2001; Draget et al., 2003), and inhibition properties (Richardson et al., 2011; Strugala et al., 2005; Wilcox et al., 2014).

There is evidence to suggest that viscosity may play a crucial role in the reduction of enzyme activity (Seal & Mathers, 2001; Shah et al., 1986), however the data here suggests that this is not the case. The data presented shows alginate with a low  $\eta_{sp}$ , is still able to inhibit pancreatic lipase activity by up to 38%. Although this data is positive the question remains as to whether the AB will be able to inhibit pancreatic lipase within an *in-vitro* model gut. The turbidity assay used here was adapted from Vogel and Zieve (1961) and works on the basis that as the fat is digested the substrate solution becomes more transparent. Although this is an effective method for determining pancreatic lipase activity, this does not however take into account other factors within the model gut such as bile, undigested bread and the plethora of other enzymes involved in digestion within the model gut. Further work is required to ascertain whether the lack of viscosity reported here has any impact upon the ability of AB to inhibit, if at all fat digestion in a model gut and *in-vivo*.



## Chapter 4: Fat Digestion

### 4.1 Introduction

Lipids are crucial for all living organisms and serve a fundamental role in a number of key functions such as thermal regulators, membrane constituents, protein modification, energy storage and the most apparent energy supply (Tucci et al., 2010). The inclusion of triglycerides into the diet is the main source of lipids due to their efficacy as a highly concentrated energy source. Each gram of triglycerides provides 9 Kcal (37kJ) compared with 4 Kcal (17kJ) for one gram of carbohydrates and proteins (Horowitz & Klein, 2000). The digestion of triglycerides can be difficult due to their non-polar structure, however the digestive system of the human is extremely efficient and is able to digest up to 95% of fat ingested (Carey, Small, & Bliss, 1983). The mechanical breakdown of digestion begins in the oral cavity (mouth) which functions as a receptacle for food. The process of mastication begins when food is mixed with saliva and the teeth break up larger pieces into small manageable pieces, which is important further along the digestion process. It is within saliva that lingual lipase begins the digestion of triglycerides, albeit a very small percentage of total digestion. However lingual lipase then continues to aid with digestion within the stomach (Pedersen, Bardow, Jensen, & Nauntofte, 2002).

It is within the stomach that further digestion of triglycerides is understood to occur, through the peristaltic contractions of the stomach and gastric lipase, which has been suggested to be responsible for between 15-20% of fat digestion (Birari & Bhutani, 2007). Armand et al. (1994) indicated that in the stomach one free fatty acid (FFA) is cleaved off the glycerol backbone, leaving a diglyceride and one FFA. It is the presence of this FFA in the small intestine that has been suggested to be responsible for the release of cholecystinin and pancreatic lipase (Hamosh, 1990). Hamosh (1990) suggested that cholecystinin may play a role in slowing down gastric emptying and thus manage the food that enters the small intestines to ensure efficient digestion. Once in the small intestines the motility of the food is controlled by a combination of peristaltic and segmental contractions, which are also responsible for the emulsification of triglycerides with bile salts (Carriere, Barrowman, Verger, & Laugier, 1993). Pancreatic lipase is responsible for between 80-90% of fat digestion (Carey et al., 1983). Although pancreatic lipase can function alone its activity increases when bound to colipase. The tri and diglycerides are digested further producing two FFA and a monoglyceride. The FFA and monoglyceride are then able to diffuse across the cell membrane of the intestinal cells (Tucci et al., 2010). Once across the cell membrane the FFA's and monoglycerides are re-

formed within the chylomicrons into triglycerides, and can then be transported around the body via the lymphatic system (Mu & Hoy, 2004). It is the digestive system's efficiency that is one of the main factors that has contributed to the obesity epidemic. As Carey et al. (1983) previously stated the human digestive system is able to digest around 95% of the fat from a consumed meal. This would suggest that if individuals consume larger amounts of fat then this additional fat within the food and drinks consumed will also be digested and stored.

Despite the need for lipids an excess, generally of triglycerides has been associated with an increased energy intake and increased levels of obesity (Hashim & Tantibhedyangkul, 1987). There are however divergent opinions about whether diets high in fat or diets high in carbohydrate is the major contributing factor towards the obesity epidemic (Bray & Popkin, 1998). Although the case of high fat intake has strong correlations with the increased incidence of obesity (Kirk et al., 2013). Kirk et al. (2013) conducted a longitudinal study over 36 months, which involved 1781 diabetes patients filling in a questionnaire for set time points and receiving dietary guidelines. The authors reported that a lower serum triglycerides level after 12 months, but more so a reduced risk of cardiovascular disease was associated with a reduced fat intake at 12 and 36 months. Bray and Popkin (1998) simply stated that when animals and humans do not consume diets high in fat they do not experience the obesity issues that are currently prevalent worldwide. There is a consensus that some triglycerides may be more predisposed to increase an individual's weight gain more than others (Aoyama, Nosaka, & Kasai, 2007; Kirk et al., 2013; St-Onge, Bourque, Jones, Ross, & Parsons, 2003). There is evidence that suggests triglycerides that have a medium chain length of between 6-12 carbon atoms (Papamandjaris, MacDougall, & Jones, 1998) may be more beneficial due to the rate at which they are absorbed, and also their lower calorific content when compared with long chain triglycerides.

Although the majority of triglycerides are absorbed in the manner described above, there are a number of factors that can affect the digestion rates. Bach and Babayan (1982) suggested that short and medium chain triglycerides may be digested at an accelerated rate compared to long chain triglycerides. Medium chain triglycerides are triglycerides with chain length of six to twelve carbons, whereas long chain triglycerides have a chain length in excess of 12 carbons (Papamandjaris et al., 1998). Aoyama et al. (2007) suggested that medium chain triglycerides are hydrolysed in the upper GI tract and are absorbed via the hepatic portal vein and directly to the liver where they undergo beta

oxidation to produce ketones, and a rapid source of energy (Aoyama et al., 2007; Lee, Tang, & Lai, 2012). Bernard and Carlier (1991) confirmed this in rats by comparing the digestion of capric acid (10 carbons) with long chain triglycerides ranging in chain length between 16 and 22 carbons long. The authors infused the different fats intraduodenally into the rats and then assessed the blood lipid profile. The authors reported that capric acid was absorbed significantly quicker and appeared in the blood earlier than long chain fatty acids. The authors suggested the absorption of medium and short chain triglycerides is due to their lower molecular weight and their solubility in water. The importance of the rate of digestibility of triglycerides may play a key role in the battle against obesity. Once digested the long chain triglycerides are packed into chylomicrons and then enter the lymphatic system. There is evidence to suggest that once reformed into chylomicrons, these triglycerides favour distribution to peripheral tissues such as muscle and adipose tissue (Figueiredo-Silva et al., 2012; Johnson, Young, Cotter, Lin, & Rowe, 1990). Papamandjaris et al. (1998) also reported that medium chain triglycerides are poorly esterified into cellular triacylglycerols in adipose tissue and do not induce adipocyte differentiation, when compared with long chain triglycerides. In addition Friedman, Ramirez, Bowden, and Tordoff (1990) reported that the triglycerides with longer chain FA had a slower uptake at the mitochondrial level, and therefore reduce the ability of cells to use the fats as an energy source. If the fats are not used as an energy source, or the functions previously mentioned then the majority will be reformed into triglycerides and stored as adipose tissue.

There is evidence to suggest that those consuming foods high in long chain triglycerides gained more weight than those consuming medium chain triglycerides, which may be due to the high calorific content of long chain triglycerides (Hashim & Tantibhedyangkul, 1987). Hashim and Tantibhedyangkul (1987) fed rats one of two diets that were identical except for the fat content. One group of rats was fed a diet including long chain triglycerides and the other included medium chain triglycerides for 20 days. The authors reported a significant increase in weight, adipose tissue and adipocytes of the rats consuming a diet high in long chain triglycerides, when compared to the diet high in medium chain triglycerides. The authors also reported here that the medium chain triglycerides were oxidised at twice the rate of the longer chain triglycerides, meaning that it would take longer to liberate the energy from the longer chain triglycerides. There is also evidence in humans that high consumption of medium chain triglycerides increases energy expenditures and reduces adiposity compared with long chain triglycerides (St-

Onge et al., 2003; St-Onge, Ross, Parsons, & Jones, 2003 (b)). St-Onge et al. (2003); St-Onge et al. (2003 (b)) fed overweight and obese men and women a diet high in medium chain triglycerides compared with olive oil (high in long chain triglycerides) for 27 days. The diets were identical except for the fat content. There was a significant decrease in upper body adipose tissue, an increase in energy expenditure and fat oxidation when consuming the diet high in medium chain triglycerides, although the latter two variables were not significantly different between the two groups. An additional study was conducted by Kasai et al. (2003) who compared the effects of medium and long chain triglycerides on the accumulation of body fat in 82 healthy subjects for 12 weeks. The authors reported a significant decrease in overall body weight in both groups, Although the authors offer no explanation for the decrease in overall body weight in the control group consuming the long chain triglycerides, a potential explanation may be that the diets that both groups were on was less calorificly dense than the subjects normal diet. Although both groups had a reduction in overall body weight those consuming medium chain triglycerides exhibited a significantly greater weight loss. This reduction in body fat was associated with a reduction in subcutaneous and visceral fat. Furthermore, the authors reported a significant reduction in serum total cholesterol. However there were no other significant differences in blood chemistry between the two groups. The data from these studies suggests that medium chain triglycerides may result in a reduction in body weight, accumulation of adipose tissue and plasma determinants associated with obesity and disease. Although the work of Kasai et al. (2003) was conducted over 12 weeks, further longitudinal work is required to assess various food types high in medium chain triglycerides in everyday life to ascertain the efficacy of consuming diets high in medium, as opposed to long chain triglycerides.

If the body is inundated with large amounts of fat that outweigh the energy requirements the body will re-form this into triglycerides through lipogenesis, where it will be distributed throughout the body as adipose tissue (Mu & Hoy, 2004). In addition to the increased amounts of fat people are consuming per meal, it is the ease of access to palatable foods, fast foods and mechanised manufacturing of foods with increased fat content that is also contributing to the increased levels of obesity (Nestle & Jacobson, 2000). The problem therefore not only appears to be related to the large amounts of food that people are consuming, but also the larger amounts of fat that are within these foods. There is evidence to suggest that medium chain triglycerides may be a healthier option for a number of reasons, although the incorporation of these medium chain triglycerides into

food production may not be possible. Long chain triglycerides are generally used to improve the consistency, appearance, taste and shelf life of products (FAO/WHO, 2003). It is unlikely that those manufacturing these foods will reduce the use of the longer chain triglycerides from foods in the foreseeable future. It is for these reasons that any weight loss treatments must target the foods that people are consuming or target food digestion once it has been consumed. Any reduction in fat digestion will be beneficial, although a treatment that may inhibit the digestion of long chain triglycerides over medium chain triglycerides may have the potential to reduce the accumulation of adipose tissue, and reduce body weight in obese and overweight individuals.

Although current obesity treatments do not target long chain triglycerides specifically, they are targeting a reduction of calories consumed and absorbed. The most common forms of treatment include pharmaceutical interventions and surgery (Santry et al., 2005) (Pacher, Batkai, & Kunos, 2006). Despite the encouraging weight loss reported in these studies, there are numerous physiological and psychological side effects. An additional drug, and potentially the most common pharmaceutical intervention is Orlistat, also known as Alli. This drug does not act upon the central nervous system, but rather by covalently binding to the active site of pancreatic lipase in the upper GI tract (Al-Suwailem et al., 2006). This binding renders the pancreatic lipase inactive and therefore ineffective at digesting fat within the small intestines, and therefore the fat is unable to pass across the cellular membrane within the small intestines and will pass through the upper GI tract undigested and out in faeces (Al-Suwailem et al., 2006; Sternby et al., 2002). Lucas (1998) and Zhi et al. (1996) have both reported weight loss in a clinical setting, and demonstrated the efficacy of the drug. Although this drug has been attributed to aiding in weight loss there are a number of side-effects caused by this drug, such as abdominal pain, diarrhoea, flatulence, fatty stools and faecal incontinence amongst others (Finer et al., 2000). Although Orlistat is effective at inhibiting pancreatic lipase there is no evidence to suggest that Orlistat is able to distinguishing between long, medium or short chain triglycerides, Therefore if all fat digestion is reduced then this may also decrease the absorption of medium and short chain triglycerides, which have been linked with health benefits (Kasai et al., 2003; St-Onge et al., 2003 (b)).

Despite the afore mentioned adverse side effects of Orlistat, this drug remains the most effective pharmaceutical treatment for obesity. If an obesity treatment can be produced that can act in a similar way to Orlistat and reduce fat digestion without the side effects, then this may have the potential to reduce the number of obese individuals worldwide.

There is *in-vitro* evidence from Richardson et al. (2011); Strugala et al. (2005); Sunderland et al. (2000) and Wilcox et al. (2013) that have all provided evidence demonstrating the ability of alginate to inhibit digestive enzymes pepsin and pancreatic lipase. The work of Richardson et al. (2011) and Wilcox et al. (2013) have reported that specific alginates are able to inhibit pancreatic lipase by up to 85%. Even though these data do suggest alginate has the potential to be used as an obesity treatment, these experiments were performed using a simplistic model for assessing pancreatic lipase activity (Vogel & Zieve, 1961). Although the adapted method from Vogel and Zieve (1961) does indicate the activity of pancreatic and co-lipase activity, it does not take into account other components within digestion, such as bile, pancreatic juice, a large range of pH's and undigested constituents that may be present during *in-vivo* digestion. Furthermore, these experiments have been conducted using dry alginate, and as previously mentioned the main issue with using alginate as an obesity treatment in a clinical setting is the delivery vehicle (Ellis et al., 1981). Although there is evidence to suggest that the addition of alginate to a beverage or food vehicle can result in a number of physiological benefits, the foremost problem with alginate in a beverage is poor palatability and thus low compliance, which is generally the main issue with any weight loss or maintenance programs (Jensen et al., 2012 (a); Paxman et al., 2008; Torsdottir et al., 1989).

## 4.2 Aims

There is *in-vivo* and *in-vitro* evidence to suggest that alginates possess the ability to attenuate the activity of digestive enzymes, and therefore reduce the available energy from calories consumed. The main issue with adding alginate to a vehicle is the palatability of the delivery vehicle. The data previously presented in this thesis indicates that there is no substantial increase in viscosity at the end of the model gut and that the alginate within the bread maintains its inhibition properties despite the cooking and digestion process. Therefore the purpose of this chapter is to:

- Utilise a model gut system that replicates digestion in the mouth, stomach and small intestine. This model allows samples to be taken at multiple times points. This will assess whether fat substrates and foods high in fat such as butter and olive oil are digested within a model gut system, and to determine if the rate of digestion is similar to what would be expected *in-vivo*.
- To determine if the fat substrates and foods containing fat are digested at a similar rate. Various publications have suggested that the rate at which fats are digested is dependent on the chain length of the fatty acids (Bach & Babayan, 1982; Bernard & Carlier, 1991). If this is the case then the model gut will help identify which fats are digested quickest and if there is a link between the digestion rate and the fatty acid chain length.
- The data presented earlier in this thesis indicates that alginate is released from the bread as it is digested in the model gut. Between 80-90% of the alginate is released in the small intestine, which is generally where fat is digested *in-vivo*. If the alginate is released from the bread as indicated here then fat digestion may be attenuated. Therefore the final aim of this chapter is to determine if the alginate enriched bread is able to reduce the digestion of fat substrate and foods containing fat within the model gut system.

## 4.3 Methods

### 4.3.1 Materials

Alginate and control bread were those used in chapter three. Fat substrates glyceryl tributyrate, glyceryl trioctanoate and glyceryl trioleate were purchased from Sigma, with their properties depicted in Table 6. Anchor butter and olive oil were purchased from Co-operatives Foods (Manchester, UK). Glycerol reagent A 40ml was purchased from Amsbio (Abingdon, UK). All materials used for the model gut were those used in chapter three.

**Table 6.** Attributes of fat substrates digested in the model gut.

Triglyceride	Fatty Acid	Fatty Acid Chain Length	Fatty Acid pKa
Glyceryl Tributyrate	Butyric Acid	4	4.84
Glyceryl Trioctanoate	Octanoic Acid (Caprylic Acid)	8	4.9
Glyceryl Trioleate	Oleic Acid	18	9.95

### 4.3.2 Equipment

All assays were carried out using BioTek 96 well plate reader at 550nm (ELx808 BioTek, Bedfordshire, UK). Two Grant GD 100 water baths were used for incubating synthetic model gut solutions and samples gut at 37°C (Fisher Scientific, Loughborough, UK). A Sci-Q 401U/D peristaltic pump was used to pump synthetic solution into samples in the synthetic model gut (Watson-Marlow, Fisher Scientific, Loughborough, UK), three IKA<sup>®</sup> laboratory egg compact mixers were used to mix digesta. A Thermo Jouan BR4i Mu Scientific Centrifuge (Basingstoke, UK) and an Eppendorf Centrifuge 5415 R (Stevenage, UK) were used for samples <4ml and >2ml, respectively.

### 4.3.3 Model Gut Procedure

The model gut was run as per chapter three, with the following experiments undertaken:

- i) Model gut solution alone
- ii) Model gut solution with 500µl glyceryl tributyrate, glyceryl trioctanoate or glyceryl trioleate.
- iii) 5.2g AB or CB alone.
- iv) 5.2g AB or CB with 500µl glyceryl tributyrate, glyceryl trioctanoate or glyceryl trioleate.
- v) Model gut solution with 1g of butter alone



- vi) 5.2g AB or CB with 1g of butter
- vii) Model gut solution with 500µl of olive oil
- viii) 5.2g AB or CB with 500µl of olive oil

During the model gut process a one ml sample was taken at 30 seconds (after mastication in the mouth), and then every 15 minutes for a total of 180.5 minutes.

#### **4.3.4 Glycerol Quantification**

##### **4.3.4.1 Glycerol Standard Curve**

To ascertain glycerol content in samples taken from the model gut a standard curve was produced for glycerol in DH<sub>2</sub>O using a 96 well plasma glycerol kit (SGA-1, Amsbio, Abingdon, UK) was used. Working reagent A was prepared at room temperature by adding glycerol reagent with DH<sub>2</sub>O in a 4:1 ratio, respectively. Glycerol control was serially diluted down from 2.5 mM in DH<sub>2</sub>O. 5µl of each concentration of glycerol was then added to a 96 well plate in duplicate, before 100µl of the glycerol working reagent was added to the appropriate wells and mixed, before being left at room temperature for 15 minutes. The plate was then read at 540nm on the plate reader, which provided free glycerol measurement.

##### **4.3.4.2 Pre Model gut**

In order to ascertain the amount of the substrate digestion that occurs within the model gut, the substrates were digested in a manner that replicated model gut digestion. Three samples were prepared replicating the solution that would be present at the end of the gastric phase of the model gut. 500µl of glyceryl tributyrate, glyceryl trioctanoate or glyceryl trioleate was added and then mixed for 60 minutes at 37°C. The fat substrates were exposed to these conditions rather than gradually adding gastric juice as minimal glycerol would be present in the gastric phase of digestion, and this experiment was to assess if digestion of the substrate was taking place, which would generally only occur in the presence of bile and pancreatic lipase. After 60 minutes bile and pancreatic juice were added, in the same volumes that would be present at the end of the model gut (180.5 minutes). The samples were then continually mixed whilst being maintained at 37°C. 1ml samples were collected after 60 minutes and then in 30 minutes intervals until glycerol had plateaued with no additional solution added. A plateau in the glycerol indicated complete digestion of the substrate. The solution from this time point is then used to create a standard curve for each individual substrate. Glycerol alone was added to model gut solution alone from 180.5 minutes and homogenised. This was then diluted to 2.5mM using DH<sub>2</sub>O to create a standard curve. This standard curve was used to quantify glycerol

present in samples AB, CB, butter and olive oil. Once collected all samples were heated at 80°C for 30 minutes to denature the enzymes and then allowed to cool to room temperature. Samples were then centrifuged at 10,000rpm for 10 minutes at 10°C. As the amount of substrate added at the start and the total solution at the end are known the concentration at each time point can be calculated. As a control model gut solution alone was used to subtract any interference from the substrate samples. The supernatant from samples were then diluted using DH<sub>2</sub>O to 2.5mM for each time point with equal dilutions for the control. 5µl of each time point was then added to a 96 well plate in duplicate, followed by 100µl of glycerol working reagent (4:1, glycerol reagent:DH<sub>2</sub>O). The plate was then read on the plate reader at 540nm every 30 seconds for 3 minutes and 30 seconds, during which time the plate was shaken prior to each measurement. The absorbance from 3 minutes was used for analysis, as the absorbance plateaued at this time point.

#### **4.3.4.3 During Model Gut**

1ml samples were extracted from the model gut at 0.5, 30, 60, 60.5 and then 30 minute intervals for a total time of 180.5 minutes. The samples were heated at 80°C for 30 minutes to denature the enzymes and then allowed to cool to room temperature. The samples were then centrifuged at 10,000rpm for 10 minutes at 10°C, before the supernatant was diluted 1:5 (v:v) in DH<sub>2</sub>O. 5µl of each sample at each time point was added to a 96 well in duplicate, and 100µl of glycerol working reagent A was added to each well. The plate was then read on the plate reader at 540nm every 30 seconds for 3 minutes and 30 seconds, during which time the plate was shaken prior to each measurement. The absorbance from 3 minutes was used for analysis, as the absorbance plateaued at this time point.

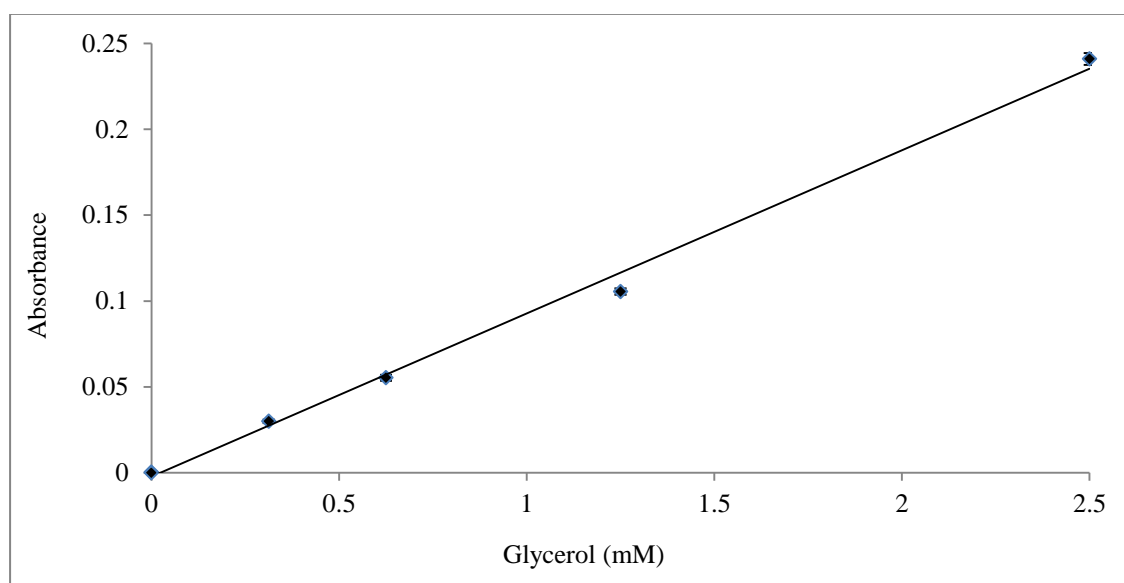
#### **4.4 Statistical Analysis**

Statistical calculations were undertaken using SPSS Statistics 19 (IBM, Predictive Analysis Software, USA). Data is presented as mean and standard error of mean (S.E.M). A Two-way Repeated ANOVA followed by a Post-Hoc Bonferroni were undertaken at a significant level ( $\alpha$ ) of 0.05 to compare differences between normal fat substrate and food digestion compared with fat substrate and food digestion with 5.2g CB or AB.

## 4.5 Results

### 4.5.1 Standard Curve for glycerol in DH<sub>2</sub>O

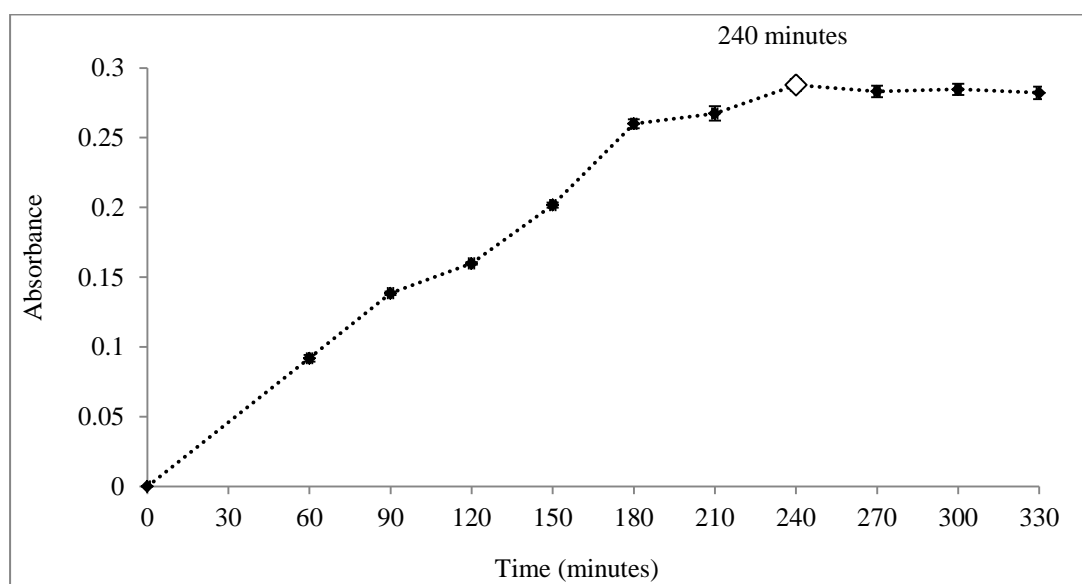
**Figure 18** illustrates the standard curve for glycerol in DH<sub>2</sub>O, demonstrating that the glycerol working reagent A is an effective method for determining free glycerol. The data in **Figure 18** demonstrates that the assay sensitivity, linearity ( $r = 0.99$ ) and a range of absorbance (0-0.24 OD) for 0.3-2.5mM glycerol concentrations.



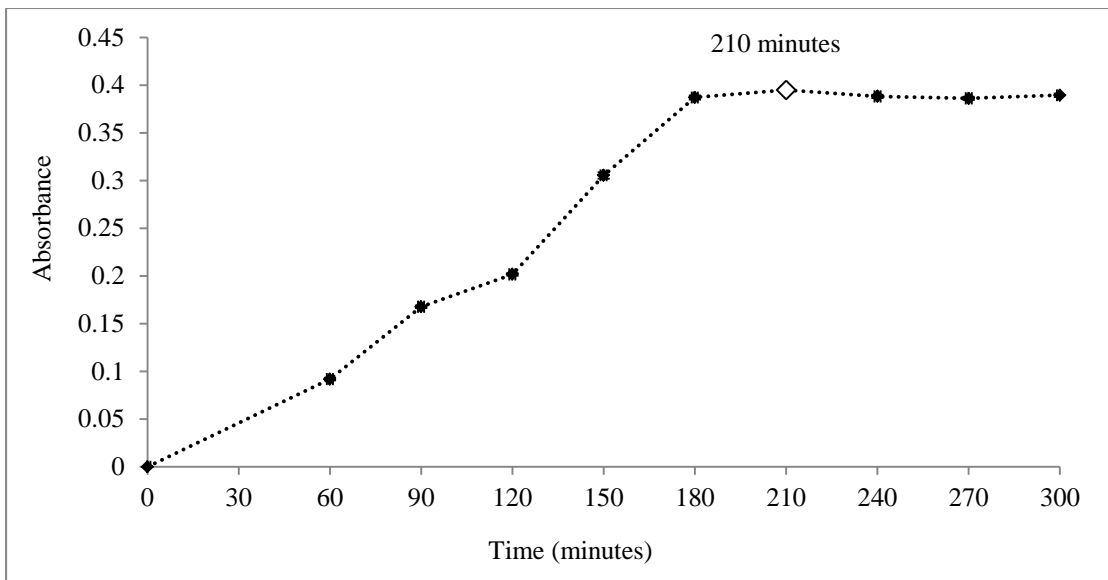
**Figure 18.** Mean standard curve (+ S.E.M) for glycerol in DH<sub>2</sub>O ( $r = 0.99$ ) using glycerol working reagent A ( $n=6$ ).

### 4.5.2 Substrate Digestion

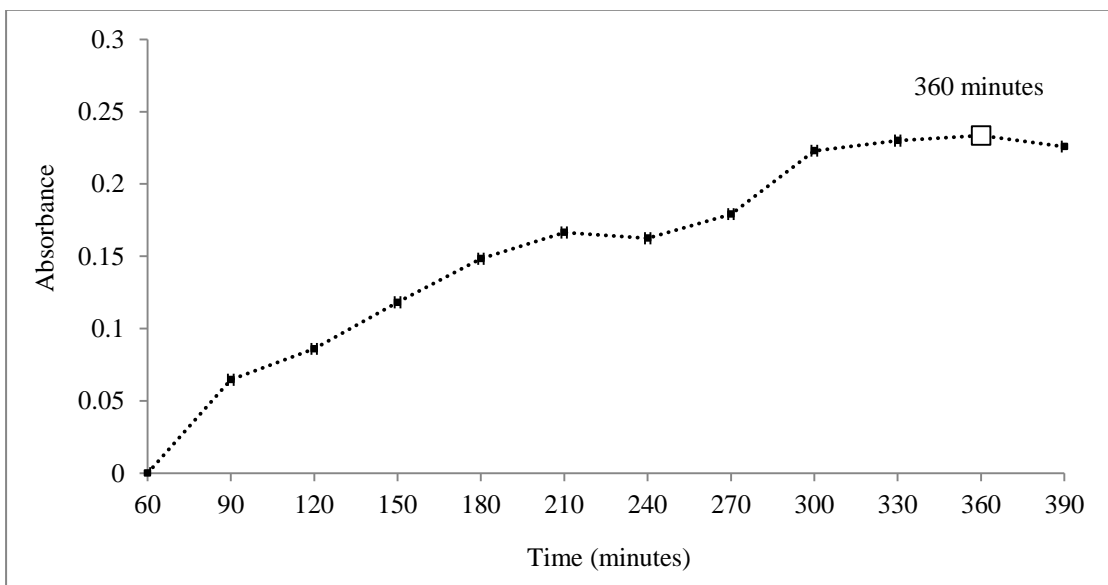
The digestion of the three substrates glyceryl tributyrate, glyceryl trioctanoate and glyceryl trioleate is depicted in **Figure 19**, **Figure 20** and **Figure 21**. The plateau in absorbance demonstrates that substrate digestion has finished after 240, 210 and 360 minutes for glyceryl tributyrate, glyceryl trioctanoate and glyceryl trioleate, respectively. The data here indicates that the fatty acid chain length appears to affect the digestibility of fat substrate. Glyceryl trioctanoate is digested first, followed by glyceryl tributyrate and glyceryl trioleate.



**Figure 19.** Mean (+ S.E.M) absorbance from glycerol released during digestion of 500 $\mu$ l of glyceryl tributyrate. A plateau in absorbance at 240 minutes identifies end point substrate digestion, highlighted by a white marker (n=6). No additional solutions were added beyond 180 minutes.



**Figure 20.** Mean (+ S.E.M) absorbance from glycerol released during complete digestion of 500 $\mu$ l of glyceryl trioctanoate. A plateau in absorbance at 210 minutes identifies end point substrate digestion, highlighted by a white marker (n=6). No additional solutions were added beyond 180 minutes.

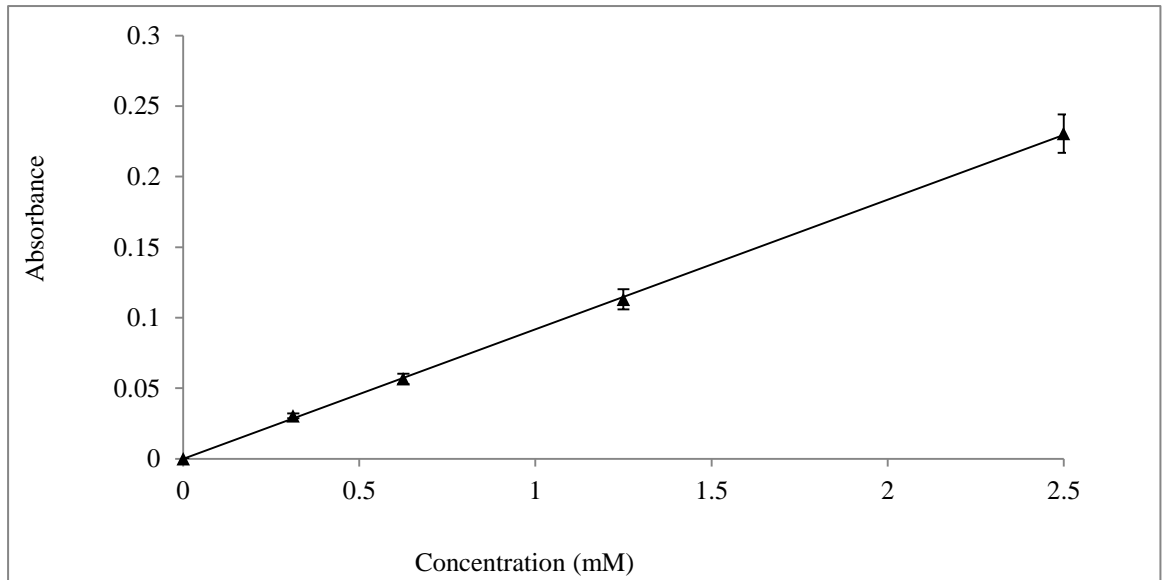


**Figure 21.** Mean (+ S.E.M) absorbance from glycerol released during complete digestion of 500 $\mu$ l of glyceryl trioleate. A plateau in absorbance at 360 minutes identifies end point substrate digestion, highlighted by a white marker (n=6). No additional solutions were added beyond 180 minutes.

### 4.5.3 Glycerol Standard Curve for Substrates

#### 4.5.3.1 Glyceryl Tributyrate

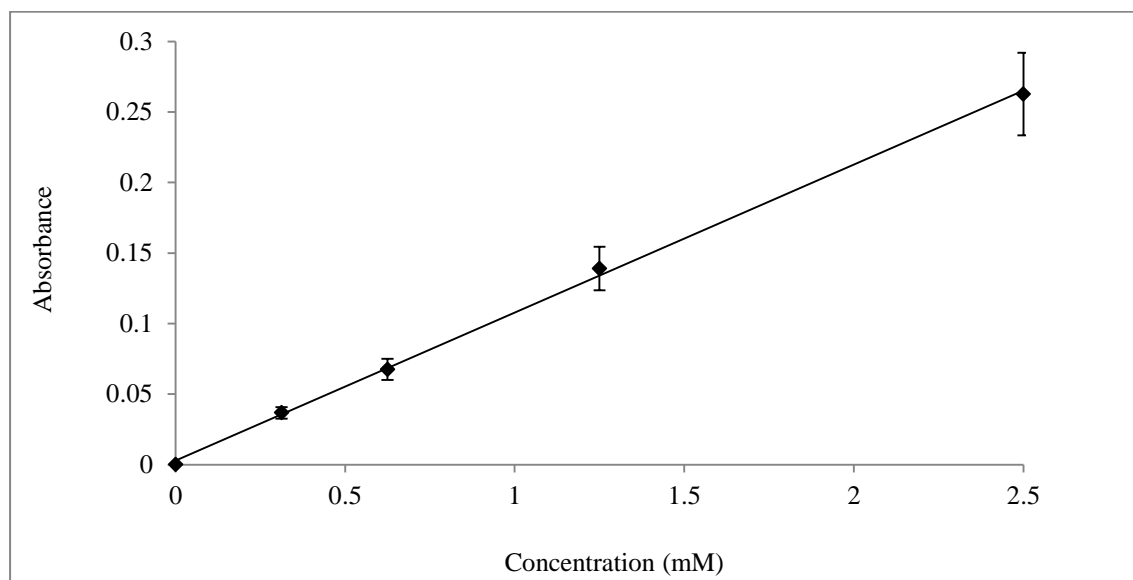
The data in **Figure 22** is the standard curve from the sample taken at 240 minutes, as this is the time point the glycerol plateaued. The data here demonstrates sensitivity (0.3mM), linearity ( $r = 0.99$ ) and an acceptable range of absorbance (0-0.24 OD).



**Figure 22.** Mean standard curve (+ S.E.M) for glyceryl tributyrate from solution taken at 240 minutes of complete substrate digestion ( $r = 0.99$ ) using glycerol working reagent A ( $n=6$ ). 2.5mM concentration was calculated by the addition of 500 $\mu$ l neat glyceryl tributyrate being added to 145ml model gut solution and then being multiplied by the molarity of the substrate. Therefore  $(0.5/145) \times 3.41 = 11.7\text{mM}$  which was then diluted down to 2.5mM and a standard curve was produced.

### 4.5.3.2 Glyceryl Trioctanoate

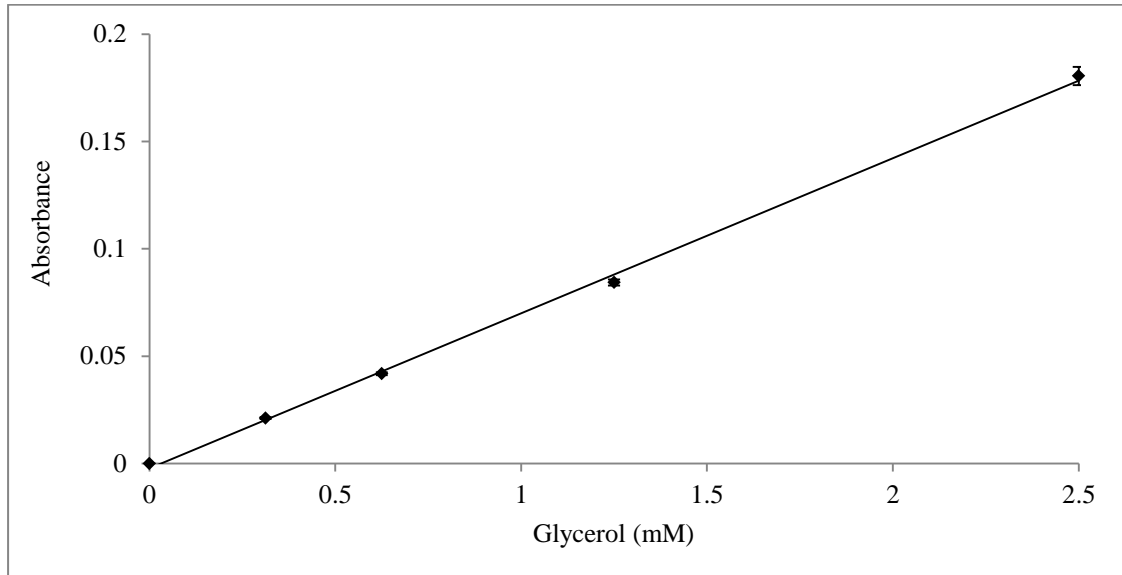
The data in **Figure 23** is the standard curve from the sample taken at 210 minutes, as this is the time point the glycerol plateaued. The data here demonstrates sensitivity (0.3mM), linearity ( $r = 0.99$ ) and a acceptable range of absorbance (0-0.26 OD).



**Figure 23.** Mean standard curve (+ S.E.M) for glyceryl trioctanoate from solution taken at 210 minutes of complete substrate digestion ( $r = 0.99$ ) using glycerol working reagent A ( $n=6$ ). 2.5mM concentration was calculated by the addition of 500 $\mu$ l neat glyceryl trioctanoate being added to 145ml model gut solution and then being multiplied by the molarity of the substrate. Therefore  $(0.5/145) \times 2.03 = 7\text{mM}$  which was then diluted down to 2.5mM and a standard curve was produced.

#### 4.5.3.3 Glyceryl Trioleate

The data illustrated in **Figure 24** is the standard curve from the sample taken at 360 minutes. The data here demonstrates sensitivity (0.3mM), linearity ( $r = 0.99$ ) and a acceptable range of absorbance (0-0.18 OD).



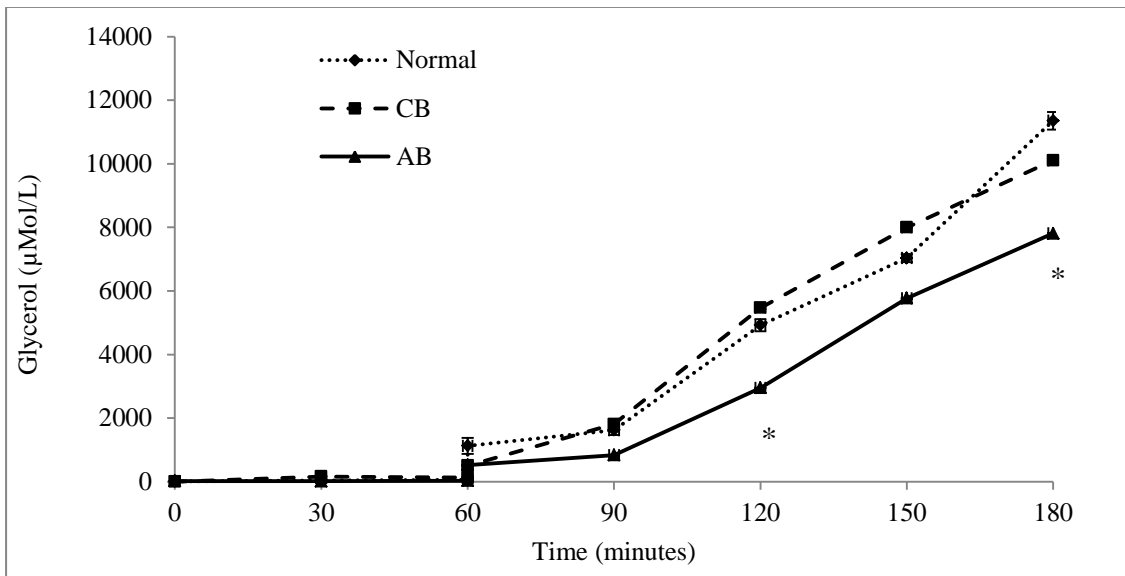
**Figure 24.** Mean (+ S.E.M) standard curve for glyceryl trioleate from solution taken at 360 minutes of complete substrate digestion ( $r = 0.99$ ) using glycerol working reagent A ( $n=6$ ). 2.5mM concentration was calculated by the addition of 500 $\mu$ l neat glyceryl trioleate being added to 145ml model gut solution and then being multiplied by the molarity of the substrate. Therefore  $(0.5/145) \times 1.03 = 3.5\text{mM}$  which was then diluted down to 2.5mM and a standard curve was produced.



## 4.6 Substrate Digestion in Model Gut

### 4.6.1 Glycerol Tributyrates

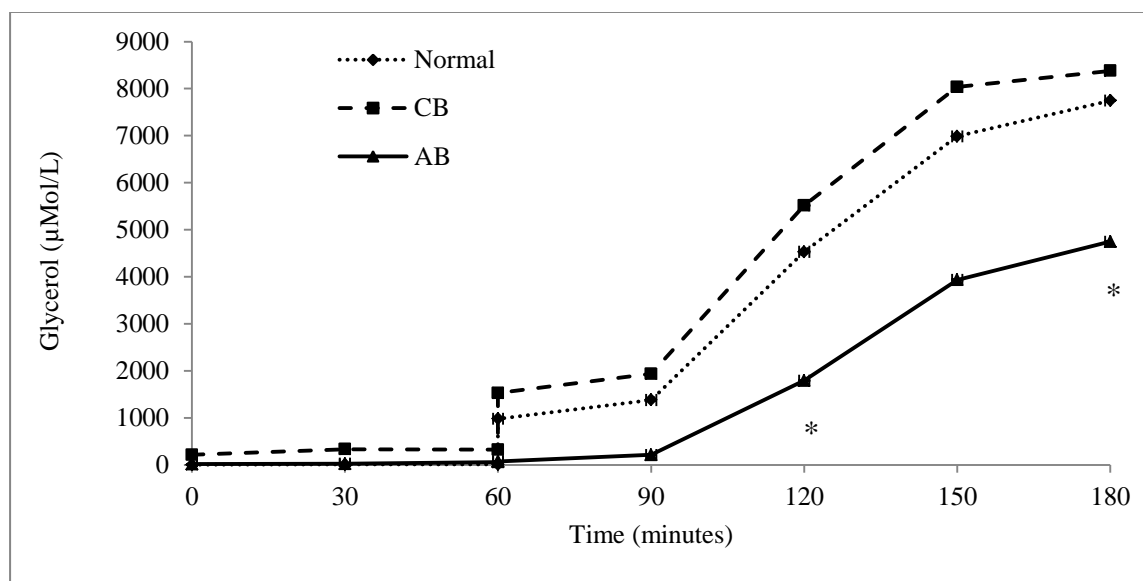
**Figure 25** shows the digestion of normal digestion of glycerol tributyrates alone, and with either CB or AB. In the gastric phase (0-60 minutes) there appears to be minimal digestion of the substrate, however in the small intestinal phase there is an exponential increase in free glycerol. There was a significant effect of treatment, demonstrating that there was a difference in the amount of free glycerol when AB and CB were present when compared with normal substrate digestion alone ( $p < .05$ ). There was a significant difference in the total mean glycerol released during the model gut between AB and substrate ( $2259.6 \pm 186.5 \mu\text{Mol/L}$ ) and substrate ( $3271.3 \pm 93.2 \mu\text{Mol/L}$ ) digestion alone ( $p < .05$ ). Although there was no significant difference between CB ( $3303.9 \pm 322.3 \mu\text{Mol/L}$ ) and normal substrate digestion ( $p > .05$ ). The glycerol released was lower at all-time points in the small intestine phase when AB was present compared to the substrate alone, although this was only significantly different at 120 and 180.5 minutes ( $p < .05$ ), with  $7811.2 \mu\text{Mol/L} (\pm 1228.4)$  and  $11356.5 \mu\text{Mol/L} (\pm 2496.2)$  glycerol released for AB and substrate and substrate alone respectively. However there were no significant differences at any other times points between normal substrates digestion when compared with AB and CB ( $p > .05$ ).



**Figure 25.** Mean (+ S.E.M) glycerol released during normal glyceryl tributyrate (500µl) digestion alone and with either 5.2g CB or AB in the model gut system. \* denotes a significant difference (n=6). To calculate potential glycerol present in 500µl of substrate alone = Density 1.032g/ml, Mr 302.36, therefore glycerol in 500µl substrate 1032g in 1L so in 500µl there is 0.516g. Therefore glycerol  $(0.516 \times 92)/302.36 = 0.157\text{g}$  and therefore moles of glycerol is  $0.157/92 = 0.0017$  moles. In moles/L =  $(0.0017 \times 1000)/145 = 0.011$  moles/L or 11,770 µmoles/L.

#### 4.6.2 Glyceryl Trioctanoate

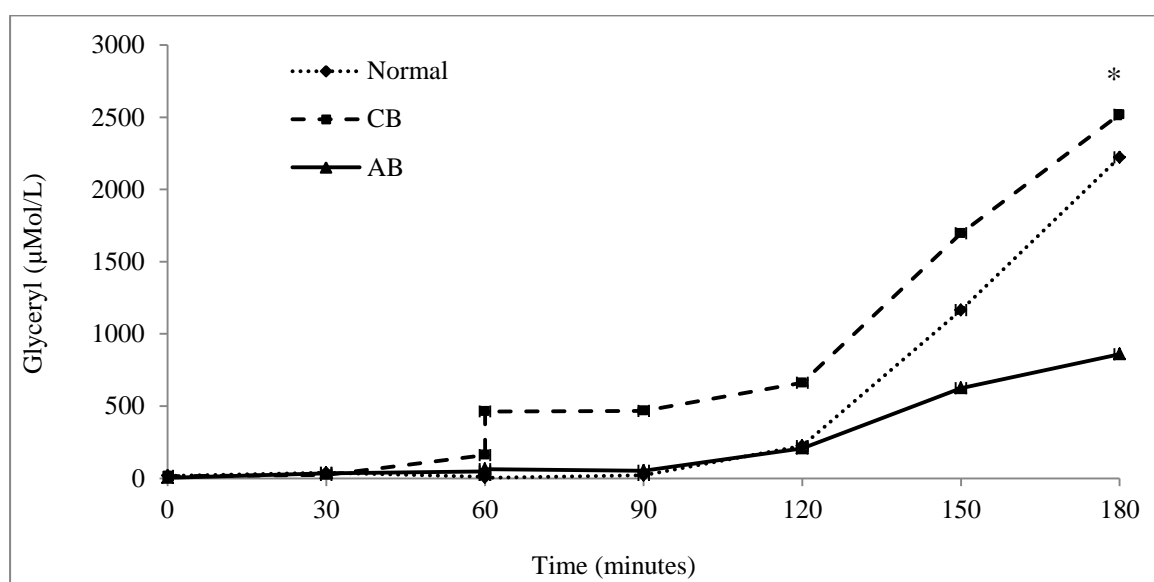
The data in **Figure 26** illustrates normal glyceryl trioctanoate digestion alone, and with CB or AB. The data suggests that there is only a small amount of free glycerol in the gastric phase of digestion (0-60 minutes). There was a significant effect of treatment on the levels of free glycerol ( $p < .05$ ). There was no significant difference between CB and normal substrate digestion ( $p > .05$ ), however there was a significant difference between both CB and normal substrate alone digestion when compared to AB ( $p < .05$ ), with a mean total glycerol release of  $5102.6 \mu\text{Mol/L} (\pm 350.4)$ ,  $4266.5 \mu\text{Mol/L} (\pm 351.5)$  and  $2174.2 \mu\text{Mol/L} (\pm 227.1)$  respectively. ). The glycerol released was lower at all-time points in the small intestine phase when AB was present compared to the substrate alone, although this was significantly different at 120 and 180.5 minutes ( $p < .05$ ). There was  $4746.3 \mu\text{Mol/L} (\pm 1167.3)$  and  $7746.3 \mu\text{Mol/L} (\pm 1563.1)$  glycerol released for AB and substrate and substrate alone respectively. However there were no significant differences at any other times points between normal substrates digestion when compared with AB and CB ( $p > .05$ ).



**Figure 26.** Mean (+ S.E.M) glycerol released during normal glyceryl trioctanoate (500µl) digestion alone and with either 5.2g CB or AB in the model gut system. \* denotes a significant difference ( $n=6$ ). To calculate potential glycerol present in 500µl of substrate = Density 0.956g/ml, Mr 470.68, therefore glycerol in 500µl substrate 956g in 1L so in 500µl there is 0.478g. Therefore glycerol  $(0.478 \times 92)/470.68 = 0.0934\text{g}$  and therefore moles of glycerol is  $0.0934/92 = 0.001$  moles. In moles/L =  $(0.001 \times 1000)/145 = 0.007$  moles/L or 7000 µmoles/L.

### 4.6.3 Glyceryl Trioleate

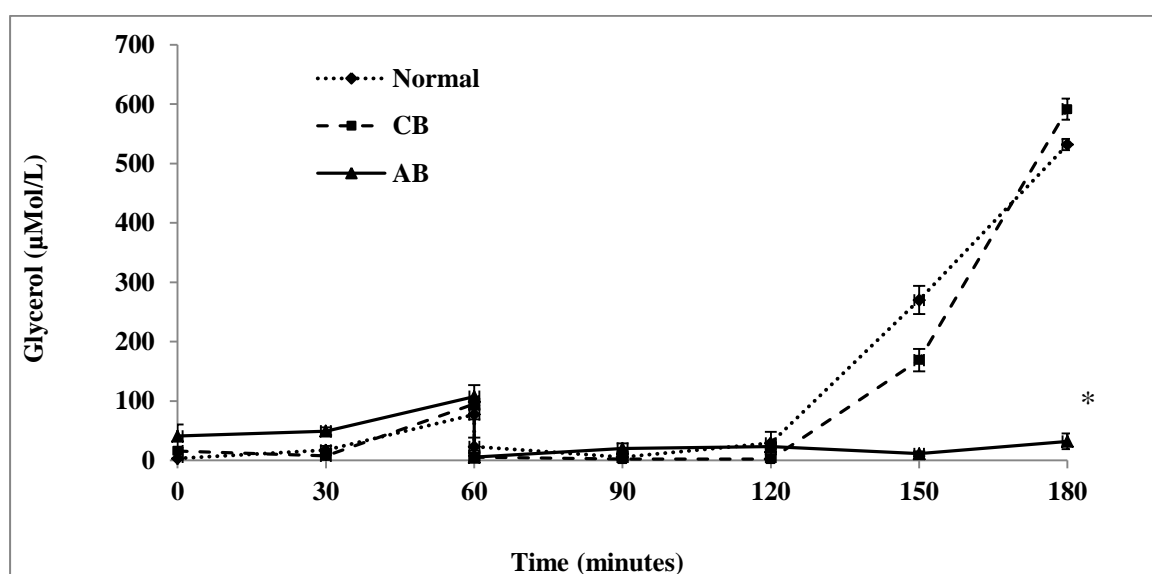
**Figure 27** shows normal glyceryl trioleate digestion alone, and with CB or AB. There is small amount of free glycerol digested within the gastric phase (0-60 minutes), however this increases as the digesta enter the small intestinal phase (60.5-180.5 minutes) of digestion. There was a significant effect of treatment, which was demonstrated by a larger mean total glycerol release in the substrate digestion alone and with CB compared with AB ( $p < .05$ ). Mean total release for substrate digestion alone was  $719.8 \mu\text{Mol/L}$  ( $\pm 43.5$ ) compared with  $665.1 \mu\text{Mol/L}$  ( $\pm 156.5$ ) and  $540.4 \mu\text{Mol/L}$  ( $\pm 34.2$ ) for CB and AB respectively. There was a significant difference at in glycerol release for normal digestion compared with substrate digestion and AB at 180.5 minutes ( $p < .05$ ) ( $2222.4 \pm 295.1$  and  $859.3 \pm 92.6 \mu\text{Mol/L}$ , respectively). There was no significant difference between normal substrate digestion, AB and CB at any other time point during digestion ( $p > .05$ ).



**Figure 27.** Mean (+ S.E.M) glycerol released during normal glyceryl trioleate (500µl) digestion alone and with either 5.2g CB or AB in the model gut system. \* denotes a significant difference ( $n=6$ ). To calculate potential glycerol present in 500µl of substrate = Density 0.91g/ml, Mr 885.43, therefore glycerol in 500µl substrate 910g in 1L so in 500µl there is 0.445g. Therefore glycerol  $(0.445 \times 92)/885.43 = 0.0462\text{g}$  and therefore moles of glycerol is  $0.0462/92 = 0.000513$  moles. In moles/L =  $(0.000513 \times 1000)/145 = 0.00354$  moles/L or 3538 µmoles/L.

#### 4.6.4 Olive Oil

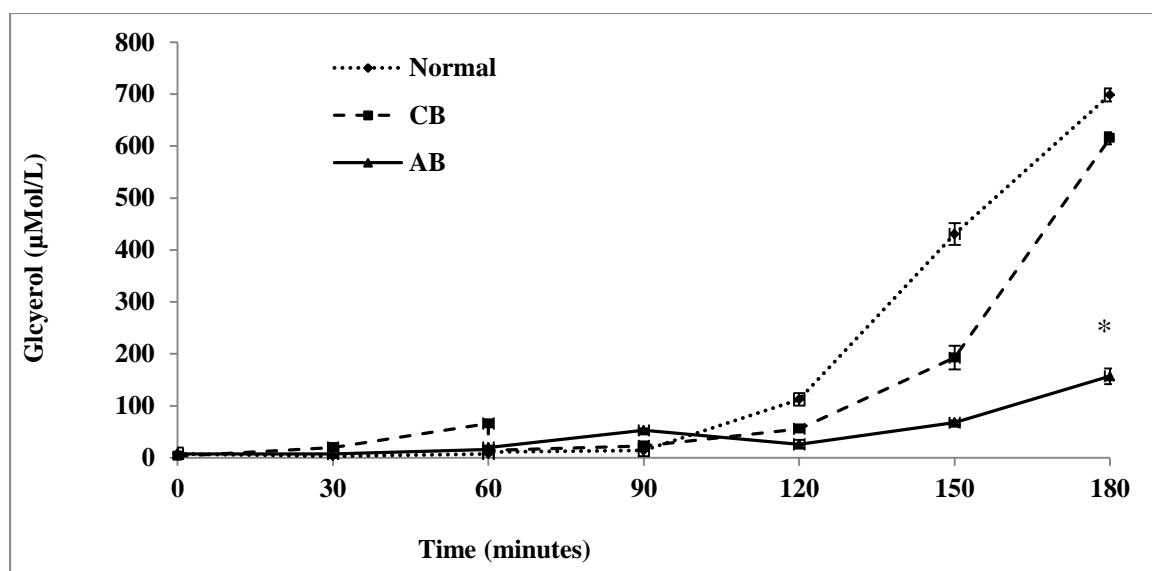
The data in **Figure 28** shows normal olive oil digestion alone, and in combination with either CB or AB. There is around 100  $\mu\text{mol/L}$  of glycerol released at the end of the gastric phase of digestion in all three samples. This then increases for the olive oil alone and olive oil with CB. There was a significant effect of treatment, which is evident in the mean glycerol released during normal olive oil digestion compared with CB and or AB ( $p < .05$ ). The mean glycerol released from olive oil digestion alone was 119.5  $\mu\text{mol/L}$  ( $\pm 48.1$ ) compared with 110.8  $\mu\text{mol/L}$  ( $\pm 25.4$ ) and 36.1  $\mu\text{mol/L}$  ( $\pm 25.4$ ) for CB and AB respectively. There was a significant difference between normal olive oil digestion and AB at 180.5 minutes ( $p < .05$ ) with 531.8  $\mu\text{mol/L}$  ( $\pm 84.4$ ) and 31.8  $\mu\text{mol/L}$  ( $\pm 118.9$ ) glycerol released respectively. Although there were no other significant differences between normal substrate digestion and CB or AB ( $p > .05$ ). For all three samples there was a lag of glycerol release until 120 minutes, thereafter glycerol increased for both olive oil alone and olive oil with CB but not with olive oil and AB.



**Figure 28.** Mean (+ S.E.M) glycerol released during normal olive oil (500 $\mu\text{l}$ ) digestion alone and with either 5.2g CB or AB in the model gut system. \* denotes a significant difference ( $n=6$ ). To calculate potential glycerol present in 500 $\mu\text{l}$  of substrate = Density 1g/ml, Mr 941 (based on the assumption that the majority of triglycerides in olive oil have C18 fatty acids), therefore glycerol in 500 $\mu\text{l}$  substrate 1000g in 1L so in 500 $\mu\text{l}$  there is 0.500g. Therefore glycerol  $(0.500 \times 92)/941 = 0.0488\text{g}$  and therefore moles of glycerol is  $0.0488/92 = 0.00053$  moles. In moles/L =  $(0.00053 \times 1000)/145 = 0.0365$  moles/L or 3650  $\mu\text{moles/L}$ .

#### 4.6.5 Butter

Figure 29 illustrates the digestion of butter in the model gut, and in combination with CB or AB. There is minimal digestion during the gastric phase of digestion (0.5-60 minutes). However glycerol release then begins to increase as the small intestinal phase of digestions begins (60.5-180.5 minutes). There was a significant effect of treatment, which was evident in the mean total glycerol released during the model gut ( $p < .05$ ). The mean glycerol released for normal butter digestion was  $160.6 \mu\text{Mol/L} (\pm 32.8)$  compared with  $123.7 \mu\text{Mol/L} (\pm 15.8)$   $44.3 \mu\text{Mol/L} (\pm 8.1)$  for 5.2g CB and AB respectively. There was a significant difference in the free glycerol between butter digestion and butter with AB at 180.5 minutes ( $p < .05$ ). There was, however, no significant differences between butter digestion and butter with CB or AB at any other time points ( $p > .05$ ).



**Figure 29.** Mean (+ S.E.M) glycerol released during normal butter (1g) digestion alone and with either 5.2g CB or AB in the model gut system. \* denotes a significant difference ( $n=6$ ). No calculation for butter as there is no data on the complete analysis of butter.

## 4.7 Discussion

The data in the present chapter demonstrates that fat substrates are digestible within the model gut and that the fat substrates, glyceryl tributyrates, glyceryl trioctanoate and glyceryl trioleate digest at varying rates. Furthermore, the data presented here indicate that the alginate enriched bread is able to attenuate fat digestion and potentially inhibit triglycerides at different rates/levels based on their chain length, whether it is as a fat substrate or fat contained within food.

Although the non-polar structure of fat implies it may be difficult to digest, the human upper GI tract is able to digest and absorb up to 95% of fat consumed (Carey et al., 1983). The majority of fat consumed is made up of triglycerides, which can either be saturated or unsaturated, which may include up to six double bonds. In addition, the length of the FA's that are attached to the glycerol backbone can vary greatly, ranging from two up to 24 carbons (Mu & Hoy, 2004). Irrespective of the chain length of the FA it is generally only the FA located at *sn1* and *sn3* that are hydrolysed, as the pancreatic lipase has specificity towards these (Lowe, 2002). This would commonly be sufficient for absorption, as this would liberate two FA, leaving a monoglyceride and two FFA which are both able to pass across the epithelial walls of the small intestines (Embleton & Pouton, 1997). Regardless of pancreatic lipase's lack of affinity, Embleton and Pouton (1997) and Mu and Hoy (2004) suggested that the FA located at *sn2* may undergo re-arrangement to the *sn1* or *sn3* position, although this re-arrangement is thought to occur with no enzymatic action. Constantin, Pasero, and Desnuelle (1960) conducted early research on whether complete hydrolysis of triglycerides can occur. The authors used synthetic pancreatic juice which included the myriad of enzymes within the small intestine. They demonstrated that complete hydrolysis of triglycerides occurred, which was evident by a significant increase in glycerol and FFA until hydrolysis plateaued. The authors attributed the alkaline environment of the small intestines for re-arrangement of the FA present at *sn-2*, which exposes the final FA for hydrolysis resulting in three FFA and a glycerol molecule. The model gut used within this laboratory has no absorption capacity, and therefore once the FA at *sn1* and *sn3* have been hydrolysed this would generally leave the FA at the *sn2* position. However the data presented here from the digestion experiments suggests that hydrolysis of all three FA is occurring as demonstrated by a plateau in absorbance and significant release of free glycerol, which corresponds well with previous research (Constantin et al., 1960; Embleton & Pouton, 1997; Mu & Hoy, 2004). Although some free glycerol may be present in the pancreatin or

bile this would not account for the substantial increase in absorbance reported here. Furthermore the glycerol that may be present within the model gut was accounted for, further strengthening the evidence that complete hydrolysis of all three FA is occurring. These data provided further evidence that the pH of the small intestines may be responsible for the re-arrangement of the third FA located at *sn2*, and thus allow this to be hydrolysed. An additional point to consider is the rate at which this re-arrangement may occur at. For example the re-arrangement may take longer when the FA chain length is longer compared with short or medium FA length, although to confirm this further research is required.

The data presented here suggests that the rate at which digestion occurs varies between different triglycerides. Luddy, Barford, Herb, Magidman, and Riemenschneider (1964) and Doreau and Chilliard (1997) indicated that the chain length and the FA that is present at the *sn2* position has significant implications on the digestion rates of triglycerides. Leyton, Drury, and Crawford (1987) reported that the longer the FFA chain length, the longer the triglyceride took to digest. Leyton et al. (1987) fed rats triglycerides that contained radioactive labelled FA, and then measured expired CO<sub>2</sub> over 24 hours. As time increased so too did the amount of radioactive CO<sub>2</sub> expired, with the smaller length FA plateauing before the longer chain FA. The evidence presented by Leyton et al. (1987) demonstrates a time dependant digestion and utilisation of triglycerides as an energy source based on their FA chain length, with shorter FA being digested quicker than long chain FA. The data presented shows that longer chain triglyceride glyceryl trioleate take longer to digest than small and medium chain triglycerides. The absorbance for glyceryl tributyrate digestion plateaued after 240 minutes; however trioleate took an additional 80 minutes, representing an increase of 44%. The digestion of triglycerides of various lengths has long been established, in as much as the chain length of the FA determines the digestion and thus the absorption rate (Bernard & Carlier, 1991; Blomstrand, 1955). Bernard and Carlier (1991) observed the digestibility and absorption of capric acid, which has a carbon tail of 10 and long chain triglycerides in rats. Either radioactive capric acid or long chain triglycerides were infused intraduodenally into the rats and blood samples were taken from the portal blood at 5 minute intervals for 1 hour. They reported that capric acid was absorbed significantly quicker than the long chain triglycerides and were therefore taken up directly via the hepatic portal vein and into the liver, and not re-constituted into chylomicrons. Although the model gut here does not have an absorption



capacity, which makes direct comparison difficult, the earlier plateau of glyceryl tributyrates suggests that digestion of this fat substrate occurs prior to glyceryl trioleate.

Although the long chain triglycerides may take longer to be re-synthesised into chylomicrons and therefore take longer to appear in the blood, an additional mechanism may be the solubility of the fats. Smits, Wilding, and Cooke (1968) indicated that although bile salts are still required for short and medium chain triglycerides, they are not as important for micelle formation as when long chain triglycerides are present. They indicated that this was due to the water solubility of short and medium chain triglycerides. The increased digestibility of capric acid containing triglycerides and glyceryl tributyrates used in the present study may also be due to the water solubility of these triglycerides. The hydrosolubility of short and medium chain triglycerides may indicate that gastric and pancreatic lipase is able to begin hydrolysis of the triglycerides earlier than long chain triglycerides. The long chain triglycerides are not water soluble and are only therefore totally solubilised at a high pH in the presence of bile salts and calcium (Bracco, 1994). If the medium and short chain triglycerides do not require these steps to release long chain FA then they can be absorbed quicker and will therefore appear in the blood quicker than long chain triglycerides. The ability of the medium and short chain triglycerides to be absorbed directly into the blood stream through the portal vein, rather than being re-constituted into chylomicrons and into the lymphatic system, and again delaying the appearance of long chain triglycerides in the blood. An additional explanation may be that pancreatic lipase has a higher affinity for the medium and short chain triglycerides when compared with the long chain triglycerides. There is no study to date that has observed the Michaelis constant ( $K_m$ ) for a triglycerides with a range of fatty acid lengths. If the medium and short chain triglycerides have a lower  $K_m$  than the long chain triglycerides then this would mean that pancreatic lipase would digest the medium and short chain triglycerides at an accelerated rate when compared with the long chain triglycerides. Although this may be a justifiable explanation for the varying digestion rates this was not confirmed in the current study and requires further research.

Interestingly the medium chain triglyceride, glyceryl trioctanoate, digested quicker than both the glyceryl trioleate and tributyrates as seen by the earlier appearance of glycerol. Yang, Kuksis, and Myher (1990) demonstrated that FA chain length played a significant role in the digestion rate of triglycerides. They incubated medium and long chain triglycerides for various timed periods in synthetic pancreatic juice including porcine pancreatic lipase in the presence of bile. They reported that medium chain triglycerides

were digested significantly quicker than long chain triglycerides as evident by the increased rate of fatty acid production recovered after each time point. Jandacek, Whiteside, Holcombe, Volpenhein, and Taulbee (1987) substantiated this further by extracting pancreatic juice using a common bile duct cannula from rats and adding various fats to the solution and then measured the release of fatty acids per minute for each of the fats. In addition the authors also fed rats the various fats before anaesthetising them and extracting the small intestines at various time points to observe the digestion rates of the fat. In both the *in-vitro* and *in-vivo* experiments the authors demonstrated that the rate of digestion was dependent on the FA chain length, with medium chain lengths having the fastest digestion rates, above both short and long chain triglycerides. Although the data presented here indicates that glyceryl tributyrate does digest at a slightly slower rate than that of glyceryl trioctanoate, which is consistent with Jandacek et al. (1987), this does not agree with earlier suggestions that medium and short chain triglycerides digest at similar rates (Bernard & Carlier, 1991; Smits et al., 1968). The methods used in this thesis are not consistent with either of the previously mentioned studies who used a rat model and humans with steatorrhoea respectively, and may explain the differences reported here. The data presented here, the physiochemical structure of short chain triglycerides and the water solubility suggests they will be digested in a similar manner to that of medium triglycerides. A potential explanation of the increased digestion rate of medium chain triglycerides over short chain triglycerides may be that the length of the FA chain of the short chains FA is too small to fit completely into the open loop of the pancreatic lipase active site resulting in weaker binding. Although the data here have not confirmed this, further work is required to ascertain if this is the case. Furthermore the majority of work conducted on short and medium FA triglycerides has been conducted in animals, ill patients *in-vivo* and *in-vitro* and therefore it is unknown whether there would be any significant difference in digestion rates in normal *in-vivo* conditions (Yang et al., 1990). To confirm whether a difference exists between the digestion rates of short and medium triglycerides further work must be conducted comparing triglycerides of varying FA lengths.

Initial digestion of triglycerides begins in the stomach where fatty acids from the *sn1* and *sn3* may be hydrolysed leaving a di-glyceride and a FFA (Tucci et al., 2010). Although digestion begins in the stomach, between 80-90% of triglycerides are digested within the small intestines (Carey et al., 1983). The data presented for the fat substrates glyceryl tributyrate, trioctanoate and trioleate digestion indicates that the model gut is digesting

them in a similar fashion as would be expected *in-vivo* digestion. Of the total amount of glycerol released during substrate digestion, 9, 12 and 2% of the total glycerol was released between 0-60 minutes for glyceryl tributyrates, trioleate and trioleate respectively. The data from the stomach phase of digestions lend support to the suggestion that short and medium chain triglycerides are more soluble rather than they are more easily digested than long chain triglycerides as depicted by the larger levels of glycerol released in the gastric phase. In a similar manner the glycerol released between 0-60 minutes from the olive oil and butter digestion which contains both long and medium chain FA was 2 and 5% respectively of the total glycerol released. Beyond 60 minutes the free glycerol increased substantially in all three substrates and both the olive oil and butter, which corresponds well with what, would be expected *in-vivo* digestion (Armand et al., 1994; Armand et al., 1996). The data presented here and earlier in this thesis shows the model gut as a suitable model to replicate digestion within the mouth, stomach and small intestines for pure triglycerides, confirming earlier studies that minimal fat digestion takes place in the stomach (Carey et al., 1983; Tucci et al., 2010). However the model gut does not appear to digest olive oil and butter to the same extent and therefore suggests that the model gut may not be effective at digesting foods high in fat. A potential explanation for this is that the end products of the olive oil and butter digestion may actually be inhibiting the release of glycerol. The rate of end product production is controlled by the level of accumulated end product (Gerhart & Pardee, 1962), and therefore if there is too much end product then this will reduce the rate of production of more products. As the model gut does not have an absorption capacity there may have been a build-up of end products and therefore a reduction in the rate of further production. However, when these products are absorbed which would occur *in-vivo* the rate of product production will not be affected. In addition within the model gut there is cholesterol, bile and FFA and all of these may be competing with the triglycerides to bind with the active site of the pancreatic lipase. Furthermore the butter and olive oil will contain a range of triglycerides ranging in medium to long chain FA and a combination of saturated and unsaturated triglycerides which may also impact upon the digestion rates when compared with pure triglycerides. Although any of these may be a viable explanation as to why olive oil and butter were not digested to the same extent as the pure triglycerides, the most important finding here was that when alginate bread was present the glycerol released was lower in both the olive oil and butter digestion.

As previously mentioned, the digestion of fat in the human digestive system is extremely efficient, digesting up to 95% of the fat consumed (Carey et al., 1983). Therein lies the problem because the amount of fat that people are consuming is increasing. Hill and Peters (1998) identified two key factors which may be exacerbating the obesity problem further, as portion size and high fat foods. Portion sizes are growing and the opportunity to consume these large portion sizes is becoming easier in fast food restaurants, with the 'super sizing' menus on offer. An additional factor is the high levels of fat that are now within foods and low costs of these high fat products. Hill and Peters (1998) suggested that these two factors are what makes designing an obesity treatment extremely difficult. It is for these reasons that an effective obesity treatment that targets food once an individual has consumed them may be the most beneficial approach to treat obesity. This is the manner by which Orlistat is able to reduce the amount of fat that an individual absorbs. Although there may be a high fat content within some foods Orlistat does not allow for complete digestion of that fat. This reduction in fat is facilitated by attenuating the activity of lipase. As an obesity treatment Orlistat is effective, but the attributed side effects are unwanted. Therefore, if alginate is able to attenuate the activity of digestive enzymes in a similar manner to Orlistat without these side effects would make alginate a potential obesity treatment.

There is a large body of evidence demonstrating that adding alginate to various delivery vehicles, such as cereal bars and drinks, may attenuate postprandial blood glucose response, reduce Kcal intake, increase satiety and increase fat excretion (Jensen, Kristensen, & Astrup, 2011; Paxman et al., 2008; Sandberg et al., 1994; Torsdottir et al., 1991). These studies all reported that alginate enriched products possess the ability to impact upon dietary intake and thus may be used to treat obesity. However, the poor palatability reported in these studies suggests that people will not consume them on a regular basis and therefore result in low adherence (Ellis et al., 1981). The bread used in the current study has undergone an acceptability study which reported no significant differences in acceptability with that of the control bread (data presented in chapter 5). In addition some anecdotal data taken from the acceptability study reported that subjects preferred the alginate bread compared to the control bread. If the alginate enriched bread is pleasant enough to eat then people will consume it on a regular basis, as they would any other product. Despite the pleasant taste of the alginate enriched bread, if it does not attenuate the activity of digestive enzymes then it is merely just bread with a larger amount of dietary fibre. The data in chapter 3 indicates that isolated alginate from

digested bread is able to inhibit pancreatic lipase activity by up to 38%, despite being exposed to the cooking process, digestion within the model gut and an isolation process. Although that demonstrates the stability of alginate, the assay used was an adapted version of the olive oil turbidity assay (Vogel & Zieve, 1961) and therefore did not take into account the myriad of factors that would be present with *in-vivo* or *in-vitro* digestion (Brownlee, Forster, et al., 2010).

This is the first study that has compared the digestion and inhibition of triglycerides ranging in chain length in a synthetic model gut. It is apparent that there is a reduction in the amount of free glycerol present during substrate digestion when AB was added. The digestion of glyceryl trioleate was reduced by 62%, which is larger than that recorded with both tributyrate and trioctanoate. Whether or not the digestion rate of glyceryl trioleate has a part to play in the larger reduction in glycerol, it appears that AB is able to attenuate the digestion of the longer chain triglycerides more than the short and medium chain triglycerides. There is previous evidence that indicates alginates or any other dietary fibres are able to attenuate the digestion of long chain triglycerides more so than short and medium chain triglycerides. One potential explanation may be that because pancreatic lipase requires longer to hydrolyse all three fatty acids of glyceryl trioleate, the alginate potentially has a longer time period in which they interact with the lipase or interfere with the digestion of the substrate, and ultimately reduce fat digestion. Additionally it may be that glyceryl tributyrate and trioctanoate are digested earlier because they do not always need to be formed into micelles for digestion to occur due to their solubility, therefore meaning pancreatic lipase is able to gain access to the substrate and cleave off the fatty acids. Alginate may have potential to inhibit the digestion of longer chain triglycerides, when compared with short and medium chain triglycerides. If alginate is binding with bile salts then this may affect the ability of the bile salts to solubilise the longer chain triglycerides and ultimately slow their digestion. Although the data presented here cannot confirm any of these suggestions, further work is required to ascertain if AB is able to attenuate the digestion of a range of triglycerides differing in FA chain length and the measure the bile salt/alginate interactions if any.

Although it is not known exactly how alginate inhibits pancreatic lipase there are a number of possibilities in which it may be binding with the enzyme or the substrate. One potential site may be the oil/water interface of the emulsification process where pancreatic lipase liberates FA, which is the suggested mechanism of how Chitosan is able to reduce the fat digestion (Han, Kimura, & Okuda, 1999; Tsujita et al., 2007). An

alternative mechanism may be through binding with the enzyme itself. The G blocks of the alginate have been shown to interact with glycoproteins eg. mucin, more specifically the exposed protein section of the mucin (Taylor, Draget, Pearson, & Smidsrod, 2005; Wilcox et al., 2014), and therefore highlights the ability of alginates to bind with proteins. This in addition may mean that the alginate is having a direct interaction with the enzyme active site and thus rendering the pancreatic lipase inactive, as is the mechanism of Orlistat. Cudrey et al. (1993) and Kumar and Chauhan (2010) postulated that the carboxyl group of the pectins may be able to protonate serine and histidine at the active site of pancreatic lipase. This could reduce or even stop the proton shuttle mechanism necessary for pancreatic lipase to become active. The similar structures present in pectins and alginates and the positioning of the carboxyl groups could make this mechanism possible. Although there is evidence for both substrate and enzyme binding it remains unknown as to what the exact mechanism of pancreatic lipase inhibition with alginate is.

The fat substrates used here ranged from 4-18 carbon long fatty acids, which are consistent with the fats that are used within the food industry to improve texture, shelf life and palatability (Satchithanandam et al., 2004). In addition the majority of foods contain a mixture of saturated and unsaturated fats, but generally tend to include triglycerides which consist of longer fatty acid chains. The use of these longer chain triglycerides is generally because they are solid at room temperature and have a higher melting point for cooking (Connor et al., 1986; Koh et al., 2011). It was for these reasons that butter and olive oil were selected for analysis as these include triglycerides, specifically butter which contains 85-88% long chain triglycerides with a small percentage of short and medium chain triglycerides (Fallon & Enig, 2000). Although the digestion rate of butter and olive oil were not digested to the same level as the pure triglycerides, they do follow the same pattern of digestion that would be expected *in-vivo* (Carey et al., 1983). There was minimal digestion in the gastric phase and 98 and 96% for olive oil and butter respectively occurred in the small intestinal phase. These data show that the model gut can be used in digesting real food sources such as bread, butter and olive oil. In addition the digestion of both the butter and olive oil were significantly reduced when digested with AB. In a similar fashion the mean glycerol released from butter digestion alone and with CB was reduced by a third with the addition of AB. Although the digestion of the olive oil was not significantly different at various time points, there was a significant difference between olive oil and butter alone when compared with olive oil and butter and AB at 180 minutes. The data here indicates that alginate enriched bread has the ability to

reduce the digestion of fat in olive oil and butter. The large reduction in butter and olive oil digestion supports the previous data suggesting AB may be able to attenuate the digestion of long chain triglycerides more so than short and medium chain triglycerides. This is the first study that has looked at the digestion and inhibition of pure triglycerides and foods high in fat content using AB and has clinical implication because if the digestion of long chain triglycerides can be reduced then this would reduce the calories absorbed from the consumed food and thus potentially have a positive impact upon obesity, and diseases associated with obesity.

As previously mentioned, the three fat substrates, butter, and olive oil consist of a mixture of short, medium and long chain triglycerides which are currently used within the food industry (Satchithanandam et al., 2004). Glyceryl triocanoate and tributyrates digestion was reduced by 39 and 32%. However glyceryl trioleate, butter and olive oil digestion was reduced by 62, 78 and 95% respectively. Glyceryl trioleate, olive oil and butter and all contain a large proportion of triglycerides with long chain FA, which are higher in calorific content than triglycerides that are made up of medium and short FA. It is for this reason that triglycerides containing long chain FA have been associated with larger weight gains than diets high in medium and short chain triglycerides (Hashim & Tantibhedyangkul, 1987). In addition, in rat and human research there is evidence that has demonstrated consuming a diet high in medium chain triglycerides resulted in greater energy expenditure when compared with long chain triglycerides (Baba, Bracco, & Hashim, 1982; Dulloo, Fathi, Mensi, & Girardier, 1996; St-Onge et al., 2003). St-Onge et al. (2003) fed seventeen obese women a diet high in medium chain triglycerides for four weeks, followed by a wash out period and then fed a diet high in long chain triglycerides for four weeks, which were matched for calorific content apart from the different fats. They reported that when the women consumed a diet high in medium chain triglycerides energy expenditure and fat oxidation were higher, which was supported by a larger reduction in body weight when compared with long chain triglycerides. Considering the large fat content in the western diets any reduction in fat digestion would be beneficial, but specifically a reduction in longer chain triglycerides. Medium and short chain triglyceride digestion begins in the stomach and they are absorbed quicker than longer chain triglycerides (Bloom, Chaikoff, & Reinhardt, 1951; Jandacek et al., 1987; Mu & Hoy, 2004). The alginate release data from the bread illustrated earlier in this thesis indicates that between 80-90% of the alginate is released in the small intestines. Therefore if the alginate is being released where the long chain triglycerides digestion is taking

place, this may account for the larger levels of inhibition reported here for glyceryl trioleate, butter and olive oil.

The data previously presented in this thesis alongside previous work in this laboratory has reported that alginates are able to attenuate the activity of digestive enzymes pepsin and pancreatic lipase (Strugala et al., 2005; Sunderland et al., 2000; Wilcox et al., 2013). Although these previous studies do provide evidence that alginates are able to reduce the activity of digestive enzymes, all of the previous work has been conducted in carefully controlled experiments and only considered individual enzymes such as pancreatic lipase and pepsin. The data from the current chapter demonstrated a reduction in fat digestion using alginate enriched bread in a synthetic model gut, which is more realistic to *in-vivo* than the afore mentioned studies. Furthermore, these data along with the earlier work in this thesis demonstrate the stability of alginate and further support the claim that alginate possess the ability to be used as an obesity treatment. The addition of alginate to a vehicle such as bread removes the poor palatability issue, which seems to be the main obstacle in using alginate as an obesity treatment (Ellis et al., 1981). Despite the encouraging results presented here further *in-vivo* and *in-vitro* work must be conducted in order to ascertain whether alginate enriched products possess the ability to be used as a treatment for obesity.



## Chapter 5 Clinical Trial

### 5.1 Introduction

The WHO and the NHS indicate that the healthiest form of weight management is to maintain a healthy diet and exercise at least 3-4 times per week. Although this is recognised as the healthiest form of weight management worldwide (Nestle & Jacobson, 2000) adherence rates to diet plans and exercise routines can be as low as 15% (Ayyad & Andersen, 2000). With adherence rates as low as this it is unsurprising that any weight lost is regained quickly during any interruption of the diet and exercise programme. In addition to the poor maintenance of a healthy diet and exercise routine, it is the large quantities of calories that individuals are consuming and the relative low cost and ease of access of palatable fast foods that are also contributing substantially to the obesity epidemic (Nestle & Jacobson, 2000). New dietary interventions to address satiety, body fat and obesity related metabolic conditions are essential to combat the obesity epidemic.

Amongst these alternative treatments is a manipulation of the stomach, which is referred to as bariatric surgery (Santry et al., 2005). Bariatric surgery encompasses a whole host of operations on the stomach, such as reducing the size of the stomach with band and removal of a portion of the stomach. Essentially these different surgeries are designed to prevent individuals from consuming as many calories as prior to the operation. Despite an average weight loss of 61.3%, these are generally a last resort due to the expensive nature and the possible complications that can be associated with them (Buchwald et al., 2004). An additional treatment is pharmaceutical interventions that target the CNS and suppress appetite. Drugs such as amphetamines and phenylpropanolamine have been used as appetite suppressants with weight loss reported in a clinical setting, although the adverse effects to drugs such as these include depression, addiction, primary pulmonary hypertension, diarrhoea and stomach pain (Fujimiya et al., 1997; Rich et al., 2000). Orlistat is an additional pharmaceutical intervention; however it does not work on the CNS. Orlistat works in the upper GI tract by covalently binding to the active site of pancreatic lipase, and therefore renders the lipase in-active (Hadvary et al., 1988). Although there is evidence to support its use in weight loss studies, adverse effects include stomach pain, flatulence, oily stools and severe diarrhoea. In retrospect all of the treatments mentioned here have long and short term benefits. However any treatment aimed at reducing obesity must have the potential to be maintained long term and adhered to without adverse side effects.

An alternative treatment may be to increase the daily intake of dietary fibre. Dietary fibre is essentially a shorthand expression for the un-digestible components of the cell walls of plants (Roehrig, 1988). The WHO recommends that 30g a day of dietary fibre should be consumed. The earliest health claims for dietary fibre came in 1953, when Hipsley travelled to Africa and reported that those consuming high levels of dietary fibre had lower rates of pregnancy toxemia (Hipsley, 1953). Since then there has been a substantial increase in the interest in dietary fibre and the potential health benefits associated with it. Such benefits include adding bulk to faeces, reduced constipation, increased satiety, decreasing gastric emptying, reducing postprandial glucose and insulin responses, decreasing fat digestion and increasing cholesterol turnover (Holt et al., 1979; Sandberg et al., 1994; Terada et al., 1999).

Early animal studies have used rats to observe the effects of cellulose, pectin, guar gum and lignin as a potential enzyme inhibitor. Burkitt et al. (1972), Trowell (1973) and Shah et al. (1986) all reported that dietary fibre resulted in attenuation in enzyme activity including trypsin, chymotrypsin, amylase and lipase. In addition to studies on rats work by Bueno, Praddaude, Fioramonti, and Ruckebusch (1981) looked at the effects of adding guar gum to the diet of dogs and reported a significantly slower transit time of 51%, and a reduced postprandial glucose response however this was not significant. A later study by Meyer and Doty (1988) observed the effect of guar gum on the digestion in dogs using fistulas. They reported that the guar gum increased the weight of the chyme, significantly increased the transit time of digesta and reduced the digestion of fat. Kimura et al. (2010) and Seal and Mathers (2001) also reported on the ability of sodium alginate to alter the digestion process. Kimura et al. (2010) demonstrated that feeding rat's alginate resulted in an increased cholesterol excretion and an attenuated glucose response when compared with the control meal. In a similar fashion Seal and Mathers (2001) reported an increase in bile acid excretion and faecal weight. In addition they also reported a reduction in plasma cholesterol when compared to the control meals. Although the previously mentioned studies highlight the potential effects of dietary fibres, the work of Calvert et al. (1985) and El Kossori et al. (2000) provided results to the contrary. Calvert et al. (1985) observed the effects of cellulose, pectin, guar gum and alfalfa on rats fed these dietary fibres for four weeks, and reported no effect on pancreatic amylase and proteolytic enzymes. Furthermore El Kossori et al. (2000) observed the effects of similar dietary fibres on *in-vitro* digestive enzymes and reported no effect on pepsin activity.

Although there is conflicting evidence *in-vitro* and in animal models there is undoubtedly a significant body of research to suggest that dietary fibre may possess the potential to be used as an obesity treatment. This is evident in the large number of *in-vivo* studies using ileostomy patients. The use of patients with ileostomies is extremely useful because digestion as far as the small intestines can be assessed with relative ease. By the end of the small intestines up to 95% of all food consumed should have been digested and absorbed, therefore any alteration in digestion through the addition of dietary fibre can be assessed in effluent fluid (Sandberg et al., 1981). A further point of interest is that there will be little or no bacterial degradation which would generally take place in the large intestines. Early work by Bosaeus and Andersson (1987) observed the effects of soya bean on bile acid and cholesterol excretion. The authors reported no significant increase in bile acids and cholesterol excretion, but failed to measure fat digestion or to make postprandial blood measurements. Furthermore although soya bean is classed as a dietary fibre the authors failed to report the amount of dietary fibre that was consumed, which may suggest the amounts used here were not sufficient to elucidate a response. Contrastingly a study by Higham and Read (1992) gave ileostomy patients 5g of guar gum three times a day for 5 days compared to 5 days with a matched diet with no guar gum. The authors reported a reduction in viscosity of effluent fluid in the guar gum, despite previous reports that dietary fibre increases viscosity in the digestive system (Blackburn et al., 1984). Despite the lack of viscosity the authors reported a reduction in both fat and protein digestion and an increase in both dry and wet weight of effluent. Supporting Higham and Read (1992) two more recent studies by Olson et al. (1997) and Ellegard and Andersson (2007) have reported positive effects of oat bran in ileostomy subjects. Olson et al. (1997) reported that a diet containing 16.3g of dietary fibre compared to 6.4g of dietary fibre resulted in an increase in cholesterol and more than a 50% increase in fat excretion. Despite not being significant the authors also reported a reduction in serum insulin, chylomicrons cholesterol and triacylglycerols, although the small sample size may have contributed to the lack of significance. In a later study by Ellegard and Andersson (2007), they compared the effect of hydrolysed oat bran with native oat bran in combination with  $\beta$ -glucans. The authors reported a significant increase in dry and wet weight effluent weight which was supported by a much larger viscosity in effluent fluid when the native oat bran when compared to hydrolysed oat bran. Furthermore the authors demonstrated that native oat bran significantly increased the sum of bile and cholesterol by 40% when compared to hydrolysed oat bran. The data from ileostomy patients in combination with the work on animals and *in-vitro* research does

appear to support the use of dietary fibres. However, there remain some inconsistencies with dietary fibres which may be due to the large range of dietary fibres used and their degree of esterification. Furthermore the use of dietary fibre as a potential treatment for obesity has come under some scrutiny which is generally due to the discrepancies within human studies, such as poor control of important factors such as body weight and total calories consumed (Franz et al., 1994). An additional point of interest is the small sample sizes used within the studies mentioned previously with sample sizes ranging from 5-12, making it difficult to assess the findings.

As previously mentioned, one of the main inconsistencies is the dietary fibre examined. Work from this laboratory has examined a number of dietary fibres, but specifically a large range of alginates. Sunderland et al. (2000) demonstrated that 58 various alginates ranging in G and M content and  $M_r$  all inhibited pepsin activity *in-vitro*. This was further supported by Strugala et al. (2005) who demonstrated a correlation between the G content of alginates and the level of inhibition used, with a range of pepsin inhibition of 39-89%. Wilcox et al. (2013) and Richardson et al. (2011) substantiated these findings further by demonstrating that certain alginates were able to reduce pancreatic lipase activity by up to 75%. These studies and the data presented in chapters 3 and 4 indicate that alginates are able to inhibit digestive enzymes in controlled conditions, but also in a system which simulates digestion in the upper GI tract. Although there is compelling evidence to suggest alginate does have the potential to be used as an obesity treatment, further *in-vivo* research is required, and an effective delivery method for alginate must be designed.

**Table 7** summarises human *in-vivo* studies that have used alginate as a source of dietary fibre. The physiological effects of adding alginate to an individual's diet has been subdivided into:

### 5.1.1 Upper GI Tract Motility

As previously mentioned alginate possess an ability to form both ionic and acidic gels, and specifically acidic gels *in-vivo* (Draget et al., 2005; Hoad et al., 2004). This gel formation within the stomach has been cited as the main reason for reducing gastric emptying (Torsdottir et al., 1991). Torsdottir et al. (1991) conducted a study observing the effects of 5g of alginate with 60% M and 40% G content added to a fruit juice drink on gastric emptying. They used radioactive labelled chromium chloride to detect gastric movement and reported a significant reduction in gastric emptying time compared to the control drink. Despite these differences the authors also failed to mention accurately whether there was any form of washout period between the test meals, meaning there may

have been some carry over between treatments. Sandberg et al. (1994) also added 7.5g of sodium alginate (60% M and 40% G content) to a milk shake and observed the physiological effects in ileostomy subjects. Total wet and dry effluent weights were significantly increased when subjects consumed the alginate enriched beverage. Although both Torsdottir et al. (1991) and Sandberg et al. (1994) reported effects of the alginate beverages, both studies had small sample sizes of 7 and 6 respectively. Mattes (2007) provided results to the contrary and reported no significant difference in bowel movements. Although Mattes (2007) did use a much larger sample size, the delivery vehicle used was a cereal bar which may have affected the ability of the alginate to form a viscous solution within the stomach. Furthermore the authors added a combination of guar gum and alginate, with only 1.1g of alginate and no details of the M:G content. The relatively low amount of alginate and the delivery vehicle used may have been an additional contributing factor to why there was no significant difference in gastric emptying between the alginate and control cereal bar.

### **5.1.2 Satiety/Hunger/Calories Consumed**

As previously mentioned alginates are able to form viscous solution in conditions where the pH drops below the pKa of alginate (Draget et al., 1997; Smidsrod, 1974). The formation of an acidic gel within the stomach has been linked with an increase in volume of the stomach (Duncan, Bacon, & Weinsier, 1983). Phillips and Powley (1996) implied that the formation of a gel in the stomach would result in an increased firing rate of stretch receptors within the stomach, due to an enlarged volume. This increased firing rate of stretch receptors has been associated with simulating the sensation of fullness. Furthermore the increased volume within the stomach has been associated with a slowing of food passing from the stomach into the small intestine. If the digesta remain in the stomach for a longer period this in turn may prolong gastric distension and therefore elongate the sensation of satiety, and ultimately reduce calories consumed (Duncan et al., 1983; Mattes, 2007). The work of Mattes (2007) as previously mentioned used a cereal bar with a relatively small amount of alginate (1.1g) and did not report any effect of the alginate cereal bar on gastric movements. It is unsurprising then that the same author reported that there was no effect on appetite and satiety following the ingestion of the treatment cereal bar when compared with the control bar. More recently there have been a number of studies that have reported a drink supplemented with alginate significantly increased the sensation of satiety and reduced Kcal intake. Pelkman, Navia, Miller, and Pohle (2007). Paxman et al. (2008) and Peters et al. (2011) all used similar amounts of

alginate ranging between 1-2.6g of alginate, with a large G content. All three of these studies reported a significant increase in satiety, reduced feeling of hunger and Paxman et al. (2008) showed a reduced intake of 134 kcal.

Further work using a beverage supplemented with alginate has been conducted by a group in Denmark. They have conducted three trials relatively recently, but have used a considerably larger amount of alginate than the studies mentioned previously. The first was a pilot study that added 15g of alginate, without mentioning the composition of the alginate. Jensen et al. (2011) administered a calorie restricted diet for subjects including either a control or alginate beverage for each group, and assessed anthropometric measures including waist circumference and body weight. Although there was a reduction in body weight and waist circumference, this was not significant between the two groups. Although there was no significant difference in weight loss this may have been because the study was only conducted over two weeks, which may not have been long enough to observe any substantial weight loss. Two later studies by Jensen et al. (2012 (a)) and Jensen et al. (2012 (b)) also used 15g of alginate added to a 500ml beverage. The first of these two studies observed the acute response of the alginate beverage on satiety and hunger feelings following consumption, when compared with the control beverage. The authors indicated that the gelling of the alginate within the stomach was sufficient to account for the significantly increased feeling of satiety and a reduced feeling of hunger. A potentially more interesting finding was reported in the third study by this group. Jensen et al. (2012 (b)) observed the effects of a beverage containing 15g of alginate and an energy restricted diet 3 times a day for 12 weeks. The authors reported a weight loss of 6.78kg when consuming the alginate beverage when compared with 5.08kg loss in body weight when participants consumed the control beverage. Although these are positive findings, there are a number of discrepancies when comparing the control and alginate group with regards to some of the anthropometric measurements. The baseline body weight and percentage of body fat were significantly higher in the alginate group than the control group. Although these may not have had a significant bearing on the overall findings of the study, this seems difficult as the authors attempt to attribute the reduction in percentage of body fat to the alginate beverage. Therefore if the alginate group have a significantly larger percentage of body fat to begin with, then they potentially have a larger proportion of fat mass to lose when compared with control group.

### 5.1.3 Blood Response and Nutrient Digestion

Although an increase in satiety is important, the ability of alginate to attenuate gastric emptying has also been linked with a reduced absorption and digestion of macronutrients, which may have a substantial impact on obesity (Brownlee, 2009). The early work of Torsdottir et al. (1991) was some of the first to observe the effects of alginate on the blood glucose and insulin response in humans. They reported a significant reduction in glucose and serum insulin response following ingestion of an alginate beverage. Any reduction in insulin response following a meal would have substantial implications for individuals suffering with diabetes. Interestingly Sandberg et al. (1994) did not assess the response in blood samples but rather the remains of digested products in the effluent fluid of ileostomy patients. There was a significant increase in the FFA recovered in the effluent samples when individuals consumed the alginate beverage. These data indicated that there was a reduction in fat absorption; however no assessment was made of plasma triglyceride levels to confirm this. Jensen et al., (2012) provided results to the contrary of both Torsdottir et al. (1991) and Sandberg et al. (1994) and reported no effect of an alginate loaded beverage on plasma triglycerides, insulin and glucose response. Interestingly the authors here only measured the plasma response once a week; however a response may have been present if the plasma samples were taken for a number consecutive hours following the meal and alginate beverage. Furthermore the authors did not assess the AUC for plasma triglyceride, which may have demonstrated a reduction in fat digestion. This assumption was confirmed by another study from the same group (Jensen et al., 2012 (b)). They used a 15g of alginate in a 500ml beverage, but they took blood samples every 30 minutes and reported a significant reduction in area under the curve (AUC) for blood glucose response. The AUC is the actual body exposure to blood glucose after the administration of food or drink consumed. The reduction in AUC of blood glucose response suggests that alginate beverage reduced the digestion rate of carbohydrates. Wolf et al. (2002) and Williams et al. (2004) provide further evidence to substantiate the use of alginate as a potential treatment to attenuate the blood plasma response following ingestion of a meal. A point of interest when comparing these two studies was that low amounts of alginate used just 1.5 and 1.6g, respectively. Despite the small amounts of alginate used both authors reported a blunting of the blood glucose response. An additional point of interest was the large sample sizes of 30 and 48 used respectively, which allows the findings to be generalised and also increases the sensitivity of the study and highlight any significant differences between the control and alginate

products. These data suggest that alginate enriched beverages are able to reduce the digestion of carbohydrates and triglycerides.

The data presented in Chapters 3 and 4 along with the research presented in **Table 7** do provide evidence that alginate may have potential in the treatment of obesity. The earlier studies in both humans and animals do provide positive results although the majority of these studies have small sample sizes. More recent findings have addressed this issue with larger sample sizes, specifically studies such as Jensen et al. (2012 (a)), and Paxman et al. (2008), with 96 and 69 respectively. An additional point to consider is the amount of alginate that is required to observe an effect, and the studies here do not allow for any conclusions to be made. There have been positive effects reported when using small amounts of alginate such as 1.5g (Wolf et al., 2002) compared to a significantly larger amount of 15g (Jensen et al., 2012 (a)). The composition of alginate used is important for a number of reasons including the costs of supplementing a product, the effect the alginate has on the properties of the product and the potential of any adverse or poor palatability associated with the addition of alginate to a food or beverage. All of the studies summarised within **Table 7** do report where they purchased the alginate from; however a number of these studies failed to include the composition of the alginate used. Studies such as Mattes (2007), Paxman et al. (2008) and Jensen et al. (2011) reported no significant effect of the alginate and control vehicle, although they did not mention the M/G ratio of the alginate used. This therefore raises the question as to whether there was a large M content incorporated and thus a weak gel would have been formed (Draget et al., 1994). The information reported here does suggest that both cereal bars and beverages elucidate physiological responses, however the response reported appears to be dependent on the delivery method. For example if there is a group of obese individuals that have type-2 diabetes then a beverage may be the best delivery method as this appears to have the greatest effect on blood glucose (Torsdottir et al., 1991; Wolf et al., 2002). Despite the various methodological differences one of the main issues reported throughout the research is the poor palatability of the alginate supplemented product. As previously mentioned alginate increases the viscosity of a solution at varying pH's (Draget et al., 2003). This is a problem when adding alginate to a beverage as the beverage can exhibit a slimy mouth feel, tooth packing and poor palatability for subjects (Ellis et al., 1981). The majority of the studies in **Table 7** have some form of adverse effects associated with them such as returning products due to poor taste (Sandberg et al., 1994), control product preferred (Mattes, 2007), stomach intolerance (Pelkman et al.,



2007; Wolf et al., 2002)(Wolf et al., 2002; Pelkman et al., 2008), poor appearance, taste and after taste (Jensen et al., 2012 (a)). The evidence here does appear to support the potential use of alginate as a treatment for obesity, however a main issue is the method in which alginate is delivered.

**Table 7.** Summary of alginate supplemented vehicle in humans.

<u>Author</u>	<u>Delivery Vehicle</u>	<u>Alginate Amount and type</u>	<u>No. Subjects</u>	<u>Satiety/Hunger</u>	<u>Plasma effects</u>	<u>Gastric Effects</u>	<u>Fat Digestion</u>	<u>Side Effects</u>
Torsdottir et al (1990)	Drink	5g – 40%G	7	No data	↓ blood glucose ↓ serum insulin	↑ gastric emptying	Not reported	Not reported
Sandberg et al (1994)	Drink	7.5g 40%G	6	No data	No mention	↑ wet effluent weight ↑ dry effluent weight	↑ Fatty acid in effluent	Alginate products returned
Wolf et al., (2002)	Drink	1.5g alginate No compositional data	17	No data	↓ AUC for serum glucose and insulin	Not reported	Not reported	Stomach intolerance
Williams et al., (2004)	Cereal Bar	1.6 alginate No compositional data. 5.5g guar gum	48	No data	↓ blood glucose	Not reported	Not reported	Not reported
Mattes (2007)	Cereal Bar	1.1g alginate No compositional data. 3.9g guar gum	25	No difference in appetite and satiety	No data	No difference	Not reported	Control bar more pleasant
Pelkman et al., (2007)	Drink	1 and 2.8g alginate No compositional data.	29	↑ satiety ↓ Kcal intake	No data	Not reported	Not reported	↑ flatulence ↑ stomach ache
Paxman et al., (2008)	Drink	1.5g alginate 65-75 G	69	↓ Kcal intake	No data	Not reported	Not reported	Not reported

Paxman et al., (2008)	Drink	1.5g alginate No compositional data.	14	No data	Only difference in overweight (5 subjects)	Not reported	No difference	No reported
Peters et al., (2011)	Drink	2 and 2.6g alginate 70% G	23	↓ Hunger	No mention	Not reported	Not reported	None
Jensen and Astrup (2011)	Drink	15g alginate No compositional data.	20	No difference	No difference	Not reported	Not reported	Poor palatability
Jensen et al., (2012)	Drink	9.9 and 15g alginate No compositional data.	20	↑ satiety ↓ hunger	No difference	Not reported	Not reported	Poor palatability
Jensen et al., (2012)	Drink	15g alginate 65-75% G	96	↑ weight loss	↑ BP No difference in insulin	Not reported	No difference	Poor palatability

## 5.2 Aims

The initial aim of the present chapter is to assess the acceptability of an alginate bread with additional fat compared with the control bread in a pilot study. Subjects will consume the alginate bread (which contains more fat, as seen in **Table 2**, or control bread at least three weeks apart in a fasted state in an attempt to ascertain the acceptability of the alginate enriched bread compared with the control bread. This will address whether the alginate enriched bread has any of the side effects that have been associated with alginate enriched products. In addition subjects will complete well-being questionnaires to ascertain if there are any differences in subjects feeling of hunger, fullness and general well-being between the alginate and control bread.

The second aim of the current chapter is to assess whether consuming alginate enriched bread will reduce fat digestion. If alginates are able to attenuate the activity of pancreatic lipase then the expected result would be a reduction in the fat digestion. This chapter will assess if there is a reduction in fat digestion between the control and alginate bread in plasma samples and effluent fluid. Post consumption of the alginate or control bread the anticipated result would be an increase in plasma triglycerides. Therefore if alginate bread has any effect on fat digestion then the triglycerides in the plasma will be lower than the control bread. Furthermore as previously mentioned if triglycerides are not broken down to monoglycerides and FFA then they are too big to pass across the epithelial walls of the small intestines. Therefore by measuring the weight and fat content of the effluent fluid this will address whether the alginate bread has reduced fat digestion in comparison to the control bread.

## 5.3 Methods

### 5.3.1 Materials

Alginate and control bread were identical to those used in chapter 3 produced by Greggs Plc. Anchor butter and standardised meals were purchased from Marks and Spencers (Newcastle Upon Tyne, UK). Glycerol reagent A and B were purchased from Amsbio (Abingdon, UK). Methanol, chloroform, sodium chloride were all purchased from Sigma-Aldrich (Poole, UK). A Thermo Jouan BR4i Mu Scientific Centrifuge (Basingstoke, UK) was used to separate red blood cells from plasma. Ultra-Turrax T18 IKA Homogeniser with dispersing tools S 18 N – 10G and 19G from Fischer Scientific (Loughborough, UK) was used to homogenise effluent samples from various time points. An Edwards Modulyo EF4-174 Freeze Dryer (London, UK) was used for isolating solid contents of effluent.

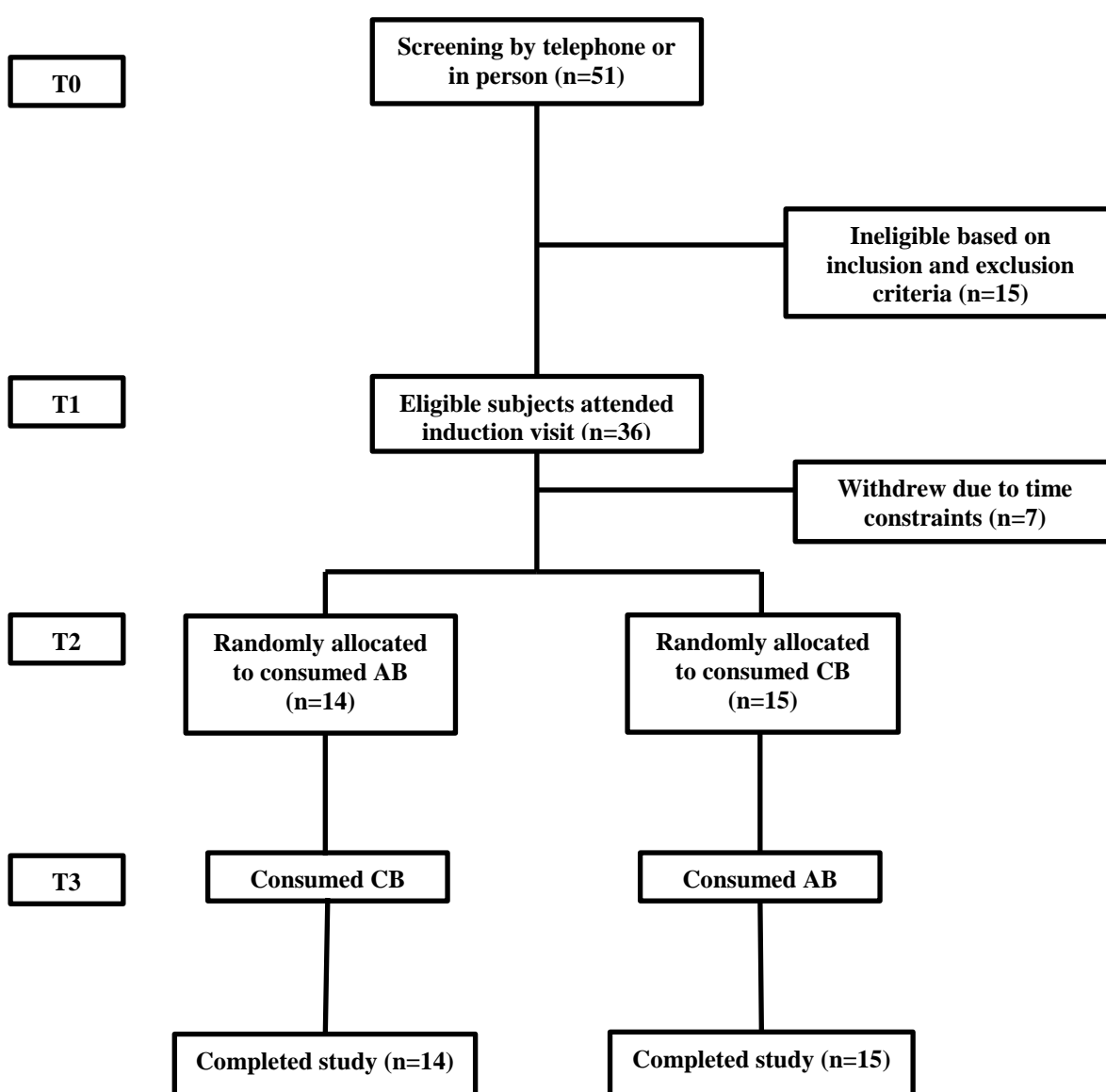
### 5.3.2 Subjects

Subjects were recruited from areas around Newcastle Upon Tyne, UK, from ileostomy meetings. Inclusion criteria were as follows: aged 18 years or above, generally healthy, able to provide multiple blood samples, able to fulfil study commitments and research burden within their usual lifestyle, have undergone an ileostomy operation at least two years prior to the commencement of the study and the ileostomy must be fully functional and stable. Exclusion criteria included: an unstable ileostomy, smoker, receiving medical treatment during the study which required them to take medication, allergic or intolerant to the foods used within the study, history of substance or alcohol abuse, currently or planning a pregnancy in the next 12 months or were planning a change in dietary habits, physical activity or change in body weight during the study. Subjects were free to withdraw from the study at any time. 36 subjects were recruited and screened. Patients within study had undergone the operation for an ileostomy for reasons including bowel cancer, ulcerative colitis and Crohn's disease.

### 5.3.3 Protocol

A double-blind, randomised, cross-over design was used. All randomisation was carried out in anonymous fashion by an independent researcher, who coded the breads and participants accordingly. 51 subjects were screened following either a telephone interview or in person to ensure subjects fulfilled all inclusion criteria and to advise them of what the research they will be taking part in would involve. Following a telephone or in person chat 36 subjects were invited into the clinical research facility (CRF), within the Royal Victoria Infirmary for an induction visit. During the induction visit subjects were

provided with full details of what the study entailed and what they would be taking part in, and asked to sign the consent form. Participants were given two standardised meals, nutritional composition provided in **Table 8** and two 500ml bottle of water to take home with them. Each of these meals would be consumed prior to their visits to the CRF (one per visit). An experienced nurse from the CRF was on hand to answer any questions and provide further information on the visit days. Of the 36 subjects recruited 29 completed the consent form and were happy to partake in the study. The final study sample was comprised of 29 adults (mean  $\pm$  S.E.M) 62.1 ( $\pm$  0.14) years of age with a BMI of 27.4 ( $\pm$  0.02). There were 15 males and 14 females. Seven subjects withdrew due to time constraints (**Figure 30**).



**Figure 30.** Consort chart for enrolment in Ileostomy study.

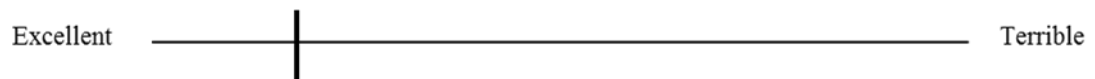
**Table 8.** Nutritional composition of standardised meals given to subjects on induction visit.

<b>NUTRITION</b>		
Typical Values	Per 100g	Per Pack
<b>Energy kJ</b>	475	1900
<b>Energy kcal</b>	115	460
<b>Protein</b>	8.9g	35.6g
<b>Carbohydrates</b>	11.7g	46.8g
<b>Fat</b>	3.1g	12.4g
<b>of which saturates</b>	1.0g	4.0g
<b>Fibre</b>	1.5g	6.0g
<b>Sodium</b>	0.25g	1.0g
<b>Equivalent as salt</b>	0.63g	2.5g

Subjects were randomly allocated to one of two treatment groups following their induction visit as depicted in **Figure 32**. Group one received 100g of alginate enriched bread, as toast with 20g of anchor butter (Test meal A), whereas group two received 100g of control bread, as toast with 20g of anchor butter (Test meal B). On the evening prior to subjects first visit they were advised to eat their standardised meal before 8pm, and refrain from all food and drink, except water before visiting the CRF. Subjects arrived at the CRF fasted for each main study visit early in the morning (10 am or before). Upon arrival, subjects were asked to fill out a Wellbeing study questionnaire on their general wellbeing and their feelings of fullness/hunger. Subjects were given instructions on how to fill in the questionnaire and provided with an example question as depicted in **Figure 31**, and then instructed to mark on a line how they were feeling at that time point. The analysis was completed by measuring in cm where on the line the subjects had marked for each question. The nearer the marked line to excellent the lower the score for the question. **Figure 31** illustrates how the score for each question was measured. The questionnaire also included two sections at the end, where subjects were able to add any unusual symptoms or discomfort experienced that were not included in the previous questions, and add any additional comments. The questionnaire was divided into two sections general wellbeing (question 1) and whether the subjects were currently experiencing any of the symptoms within the questionnaire (question 2). The questions

that subjects were asked can be seen in **Table 9** and **Table 10**. Subjects were informed that the questionnaire should take no more than 5 minutes to complete. The questionnaire has been used previously in whole grain research and is valid and repeatable (Aitken, Zealley, & Rosenthal, 1969; Brownlee, Moore, et al., 2010).

*How do you feel?*



**Figure 31.** Example question provided to subjects as part of the wellbeing questionnaire. The line depicts a potential answer to the question.



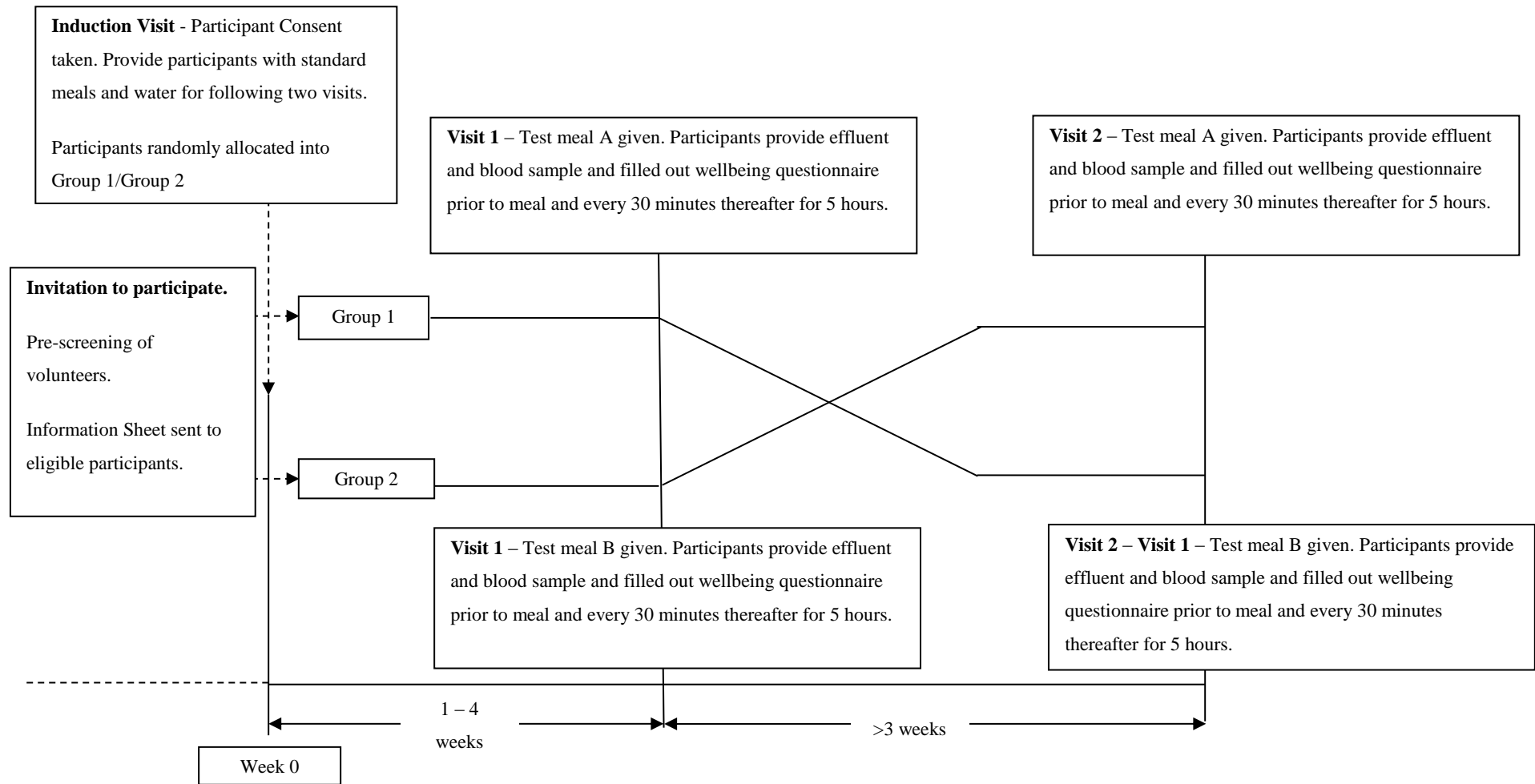
**Table 9.** Question 1 of wellbeing questionnaire assessed on an analogue scale.

Alert		Sleepy
Fine		Nauseous
Full		Starving
Not Bloating		Bloating
Not flatulent		Flatulent
Calm		Irritable
Relaxed		Anxious

**Table 10.** Question2 of wellbeing questionnaire assessed on an analogue scale.

<b>Light-headedness or dizziness?</b>		
Not at all		Very
<b>Blurred Vision?</b>		
Not at all		Very
<b>A difficulty to concentrate?</b>		
Not at all		Very
<b>A difficulty to think?</b>		
Not at all		Very
<b>Excessive Thirst?</b>		
Not at all		Very
<b>Headaches/Migraines?</b>		
Not at all		Very
<b>Cravings for sweets?</b>		
Not at all		Very
<b>Abdominal Discomfort?</b>		
Not at all		Very

In addition the complete contents of effluent bags were collected. A nurse from the CRF identified an appropriate vein via palpitation and visual inspection from both arms to assess the most appropriate antecubital vein of the forearm. The nurse then confirmed subject's particulars and cleaned the site from which the blood samples were taken from. A tourniquet was then applied to the arm approximately 7-10cm above the puncture point. The subject's arm was then placed in a downward position and a 21 ml gauge needle (S-Monovette, Sarstedt, Germany) was placed into the antecubital vein. The vacutainer tube was then placed into the needle holder and a six millimetres blood sample was extracted, during which time the tourniquet was released. The remaining blood samples were taken using a cannula to ensure patient comfort. All procedures were consistent with NHS standards. The initial questionnaire, effluent sample and blood sample were baseline. Subjects then consumed their allocated toast and were allowed to consume water ad libitum. Upon finishing the toast subjects filled in another wellbeing questionnaire, provided a 6ml blood sample and emptied their effluent bag of its contents. Subsequent questionnaires, blood samples and effluent samples were taken every 30 minutes for a total of five hours. All effluent and blood samples were stored on ice until analysis. Upon completing the study subjects were provided with a lunch of their choice. Subjects then returned at least three weeks after the initial visit and received the alternative bread from the first visit. Both visits were identical apart from the different bread provided (**Figure 32**). Following completion of both visits, subjects received travel expenses and a study honorarium (in the form of vouchers).



**Figure 32.** Double blind randomised ileostomy study protocol.

### 5.3.4 Effluent Sample Analysis

Effluent samples were weighed for each time point and then frozen at  $-80^{\circ}\text{C}$  until analysis. During analysis samples were diluted 1:1 (w:w) in  $\text{DH}_2\text{O}$ . As not all participants had an effluent sample at each time point, the 12 time points were subdivided into four separate points as follows:

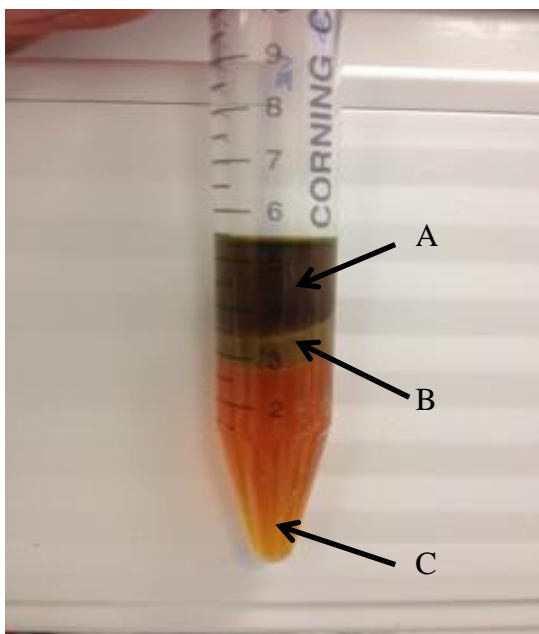
1 - 0 and 30 minutes

2 - 60, 90 and 120 minutes

3 - 150, 180 and 210 minutes

4 - 240, 270 and 300 minutes

Samples were then homogenised for five minutes, and 4ml samples for both test meals AB and CB at each of the above four time point were then placed into separate pre-weighed 15 ml universals. The effluent samples were then re-frozen at  $-80^{\circ}\text{C}$  before being placed in a freeze dryer for 48 hours. Following freeze drying samples were left at room temperature for 30 minutes before being weighed again. 4ml of a chloroform and methanol solution (2:1) was added to each sample and mixed for 5 minutes by hand. 1ml of 0.73% sodium chloride was then added and mixed for a further 5 minutes on a vortex mixer. Samples were then centrifuged at 4100 RPM for 10 minutes at  $4^{\circ}\text{C}$ . The above process produced three layers, as evident in **Figure 33**. The top layer was discarded and the middle layer was pierced to extract the lower layer using a glass pasteur pipette. The lower layer of each sample was transferred to a pre-weighed 15ml universal. 4ml chloroform/methanol (2:1) was then added to the middle layer and the process from this point was repeated a further two times, which is consistent with previous research. Repeating this procedure three times is to ensure complete extraction of all lipids is achieved. This method has been demonstrated to be efficient at recovering up to 98% of lipids (Folch, Lees, & Sloane Stanley, 1957). Following three repeats of the procedure the universals containing the pooled lower layers were then placed into an incubator at  $50^{\circ}\text{C}$  for 48 hours to evaporate the contents. The remaining contents of the tube were then weighed to ascertain total fat content. To ascertain total fat content from complete effluent samples **Equation 7** was used.



A = non lipid contaminants.

B = undigested digesta.

C = lipids.

**Figure 33.** Fat extraction remnants from an effluent fluid sample following mixing with chloroform/methanol mixture, and centrifugation.

$$\frac{TF\ 4ml \times TW\ of\ EF}{4} = Total\ Fat$$

TF 4ml = Weight of sample from 4ml sample following extraction process.

TW of EF = Total weight of effluent sample for each of the 4 time points (e.g. 60 + 90 + 120 minutes, time point 2).

The assumption that 1g of effluent = 1ml was made. This was confirmed by weighing 1ml of three fat substrates, glycerol tributyrates, glyceryl trioctanoate and glyceryl trioleate.

**Equation 7.** Calculation for total fat from effluent samples.

### 5.3.5 Plasma Samples

Samples were centrifuged at 4100rpm for 10 minutes at 4°C to separate the serum from blood cells. Two aliquots (1.2ml each) of the resulting serum samples were taken and stored at -20°C for later analysis. To ascertain total plasma triglycerides a total serum triglyceride assay kit (STG-1-NC, Amsbio, Abingdon, UK) was used. Working reagent A was prepared using glycerol reagent with deionised H<sub>2</sub>O in a 4:1 ratio, respectively. Working reagent B was prepared using triglyceride reagent and glycerol reagent on a 1:4 ratio, respectively. Both working reagents A and B were reconstituted at room temperature. 5µl of serum from each time point for each subject was added to a 96 well plate in duplicate, before 100µl of working reagent A was added to the appropriate wells and mixed, before being left at room temperature for 15 minutes. The plate was then read at 540nm on the plate reader, which provided free glycerol measurement. 5µl of serum samples was then added to a separate 96-well plate for each time point for each subject in duplicate. 100 glycerol reagent B was added to the appropriate wells and mixed before being left at room temperature for 15 minutes. Working reagent A quantifies any glycerol present in plasma and working reagent B breaks down triglyceride into glycerol and ensures complete digestion of all plasma triglycerides. The plate was then read at 540nm on the plate reader which provided total triglyceride measurement. The absorbance from glycerol reagent A was subtracted from glycerol reagent B, giving total plasma triglycerides, as seen in **Equation 8**. A standard curve was produced using working reagent A for glycerol (**Figure 18, Chapter 4**).

$$OD\ Serum\ B - OD\ Serum\ A = Total\ TG$$

OD Serum B = Absorbance from serum sample using working reagent B

OD Serum A = Absorbance from serum sample using working reagent A

**Equation 8.** Total triglyceride calculation using serum triglyceride assay kit.

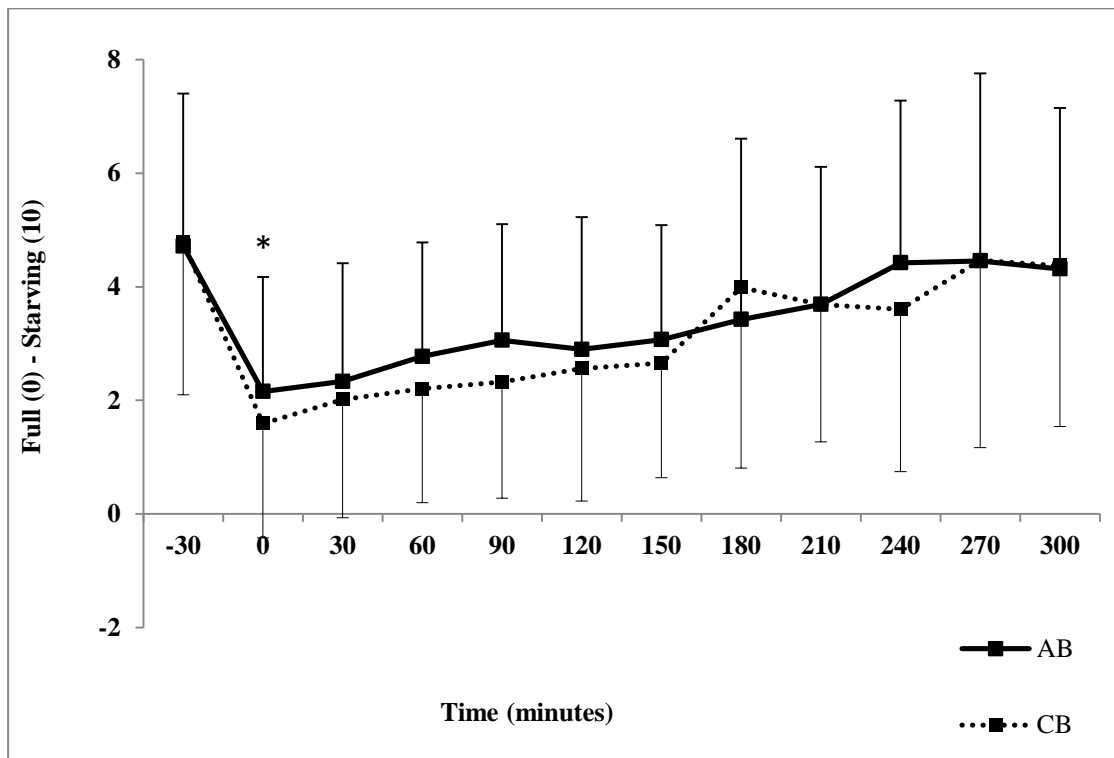
## 5.4 Statistics

Statistical calculations were undertaken using SPSS Statistics 19 (IBM, Predictive Analysis Software, USA). Data is presented as mean and standard error of mean ( $\pm$  S.E.M) or standard deviation ( $\pm$  SD). A paired t-test was undertaken at a significant level ( $\alpha$ ) of 0.05 to compare effluent weights at four time points and total effluent weight, fat at four time points and total fat in effluent samples and compare subject's response from the wellbeing questionnaires between control and alginate bread. A bivariate Pearson's correlation was carried out to observe any relationship between effluent weight and fat in effluent samples in alginate and control bread. A One Way Repeated ANOVA followed by a Post-Hoc Bonferroni was undertaken at a significant level ( $\alpha$ ) of 0.05 to compare plasma triglyceride levels.

## 5.5 Results

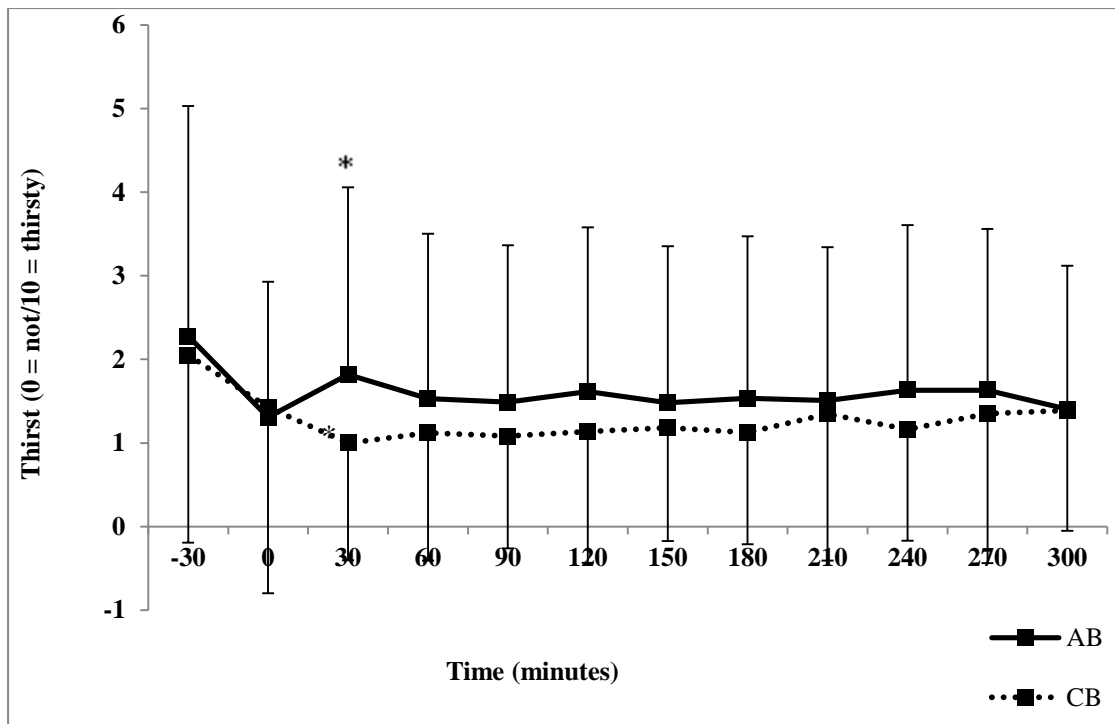
### 5.5.1 Wellbeing questionnaires

The postprandial changes for question 1 and 2 from subjects reported a significant difference in fullness (**Figure 34**) and excessive thirst (**Figure 35**) between the alginate and control bread ( $P < 0.05$ ) at 0 and 30 minutes respectively. There were no other significant differences between control and alginate bread for any other questions at any other times points ( $P > 0.05$ ) (**Table 11**, **Table 12**). However there was a trend that subjects were thirstier after consuming alginate bread compared to the control bread. There were no significant differences for area under the curve (AUC) between control and alginate bread at any time points for any questions ( $P > 0.05$ ).



**Figure 34.** Mean ( $\pm$  S.D) response from subjects for question 1 from the wellbeing questionnaire (Full (0) or Starving (10)). (\* indicates a significant difference at  $P < 0.05$ ) (-30 represents Baseline) ( $n=29$ ).





**Figure 35.** Mean ( $\pm$  S.D) response from subjects for question 2 from the wellbeing questionnaire (Not thirsty (0) or Thirsty (10)). (\* indicates a significant difference at  $P < 0.05$ ) (-30 represents Baseline) ( $n=29$ ).

**Table 11.** Significant values comparing subject's response between control and alginate bread for question 1 of wellbeing questionnaire.

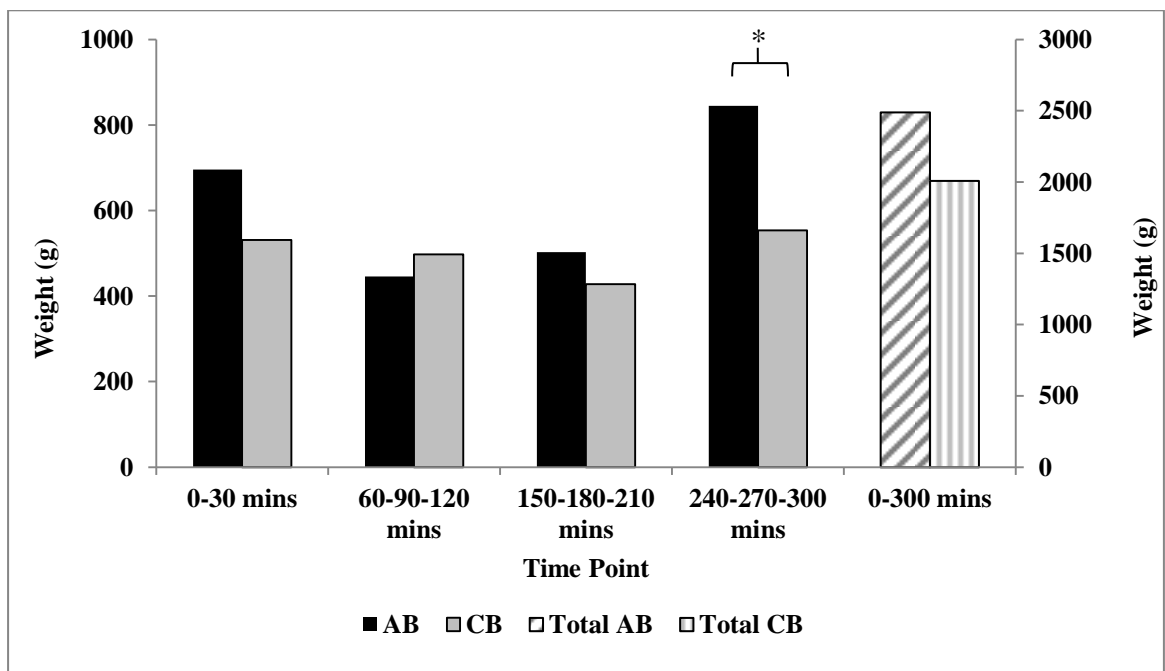
<b>Time (minutes)</b>	<b>Baseline</b>	<b>0</b>	<b>30</b>	<b>60</b>	<b>90</b>	<b>120</b>	<b>150</b>	<b>180</b>	<b>210</b>	<b>240</b>	<b>270</b>	<b>300</b>
<b>Alert (0) –Sleepy (10)</b>	0.32	0.17	0.27	0.28	0.97	0.86	0.99	0.38	0.47	0.32	0.96	0.47
<b>Fine (0) – Nauseous (10)</b>	0.35	0.37	0.98	0.94	0.66	0.31	0.93	0.59	0.63	0.77	0.94	0.23
<b>Full (0) – Starving (10)</b>	0.72	0.05	0.65	0.33	0.21	0.77	0.85	0.32	0.63	0.30	0.85	0.87
<b>Not bloated (0) – Bloating (10)</b>	0.45	0.67	0.31	0.73	0.73	0.77	0.50	0.18	0.67	0.97	0.81	0.91
<b>Not flatulent (0) – Flatulent (10)</b>	0.07	0.17	0.10	0.26	0.17	0.51	0.66	0.72	0.43	0.49	0.48	0.22
<b>Calm (0) – Irritable (10)</b>	0.59	0.96	0.98	0.77	0.42	0.41	0.27	0.30	0.35	0.82	0.41	0.10
<b>Relaxed (0) – Anxious (10)</b>	0.56	0.63	0.96	0.53	0.30	0.35	0.32	0.49	0.45	0.84	0.49	0.61

**Table 12.** Significant values comparing subject's response between control and alginate bread for question 2 of wellbeing questionnaire.

<b>Time (minutes)</b>	<b>Baseline</b>	<b>0</b>	<b>30</b>	<b>60</b>	<b>90</b>	<b>120</b>	<b>150</b>	<b>180</b>	<b>210</b>	<b>240</b>	<b>270</b>	<b>300</b>
<b>Light Headedness or Dizziness</b>	0.90	0.24	0.72	0.68	0.65	0.26	0.68	0.81	0.93	0.37	0.25	0.48
<b>Blurred Vision</b>	0.52	0.27	0.16	0.76	0.62	0.27	0.97	0.88	0.38	1.00	0.45	0.13
<b>A difficulty to concentrate</b>	0.91	0.33	0.58	0.52	0.87	0.16	0.39	0.24	0.33	0.95	0.43	0.24
<b>A difficulty to think</b>	0.70	0.10	0.12	0.55	0.40	0.38	0.87	0.26	0.99	0.81	0.33	0.41
<b>Excessive thirst</b>	0.82	0.75	0.04	0.30	0.20	0.30	0.36	0.16	0.67	0.33	0.62	0.97
<b>Headaches/Migraines</b>	0.34	0.24	0.51	0.31	0.91	0.45	0.91	0.97	0.91	0.96	0.36	0.75
<b>Cravings for sweets</b>	0.70	1.00	0.33	0.70	0.35	0.16	0.59	0.87	0.87	0.75	0.61	0.10
<b>Abdominal discomfort</b>	0.55	0.52	0.32	0.26	0.49	0.45	0.13	0.66	0.56	0.36	0.28	0.13

### 5.5.2 Wet Effluent Weights

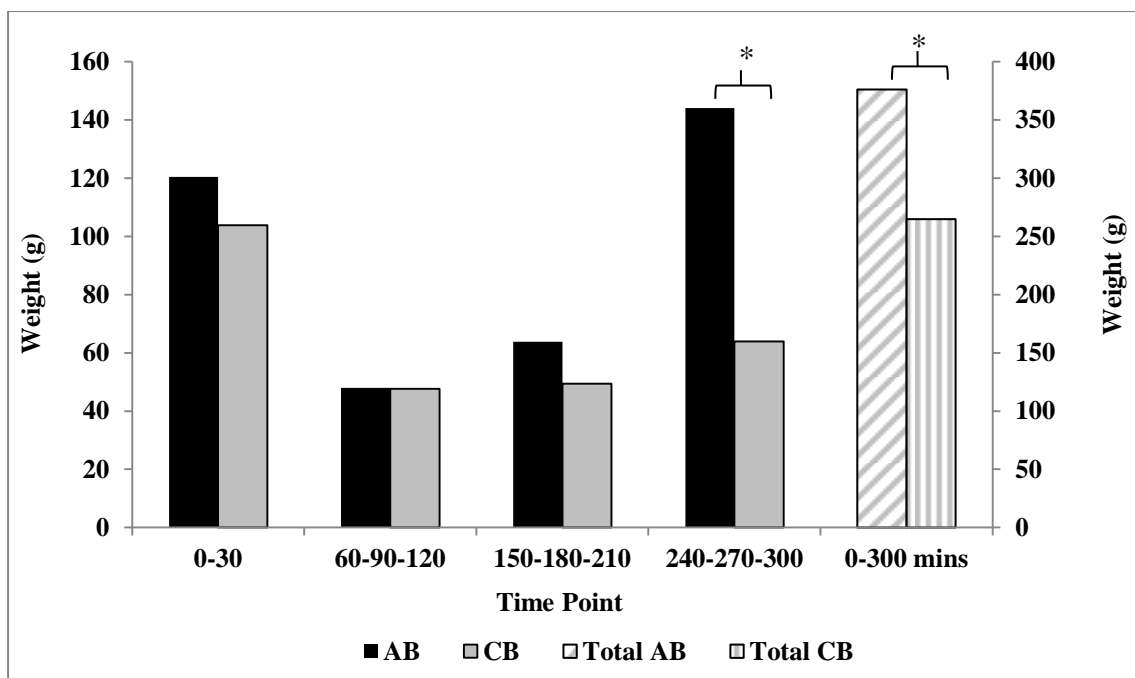
The baseline samples were removed from the analysis of both the control and alginate bread as these samples would only contain digesta from the standardised meal, mucus and shed cells. **Figure 36** illustrates the combined effluent weights for subjects at the four time points, and the combined effluent weights for alginate and control bread across all four time points. A paired samples t-test reported a significant difference in effluent weight between alginate and control bread at 240-270-300 minutes ( $P < 0.05$ ). There was an increase in effluent weight of 292g between the alginate and control bread at 240-270-300 minutes (time point 4). There was no significant difference in effluent weight at any other time point between alginate and control bread ( $P > 0.05$ ), although at time points 1 and 3 the weights were larger when subjects consumed alginate bread. The combined effluent weight for alginate bread was 2489 ( $\pm 182$ ) grams, which was 20% larger than the 2010 ( $\pm 54$ ) grams in the control bread samples. Despite these changes between the breads the paired t-test revealed no significant difference in the combined effluent weights between alginate and control bread ( $P > 0.05$ ). There was no significant difference in AUC for effluent weights between alginate and control bread ( $P > 0.05$ ).



**Figure 36.** Total effluent weights for four time points and combined total effluent weights for alginate and control bread. \* indicates at significant difference at  $P < 0.05$ . (0-300 mins is the total effluent for all four time points and for all volunteers) ( $n=29$ ). The number of effluent samples for each time point was 29, 25, 26 and 24 for AB and 26, 24, 22 and 23 for CB.

### 5.5.3 Effluent Fat

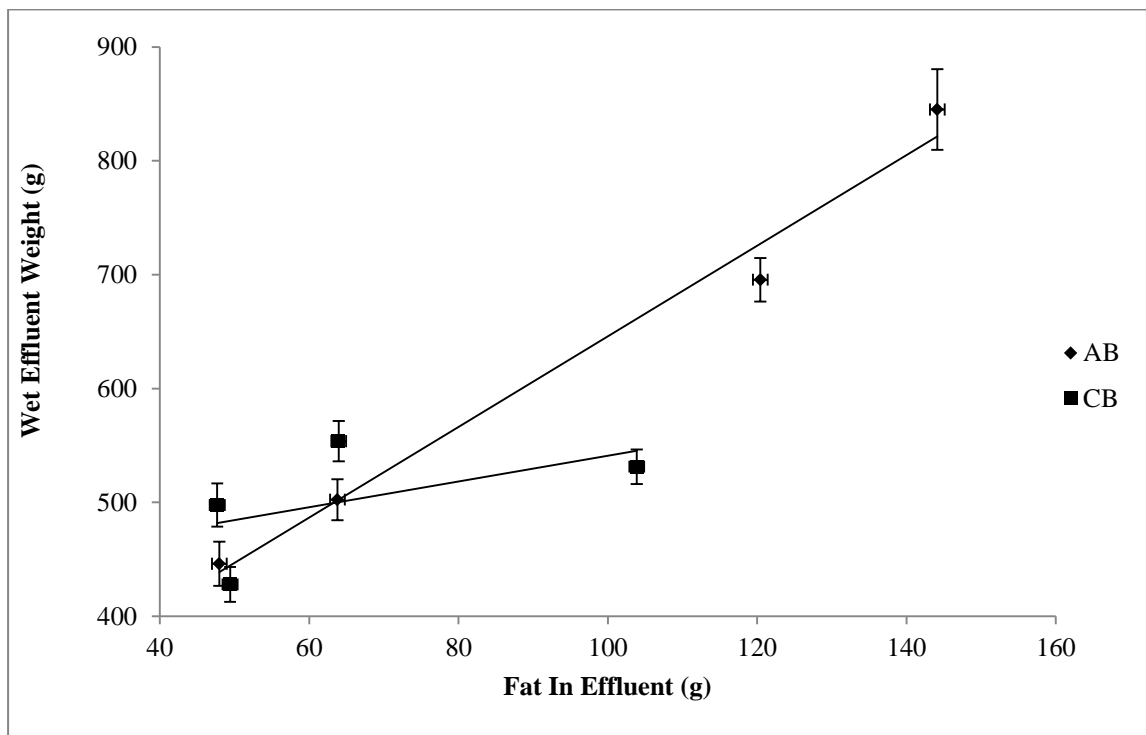
The baseline samples were removed from both the control and alginate bread as these samples would only contain digesta from the standardised meal, mucus and shed cells.. **Figure 37** illustrates the combined fat in effluent samples for subjects at the four time points, and the combined fat from effluent for all time points for alginate and control bread. There was more fat at time points 1, 3, 4 and the combined total fat weights when alginate bread was consumed when compared to the control bread. The amount of fat found in effluent sample when consuming alginate bread was 81 and 112g larger at 240-270-300 and total fat for combined time points respectively than the fat found in effluent samples when consuming control bread. A paired t-test revealed a significant increase in fat from effluent fluid at 240-270-300 minutes and total fat weights for combined time points ( $P<0.05$ ) (**Figure 37**). The paired samples t-test revealed no significant difference between fat from effluent samples at any other time point between alginate and control bread ( $P>0.05$ ). There was also a significantly larger AUC for the alginate bread when compared to the control bread ( $P<0.05$ ).



**Figure 37.** Total weights of fat in effluent for four time points and total fat weight from effluent combined for alginate and control bread. \* indicates at significant difference at  $P<0.05$ . (0-300 mins is the total fat weight from effluent for all four time points and for all volunteers) ( $n=29$ ). The number of effluent samples for was time point were 29, 25, 26 and 24 for AB and 26, 24, 22 and 23 for CB.

### 5.5.5 Wet Effluent weight and fat content from effluent

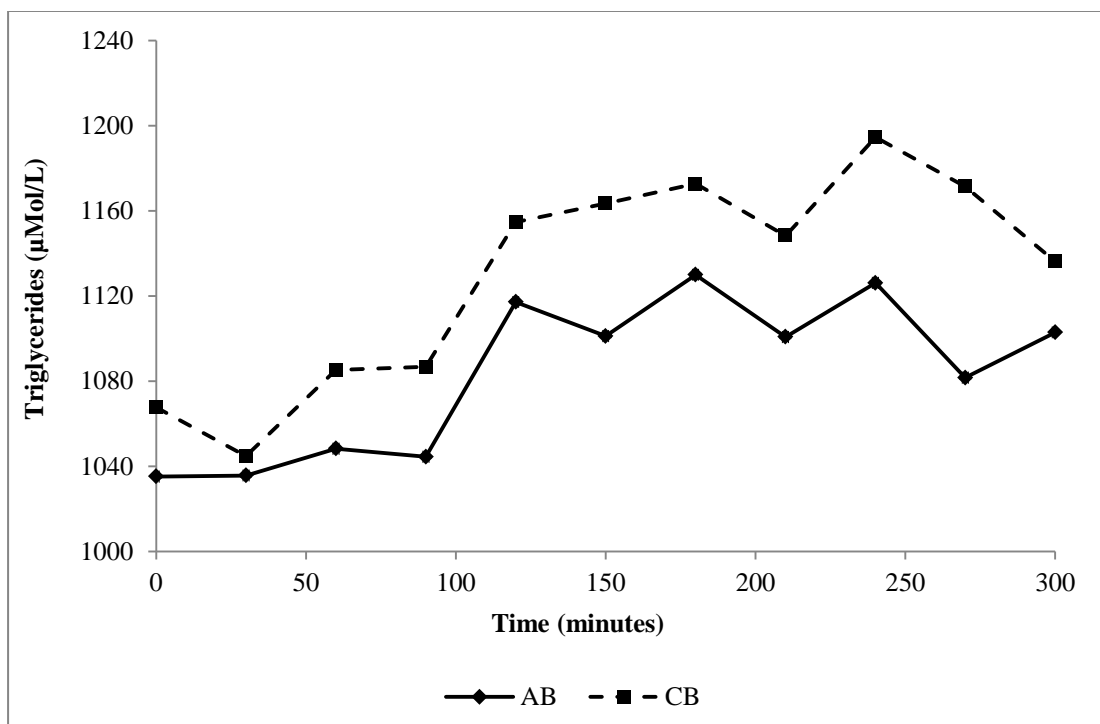
The data in **Figure 38** illustrates the relationship between wet effluent weight and fat extracted from effluent samples. A bivariate Pearson's correlation test revealed a significant relationship between wet effluent weight and fat in effluent samples when subjects consumed alginate bread ( $P < 0.05$ ), which was confirmed with an  $r$  of 0.99. The bivariate Pearson's correlation revealed no significant relationship between wet effluent weight and fat in effluent samples when subjects consumed control bread ( $P > 0.05$ ), which was also evident by the  $r$  of 0.53 (**Figure 38**).



**Figure 38.** Mean (+ S.E.M) relationship between wet effluent weight and fat from effluent for four time points for alginate bread ( $r = 0.98$ ) and control bread ( $R^2 = 0.28$ ) ( $n=29$ ).

### 5.5.6 Plasma Samples

Figure 39 illustrates total plasma triglycerides following ingestion of alginate and control bread. Baseline data was removed as this contained fasting plasma samples. Although there was less triglyceride in the alginate bread samples the one way repeated ANOVA showed no interaction between control and alginate bread, or time interaction ( $P < 0.05$ ). The ANOVA reports that there was no significant difference between the alginate and control bread at any time point, or the combined plasma triglycerides for all time points. The post-hoc bonferroni indicated that there was no significant differences between alginate and control bread at any time point ( $P > 0.05$ ). A paired t-test revealed no significant difference in AUC between alginate and control bread.



**Figure 39.** Mean (S.E.M) plasma triglycerides following consumption of alginate and control bread at 30 minute intervals (n=29).

## 5.6 Discussion

The data presented here indicates that bread enriched with alginate and additional fat is able to increase effluent weight and increase fat content in effluent samples in ileostomy patients. There was also a relationship between the increased wet effluent weight and the amount of fat found in effluent samples. In addition the alginate bread reduced the plasma triglycerides levels in ileostomy patients, although this was not significant. There were no substantial differences in palatability between the two breads, apart from one time point where subjects reported an increase in thirst and reduced fullness following consumption of alginate bread.

The incorporation of alginate into beverages has previously been shown to increase satiety and reduce the feeling of hunger (Jensen et al., 2012 (b); Paxman et al., 2008; Pelkman et al., 2007). The data from the Wellbeing Questionnaire used in the current study included a question on whether the subjects were full or starving (**Table 9**) and whether subjects were craving sweets (**Table 10**). There was no significant difference in the cravings for sweets between alginate and control bread. There was however a significant difference between the alginate and control bread, with regards to the feeling of fullness. In the current study subjects experienced a larger feeling of hunger 30 minutes after consuming the alginate compared to the control bread. These findings contradict previous work that has reported a significant increase in satiety and a reduction in hunger (Jensen et al., 2012 (b); Paxman et al., 2008; Pelkman et al., 2007).

Furthermore the findings of Pelkman et al. (2007) and Paxman et al. (2008) also reported that this increase in satiety and reduction in hunger was matched with an average reduced kcal consumption of 138 per day (Paxman et al., 2008). The previously mentioned studies all used a beverage as a delivery vehicle. However the current study and the work of Mattes (2007) and Williams et al. (2004) used food as a delivery vehicle, with both the afore mentioned authors in accordance with the current study reporting no significant difference in appetite or food intake. One potential explanation for this may have been the presence of the alginate in the bread, and its ability to alter digestion of the bread. If the control bread was digested quickly it would resemble a watery chyme as the bread is broken down in the stomach creating a large volume within the stomach. Data from the current laboratory has been shown that alginates are able to reduce the activity of pepsin (Strugala et al., 2005; Sunderland et al., 2000). If the activity of pepsin is reduced in the presence of the alginate bread then the gluten that holds the bread together may not be digested as quickly as normal. If this is the case then the alginate bread will resemble



more of a solid chyme and thus may not provide the same volume as the control bread in the stomach. This may ultimately affect stretch receptors, which have been linked with the feeling of fullness. Although this has not been confirmed in the current thesis further work is required to ascertain whether alginate bread is able to attenuate the activity of pepsin in a model gut.

The use of the visual analogue scale (VAS) used here has been validated as a repeatable and valid method to quantify any changes in an individual's feelings regarding a specific question (Aitken et al., 1969). There are however questions marks over the sensitivity of the VAS and whether it can highlight any significant differences between people's feelings (McCormack, Horne, & Sheather, 1988). Hornblow and Kidson (1976) reported that although the VAS may be user friendly and repeatable, there may also be problems with a clumping effect of responses. For example subjects may either put their answers in the middle or at one end of the scale. This appeared to be the case for the questions of whether subjects were full or thirsty. For example after an overnight fast the anticipation would be that subjects would be starving, however the mean answer for whether the subjects were starving was only 4.8. However, when looking at the raw data there were a number of subjects that gave a response of between 8 and 9. Although there was a clear clumping of the responses, as evident in the mean, the subjects who gave a response nearer to 10 would strongly influence the SD. The same response was evident in the thirst question with the majority of subjects giving a mean answer of 2.3. However, a number of subjects gave a much larger answer, which is surprising considering all subjects consumed the same food and drink. This clumping may have been the cause for the large variability in subjects response reported in the current study. This meant that reporting SD in **Figure 34** and **Figure 35** was difficult, and the reason for reporting SEM, despite the small size of this. An additional reason for the clumping effect may have been a poor explanation of the marking scheme, or subject's failure to remember the instructions given. An alternative option may be to shorten the scale from 0-10 down to 0-5. This would make the scale smaller and would potentially be more sensitive as the subjects only have a small scale to mark a response on and potentially increase the sensitivity of the scale. Despite what the scale is that is used the emphasis must be on a detailed and repeated explanation of the marking criteria for the VAS in future work to ensure understanding from the subjects.

DiMeglio and Mattes (2000) conducted a study over 4 weeks and compared the effects of liquid and solid meals on fullness and energy intake. The authors reported that the solid

foods have a greater impact upon increasing fullness than beverages. As food may take longer to be homogenised into a chyme in the stomach it may stay there for longer, which may attenuate the ability of the stomach to empty and thus contribute to prolonging gastric distension. Prolonged gastric distension has also been linked with increasing fullness (Phillips & Powley, 1996), which has also been linked to a reduction in calories consumed (Paxman et al., 2008; Pelkman et al., 2007). The lack of response on the feeling of hunger reported here and by Mattes (2007) and Williams et al. (2004) do not support the suggestion that foods may increase satiety greater than beverages. Although this may be valid for beverages that do not contain alginate, it appears that the alginate could be modifying the suggestions of DiMeglio and Mattes (2000) that a solid food may increase the feeling of fullness more than a liquid meal. The authors reported that when consuming the liquid meals subjects had a significantly larger BMI and body weight after 4 weeks, compared with the solid meal. The authors attributed this increase in body weight and BMI to increase calorie consumption when consuming the liquid meal. The formation of a gel in the stomach as reported by Hoad et al. (2004) may give the alginate enriched beverage more of a food like consistency and this could reduce gastric emptying. The fact that there was not an increase in fullness in the present study may have been due to the inability of the alginate to form a viscous acid gel within the stomach. If the alginate is properly homogenised and cooked into the delivery vehicle this may delay the release of the alginate as reported earlier in this thesis. The main constituents of the bread used here and other alginate enriched foods would not be digested until the small intestines, and thus the alginate in the alginate bread would also be released there. If the alginate is not released until the small intestines, as suggested by earlier work in this thesis, there may be no increase in the volume within the stomach as a result of increased viscosity or gel formation, and thus no reduction in hunger was reported. These data suggest that alginate enriched foods may not be suitable if the purpose of the study is to promote satiety. However it must be noted that there were no significant differences in palatability between the alginate and control bread product as reported in alginate enriched beverages. Furthermore in the current study subjects only consumed two slices of toast with butter, however in previous studies such as Pelkman et al. (2007) and Paxman et al. (2008) subjects were given an alginate beverage in addition to breakfast, which makes a direct comparison on fullness with the present study difficult.

An additional reason for the increase in hunger at 30 minutes may have been due to the dimensions of the breads when consumed. Post defrosting and toasting the alginate bread

appeared to reduce in size, when compared to the control bread. Visual cues have been reported to play a significant role in the feeling of hunger. Leidy, Apolzan, Mattes, and Campbell (2010) fed 43 subjects meals over eight days that were similar in calorie content but differed in size and form, such as drink vs solids. The authors indicated that there was reduced satiety and increased hunger when subjects consumed beverages and the smaller portion sizes, despite the calorie content being similar. These findings confirm the suggestions that if the bread size is reduced post freezing and toasting then this may have affected the early response in hunger as reported here. Leidy et al. (2010) also went on to suggest that people already have a pre-conceived idea of what should or should not make them full just by looking at the food they are about to consume. Therefore if the alginate bread does reduce in size after defrosting and toasting then this may have an impact upon the subjects feeling of fullness. This is important when designing a vehicle for delivering alginate as the delivery vehicle should not be making an individual feel hungrier than when they consume a control vehicle. Although the findings from the current study may be merely an inconsistency within the data as beyond this time point there were no other significant differences in the feeling of hunger, this must still be taken into account when designing any future alginate delivery vehicles and trials to ensure the control and treatment vehicles are identical apart from the alginate.

An additional mechanism by which alginates may be used to treat obesity is their ability to alter the digestion and absorption of metabolizable nutrients (Englyst, Quigley, & Hudson, 1994). Although the exact mechanism by which this occurs is unknown there is evidence to suggest that alginates are able to attenuate the postprandial blood glucose response (Williams et al., 2004; Wolf et al., 2002) and fat digestion (Sandberg et al., 1994). The implications for this are substantial not only in reducing obesity but also diseases associated with obesity, such as diabetes. Salmeron et al. (1997) conducted a longitudinal study over six years and looked at the diet of over 46,000 subjects, and how this may link with type-2 diabetes. They reported that individuals who consume large amounts of high glycaemic, easily digestible carbohydrates were almost six times more likely to develop type-2 diabetes. In addition Jenkins, Storlien, Chisholm, and Kraegen (1988) reported that diets high in saturated fats may alter membrane structure. Any alteration in cell membrane structure has been suggested to attenuate the cells insulin receptor activity, and therefore contribute to an increased risk of type-2 diabetes. A potentially more obvious effect of diets high in fat may be due to their high calorific content. One gram of fat contains 9 Kcal (37kJ) compared with 4 Kcal (16kJ) in 1 gram

of protein and carbohydrates (Horowitz & Klein, 2000). An excess of fat consumption will ultimately outweigh energy expenditure and the excess calories will then be stored as adipose tissue. Increased adipose tissue is closely associated with an increased BMI, elevated levels of cholesterol, and raises the risk of developing type 2 diabetes (Gostynski et al., 2004). Although both the increased consumption of fat and carbohydrates may contribute to the increased incidence of obesity worldwide, the current study concentrated on the effect of alginate enriched bread on fat digestion within ileostomy patients.

The data from the current study indicated that when subjects consumed the alginate enriched bread there was a significant increase in the amount of fat in the effluent fluid when compared with when subjects consumed the control bread. More fat was recovered in the effluent fluid at all four time points when subjects consumed alginate bread when compared with the control bread. However this was only significant at 240-270-300 minutes. There was an increase of 57% (81g) at 240-270-300 minutes between the alginate and control bread effluent fluid. In addition the AUC and combined fat for all four time points was significantly larger when alginate enriched bread was consumed compared to the control bread. The total weight of the four time points combined was 264g for the control bread, compared with 376g for the alginate bread, which was a difference of 30%. These results were consistent with the work of Sandberg et al. (1994), who conducted a two week study with six subjects and fed them a constant low fibre diet for four days with 7.5g of alginate with each meal, followed by a wash out period and then four days of a low fibre diet alone. The authors reported that over the four days when subjects consumed alginate there was an increase of 140% in the fat content recovered in the effluent fluid. Although this increase was substantially more than the current study, it must be noted that the Sandberg et al. (1994) study was conducted over four days and administered a larger amount than here. Furthermore two of the subjects used by Sandberg et al. (1994) had substantially larger FFA concentration in effluent fluid than the remaining 4, and because of the small sample used this may have biased the results. In addition to alginate other dietary fibres have elicited a similar response in fat excretion. Both Bosaeus, Carlsson, Sandberg, and Andersson (1986) and Higham and Read (1992) administered subjects 15g of pectin and guar gum respectively and observed the effects on fat content found in effluent fluid. Both studies reported a significant increase in fat content in effluent fluid which supports the data in the current study although both studies used a substantially more than the 4g of dietary fibre used in the current study. Interestingly Higham and Read (1992) reported a significant reduction in viscosity of the

effluent fluid when subjects consumed the guar gum. This was also accompanied by an increased weight of wet effluent fluid and a reduced transit time through the upper GI tract. Despite the small sample sizes and larger quantities of dietary fibre these data support the findings of the current study. These data combined would seem to suggest that the presence of alginate and other dietary fibres within the upper GI tract can reduce the absorption of fat.

Previous data from this laboratory and previously presented data in this thesis suggest that alginates possess the ability to attenuate the activity of pancreatic lipase (Wilcox et al., 2013), and therefore reduce fat absorption. Although the previous data suggests that the alginate is reducing fat digestion, this may not be the case in clinical studies. Although Sandberg et al. (1994) reported a significant increase in fat content in effluent fluid, the majority of this was FFA as confirmed by high performance liquid chromatography. These findings may not support the work from this laboratory that suggest alginates are attenuating digestive enzymes, but potentially encapsulating the digested FFA, and thus reducing the absorption potential of the small intestines. In the same study Sandberg et al. (1994) reported a significant increase in effluent weight, which may support the encapsulation of FFA, as the additional weight in part may be due to the increased FFA. The data from the current study lends some support to this idea as there was an increase in fat in the effluent fluid. However the methods used here did not distinguish between tri, di, monoglycerides or FFA. It is therefore difficult to conclude that this was the mechanism for the increased fat found in the effluent fluid in the current study. The potential of encapsulation of digestible nutrients is a feasible idea but further work is required to substantiate this suggestion.

Although encapsulation of digested nutrients may be a feasible explanation for the increased fat found in the current study there may be other mechanisms involved. An additional mechanism which may have accounted for the increased fat in the effluent fluid reported here could have been as a result of a reduced transit time. A review by Brownlee (2009) indicated that as the dietary fibre passes through the upper GI tract undigested it may add to luminal bulk. If the luminal bulk is increased the mechanoreceptors within the upper GI tract will respond by increasing muscular contractions and move the digesta through the small intestines at an accelerated rate. This increase in luminal bulk has been suggested to be due to the water binding capacity of the dietary fibre (Chaplin, 2003). The data from the current study reported a significant 35% increase in the wet effluent weight at 240-270-300 minutes, and although not significant

there was also an increase in the total combined wet effluent weight when alginate bread was consumed compared to the control bread. The total effluent weight for the combined time points was 20% larger in the alginate bread when compared to the control bread. The mean wet effluent weight for each subject consuming alginate bread was 85g (2489g/29 subjects), which is consistent with previous studies observing the effects of a range of dietary fibres 106g (Sandberg et al., 1994), 76g (Sandberg et al., 1981). The data from the current study support the suggestions of Brownlee (2009) and Chaplin (2003) that dietary fibre may bind with water and other constituents within the upper GI tract and thus increase faecal weight.

An additional point to consider is that fat in effluent of ileostomy patients should be minimal in a normal diet as it should have been digested and absorbed, therefore some of the difference in wet effluent weight could be accounted for by the presence of alginate. Furthermore dietary fibres have been shown to bind with large amounts of water (Chaplin, 2003) and therefore this may also have contributed to the wet weight of the effluent fluid, although it is difficult to ascertain whether the amount of alginate used can account for the total difference in weight. A comparison of wet and dry weight would be beneficial in future work, which would take the additional weight from water content out of the equation. This would therefore mean that any additional weight could be attributed to an additional fat content and dry alginate in the effluent fluid. Contrastingly (Mattes, 2007) reported no difference in bowel movements when administering an alginate enriched cereal bar. Similar to the current study Mattes (2007) used food as a delivery vehicle and a similar sample size. Although Mattes (2007) reported that there was no significant difference between the alginate and control bar in alteration of bowel movements the assessment of gastric motility used was subjective. They used questionnaires, rather than any direct assessment such as wet effluent weight used in the present study. Additionally the authors only administered a cereal bar of 55g which is nearly half the amount of bread administered here. The small cereal bar means that this would be well dispersed and therefore may not have had any significant impact upon gastric movements. The authors also used a combination of alginate (1.1g) and guar gum (3.9g). The amount of alginate used was less than in the current study, suggesting 1.1g of alginate may not have been sufficient to elicit a response. The use of guar gum may also have contributed as the ability of guar gum to both inhibit digestive enzymes and form a viscous solution and alter the digestion process is questionable (Higham & Read, 1992). Although the current study reports an increase in wet effluent weight, this study and

previous studies that have reported an increase in faecal weight such as Sandberg et al. (1994) would only be able to confirm a decreased transit time by measuring wet effluent weight for longer time periods after ingestion of the meal/beverage. If in fact the alginate caused a decreased transit time as suggested previously then the effluent weight of control bread would eventually be larger than the alginate bread if later time points were collected, as it would take longer to pass through the upper GI tract, but this was not confirmed in the current study.

A decreased transit time in the upper GI tract and an encapsulation of digestible nutrients may very well be enough to account for the increased fat in the effluent fluid alone, but it may be more realistic that it is a combination of the two mechanisms. If the fats consumed by the subjects here and in Sandberg et al. (1994) study were in fact digested, then the expected result would be for them to be absorbed as monoglyceride and FFA (Carey et al., 1983), although this does not appear to be the case. The increased fat in effluent fluid in the current study was closely related to an increase in wet effluent weight. A similar relationship was reported by Sandberg et al. (1994), who also reported an increased fat content in effluent fluid with an increase in wet effluent weight. The relationship between effluent fat and wet effluent weight provides evidence to support the idea of encapsulation of fat and a decreased transit time in the upper GI tract. If the transit time alone was reduced then the small intestines would still be expected to be able to absorb the fat that subjects consumed. Therefore a potential encapsulation may also have contributed to the increased fat in effluent weight reported here. Lin, Zhao, Chu, Lin, and Wang (1997) contradicted the suggestion that dietary fibre decreases transit time. The authors suggested that when the sensory receptors within the small intestine are exposed to a meal with a large nutritional content, the movement through the small intestines is reduced, which has been defined as the ileal break. The ileal break is essentially an action of the small intestines which reduces movement through the small intestine when undigested nutrients, specifically triglycerides are sensed in the ileum. Marciani et al. (2001) supported the findings of Lin et al. (1997) and indicated that movement through the upper GI tract is due to the nutritional content of the meal and not the viscosity of the digesta. They fed twelve healthy volunteers four 500 ml beverages ranging in viscosity and nutrient content over a 4 day period. Using MRI to monitor movement through the upper GI tract the authors reported that both nutrient and viscosity make-up of the beverages reduced movement through the upper GI tract. However when viscosity and nutrient content were compared the beverages that contained a larger nutritional content

resulted in a more substantial reduction in movement through the upper GI tract when compared with viscosity alone. Therefore if the meal consumed in the present study had more fat present by the time the digesta reached the ileum then the expected result would be a decreased movement through the small intestines, and a smaller effluent weight when subjects consumed alginate bread. The increased wet effluent weight and fat content in effluent fluid supports the suggestion that alginate bread may be encapsulating the fat in the toast and butter. If, in fact, the alginate bread is encapsulating the fat, then the sensory receptors of the small intestines may not be able to sense the fat, and therefore movement through the small intestines would not be reduced as suggested by Marciani et al. (2001) and Lin et al. (1997). Although it remains to be seen if the amount of alginate used in the current study is enough to encapsulate the fat found in the effluent fluid, further in-vivo research is required to confirm if in fact this is the case.

A further point to consider is that movement through the upper GI tract determines the digestion rates of nutrients and the control of movement of digesta through peristalsis, which ensures complete digestion and absorption of the digesta (Brownlee, Forster, et al., 2010). If in fact the transit through the small intestines is reduced, as suggested in the present study, potentially due to encapsulation of fat and an increase in digesta bulk then this alone may account for the increased fat in the effluent fluid. The work of Jensen et al. (2012 (a)) suggests that the viscosity of an alginate enriched product may also increase the bulk of the chyme entering the small intestine from the stomach. This increased bulking of the chyme entering the small intestine has been suggested to result in a thickening to the unstirred water layer at the luminal surface of the small intestine, and thus reduce the removal of nutrients (Lin et al., 1997). If in fact the alginate bread increases the bulk of the chyme in the small intestine, then this may increase the unstirred water layer at the luminal surface, and ultimately a reduction in postprandial plasma triglyceride response. Although initial observation of the plasma triglycerides may suggest that alginate bread is attenuating absorption of triglycerides this was not significant. There was no significant difference in plasma triglycerides at any time points or AUC between alginate and control bread. These data confirm earlier work by Paxman et al. (2008) who reported no significant difference in plasma triglycerides when administering an alginate enriched beverage compared to a control beverage.

In a more recent study by Jensen et al. (2012 (a)) they assessed the ability of an alginate enriched beverage to alter the postprandial response to a meal in 96 obese individuals. The authors reported a slight reduction in plasma triglycerides, however this was not



significantly different between the alginate and control beverages. An interesting point of the study though was that they only compared the plasma triglycerides level to baselines after a 12 week intervention. Therefore if the study had assessed plasma triglycerides directly post consumption of the beverage and then for a number of hours post consumption of the meal they may have reported an affect. Furthermore it is difficult to ascertain whether fat digestion was truly affected in either the Paxman et al. (2008) or Jensen et al. (2012 (a)) studies because there were no faecal or effluent samples taken. An additional point of interest to add is that the fat content of the two breads in the current study were not identical. The fat content of the alginate bread was 14.1g/100g compared to 1.7g/100g, which is substantially larger. The purpose of the study was to compare two breads that were matched for calorific content, apart from the addition of alginate, which was the information given to Greggs Plc. The breads were designed by Greggs Plc master baker to ensure the bread was of a high standard. During this process the master baker incorporated unwanted additional fat to the alginate bread. Although it is unclear why the alginate bread was produced with extra fat, one possibility is that the alginate absorbed the water in the bread making process and therefore dried out the bread. Therefore if a fat in liquid form was added this may give the bread a more doughy and moist texture and therefore increase palatability. Although there was considerably more fat in the alginate bread the plasma triglyceride levels were lower when subjects consumed alginate bread compared to the control bread, suggesting that alginate attenuated fat absorption. In addition it cannot be excluded that the additional fat in the alginate bread may have impacted upon the increased wet weight in the effluent fluid and the increased fat content in effluent fluid. Even though this may be the case, the fact of the matter remains that if alginate was not present in the bread the additional fat in the bread should have been digested and absorbed. Particularly as the subjects were in a fasting state when given the test meal. This therefore suggests that the alginate bread did have an impact upon fat digestion. Consequently if the two breads were matched for fat content then the anticipation may have been a significant reduction in plasma triglycerides, which would correlate with an increase in fat content in effluent samples. Any future studies must ensure that the calorific content and the fat content of the alginate and control vehicles are identical, other than alginate content.

Although the data presented here does not conclusively prove that alginate enriched bread are able to attenuate the plasma triglyceride response, there is evidence from the literature that indicates alginate enriched products may be able to attenuate the blood glucose and

insulin response following a meal. Alginate enriched products including beverages (Paxman et al., 2008; Torsdottir et al., 1991; Wolf et al., 2002) and cereal bars (Williams et al., 2004) have reported significant reductions in both glucose and insulin response. In contrast more recent work by Jensen et al. (2012 (a)); (Jensen et al., 2012 (b)) reported no significant difference in blood glucose or insulin response. A potential reason for the lack of effect reported by these authors could have been due to the large amount of alginate and the composition of the alginate used. A large concentration made up of 65-70% guluronic acid would result in a much more rigid gel (Draget et al., 1997), and thus a clumping effect may have occurred. This clumping effect may have reduced the interaction with digesta in the upper GI tract, and thus no reduction in blood glucose or insulin response was reported. The question however remains as to why in the previously mentioned studies alginate enriched products resulted in a blunting of the glucose and insulin response, however no significant reductions in plasma triglycerides were reported in this thesis. This may have been due to the digestion process of carbohydrates compared to triglycerides. Carbohydrates and short/medium chain triglycerides are generally digested and absorbed earlier than long chain triglycerides (Bernard & Carlier, 1991; Blomstrand, 1955).

The plasma triglyceride response reported here is consistent with the plasma response of previous research following ingestion of a meal which included fat (Daly et al., 1998; Sasahara, Burns, Miyashita, & Stensel, 2012). In the current study there was a small spike prior to 90 minutes which is consistent with the afore mentioned studies. However, beyond this point the increase in plasma triglycerides was not to the same extent as Sasahara et al. (2012). The authors administered subjects with a meal containing butter, but did not mention the total amount which makes it difficult to compare with the current study. Despite this the authors reported an increase of 50% in plasma triglycerides between 0 and 120 minutes. Contrastingly in the current study and the work of Daly et al. (1998) the increase was only 8 and 12% respectively between 0-120 minutes. The small rise of 8% reported here in the alginate bread samples may suggest a reduction in absorption, however the control bread only increased by 10% over the same time period. Although the exact amounts of short, medium and long chain triglycerides in butter are unknown, Fallon and Enig (2000) indicated that most butter contains 12-15% short or medium chain triglycerides. The remaining 85-88% must be made up of long chain saturated triglycerides, which allows the butter to become a solid. The amount of short and medium chain triglycerides would be much lower, but is also important to ensure that

when the butter is spread on bread or toast that it is able to be spread and does not remain a solid. The composition of the butter may have had an impact upon the small peak in plasma triglyceride reported here beyond 90 minutes may have been due to the absorption of short and medium triglycerides which do not require the same level of digestion, and thus less fat was absorbed. Furthermore these saturated long chain triglycerides would enter the small intestine as a coarse emulsion created by acid and contractions within the stomach. Bile salts are then released from the liver, which are detergents with polar side chains which allow them to interact with both water and lipids, and therefore create water soluble droplets. If dietary fibre alters this process in any way then the digestion of fat may be affected.

There is currently no research which has observed the Michaelis-Menten kinetics ( $K_m$ ) of pancreatic lipase with a range of triglycerides, including short, medium and long chain triglycerides. If the medium and short chain triglycerides have a smaller  $K_m$  than long chain triglycerides then this would mean that they may be digested faster and therefore absorbed quicker. Another potential explanation for the increased absorbance of the short and medium chain triglycerides may be because of the solubility of them. As previously mentioned Smits et al. (1968) indicated that bile salts and micelle formation was not crucial for digestion of short and medium chain triglycerides. Essentially if short and medium chain triglycerides do not require the same lengthy digestion process involved with long chain triglycerides, then pancreatic lipase can begin digestion earlier.

Furthermore Camire and Dougherty (2003) reported that dietary fibres are able to bind with bile and therefore reduce their interaction with triglycerides. Any reduction in bile function would ultimately reduce the digestion of triglycerides, and may have contributed towards the increased fat in the effluent fluid here, but no major difference in plasma triglycerides. Although the increased fat found in the effluent fluid does suggest that the alginate enriched bread may have reduced fat digestion, however the blood response does not confirm this. Although both the  $K_m$ , solubility of the medium and short chain triglycerides and binding of bile salts may explain the earlier appearance in the blood of medium and short chain triglycerides, an additional reason may be the manner in which they enter the blood stream. Due to the size of the long chain triglycerides they are too big to cross the epithelial cells of the capillaries and therefore must be reformed as chylomicrons and enter the lymphatic system. They then travel through the lymphatic system and enter the blood stream at the subclavian vein via the thoracic duct. Short and medium chain triglycerides are able to be absorbed directly into the portal vein and will

therefore appear in the blood earlier than long chain triglycerides. Any future work observing the effects of alginate on fat digestion must consider the fat source used in the study to ensure a larger peak in plasma triglycerides.

An additional explanation for the lack of difference between the plasma triglyceride levels for the control and alginate bread may have been due to the “second meal response” (Lambert & Parks, 2012). The second meal response suggests that triglycerides that are consumed during an initial meal are not completely absorbed into the bloods to be used by the body, but rather stored in enterocyte cells. Following the consumption of a second meal these triglycerides are then reformed into chylomicrons before passing through the liver and into the blood. Robertson, Henderson, Vist, and Rumsey (2002) reported that the increase in plasma triglycerides generally begins after 10-30 minutes of meal consumption, with the peak rise in plasma triglycerides occurring 3-5 hours later. Therefore the early rise in triglycerides that appear in chylomicrons occurs before the meal containing fat can be digested and absorbed. This was also supported by Chavez-Jauregui, Mattes, and Parks (2010) who used stable isotopes to confirm that up to 12% of the triglycerides contained within the chylomicrons appeared 15-20 minutes following meal ingestion, and therefore could not have been from the meal subjects had just consumed. Heath et al. (2006) observed the effects of meal at breakfast and one at lunch where the fats were labelled. The authors reported a normal peak after each meal which began to return back to normal, however following lunch the peak in plasma triglycerides was larger than the peak at breakfast despite matching for calorific content. These data support the suggestion that consumption of subsequent meals results in larger concentration of triglycerides within chylomicrons than that which would occur following the first meal consumed. The “second meal response” may have been the cause for the blunting of the plasma triglycerides response reported here in both control and alginate bread samples. Data from Lambert and Parks (2012) would suggest that the plasma triglyceride response reported here may have been from the standardised meal consumed the evening before and not from the toast and butter. Therefore if an additional meal had been consumed by both groups three hours after the toast which was matched for calorific content as the toast and butter, then the anticipated result would be a lower plasma triglyceride response in the alginate bread samples. This would correspond with the increased fat content of the effluent samples, and support the data here that fat digestion was reduced when subjects consumed alginate bread. An additional point of interest was promoted by Robertson et al. (2002), who demonstrated that carbohydrates and fat can

adversely influence each other's metabolism after a meal. Therefore the subjects in the Sasahara et al. (2012) study consumed a meal high in fat then this may explain the large increase in plasma triglycerides compared with the current study. Subjects in the current study consumed a standardised meal containing 47g of carbohydrates and only 12g of fat, which may have resulted in the muted plasma triglyceride response as suggested by Robertson et al. (2002). Although here a number of suggestions have been proposed for the small rise in the plasma triglycerides it is difficult to draw strong conclusions from the current study because of the different fat contents of the two breads.

The data in the present study reports an increase in wet effluent weight and fat content when consuming the alginate bread. However there was no significant attenuation in the plasma triglyceride levels or hunger. Although here there was no reduction in hunger the test meals given were small with just two slices of toast given, which may not have been enough to reduce the feeling of hunger, as portion size has been related to hunger (Leidy et al., 2010). A potentially important result of the current study was that there were no significant adverse side effects of the alginate enriched bread. A minor effect was that subjects were more thirsty 60 minutes after consuming the alginate bread than when compared to the control bread. Thirst is the craving for fluids which can be brought about by the loss of water. The alginate that is present in the bread is sodium alginate which may have resulted in the sensation of thirst. Although the sodium is bound to the alginate some of the sodium may be absorbed and increase osmotic pressure of the plasma. This can initiate the cravings on thirst and make people drink. An additional point of interest is that soluble dietary fibres possess the ability to retain large amounts of water (Chaplin, 2003) which may also contribute to the increased thirst. Beyond this time point there were no significant differences in thirst. Despite this small increase in thirst there were no other adverse effects, which have previously been reported when consuming alginate enriched products. Adverse effects have previously included products being returned (Sandberg et al., 1994), burping, nausea (Wolf et al., 2002), flatulence, stomach ache (Pelkman et al., 2007) and subjects preferring the control products over alginate products due to poor palatability (Jensen et al., 2012 (a); Jensen et al., 2012 (b); Mattes, 2007). The previously mentioned studies all use varying amounts of alginate ranging from 1.1g up to 15g, which suggests that the amount of alginate may not have been the main factor contributing to the adverse side effects mentioned. All of these studies, except Mattes (2007) used a drink as a delivery vehicle, suggesting that the delivery vehicle may be the main contributing factor. Mattes (2007) used a cereal bar and the main problem reported by the author was a

poor taste between the alginate and control bar, but no gastrointestinal adverse side effects. These data indicate that if the alginate is not mixed thoroughly within a delivery vehicle then this may have an impact upon adverse side effects in the upper GI tract. Although the adverse effects of these alternative alginate enriched products may not be severe, they will all contribute to poor adherence, and subjects are less likely to consume products that have adverse side effects and poor palatability (Ellis et al., 1981).

This is the first pilot study to date that has assessed the effects of an alginate enriched product on fat digestion *in-vivo*. The data in the current study suggests that alginate enriched products are able to attenuate the digestion of fat, yet a direct comparison between the current study and previous work is difficult due to the variance in methodologies. For example the sample size and subjects observed, various amounts and composition of alginate used, delivery vehicles used and varying meals consumed post consumption of alginate enriched product. The addition of alginate to bread in the current study increased effluent weight and fat content. The findings from the current study support a food vehicle as a delivery method for alginate in reducing adverse effects associated with alginate enriched products. There is only one longitudinal study to date that has reported weight loss with an alginate supplemented product. Therefore it is too early to make conclusions on whether alginate enriched bread may be able to aid in weight loss, however further longitudinal studies in healthy populations will answer this question. Future research must observe the effects of an alginate enriched product of equal fat content between test meals on healthy subjects in order for any health claim to be associated with alginate.

## Chapter 6 Overall Conclusion

### 6.1 Alginate Quantification

Alginates are extracted from the cell walls of a number of brown seaweed species (Brownlee et al., 2005) using a purification step involving an acid treatment that precipitates alginate as alginic acid (Draget et al., 1997). Although used within a number of industries, alginate has received considerable interest specifically over the last 10-15 years for its beneficial physiological effects *in-vivo*; such as a reduced postprandial insulin response, reduced fat digestion, increased satiety and reduce absorption of nutrients consumed (Jenkins et al., 1978; Paxman et al., 2008; Sandberg et al., 1994; Torsdottir et al., 1991). Furthermore *in-vitro* work from this laboratory has demonstrated the ability of a wide range of alginates varying in  $M_r$  and composition to attenuate the activity of proteolytic enzymes by up to 85% (Strugala et al., 2005; Sunderland et al., 2000; Wilcox et al., 2013)

In combination with the beneficial physiological effects reported from adding alginate to a food or beverage and the work from this laboratory, these data suggest that alginate has the potential to be used as an obesity treatment. If alginate is to be used as an obesity treatment it must be incorporated with minimum effort into an individual's normal diet. Although the addition of alginate to a food or beverages has the potential to treat obesity, there is one common problem which has plagued alginate enriched products, namely palatability. The ability of alginate to form both ionic and acidic gels makes alginates unique (Draget et al., 1997; Smidsrod, 1974). However it is the gel formation that has contributed to the problem of poor palatability and the addition of alginate to a food or beverage has caused a slimy mouth feel of the product and poor taste of these products. Consequently, despite the potential beneficial effects of alginate enriched products previously discussed the poor palatability makes them difficult to incorporate into a day to day diet. The alginate enriched bread used here removed the problem of poor palatability, as subjects actually preferred the alginate enriched bread compared to the control bread during the ileostomy study.

In addition to producing a product which is palatable enough that subjects may consume it on a daily basis, there is no *in-vivo* or *in-vitro* study to date that has reported the release rate of alginate from the enriched products. The work of Hoad et al. (2004) demonstrated that alginate gels in the stomach, yet no assessment was made on the ability of the alginate to form a gel within the small intestines. The small intestines is where the

majority of macronutrients are digested and absorbed, therefore if alginate is to attenuate the digestion and absorption in the upper GI tract the release rate of alginate throughout the whole of the upper GI tract from a delivery vehicle must be determined.

Current techniques to quantify alginate are lengthy processes, requiring extensive sample preparation, and are unable to work throughout a range of conditions that would be present during *in-vivo* digestion. The work of Richardson et al. (2004) developed a method using DMMB and suggested that this method was suitable for quantifying alginate. The data provided here does not support the use of DMMB or a number of other dyes for quantifying alginate in a solution or in samples taken from a synthetic model gut, which could potentially be due to low positive charge density of the dyes.

The modified PAS assay is able to quantify alginate in  $DH_2O$ , with excellent sensitivity and linearity (0.5mg/ml and  $R^2$  0.99, respectively). Furthermore the PAS assay was able to quantify alginate in samples taken from the model gut once interference was removed. The PAS assay also allowed the release rate of alginate from a bread vehicle to be determined during digestion in the model gut. The data indicate that between 80-90% of the alginate is being released in the small intestinal phase of digestion. The quantification of alginate released from delivery vehicle is essential if alginate is to be used as an obesity treatment. These data correlate with the digestion of carbohydrates and fats within the diet, which are the main constituents of the bread. Although the release of the alginate in the small intestine is not crucial, these data confirm that alginate is released in the small intestines where between 85-90% of triglycerides are digested and absorbed. The PAS assay is a suitable method for quantifying alginate in a solution and for determining the release rate from a delivery vehicle.



## 6.2 Biological Activity of Alginate

Alginates have been reported to be able to pass through the upper GI tract undigested, and thus resemble characteristics of dietary fibre, which can be loosely defined as “unavailable plant material, which is not digested or absorbed in the upper GI tract” (Asp, 1987). The ability of alginate to evade digestion has been reported *in-vivo* by Sandberg et al. (1994) who recovered 96% of uronic acids within the effluent fluid from ileostomy patients, and thus further support the classification of alginate as a dietary fibre. *In-vitro* work presented here demonstrates that alginate is recovered in the model gut simulating digestion in the upper GI tract, and further demonstrates the robustness of alginate.

Although alginate appears to be able to pass through the upper GI tract undigested, there is evidence that suggests when alginates are exposed to temperatures in excess of 100°C they are broken down (McDowell, 1977). If the alginate structure is affected during cooking of the bread, which can reach temperatures in excess of 180°C, then this may affect the ability of the alginates to form gels or attenuate the activity of digestive enzymes.

The evidence presented here in chapter 3 indicates that although alginate is being released, there is no substantial increase in viscosity, as would be expected when alginate is exposed to a range of pH's in the upper GI tract (Draget et al., 2005; Hoad et al., 2004). Furthermore the heating of alginate confirms that temperatures in excess of 100°C may alter the structure of alginate as suggested by McDowell (1977), and therefore reduce the ability of alginate to form viscous gels. The ability of alginate to form viscous gels in both the stomach (Hoad et al., 2004) and small intestines (Seal & Mathers, 2001) has been suggested as a potential mechanism responsible for a reduction in nutrient digestion and absorption. Therefore if the alginate bread is unable to increase the viscosity within the upper GI tract, the expectation may be that alginate bread is unable to alter the digestion and absorption process of macronutrients, specifically fat.

Although there was a small increase in viscosity at the end of the end of the model gut when all of the alginate has been released this does not correspond with the viscosity that would be expected. Despite the small increase in viscosity the data presented here demonstrate that viscosity may not be the determining factor contributing to a reduction in enzyme activity. The alginate released from the bread as reported here has been exposed to cooking into the bread, digestion in the model gut and an isolation process, yet it still retained the same level of inhibition as alginate that had not undergone any of the

aforementioned processes. Furthermore when dry alginate was exposed to temperatures up to 180°C the alginate retained an ability to inhibit pancreatic lipase activity. Although at 180°C the level of inhibition was low compared with alginate heated at 37°C. The level of inhibition remained relatively consistent up to 150°C when compared with alginate heated at 37°C, however it then falls off up to 180°C. These data suggest that viscosity may not be crucial in relation to the ability of alginate to inhibit pancreatic lipase.

These data provide evidence that alginate enriched bread may be a suitable delivery vehicle to treat obesity. Not only is alginate able to withstand a number of processes and still reduce the activity of pancreatic lipase, but the lack of viscosity as reported here suggests that poor palatability due to slimy mouth feel of other alginate enriched products (Ellis et al., 1981) may not be a problem. The data from the ileostomy study confirms this with subjects preferring the alginate bread to the control bread. In addition these findings suggest that viscosity may not have a substantial bearing on the digestion and absorption of macronutrients in the small intestines, and suggest that another mechanism is responsible for the attenuated activity of pancreatic lipase activity.

### 6.3 Triglyceride Substrate and Food Inhibition by Alginate Bread

The *in-vitro* data presented by Richardson et al. (2011) and Wilcox et al. (2014) indicate that dry alginate is able to attenuate the activity of pancreatic lipase in an olive oil turbidity assay. In addition using the same olive oil turbidity assay the isolated alginate from the digested bread retains its inhibition ability despite being cooked into the bread, digested in the model gut and isolated. Although these findings do support the use of alginate as an obesity treatment, the olive oil turbidity assay does not take into account the plethora of variables within the model gut and *in-vivo* digestion.

These data indicate that the model gut is a suitable model for simulating digestion in the upper GI tract and provides optimal conditions for digestion to occur. This was demonstrated by the pH reported here and also by the increase in glycerol released throughout the model gut when fat substrates were digested. This was further substantiated when two food sources high in fat content (butter and olive oil) were digested in a similar manner as the triglyceride substrates. Both the triglyceride substrates and foods demonstrated an increase in glycerol throughout the model gut, peaking in the small intestinal phase. The majority of glycerol was released in the small intestine, which is consistent with normal *in-vivo* digestion of triglycerides (Carey et al., 1983). This release of glycerol is harmonious with the release of alginate from the bread as described earlier.

As previously mentioned alginate bread removes the issue of poor palatability as described with previous alginate enriched products and is able to reduce pancreatic lipase activity in a strictly controlled olive oil turbidity assay. The data here demonstrates that alginate enriched bread is able to reduce the amount of glycerol released during triglyceride substrate and food digestion. These levels ranged from 32-62% in triglyceride substrates and 79% and 90% for butter and olive oil, respectively. Furthermore there appears to be a greater inhibition of triglycerides that have a longer FA chain length. Although any inhibition of fat digestion using alginate enriched bread would be beneficial in treating obesity, a reduction in digestion of longer chain triglycerides would be a significant finding due to the high calorific content of these triglycerides and also the impact these triglycerides may have on obesity and diseases associated with obesity. Further research is required to ascertain whether alginate is able to inhibit fat digestion *in-vivo* and to assess whether alginate enriched products are able to inhibit the digestion of long chain triglycerides more than medium and short chain triglycerides in the model gut and *in-vivo*.

## 6.4 Clinical Implications

There is strong evidence that suggests alginate possesses the potential to be used as an obesity treatment. The data presented here support the use of bread as a delivery vehicle and something that individuals can incorporate into their diet on a regular basis. This easy incorporation into the current diet removes the adherence problems commonly associated with current obesity treatments (Ayyad & Andersen, 2000). Furthermore the previous *in-vitro* work from this laboratory and the data presented here indicate that alginate is robust enough to withstand a number of processes and still inhibit fat digestion on a 96-well plate and also in more realistic version of digestion, such as the model gut.

Ileostomy patients were used here due to the similarities between their digestive system and the synthetic model gut and ease of access to effluent samples. Ileostomy patients generally have normal digestion up until the end of the small intestine which corresponds well with the model gut. The data presented here demonstrate a substantial increase in effluent weight and fat in the effluent samples when subjects consumed the alginate bread. Furthermore there was a reduction in the plasma triglyceride concentrations, although this was not significant. These data indicate that regular consumption of an alginate enriched product with each meal may result in a reduction in the amount of fat digested. Two potentially important factors that were not taken into account in the current study were consistent levels of fat in the bread and the second meal theory. There was 12.4g more fat in the alginate bread compared to the control bread, which is substantially larger. Furthermore there is evidence to suggest that fat that appears in the blood immediately after a meal is not from the meal they have just consumed. The second meal theory indicates that fat is stored in the enterocyte cells and then released when more fat is digested and absorbed, and therefore the fat from the meal consumed will be stored in enterocytes cells ready for the following meal (Lambert & Parks, 2012). Both of these factors must be taken into account when considering future research. Even though the alginate bread had a larger fat content the plasma triglyceride levels were still lower when consuming alginate bread compared with the control bread. A further point of interest may be that the fat that appeared in the plasma of the ileostomy patients may have been from the standardised meal consumed the evening prior to testing. When taking into account these two potential factors there was still significantly more fat in the effluent fluid when subjects consumed the alginate bread, indicating that the alginate bread reduced the absorption of fat consumed.

Although these findings do demonstrate the ability of alginate to reduce fat digestion in ileostomy patients these findings must be confirmed in a healthy population group. If similar positive findings can be repeated in a healthy group of subjects then this will support any health claims that may be attributed to alginate and the potential for its use as an obesity treatment. A longitudinal study is also required assessing weight loss and observing the effects of a number of alginate meals to account for the second meal response.

## 6.5 Final Conclusion

The release rate of alginate from a bread vehicle during in-vitro digestion is determined here using a modified PAS assay. This assay reports that the majority of alginate is released in the small intestine phase of digestion. Furthermore alginate appears to evade digestion in the upper GI tract where it is able to attenuate the activity of pancreatic lipase. This was demonstrated by a reduction of glycerol released in the model gut when normal substrate and foods high in fat content were digested. Furthermore there was a small reduction in plasma triglyceride levels and a significant increase in effluent weight and fat content of effluent samples.

Although the alginate enriched bread appears to have an acute response in ileostomy patient's further work is required in healthy populations. The delivery vehicle selected here was bread, which did not have any significant adverse side effects and was palatable for subjects. Furthermore it appears that certain alginate enriched products may elicit certain physiological effects and depending on what the main aim of the study is will also determine the selected delivery vehicle. For example alginate beverages appear to increase satiety and reduce the blood insulin response, however alginate enriched bread did not seem to affect hunger levels, but reduced fat digestion. Future studies may want to compare a number of delivery vehicles for alginate that people are already consuming.

An additional concept would be to select the appropriate alginate as this appears to be one of the main issues when observing the effects of alginate enriched products. Strugala et al. (2005) demonstrated that alginates with a large M:G content has a larger inhibition of pepsin, and although Wilcox et al. (2013) does provide some evidence that alginates high in G content may be more effective at inhibiting pancreatic lipase. Therefore further research is required to determine which alginates are more effective at inhibiting pancreatic lipase. Furthermore, as well as the Mr and composition the amount of alginate used will also be crucial. Here only a small amount was used, however further work is required to assess if there is a dose response and also what the cut-off point is for the amount of alginate that can be added before adverse side effects are experienced, whilst maintaining the beneficial physiological responses reported here.

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