

**Determination of the effect of lyophilised kiwifruit on
digestion of protein: *in vitro* and *in vivo***

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Abbreviations

% ₀	parts per thousand
¹³ C	stable isotope of carbon
¹³ C _{VPDB}	carbon-13 expressed in relation to standard Pee Dee Belemnite
AUT	Auckland University of Technology
AUTEC	Auckland University of Technology Ethics Committee
BMI	body mass index
BMR	basal metabolic rate
C	control
CCK	cholecystokinin - GI hormone
CDR	cumulative dose recovered
CHO	carbohydrate
CO ₂	carbon dioxide
CT Scan	x-ray computed tomography
CV%	coefficient of variation expressed as a percentage value
CYP450	cytochrome P450 - hemoprotein enzyme
DOB	delta over base
EE	energy equivalent
FAO	Food and Agriculture Organisation
FBD	functional bowel disorder
FDM	freeze dried meat
FDR	fraction of dose recovered
GERD	gastro-oesophageal reflux disease
GI	gastrointestinal
<i>H. pylori</i>	<i>Helicobacter Pylori</i> bacteria
HCl	hydrochloric acid
IBS	irritable bowel syndrome

IRMS	isotope ratio mass spectrometer
IRU	isotope ratio value
k	terminal slope of a semi logarithmic plot of the dependent variable on the independent variable
kcal	kilo calorie
KFI	kiwifruit isolate - Zyactinase™
m	value of theoretical dose recovered at infinity
MRI	magnetic resonance imaging
N	nitrogen
n3	omega 3 poly unsaturated fatty acid
n6	omega 6 polyunsaturated fatty acid
NaHCO ₃	sodium bicarbonate
NaOH	sodium hydroxide
NDIRS	non-dispersive infrared spectrometry
OABT	octanoic acid breath test
P	probability
PAL	physical activity level
PDB	Pee Dee Belemnite
PG I/II	pepsinogen 1 and 2
pH	acidity or power of H ⁺ ions
PPIs	proton pump inhibitors
ppm	parts per million
R ²	coefficient of determination
SD	standard deviation
SEE	sedentary energy expended
T	treatment
TEE	total energy expended

$T_{1/2}$	time from ingestion until 50% of the biomarker is retained in the stomach
T_{lag}	the length of time from ingestion to peak gastric emptying rate of the biomarker
USDA	United States Department of Agriculture
WHO	World Health Organisation
β	variable, responsible for generating the calculated lag value of gastric emptying
Δ	change
δ	delta unit used to describe the carbon isotope ratio
σ	population standard deviation

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgments), nor material which to a substantial extent, has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed.....

Date.....30 November 2013.....

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Winger, a graduate of Wisconsin - Madison University, spent his early years with the NZ Meat Industry Research Institute and later with one of NZ's leading Agri-Food companies. As Chair in Food Technology, Massey University he was an executive director of the Food Technology Research Centre, a board member of Massey University and Food Standards Australia-NZ. He served on the Executive of the NZ Institute of Food, Science and Technology, the IFT global strategy advisory panel, was instrumental in setting up the South Auckland pilot plant for food and beverage manufacturers that has become the FoodBowl, and is currently Managing Director of Cousine for Me, a specialty manufacturer of ready-made meals for people with renal diseases in the UK. The generous and uncompromising support, insight, advice and encouragement of these outstanding individuals, as teachers and friends, has made this an exciting, enlightening and thoroughly enjoyable process and I thank them each from the bottom of my heart for all they have done for me.

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Abstract

Consumption of the green flesh kiwifruit (*Actinidia deliciosa*, Hayward variety) is known to relieve constipation and provide relief from symptoms of digestive dysfunction, but the effect is poorly researched and a credible explanation and mechanism has not been documented. This body of work aimed to increase the understanding of the effects of kiwifruit on the digestion process.

There is some evidence that the mechanism may be due to the proteolytic properties of actinidin, a unique protease in kiwifruit. Actinidin has a wide pH-dependent reactivity between pH 2.5 - 6 with an optimum at pH 3.0 - 3.5; equivalent to low acid conditions more associated with hypochlorhydria than the pH range of 1.5 - 2.5 normally associated with efficient gastric hydrolysis of protein. It was hypothesised that *in vitro* and *in vivo* the digestion of protein would be facilitated by the presence of the kiwifruit protease, particularly when pH exceeded normal fasting gastric pH. A sequence of studies to measure the effects of lyophilised kiwifruit (KFI), rich in actinidin, on the digestion of protein was undertaken to test this hypothesis.

Initially, proof of principle of the kiwifruit enzyme activity *in vitro* was investigated. Then, the practicality and reliability of a stable isotope technique to measure the rate of gastric protein-digestion *in vivo* was tested in three studies that compared the effect on gastric emptying dynamics of a protein meal with and without the protease. *In vitro*, homogenised freeze-dried meat powder (the protein) was incubated with a series of dilutions of hydrochloric acid (pH 1.3 - 6.4), pepsin (0 - 1.2 % w/w) and KFI (0 - 0.8 % w/w). Simulated gastric digestion involved incubation at 37°C for 60 minutes, after which the pH was adjusted to 6.4, and simulated duodenal incubation continued for a further 120 minutes. The degree of protein hydrolysis was determined using the Kjeldahl technique. The addition of KFI to the gastric milieu was

associated with an increase in the proportion of protein substrate hydrolysed, but only when the concentration of pepsin was sub-optimal for efficient protein hydrolysis and pH of the digestate was elevated above normal fasting gastric pH of >2.5 but <6.4. Under these conditions the addition of KFI to the milieu doubled protein hydrolysis from a low of $20.7 \pm 0.7\%$, to a high of $55.2\% \pm 1.2\%$ (mean \pm SD).

The aim of the next step described in the thesis was to test the hypothesis that KFI facilitated gastric protein digestion *in vivo* in a manner similar to the *in vitro* observations. As the *in vitro* study had shown KFI activity was minimal in a simulated duodenal pH > 6.0, this step required a method of measuring the change in the extent of hydrolysis of protein as a result of its passage through the stomach only. A literature review highlighted many of the problems associated with measuring *in vivo* factors likely to influence gastric protein-digestion efficiency, including fasting gastric pH, pepsin concentrations and post-prandial gastric re-acidification capacity. The complexity of measuring any of these factors, combined with limited resources, led to testing the feasibility of measuring an individual's gastric protein-digestion efficiency through an adaptation of the carbon-13 octanoic acid breath test (^{13}C -OABT), as a proxy for protein digestion efficiency.

In the first study, eleven healthy participants aged 58–80 agreed to consume, on two separate occasions one week apart, a three egg white, one yolk omelette with 100 mg of ^{13}C labelled octanoic acid with and without the addition of 2.160 g of KFI (the treatment) taken in capsule form. On each occasion, two expired breath samples were collected from each participant before they commenced the meal and nine more postprandial over three hours. The rate of appearance of $^{13}\text{CO}_2$ on the breath, an accepted measure of gastric emptying, is expressed as T_{lag} (time to maximum rate of detection of isotope on the breath) and $T_{1/2}$ (time at which 50% of the isotope is retained in the stomach). The difference in these parameters between the two test meals was

hypothesised to reflect the change in gastric protein-digestion efficiency due to the effect of KFI. Recruitment of participants was not difficult and the procedure was well tolerated. Based on the findings of the *in vitro* study, the hypothesis tested was that for participants with normal digestion, treatment would have little or no effect on the rate of protein hydrolysis and would therefore record little or no change in the rate of gastric emptying between control and treatment; whereas protein digestion, for participants with diminished gastric acidification, would be enhanced by the treatment and this would reflect in a one-way change in the dynamics of gastric emptying.

Of the eleven participants, gastric emptying parameters increased for seven and decreased for four. The inter-individual variability brought into question the reliability of the test method and the measures were repeated four months later with six participants from the first study. Variation in repeat measures in the control condition T_{lag} and $T_{1/2}$ did not exceed 13% and with the addition of KFI to the meal variation was slightly more at 20–24%. Again the pattern of some participants exhibiting accelerated gastric emptying and some recording delayed gastric emptying as a result of treatment, was observed.

A third and final *in vivo* study undertaken in China, employed a non-dispersive infrared spectrometer to measure isotope ratios, as opposed to the isotope ratio mass spectrometer used in the two earlier studies. It was hypothesised that neither the participant's ethnicity nor the equipment used to measure gastric emptying would significantly alter the measures when compared to studies in New Zealand. Parameters measured in the twelve volunteers were closely aligned to those of the first study and measures of differences of T_{lag} and $T_{1/2}$ with and without treatment were combined ($n = 22$) into a small meta-analysis. Treatment with KFI increased T_{lag} by 7 minutes (95% CI [0, 19], $p = 0.060$) and $T_{1/2}$ by 10 minutes (95% CI [0,14], $p = 0.027$). However for the 16/22 participants not consuming proton pump inhibitors (PPIs), the effect was

more marked: T_{lag} was increased by 14% or 11 minutes (95% CI [4, 18], $p = 0.050$) and $T_{1/2}$ was increased by 10% or 13 minutes (95% CI [4, 23] $p = 0.027$) = 0.050) and $T_{1/2}$ was increased by 10% or 13 minutes (95% CI [4, 23] $p = 0.027$). Multiple regression showed that the percentage increase in T_{lag} with KFI treatment was positively predicted by body weight and negatively predicted by consumption of PPIs.

Despite trial design shortcomings, the combination of three small *in vivo* studies, involving 58 individual meals and the collection and analysis of 580 breath samples, indicated KFI had a significant and delaying effect on gastric emptying times (T_{lag} and $T_{1/2}$). This may indicate that for the majority of participants in these studies, KFI improved gastric digestion efficiency, as peptide products of protein hydrolysis are known to delay gastric emptying. As a result it was hypothesised that the inter-individual variability in response to treatment reflected the underlying gastric protein-digestion efficiency of the individual trial participants, but this hypothesis should be tested in future studies.

While the novel test method employed in this study requires considerable further research to confirm the validity of the findings, what has emerged from this body of work is evidence that the protease content of the fruit is the likely active ingredient; that the stomach is the likely site of activity and that the effect is likely to be associated with existing gastric protein digestion inefficiency due to sub-optimal peptic hydrolysis.

Thesis chapter flow plan



CHAPTER 1. Preamble and Background

1.1 What is it about kiwifruit that makes it a Kiwi icon?

Kiwifruit doesn't look very attractive, it doesn't even taste very nice, yet year after year, worldwide more and more people are consuming kiwifruit. Is it for the fruit's reputed health benefits?

This chapter outlines the author's journey in his quest to understand the special characteristics of this fruit that have given rise to its reputation for positively impacting gut health and improving human digestion.

1.2 Preamble

In 1971 the author graduated with a bachelor's degree in agricultural science from Lincoln University and then took up a position as a farm advisory officer with the New Zealand Department of Agriculture in Hamilton. This afforded considerable interaction with members of the Ruakura Research Centre's science community. Foremost amongst them was Dr. Douglas Lang, a leading animal physiologist. Over the ensuing years Lang and Donaldson collaborated on numerous innovative agricultural-based projects designed to add value to New Zealand's primary products, some of which have been recognised with international patents. (Improved protein recovery, NZ Patent 544925; Animal medicament and method of manufacture, US Patent 20080248014; Digestive/laxative composition, US Patent 8057831B2; Extraction and processing of kiwifruit seeds for oil, NZ Patent 539698).

Farm advising also brought the author into contact with some of New Zealand's leading industrialists of the day, resulting in a change of direction to management roles in corporate funded agricultural investments. Experience was gained in the diverse and complementary areas of livestock farming, embryo transfer technology, cash cropping,

equine breeding and various horticultural disciplines including pomme fruit, stone fruit, floriculture and kiwifruit.

At the same time, a proliferation of individual and corporate investment during the late 1970s through to the mid 1980s kindled the growth of the then fledgling kiwifruit sector, into becoming one of New Zealand's predominant primary export industries. Involvement in the practical and business side of the growth and refinement of the kiwifruit industry fuelled the author's lifelong passion to add value to primary products. In particular the author now has more than twenty-five years involvement with processing the non-exportable portion of the annual kiwifruit crop into high value exportable products.

1.3 The growth and importance of the kiwifruit industry to New Zealand

Over the past century, New Zealand's expertise in all things agricultural has provided the principal underlying platform that has supported the country's economy. In the past, cheap fuel and labour overcame the associated cost of transport to export markets for the country's primary products, but as the cost of these inputs inexorably increased with time, farmers' margins were constantly being eroded. This is apparent today in all primary products and has resulted in the amalgamation of farms to enable fewer people to produce ever more product to simply maintain the *status quo*. New Zealand's economic situation will continue to deteriorate unless ways are found to add value to its primary products currently being sold as bulk commodities to the highest bidder. Tatua Co-operative Dairy Company is an outstanding example of a company that has embraced this strategy and leads the way in innovative, value added dairy-products ("Tatua payout highlights benefits of adding value". NZ Herald, 27 July, 2006).

The high quality animal and plant husbandry skills of New Zealand farmers ensures agricultural output results in a very high premium grade pack-out of products

able to carry the transport costs of export, but inevitably, due to weather and other factors, second grade and 'waste' product is also generated. In counties with large domestic or peripheral markets, returns for second grade produce still provide a profit to growers, but the limited size of the New Zealand domestic market does not afford its growers that luxury, and as a result the non-export grades often sell at less than cost. However, the relatively low cost of second grade produce in New Zealand provides the perfect opportunity to initiate the development of products with added value. The Apple and Pear industry did this very effectively back in the 1960s with the development of Fresh-Up juice made from the non-exportable portion of the apple and pear crop. However, that industry subsequently sold off its processing business to a multinational and no longer benefits from that revenue stream.

In the case of the kiwifruit industry in New Zealand, every effort has been made to maximize the exportable portion of the annual crop, but minor skin blemishes still result in a portion of the crop being downgraded and excluded from export. The uneconomic returns prompted the author to investigate ways to add value to kiwifruit products in terms of convenience, storage, transport and year round availability.

The first commercial product was a frozen form of kiwifruit juice, Kiwi Crush™. In 1991, the product won the premier New Zealand Food Award (then sponsored by Carter Holt Harvey Industries Ltd, but now by Massey University) and also the National Heart Foundation Award. The result was that Kiwi Crush was accepted into supermarkets where it is still sold more than 20 years later. More importantly, the Food Awards gave the product a degree of legitimacy that encouraged a hospital dietician to introduce the product to the hospital's menu. Fresh kiwifruit was, at that time, being administered to patients in the hospital's burns unit to prevent constipation following microsurgery. The logistics of sourcing and preparing fresh ripe kiwifruit year-round and gaining patient consumption compliance, proved to be a challenge, which Kiwi

Crush was able to mitigate. Kiwi Crush proved to be as efficacious as fresh kiwifruit but more convenient for the catering staff, easier to administer for nursing staff and more acceptable to the patients.

People admitted to hospital for surgery are generally administered antibiotics and opiate-based analgesics, both of which have a tendency to induce constipation as does the change of diet and immobility from enforced bed rest. Anecdotal reports that Kiwi Crush relieved constipation, without the less pleasant aspects associated with laxatives, spread through the Association of Dieticians. Because of its legitimacy as a whole fruit extract, and its 'natural' status, hospitals throughout New Zealand gradually adopted Kiwi Crush as the preferred treatment for constipation. Many older people with chronic digestive conditions, and people prescribed medication for some terminal conditions, also discovered Kiwi Crush relieved digestive discomfort and helped maintain regularity: as a result, sales increased.

While the proven efficacy of the product (Wyeth, 2011) makes it one of the few truly 'functional' foods, until now the mode of action has not been fully elucidated. Relief from constipation was originally assumed to be the effect of fibre increase, despite the product not qualifying as a 'good source' of dietary fibre. On average a single 100 g Hayward kiwifruit contains 1.6 g of fibre, 80 mg of vitamin C, 0.8 g of protein and 9.6 g of sugar. Kiwifruit protein however, is unique in that it hydrolyses protein e.g. kiwifruit is used as a meat tenderizer and cannot be placed on a pavlova (egg white protein) until just before serving.

More recently, to convenientise Kiwi Crush and enhance its exportability, the active protein component of kiwifruit (and therefore Kiwi Crush), Zyactinase™, was isolated and stabilized into a powder form by lyophilisation; a form of freeze drying. This dry powder is now marketed and sold in capsule and tablet form to deliver the

same benefits as Kiwi Crush, but in a more portable and convenient form. In New Zealand these products are currently branded as Phloe™ and in Australia as Kivia™.

It was from this background the author set out on an academic journey to identify a scientifically credible mode of action to explain how kiwifruit influences digestion, with the thought this could add to the body of knowledge and possibly lead to the development of new forms of medication to prevent digestive dysfunction. This thesis is a record of that journey from 2008 to 2012.

1.4 Background

Kiwifruit was so named in the late 1950s to develop an export market for the New Zealand-cultivated fruit *Actinidia deliciosa* and distinguish it from the popular garden plant the Chinese gooseberry, a native of the Yangtze valley in Central China. Today kiwifruit is cultivated in Europe, North and South America, Asia and Australasia and is almost as widely available as pomme, citrus and stone fruit.

Although an 'apple a day' has long promoted the health benefits of apples, the health attributes of kiwifruit are legendary, particularly its reputation for relieving constipation and indigestion. Yet, despite its reputation, very little is known about the mechanism that enables kiwifruit to relieve the discomfort of these digestive afflictions.

The first step in this study was to review the existing literature to establish what was known and what questions still needed to be answered; including the possible mechanism that explains how consumption of kiwifruit could influence gastro-intestinal digestion.

CHAPTER 2. Literature Review: kiwifruit / digestion associations

Kiwifruit, pineapple and papaya are unusual amongst soft fruits in that they contain protein. Unlike pulses (peas, beans, lentils and lupines), which contain 20–25% protein, these fruits may only contain 1–3%, but much of this protein is of a proteolytic nature. The purpose of this review was to identify existing literature that might indicate an association between proteolysis, or any other constituent or biological property of kiwifruit, and the relief of digestive dysfunction.

All organisms contain enzymes to catalyse physiological reactions. Enzymes that catalyse reactions by dissociating peptide bonds are termed proteases. They are an essential component of the life cycle and play a fundamental role in all aspects of protein metabolism.

Protease from pineapple, known as *bromelain*, and *papain*, sourced from papaya, are commercially produced fruit proteases, which are refined into dry powder form suitable for use in many biological and industrial processes. Kiwifruit contains a group of six individual proteases collectively referred to as *actinidin*, but the difficulty of processing kiwifruit results in a low yield high cost product, which is used mostly in small quantities for highly specialized processes including gene technology.

Anecdotal reports and common knowledge suggest that kiwifruit, and some products made from the fruit, relieve constipation and improve digestive health. A review of the literature relating to kiwifruit and digestion, proteolytic action of the kiwifruit enzymes on protein, and the importance of the digestion of protein in relation to human health was undertaken. Key words and phrases searched included: protease, protein, digestion, maldigestion, digestive dysfunction, constipation, functional bowel disorder, dyspepsia, irritable bowel syndrome, gastric pH, mitochondrial degradation and sarcopenia.

2.1 Kiwifruit, constipation and digestion

In this literature review, three studies were found that associated the consumption of kiwifruit or kiwifruit extract with improved frequency and ease of laxation (Chang, Lin, Lu, Liu, & Liu, 2010; Rush, Patel, Plank, & Ferguson, 2002) and the relief of constipation (Uebaba et al., 2009). The 2002 three-week randomised cross-over trial (Rush, Patel, Plank, & Ferguson, 2002) with 38 elderly subjects, reported that inclusion of one fresh kiwifruit a day for every 30 kg of body weight improved the ease of laxation from the first week and an increase in frequency of laxation was not reported until the third week. The authors noted that four out of five subjects reported that kiwifruit improved their regularity while the others reported no difference. A more recent Japanese study (Uebaba et al., 2009) noted an improvement, for 42 elderly patients (60 to 84 years) experiencing mild to moderate constipation, after ingesting six capsules of Zyactinase™ (a freeze-dried kiwifruit juice extract from the Hayward variety of kiwifruit) per day for four weeks. Improvement was measured by bowel evacuation time and stool consistency as per the Bristol Stool Chart, and generic quality of life questionnaires using an 11-point Likert scale (0–10). Improvement in stool consistency and evacuation times was observed from about the 14th day and continued for 28 days compared to the control patients. Quality of life in terms of skin rashes, oedema, lumbago and headaches were also reported to improve over the time. The purpose of the third study involving 54 subjects diagnosed with IBS-C (constipation dominant irritable bowel syndrome) and 16 healthy subjects was to observe the impact of a four-week intervention of kiwifruit on bowel function of the IBS subjects. The kiwifruit intervention significantly increased defecation frequency and reduced bowel transit time for the IBS subjects.

Anecdotal evidence from an unpublished, but independently conducted survey of 1200 customers who purchased kiwifruit-based supplements for digestive health (Vital Foods, Auckland, 2012), supports the hypothesis that kiwifruit consumption can have a relieving influence on indigestion, bloating, constipation and irritable bowel syndrome (IBS), collectively referred to in the literature as functional bowel disorder (FBD) and in this thesis as dysfunctional digestion (Malagelada & Malagelada, 2010).

A review of functional gastrointestinal disorders in New Zealand (Wyeth, 2011) revealed that in a community survey of 1,000 adults 45.2% had either reflux or dyspepsia. The majority had used 'over the counter' (OTC) or non-pharmaceutical medication, and only 17% had consulted a medical practitioner in relation to the problem. An observation from the article was that kiwifruit products were routinely used to manage functional constipation in New Zealand hospitals and rest homes, and further research was being undertaken to explore potential mechanisms for the observed beneficial effect of kiwifruit.

An unpublished audit of hospital inpatients diagnosed with constipation confirmed the practice of hospitals using kiwifruit products to manage functional constipation, and showed this to be an effective option to reduce the frequency of constipation and the use of other laxative products (Wyeth et al., 2000 - unpublished).

Constipation is a symptom of digestive dysfunction, not a disease. The Merck Manual (2010), under the heading *Symptoms and Diagnosis of Digestive Disorder* states '...some symptoms, such as diarrhea, constipation, bleeding from the digestive tract, regurgitation, and difficulty swallowing, usually suggest a digestive disorder' (Shaheen, 2007). Constipation is a condition in which a person has uncomfortable or infrequent bowel movements. Constipation can result when the passage (transit) of the stool through the large intestine is slowed by disease or by certain drugs. Sometimes constipation is caused by dehydration or a low-fibre diet. Pain and mental disorders

such as depression may also contribute to constipation. In many cases, however, the cause of constipation is unknown (*The Merck Manual of Diagnosis and Therapy*, 2010).

To summarise: constipation is essentially the slow passage of food through the gastro-intestinal system, particularly the colon. Generally accepted causes of constipation are insufficient fibre and liquid in the diet; certain medications, especially opioids; and a lack of exercise. However, there are also unknown causes as highlighted above. The quantity of protein in a meal is known to be a factor that delays gastric emptying (Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011). Whether a delay in gastric emptying also delays transit through the rest of the GI system is not clear, but may be one of the unknown factors referred to in the Merck Manual.

The concentration of dietary fibre present in fresh, whole kiwifruit (Rush et al., 2002) may be associated with improved laxation as it is accepted that kiwifruit usually contain approximately 3 g of dietary fibre per 100 g of fresh fruit. This means an average sized kiwifruit, without skin and weighing 65–75 g, will contain about 2 g of dietary fibre of which 75% is soluble and 25% is insoluble (USDA National Nutrition data base). However, the Japanese trial conducted by Uebaba et al using six capsules of freeze-dried kiwifruit powder, the equivalent to about 30 g of fresh kiwifruit or approximately 1 g of dietary fibre, also showed kiwifruit's effectiveness for improving laxation. This leads to the question: Is it the fibre in kiwifruit that contributes to the effect on laxation and reports of relief from digestive disorder, or is it other physiological aspects of the fruit that are responsible?

2.2 Protease

While the clinical literature review revealed very little information about the mechanism that linked the fruit to the largely anecdotal reports of health benefits, a search of the plant physiology literature (Nieuwenhuizen et al., 2012) revealed that one

of the more unusual features of some varieties of kiwifruit (plants of the *Actinidia* genus) is the presence of protein digesting enzymes in the structure of the mature fruit.

Although proteases are virtually absent in the commercial yellow Zespri Gold variety (*Actinidia chinensis* Hort.16A), they are abundant (1–2%) in the green Hayward variety (*Actinidia deliciosa*) which is the principal commercial variety worldwide.

Studies undertaken by Sugiyama et al. (1976) demonstrated the presence of six proteases in the Hayward variety of kiwifruit. Predominant among these is the well-documented thiol cysteine protease *Actinidin* (Baker, Boland, Calder, & Hardman, 1980). *Actinidin*, like other fruit-derived proteases such as bromelain (pineapple), papain (papaya) and ficin (figs) is known to hydrolyse a wide range of food proteins. As with other enzymes, the extent of hydrolysis is determined largely by the ratio of enzyme to substrate, the temperature at which the reaction takes place, the pH of the medium and the nature of the bonding between the substrate and matrix.

Given the paucity of literature on the mechanism between the fruit and its effect on human digestion, and the unusual feature of the presence of protease, I undertook a literature search on the role of proteases in digestion of protein in humans.

2.3 Protein digestion, absorption and turnover

Although the importance of protein quality on biological processes beyond the requirements for growth and nitrogen balance have not been extensively researched (Millward, Layman, Tomé, & Schaafsma, 2008), the quantity of dietary protein required to build or maintain body protein mass has been well documented by researchers in journal articles and text books.

At a cellular level proteins are continually catabolized, and the amino acids recycled contribute to either the synthesis of new protein or energy metabolism. Overall there is a continual and net loss of protein metabolites, mainly urea, via the kidneys. Protein

balance can only be restored by the absorption of peptides and amino acids from the digestion of dietary protein (Dangin, Boirie, Guillet, & Beaufrère, 2003). Exact protein requirements for positive nitrogen balance are the subject of some debate (Pencharz & Ball, 2003), but Millward et al (Millward, Fereday, Gibson, & Pacy, 1997) estimated a normal healthy adult requires approximately 0.75–1.0 g/kg of body weight per day of good quality, highly digestible protein to maintain positive nitrogen balance and to provide the essential amino acids. The average daily Western diet is purported to contain between 70 and 100 g of exogenous protein, but quality may vary depending on the source and processing. This is important as it is the essential amino acids that the body requires rather than protein per se. Fecal excretion of nitrogen is estimated to be 6–12 g of protein per day for a normal healthy adult (Erickson & Kim, 1990).

Endogenous sources of protein are thought to contribute almost a third of the daily requirements depending on the health and possibly the age of the individual. As a result, approximately 70% of the body's protein requirement is dietary sourced. The protein requirements of pregnancy, childhood growth, extreme-pursuits and adult maintenance are all well understood, with that understanding coming from extensive *in vitro* trials as well as from both animal and human prandial trials. As a result, the protein requirements of domestic animals to optimise meat, wool and milk production are also well understood.

Skeletal muscle makes up 45 to 50% of the body mass of the mature man; less for women and the obese. From about the age of 45 years, muscle mass declines in both men and women, and after about 60 the rate of decline accelerates, so that by age 80 it is not uncommon for skeletal muscle mass to comprise less than 30% of body mass. A cross sectional study (Bammens, Evenepoel, Verbeke, & Vanrenterghem, 2004) of a heterogeneous sample of 486 men and women between the ages of 18 and 88, using whole body magnetic resonance imaging (MRI) to measure total skeletal muscle mass,

Bammens et al. (2004) concluded that aging is associated with a decrease in skeletal muscle mass, mainly explained by a decrease in lower body skeletal muscle mass after the fifth decade of life.

Muscle wasting, or atrophy, associated with frailty and infirmity is referred to as sarcopenia (Greek sarco meaning flesh, penia meaning deficiency). Skeletal muscle mass loss resulting in frailty is almost synonymous with ageing; it is considered the major contributing factor to the falls and fractures that result in the loss of independence for the elderly (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2003). The proportion of populations in New Zealand and many developed countries attaining elderly status will increase markedly over the next 15 years. Enhancing the ability of elderly people to maintain their independence would have a profound fiscal influence on the health care systems of all developed countries. Lynch et al. (2005) also concluded sarcopenia is likely to become one of the most significant health issues confronting health care providers of developed countries over the next decade.

Arguments advanced as to the cause of sarcopenia include altered metabolic rate, mitochondrial free radical production, hormonal imbalances, amino acid deficits and lack of exercise. Attempts to prevent or reduce the rate of muscle loss by way of therapies designed to manipulate one or more of these proposed causes have met with varying degrees of success. Indeed the positive effects of such therapies may be by indirect means. Hormone replacement therapy has shown significant promise, but side effects have hindered its universal adoption (Panay, Hamoda, Arya, & Savvas, 2013). Amino acid supplementation also has potential to prevent or limit the loss of skeletal muscle mass (Dillon et al., 2009; Volpi, Mittendorfer, Wolf, & Wolfe, 1999) in that high concentrations of leucine in the diet have been shown to stimulate protein synthesis and inhibit degradation of skeletal muscle mass (Garlick, 2005). Leucine appears to act as a messenger to signal when sufficient concentration of amino acids and insulin are

present to stimulate protein synthesis, and insulin sensitivity is improved. However, Garlick (2005) suggests that high concentrations of supplemented leucine to enhance insulin sensitivity may paradoxically result in insulin resistance. Dideriksen et al (2013) showed the effect of increased protein intake on muscle protein accretion is additionally stimulated by exercise prior to consumption (Dideriksen, Reitelseder, & Holm, 2013). They speculated that physical exercise for the ageing population, in conjunction with an increased proportion of protein in the diet, might counteract the development of anabolic resistance that gives rise to sarcopenia. However, they note that current knowledge is based on measures obtained in standardized experimental settings, or during long-term controlled intervention periods. In order to improve coherence of the understanding of the evidence, other investigative approaches than those presently used are needed to increase the understanding of the effects of protein ingestion on muscle synthesis.

Parker et al. (2004) attributed the cause of sarcopenia to the influence of hormonal diminution on appetite, while Balagopol et al. (1997) demonstrated that increasing age was strongly correlated with a decline in the myosin heavy chain synthesis rate. Fujita et al. (2004) acknowledged that advancing age was associated with progressive loss of muscle mass, but observed that the mechanisms of sarcopenia were still not well understood. They went on to review protein metabolism in the elderly and concluded, as did Cuthbertson et al. (2005), that a diminished anabolic response to nutritional stimuli may be a significant contributing factor (Cuthbertson et al., 2005; Fujita & Volpi, 2004; Parker & Chapman, 2004). The success of exercise as a therapy to reduce the rate of age related muscle mass loss has been highlighted by several researchers (Adamu, Weck, Rothenbacher, & Brenner, 2011; Hebuterne X, Bermon S, & Schneider SM, 2001). In contrast, Katsuhiko et al (2005) concluded from their animal study that the anabolic response to muscle stimulation is attenuated with aging and may contribute to the limited capacity for muscle hypertrophy in aged

animals (Katsuhiko, Parkington, Carambula, & Fielding, 2005). A later study by Hulmi et al (2010) leaves little doubt that a combination of resistance exercise and amino acid supplementation reduces the rate of age-associated muscle degradation (Hulmi, Lockwood, & Stout, 2010). However when Fry et al. (2011) examined the role of the nutrient/energy/redox signaling protein rapamycin complex 1 (mTORC1) which is critical to the rate of protein synthesis, they concluded that the efficiency of this signal is diminished with aging, (Fry et al., 2011) and that this may contribute to the blunted hypertrophic response of skeletal muscle to resistance-exercise training in older compared to younger adults. They highlighted the mTORC1 pathway as a key therapeutic target to prevent sarcopenia.

At an intracellular level mitochondrial degradation and increased free radical production have also been cited as possible causes of sarcopenia in the elderly (Navarro, López-Cepero, & del Pino, 2001). While this is a complex topic, it is noted (Katsuhiko et al., 2005) that mitochondrial function depends on the availability of intracellular enzymes and amino acids. Mitochondrial density and functionality in skeletal muscle decline with age (Katsuhiko et al., 2005; Short et al., 2005; Zubkova & Robaire, 2004) as do enzyme concentrations (Short et al., 2005). Could a greater reliance on amino acids from endogenous protein catabolism, as opposed to exogenous protein from dietary sources, be associated with this reduced cytosol concentration of protein resulting in cellular apoptosis, diminished mitochondrial concentration and impaired muscle function?

2.4 Gastric acidity

Gastric acidity achieves highest concentration in the absence of food. Food generally exerts a buffering effect and increases gastric pH. The standard measure of maximal acid concentration is after an overnight fast. The range of fasting gastric pH is

from 1.5–2.5 in normal, healthy adults (Andersen, Naesdal, & Strom, 1988).

Subsequent olfactory stimulation increases parietal cell secretions of gastric acid and the volume of gastric acid in the stomach, but not the pH. Food entering the stomach increases both the volume of the gastric content and also the pH. The pH may rise to >pH 5 as the food, having a more neutral pH, exerts a buffering effect on pH of the total gastric content. The time food is retained in the stomach prior to the commencement of gastric emptying is determined by a number of factors, some understood and some yet to be quantified. It was initially thought that gastric emptying of the stomach was determined by the calorific content of the chyme entering the duodenum (Hunt & Stubbs, 1974), but it was later determined that the volume of the meal, its energy density (kcal/ml), and the proportions of fat, carbohydrate, and protein in the meal had relatively minor effects and that gastric emptying was regulated through the osmotic effect and calcium binding of the products of digestion in the duodenum (Hunt, 1983). Certainly food quantity and food digestibility affect the dynamics of an individual's gastric emptying, but there are inter-individual differences in the dynamics of gastric emptying for identical meals. This is thought to be a reflection of the individual's gastric digestion efficiency which involves both physical and chemical breakdown of the food. The rate at which a standard meal is digested will be influenced by both the fasting gastric pH and the re-acidification capability of the individual. For example, if fasting gastric pH for one individual is pH 1.5 and the standard meal buffers this to pH 4.5, the efficiency of protein digestion is expected to be greater than for the individual with a fasting gastric pH of 3.5 and the meal buffers the gastric pH to 6.5. This is due to both the presence of more concentrated acid, (lower pH) and the protease, pepsin, which is considerably more active at low pH (concentrated acid) than at a higher pH (dilute acid). The pH of the gastric milieu may be a cause of delayed gastric emptying. Delayed gastric emptying is a feature of advancing age i.e. food is retained

in the stomach for longer to increase exposure to the acid and enzymes necessary to effect adequate digestion to enable the mechanisms of gastric emptying to function normally. More recent studies (Inui et al., 2004) have identified that the mechanisms controlling gastric emptying are more complex than previously thought and involve physical, neural and hormonal receptors and feedback reactions to control appetite and optimise digestion of food (particularly proteins) prior to gastric emptying. However these mechanisms are eventually over-ridden by the uninterrupted phasic contractions of the migrating motor complex (MMC) that forces the stomach contents into the duodenum regardless of the extent of its digestion. It may be that in the case of extreme delayed gastric emptying this is the mechanism that most effects gastric emptying.

Despite the wealth of research into causes of sarcopenia, the relationship between age-related changes in gastric acid production and digestive enzyme levels and the effect this may have on nutrient absorption and sarcopenia appeared to have attracted limited research. Lee & Feldman (1997) concluded from a review of human and animal studies that little or no change in gastric luminal aggressive factors was observed with normal ageing, but that advancing age was associated with significant changes in gastric mucosal defense mechanisms resulting in diminished ability to respond to gastric assault and injury (Lee & Feldman, 1997). In contrast, Ali et al (2009) observed that subtle changes in the composition of the gastric medium do occur with ageing, resulting in the diminution of gastric acid secretion associated with advancing age, gastric atrophy and pharmacological acid suppression. They advanced this as a possible factor in protein malnutrition (Ali, Roberts, & Tierney, 2009). This raises the question: could a decline of gastric acidity be a contributing factor to sarcopenia? Although gastric digestion of protein is not regarded by all researchers (Pohl et al., 2008) as essential for efficient digestion of protein, across vertebrates there are similarities in the gastric production of hydrochloric acid.

Gastric secretion of hydrochloric acid appears to be unique to vertebrates and is almost ubiquitous in all fish, amphibians, reptiles, birds and mammals. Comparative anatomy and physiology suggest that gastric acid evolved approximately 350 million years ago. The similarity of the acid-secreting mechanism across all classes of vertebrates implies a major advantage for selection, but the evidence regarding its precise purpose remains inconclusive (Koelz, 1992).

Pancreatic and intestinal enzymes are assumed by some researchers (Pohl et al., 2008) to have the capacity to complete normal digestion in the absence of gastric acid. This is supported by the prevalent use of pharmacological acid suppressing medication including proton pump inhibitors (PPIs), which by 2005 constituted 55% of the \$US20 billion worldwide gastrointestinal medication market (Raghunath, O'Morain, & McLough, 2005). PPIs inhibit the H^+/K^+ ATPase enzyme system that produces the hydrogen ions to make gastric hydrochloric acid, while the earlier H_2 -receptor antagonists (H_2 blockers) prevent the H_2 receptors on the gastric parietal cells from reacting to histamine.

While expedient, the assumption that normal digestion can be completed in the absence of gastric acid may deserve further investigation as gastric acidity is essential to activate pepsin. Although in the present-day the need for such an intricate mechanism as gastric acidification and peptic hydrolysis of protein may be open to conjecture, as attested to by Pohl et al. (2008), it is far from clear whether peptic hydrolysis of protein is redundant.

Our results, I hope, have for ever done away with the crude and barren idea that the alimentary canal is universally responsive to every mechanical, chemical, or thermal agency, regardless of the particular requirements of each phase of digestion. Instead of this hazy conception, we now see delineated an intricate mechanism which, like everything else in nature, is adapted with the utmost delicacy and precision to the

work which it has to perform. Ivan Pavlov, *The Work of the Digestive Glands* (Quoted by (Rothman, 1977))

Several researchers have observed that elevated fasting gastric pH (>pH 3.5) seems to be associated with advancing age (Bammens et al., 2004; Gidal, 2006), while others observe that gastric emptying takes twice as long for older people compared with the young (Clarkston et al., 1997). Physiologically, delayed gastric emptying is consistent with less-than-optimal gastric digestion as an outcome of elevated pH and/or diminished pepsin concentration or activity. As pepsin activity diminishes with increasing pH, elevated gastric pH is likely to result in maldigestion of protein (i.e. incomplete digestion) in the gastric phase of digestion, regardless of the pepsin concentration.

Unfortunately the invasive nature of gastric sample collection to measure acidity at all stages of digestion limits the extent of available data, making it difficult to confirm that elevated gastric pH is a result of advancing age and not declining health status from environmental factors, medication or *Helicobacter pylori* infection. As many older people also have health issues, the correlation may relate more to pathophysiological factors rather than chronological ageing (McLean & Le Couteur, 2004). The incidence of some diseases has changed over time, for example in 1973 chronic gastritis was thought to be prevalent in 28% of the American adult Caucasian population (Strickland & Mackay, 1973). Strickland et al described two distinct types of gastritis – Type A (serologic test for parietal cell antibodies is positive) and Type B (test for parietal cell antibodies is negative). The prevalence of pernicious anaemia, related to deficiency of intrinsic factor, associated with the Type A gastritis which has diminished since then, but at the time Type B gastritis, the more important precursor of gastric carcinoma, was thought to be four times more prevalent than Type A gastritis

suggesting approximately 20% of the adult Caucasian population was affected by Type B gastritis. Many of this group would be included amongst today's 'elderly'.

The possible association of ill health with reduced gastric acid production is supported by a study of 742 intubated hospitalised patients of mixed ages with a mean fasting gastric pH of 4.06 (Metheny et al., 1997), whereas the accepted fasting gastric acidity of a healthy young adult is in the order of $\text{pH } 2.0 \pm 0.5$. While fasting gastric acid pH can be viewed as a measure of digestive health, a more relevant measure may be the re-acidification capacity of the gastric mechanism as the buffering effect of food can raise the gastric pH by 2 to 3 points (a rise of one pH units is a 10-fold decrease of the H^+ ion concentration; two units is 100-fold decrease). Efficient gastric digestion, especially of proteins, is likely to be more reliant on the post-prandial reacidification capability of the gastric mucosa than it is on fasting gastric acidity *per se* (personal correspondence with supplier of Heidelberg radio telemetry capsule).

Protein maldigestion, variously caused by elevated gastric pH, pepsin deactivation or diminished pepsin concentration due to reduced excretion into the stomach, may reduce the subsequent absorption of peptides and amino acids (and increase the quantity of undigested protein entering the bowel and possibly the faeces) and thus depress the supply of circulating free amino acids to fewer than that required for the effective maintenance of muscle anabolism and immune response. Supporting this hypothesis — but contrary to the 1985 FAO/WHO/UNU Joint Expert Consultation that concluded older adults required lower protein intake than younger adults — Campbell et al. (1994) found that older people had higher protein requirements than younger adults. This finding is consistent with protein malabsorption, meaning that older people may experience protein malnutrition because of their inability to efficiently digest protein rather than an innate requirement for a greater quantity of dietary protein.

Amino acid supplementation, in the absence of additional exercise, was associated with increased muscle anabolism in the elderly (Volpi et al., 1999), suggesting the existence of an amino acid deficit. Enhanced nutrition failed to replicate the effects of amino acid supplements (Volpi, Mittendorfer, Rasmussen, & Wolfe, 2000; Welle & Thornton, 1998) indicating protein digestion as a potential core problem. The finding that specific essential amino acids (especially lysine) stimulated muscle anabolism in elderly subjects, as opposed to amino acids *per se* (Volpi, Kobayashi, Sheffield-Moore, Mittendorfer, & Wolfe, 2003), is also consistent with protein malabsorption due to gastric protein maldigestion in that high quality protein and efficient digestion will make available all essential amino acids whereas maldigestion may release some but obscure others that may be essential.

Gastric protein-maldigestion is likely to have several other outcomes including deficiencies in Vitamin B6 and B12 (Adamu et al., 2011; Bammens et al., 2004; Carmel, 1994). Vitamin B12 deficiency is associated with elevated blood plasma homocysteine concentrations which are positively and highly correlated to cardiovascular disease (Brattström & Wilcken, 2000). A further effect of the reduced gastric digestion is an increased reliance on the proteolytic action of pancreatic and brush border sourced proteases (Erickson & Kim, 1990) to affect protein hydrolysis to tri-peptides, di-peptides and amino acids for transport across the small intestinal epithelium. The inability of these latter processes to compensate for incomplete gastric digestion is likely to result in increased polypeptide fermentation by the colonic flora that may have a number of health implications including inflammatory diseases and immunosuppressant effects (Hughes, Magee, & Bingham, 2000).

Further support for the importance of a low pH for the gastric digestion of protein comes from a study of gastric pH and micro-flora of normal and diarrhoeic infants (Maffei & Nobrega, 1975), collected using an intubated catheter and tested using

a glass electrode pH meter. Maffei et al noted that the diarrhoeic infants had significantly elevated gastric pH when compared with normal infants. While the study assumed elevated gastric pH resulted from pathogenic infection of the gut, the possibility remains that elevated gastric pH resulted in protein maldigestion, and that this provided the stimulus for the proliferation of protein-fermenting bacteria in the colon which then invaded the small intestine and ultimately, the stomach.

Several research reports include discussion of age-related declines in both intracellular enzymes due to reactive oxygen metabolites (Evereklioglu et al., 2003) and factors that affect digestive enzyme concentrations (Greenberg & Holt, 1986; Layer, Peschel, Schlesinger, & Goebell, 1990). An interesting observation that supports this concept was referred to by Russell (R. M. Russell, 1999) who noted that in a trial (reported by Feibusch and Holt (Feibusch & Holt, 1982)) comparing carbohydrate digestion between young and elderly subjects, that increasing dietary carbohydrate to 200 g /meal had no apparent effect on the appearance of hydrogen (a measure of microbial carbohydrate fermentation) on the breath of young subjects. However, 80% of the elderly subjects had higher concentrations of breath hydrogen than the younger subjects which was interpreted as meaning most of the elderly subjects were lacking the carbohydrase reserves necessary to efficiently hydrolyse the carbohydrate (Bammens et al., 2004). Can it be extrapolated from this evidence that if carbohydrase secretion is limited to the extent that carbohydrate digestion is affected, the possibility might also exist in this same population that secretion of other digestive enzymes might limit digestion of protein? Certainly this is a conclusion that can be drawn from the review article of Bitar et al. (2011) on aging and gastrointestinal neuromuscular function (Bitar, Greenwood-Van Meerveld, Saad, & Wiley, 2011).

2.5 Summary

The growing body of evidence (albeit modest) indicates that the inclusion of kiwifruit in the diet may reduce symptoms of digestive dysfunction, relieve constipation and increase laxation (Chang, Lin, Lu, Liu, & Liu, 2010; Rush et al., 2002; Wyeth, 2011) (and unpublished studies by Wyeth, 2000 and Weir, 2008,). There is also evidence that the protease from *Actinidia deliciosa* can, under controlled conditions, hydrolyse meat to a liquid consistency (Donaldson & Lang, 544925 (23.01.2007)/2006). While these reports are independent of each other, the possibility that the proteolytic activity of kiwifruit protease might explain the effects on the digestive tract, formed the basis of the hypothesis formulated at the start of this doctoral research journey.

In other words, if there is an age-associated decline in the concentration or activity of digestive enzymes, particularly pepsin, especially if it is associated with a decline in gastric acidity, it may be gradual and unrecorded. A decline of this nature may explain the progressive loss of cellular and skeletal muscle function observed in many elderly, but evidence from 'molecule to malady' is needed to endorse this hypothesis.

The conclusion from this literature review is that there is some evidence that gastric reacidification efficiency diminishes with diminished functionality associated with aging, but not necessarily as a result of age per se, and furthermore that secretion of other digestive enzymes might similarly be impaired.

Although no evidence was found to support the hypothesis that maldigestion of protein is a contributing factor to digestive discomfort or more specifically, constipation, no evidence was found to the contrary.

Based on the fact that kiwifruit contain a powerful protease component and the clinical and anecdotal evidence that kiwifruit improves laxation and relieves constipation in the elderly, the following sequence of studies (**CHAPTER 3**) was

designed to test the hypothesis that kiwifruit may relieve digestive dysfunction by improving protein hydrolysis efficiency.

CHAPTER 3. Kiwifruit hydrolysis of protein — an *in vitro* study

3.1 Introduction

Scientific evidence, albeit meagre, supports a growing body of anecdotal evidence that consumption of kiwifruit, specifically the Hayward variety (*Actinidia deliciosa*), relieves some of the symptoms of functional bowel disorder (Chang et al., 2010) and constipation (Wyeth, 2011), collectively referred to in this thesis as symptoms of digestive dysfunction (Malagelada & Malagelada, 2010). Enforced immobility, autoimmune responses, microbial influx, inflammatory bowel disease, irritable bowel syndrome, an imbalance in (or change of) diet and some forms of medication (including analgesics, antibiotics and chemotherapeutics) are factors associated with these symptoms (Miszputen, 2008), particularly among the elderly (Tiihonen, Tynkkynen, Ouwehand, Ahlroos, & Rautonen, 2008) but also among an increasing number of younger people (Henderson et al., 2011; Lehtinen et al., 2011).

An unusual feature of the green-fleshed Hayward variety of kiwifruit is the presence of protein in the mature fruit at a rate of about 3.0 g/kg of fruit. Approximately 40% (≈ 1.2 g/kg of fruit) of this comprises a clade of six cysteine proteases referred to as actinidin (Lewis & Luh, 1987); thus explaining the reputation of the fruit as a meat tenderiser. In contrast, the yellow-fleshed *Actinidia chinensis* variety, Zespri Gold (Hort.16A), contains similar concentrations of total protein but <0.03 g/kg of this is protease (Nieuwenhuizen et al., 2012).

The purpose of this study was to investigate a possible mode of action to explain how kiwifruit may relieve digestive dysfunction. The first step (this chapter) was to measure *in vitro* the effect of the addition of kiwifruit protease on the rate of protein hydrolysis with changing pepsin and acid concentrations that simulated individual variation in gastric efficiency. It was hypothesised that the addition of actinidin to an *in*

vitro simulation of the gastric and duodenal milieu would increase the rate of protein hydrolysis.

3.2 Materials and Methods

Substrate, protease and acid

The first requirement to simulate the gastric milieu for the experiments was to prepare a homogeneous proteinaceous substrate that would mimic a standardised protein meal. Secondly, both pepsin and hydrochloric acid were required in concentrations that were physiological, and thirdly, the kiwifruit enzyme should be added in a quantity that was realistic, such as equivalent to consuming kiwifruit as a snack or with a meal.

Preparation of the substrate

Several sources of protein were considered and red meat was selected because it is a major source of complete protein in the Western diet and is recommended for increasing muscle mass through diet. Raw meat hydrolysed more readily than cooked meat (Gatellier & Santé-Lhoutelliera, 2008); however, as the overall aim of this work was to explore the effects of kiwifruit on human digestion, the meat was prepared in a manner consistent with a normal meal served in a residential setting.

Forequarter shoulder steak, which contains a high component of connective tissue and represents a medium price range cut, was selected. All visible fat was trimmed to waste and the meat double-passed through a 6 mm industrial mincing plate. Homogeneous freeze dried meat (FDM) was prepared as follows: the minced meat was boiled in water for 10 minutes, the cooked meat and fluid were freeze dried together, the product was milled to a powder and thoroughly mixed prior to packing into 50 g foil bags for refrigerated storage. The yield of freeze-dried meat powder to wet weight of

cooked meat before drying was 30% (600 mg FDM was equivalent to 2 g hydrated cooked meat).

Protease selection

Two proteases were used: porcine pepsin (Sigma-Aldrich Australia, Product # P7125, with ≥ 400 Sigma units/mg protein) and a proteolytically active freeze-dried kiwifruit isolate.

The variation between individual kiwifruit, in terms of stage of maturity and nutrient and enzyme content, was such that it could introduce sample errors to an experiment. Also, after processing to achieve a usable kiwifruit pulp, the material is notoriously difficult to stabilise due to the reactive nature of the protease component. Antioxidants will stabilise the product, but because this study was to mimic the *in vivo* effects of consuming fresh fruit this option was not considered. Instead of fresh fruit, a powdered, freeze dried, proteolytically active kiwifruit isolate (supplied as Zyactinase® by Vital Food Processors Ltd, Auckland) was selected. According to the manufacturer's specification Zyactinase® is a shelf-stable, nutritionally homogeneous material that replicates the fresh fruit (including fibre content), but the water content has been reduced by 97% and the protease activity standardised at >3000 U/mL. The protease activity was assessed using a casein substrate according to the manufacturer's specification. In this study, rather than use and potentially promote the name Zyactinase, it is simply referred to as kiwifruit isolate (KFI).

Protein concentration

The protein concentration was determined for FDM, pepsin and KFI from six replicate Kjeldahl analyses for nitrogen, assuming a conversion factor of 6.25 g protein/g nitrogen. The concentrations of protein (g/100 g) and standard deviation for

each were: FDM 85.34 ± 0.01 ; pepsin 60.20 ± 0.02 ; and for KFI 4.34 ± 0.00 respectively.

Primary and secondary digestions

The *in vitro* model digester constructed for this study comprised 8 x 75 mL screw-top glass tubes held in a reciprocating flask shaker set at approximately 240 shakes/minute immersed in a water bath at 37 °C. In these experiments, the simulated gastric digestion was designated as the ‘primary digestion’, while a subsequent simulated duodenal digestion at pH 6.4 was referred to as the ‘secondary digestion’. FDM (600 mg) plus one or both enzymes were weighed into the tubes, a 20 mL aliquot of hydrochloric acid (of predetermined molar concentration) was added and the mixture was then incubated at 37°C for 60 minutes for the primary digestion. At this time, the pH was adjusted by addition of phosphate buffered NaHCO₃ (pH 6.4, 0.074 M) to the primary digests, and then the secondary digestion commenced with shaking for a further 120 minutes. Because small intestine pH and food transit time for same type meals vary little in healthy individuals (McCloy, Greenberg, & Baron, 1984), the simulated duodenal incubation that followed primary gastric digestion was maintained at a constant pH 6.4 for 120 minutes. After incubation, the mixtures were centrifuged at 2080 relative centrifugal force for 10 minutes and the Kjeldahl method (see later) used to measure ammoniacal nitrogen in the supernatant. A minimum of two replicate digestions were performed for every experiment. For clarity, data are presented as means only in tables, but with error bars in one of the figures.

Determination of pH

A Radiometer (Denmark) PHM201 meter was used to measure pH. In the absence of a standard gastric pH model, the study used 0.074 M HCl for the baseline gastric medium; its calculated pH in water was 1.13 at 0°C, but this would be higher in

the presence of FDM and the enzymes, which themselves have buffering capacity. Because of meter inaccuracies at low pH, the acidity of the gastric medium prior to inclusion of FDM and enzymes is reported as a molar concentration (M) where the measured pH was ≤ 3.0 pH.

Description of the hydrolysates and supernatants from the digestions

After centrifugation of the hydrolysates from the individual incubations, the quantity of ammoniacal nitrogen (N) in each supernatant was determined by the Kjeldahl method. Ammoniacal N could derive from the FDM in the absence of any protease from the added enzymes, and from enzyme-dependent hydrolysis. The first of these three sources was found to be as follows after 60 minute incubations of 600 mg of FDM: 13.2% in 0.074 M HCl; 12.0% in 0.022 M HCl; and 10.7% after incubation in deionised water and at pH 6.4. On average this comprised about 62 mg of protein from the 511 mg of FDM protein, in each incubation. The likely contribution from pepsin at its highest addition rate (40 mg) in this sequence of experiments would be a further 26 mg, or from KFI at its highest addition rate (350 mg) would be a further 15 mg. Ammoniacal N in the supernatant was typically above 30% rather than 13.2% or lower, so pepsin and KFI would make only minor contributions. In terms of human digestion it is likely that the supernatant fraction would represent the most easily digestible fraction, whatever its source, so in this report hydrolysis refers to the total ammoniacal N in the supernatant from the various digestions.

Kjeldahl determination of ammoniacal N

Following digestion, the supernatant was decanted into 250 mL digestion tubes; the precipitate was then washed with 10 mL of deionised water, re-centrifuged and the supernatant combined with the initial supernatant. To this was added 10 mL of concentrated H₂SO₄, 10 mL of 35% hydrogen peroxide (H₂O₂) and 7.5 g of catalytic

mineral salts. The tubes were subjected to 420 °C to effect digestion. After digestion 50 mL of deionised water was added to the tubes plus 50 mL of 35% NaOH and the contents were steam distilled using VELP equipment (VELP Scientifica UDK126A). The condensate was trapped in a 4% boric acid solution and titrated with standardised HCl (0.1282 M) using Tashiro's indicator.

The Kjeldahl method is still the 'gold standard' of nitrogen determination and is commonly used in the food industry because of its accuracy and reliability. The method measures total Kjeldahl nitrogen which includes total organic nitrogen, ammonia and ammonium; it does not measure nitrite or nitrate nitrogen. It does not measure protein as such, but by multiplying the nitrogen in the supernatant of the centrifuges meat digests by a factor of 6.25 (Hiller, Plazin, & Van Slyke, 1948). The water soluble protein on the basis that $1/6.25 = 0.16\text{g}$ of N per gram of meat protein was able to determined accurately.

3.3 Results

This series of experiments explored the effect of varying pH, enzyme concentrations and time on protein hydrolysis, determined as ammoniacal N in the supernatant.

After a 60-minute incubation of 20 mL 0.074 M HCl with 600 mg FDM and 100 mg of KFI, 13.5% of the ammoniacal N was recovered in the supernatant, showing that under these conditions KFI had little or no effect on net hydrolysis because the no-enzyme control value was 12.6%. However, decreasing the acidity (pH increase) increased hydrolysis (**Table 1**) which peaked in the acidity range of 0.02 to 0.03 M HCl; 0.022 M HCl achieved the highest N yield of 37.7% in 60 minutes. Reacting 600 mg of FDM with 100 mg of KFI in water alone resulted in 27.8% N in the supernatant after 60 minutes incubation.

Table 3.1 Effect of dilution of HCl (0.074M concentration) on protein hydrolysis activity of kiwifruit protease (100 mg KFI) on 600 mg of FDM (freeze dried meat) measured by the percentage of ammoniacal nitrogen in the supernatant of the hydrolysates. (mean results of 2 repeats from 2 separate experiments)

Dilution HCl : H ₂ O (mL)	Concentration HCl (M)	Ammoniacal Nitrogen in the supernatant				
		Expt 1A (%)	Expt 1B (%)	Expt 2A (%)	Expt 2B (%)	Mean ± SD (%)
20:00	0.0744 M	13.75	13.38	-	-	13.56 ± 0.27
10:10	0.0372 M	18.81	19.29	-	-	19.05 ± 0.34
9:11	0.0335 M	-	-	29.36	28.89	29.12 ± 0.33
8:12	0.0298 M	-	-	35.98	35.26	35.62 ± 0.51
7:13	0.0260 M	-	-	36.94	36.41	36.67 ± 0.38
6:14	0.0223 M	-	-	37.52	37.78	37.65 ± 0.19
5:15	0.0186 M	37.11	36.98	-	-	37.04 ± 0.09
4:16	0.0149 M	-	-	32.36	32.82	32.59 ± 0.32
3:17	0.0112 M	-	-	31.11	30.76	30.93 ± 0.25
2.5:17.5	0.0093 M	30.51	30.31	-	-	30.41 ± 0.14
1.25:18.75	0.0047 M	29.94	30.06	-	-	30.00 ± 0.09
00:20	Includes KFI	28.67	27.46	27.67	26.92	2768 ± 0.73
00:20	Excludes KFI	12.03	11.97	13.25	13.15	12.60 ± 0.69

SD standard deviation; Expt experiment

At the optimal acidity for KFI activity (0.022 M HCL), increasing KFI concentration from 0 to 350 mg per 600 mg of FDM (**Table 3.2**) resulted in progressively increased hydrolysis with saturation occurring at concentrations higher than 250 mg.

Table 3.2 Effect of KFI (kiwifruit protease) concentration on protein hydrolysis of FDM (freeze dried meat) measured by percent of Kjeldahl nitrogen in the supernatant after 60 minutes incubation in 0.022 M HCl. (mean results of 2 repeats)

Experiment 1		Experiment 2		Mean ± SD	
KFI (mg)	Nitrogen (%)	KFI (mg)	Nitrogen (%)	KFI (mg)	Nitrogen (%)
0	12.02	0.0	12.86	0.00 ± 0.00	12.44 ± 0.59
51.9	27.56	50.4	26.40	51.15 ± 1.06	26.98 ± 0.82
75.3	34.26	75.8	35.08	75.55 ± 0.35	34.67 ± 0.58
104.5	36.93	101.4	37.58	102.95 ± 2.19	37.25 ± 0.46
125.8	37.88	126.2	38.76	126.00 ± 0.28	38.32 ± 0.62
150.7	40.04	150.3	41.59	150.50 ± 0.28	40.81 ± 1.10
175.1	40.75	175.9	42.68	175.50 ± 0.57	41.72 ± 1.36
202.3	42.68	201.6	43.08	201.95 ± 0.49	42.88 ± 0.29
252.3	45.18	250.7	45.78	251.50 ± 1.13	45.48 ± 0.43
306.3	45.27	304.7	47.23	305.50 ± 1.13	46.25 ± 1.38
350.4	45.38	352.4	46.34	351.40 ± 1.41	45.86 ± 0.68

SD - standard deviation

The effect of incubation time was also explored (**Table 3.3**). Increasing the primary digestion time from 60 to 120 minutes in 0.022 M HCL increased supernatant ammoniacal N from 37.2 to 44.1%. Extending digestion for a further 60 minutes caused

a negligible increase, to 45.1%. However, extending the primary digestion in 0.074 M HCl from 60 to 180 minutes increased N in the supernatant from 13.5 to 30% (**Table 3.3**), although that could have been caused by acid-catalysed hydrolysis with time.

Table 3.3 Effect of incubation time on hydrolysis of FDM (freeze dried meat) by KFI (kiwifruit protease) measured by the percentage of ammoniacal nitrogen in the supernatant (SN) of the hydrolysate. The reaction mixtures contained 600 mg FDM, 20 mL of 0.022M HCl and 100 mg KFI. (mean results of 2 repeats)

Incubation Time (minutes)	Ammoniacal Nitrogen in the supernatants		
	Experiment 1 (%)	Experiment 2 (%)	Mean \pm SD (%)
15	22.95	21.28	22.12 \pm 1.19
30	31.09	30.13	30.61 \pm 0.68
45	34.55	35.18	34.87 \pm 0.44
60	36.60	37.82	37.21 \pm 0.86
75	40.42	40.70	40.56 \pm 0.19
90	41.06	41.45	41.26 \pm 0.28
105	43.13	42.59	42.86 \pm 0.38
120	44.23	43.89	44.05 \pm 0.26

SD - standard deviation

The experiments described above were all concerned with primary digestion with KFI. The effect of subsequent secondary digestion was also explored in two experiments that simulated the transition from the gastric to duodenal conditions. First, in 0.074 M HCl – ineffective for KFI (**Table 3.1**) – a subsequent increase in pH to 6.4 by addition of bicarbonate marginally increased the ammoniacal N from 13.5 to 15.2% after 120 minutes of further secondary digestion (data not tabulated). The results obtained with the addition of the more effective 0.022 M HCl are shown in Table 3.4.

Table 3.4 Effect of 120 minutes of secondary digestion at various pH between 3.1 and 6.4 on the hydrolysis of FDM (freeze dried meat) by KFI (kiwifruit protease) measured by percent ammoniacal N in the supernatant of the hydrolysate, following 60 minutes of primary digestion in 0.022 M HCl. (mean of 2 repeats)

Secondary Digestion (pH)	Ammoniacal Nitrogen in the supernatants		
	Experiment 1 (%)	Experiment 2 (%)	Mean \pm SD (%)
3.1	45.11	44.94	45.03 \pm 0.11
3.5	45.62	45.44	45.53 \pm 0.13
3.8	41.44	40.97	41.20 \pm 0.33
4.2	40.65	39.91	40.28 \pm 0.52
4.6	39.67	39.30	39.49 \pm 0.26
5.1	39.58	39.77	39.68 \pm 0.14
5.7	39.52	40.57	40.05 \pm 0.75
6.1	38.94	40.69	39.81 \pm 1.23

SD - standard deviation

As the pH of the secondary digestion was increased serially to a maximum of 6.4, there was a clear tendency for ammoniacal N in the supernatant to decrease (**Table 3.4**). Thus it appears that KFI is ineffective at further solubilising the protein at higher pH values (pH > 6.1).

Having established the basic activity parameters for the KFI, attention was directed at pepsin, the native gastric enzyme. Pepsin activity was tested at two acidities 0.074 M HCl and 0.022 M HCl (**Table 3.5**) the former being the AOAC standard for *in vitro* gastric digestion, the latter being the optimum acid concentration for KFI activity.

Table 3.5 Effect of high and low acid concentration on pepsin hydrolysis of FDM (freeze dried meat) measured by percent of ammoniacal N in the supernatant of the hydrolysate. (mean of 2 repeats)

Ammoniacal Nitrogen in supernatants						
Experiment	HCl	FDM	Pepsin	Expt 1 & 2	Mean ± SD	CV
	(mL)	(mg)	(mg)	(%)	(%)	(%)
0.0744 M						
1	20	600.3	30.6	80.49	80.03 ± 0.64	0.8
2	20	601.1	30.1	79.58		
0.0223 M						
1	20	601.6	30.1	39.34	39.49 ± 0.21	0.5
2	20	603.3	30.4	39.64		

SD - standard deviation; CV - coefficient of variation.

For 0.074 M HCl, the ammoniacal N in the supernatant peaked at 80% with 30 mg pepsin per 600 mg of FDM. However, in 0.022 M HCl, pepsin hydrolysis resulted in only 39% \pm ammoniacal N in the supernatant.

The effect of pepsin concentration over the range 0 to 40 mg per incubation was also investigated (**Figure 3.1**) at both the high (0.074 M HCl) and low acid concentrations (0.022 M HCl). A combination of low pepsin concentration (5 mg per 600 mg FDM) and low acid concentration (0.022 M HCl) reduced the ammoniacal N in the supernatant to a mere 21%.

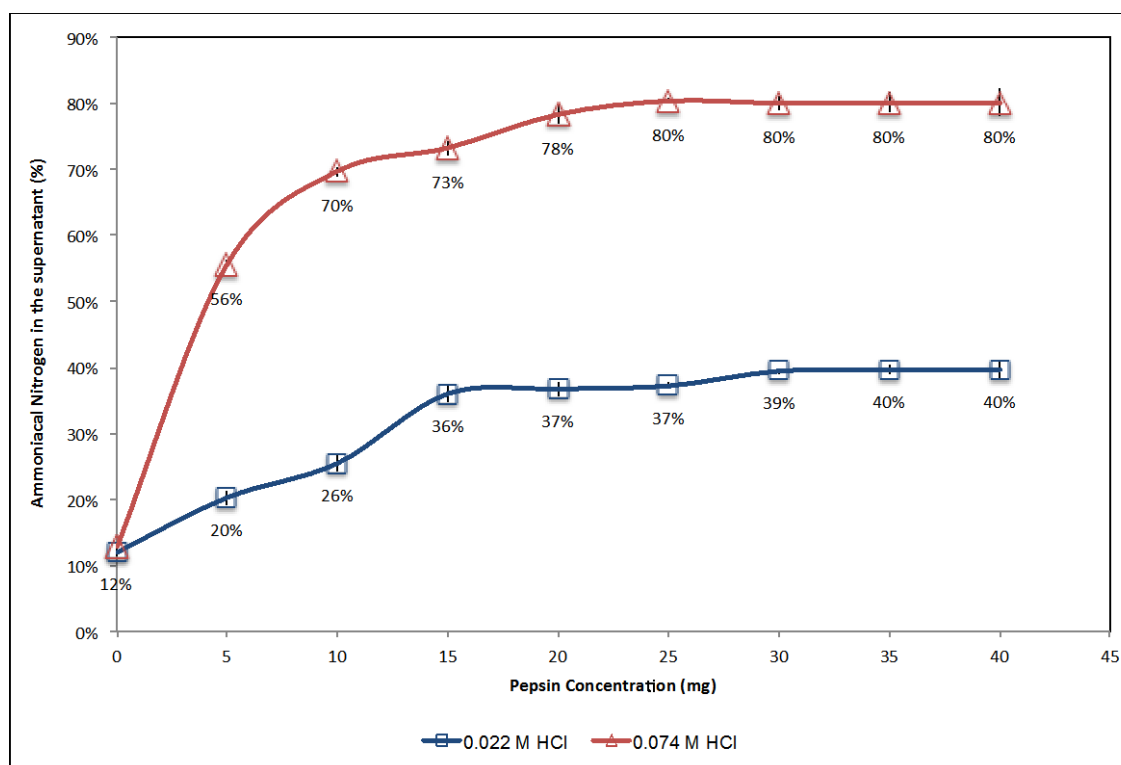


Figure 3.1 Effect of pepsin concentration on protein hydrolysis of freeze dried meat at two acid concentrations measured by percent of ammoniacal N in the supernatant of the hydrolysate. (means \pm SD for 2 determinations)

The question was then posed: How is hydrolysis affected by the enzymes in combination?

It was established (**Figure 3.1**) that where pH was optimal for pepsin activity (20 mL of 0.074 M HCl), an enzyme to substrate ratio of 30 mg of pepsin to 600 mg FDM resulted in 82.2% of N in the supernatant after 60 minutes incubation. When this experiment was repeated with the addition of 100 mg of KFI a similar result was obtained (data not shown) demonstrating that the addition of KFI had little effect on protein hydrolysis under conditions of low pH that were optimal for normal gastric

pepsin digestion. This result was predictable given that the enzyme-independent ammoniacal N was 13.2% in 0.074 M HCl and only 13.5% when KFI was added. However, in the less acidic conditions of 0.022 M HCl, a combination of 5 mg pepsin (low concentration) and 100 mg of KFI resulted in increased, but not perfectly additive, hydrolysis (**Table 3.2**). Thus pepsin alone gave 20.5% hydrolysis, but with added KFI the ammoniacal N increased to 48.3%. At higher pepsin concentrations (10, 15 and 30 mg/600 mg FDM) the protein hydrolysis increased from 48.6% to 55.1% and then to 55.9% respectively. However, the percentage increase for pepsin alone was greatest with 5 mg pepsin (139%), followed by 10 mg (89%), 15 mg (50%) increase. These metabolically important effects were all statistically significant.

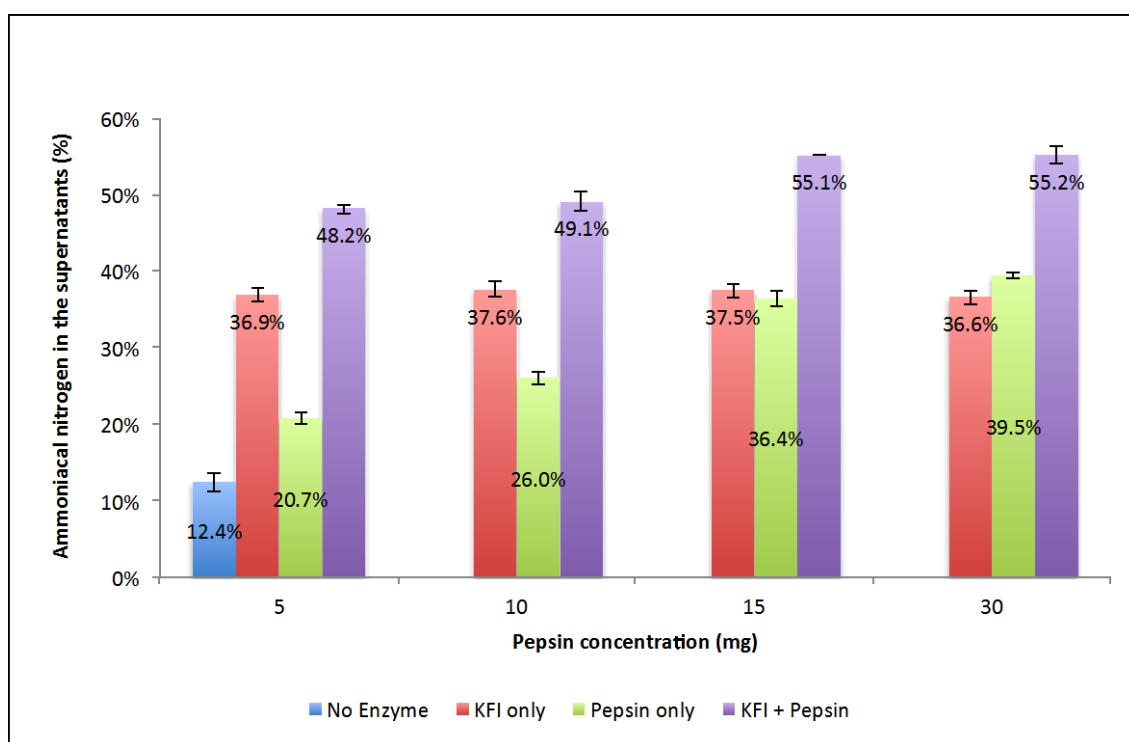


Figure 3.2 Effect of pepsin, alone and in combination with kiwifruit protease (KFI), on hydrolysis of freeze-dried meat (FDM) in primary digestion. Incubations each contained 20 mL 0.022 M HCl plus various combinations of pepsin, 5, 10, 15 & 30 mg/incubation alone or in combination with 100 mg KFI. Bars are means of duplicates and errors are standard deviations.

3.4 Summary

This series of experiments was conducted to determine the effect of KFI on protein digestion over a pH range that encompassed those of human gastric and duodenal digestion. The main findings of each are summarised in Table 3.6.

Table 3.6 Summary of the key findings from the seven chronologically sequential experiments, with Table and Figure references.

Experiment 1 Table 3.1	Hydrolysis efficiency of KFI was optimal in the HCl acidity range 0.02–0.03 M and declined as acidity was increased or decreased. Hydrolysis of KFI was minimal in 0.074M HCl
Experiment 2 Table 3.2	Hydrolysis efficiency increased as KFI concentration increased from 0–250 mg per 600 mg FDM, but saturated above this concentration of 1.8% w/w KFI protein to FDM.
Experiment 3 Table 3.3	Extending primary incubation from 60 minutes to 120 minutes increased KFI hydrolysis of FDM in all pH media tested.
Experiment 4 Table 3.4	Following 60 minutes primary digestion in either high or low gastric acidity, KFI demonstrated little activity in a simulated duodenal medium (6.4 pH).
Experiment 5 Table 3.4	The primary digest that produced maximum hydrolysis from 100 mg of KFI, contained 20 mL of 0.022 M HCl in the presence of 600 mg of FDM had a measured acidity of pH 3.1 at the conclusion of the 60 minute incubation. KFI hydrolysis of FDM was greatest when secondary digestion pH was in the range 3.1–3.6; as secondary digestion pH increased beyond pH 3.6, secondary digestion hydrolysis was diminished.
Experiment 6 Table 3.5 & Figure 3.1	Hydrolysis efficiency of pepsin diminished as pH increased and also as pepsin concentration decreased from 40 mg (4%) to 5 mg (0.5% w/w pepsin protein to FDM).
Experiment 7 Figure 3.2	Where low acid (0.022 M HCl) and pepsin concentrations limited hydrolysis of FDM, the addition of KFI increased primary protein digestion over the range 0–15 mg pepsin/600 mg FDM.

3.5 Discussion

This series of experiments demonstrated that the addition of KFI to the gastric milieu was associated with an increase in the proportion of protein substrate hydrolysis (Experiment 7, **Figure 3.2**), but only when the concentration of pepsin was sub-optimal for efficient protein hydrolysis and pH of the digestate was elevated above normal

fasting gastric pH (1.2–2.0 pH) (McLauchlan, Fullarton, Crean, & McColl, 1989).

There was no evidence that KFI significantly increased protein hydrolysis in the simulated duodenal medium. The main finding was that protein hydrolysis was doubled (20.5% to 48.3% of the ammoniacal nitrogen in the supernatant of the hydrolysate) when kiwifruit protease was added to the milieu in which pepsin concentration and gastric pH were less than optimal for normal gastric digestion of protein (**Figure 3.2**).

Selecting an appropriate baseline pH for *in vitro* studies of gastric digestion can be contentious because studies show gastric pH varies significantly between individuals, and between young and elderly people (Clarkston et al., 1997). In a study of 79 healthy elderly people (T. L. Russell et al., 1993), 11% of participants recorded fasting gastric pH > 5.0 throughout the trial. Chronic gastritis, a condition not uncommon among the elderly (Adamu et al., 2011), can result in hypochlorhydria (gastric acid insufficiency) which may explain this finding. While achlorhydria (an absence of gastric acid) represents the extreme outcome of gastritis, results from our study indicated that even small variations in gastric pH and pepsin concentration had a marked influence on protein digestion efficiency. As acid and pepsin concentration diminished, primary protein hydrolysis became progressively less efficient. This is a simple but fundamentally important result.

The need for adequate quantities of pepsin is in agreement with a study on the effects of pH on pepsin activity conducted by Al-Janabi et al. (1972) who demonstrated that pepsin was 100 times more active at pH 1.0 than at pH 4.6. If both the pH and pepsin concentration were compromised *in vivo*, this finding may have implications for some age associated diseases. (In this respect porcine pepsin, as used in this study, has been observed to display greater activity than human pepsin (Eriksen et al., 2010). As a result, *in vivo* hydrolysis by kiwifruit and its interaction with human pepsin may not equally reflect the current *in vitro* results.)

The purpose of the seven experiments that made up this *in vitro* study, summarised in Table 3.6, was to investigate the effects of kiwifruit on protein digestion in an *in vitro* model of a gastric and duodenal environment of the human GI system with the intention this might help identify a biochemical mode of action to support a meagre body of scientific evidence that kiwifruit increased laxation and relieved digestive discomfort and constipation.

The literature review led to the hypothesis that kiwifruit protease might increase protein digestion efficiency, possibly at the interface of gastric and duodenal digestion, but the complementary effect of kiwifruit protease with pepsin was not expected and had not been recorded before.

3.6 Strengths of this research

This investigation demonstrated a principle *in vitro*, i.e. proof of principle, before undertaking an *in vivo* investigation. More specifically, it demonstrated for the first time that kiwifruit increased protein hydrolysis in a simulated gastric medium only when the extent of protein hydrolysis was impaired by the acid and pepsin concentrations associated with normal, healthy gastric digestion were not optimal. However, this finding needs to be replicated *in vivo* to make a meaningful contribution to the understanding of how kiwifruit consumption may relieve symptoms of digestive dysfunction. More importantly, an association between impaired gastric digestion of protein and constipation would also need to be established to support the hypothesis that kiwifruit prevents constipation by improving primary protein digestion.

Research on within-individual protein digestion efficiency is constrained to the extent that there is no accepted standard for *in vitro* studies of the dynamics of gastric digestion. The AOAC 1995 standard procedure for determining protein digestibility (AOAC, 1995), specifies 0.0744 M HCl (Bellaver, Zanotto, Guidoni, & Klein, 2000),

which equates to a simulated gastric medium with a pH value of 1.13. Kaur et al (Kaur, Rutherford, Moughan, Drummond, & Boland, 2010a) maintained their digestate at pH 1.9 (0.0126 M HCl) throughout the digestion of the protein. Takumi et al. (2000) selected 0.1 M HCl (pH 1.0) for a pH-controlled study but emphasised a need to establish standard guidelines for *in vitro* gastric pH research. The analysis does not appear to make allowances for the buffering effects of food that can be expected to elevate gastric pH to >4.0 for >60 minutes post prandial.

Regular measurement of individual's fasting gastric pH and re-acidification efficiency are not currently a feature of routine health checks, making it conceivable that a pernicious deterioration in protein digestion efficiency with ageing could go undetected. This raises the possibility of inefficient primary protein digestion as a contributing factor in the onset of sarcopenia and other age associated conditions.

3.7 Limitations of this research

These *in vitro* experiments indicate a measureable effect of kiwifruit on one aspect of digestion; however, they are preliminary in nature and do not equate with a clinical outcome. In the design of any further studies of this nature, the model used to simulate *in vivo* gastric digestion should be considered. A dynamic model of the upper human gastrointestinal tract (Mainville, Arcand, & Farnworth, 2005) was beyond the scope of this study. A dynamic model would maintain the pH of the digestate at a constant level throughout the incubation; however, this may have masked one of the more important findings of this current study. The optimal acid concentration for KFI activity in this study was found to be 0.0223 M HCl (**Table 3.1**) equivalent to pH 1.66 before addition of FDM and enzymes, but the measured pH was 3.1 after inclusion of the FDM and KFI (**Table 3.4**). A more sophisticated digestion model used in similar studies by the Riddet Institute (Massey University, New Zealand) for Zespri

International Ltd., maintained a constant gastric pH of 1.9 by means of an automated acid pump (Kaur et al., 2010a). That study confirmed our earlier unpublished finding that kiwifruit increased protein digestion efficiency at pH 1.9. However, our study showed, kiwifruit protease achieved considerably greater activity at pH 3.1 than pH 1.9.

Our results also indicated that kiwifruit is unlikely to have any effect on protein hydrolysis in the small intestine where the normal pH is 6.4–7.0 (**Table 3.4**). This result conflicts with that of Kaur et al. (2010) (Kaur, Rutherford, Moughan, Drummond, & Boland, 2010b) who observed further hydrolysis of some proteins, by kiwifruit, occurred at the higher pH of the small intestine but this may be explained by the different protein types.

The observed decline in activity as acidity concentration decreased above pH 3.6 (**Table 3.4**) supports the hypothesis that elevated gastric pH and pepsin insufficiency are the principal factors that determine kiwifruit's effect on digestion.

The increase in protein digestion efficiency, observed when KFI concentration was increased, (**Table 3.2**) may indicate that *in vivo*, where consumption of one kiwifruit fails to relieve digestive discomfort, increasing the dose might improve the outcome. (In this respect, the effect of pepsin on kiwifruit protease activity and vice versa is unknown: both are proteins and both are proteases.)

3.8 Further Research

This series of experiments demonstrated that kiwifruit, under certain conditions, is likely to increase meat protein hydrolysis *in vivo*. However this does not explain how or why kiwifruit relieves symptoms of digestive dysfunction, unless it can be shown that impaired gastric digestion of protein is a causal factor in digestive dysfunction, including constipation.

Given that some causes of constipation are unknown (*The Merck Manual of Diagnosis and Therapy*, 2010); the incidence of constipation increases with age (Gallagher & O'Mahony, 2009); sarcopenia is associated with advanced aging (Chernoff, 2004); and the diminished efficiency of gastric digestion with age which is likely, due to detrimental changes to cell structure and function resulting from the cumulative effects of exposure to corrosive or mutagenic agents (Lee & Feldman, 1997), this line of reasoning warrants further investigation.

Extrapolations from the findings of this study and the literature reviewed, indicate that relief from digestive discomfort and constipation, associated with kiwifruit consumption, may be a result of three factors alone or in combination: *first*, improved primary protein hydrolysis efficiency when gastric acidity and pepsin concentration are depressed, resulting in an overall improvement of gastric digestion; *second*, a reduction in the quantum of undigested protein entering the duodenum, thus reducing reliance on pancreatic digestion to compensate for impaired gastric digestion; *third*, reduced protein in the chyme entering the colon, resulting in beneficial compositional changes in gut micro-flora populations.

The literature review (**CHAPTER 2**) indicated that elevated gastric pH could be extensive amongst the elderly as a result of pathologies and medication rather than physiology, (Lovat, 1996; Pohl et al., 2008). Constipation, a symptom of digestive dysfunction, is also more prevalent among the elderly, possibly for the same reasons (Schaefer & Cheskin, 1998).

A correlation between sub-optimal protein digestion and constipation is not currently supported by the literature. However, the literature is clear that the bioactivity of peptic protein hydrolysates determine the release of gut hormones such as CCK (Nishi, Hara, Hira, & Tomita, 2001) and these control the rate of gastric emptying to a

greater extent than dietary fats or carbohydrates. Other scientific findings (Chan, Leung, Tong, & Wong, 2007; Chang et al., 2010) showing that kiwifruit increases laxation and relieves constipation, in combination with the current *in vitro* results, that kiwifruit increases protein hydrolysis where this is constrained by sub-optimal peptic proteolysis, justifies an *in vivo* investigation of the gastric digestion efficiency of dietary protein.

Another area that may warrant further research is the issue of a 'gold standard' for acidity in studies of gastric digestion. Given the prolific use of PPI medication it may no longer be appropriate to assume gastric digestion necessarily involves exposure of chyme to low pH.

CHAPTER 4. Background and Literature Review

The initial literature review (**CHAPTER 2**) focused on factors that might pertain to an association between symptoms of digestive dysfunction and relief of those symptoms due to kiwifruit consumption. As a result of the literature review, a study was conducted (**CHAPTER 3**) that explored the effects of kiwifruit on protein digestion *in vitro*. This indicated that kiwifruit increased primary protein digestion where simulated gastric acidity was insufficient to achieve normal digestive efficiency. Protein digestion efficiency is an important consideration in animal breeding for productivity but is not a topic well traversed in the scientific literature pertaining to human health.

To build on the findings of the *in vitro* study and support the hypothesis *in vivo* that kiwifruit relieves digestive dysfunction by improving protein digestion efficiency, a method of measuring primary protein digestion *in vivo*, appropriate in the context of this study, was required. Reviewing the literature to identify a suitable method of measuring gastric protein digestion efficiency was the purpose of this chapter.

4.1 Measuring Protein Digestion Efficiency

This chapter reviews relevant literature on the process and efficiency of protein digestion and its importance to optimal function, across the life-course. Ways to measure the completeness and timing of the *in vivo* digestion of proteins are also explored and critiqued. Specifically, the use of Carbon-13 stable isotope breath testing as a tool to measure the dynamics of digestion absorption and metabolism is examined, and the rationale for an experimental study that uses an adaptation of the ^{13}C -octanoic acid breath test to measure the effects of kiwifruit on protein digestion dynamics *in vivo*.

4.2 Introduction

Of the three macronutrients, carbohydrate and lipid usually provide the energy that fuel biological processes of the body. Essential macronutrients (must obtain from dietary sources) include n3 and n6 fatty acids and eight of the twenty amino acids. While protein, and amino acids derived from protein, can also provide energy, the fundamental role of protein is as a source of amino acids for the programmed synthesis of new proteins that translate the information coded in cellular deoxyribonucleic acid into diverse functions (Petsko & Ringe, 2004).

Recommended dietary intake of the macronutrients carbohydrate, protein and fat, for long-term sustenance (Australian NHMRC & New Zealand MoH, 2006) is for 60% of total energy expenditure (TEE) to be contributed by carbohydrate, 25% by fat and 15% by protein. Whilst a wide variation to this ratio appears to be tolerated without increasing the risk of chronic disease, sustained absence of any one of these macronutrients in the diet has detrimental health implications. This thesis focuses principally on the role of protein in maintenance of body homeostasis. The following quote exemplifies this:

Dietary proteins and their digested products interact with the regulatory functions of the gastrointestinal (GI) tract in a source dependent manner. Within the GI tract, dietary proteins and their products of digestion affect several regulatory functions by interacting with receptors releasing hormones, affecting stomach emptying and GI transport and absorption, transmitting neural signals to the brain, and modifying the microflora. The characteristics of proteins including their physico-chemical properties, amino acid composition and sequence, bioactive peptides, digestion kinetics and also non-protein bioactive components conjugated with them influence their interaction with the GI tract. Yet, there are currently no

assessment methods designed to evaluate the physiological quality of proteins beyond providing indispensable amino acids and contributing to protein synthesis. Clearly, the role of dietary proteins as determinants of health have to be understood beyond that provided by the traditional methods of assessment of protein quality. (Jahan-Mihan et al., 2011)

Formulae used in this thesis to calculate parameters of gastric emptying to determine protein digestion, are driven by the calculations of an individual's total energy expenditure (TEE). The following table uses FAO data (**Table 4.1**), to give an indication of the energy requirements for males and females over a range of ages (FAO/WHO/UNU, 2001)

Table 4.1 Energy requirements for males and females of the same body weight — both 50 kg and 80 kg over three age ranges but one common physical activity level (PAL)

Age Range		18-29	30-59	60+
Gender	Body mass (kg)	TEE (kJ/kg/d)	TEE (kJ/kg/d)	TEE (kJ/kg/d)
Male	50	175	175	140
Female	50	150	150	135
Male	80	145	135	115
Female	80	125	115	105

PAL = Total Energy Expenditure (TEE)/Basal Metabolic Rate (BMR); TEE = Total energy expenditure; BMR = Minimum energy expenditure required to maintain body equilibrium at rest in a supine position

The body mass of a 70 kg man is composed of approximately 60% water and 19% fat, while protein accounts for approximately 10 kg of the remaining 14 kg (70%). Almost half of this protein is in the form of actin, myosin, collagen and haemoglobin that together make up the body's muscle mass. The body has very limited amino acid storage structures (Waterlow, 1984) and labile reserves of protein other than skeletal muscle constitute less than 1% of total body protein. As a result skeletal muscle is catabolised to provide essential amino acids in situations where dietary protein is insufficient to maintain blood plasma amino acids at appropriate concentrations to meet

body requirements. As a consequence a diet deficient in protein or essential amino acids will, over time, result in a loss of muscle mass. Sickness or disease also increases the body's requirement for protein (Hoffer & Bistrian, 2013) and can result in muscle loss in the case of prolonged elevated body temperature caused by fever. From conception through to adulthood, the rate of protein anabolism exceeds the rate of catabolism, hence infants grow to be adults. With maturity comes a change and for a period the rate of anabolism equates with the rate of catabolism until a point is reached when the rate of muscle mass anabolism fails to match the rate of catabolism and the muscle mass starts to decline with advancing age, a decline which appears to accelerate from about age 60 years on (Vandewoude, Alish, Sauer, & Hegazi, 2012). Extreme loss of muscle mass, sarcopenia, is associated with frailty in the elderly (Lynch, Shavlakadze, & Grounds, 2005). The extent of muscle mass decline between the age of 20 and 80 was estimated by Janssen et al. (2000) to be in the order of 30 to 40% (I. Janssen, S. B. Heymsfield, Z.M. Wang, & R. Ross, 2000). Sarcopenia has serious future health and welfare implications due to its widespread prevalence amongst the elderly (Janssen et al., 2003). Furthermore a clear positive correlation exists between the percentage of body mass that is skeletal muscle and bone mineral density (Felson, Zhang, Hannan, & Anderson, 1993). As both indices decline with age, frailty, instability and the incidence of falls and bone fractures increase and may ultimately result in the loss of independence and the need for supported care (Fujita & Volpi, 2006a). On average women have 30% less skeletal muscle mass and more fat mass than men, which may explain why women are more prone to osteoporosis than men (Lang, 2011).

Urinary calcium loss has been associated with high protein intakes (Heaney, 1998), which is difficult to explain if there is a correlation between protein malnutrition, sarcopenia and osteoporosis, unless there is a link between calcium loss and impaired primary protein digestion . This is not beyond the realms of possibility as excessive

protein intake may impair gastric protein digestion by depleting acid and pepsin secretion capability, resulting in increased demand on pancreatic protease hydrolysis to compensate. Alternatively, an increase in colonic bacterial protein digestion may be a related factor.

As a general rule, adults (under normal conditions excluding stress due to tissue damage or disease) require 13 to 15% of their energy intake in the form of protein. However the need for elderly (>65 years) to increase the quality and intake of dietary protein has been recognised (Australian NHMRC & New Zealand MoH, 2006). Unfortunately, a consequence of aging may be diminished appetite (Clarkston et al., 1997) and loss of taste and smell, which may lead to a reduction in energy intake (Parker & Chapman, 2004). Furthermore an increased preference for carbohydrate (Morley, Kaiser, & Raum, 1997) may combine to set the scene for protein energy malnutrition in the elderly. If protein digestion is a cause of digestive discomfort for some elderly, it is understandable that they limit their protein intake, either voluntarily in an independent living situation, or by a reluctance to eat, in an institutional setting, in favour of a carbohydrate rich diet.

Researchers, (Bross, Javanbakht, & Bhasin, 1999; Giunta et al., 2008) refer to an age-related decline in the concentration of enzymes that catalyse many of the body's biological processes. If the extent of this diminution extends to digestive enzymes as suggested by R. M. Russell (1992), particularly the main digestive proteases, pepsin, trypsin and chymotrypsin, it follows that protein digestion in the elderly is likely to be compromised regardless of dietary protein intake (Whitcomb & Lowe, 2007). Diminishing concentrations and activity of digestive enzymes may induce gradual protein malnutrition despite the quantum of dietary protein appearing to be adequate. Whether gastric pH increases as a consequence of age or gastropathy induced by other etiologic factors, is still controversial (Haruma et al., 2000). Certainly chronic gastritis,

caused by long-term exposure to *Helicobacter pylori*, is known to damage the glandular tissue of the gastric mucosa, which can result in diminished gastric acid and pepsinogen production capability (Asfeldt et al., 2009). Furthermore, older age and *Helicobacter pylori* infection are compounding factors in the development of chronic atrophic gastritis (Adamu et al., 2011).

The observation that protein digestion influences the rate of gastric emptying (Erickson & Kim, 1990; Jahan-Mihan, Luhovyy, El Khoury, & Anderson, 2011) and the more difficult a protein is to hydrolyse, i.e. casein protein cf whey protein, the longer it takes to absorb (Dangin et al., 2001; Low, 1990), may relate to the finding of the *in vitro* study (**CHAPTER 2**). That study demonstrated that pepsin activity was reduced two fold when acidity was decreased from 0.074M HCl to 0.023M HCl and resulted in progressively less efficient protein digestion.

Extrapolating from the literature and the study findings, I hypothesised that the slower the process of gastric hydrolysis of protein in a protein rich meal, the longer it would be retained in the stomach. Therefore the rate of gastric emptying of a high protein content meal may be a predictor of the ability of the stomach to digest protein and subsequently a reflection of the “health” of the stomach in terms of its capacity to secrete pepsin and hydrochloric acid to effect normal protein hydrolysis.

Protein hydrolysis is not generally considered a determining factor of the dynamics of gastric emptying either in terms of the time from ingestion to the commencement of emptying (T_{lag}) or the rate at which the stomach empties ($T_{1/2}$). However, the control mechanisms of gastric digestion have not been fully elucidated. For example, the role of ghrelin, a 28 amino acid peptide and hormone, produced by cells lining the fundus of the stomach (and by other organs) amongst other functions is thought to regulate appetite (Cummings et al., 2002) and was only recognised in 1999.

Likewise obestatin, a putative hormone thought to decrease appetite (Depoortere, Thijs, & Peeters, 2008), was only discovered in 2005.

Delayed gastric emptying, a feature of aging (Clarkston et al., 1997), may reflect a progressively inefficient gastric acid and protease secretion capacity; however, the phenomena as such has no defined health implications. Furthermore, in the presence of diminished protease activity, increasing dietary protein (rather than amino acids) to compensate for impaired gastric hydrolysis, may compound the problem, resulting in longer gastric transit times and/or increased levels of undigested protein depositing into the large intestine. Increased quantities of colonic protein fermentation are known to influence the micro-biota population composition of the lower bowel and encourage multiplication of sulphide producing bacteria which exacerbate conditions conducive to inflammatory bowel diseases (Magee, Richardson, Hughes, & Cummings, 2000) and give rise to a proliferation of malignant precursors such as 'mucosal ulceration, goblet cell loss, apoptosis and distortion of the crypt architecture.' (Magee et al 2000)

4.3 Protein Digestion Efficiency

Protein digestion efficiency, a key determinant of livestock productivity, is measured in a number of ways including day-old chick growth rates, livestock daily weight gain, as well as egg, wool and milk production (Yoruk, Gul, Hayirli, & Macit, 2004). As a consequence many techniques have evolved in the agricultural sector to allow the digestibility of proteins to be optimised and commercial livestock feeds, with the appropriate nutrient composition, to be formulated for least cost (an early use of linear programming). Measuring protein digestibility generally involves a combination of animal feeding trials to determine growth rate, and nutrient profiling to verify the protein quantum and amino acid composition of the feed. As the aim of growth rate trials is to establish the protein's digestibility, selection criteria minimise inter-

individual variability by using same age, breed and gender animals. Decades of selecting for productivity within-breeds has minimised inter-individual variability to the extent productivity response to a known protein source is predictable, given animals of the same age and gender.

The same does not apply however to humans. Whilst there is comprehensive understanding of the digestibility of protein in the human diet, much of this has been derived from animal growth trials. There is a wide inter-individual variation in human protein digestion efficiency not found in productive livestock, due in part to greater genetic diversity as a result of non-selective breeding; higher exposure to environmental pollutants from confined living space, workplace hazards, exposure to tobacco smoke, alcohol, bacteria, food contaminants and some pharmaceuticals; and increased longevity because the principal determinants of death are from 'natural' causes.

A tendency to view human performance in terms of athletic strength and endurance perhaps masks everyday health as a fundamental measure of human performance. It is well accepted that advancing age is accompanied by increasing physical frailty and greater susceptibility to disease factors (Lovat, 1996). It is also well recognised that sarcopenia is an affliction associated with advanced age. The prevalence of sarcopenia in the North American population is estimated to be as high as 45% of those over the age of 65 years (Janssen et al., 2003).

Muscle mass is maintained by a balance of the dynamic anabolic and catabolic reactions that regulate protein synthesis, but the molecular pathways that control the process are not fully defined. Muscle atrophy from lack of use is well documented, and increased exercise has been shown to reverse the rate of muscle atrophy thus retarding the rate of muscle loss but it has not been able to halt it (Balagopal P, Schimke PK, Ades PA, Adey DB, & Nair KS, 2001). It is not known if the process of anabolism and catabolism act in isolation or in unison (Cuthbertson et al., 2005) but elderly people, in

contrast to younger people, do appear to retain protein mass better when consuming fast digesting proteins such as whey compared with slower digesting proteins such as casein (Dangin et al., 2003).

The absence of any one essential amino acid will impair the process of protein synthesis (Narita et al., 2011; Shikata et al., 2007). It is also known that a restriction on amino acid availability due to acute dietary deficiency, or increased demand in the case of severe disease, will result in loss of muscle mass. What is not clear is whether a lack of use, resulting in muscle atrophy, is the primary cause of sarcopenia, or if restricted access to one or more amino acids in a timely manner, constrains muscle anabolism and results in sarcopenia (Drummond et al., 2008). The efficiency with which older people (>60 years) digest protein has been the subject of debate for many years with some authors contending the ability to digest protein declines with advancing age (Bross et al., 1999) and others claiming age has little effect (Tiihonen, Ouwehand, & Rautonen, 2009).

4.4 Measuring protein digestion efficiency

Several methods have been used over the years to investigate the kinetics of human digestion, particularly gastric digestion. Earlier methods included gastric gavages, nasogastric suction, insertion of pH electrodes, radio telemetry (Heidelberg capsule) plasma amino acid analysis (Scriver, Clow, & Lamm, 1971) and radio isotope scintigraphy. All involve a degree of patient distress or safety and all have features that limited their use for routine assessment of protein digestion efficiency. Until recently gamma ray scintigraphy has been the principal technology for measuring gastrointestinal function, but a growing awareness of the risk to patient health from over exposure to ionizing radiation has limited the use of the technique.

X-ray computed tomography (CT scan) (Räty, Sand, Lantto, & Nordback, 2006) has also been used but again the exposure risk to ionizing radiation has reduced the use of this method to situations requiring urgent diagnosis.

Magnetic resonance imaging (MRI) has become a more acceptable method to measure features of digestion such as gastric emptying. MRI uses magnetism of atomic nuclei to create radio frequency fields that are captured by the scanner and converted to images rather than employing ionizing radiation. The cost of MRI however limits its use for everyday monitoring of digestive efficiency.

Ultrasonography is gaining greater acceptance in this field as the equipment is less expensive and more portable than MRI. It is generally accepted as a safe technology although over exposure has raised the possibility of neurological implications. However, the quality of ultrasound imaging is largely dependent on the skill of the operator which confines it to specialists' use rather than as an office based tool for routine diagnostic use. Also limitations on depth of imaging with this technology lessens its effectiveness on obese subjects.

Since its inception scintigraphy has been the 'gold' standard for measuring gastric emptying which is a complex, coordinated feature of digestion that controls the length of time food is retained in the stomach and the rate at which it is released into the duodenum. The complexities of the various mechanisms that determine the kinetics of gastric digestion make gastric emptying a victim of a number of pathological conditions. Identifying the precise kinetics of gastric emptying can provide a diagnostic insight into the presence and extent of some of these conditions, including liver function, fat absorption, CYP450 oxidase abnormalities, bacterial overgrowth, oro-caecal transit time in relation to nausea, vomiting, pain, bowel movement aberrations and small intestine absorption issues. Consequently, an accurate identifying of gastric emptying kinetics is an important diagnostic tool.

While the various techniques referred to are capable of measuring the kinetics of digestion, their everyday use is limited by factors such as the availability of equipment, skilled technicians, cost and patient safety issues. Furthermore, they do not measure protein digestion efficiency in a manner that would enable a physician to determine if a patient's protein digestion efficiency was deteriorating over time. More recent advances in plasma amino acid blood profiling (Higashiyama et al., 2011; Hurwitz et al., 1997) may provide a suitable option in the future, but the opportunity to regularly and cost effectively measure an individual's protein digestion efficiency is currently limited and as a result the debate as to whether protein digestion efficiency diminishes with age is unresolved.

Stable isotope breath testing is becoming a preferred method of assessing the kinetics of gastric emptying. The main advantage of this technology over the others, discussed above, is patient acceptance as the method is non-invasive and safe to use even during pregnancy. As there is no ionizing radiation involved, repeated tests can be performed without risk to patients or operators. Non-dispersive infrared spectrography (NDIRS), is now replacing isotope ratio mass spectroscopy (IRMS) for measuring isotope concentration because the equipment does not require a highly skilled technician to operate it; the equipment is readily portable, and the capital and operating costs are modest (Modak, 2007).

While there is currently no defined test method for measuring protein digestion efficiency *per se* with this technology, in the course of this study I have identified an existing test that with adaptation might prove effective and enable me to further investigate the role of kiwifruit protease on protein digestion *in vivo*. To this end I applied to the AUT Ethics Committee (Appendix 1) for approval to conduct a pilot study involving up to 15 participants, with the purpose of the study being to determine

if an adaptation to an existing approved ^{13}C Carbon breath test could accurately predict protein digestion efficiency.

4.5 Carbon-13 Breath Testing

While the number of protons in the nucleus of an atom defines the element, the number of neutrons may vary. These variations in number of neutrons, and therefore atomic mass, are known as isotopes of an element. Carbon-13 (^{13}C) is one of the 16 known isotopes of the carbon atom. Some isotopes are atomically unstable. An unstable isotope emits ionizing radiation when it degrades whilst a stable isotope does not. The differences in masses between isotopes mean they can be physically separated by diffusion and fractional distillation.

Carbon-13 makes up approximately 1% of the naturally occurring carbon on the planet. The ratio of specific isotopes in a given substance is termed enrichment. One of the standards for ^{13}C enrichment is a limestone derived from the marine fossil *Belemnitella Americana* and found in the Pee Dee formation in South Carolina. This material called Pee Dee Belemnite (PDB) has an unusually high ratio of ^{13}C to ^{12}C and is used as an international standard value against which ^{13}C enrichment is measured. As a consequence, because most enrichment is less than the PDB standard, the difference between ^{13}C enrichment of tested samples and PDB, tends to be a negative values. Measurement of ^{13}C abundance is expressed as a ratio $\delta^{13}\text{C}_{\text{VPDB}}$, ‰, which is the difference between the ratios of ^{13}C to ^{12}C atoms in the sample measured against the ratio of the ^{13}C to ^{12}C atoms in the standard PDB. The difference from the standard is expressed as a ratio in delta (δ) units as per the formula below:

$$\delta^{13}\text{C} (\text{‰}) = \left(\left(\frac{R_u}{R_s} \right) - 1 \right) \times 1000 \text{ ‰}.$$

In the formulae, R is the $^{13}\text{C}/^{12}\text{C}$ isotope ratio; U is the sample being measured; S is the standard PDB; ‰ is parts per thousand or atoms per thousand atoms. The

standard Pee Dee Belemnite Limestone (PDB) has an absolute value in terms of atomic ratios of 0.011273 or 11273 ppm also known as parts per million. For ^{13}C , one atom per million atoms change, $((11274/11273) - 1) \times 1000$, is equal to a change of 0.089 δ units. So if a typical breath sample returns a $\delta^{13}\text{C}$ (‰) reading of -25.00 this means there is 10,991-ppm of ^{13}C and 989009-ppm of ^{12}C , in the sample or roughly 1% ^{13}C .

The principle of ^{13}C breath testing consists of the usually oral administration of a ^{13}C labeled substrate that is metabolized by a specific enzyme system resulting in $^{13}\text{CO}_2$ as the end product. To monitor the enzyme response ^{13}C enrichment is measured in expired breath CO_2 (Ventrucci, Cipolla, Ubalducci, Roda, & Roda, 1998). If the substrate is metabolised in the liver for example, the ^{13}C will enrich CO_2 and be carried as $^{13}\text{CO}_2$ in the systemic venous blood, released in the lungs and detected in timed samples of exhaled breath. By measuring the concentration of ^{13}C exhaled in breath prior to ingesting a known dose of ^{13}C and sequentially measuring the concentration in breath samples after ingestion, the kinetics of the digestion of the compound can be determined. By comparing the curve of concentration versus time to 'normal' kinetics the existence of a specific disease or infection and the extent of the damage caused may be indicated. The standard test for measuring the kinetics of gastric emptying is the ^{13}C -octanoic acid breath test (^{13}C -OABT). While it is acknowledged that diet also effects the ^{13}C concentration of individuals, the principle of the ^{13}C breath test is to measure the difference in the concentration before and after the intervention. The pre-existing concentration has no influence on the rate at which the administered dose is exhaled.

4.6 ^{13}C -Octanoic Acid Breath Test (OABT)

Octanoic acid is an eight carbon saturated fatty acid and a naturally occurring component of mammalian milk, especially sheep and goats, and some plant based oils

(coconut). Its relatively short chain structure enables it to be rapidly absorbed across the duodenal mucosa. In the gastric environment octanoic acid is retained in the solid phase of a meal, but the solid phase rapidly disperses in the duodenum and octanoic acid is absorbed into the blood, delivered to the liver and oxidized to $^{13}\text{CO}_2$. This is then transported to the lungs and expelled in exhaled breath which may be sampled for analysis. Only about 50% of the original dose is ever recovered in the breath with the balance lost to the bicarbonate reserves and later excreted. The rate limiting step in the timing of the process from mouth to breath is the retention time in the stomach; absorption from the duodenum and oxidation in the liver have been shown to be very rapid (Ghoos et al., 1993).

4.7 Validation of ^{13}C -OABT as a reliable *in vivo* measure of protein digestion efficiency

Concept

Mass isotopomer distribution analysis (Hellerstein & Neese, 1992), developed in the early 1990s, was the forerunner of the ^{13}C -octanoic acid breath test, developed to measure the kinetics of gastric emptying (Ghoos et al., 1993; Maes et al., 1994; Sidossis, Coggan, Gastaldelli, & Wolfe, 1995). Octanoic acid binds well to egg yolk which holds the ^{13}C labelled acid in the solid phase during passage through the stomach. Octanoic acid is not digested or absorbed in the stomach so no ^{13}C appearing in the exhaled breath originates from this phase of digestion.

While there is currently no defined standard meal to accompany this test method, most researchers use the same or a similar test meal as the originators of the method (Ghoos, Y.F. and Maes, B.D. et al). This consisted of two scrambled eggs on two slices of white bread spread with five grams of margarine and accompanied by 150 mL of

water. The ^{13}C -octanoic acid in powder form (100 mg) is initially mixed with the egg yolk to bind it in the solid phase, then scrambled with the egg whites prior to cooking.

Gastric emptying of this meal would proceed as for a reasonably balanced meal containing the three macronutrients, with bread providing carbohydrate, margarine and 50% of the egg yolk providing fat and the other 50% of the egg yolk and the egg albumin providing protein (Jonderko, Kasicka-Jonderko, Syrkiewicz-Trepiak, & Blonska-Fajfrowska, 2005; Maes et al., 1998)

Data Analysis of ^{13}C breath samples

Breath samples submitted for IRMS analysis are returned as delta values in the form $\delta^{13}\text{C}_{\text{VPDB}}$, ‰. To generate the parameters of gastric emptying the delta values are transposed and express as instantaneous fractions of the ^{13}C dose that was administered in the meal and subsequently recovered in each breath sample. This fraction of the dose recovered (FDR) was then plotted on the y-axis of a graph against time on the x-axis. The resulting curve (**Figure 4.1**) shows that at time zero, which represents the mean value of the two pre-prandial samples, the FDR was zero. With each subsequent breath sample the fraction of the dose recovered increased to a maximum. The maximum or peak represents the maximum rate of gastric emptying. From the peak, the fraction of the dose recovered in subsequent breath samples diminished gradually over time.

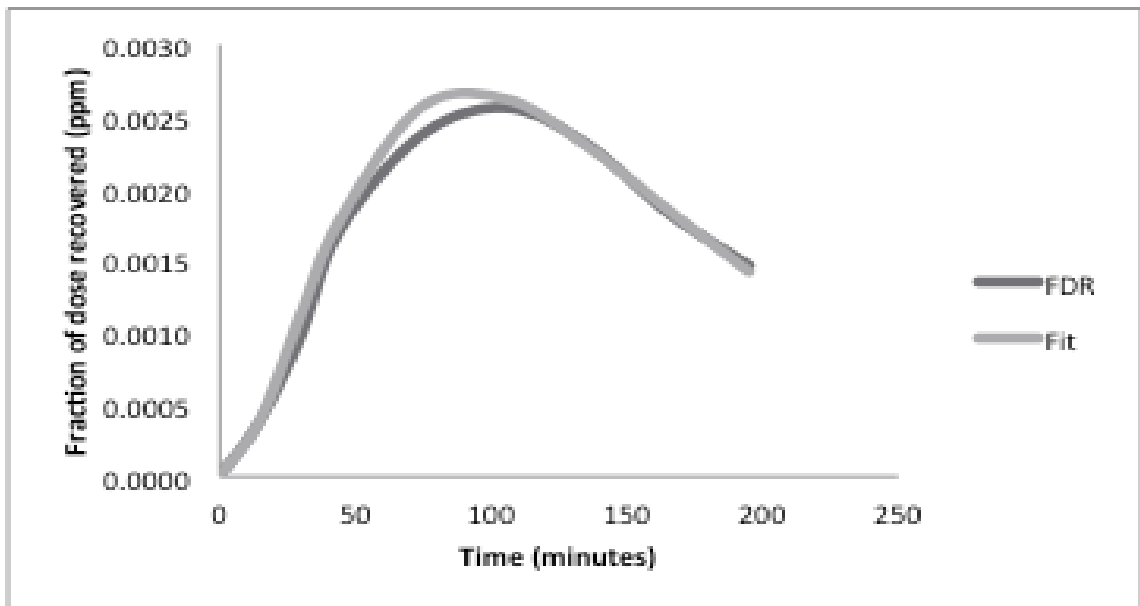


Figure 4.1 Instantaneous dose recovered is referred to as the fraction of dose recovered (FDR) and is the measured fraction of the ^{13}C dose recovered in the breath over time. Fit is the formula derived calculation of the recovery curve based on the individual's physiological characteristics.

Parameters of Gastric Emptying

There are two principle parameters of gastric emptying used to compare inter-individual and intra-individual effect of interventions and as a stand-alone diagnostic tool. The first is the time taken from the start of the test (post-prandial) to the maximum rate of ^{13}C gastric emptying, which corresponds with the peak of the graph. This is referred to as the lag time or T_{lag} and is represented as T_{lag} . The second is the calculated time from the start of the trial (post-prandial) until, theoretically, only 50% of the dose administered remains in the stomach. This is referred to as time half or T_{half} and represented as $T_{1/2}$.

Calculating T_{lag} and $T_{1/2}$

Parameters and formulas used in the calculation

Participants weight (kg)	P.wt
Participants height (m)	P.ht
Participants age (y)	Age
Participants gender	M or F
Dose ^{13}C -octanoic acid (mmol)	Mass (mg)/145 mg/mmol
^{13}C standard PDB (ppm)	11237.2
TEE	Total energy expenditure
PAL	Physical activity level
BMR	Basal metabolic rate
BMR (Schofield's equation) (MJ/d) Male	$((15.057 * \text{P.wt}) + (0.1 * \text{P.ht}) + 705.8) * 0.0041868$
BMR (Schofield's equation) (MJ/d) Female	$((12.623 * \text{P.wt}) + (2.83 * \text{P.ht}) + 98.2) * 0.0041868$
TEE (kJ/min) (PAL 1.1)	$1.1 * \text{BMR} / 24 / 60 * 1,000$
Energy Equivalence (EEq) (kJ/L)	$15.48 / 0.85 + 5.55$
CO_2 prod (mmol/min of CO_2 produced)	$\text{TEE} / \text{EEq} / 22.4 * 1,000$
ppm	$((\delta^{13}\text{C}_{\text{VPDB}}, \text{‰} / 1,000) + 1) * \text{PDB}$
ppm excess (ppm xs)	Difference between pre-prandial and post-prandial ppm
FDR	Instantaneous fractional recovery of label, fraction of dose recovered
CDR	Cumulative fractional recovery of dose or cumulative dose recovered

$\text{FDR} = (\text{ppm xs} / 1,000,000 * \text{CO}_2 \text{ prod} / \text{dose})$. This was plotted on the y-axis against time on the x-axis to produce the graph illustrated above (**Figure 4.1**)

The fitted curve can be generated from either the fraction of dose recovered over time or the cumulative dose recovered over time. In this case the former was used. The instantaneous fractional recovery of label was determined by integration of the area under the curve by applying the trapezium rule; to give the cumulative fraction of dose recovered, as illustrated below (**Figure 4.2**).

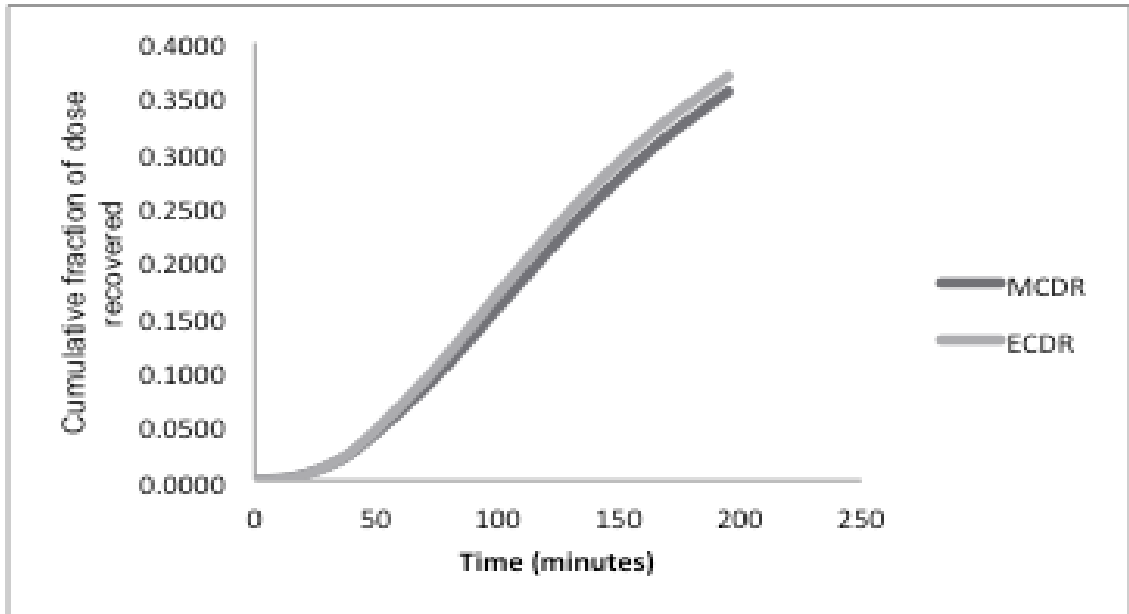


Figure 4.2 An example of the curve of the cumulative fraction of the dose recovered over time. MCDR is measured cumulative dose recovered; ECDR is the extrapolated cumulative dose recovered.

The theoretical fraction of dose recovered is referred to as Fit FDR and this is calculated using the following formula (source Bluck, L. Medical Research Council Human Nutrition Research, Elsie Widdowson Laboratory, Cambridge, United Kingdom):

$$\text{Fit FDR} = m \cdot k \cdot \beta \cdot (1 - \text{EXP}(-k \cdot T))^{(\beta - 1)} \cdot \text{EXP}(-k \cdot T).$$

Where m , which affects the amplitude of the curve, is the theoretical total dose recovered at infinity and is usually about 50% of the dose administered, the balance is lost in the tricarboxylic or citric acid cycle (Sidossis, Wolfe, & Coggan, 1998); k is the terminal slope of a semi logarithmic plot of the dependent variable, FDR (y axis) on the independent variable, time (x axis); and β is responsible for the 'lag' at the beginning of the curve. While the variables, k and β have no simple physiological meaning they do influence the shape of the curve. The sums of the squared residuals between the observed and calculated values for FDR are calculated to give a figure that indicates the closeness of fit between observed and predicted values. Using nonlinear regression analysis the values of m , k and β can be varied to minimise the difference between the

sums of the squared residuals and create a curve that best fits the observed data. Initial values used for the variables in the regression formula are:

$$m = 0.5; k = 0.01; \beta = 2.5$$

The final values of k and β are then used to calculate the two parameters of gastric emptying T_{lag} and $T_{1/2}$ described above using the following formulae:

$$T_{lag} = \text{LN}(\beta)/k$$

$$T_{1/2} = -\text{LN}(1 - 0.5^{(1/\beta)})/k$$

4.8 Discussion

The aim of this thesis was to investigate if kiwifruit protease has a measurable effect on digestion and, if so, to determine the mode of action. The *in vitro* study (**CHAPTER 3**) found that kiwifruit protease had little effect on protein digestion at pH concentrations equivalent to those found in the small intestine (pH 6.4–7.0).

As a result, the next stage of the investigation would concentrate on measuring the effects of kiwifruit on the hydrolysis of protein in an *in vivo* gastric environment. The challenge was to find a method of measuring gastric digestion of protein *in vivo* that was ethical, affordable, and acceptable to people willing to participate in a trial. The criteria, established to ensure participant safety and ongoing compliance but also to accommodate fiscal constraints, negated methods used by other researchers in this field. The purpose of this chapter was to identify a method of measuring gastric protein digestion efficiency *in vivo* that could meet the criteria. As the products of gastric digestion of protein are thought to influence the kinetics of gastric emptying (Erickson & Kim, 1990), measuring the changes in the rate of gastric emptying of a proteinaceous meal, with and without the addition of kiwifruit protease, could provide a method to determine if the added kiwifruit protease influenced protein digestion *in vivo*. This led to an exploration of the possibility of using of the ^{13}C -octanoic acid breath test to

measure the kinetics of gastric emptying as a proxy for protein digestion, by modifying the meal component of the test from a nutritionally acceptable balance of macronutrients as used by other researchers, to a predominance of protein. Maes (2002) and Ghooos (1993) had used the same balanced nutrient meal composition; Peracchi (2000) used a similar composition although fat and protein composition was increased, whereas Bromer (2002) pioneered a largely carbohydrate dominant meal. All achieved comparable results.

Table 4.2 Comparison of the meal composition designed for this study with those used by other researchers, demonstrating the protein dominance

Article	Meal	Composition (%)			Mean Age	Gender
		CHO	Fat	Pro		
Peracchi (2000)	250kcal	42	40	18	28 years	5F/5M
Bromer (2002)	350kcal	82	9	9	34 years	6F/4M
Maes (2002)	250kcal	47	33	10	23 years	10F/10M
Ghooos (1993)	250kcal	47	33	10	22 years	20F/20M
Donaldson (2013)	110kcal	0	40	60	70 years	9F/2M

CHO = carbohydrates; Pro = protein; F = female; M = male

The ^{13}C -octanoic acid breath test is a proven method of measuring gastric emptying and it met the criteria for a trial method, but the literature review did not disclose any previous use of the technique for measuring protein digestion efficiency of a protein-dominant meal. The next chapter investigates the feasibility of using the ^{13}C breath test to measure kiwifruit protease influences on protein digestion efficiency *in vivo*.

CHAPTER 5. Proof of principle — an *in vivo* feasibility study

The initial hypothesis that the protease in kiwifruit was likely to be the active ingredient responsible for the fruit's reputation for relieving constipation and the discomfort of digestive dysfunction (Wyeth, 2011) was supported by the chemistry of the *in vitro* study. Kiwifruit protease (KFI) appeared to have maximum activity on the *in vitro* hydrolysis of meat protein during 60 minutes incubation at 37°C, when the pH of the matrix was greater than 2.5 and less than 6.0 (**CHAPTER 3**). From this I concluded that if the action of kiwifruit protease on dietary protein digestion was responsible for the *in vivo* effect, then protease hydrolysis of the protein would most likely start in the stomach because the *in vitro* study indicated the pH of the small intestine could be expected to negate the activity of the protease (KFI).

After consumption of a solid meal, gastric emptying into the duodenum is, in part, dependent on pH, meal volume, particle size and the osmolarity of the digestate (Low, 1990). Osmolarity is increased during the hydrolysis of protein to peptides and amino acids. Lipids are known to delay gastric emptying (Low, 1990), but polypeptide products of protein digestion are also known to have a controlling effect on gastric emptying (Erickson & Kim, 1990; Freeman & Kim, 1978; Jahan-Mihan et al., 2011). Therefore it was hypothesised that after a standardised protein-based meal the rate of hydrolysis of the protein would have a controlling influence on the rate of gastric emptying. Furthermore, if the addition of kiwifruit to a meal altered the rate of protein hydrolysis, the effect should be able to be observed by comparing the rate of gastric emptying after the consumption of two identical meals, one with and one without the inclusion of the kiwifruit protease.

A method of measuring, the effect of kiwifruit protease on gastric emptying kinetics, *in vivo* was sought that met the criteria of (i) non-invasiveness, (ii) minimal

burden of discomfort for participants, (iii) ready availability of the technology required and (iv) an affordable price. Of the methods considered, none met all five criteria other than the octanoic acid stable isotope breath test for measuring the kinetics of gastric emptying (Modak, 2007; Perri, Pastore, & Annese, 2005), but this test method had not previously been used specifically to measure protein digestion. The aim of this part of the study was to explore the feasibility of using the ^{13}C breath test to measure gastric emptying as a proxy for gastric digestion efficiency of protein.

5.1 The principle of the ^{13}C -octanoic acid breath test

Briefly, the principle of the ^{13}C -octanoic acid breath test (^{13}C -OABT) is that ^{13}C -labelled octanoic acid, mixed with lipid as part of a solid meal, is retained in the stomach during the passage of the meal but when the meal particles reach the duodenum the octanoic acid is released, absorbed and then oxidized to $^{13}\text{CO}_2$ in the liver. The rate-limiting step is the emptying into the duodenum because absorption into the portal vein and oxidation in the liver is almost immediate. The concentration of ^{13}C is measured before consumption of the meal and then at regular (15 minute) intervals after consumption and for up to three hours. The percentage of the dose of ^{13}C recovered over time is a measure of gastric emptying kinetics.

However, no reports of the use of the ^{13}C -OABT to measure the gastric emptying dynamics of a protein-based meal were found after an extensive literature search. Therefore one of the aims of this study was to search the literature relating to ^{13}C -OABT and alternative tests and assess the feasibility of using the ^{13}C -OABT with a protein based meal in terms of recruitment and compliance by volunteers with the test, meal preparation and palatability, the reliability of measures and the analysis of the data. The repeatability of the test results is investigated in Chapter 6.

Measuring the rate of gastric emptying of a known dose of ^{13}C Carbon blended with a protein-dominant meal, rather than a meal balanced for macronutrients, as is normally the case for the ^{13}C -OABT, it was hypothesised that any changes in the kinetics of gastric emptying due to the inclusion of kiwifruit protease to the meal would be a measure of an effect from the protease.

5.2 Rationale for choosing to test the feasibility of measuring gastric emptying using ^{13}C -OABT to determine protein digestion efficiency

Release of nutrients from the stomach to the duodenum (gastric emptying) is determined by the chemical and physical characteristics of the ingested food (Jahan-Mihan et al., 2011) including the osmolarity, pH, temperature, viscosity, energy density and volume. However Khoshoo et al (2002) demonstrated protein digestion has an overriding effect on controlling gastric emptying that exceeds these other controlling factors. This was ably demonstrated by Khoshoo et al.(2002) when they fed two different formulations containing the same whey protein to two groups of children (Khoshoo & Brown, 2002). Despite having different osmolarity (270 and 450 mOsm/kg and energy content (4.18 kJ/ml and 6.27 kJ/ml) the two formulations emptied at the same rate determined at 30, 60, 90 and 120 minutes. Jahan-Mihan et al. (2011) explain that peptic digestion of protein results in a range of peptides some of which display bioactivity. These bioactive peptides (BAP) influence the rate of nutrient release from the stomach to the duodenum by the effect they have on the release of the gut hormones cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide-1 (GLP-1). The BAP /gut hormone trigger delays gastric emptying by regulating pyloric pressure and gastric motility. Furthermore GLP-1 also moderates gastric emptying by way of the 'ileal break' mechanism.

The validity of the ^{13}C -Octanoic Acid Breath test for measuring the dynamics of gastric emptying is well established and supported by the literature. The overriding effect of protein digestion on the dynamics of gastric emptying is also supported by the literature (Jahan-Mihan et al., 2011). Therefore, for this study it was considered necessary to test the feasibility and repeatability, but not the validity of the method, to measure gastric emptying determined by protein digestion.

Criteria considered in relation to the feasibility of the ^{13}C -OABT were:

- To expedite recruitment of participants, the method must be non-invasive — this eliminated endoscopy, biopsy, phlebotomy and possibly the Heidelberg capsule (Mojaverian, 1996) although cost and availability were also issues with that technique.
- To protect the participants, safety was paramount. This eliminated scintigraphy, which uses a meal labeled with metastable technetium-99 that emits gamma ionizing radiation (Kowalsky, 2006)
- With a limited budget the method had to be affordable, especially as its later use in an expanded study was anticipated — this eliminated MRI — magnetic resonance imaging.
- The method had to meet ethical standards and be acceptable to the AUT University's Ethics Committee.

These criteria eliminated most of the methods previously used to measure changes in protein digestibility and as a result encouraged the consideration of alternatives. While no evidence could be found of previous validation or use of stable isotope breath testing for the purpose of measuring protein digestion efficiency, it was, however, well accepted (Maes et al., 1998) as a viable alternative to scintigraphy for

measuring gastric emptying. Having established from the literature that protein digestion has a controlling effect on gastric emptying (Jahan-Mihan et al., 2011) even when fat is present in the formulation, the rationale for using the ^{13}C -OABT to measure the efficiency of gastric emptying was based on the fact that the standard meal used in the proposed tests would be protein dominant (three egg whites, with only sufficient fat (one egg yolk) to bind the octanoic acid during passage through the stomach. Furthermore, the diminutive size and energy content of the test meal (155 mL; 170 kcal) was not expected to impose a volume effect on the stomach that might influence gastric emptying dynamics, as might be expected of a more substantial meal of >1000 mL.

5.3 Study design

The experimental study design was a pre- and post-test. This required two visits where the participant was asked to fast overnight and present to the laboratory in the early morning for breakfast. On the first visit, a breakfast meal consisting of an omelette containing a measured dose of ^{13}C ; on the second visit, seven days later, an identical meal was eaten with the addition of six capsules of the kiwifruit protease. Each 360 mg capsule contained 330 mg of Zyactinase™ a shelf-stable, nutritionally homogeneous, freeze dried kiwifruit powder, the same product used in the *in vitro* study and profiled in CHAPTER 3. Rather than use and potentially promote Zyactinase or the capsule composition, it is simply referred to as kiwifruit isolate (KFI) in the following chapters.

The meal prepared was a four egg whites (100% protein energy as dried mass) mixed with one yolk (33% protein, 67% fat, energy) plus the measured dose of ^{13}C -octanoic acid. Because egg yolk is 67% fat and 33% protein by dried weight, only one yolk was included in the omelette that was intended to be a high protein meal. From the New Zealand food composition tables (Lesperance, 2009) using grade 7 eggs (62 g

edible), the approximate composition of the meal would be: ~19.7 g protein, (14.7 g from the egg whites and 5 g from the yolk) and ~10 g of fat. In terms of energy the meal constituted 170 kcal of energy, 47% from protein and 53% from fat.

The concentration of KFI in the *in vivo* study was half that used in the *in vitro* study i.e. the ratio of KFI protein to meal protein in the *in vivo* study was approximately 1:232 (wt/wt) whereas the rate of KFI protein to meat protein used in the *in vitro* study was 1:118.

To prepare the dosed omelettes, exactly 100 mg of powdered ^{13}C -octanoic acid was whisk-blended with the egg yolk. The whites were then blended in with the yolk prior to cooking for approximately three minutes at a medium heat in a 'non-stick' fry pan. No other food was to be offered with the meal, but water (250 mL) was to be available to consume at will. Before consumption of each meal (pre-prandial), two breath samples were collected, 15 minutes apart. The mean was the ^{13}C enrichment of the individual's baseline exhaled breath.

Baseline $^{13}\text{CO}_2$ enrichment can alter depending on diet as the enrichment of ^{13}C in plant and animal derived foods depends on the number of steps through the biosynthesis pathway. For example, maize (corn) and cane sugar are so called C4 plants that have fewer steps in their photosynthetic pathway than C3 plants like wheat, and, for reasons unimportant to this study, have a higher $^{13}\text{C}/^{12}\text{C}$ ratio in the resulting hexose sugars.

Eight post-prandial breath samples were collected over the following 180 minutes with the first collected 15 minutes after completion of the meal. Breath samples were analysed using isotope ratio mass spectroscopy (IRMS) to determine ^{13}C enrichment. From these measurements key parameters of the kinetics of gastric emptying were computed using non-linear regression analysis as detailed in the previous chapter. Two parameters were then to be used to assess the dynamics of

appearance of the ^{13}C biomarker in the carbon dioxide of expired breath. These parameters were T_{lag} (the time from ingestion of the ^{13}C dose until the maximum rate of output of $^{13}\text{CO}_2$ was detected on the breath) and $T_{1/2}$ (the time from ingestion of the ^{13}C dose until only 50% remained in the stomach), as detected in the breath.

The rationale and procedure for this *in vivo* intervention study was approved by the AUT Ethics Committee - AUTECH 09/284 (Appendix 1)

5.4 Method for Study 2 - an *in vivo* study to test method feasibility

Volunteers for the study were recruited from (apparently) healthy older adults. Recruitment was mainly from a group of older adults (58–80 y) attending the AUT University YMCA fitness program known as Never Too Old™. Exclusion factors included previous gastric surgery including lap-banding, and known kiwifruit allergy.

Prior to the commencement of an exercise programme at the university gymnasium, I was given the opportunity to speak to the group to explain the study and invite volunteers. Those who were interested were given a written information sheet, that explained the purpose of the study and what was required of the participants. Those who agreed to proceed provided their contact details, and appointments were arranged for them to attend the AUT Nutrition Laboratory on a date of their choice. Each participant was phoned the night before to confirm their attendance and to remind them of the requirement to fast overnight and prior to the trial i.e. no breakfast, tea, coffee or juice — only water. No inducements were offered to the participants other than a commitment to share with them any knowledge gained from the study that may benefit them. Opportunities were given for further explanations and to answer questions throughout all interactions. All participants signed consent forms before any testing was undertaken. Consent forms were filed in a secure cabinet with access restricted to the researcher and supervisor to ensure confidentiality. Each participant

was allocated a trial alphanumeric code number to maintain the anonymity of the data collected.

Participants arrived at the laboratory at 0800 hours and confirmed that they were fasting. Demographic and anthropometric data were recorded for each participant which included, age, gender, weight, height, waist measurement, grip strength and blood pressure (**Table 5.1**). In total, 11 people participated in the study, nine women and two men, all Caucasian. Each person provided details of their routine medication regimes as well as an informal assessment of their overall fitness based on their reported exercise programme. Participants were invited to relax in comfortable seating for at least 15 minutes before any breath was collected.

Two expired breath samples were collected 15 minutes apart from each subject to establish fasting breath $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratios for each person. At the end of a normal breath the participant was asked to breathe in and then halfway out before blowing through a straw into a nominally 400 mL foil bag with a three-way tap. The straw was then removed and a hypodermic needle was fitted to the tap. Two identical 10 mL Exetainer tubes (Labco, Ceredigion, UK), pre-labelled with the participant's code and sample number, were filled with expired air from the foil bag. The needle on the tap of the foil bag was then inserted through the rubber seal of the tube. The tap on the foil bag was then opened and gentle pressure applied to the bag to expel the expired breath sample into the tube. A second hypodermic needle was then inserted into the tube to act as a one-way relief valve to allow some of the sample air to escape from the tube under pressure thus ensuring the tube was thoroughly flushed and filled with sample air. The relief valve needle was then drawn out of the tube seal while still maintaining pressure on the sample bag. Finally, the sample bag needle was drawn from the Exetainer tube and the sample bag tap closed. This procedure ensured that the CO_2 content of the sample was maximised and the gas maintained under slight pressure in the tube.

The participants were then asked to consume their freshly prepared meals in less than 10 minutes. The first post-prandial breath sample was collected 15 minutes after the meal was finished; five more samples were collected at 15 minute intervals after the first post-prandial sample and a further three were collected at 30 minute intervals, giving a total of nine samples collected over three hours post-prandial, plus the two pre-prandial samples, making 11 samples per participant in all. Some duplicate samples were taken which were non-sequentially labeled and sent for analysis as a check on the accuracy of the analysis results.

One week after the first test meal the participants returned to the laboratory and repeated the procedure outlined above with the addition of 6 x 360 mg capsules of KFI consumed immediately prior to consuming the meal.

One Exetainer tube from each breath recorded was retained in the laboratory under refrigeration at 4°C and the second was couriered to the Department of Chemistry — University of Otago for analysis using a Finnigan Thermo DeltaPlus XP isotope ratio mass spectrometer coupled to a Trace GC Ultra with a combination PAL auto-sampler.

The following laboratory reference materials were used to determine isotopic values: $\delta^{13}\text{C}$ (‰) IRU-Bicarbonate -5.48 ± 0.12 and IRU-Marble 2.10 ± 0.06 . IRU-Marble was used to determine the precision and accuracy. Its accepted $\delta^{13}\text{C}$ (‰) value is 2.10 ± 0.06 but the measured value was 2.03 ± 0.10 ($n = 2$). Drift correction and peak area calculations were performed by customised software supplied by the mass spectrometer manufacturer.

IRMS analysis of breath samples was returned from the laboratory in the form of δ units for each breath. For example: $\delta^{13}\text{C}_{\text{VPDB}}$, ‰ = -25.67, this value can be converted to atoms percent using the following formula:

$$[(-25.67 \delta^{13}\text{C}_{\text{VPDB}}, \text{‰ units} / 1,000) + 1] * 11,237.2 (\text{PDB}) = 10,948.7 \text{ ppm} = 1.095\%$$

That is, 1.095% of the carbon atoms were ^{13}C and the balance, were ^{12}C .

Gastric emptying parameters T_{lag} and $T_{1/2}$ were calculated from the ^{13}C enrichment analysis using the method described in the previous chapter.

5.5 Post-prandial breath sample analysis

From the physical characteristics of each individual participant, the carbon dioxide output in mmoles/minute is estimated (**Table 5.1**) using basal metabolic rate predicted from their weight age and gender (Schofield, 1985) with 10% added to account for the dietary induced thermogenesis of the meal. This estimate is required to be able to calculate the fraction of dose recovered (FDR) over the course of the test and from this to calculate the parameters of gastric emptying i.e. T_{lag} and $T_{1/2}$ from the shape of the curve of the plot of the concentration of $^{13}CO_2$ vs. time (**Figure 4.1**). The use of a predictive equation to determine an individual's CO_2 production was considered preferable to the use of metabolic measurement systems such as Metabolic Carts, CPX systems or Ergo spirometers which require calibration that can introduce errors due to equipment faults and operator inexperience which give inaccurate results. The Douglas Bag technique, (introduced in 1911 and for some time regarded as the 'gold standard') was also considered. However, poor accuracy of the Douglas bag method (Shephard, 1955) resulting from two-way breathing valve leaks, inaccurate assessment of the captured gas volume even with use of large and perfectly maintained spirometers and conditions precluding proper measurement of gas concentrations due to diffusion of gases and dilution of CO_2 in water wetting the inner walls of the spirometer as well as condensation developing in the collection bags, have the potential to introduce errors the extent of which can not be determined. As a result the prediction of the rate of CO_2 production was employed. Furthermore, the effect of changing the predicted BMR on the calculation made very little difference. The following table demonstrates the information required to calculate an individual's CO_2 production.

Table 5.1 Example of each participant's physical characteristics required to calculate the predicted CO₂ content of an exhaled breath, in a resting state

Subject ID	OK04	¹³ C Carbon dose (mmol)	0.710344
Test	1 of 2	Pee Dee Belemnite (ppm)	11,237.2
Weight (kg)	49	Basal metabolic rate (MJ/day)	4.92
Height (m)	1.616	Total energy expended kJ/min)	3.75
Age (years)	67	Physical activity level 1.1 (MJ/day)	5.40
Gender	F	Energy equivalence (kJ/L)	23.76
Dose of octanoic acid (mg)	103	CO ₂ production (mmol/min)	7.054628

The formula used to calculate the fraction of dose recovered (FDR) is as follows:

$$\text{FDR} = (\text{ppm } ^{13}\text{C excess} * 1,000,000) * (\text{CO}_2 \text{ produced} / ^{13}\text{C dose administered})$$

The calculation of ¹³C excess (ppm), is derived from the ¹³C (ppm) recorded in the post-prandial breath samples over time in excess of that recorded in the pre-prandial breath samples, which establishes the individual's baseline breath ¹³CO₂ concentrations prior to consuming the test meal containing the ¹³C-octanoic acid dose. Cumulative dose recovered (CDR) can then be calculated at each sampling point. This is followed by curve fitting using non-linear regression analysis described in Section 4.1. A typical result for an individual participant is shown in the following table.

Table 5.2 Example of the table format used to generate FDR and CDR values, which are then plotted against time to produce the curves (Figures 4.1 and 4.2 previous Chapter) depicting the kinetics of gastric emptying of the ¹³C enriched meal.

Time (min)	δ ¹³ C PDB*	Breath ¹³ C (ppm)**	Excess ¹³ C (ppm)***	FDR measured†	FDR fitted††	CDR measured°	CDR fitted°°
0	-26.70	10937	0	0.0000	0.0000	0.000	0.000
15	-20.88	11003	65	0.0006	0.0008	0.005	0.005
30	-11.77	11105	168	0.0017	0.0016	0.022	0.023
45	-5.43	11176	239	0.0024	0.0022	0.053	0.053
60	-4.51	11186	249	0.0025	0.0025	0.089	0.088
75	-4.70	11184	247	0.0025	0.0026	0.126	0.127
105	-6.42	11165	228	0.0023	0.0023	0.197	0.200
135	-11.04	11113	176	0.0017	0.0018	0.257	0.261
165	-14.10	11079	142	0.0014	0.0013	0.304	0.307

*Recorded breath sample delta values; ** Calculated parts per million of ¹³C; ***Difference between post and pre-prandial values; † Measured fraction of ¹³C dose recovered; ††Calculated FDR from curve fitting; °Measured cumulative ¹³C dose recovered (CDR); °° Calculated CDR from curve fitting.

Statistical analysis

Raw baseline $\delta^{13}\text{C}$ delta values and calculated T_{lag} and $T_{1/2}$ values derived using non-linear regression analysis were compared within an individual using paired t tests. Measurement error (biological and methodological) was determined from one-way analysis of variance with participants as the factor. Unless otherwise stated, data in the text is expressed as means \pm standard deviation (SD). Statistical significance was set at $p < 0.05$. Analyses were carried out using a combination of IBM[®] SPSS Statistics version 19.0, Sigma Plot Version 10, Microsoft[®] Excel[®] Version 14.2.3 and Addinsoft[™] XLSTAT version 2013 1.01 packages.

5.6 Results of the *in vivo* Feasibility Study

Participant characteristics

Participants, nine women, two men, were aged between 58 and 80 years and were all Caucasian and apparently healthy (**Table 5.3**). Three of the participants had body mass indexes $>25 \text{ kg/m}^2$, and therefore could be classified as overweight, while one woman and one man were borderline centrally obese (waist/height ratio >0.5). No participant had high blood pressure (systolic $>140 \text{ mmHg}$) although four (three women and one man) were prescribed ACE inhibiting medication (angiotensin-converting-enzyme inhibitor) and four (three women and one man) were consumers of prescribed acetylsalicylic acid. The most frequently prescribed medication (four women and one man) was statins (or HMG-CoA reductase inhibitors) to reduce blood plasma cholesterol. Three participants (all women) were taking prescription proton pump inhibitor (PPI) medication.

Table 5.3 Physical characteristics of the 11 participants (nine women, two men)

Measure	Mean \pm SD	Range
Age, years	70 \pm 7	(58, 80)
Weight, kg	63 \pm 10	(49, 86)
Height, cm	162 \pm 7	(151, 174)
Body Mass Index, kg/m ²	24.2 \pm 2.7	(18.8, 28.2)
Waist, cm	78 \pm 8	(61, 88)
Waist/Height	0.48 \pm 0.05	(0.38, 0.53)
Blood pressure – Systolic, mmHg	132 \pm 8	(113, 143)
Blood pressure – Diastolic, mmHg	77 \pm 6	(66, 89)
Pulse, beats per minute	66 \pm 7	(51, 73)
Proton Pump Inhibitor	3/11	3 women, 0 men

Variation in pre-prandial breath ¹³CO₂

Within each individual there was no difference between the ¹³CO₂/¹²CO₂ in fasting expired breath samples taken within 15 minutes of each other viz. C1 and C2 and, a week later, T1 and T2; (**Table 5.3**). The average value of the paired samples was not different between the first and second visits ($P = 0.65$, **Table 5.1**). Variation in the ¹³CO₂/¹²CO₂ of fasting breath samples among participants and between visits was trivial; the average of the paired samples ranged from -26.7 to -23.7 δ units. The control visit mean was -25.4 δ units (95% CI -25.9 to -24.8) and the second (treatment) visit mean was -25.2 δ units (-25.8 to -24.6) and these were not statistically different.

Table 5.4 Fasting, pre-prandial ¹³CO₂/¹²CO₂ content of breath samples

	n	Mean \pm SD	Mean diff \pm SD	95% CI mean diff	P*
First visit					
Pre-prandial C1	11	-25.32 \pm 0.88	0.08 \pm 0.05	-0.71, 0.86	0.84
Pre-prandial C2	11	-25.39 \pm 0.89	0.32%		
One week later					
Pre-prandial T1	11	-25.18 \pm 1.01	0.019 \pm 0.00	-0.82, 0.83	0.99
Pre-prandial T2	11	-25.18 \pm 0.84	0.08%		
Average of paired samples					
C1 + C2 Mean	11	-25.35 \pm 0.86	-0.17 \pm 0.12	-0.95, 0.61	0.65
T1 + T2 Mean	11	-25.18 \pm 0.89	0.67%		

*Paired t test; C control test; T Treatment test

The mean $-25.27 \delta^{13}\text{C}_{\text{VPDB}}$, ‰ units can be expressed as follows:

$$[(-25.27 \delta^{13}\text{C}_{\text{VPDB}}, \text{‰ units} / 1,000) + 1] * 11,237.2 (\text{PDB}) = 10,952.9 \text{ ppm} = 1.095\%$$

That is, 1.095% of the carbon atoms in the baseline sample were ^{13}C , the balance were ^{12}C . As the delta value increased (became less negative) the percentage of ^{13}C increased. Two standard deviations i.e. $\pm 1.6 \delta^{13}\text{C}_{\text{VPDB}}$, ‰ units either side of the mean gave a range of 10,935 - 10,971 ppm or 1.094 - 1.097% (PDB enrichment is 11,237.2 ppm or 1.124%).

Calculations of T_{lag} and $T_{1/2}$ with and without kiwifruit treatment

Overall the effect of KFI treatment was not apparent from the results generated (**Table 5.4**). There was a slight increase in mean T_{lag} by 7.4% ($P = 0.21$) and the mean increase in $T_{1/2}$ of 13 minutes from 131 to 144 minutes equated to a 10% increase and this was statistically significant ($P < 0.05$). For five of the participants, the effect of treatment was to shorten T_{lag} and for three of the five, $T_{1/2}$ was also shorter. (**Table 5.4, Figure 5.1**). For the six participants for whom treatment resulted in delayed gastric emptying (positive responders), the mean T_{lag} -Control was 74.5 minutes and the mean delay due to the treatment was 16.6 minutes. In other words, the treatment appeared to cause a 22% delay in the time to peak $^{13}\text{CO}_2$ excretion.

Table 5.5 Gastric emptying parameters T_{lag} and $T_{1/2}$ as difference in minutes between control meal (C) and meal with kiwifruit (T) and percentage change

Participants	T_{lag} -C min	T_{lag} -T min	ΔT -C min	% Δ	$T_{1/2}$ -C min	$T_{1/2}$ -T min	ΔT -C min	% Δ
OK 01	84.80	87.70	2.90	3	135.90	166.00	30.10	22
OK 02	92.40	82.80	-9.60	10	132.30	113.80	-18.50	-14
OK 03	77.60	109.40	31.80	41	129.70	172.80	43.10	33
OK 04	70.80	83.40	12.70	18	105.90	117.20	11.20	11
OK 05	81.20	103.00	21.80	27	127.10	158.60	31.50	25
OK 06	80.00	93.60	13.60	17	126.10	143.60	17.50	14
OK 07	82.50	68.70	-13.70	-17	161.70	150.60	-11.10	-7
OK 08	80.30	74.10	-6.20	-8	117.70	143.10	25.40	22
OK 09	69.30	65.80	-3.60	-5	167.10	164.30	-2.80	-2
OK 10	52.80	69.40	16.60	31	109.20	122.80	13.70	13
OK 11	87.30	85.40	-1.90	-2	127.80	130.70	3.00	2
MEAN	78.09	83.94	5.85	9	130.95	143.95	13.01	11
SD	10.69	14.11	14.40	19	18.99	20.59	19.08	15
P value		0.208				0.047		
CI		(-3.83, 15.52)				(0.18, 25.82)		

% $\Delta T_{lag} = ((T_{lag}-T - T_{lag}-C)/T_{lag}-C) * 100$; CI = 95% confidence interval on the difference between the means; P = 2 tailed t test; SD = standard deviation

For the five participants who recorded accelerated gastric emptying due to the treatment (negative responders), the mean T_{lag} -Control was 82.4 minutes but the mean difference due to the treatment was only 7.0 minutes, or 8.5% acceleration.

Removing data that recorded a lower than 10% response to the treatment (positive and negative) on the basis that the difference may be due to measurement errors, the $T_{1/2}$ change due to treatment resulted in one participant with reduced T_{lag} of ~14% and seven increases in the timing of T_{lag} , with a mean change of 20%.

In total 8 of the 11 participants (73%) had greater than a 10% response to treatment and for 64% of participants, treatment delayed gastric emptying by >10%.

On the following page (**Figure 5.1**), individual's T_{lag} and $T_{1/2}$ result for the Control test are matched with those from the Treatment test, to visually demonstrate the magnitude of the difference, but more particularly the direction of the individual responses to treatment i.e. accelerated gastric emptying being a negative slope and delayed gastric emptying being a positive response.

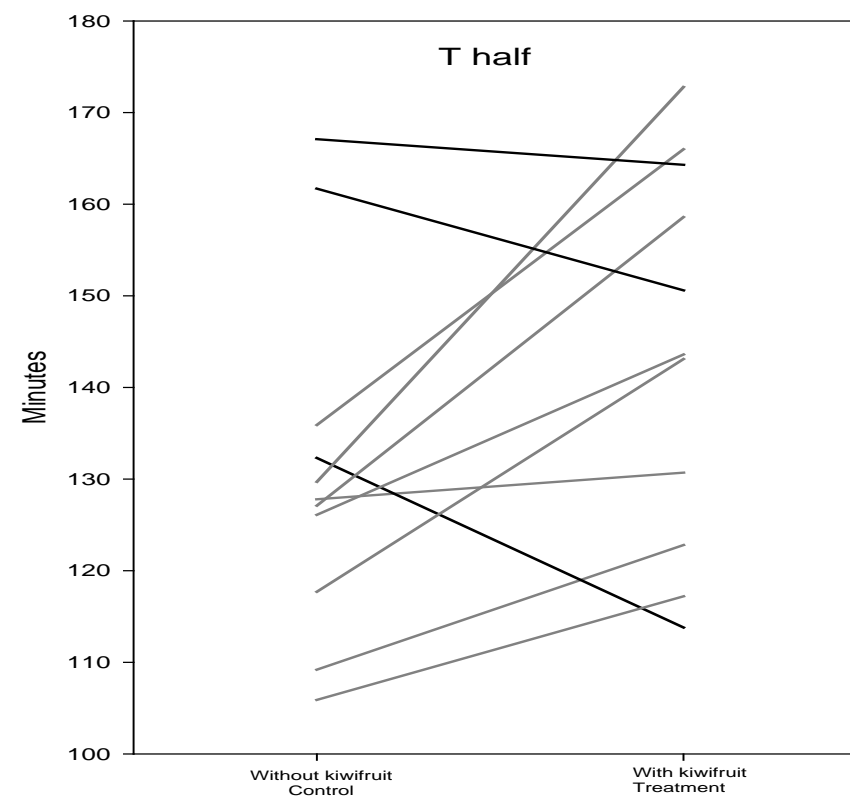
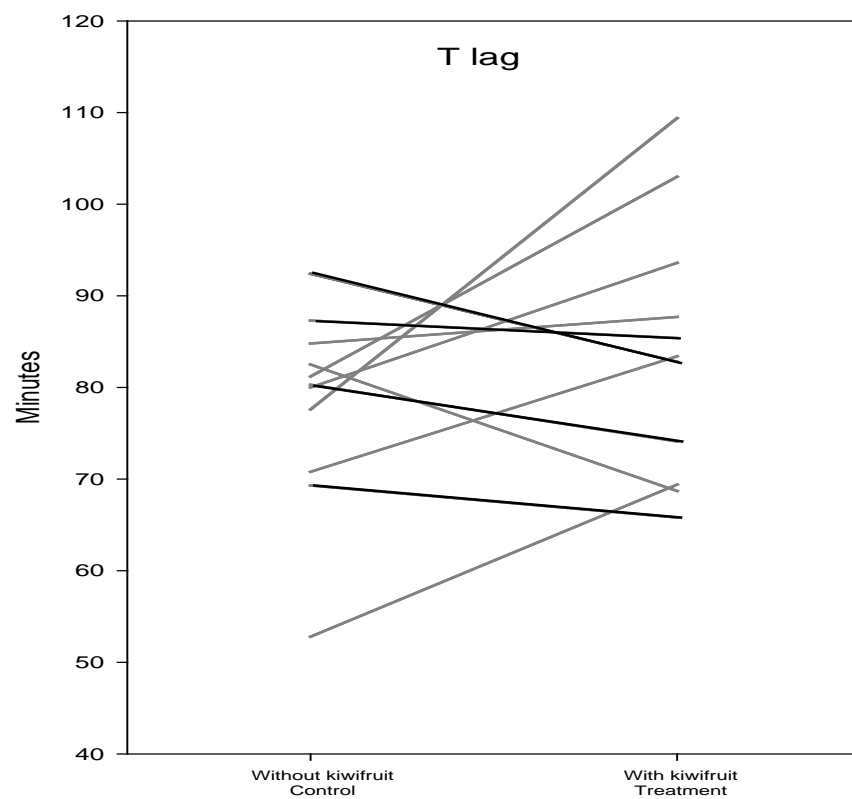


Figure 5.1 Graphical presentation of the Feasibility trial results (n = 11) showing the magnitude (in minutes) and the direction (positive or negative slope) of individual's response to kiwifruit protease treatment, measured as T_{lag} and $T_{1/2}$ calculated from $^{13}\text{CO}_2$

Correlation between physical characteristics and parameters of gastric emptying

Associations between changes in expired ^{13}C concentration and anthropometric measures were explored. (**Table 5.6**); T_{lag} and $T_{1/2}$ were positively correlated. A decrease in percentage change in $T_{\text{lag}}\text{-C}$ and $T_{\text{lag}}\text{-T}$ was associated with increased weight and BMI. Similarly the percentage change in T_{lag} was lessened with increasing weight ($r = -0.740$, $p = 0.009$). Height and BMI were also negatively associated but did not reach significance. Overall, with larger body size and more central fat, the association of change in gastric emptying time was shorter.

Table 5.6 Correlations (Pearson r value) between anthropometric measures and percentage change in gastric emptying parameters with treatment of meal with kiwifruit (n=11) Pearson r (p)

	% Δ $T_{1/2}\text{-T}$	% Δ $T_{\text{lag}}\text{-T}$	Waist	Waist:Ht	Weight	Height	BMI	Age
% Δ $T_{\text{lag}}\text{-T}$	0.72* (0.01)		-0.31 (0.36)	-0.12 (0.73)	-0.72* (0.01)	-0.49 (0.13)	-0.65* (0.03)	0.27 (0.43)
% Δ $T_{1/2}\text{-T}$		0.72* (0.01)	-0.52 (0.10)	-0.31 (0.35)	-0.74** (0.01)	-0.57 (0.07)	-0.59 (0.06)	0.06 (0.86)

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Proton pump inhibiting medication

Three of the participants in the Feasibility study were taking proton pump inhibiting medication (PPIs). These participants have been identified with dark lines in Figure 5.1. The role of PPIs in the treatment of gastro oesophageal reflux is to suppress activity of the gastric acid producing parietal glands in the stomach, thereby elevating fasting gastric pH. The initial *in vitro* study demonstrated $\text{pH} > 6.0$ suppressed the protease activity of kiwifruit. It was therefore hypothesised, *post hoc*, that PPI medication may have the same effect *in vivo* and that treatment with KFI would shorten the time spent in the stomach. For two of the three participants consuming PPIs, T_{lag} and $T_{1/2}$ times shortened with protease added to the meal. When the three participants

who were treated with PPIs were removed from the data the trend for both T_{lag} and $T_{1/2}$ times to increase, remained (**Table 5.7**).

Table 5.7 Summary of treatment effect on the parameters of gastric emptying with and without inclusion of participants using proton pump inhibiting medication (PPIs)

Parameters	Control meal, no protease	Treatment meal with protease	Difference (95% CI)	*P Value difference
Including PPIs (n=11)				
T_{lag} (minutes)	78.1 \pm 10.7	83.9 \pm 14.1	5.8 (-3.8, 15.5)	0.21
$T_{1/2}$ (minutes)	131.0 \pm 18.9	144.0 \pm 20.6	13.0 (0.2, 25.8)	0.05
Excluding PPI medication (n=8)				
T_{lag} (minutes)	78.7 \pm 12.3	86.2 \pm 14.3	7.6 (-5.7, 20.8)	0.22
$T_{1/2}$ (minutes)	128.7 \pm 17.1	141.6 \pm 23.2	12.9 (-5.6, 30.8)	0.13

* paired T test 2 tailed; PPI = proton pump inhibitor

5.7 Discussion

The primary purpose of this study was to evaluate the feasibility of using a ^{13}C -octanoic acid breath test (^{13}C -OABT) and a protein based meal, in terms of recruitment and compliance of volunteers with the test, meal preparation and palatability, reliability of measures and the analysis of the curves.

A secondary aim was to observe if the addition of kiwifruit to the meal changed the gastric emptying dynamics.

In hindsight both the feasibility and repeatability of the method should have been considered in the one study. This could have been achieved by extending the study from two meals to three with the second being a repeat of the first (Control) and used to validate the Control results prior to embarking on the Treatment.

The ^{13}C -OABT method has been validated by numerous researchers as a viable alternative to scintigraphy, the 'gold standard' method of measuring the kinetics of gastric emptying (Delbende et al., 2000; Maes et al., 1998). However, no record of the prior use of the technique for measuring the gastric digestion of a high protein meal was found in an extensive literature search. This is not to say such papers do not exist, simply that they were not found.

For the ^{13}C -OABT to accurately reflect the dynamics of gastric emptying of a protein meal, the presumption that the extent of protein hydrolysis in the gastric milieu has a predictable influence on the mechanics of gastric emptying must hold true. Amongst the literature reviewed, this was certainly the opinion of some researchers (Erickson & Kim, 1990; Jahan-Mihan et al., 2011; Low, 1990).

As octanoic acid binds well to fat, it was considered necessary to include egg yolk (33% protein, 67% fat) in the omelette, which in total was 47% protein and 53% fat. What is not known is whether the fat, the protein, or neither, influenced the observed changes in gastric emptying although the literature is emphatic that protein has a greater influence on gastric emptying than other factors known to influence gastric emptying. Ghrelin, a gastric peptide that stimulates gut motility, is suppressed more by dietary protein than fats or carbohydrates (El Khoury, Obeid, Azar, & Hwalla, 2006).

The only difference between the two test meals was the addition of 2.0g of KFI (Zyactinase) to the treatment meal. KFI is approximately 95% carbohydrate and 5% protein. Again, in hindsight, the Control should have contained a deactivated replica of the Treatment to demonstrate that any effect of the Treatment was in fact due to the protease activity and not simply the protein or carbohydrate content. However, the protein content of the treatment was approximately 86 mg and the carbohydrate content of the treatment was 1.7 g, neither would be expected to influence the dynamics of gastric emptying.

Bromer et al. (2002) recorded gastric emptying dynamics using both scintigraphy and ^{13}C -OABT of a 350 kcal muffin meal and a scrambled egg sandwich meal labelled with technetium (Bromer et al., 2002). Tests were less than four weeks apart. Each meal had a similar fat content but the egg meal at 12.8 g had 83% more protein by weight than the muffin meal at 7 g. Measured by scintigraphy, the $T_{1/2}$ for the muffin meal was 76 ± 26 minutes; for the egg meal it was 90 ± 36 minutes and the

difference was significant ($p = 0.015$). The point should be made here that scintigraphy records the actual rate of gastric emptying while the breath test measures the rate from ingestion to the appearance of the isotope biomarker on the breath. While the transfer of the isotope from the duodenum to the liver is almost instantaneous, transfer of $^{13}\text{CO}_2$ from the liver to the lungs is not. As a consequence gastric emptying times recorded by scintigraphy are in the order of 50% less than the gastric empty time recorded from a breath test (Peracchi et al., 2000). The following table provides a comparison of results from different studies.

Table 5.8 Measurement of gastric emptying rate of solids from meals of different nutritional composition (carbohydrate, protein & fat) by means of ^{13}C -octanoic acid breath test in healthy participants.

Article	Meal	Composition	T_{lag} (min)	$T_{1/2}$ (min)	Subjects
Peracchi (2000)	250kcal	42% CHO 40% fat 18% pro	110	155	28 years 5F/5M
Ghoos (1993)	250kcal	17% CHO 33% fat 10% pro	32 ± 20	72 ± 22	22 years 20F/20M
Bromer (2002)	350kcal	82% CHO 9% fat 9% pro	86	138 ± 15	34 years 6F/4M
Donaldson (2013)	170kcal	47% pro 53% fat	78 ± 11	131 ± 19	70 years 9F/2M

CHO = carbohydrate; pro = protein; F = female; M = male

The results reported by Ghoos et al. (1993) were adjusted by a formula designed to equate breath test results with scintigraphy recordings of gastric emptying. The formula used was found to give false readings for some outcomes and fell into disrepute, but the correlation between the two methods is not in dispute and revised formulae have been proposed (Sidossis et al., 1995). Most studies today employing the ^{13}C -OABT express results for T_{lag} and $T_{1/2}$ as the times measured by the method with no attempt to convert the times to scintigraphy equivalents, although a new method using the Bayesian technique in conjunction with a hierarchical modeling process has been

developed recently that is reported to overcome failings of earlier method (Bluck, Jackson, Vlasakakis, & Mander, 2011).

Compliance

Because all participants returned for the second day of testing and no one reported an aversion to the meal, the method of recruitment and testing requirements were feasible for a bigger study. Furthermore up to three people could be measured at the same time by one operator.

The repeatability of the pre-prandial measures of expired $^{13}\text{CO}_2/^{12}\text{CO}_2$ ratio within 15 minutes and one week later for an individual suggested that measures of enrichment of the isotope were reliable (**Table 5.4**). Furthermore the group means recorded for both tests were in agreement with the known normal $^{13}\text{CO}_2/^{12}\text{CO}_2$ enrichment in nature of approximately 1%.

Bromer et al. (2002) reported that using the ^{13}C -OABT with a muffin meal, the $T_{1/2}$ time for 10 apparently normal subjects aged 18–54 years was 150 ± 25 minutes (range 116 - 201 minutes); while for 23 patients all diagnosed with dyspepsia, aged 20–72 years the $T_{1/2}$ was longer 175 ± 96 minutes (range 78 - 488 minutes). This compares, for the present study, with a $T_{1/2}$ of 131 ± 19 minutes (range 106 - 167 minutes) without protease and 144 ± 21 minutes (range 113 - 172 minutes) with protease. Thus the calculations of T_{lag} and $T_{1/2}$ in the present study are of the same magnitude as those of other researchers. The dyspeptic patients in the Bromer et al (2002) study appeared to have delayed gastric emptying compared to the normal subjects, which is similar to the observation that one of the three participants in this study who were prescribed PPIs had the longest $T_{1/2}$ observed in the control condition (**Table 5.5**).

Increased body fatness (Delgado-Aros et al., 2004) and central obesity (Chirila, Drug, Petrariu, & Gavat, 2012) are known to be associated with indigestion and

gastrointestinal symptoms, and we found some evidence of a decrease in T_{lag} with increasing body size

A systematic review of the effects of PPIs on gastric emptying in 2010 (Sanaka, Yamamoto, & Kuyama, 2010) concluded that the use of PPIs delays the gastric emptying time of a solid meal. The stated mechanism was impairment of acid-dependent peptic activity and delayed hydrolysis of protein. This is in accordance with the observations in the *in vitro* study, and certainly in the present study the participants using PPIs recorded. Yet three of the eleven participants (27%), were prescribed PPIs and this is an indication of the widespread prevalence of gastric dysfunction in the apparently well, older population. In the 2006-2007 national health survey of New Zealand (Gerritsen, Stefanogiannis, & Galloway, 2008) stomach/gastric ulcers were reported for 3% of the population aged over 15 years. The prevalence of prescribed use of PPIs in New Zealand is high and increasing (Lampen-Smith, Young, O'Rourke, Balram, & Inns, 2012) with PPIs being the second most prescribed medication. The effect of both PPIs and kiwifruit on gastric emptying warrants further investigation in larger cohort studies with better characterisation of gastric symptoms.

Two or possibly three categories or subgroups of response to kiwifruit were observed. The *in vitro* study (**CHAPTER 3**) indicated KFI would be unlikely to have any appreciable effect where gastric digestion of protein was 'normal'. Speculating that for some people KFI does have a measurable effect on protein digestion, if the breath test results are an accurate reflection of protein digestion efficiency, then those participants who recorded 10% or lower response to treatment (either positive or negative) could possibly be assumed to enjoy 'normal' digestion. This then leaves the digestion efficiency of the greater than 10% responders to be accounted for. The Bremer et al. (2002) $T_{1/2}$ results indicated the dyspeptic patients ($n = 23$) had delayed gastric emptying compared to the, so-called, normal patients ($n = 10$); confirming the

earlier work of Maes et al (1997). This raises the question: Could there be a group of people whose gastric digestion is less efficient than 'normal' but not to the extent that they experience the classic symptoms of dyspepsia to the point that they seek medical counsel? In other words, could the > 10% positive responders to the KFI intervention (those who recorded delayed gastric emptying as a result of treatment) have subclinical dyspepsia, and those who recorded a >10% negative response be clinically dyspeptic? Furthermore, could the dyspepsia be associated with gastric acidity or gastric reacidification capacity? Protein in a meal has been shown to delay gastric emptying when compared with a meal devoid of protein (El Khoury et al., 2006).

A study by Keller et al. (Keller, Andresen, Wolter, Layer, & Camilleri, 2009), which compared scintigraphy and breath test results from 1279 patients and 19 healthy controls acknowledged the possibility of subsets of patients whose gastric emptying characteristics were determined by a common condition. For example, they found factors that had a consistent and significant but small effect on gastric emptying dynamics included gender, age, diabetes mellitus, IBD, bacterial overgrowth and some malignant diseases. Patients with IBD recorded decreased cumulative $^{13}\text{CO}_2$ excretion and prolonged $T_{1/2}$, whereas patients with malignant diseases recorded increased cumulative $^{13}\text{CO}_2$ excretion and decreased $T_{1/2}$. Perri et al. (2005) also confirmed the ^{13}C -OABT has been used to identify different pathological conditions (Perri et al., 2005).

No alternative method of measuring gastric acidity or gastric emptying was employed in the Feasibility study for the reasons discussed earlier. Consequently there were no standards against which the results could be compared. This was a weakness in the study design. In hindsight the study could have comprised three test meals rather than two. All three could have been with the inclusion of ^{13}C , with only the third including the kiwifruit treatment. Such a design would have allowed the intra-individual gastric

emptying repeatability to be evaluated prior to the observations being confounded by the treatment. As it was, each test took almost four hours of participant's time, plus travel time — a total for the two tests of about ten hours. Fortunately, there were no dropouts over the two tests, but compliance may have suffered had there been three tests. However, in an attempt to clarify the reason for the less than conclusive results it was agreed the study should be repeated to determine the reliability of the method. Rather than entering into further discussion on the outcome of the Feasibility study at this stage, the next two chapters (**CHAPTERS 6 & 7**) will describe the rationale, methods, protocols and results for two further *in vivo* studies and the findings will be considered in greater depth in Chapter 9 under the heading of Discussion.

CHAPTER 6. Precision of measurement of gastric emptying dynamics using the ^{13}C -Octanoic acid breath test

6.1 Introduction

The proof of principle *in vivo* study (**CHAPTER 5**) tested the feasibility of using the standard ^{13}C -Octanoic acid breath test to measure if the inclusion of kiwifruit (KFI) with a protein-dominant meal altered the rate of gastric emptying when compared with the gastric emptying rate of an identical meal without KFI. The proposition tested was that the dynamics of the appearance of $^{13}\text{CO}_2$ on the breath would change if the individual's protein digestion efficiency altered by the inclusion of KFI with the second meal. The outcomes of that small study ($n = 11$) were that the procedure was acceptable to the participants, there were no adverse events and for some of the participants there appeared to be a change as a result of the KFI treatment. However, it was unclear if the variability of the inter-individual response among participants to the addition of KFI was an accurate reflection of their protein digestion efficiency or a result of biological variations or measurement errors. An intensive search of the literature was unable to find any reports of a meal with protein energy content greater than 18% (**Table 5.8**), or measurements of intra-individual change with treatment. Only one study of the reliability of the ^{13}C -OABT by repeated measures of gastric emptying of two identical meal on separate days was found (Perri et al., 2010). Others were comparative studies of OBT with scintigraphy or ultrasound (Peracchi et al., 2000).

It had been assumed that the group response to the treatment would be relatively homogenous given the age profile (58 to 80 years) and the apparent good health of the participants. What was demonstrated however was that the differences in the rates of gastric emptying between the treatment and control tests, measured by the time from

ingestion of the test meal to the stage at which the rate of gastric emptying peaked (T_{lag}), ranged from an increase of 13.7 minutes to a decrease of 31.7 minutes.

The quality of these diverse assessments of gastric emptying required that the intra-individual reliability (repeatability, precision) of the modified breath test to measure gastric protein digestion efficiency had to be reassessed. A repeat of the measurements using the same participants as in the Feasibility study was proposed to establish the intra-individual reliability of the ^{13}C -OABT to detect gastric emptying time under both control and treatment conditions. Ethics approval was extended and the same subjects were invited to undergo a repetition of the initial investigation, with the aim of measuring the repeatability; albeit a number of months after the first tests.

6.2 Aim and hypothesis

As the *in vivo* study described in Chapter 5 produced what initially appeared to be conflicting results, the aim of the study described in this Chapter (6) was to investigate the repeatability of the results from the previous study by retesting as many of the original participants as were able and willing to participate in a second study. The hypothesis to be tested was that similar results would be obtained from a repeat study for participants common to both studies.

6.3 Method

Testing to determine the precision of the intra-individual measurements commenced four months after completion of the Feasibility study. One change was made to the original protocol. Treatment in the Feasibility study consisted of 6 x 440 mg capsules of KFI consumed with the second test meal (Treatment). In this Repeat study the researcher asked the participants to consume the same number of capsules (6 x 440 mg) but on a daily basis (two with each meal), over the seven days between the Control test and the Treatment test, including on the day of the Treatment test. The

rationale for this was anecdotal evidence (survey of 1,200 consumers — Vital Foods) that the treatment effects of kiwifruit linger for several weeks after cessation of consumption and therefore longer exposure to the kiwifruit prior to testing might provide a more uniform response than observed in the Feasibility study and in line with expectations, especially if the treatment effect was not direct.

It was recognised that changing the treatment between studies may produce different results and invalidate a comparison between studies, but because this was still a pilot study, the researcher, in consultation with the supervisors, determined the change was warranted to provide greater clarity for future treatment regimes. All other aspect of the Repeat study were the same as for the Feasibility study.

All participants were contacted and the need for a repeat test explained. Of the eleven original participants, six agreed to repeat the assessment and five were unavailable.

Statistics

The measurements from all the 11 participants in the Feasibility study and the repeat measurements from the six participants were analysed. Paired t tests were used to compare measurements within the same individual. Precision error (biological and methodological) was determined from a one-way analysis of variance (ANOVA) with subjects as the factor. The ‘standard deviation’ within subjects was the square root of the residual mean square from the ANOVA results table with the precision errors calculated as a within subjects coefficient of variation ($SD/mean * 100$) (Bland & Altman, 1986). Statistical analysis was performed using the IBM Statistics program (SPSS version 19, IL), Sigma Plot Version 10, Microsoft™ Excel Version 14.2.3 and Addinsoft™ XLSTAT version 2013 1.01 packages.

6.4 Results

Pre-prandial or baseline breath $\delta^{13}\text{C}$ intra-individual variability

In both studies two breath samples were collected from each participant prior to their consuming each of the test meals to establish their baseline $^{13}\text{C}:^{12}\text{C}$ ratio before they consumed the meal with ^{13}C -octanoic acid added. These pre-prandial samples were collected after the participants had arrived at the laboratory and had rested for about 15 minutes to allow for metabolic rate stabilisation; there was a 15-minute interval between collection of the first and second sample.

Table 6.1 shows the coefficient of variation for the $^{13}\text{CO}_2$ in pre-prandial breath samples taken 15 minutes apart was less than 2% on each of the four testing days and within individuals there was no significant difference between measurements on the same day or between tests on different days.

Table 6.1 Variability of the pre-prandial baseline $\delta^{13}\text{CO}_2$ duplicates, sampled at 0 and 15 minutes apart on each test day for the Control and Treatment tests

	Mean \pm SD	Mean diff \pm SD	IQ CI diff	95% CI mean diff	CV%	P*
Feasibility (n = 11)						
Control 1	-25.44 \pm 0.76	0.12 \pm 0.39	-0.06, -0.46	-0.15, 0.38	1.1	0.34
Control 2	-25.56 \pm 0.63					
Treat 1	-25.09 \pm 0.99	0.19 \pm 0.72	-0.25, 0.86	-0.29, 0.67	1.9	0.40
Treat 2	-25.27 \pm 0.85					
Repeat 4 months later (n = 6)						
Control 1	-24.19 \pm 0.68	0.16 \pm 0.32	-0.06, 0.32	-0.17, 0.49	0.95	0.28
Control 2	-24.35 \pm 0.79					
Treat 1	-25.59 \pm 1.41	-0.07 \pm 0.18	-0.24, 0.09	-0.28, 0.14	0.50	0.42
Treat 2	-24.51 \pm 1.52					

*Paired t-test; Treatment test was conducted seven days after the Control test in each study. The Repeat study (n = 6) was conducted four months after the Feasibility study (n = 11); IRMS - isotope ratio mass spectroscopy; $\delta^{13}\text{C}$ - IRMS readout

Similarly, when the pre-prandial means of $\delta^{13}\text{CO}_2$ within the two trials were compared (**Table 6.2**), the coefficients of variation were small (1.6 and 2.6%) and were not different. The difference between the Control and Treatment means in the Feasibility study was close to significance (p = 0.06) yet the difference between the

means was only 0.32 of a δ unit. The mean $\delta^{13}\text{CO}_2$ for the Repeat study appeared to be approximately one δ unit higher than the means of the Feasibility study.

Table 6.2 Intra-individual variability between test days, within trial, in baseline mean $\delta^{13}\text{CO}_2$ derived from two measurements taken within 15 minutes.

	Mean \pm SD	Mean diff \pm SD	IQ CI Diff	95% CI Mean diff	CV%	P*
Feasibility (n = 11)						
Control 1&2	-25.50 \pm 0.67	-0.32 \pm 0.15	-0.93, 0.05	-0.65, 0.01	1.6	0.06
Treat 1&2	-25.18 \pm 0.85					
Repeat (n = 6)						
Control 1&2	-24.27 \pm 0.72	0.19 \pm 0.97	-0.50, 0.85	-0.82, 1.21	2.6	0.64
Treat 1&2	-24.47 \pm 1.33					

*Paired t-test; Treatment test was conducted seven days after the Control test in each study. The Repeat study (n = 6) was conducted four months after the Feasibility study (n = 11)

Variability in Post-prandial T_{lag} and $T_{1/2}$ Analysis

As with the Feasibility study, the parameters of gastric emptying (T_{lag} , the time from ingestion to the peak rate of gastric emptying; and $T_{1/2}$, the time from ingestion to the time when 50% of the dose remains in the stomach) were calculated for the six participants common to both studies. The measurements for the Repeat Study (**Table 6.3**) showed there was no significant difference between the Treatment and the Control but individual T_{lag} and $T_{1/2}$ differences showed a positive response to the treatment for three trialists and a negative response for the other three.

Table 6.3 Gastric emptying parameters (T_{lag} and $T_{1/2}$) and differences between the Control and the Treatment from the Repeat study (n = 6)

Participant	$T_{lag}C$	$T_{lag}T$	$T_{1/2}C$	$T_{1/2}T$
OK03	79.0	108.6	131.0	163.5
OK04	72.1	127.4	125.1	214.7
OK05	104.3	77.7	147.5	124.1
OK06	73.4	62.5	120.4	111.2
OK10	75.0	82.3	143.8	177.4
OK11	102.1	78.6	157.8	124.8
Range	72.1–104.3	62.5–127.4	120.4–157.8	111.2–214.7
Mean	84.3	89.5	137.6	152.6
Standard Deviation	14.8	23.9	14.4	39.7
Difference in means	5.2		15.0	
95% confidence limits	-28.7, 39.1		-33.3, 63.3	
P value (2 tailed t test)	0.71		0.46	

Repeat measurements of T_{lag} and $T_{1/2}$ in both Control and Treatment conditions after four months showed high variability in response with a CV of up to 25% for $T_{1/2}$ with kiwifruit (**Table 6.4**). The intra-individual differences in the treatment effect for the Feasibility study and the Repeat study led to large coefficients of variation for the difference in time with treatment; 290 and 180% for T_{lag} and $T_{1/2}$ respectively.

Table 6.4 Repeat determination of intra-individual changes in T_{lag} and $T_{1/2}$ in protein meals with and without kiwifruit protease (n=6)

N = 6	Feasibility	Repeat	Difference	CV, %	*P
T_{lag} (minutes)					
Without kiwifruit	75.0 ± 12.1	84.3 ± 14.8	9.4 ± 12.4	13.1	0.12
With kiwifruit	90.7 ± 14.5	89.5 ± 23.8	-1.1 ± 27.4	19.6	0.92
$T_{1/2}$ (minutes)					
Without kiwifruit	121.0 ± 10.5	137.6 ± 14.4	16.6 ± 15.9	12.1	0.05
With kiwifruit	141.0 ± 21.6	152.6 ± 39.7	11.7 ± 53.0	24.0	0.65
Difference in times with and without protease					
	Feasibility	Repeat trial	Difference between trials		
T_{lag} (minutes)	-15.8 ± 11.1	-5.2 ± 32.3	-10.6 ± 30.5 (-42.6, 21.5)	289	0.44
$T_{1/2}$ (minutes)	-20.0 ± 14.7	-15.0 ± 46.0	-5.0 ± 48.0 (-55.3, 45.4)	180	0.81

*Paired t-test

Isolating the gastric emptying parameters from the Feasibility study for the six participants common to both studies (**Table 6.5**) showed that in the Feasibility study the treatment appeared to have a significant effect on both the change in T_{lag} ($p = 0.018$) and in $T_{1/2}$ ($p = 0.021$) for those six participants. Whereas in the Feasibility study as a whole ($n = 11$), the change in $T_{1/2}$ due to treatment was significant ($p = 0.047$) but the change in T_{lag} was not ($p = 0.21$).

Table 6.5 Gastric emptying parameters from the Feasibility study for the six participants who had repeat measurements. Control (C), Treatment (T)

Measure	Mean \pm SD	Diff \pm SD	95% CI	P
T_{lag} -C	74.9 \pm 12.1	15.8 \pm 11.1	6.8, 24.7	0.018
T_{lag} -T	90.7 \pm 14.5			
$T_{1/2}$ -C	121.0 \pm 10.5	20.0 \pm 14.7	8.3, 31.8,	0.021
$T_{1/2}$ -T	141.0 \pm 21.6			

Combining the measurements from both studies (Feasibility and Repeat) for the six participants common to each gave the following mean outcome (**Table 6.6**)

Table 6.6 Variance in mean of the T_{lag} and $T_{1/2}$ for the Control and Treatment tests when measurements from participants common to both studies were combined ($n = 6$)

Measure	Mean \pm SD	Diff \pm SD	95% CI	P
T_{lag} -C	79.6 \pm 12.0	10.5 \pm 7.4	-4.5, 25.5	0.23
T_{lag} -T	90.1 \pm 14.2			
$T_{1/2}$ -C	129.3 \pm 9.8	17.5 \pm 12.4	-1.94, 37.0	0.14
$T_{1/2}$ -T	146.8 \pm 17.9			

6.5 Discussion on the findings from the Repeat Study

This step-wise exploration of the reliability of the measurements of gastric emptying using ^{13}C -OABT with a protein-dominant meal has demonstrated that individual repeated measurements of fasting $\delta^{13}\text{CO}_2$ 15 minutes, seven days and four months apart had $<2\%$ variation. When the same meal was consumed four months later the variation in times for T_{lag} and $T_{1/2}$ did not exceed 13%, and when the meal was consumed with kiwifruit the variation was slightly more at 20–24%. In real terms this means that if the time was 100 minutes then the variation in response of an individual in

T_{lag} and $T_{1/2}$ without kiwifruit would be within around ± 26 minutes, and with kiwifruit it would be approximately ± 48 minutes (in 95 cases out of 100). As variance is additive this translated to a large variation of difference in times with and without kiwifruit, but the mean increases in times for these six participants were 10 and 5 minutes respectively, reflecting the large, presumably biological, variation in response. Perri et al. (2010) conducted the only replication study found in the literature. In that study 131 healthy volunteers (47 ± 18 years) completed the ^{13}C -OABT. The following day 30 of them repeated the test. The CV between the $T_{1/2}$ measurements for the same subjects in the two tests was 17%, reported by the researchers to indicate the low within subject variability of $T_{1/2}$.

The CV% of $T_{1/2}$ Control in the Repeat study ($n = 6$) was 12.1% (**Table 6.5**) and the CV% T_{lag} Control was 13.1% indicating these results also demonstrated a low within subject variability.

Variation in gastric emptying times within an individual, over time, is mediated by technical and external factors such as caloric composition and size of the meal (Peracchi et al., 2000), solid or liquid phase of the meal, duration of the collection time and the confidence limits of the data (Perri et al., 2010).

Within an individual the response may vary according to BMI (Cardoso et al., 2007), the amount of chewing and speed of eating (Santangelo, Peracchi, Conte, Fraquelli, & Porrini, 1998), thermogenesis, gastric tone, anxiety, recent diet and other gastric and digestive pathologies (D'Souza, 2007) as well as both current and recent exercise (Markey & Shafat, 2013). External factors were controlled in this reliability study but given that the repeat test was four months later. there may have been other factors that may not have been able to be measured.

The accuracy of the ^{13}C -OABT method to measure gastric emptying when compared to the more direct measure of scintigraphy is well established (Modak, 2007)

and shows the ability to gain meaningful diagnostic and explanatory measures of gastric emptying dynamics. A strong correlation between the ^{13}C -OABT and scintigraphic measures of gastric emptying for a wide range of macronutrient variation in the composition meals, from 18% protein, 40% fat and 42% carbohydrate (Peracchi et al., 2000), to 9% protein, 9% fat and 82% carbohydrate (Bromer et al., 2002). This indicates the test method is not unduly influenced by the macronutrient composition of the meal. Given this evidence it is possible, but unlikely, that the apparent inconsistencies observed between the Feasibility and Repeat trials T_{lag} and $T_{1/2}$ times were due to the dominance of protein in the meals.

Peracchi et al. (200) demonstrated that the caloric composition of a 550kcal meal increased gastric emptying parameters compared with a 250 kcal meal but the correlation between ^{13}C -OABT and the measurement of gastric emptying by ultrasonography remained consistent when the food composition was the same. In the Feasibility and Repeat studies the caloric content of the meals were identical and would therefore be unlikely to have influenced the outcomes.

Not anticipating the need for a Repeat study, the participants were informed at the completion of the Feasibility study of the anecdotal evidence of kiwifruit relieving digestive discomfort. If in the intervening time (four months) between studies participants common to both studies changed their diets as a result of that information and consumed kiwifruit on a daily basis, their Repeat study Control test results may change relative to those for the Feasibility study if, indeed, kiwifruit imparts some ongoing benefits in terms of protein digestion efficiency. This is consistent with anecdotal evidence that digestive discomfort relieved by kiwifruit consumption does not re-emerge immediately kiwifruit consumption ceases. It has been reported that the discomfort can gradually re-establish over several days and even weeks after regular kiwifruit consumption ceases (Vital Foods survey of 1200 customers).

In summary, there was excellent reliability in the laboratory measures of breath $\delta^{13}\text{CO}_2$ after four months. Within subject variation in gastric emptying parameters was slightly greater with the kiwifruit treatment but overall was less than 24%. The observation that an individual's response varies suggests that a greater understanding of other factors that may influence gastric emptying dynamics is required to determine a treatment effect greater than the biological variation for that individual.

6.6 Further study required

The minimal variation in the six participants' pre-prandial baseline $\delta^{13}\text{CO}_2$ recordings between the Feasibility study and the Repeatability study four months later support the proposal that the test method was conducted appropriately. The coefficient of variation for T_{lag} and $T_{1/2}$ for the Control measures of 13.1% ($P = 0.12$) and 12.1% ($P = 0.05$) respectively (**Table 6.4**) indicate a possible change in the group's digestion efficiency between studies. This may have been a feature of a change in seasonal diet or inclusion of kiwifruit in the diet; however, it may also be a reflection of the true intra-individual variability of gastric emptying. More robust studies with appropriate controls are required to determine the validity of this test method, designed for measuring gastric emptying, to be used specifically with a protein dominant meal as a proxy for protein digestion efficiency.

CHAPTER 7. A third *in vivo* study conducted in China

The investigations that informed this thesis sought to identify a credible mode of action in the digestive process that may explain why kiwifruit is reported to relieve constipation and other symptoms of digestive dysfunction. The first study (**CHAPTER 3**) investigated the effects of kiwifruit protease on meat protein hydrolysis in an *in vitro* human digestion setting. Findings from that study indicated that the protease had little activity at normal fasting gastric pH (pH 2.0) or in a simulated duodenal pH (pH 6.4), but had the greatest activity in a simulated gastric environment where pH was between these two extremes and the pepsin concentration was less than optimal for efficient protein hydrolysis. It was also demonstrated that the protease activities of both pepsin and kiwifruit were increased or diminished by relatively small changes in the acidity of the simulated gastric milieu. This gave rise to the hypothesis that kiwifruit increases gastric protein hydrolysis when the efficiency of gastric digestion is less than optimal due to insufficient pepsin and/or elevated gastric pH. To test the hypothesis *in vivo* a method of measuring gastric protein digestion efficiency was sought. The method requirements were that it was ethical and safe from the point of view of invasiveness in healthy participants, and simple enough to meet the fiscal constraints of this research project and would also encourage people to volunteer. No existing measurement method meeting these criteria could be found, so an adaptation of a standard stable isotope breath test was proposed and the feasibility of this test method was investigated. (Feasibility *in vivo* study, **CHAPTER 5**).

The principle underlying the use of the ^{13}C -OABT method was: If a biomarker such as ^{13}C labelled octanoic acid is ingested with a meal, the dynamics of gastric emptying will be reflected in the rate at which the 13-carbon dioxide appears on the exhaled breath. Because the extent of protein hydrolysis has a controlling effect on the

kinetics of gastric emptying (Low, 1990), if the meal was predominantly protein then it was hypothesised that the dynamics of gastric emptying would be a measure of the efficiency of the gastric digestion of protein.

Firstly, the compliance and comfort of the eleven volunteers with the test regime indicated that recruitment for a larger study would be feasible. Secondly, when kiwifruit was added to the Treatment test meal there was an increase in the group mean change in time from ingestion of the ^{13}C labelled meal until half the ^{13}C was calculated to be retained in the stomach ($T_{1/2}$). The increase in time was 13 minutes (95% confidence interval 0.2, 25.8) and this was found to be statistically significant (effect size 0.66, $P = 0.047$). However, no significant change was observed in the time from ingestion of the ^{13}C dose to the maximum rate of appearance of ^{13}C on the breath (T_{lag}), but the size effect was moderate at 0.46. Individual responses to treatment compared to the control were right skewed, i.e. for most participants with treatment T_{lag} and $T_{1/2}$ increased.

While the feasibility of the ^{13}C -OABT to measure protein digestion efficiency was shown, due the small sample size of the Feasibility study the reliability of the test method was still in question.

Six of the original participants from the first study volunteered to take part in a Repeat trial (**CHAPTER 6**) conducted four months after the Feasibility trial. The Repeat trial had a modified kiwifruit treatment where participants took a daily dose of KFI for the seven days between the Control and Treatment tests. For the six participants measured again it was demonstrated that the intra-individual, pre-prandial measure of $^{13}\text{CO}_2$ and the measurements of T_{lag} and $T_{1/2}$, were reliable. If the change in T_{lag} or $T_{1/2}$ due to treatment was more than 20% and 24% respectively, then it was likely that the treatment effect was meaningful for that individual (**Table 6.4**).

The group mean $T_{1/2}$ Control for the Repeat study was almost 16.6 minutes longer (13.7%) than for the same group in the Feasibility study, and this was almost significant ($P = 0.05$, 95% CI -0.02, 33.3). The coefficient of variation of $T_{1/2}$ Control, between the studies (**Chapter 6**, $n = 6$) was 12.1% (**Table 6.5**), indicating a low intra-individual variability of $T_{1/2}$ over time (four months). The group mean $T_{1/2}$ Treatment for the Repeat study was also longer than for the Feasibility study by 11.7 minutes (8.3%) but this was not significant ($P = 0.65$).

Several factors may have contributed to the individual and group variation in measurements between the studies and by treatment. Variation can include measurement error or result from the small sample size of both *in vivo* studies in combination with the change in the treatment between studies. Additional variation may be related to unknown dietary changes by participants, change in seasons, or inclusion of additional kiwifruit in their diets as a result of discussions about kiwifruit and digestion in the course of the Feasibility study.

Given these uncertainties, a third, larger *in vivo* study, the subject of this chapter, was proposed.

7.1 Aim and hypothesis

A larger *in vivo* study than those described in Chapters 5 and 6 was planned, with the primary aim of validating the results of the earlier studies, and secondarily to further investigate the indications from those studies that PPI medication affects gastric emptying dynamics. The hypotheses to be tested were: firstly, that the effects of KFI on gastric emptying for Chinese participants in the proposed study would be similar to those recorded for the participants in the New Zealand study; secondly, that the high degree of correlation referred to by other researchers between measurements of stable isotopes with a NDIR spectrometer and IRM spectrometer would not prejudice a

comparison between results from the New Zealand studies and those from the proposed China study; and thirdly, that selecting participants with pre-determined degrees of severity of hypochlorhydria due to *H. pylori* exposure would be a proxy for investigation of the influence of PPI medication in conjunction with protease supplementation. Unfortunately the size and sophistication of this study had to be curtailed with the result that the *H. pylori* hypothesis could not be pursued. Furthermore, the size of the study also had to be curtailed with the outcome that this study was more closely aligned to a repeat of the initial study (**Chapter 5**) albeit using a NDIRS unit to measure results rather than IRMS unit.

7.2 Rationale for a third *in vivo* study

Method of ¹³C measurement, time and cost.

Breath samples for both the Feasibility and Repeat studies were analysed for their ¹³C:¹²C ratio, under contract by the University of Otago's Department of Chemistry, using an isotope ratio mass spectrometer (IRMS). Each trial participant provided 11 breath samples for the Control test and a further 11 samples for the Treatment test — in all, 22 samples. These were couriered in batches to Otago University, and the analytical results were received a number of weeks later.

For each sample analysed using IRMS the cost was \$NZ35.00, making a total cost for ¹³C analysis in the order of \$770.00 for each trial participant. As there were two arms planned for this next study, the intention was to recruit 165 participants. The sample size was estimated using the following formula:-

$$n = (1.962 \sigma^2) / \epsilon^2$$

Where n = the sample size

σ = the population standard deviation (Combined NZ studies, Feasibility and Repeat, data combined the T_{lag} SD = 20.71 minutes)

$$\varepsilon = \text{the standard error} = \sigma / \sqrt{n} \text{ (Combined NZ data SD}_p / \sqrt{17} = 4.123$$

In a normal distribution, 95% of the values lie within 1.96 standard deviations each side of the mean. Therefore, for the proposed trial the estimated sample size was:-

$$n = (3.92 * 428.9) / 25.23 = 67$$

Allowing for a 15% drop-out rate, $n = 78$. But as the trial was designed to look at the effect of treatment on either side of the gastric atrophy score (explained **7.3 Design of the China Study**), the trial needed twice the number of participants, so $n = 156$

For a study of this size IRMS analysis of breath samples was estimated to cost in the region of \$120,000.

Alternative method

An alternative method of analysis was to use the more recently developed non-dispersive infrared spectroscopy (NDIRS). The capital cost of the spectrometer for this process is considerably less than for an IRMS unit (\$35,000 cf. \$500,000) and has the advantage that it may be installed on a desktop, is portable and relatively simple to operate. As a result, the cost of sample analysis using NDIRS was almost negligible. This then was an attractive option that was investigated. However, at the time of this work there were no NDIRS units in New Zealand. So, in collaboration with Massey and Auckland Universities, I made an application to the Lotteries Commission for a grant to purchase a new NDIRS unit from Germany, initially to be used on this project but later to be shared by the other universities, but the application was declined.

In subsequent correspondence a contact in China, with connections to the Wuhan Hospital of Traditional Chinese Medicine, advised that their hospital had recently acquired a new NDIRS unit specifically to diagnose *Helicobacter pylori* infection using the ^{13}C -Urea breath test. Previously, a combination of laboratory culture of gastric biopsies and endoscopy had been the main method employed by the hospital's

gastroenterologists to diagnose *H. pylori* infection and gastritis. Since the introduction of the urea breath test, analysed with the NDIRS unit, the use of endoscopic diagnosis was reportedly reduced by 75%.

The hospital administration advised that the hospital was willing to collaborate on a kiwifruit study by recruiting patients for the trial and providing equipment at no cost. This attractive offer prompted an application to the AUT Ethics Committee to approve the study, which was granted (AUTEC 11/268). The same application was translated into Chinese and subsequently submitted to the Wuhan Hospital's own Ethics Committee, and the necessary regulatory approvals to conduct the study were obtained. Participant information sheets and consent forms were translated and printed, and personnel were recruited and trained to assist with the study. Then the Wuhan hospital administrators, in spite of a signed contract to the contrary, demanded a substantial fee for each patient the hospital introduced to the study. This caused an impasse for several days, and eventually the formal study had to be abandoned. In its place, using a network of contacts, eighteen middle-aged people with a history of digestive dysfunction were invited to participate in the study. Measurements were undertaken at the hospital on the same floor that housed the NDIRS unit and at no cost except for the disposable breath sample bags. The rest of this chapter describes this study (China study), which had the aim of demonstrating that with kiwifruit added to a protein-based meal there would be changes in both the half and lag time measurements of gastric emptying. Mediators such as gastric atrophy due to *Helicobacter pylori* would be accounted for with the aid of the endoscopy endorsed gastric atrophy score.

7.3 Design of the China Study

The China Study was an experimental design with pre- and post-treatment with a week between tests. This was the same design as the Repeat study (**CHAPTER 6**),

where treatment was six 440 mg KFI capsules taken daily between test meals. There were three further modifications.

The first, a change to the inclusion criteria, required participants to have a history of digestive discomfort that comes under the umbrella of the definition of a functional bowel disorder (FBD). FBD is a general term that describes an unspecified problem associated with an occasional malfunction of the gastrointestinal system despite all organs appearing to be normal (Thompson et al., 1999). The malfunction can result in a variety of symptoms usually associated with pain or a sense of urgency to void the bowels. Upper gastrointestinal symptoms of FBD include nausea, bloating and stomach pain, while lower GI symptoms can include constipation, diarrhoea and pain. Both irritable bowel syndrome and functional dyspepsia are included under the general heading of FBD. The intention for the China study was to only recruit volunteers with a history of FBD. Participant's FBD symptoms were to be recorded as part of the study, then following treatment any changes in those symptoms as a result of the treatment were also to be recorded.

The second modification was that all participants were to have been pre-examined for the presence or pre-existence of *Helicobacter pylori* with the severity of concomitant gastric atrophy confirmed by endoscopic examination on a scale of 0 - 5, ranging from nil at grade 0 to severe at grade 5. The rationale was that as gastric atrophy increased, fasting gastric pH would also be increased and gastric reacidification capability would be diminished. The hypothesis was that KFI would accelerate gastric emptying for those with the highest gastric atrophy score (i.e. the more severe gastric atrophy) and delayed gastric emptying for those with the lowest score. This thinking was a result of the initial *in vitro* study which indicated kiwifruit protease was least active at $\text{pH} \leq 2.0$ equivalent to normal fasting gastric pH — no gastric atrophy — grade 0, and also at $\text{pH} \geq 6.0$ — severe gastric atrophy — grade 5 — where the patient

is likely to be achlorhydric (an inability to secrete gastric acid). Extrapolating from the results of the *in vitro* study and the two *in vivo* studies, Kiwifruit treatment was expected to be most effective in increasing protein hydrolysis in a range between grade 2 and grade 3.

However, the impasse with the hospital meant this second modification to the participant's inclusion criteria (the requirement for all participants to be pre-screened for *H. pylori* infection, whether positive or negative; gastric atrophy arising from the infection noted) was not possible.

The third modification to the protocols of the two earlier *in vivo* studies was the employment in the China study of a non-dispersive infrared spectrometer (NDIRS) to analyse $\delta^{13}\text{C}$ (‰) in the breath samples in place of the isotope ratio mass spectrometer (IRMS) used in both the Feasibility and Repeat studies. The NDIRS analysis differs from IRMS in a number of ways detailed below.

7.4 Non-dispersive Infrared Spectrometry (NDIRS)

The analysis of expired breath $^{13}\text{CO}_2$ by isotope ratio mass spectroscopy (IRMS) requires that the gas is ionised and the ^{13}C and ^{12}C ions are differentiated by passing them through a magnetic field to a detector configured to measure the ratio of each isotope in the gas sample. The relative concentration of the isotopes is then compared with the isotope concentration of a known standard reference material, in this case, Pee Dee Belemnite (PDB). The difference between the two is reported in delta units expressed as $\delta^{13}\text{C}_{\text{VPDB}}$, ‰ values.

Non-dispersive infrared spectroscopy (NDIRS), in contrast, directs energy in the form of isotope-selective wavelengths of infrared light through two separate samples of breath and compares the absorbance of two samples. For ^{13}C breath sampling the baseline (or reference) sample of expired air is collected prior to ingestion of the ^{13}C

dose and the timed comparison sample is collected post-prandial and should contain the higher ratio of ^{13}C in the exhaled breath. Because the two isotopes (^{12}C and ^{13}C) have different absorbance characteristics, the isotope ratio can be calculated and is expressed as the difference in parts per million between the two samples (delta over baseline or DOB). This method (NDIRS) is possibly less accurate than IRMS as no specific value is established for the baseline sample (Barth et al., 1998).

However, in the Feasibility study (n = 11, **CHAPTER 5**) pre-prandial samples (summarised below) demonstrated very little inter-individual difference between the means of the two pre-prandial breath samples taken for each of the two tests (Control — denoted C1 & C2 and Treatment — denoted T1 & T2) taken seven days after the Control samples.

Table 7.1 Group mean $\delta^{13}\text{C}$ (‰) observations (IRMS) in pre-prandial breath samples taken in the Feasibility study to establish baseline carbon isotope ratios (C = Control test; T = Treatment test)

Pre-prandial test	N	MEAN \pm SD	Diff \pm SD	95% CI	*P value
C1 + C2 Mean	11	-25.35 \pm 0.86	-0.17 \pm 0.122	-0.95, 0.61	0.65
T1 + T2 Mean	11	-25.18 \pm 0.89			

* Two tailed paired t-test; C = Control test; T = Treatment test

When assessing $\delta^{13}\text{C}$ with NDIRS and then deriving gastric emptying parameters, all calculations are based on the difference between the pre-prandial (baseline) breath carbon isotope ratio and subsequent post-prandial sample ratios. Ideally any one study should use one or other isotope measurement options, either NDIRS or IRMS, as validation. The Feasibility and Repeat studies used IRMS while the China study used NDIRS. (**CHAPTER 8** will consider an analysis from combining the measurements of the Feasibility and China studies.)

7.5 Method (using NDIRS in the China Study)

All participants in the China study were volunteers and gave signed, informed consent after being provided with both written and verbal information (both in Chinese)

and answers to any queries. On the first visit each participant's height, weight and waist were measured in duplicate using standard clinic equipment (manufacturer unknown). The accuracy of the stadiometer and scales were checked by measuring myself on similar equipment on another floor of the hospital, and reliability by checking my own measurements daily.

At 0800 hours on the day of each test, after an overnight fast, participants reported to the Gastroenterology Department on the 7th floor of the Wuhan Hospital. After a 30-minute period of relaxation and paper-work completion, twelve pre-prandial expired breath samples were collected from each participant (details below) before consuming in less than 10 minutes, an omelette breakfast incorporating the 100 mg dose of ^{13}C -octanoic acid.

Special 125 mL foil bags with a fitted tube and cap were required to collect and analyse the expired breath samples on the NDIRS unit. The tube had a plastic sealing cap to stop air escaping once the bag was full. The particular NDIRS unit employed by the Wuhan Hospital had six pairs of ports arranged in two parallel rows one above the other. The uppermost row accommodated the post-prandial sample bags, while its twin in the lower row of ports accommodated the pre-prandial baseline sample bags. Sample bags were attached to each port by removing the cap from the bag's tube, fitting the mouth of the tube over the smaller tapered end of the port (which protruded about 10 mm from the body of the machine) and securing it in place by a gas-tight friction fit. The first and last pre-prandial breath samples were analysed on the NDIRS unit to check that no significant drift in the isotope ratios had occurred over the elapsed time between the samples. The other 10 pre-prandial samples were retained for use as comparative references of isotope ratio for the 10 post-prandial breath samples.



Figure 7.1. The Wuhan Hospital's NDIRS unit showing the two rows of parallel ports with sample bags attached

Each sample bag was coded with the participant's ID and sample number and these were entered into the computer attached to the NDIRS unit, along with the corresponding port number to which each sample bag was attached. Once the ports were loaded the machine was switched on and analysis commenced sequentially, starting with port 1 a vacuum was applied to draw air from each sample bag into a quartz chamber where absorption measurements were undertaken. The automated sequence ended when the last sample had been analysed. As each sample run was completed (approximately 2–3 minutes per sample) the difference in the isotope ratio in relation to the baseline sample, designated DOB (delta over base), was displayed and a report for each sample printed.

7.6 Participants in the China Study

Recruitment was initially slow due to an element of apprehension about a European seeking to conduct a study with Chinese participants. However, once the first three volunteers had completed the first test meal and measurements and been given the kiwifruit capsules to consume over the time prior to their second test, word of mouth endorsement of the study spread and recruitment become considerably easier. As the gastroenterology staff at the hospital observed and became familiar with the protocols they presented three patients for inclusion, albeit without endoscopy reports.

In all, 17 participants signed informed consent forms for the study, two failed to turn up for the second test and three had eaten breakfast at home prior to presenting for the second test. More volunteered for the study as it progressed; unfortunately, time and space constraints precluded their inclusion. More than four study participants at the hospital on any one day was too disruptive for the gastroenterology staff as space was very limited. As well as a steady stream of people presenting for diagnosis in a very small suite of offices at one end of the central corridor, the Gastroenterology floor of the hospital accommodated approximately 60, obviously very ill, bed patients, with five or six occupied beds routinely located in the corridor. The time constraint was such that I conducted the second test for the last three participants in the morning prior to my mid-afternoon departure for New Zealand.

7.7 Calculation of T_{lag} and $T_{1/2}$ from NDIRS measurements

The NDIRS analysis reports the difference between the pre-prandial and the post-prandial breath samples as delta over base (DOB), but unlike the IRMS that reports the actual isotope ratio between the pre-prandial or baseline samples and the isotope ratio of the reference material (for example $-25.34 \delta^{13}C_{VPDB}$, ‰), the NDIRS baseline isotope ratio is zero. As a result, to determine the excess ^{13}C in a post-prandial sample

using the same calculation as used previously in the Feasibility and Repeat studies, I made the assumption that the baseline value for all participants and for both tests was -24.00 $\delta^{13}\text{C}_{\text{VPDB}}$, ‰. I based this assumption on the results of the two previous trials which demonstrated that the mean pre-prandial baseline isotope ratio for both studies ranged from -24.19 to -25.59 $\delta^{13}\text{C}_{\text{VPDB}}$; while coefficient of variation ranged from 0.5 to 1.1% and the differences were not significant (Repeat study, **Table 6.1**). Subsequent recalculation of the ^{13}C excess, assuming a baseline of -25 $\delta^{13}\text{C}_{\text{VPDB}}$, ‰ had trivial if any effect on the measures of T_{lag} and $T_{1/2}$.

Statistical analysis

T_{lag} and $T_{1/2}$ values were derived using non-linear regression analysis. Control and treatment measurements were compared within an individual using paired t-tests. The correlation between gastric emptying parameters and anthropometric measurements was assessed by Pearson r. Unless otherwise stated, data in the text are expressed as means \pm standard deviation (SD). Statistical significance was set at $p < 0.05$. Analyses were carried out using a combination of IBM® SPSS Statistics version 19.0, Sigma Plot Version 10, Microsoft® Excel® Version 14.2.3 and Addinsoft™ XLSTAT version 2013 1.01, packages.

7.8 China Study Results

Descriptive characteristics

More women than men participated in the study (**Table 7.2**). The BMI of ten of the twelve participants was between 20 and 25.3 kg/m^2 ; one was obese and one was thin.

Table 7.2 Physical characteristics of the 12 China study participants (eight women, four men)

Measure	Mean \pm SD	Range
Gender	67% F, 33% M	F-8, M-4
Age, years	54 \pm 10	(40, 79)
Height cm	165 \pm 9	(150, 178)
Weight, kg	64 \pm 16	(47, 108)
Body Mass Index, kg/m ²	23.5 \pm 4.2	(19.1, 35.3)
Waist, cm	80 \pm 15	(63, 112)
Waist/Height ratio	0.48 \pm 0.07	(0.40, 0.64)
Medication — PPI	3 / 12	3 F, 0 M

F female; M male; PPI Proton pump inhibitor

Gastric emptying

Without treatment, i.e. Control, T_{lag} ranged four-fold, from 53 to 209 minutes (**Table 7.3**) and a similar range was seen with $T_{1/2}$. T_{lag} was positively correlated with $T_{1/2}$ ($r = 0.97$, $P < 0.0001$). No meaningful correlations were observed between T_{lag} or $T_{1/2}$ and the other anthropometric measurements.

Three of the participants recorded a difference in T_{lag} and $T_{1/2}$ due to treatment of 10 minutes or less (<11%) this was considered no change (**Table 7.3**). Measured by a decrease in $T_{lag} > 10$ minutes, three participants recorded accelerated gastric emptying due to treatment, and six participants recorded delayed gastric emptying >10 minutes due to treatment measured by T_{lag} (**Table 7.3** and **Figure 7.2**). When considered as a group, any change in the combined mean difference between Control and Treatment for both T_{lag} and $T_{1/2}$, for all 12 participants was modest and not statistically significant (**Table 7.3**). One participant, AR-1, had a very marked difference between treatment and control and the longest $T_{1/2}$ C calculated in all tests (386 minutes compared to the China group mean, excluding AR-1, of 137 minutes). This extreme outlier was removed from further analysis.

Table 7.3 China study T_{lag} and $T_{1/2}$, for Control and Treatment showing results for all 12 participants

	T_{lag} -C (minutes)	T_{lag} -T (minutes)	ΔT_{lag} (minutes)	$\Delta T_{lag}/T_{lag}$ -C %	$T_{1/2}$ C (minutes)	$T_{1/2}$ T (minutes)	$\Delta T_{1/2}$ (minutes)	$\Delta T_{1/2}/T_{1/2}$ -C %
AA-1	82.00	61.00	-21.00	-25.60	133.50	116.60	-16.90	-12.70
AB-1	121.00	136.60	15.60	12.90	174.40	207.60	33.20	19.00
AC-1	147.60	121.50	-26.10	-17.70	232.00	194.50	-37.50	-16.20
AD-1	63.10	69.90	6.80	10.80	111.70	105.20	-6.50	-5.80
AF-1	72.60	72.30	-0.30	-0.40	111.00	114.70	3.70	3.30
AH-1	52.90	70.20	17.30	32.70	94.10	105.40	11.30	12.00
AL-1	90.60	99.50	8.90	9.80	156.90	155.60	-1.30	-0.80
AM-1	67.90	87.30	19.40	28.60	110.90	127.80	16.90	15.20
AO-1	82.70	97.40	14.70	17.80	123.70	146.80	23.10	18.70
AP-1	79.50	114.20	34.70	43.60	159.30	188.20	28.90	18.10
AQ-1	72.90	83.70	10.80	14.80	102.40	129.30	26.90	26.30
AR-1	203.40	113.20	-90.20	-44.30	386.40	167.50	-218.90	-56.70
MEAN	94.68	93.90	-0.78	6.92	158.03	146.60	-11.43	1.70
STDEV	42.94	23.83	32.80	25.33	81.71	35.99	68.56	22.87
P value			0.94				0.58	
95% CI			-21.62, 20.06				-54.99, 32.14	

Δ = Difference of T - C; P value t-test 2 tailed; CI = 95% confidence interval; § PPI medication users in BOLD

The individual percentage change in T_{lag} and $T_{1/2}$ with treatment was variable (**Table 7.3**), but for the group it was not statistically significant, i.e. not different from zero. The difference in $T_{lag}\%$ was 7.0 with 95%CI of -9.0 to 23.0 ($P = 0.35$) and the difference in $T_{1/2}\%$ was 1.7 with 95%CI of -12.8 to 16.2 ($P = 0.80$).

The removal of the outlier AR-1 from the combined data resulted in a mean difference in $T_{lag}\%$ of 11.7% with 95% CI of -1.9 to 25.3 ($P = 0.09$) and $T_{1/2}$ of 7.0% with 95% CI of 12.5 to 16.6 ($P = 0.13$).

Six of the eleven participants in this trial (55%), showed an increase of $T_{1/2}$ of more than 10% (i.e. gastric emptying was delayed by >10%) and seven of the eleven (64%) recorded T_{lag} of >10%, and three of the eleven (27%) demonstrated a reduced T_{lag} and $T_{1/2}$ (gastric emptying accelerated by >10%). This suggests that the response to KFI treatment may be mediated by other factors in this heterogeneous group, but overall the outcome was an increase in both T_{lag} and $T_{1/2}$. The pattern of the individual responses were examined for positive and negative responses as seen in the New Zealand studies (**Figure 7.2**).

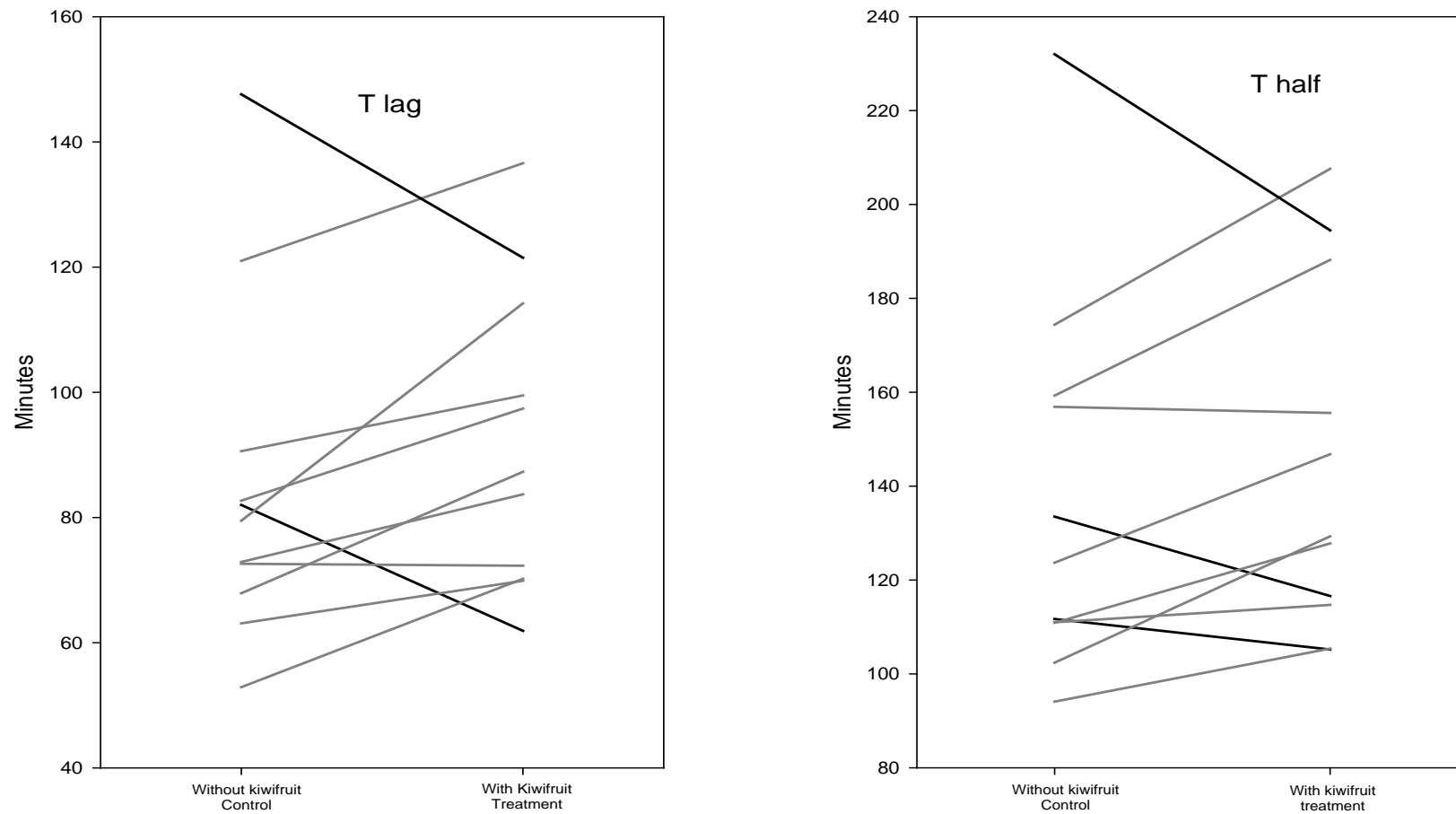


Figure 7.2 Individual variation in gastric emptying kinetics. T_{lag} and $T_{1/2}$ response to treatment of a protein dominant-meal, with kiwifruit protease. The dark lines are the negative responders (accelerated gastric emptying) and lighter lines the positive responders (delayed gastric emptying)

PPI Medication - China Study

Including the outlier, AR-1, three of the twelve participants in the China study (AA-1, AC-1 & AR-1) were medicated with proton pump inhibiting medication (PPIs), known to increase gastric pH. High gastric pH ($> \text{pH } 5$) was shown in the *in vitro* study to have a deactivating effect on kiwifruit protease. These three participants demonstrated decreased time for both T_{lag} and $T_{1/2}$, with KFI treatment. Analysis of the effect of KFI treatment for the remaining nine participants resulted in a substantial mean percentage change in both T_{lag} and $T_{1/2}$ of 19.5% with a 95% CI of 7.4 to 31.5 ($P = 0.007$) and for $T_{1/2}$ of 10.0% with a 95% CI of 1.8 to 18.1 ($P = 0.023$).

7.9 Discussion about the China Study Findings

The findings from this study indicated that the gastric emptying kinetics measured by NDIRS closely resembled those recorded using IRMS and that despite the ethnic difference between the participants in this study and the earlier NZ studies, response to the KFI treatment appeared to be very similar.

While the China study was unable to fulfill its purpose of providing the quantity or quality of data to confirm the effect of kiwifruit on gastric emptying with different degrees of gastric atrophy as originally intended, it did nevertheless provide evidence of an effect from the kiwifruit treatment that added to the New Zealand studies. It also demonstrated that with the PPI medication users excluded, the kiwifruit treatment had a substantial and significantly delaying effect on the kinetics of gastric emptying.

The magnitude and range of the China study measurements did not appear to be different from those recorded in the New Zealand study and were similar to those recorded in other studies (Bromer et al., 2002).

There were two main reasons for conducting the study in China. One was the opportunity to conduct a larger trial at minimal cost using the NDIRS unit. The other

was the reportedly high prevalence of hypochlorhydria and achlorhydria as a result of *Helicobacter pylori* especially among the adult and older population (>35 years) (Li, Hu, Du, & S.L., 1991). Being asymptomatic, *H. pylori* infection is usually only detected in a diagnosis of related disease symptoms usually associated with digestive tract irregularities such as functional bowel disorder (Thompson et al., 1999). Long-term chronic infection can result in atrophy of the gastric glandular tissue and irreversible mutagenesis of chief and parietal cells. As a consequence mild damage can be associated with hypochlorhydria while severe damage almost certainly results in achlorhydria (Kuipers et al., 1996). PPI medication has a similar outcome in that as a powerful agent for suppressing gastric acid and pepsin secretion it can, at lower dose rates, effectively emulate hypochlorhydria, and at higher dose rates has an achlorhydric effect (Yearsley et al., 2006). The literature confirms the role of PPI medication is to disable the proton pump mechanism that acidifies the stomach contents (Ali et al., 2009). As a consequence acid concentration is reduced from normal fasting gastric pH 1.5 - 2.0 to a targeted pH >5.0 and pepsin secretion is also suppressed (Mejia & Kraft, 2009). Furthermore, PPI medication has been identified by others as an agent that retards gastric emptying (Tougas, Earnest, Chen, Vanderkoy, & Rojavin, 2005). The *in vitro* study (**CHAPTER 3**) demonstrated that pH >5.0 severely suppressed kiwifruit protease activity.

While a similar trial was considered and could have been conducted in New Zealand with participants prescribed PPI medication, under the circumstances the cost would have been prohibitive and this was a compelling reason to undertake the study in China where *H. Pylori* induced hypochlorhydria or achlorhydria could be used as a proxy for the effects of PPI medication on peptic digestion. The indication from the limited sample size is that KFI accelerates the lag phase of gastric emptying, but this finding needs to be validated with a larger sample size in further studies.

Limitations and strengths

The dose regime in the China study differed from that of the Feasibility study and could be considered a weakness in the trial design. However, treatment between the Feasibility and Repeat studies also differed in that treatment in the former consisted of a single dose of kiwifruit protease (six, 440 mg capsules of KFI) with the second test meal, whereas in the latter, treatment was a daily dose of kiwifruit protease (six, 440 mg capsules of KFI) each day for seven days between the first test meal (Control) and the second (Treatment). Despite this dose regime change between the studies there was no apparent intra-individual difference in the response to treatment between the two studies (Feasibility and Repeat) that would indicate the data sets were not homogeneous. This limited evidence indicates that multiple treatment on a daily basis prior to the test meal (as per the Repeat and China studies) is no more effective in terms of gastric emptying dynamics than a single treatment with the test meal as administered in the Feasibility study. However, the treatment dose regime was not measured in this series of trials and there may be reason to consider alternative dose regimes in future studies as a single treatment of six capsules with a meal is compliantly impractical.

Sample size ($n = 12$) and lack of characterisation in terms of gastric function were weaknesses of the China study. This is particularly relevant if indeed there were three subgroups of responders, but results from a larger sample would be needed to support this proposal. To this end the next chapter in this thesis investigates whether data from the China study ($n = 11$) and the New Zealand Feasibility study ($n = 11$) could be combined into a larger sample size ($n = 22$).

In conclusion, the response of Chinese participants to treatment with KFI as part of a protein-dominant meal was not apparently different from the NZ Feasibility study and suggests that there is both a variable and an individual response. Furthermore, there was a suggestion that individuals who receive PPI medication respond to kiwifruit

treatment by decreases in T_{lag} and $T_{1/2}$ while those not receiving PPI medication with KFI treatment had an increase in T_{lag} and $T_{1/2}$. However, further studies are required for these findings to be validated.

CHAPTER 8. Putative effect of kiwifruit on gastric digestion dynamics: evidence from two trials

8.1 Introduction

The purpose of this chapter is to gain insight into how the original research question — “how does kiwifruit influence gastric digestion?” was answered. To do this the measurements of the dynamics of the gastric phase of digestion from three small *in vivo* studies, using the ^{13}C -OABT and a high-protein meal (**CHAPTER 5 to 7**) are examined, and the validity of combining the data sets into a meta-analysis is explored. The hypothesis being tested was that between the New Zealand and China studies, for differences in ethnicity, measuring methods and diet, the data would be compatible and could be combined in a meta-analysis.

The measurements taken were the time from ingestion of a stable isotope biomarker to its maximum rate of output of $^{13}\text{CO}_2$ detected on the breath (T_{lag}), and the time from ingestion of the biomarker until 50% remained in the stomach ($T_{1/2}$). Changes in these measures when kiwifruit enzyme was consumed with the meal were assessed.

Despite the small sample sizes of the three studies the observation was made that there may be more than one outcome, i.e. kiwifruit appeared to delay gastric emptying for some participants (positive response), accelerate it for others (negative response) and for others there appeared to be little, if any, response. All three sets of measurements indicated the positive response (delay) occurred more often than the negative response (accelerate) both in numbers of participants and in magnitude of effect. Proton pump inhibitor medication was shown to influence the response to treatment in both the NZ and the China studies (**CHAPTER 5** and **CHAPTER 7**).

Proton pump inhibiting medication (PPI) is reported in the literature to delay gastric emptying (Sanaka et al., 2010; Tougas et al., 2005). The reason is not apparent

but Sanaka et al (2010) speculated that it might be associated with a reduced rate of production of gastric acid delaying the peptic hydrolysis process. The *in vitro* study (**CHAPTER 3**) demonstrated that hydrolysis of meat protein with kiwifruit decreased inversely with the pH of the hydrochloric acid when dilution of the acid exceeded 0.0223 M. (equivalent to pH 3.1 in the hydrolysate.)

While a larger sample size, originally anticipated from the China study, was not forthcoming, this current chapter (**CHAPTER 8**) uses a very small ‘meta analysis’ approach to explore the effect of combining the raw data collected from the China study ($n = 12$ but subsequently reduced to $n = 11$) with that of the New Zealand Feasibility study ($n = 11$) to achieve a larger sample size e.g. $n = 23$. For the rest of this chapter the Feasibility study will be referred to as the New Zealand study and denoted (NZ), as distinct from the China study.

The aims of this meta analysis were firstly to investigate the effect on gastric emptying dynamics, T_{lag} and $T_{1/2}$, of adding kiwifruit to a protein-based meal and secondly, to explore the effect of PPI medication on the response to the kiwifruit treatment.

8.2 Study design and method

Both the study design and method were previously described in detail for the Feasibility study (**CHAPTER 5**) and the China study (**CHAPTER 7**). However, the feasibility of combining gastric emptying measures derived from analysis of $^{13}\text{CO}_2$ by non-dispersive infrared spectroscopy (NIRS) and that derived by isotope ratio mass spectroscopy (IRMS) into a single data set was explored.

Comparison of NIRS and IRMS measures.

Isotope ratio mass spectroscopy (IRMS) was used in NZ to determine the isotope ratio in the breath samples and relies on physically separating the two carbon

isotopes, calculating the atomic ratio of ^{12}C to ^{13}C in the sample and reporting the outcome as a percentage in relation to a known standard (in this case Pee Dee Belemnite) in the format $\delta^{13}\text{C}_{\text{VPDB}}$, ‰ (delta ^{13}C value). An analysis sheet used to plot the fraction of ^{13}C dose recovered over time and the calculation of T_{lag} and $T_{1/2}$ is shown in the following table for the Control test of the NZ study.

Table 8.1 NZ Feasibility trial analysis sheet used for IRMS variables to calculate the fraction of dose recovered over time from which the gastric emptying curves are generated and T_{lag} and $T_{1/2}$ calculated using non-linear regression analysis

Subject	YY							
Control		Dose	0.70206	mmol	m	0.4500		
Run	1	PDB	11237.2	ppm	k	0.0100		
Wt kg	62	BMR	5.6251	MJ/day	beta	2.3340		
Ht cm	163.5	TEE	4.2969	kJ/min	error	5.19E-08		
Age y	60	Energy eq	23.7618	kJ/L	T_{lag}	84.8		
Sex M/F	F	CO ₂ prod	8.0730	mmol/min	$T_{1/2}$	135.9		
Dose mg	101.8							
Date	YY							
Time	δPDB	PPM	PPM XS	FDR	Cum	FDR Fit	Cum Fit	
0	-25.45	10951	0	0.0000	0.000	0.0000	0.0000	
15	-19.97	11013	62	0.0007	0.005	0.0007	0.0045	
30	-14.45	11075	124	0.0014	0.021	0.0013	0.0193	
45	-12.29	11099	148	0.0017	0.045	0.0017	0.0421	
60	-10.24	11122	171	0.0020	0.072	0.0020	0.0702	
75	-8.74	11139	188	0.0022	0.103	0.0021	0.1012	
90	-8.19	11145	194	0.0022	0.136	0.0021	0.1331	
115	-9.47	11131	180	0.0021	0.190	0.0020	0.1851	
150	-13.35	11087	136	0.0016	0.253	0.0017	0.2496	

Peak output in this example was at 90 minutes - δPDB highlighted in bold

Calculation of PPM from δPDB used the following formula:-

At time 0 minutes, $\text{PPM} = (\delta\text{PDB at 0 min}/1000 + 1) * \text{PDB (ppm)} = 10,951 \text{ ppm}$

At time 0 minutes, $\text{PPM} = (-25.45/1,000 + 1) * 11,237.2 = 10,951 \text{ ppm}$

At time 15 minutes $\text{PPM} = (-19.97/1,000 + 1) * 11,237.2 = 11,012.79$

$\text{PPM excess (XS) at 15 minutes} = (\text{PPM at 15 min}) - (\text{PPM at 0 min}) = 62 \text{ ppm}$

$\text{FDR at 15 min} = \text{PPM XS at 15 min}/1,000,000 * \text{CO}_2 \text{ produced (mmol/min)}/^{13}\text{C dose administered (mmol)}$

The China study, however, used NDIRS (non-dispersive infrared spectroscopy) to determine the ^{13}C concentration in the breath sample submitted for analysis. As discussed in Chapter 7, this method relies on reading the absorbance difference between

the absorbance of a known energy source (infrared light) between the baseline and a treated sample enriched with ^{13}C where one sample is a baseline sample taken prior to ingestion of a known dose of ^{13}C . Because the two isotopes (^{12}C and ^{13}C) have different absorbance characteristics, the isotope ratio can be calculated from the difference in the light absorption and is expressed as the difference in parts per million between the two samples (delta over baseline or DOB). Unlike the IRMS method, the NDIRS unit does not provide a specific value for the baseline sample.

Table 8.2 China trial analysis sheet used with NDIRS variables to calculate the fraction of dose recovered over time from which the gastric emptying curves are generated and T_{lag} and $T_{1/2}$ calculated using non-linear regression analysis

Subject	XX						
Control		Dose	0.696551724	mmol	m	0.468	
Run	1	PDB	11237.2	ppm	k	0.01	
Wt kg	65	BMR	5.8251	MJ/day	beta	2.27	
Ht cm	167	TEE	4.4497	kJ/min	Error	3.46E-07	
Age y	45	Energy eq	23.7618	kJ/L	T_{lag}	81.98	
Sex M/F	F	CO ₂ pro	8.3600	mmol/min	$T_{1/2}$	133.51	
Dose mg	101						
Date	XX						

TIME	DOB	PPM	PPM EX	FDR	Cum	FDR Fit	Cum Fit
0	0.0	10967.5	0.0	0.0000	0.0000	0.0000	0.0000
15	2.6	10996.0	28.5	0.0003	0.0026	0.0007	0.0053
30	13.4	11114.5	147.0	0.0018	0.0184	0.0014	0.0218
45	14.2	11123.2	155.7	0.0019	0.0456	0.0019	0.0467
60	15.5	11137.5	170.0	0.0020	0.0749	0.0021	0.0768
75	16.1	11144.1	176.6	0.0021	0.1061	0.0022	0.1096
100	15.3	11135.3	167.8	0.0020	0.1578	0.0022	0.1652
120	16.0	11143.0	175.5	0.0021	0.1990	0.0020	0.2075
150	12.7	11106.8	139.3	0.0017	0.2557	0.0017	0.2638
180	11.2	11090.3	122.8	0.0015	0.3029	0.0014	0.3105

Peak output in this example was at 75 minutes - DOB if highlighted in bold

Calculation of PPM from δPDB used the following formula:-

At time 0 minutes, $\text{PPM} = (1 - 0.024) * \text{PDB (ppm)} = 10,967.5 \text{ ppm}$

At time 15 minutes, $\text{PPM} = 10,967.5 * (1 + \text{DOB at 15 min}/1000) = 10,996.0 \text{ ppm}$

At time 30 minutes, $\text{PPM} = 10,967.5 * (1 + \text{DOB at 30 min}/1000) = 11,114.5 \text{ ppm}$

PPM EXcess and FDR were calculated in the same way as in the NZ trial (Table 8.2)

In the NZ study the mean $\delta^{13}\text{C}_{\text{VPDB}}$ (baseline) \pm SD for four samples for each of the 11 participants was -25.27 ± 0.12 . For the six participants in Proof of Principle study (**CHAPTER 6**) the mean SD $\delta^{13}\text{C}_{\text{VPDB}}$ baseline \pm was -24.37 ± 0.14 . For the purpose of the China study the baseline $\delta^{13}\text{C}_{\text{VPDB}}$ for all participants in the study was set at -24.00 i.e. -24.00/1000 (0.024) as no baseline value was available to derive the formula. When the value of baseline was changed from -24 to -25 it had no appreciable effect on the derivation of the T_{lag} or $T_{1/2}$ times.

Statistical analysis

Anthropometry and T_{lag} and $T_{1/2}$ measures from the two studies (China and NZ) were compared using unpaired t-test analysis. The treatment effect of the combined study was also analysed using unpaired t-test. T_{lag} adjusted for body weight was modelled using analysis of covariance (ANCOVA) with PPI as a fixed effect. Unless otherwise stated data in the text are expressed as means \pm standard deviation (SD) and statistical significance was set at $p < 0.05$. Graphical representations to visually show intra-individual responses to treatment were examined using Sigma Plot Version 10. Analyses were carried out using a combination of IBM® SPSS Statistics version 19.0, Microsoft® Excel® Version 14.2.3 and Addinsoft™ XLSTAT version 2013 1.01, packages.

8.3 Results

Physical characteristics and Health

Both trials had more women than men participants (China - eight women, four men; NZ - nine women, two men). All participants were older than 40 years with a maximum age of 80 years (**Table 8.3**). Participants were not different by country in any anthropometric characteristic other than age where the Chinese participants were 17 ± 8

years ($p < 0.001$) younger than the NZ participants. None of the men, but 35% (6/17) of the women were medicated with PPIs. Each country had three women who were prescribed proton pump inhibitors (PPIs) but one was excluded from this report due to an excessively long T_{lag} time (>6 hours). All NZ trialists were Caucasian, self-reported as healthy, and nine of the 11 participated in an activity programme for older people (>60 years). In contrast the China study participants were all of Chinese ethnicity and all, self-reportedly, had digestive problems that were assessed from the individual's response to a written questionnaire to be consistent with upper functional bowel disorder (FBD).

Table 8.3 Comparison of anthropometric measurements of New Zealand study ($n = 11$) and China study ($n = 11$, as AR regarded as an outlier and excluded from the data set)

Measure	NZ Mean \pm SD	China Mean \pm SD	Difference	95% CI	P value
Age, years	70 ± 7	53 ± 10	-16.5	-22.0, -11.1	<0.0001
Weight, kg	63 ± 10	65 ± 17	1.7	-8.2, 11.6	0.74
Height, cm	162 ± 7	165 ± 9	3.6	-2.2, 9.4	0.22
Waist, cm	77 ± 8.4	81 ± 14.2	4.4	-6.4, 15.1	0.43
Waist/Height	$0.48 \pm .05$	0.4 ± 0.1	0.04	-0.04, 0.07	0.63
BMI, kg/m ²	24.2 ± 2.7	23.6 ± 4.3	0.60	-3.5, 2.3	0.69
PPI	3-W, 0-M	2-W, 0-M	1.7	-8.2, 11.6	0.74

Outlier excluded, 95% CI confidence interval on the difference between the means; BMI - body mass index calculated as (weight in kg / height in meters squared); PPI proton pump inhibitor medication users, W women, M men.

Neither the NZ study's anthropometric measurements nor those of the China study showed any significant statistical relationships with the parameters of gastric emptying (T_{lag} and $T_{1/2}$) from their respective studies, but the percentage change in response was negatively correlated (**Table 8.4**).

Table 8.4 Correlation of percentage change of T_{lag} and $T_{1/2}$ with treatment and anthropometric measurements - Pearson r (probability)

	% change T_{lag}		% change $T_{1/2}$	
% change $T_{1/2}$	0.72	(<0.001)	-	-
Weight (kg)	-0.42	(0.049)	-0.53	(0.011)
Height (cm)	-0.34	(0.12)	-0.47	(0.027)
BMI (kg/m ²)	-0.39	(0.08)	0.43	(0.048)
Waist (cm)	-0.29	(0.219)	-0.62	(0.003)
Waist/Height ratio	-0.02	(0.34)	-0.55	(0.01)

Treatment effect for the combined data set

Comparison of the effect of the addition of KFI to the meal compared with the control meal demonstrated an overall delay in gastric emptying for the group (n = 22). The difference in T_{lag} was an increase of 7 ± 15 minutes, which was almost significant (P = 0.06) and $T_{1/2}$ increased by 10 ± 20 minutes, which was significant (P = 0.027) (Table 8.5).

When the data were split to explore the effect of with and without PPI medication, an even clearer treatment effect was observed, with an increase in both T_{lag} and $T_{1/2}$ times for the 16 participants with no PPI medication but not for the six medicated participants (Table 8.5).

Table 8.5 Comparison of the treatment effect on T_{lag} and $T_{1/2}$ when PPI medication users were isolated

	Control	Treatment	Difference	95% CI	P
All Data n=22					
T_{lag} , minutes	81 ± 20	88 ± 20	7 ± 15	0, 19	0.060
$T_{1/2}$, minutes	134 ± 31	144 ± 29	10 ± 20	0, 14	0.027
Excl'dg PPI n=16					
T_{lag} , minutes	79 ± 16	90 ± 19	11 ± 13	4, 18	0.005
$T_{1/2}$, minutes	129 ± 23	143 ± 31	13 ± 17	4, 23	0.009
PPI only n=6					
T_{lag} , minutes	89 ± 24	80 ± 29	-9 ± 14	-24, 6	0.180
$T_{1/2}$, minutes	146 ± 31	150 ± 44	4 ± 23	-28, 20	0.690
PPI only n=5					
T_{lag} , minutes	91 ± 25	77 ± 32	-14 ± 10	-26, -2	0.036
$T_{1/2}$, minutes	154 ± 35	146 ± 48	-8 ± 23	-37, 20	0.470

The only PPI consumer to record a positive response, OK-06, did so in the Feasibility study; however, OK-06 was the only PPI consumer to also participate in the Repeat study as OK-06B; in the Repeat study OK-06B recorded a strong negative response to the intervention. Had OK-06 refrained from consuming PPI medication on the day of the Feasibility study but not on the day of the Repeat study? This may explain the anomaly, but this is a conjecture. However, in Table 8.5, the effect on the PPI group measurements, were investigated by removing OK-06 from the data set but retaining OK-06B. The result (**Table 8.5**), shown as PPI only ($n = 5$), accelerated gastric emptying as measured by T_{lag} , by -14 ± 10 minutes ($P = 0.036$), as compared to PPI only ($n = 6$), where the T_{lag} difference was -9 ± 14 minutes and was not significant. Treatment appeared to have a significant effect on $T_{1/2}$ for the non-users of PPIs increasing $T_{1/2}$ from 129 minutes to 143 minutes, while the effect was opposite for the PPI users in that $T_{1/2}$ was reduced from 154 minutes to 146 minutes, although this was not significant.

Observation of trends - a graphic representation

Of the 22 participants in the combined study (NZ and China), 16 (72%) recorded a difference in gastric emptying kinetics greater than 10% as a result of treatment, and 6 (27%) recorded less than a 10% effect. These latter were regarded as non-responders. Of the 16 that recorded >10% effect, 12 (75%) experienced delayed gastric emptying and four experienced accelerated gastric emptying as a result of treatment.

Of the 22 participants in the combined study, 15 recorded delayed T_{lag} (Figure 8.1) and seven recorded accelerated T_{lag} in response to the treatment. Of these seven, five (71%) were taking PPI medication.

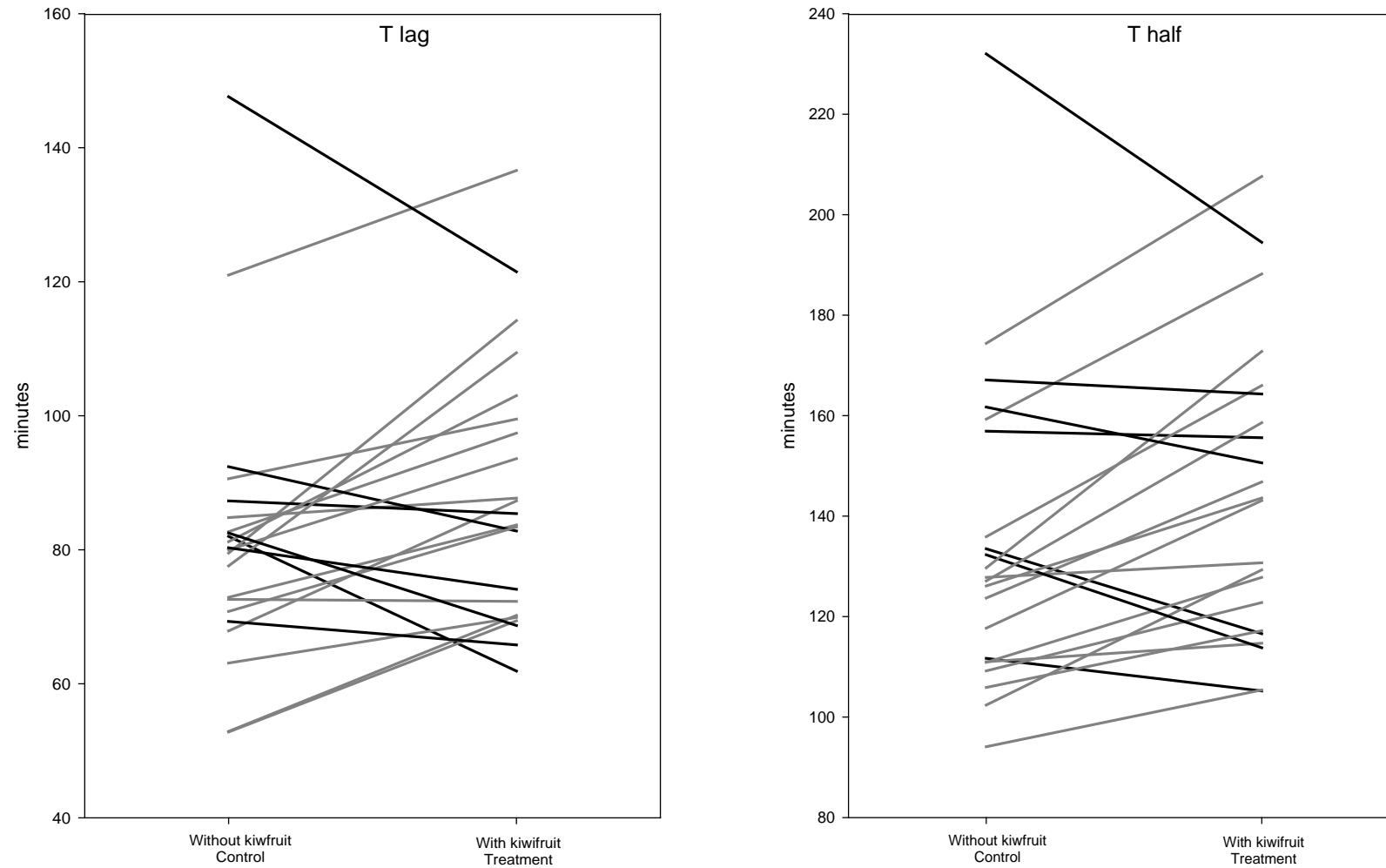


Figure 8.1 Individual variation in the gastric emptying parameters, T_{lag} and $T_{1/2}$ in response to treatment of a protein meal with kiwifruit protease. Combined NZ and China Data ($n = 22$). The black lines represent negative responders (accelerated gastric emptying) and grey lines positive responders

In summary: 55% were +ve responders >10%
 18% were -ve responders >10%
 27% were non-responders $\pm <10\%$

The percentage change of T_{lag} due to treatment indicated a normal distribution with a mean >0 . The following histogram illustrates the density of the % T_{lag} differences for the combined data set ($n = 22$) compared to the normal distribution at 95% confidence level for confidence limits of 0.02 and 13.2.

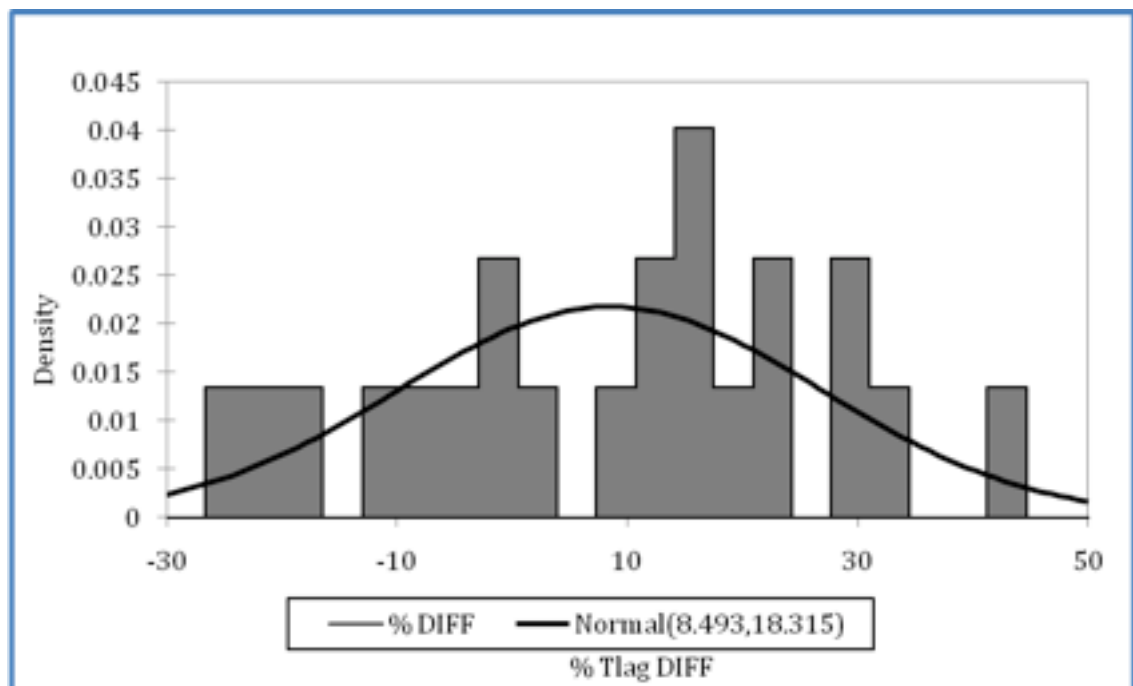


Figure 8.2 Histogram showing the actual density of the % T_{lag} difference between Treatment and Control compared with the expected normal distribution for the mean and standard deviation of 8.5 ± 18.3 ; Combined study ($n = 22$)

When the percentage change in T_{lag} was modelled using ANCOVA with PPI as a fixed effect and adjusting for body weight, both weight and PPI were significant predictors of the percentage change in T_{lag} . At a constant weight of 64 kg mean difference in the percentage T_{lag} increase accounted for was 16 (7 to 37)%. As a prediction equation:

$$\% \Delta \text{ in } T_{lag} = 0.7 * \text{weight} - 21.8 * \text{PPI} + 60.4 \quad R^2=0.44 \quad \text{SEE} = 15.1\%$$

Where PPI medication = 1 and no medication = 0; SEE is the standard error of the estimate and R^2 is the coefficient of determination.

Similar analysis of percentage change in $T_{1/2}$ showed that weight was a significant factor (as expected from the correlation reported earlier) but PPI medication was not; however, in overall terms these changes were relatively minor. Furthermore, they reflect the measurements of a specific population sample and may not apply to a young healthy population sample.

8.4 Discussion on the outcome of combining the two data sets

Despite differences in age, ethnicity and treatment regime, in general the gastric emptying parameters for the 22 participants derived from each of the two studies (NZ and China) were not different in their pattern of response or the magnitude of the measurements T_{lag} and $T_{1/2}$.

The use of two different methods of measuring the $^{13}\text{C}/^{12}\text{C}$ ratios of breath samples i.e. the use of IRMS in the NZ study and NDIRS in the China study, was considered a potential source of error in combining the data in the “meta analysis”. However, Braden et al. (1999) clearly demonstrated excellent agreement on baseline-corrected $^{13}\text{CO}_2$ -exhalation values between IRMS and NDIRS. The mean difference between both methods in their study was 0.28 ± 1.93 delta/1000; with IRMS as the reference, the NDIRS results had a sensitivity of 97.8% and a specificity of 98.9%. (Braden, Caspary, & Lembcke, 1999). Therefore it was considered reasonable to combine the data sourced from the two different methods of measurement, into this meta-analysis.

Excluding the outlier from the China study, the pattern of response; viz a negative response, no change and a positive response to treatment seen in the combined study (Figure 8.1) reinforced the pattern observed separately in all three *in vivo* studies.

Overall, for the 22 participants, treatment of the meal with kiwifruit resulted in a statistically significant increased time for both T_{lag} and $T_{1/2}$. Furthermore, medication with PPIs and participant weight partially mediated the increased time of gastric emptying observed with kiwifruit treatment. Tougas et al. (2005) used a similar graphic representation as Figure 8.1 to demonstrate, by individual, the effects of 14 days treatment with 20 mg b.d. of omeprazole, a PPI medication, on gastric emptying using scintigraphy in 20 healthy males. For the group, treatment delayed gastric emptying, $T_{1/2}$, by around 11 minutes \pm 3%, yet six of the participants had demonstrated accelerated gastric emptying as a result of treatment. The authors did not comment on the variability of response among participants (Tougas et al., 2005).

While a negative association between percentage change in gastric emptying parameters and both waist to height ratio and BMI was observed in these studies, and were expected to be significant, there is one report (Chen et al. 2003) for 44 healthy subjects that there was no effect of age, gender, BMI or smoking status on gastric emptying or body proportion and size on T_{lag} or $T_{1/2}$. Yet, increasing age, male gender and some disease states, such as diabetes mellitus, are associated with longer times of gastric emptying kinetics. While the effect in the sample of almost 1300 subjects was small, it was significant (Keller et al., 2009). Furthermore, the obese in general have a larger gastric volume and therefore greater mucosal surface area (Wisén & Hellstom, 1995).

Chen et al. (2003) using both scintigraphy and NDIRS to monitor the gastric emptying of a meal (one egg yolk, two egg whites, two slices of white bread, 7 g of margarine, 8 g of grape jelly and 150 mL of water - 260 kcal) found no correlation between the parameters of gastric emptying (T_{lag} and $T_{1/2}$) with age, gender or BMI. Wisén et al (1995) reviewed the relationship of gastric motility with obesity and found conflicting reports of more rapid nutrient absorption and less satiety due to reduced

response to cholecystokinin (CCK) and lower concentrations of somatostatin and neurotensin, but no difference in overall gastrointestinal transit times. Moore et al (1983) used scintigraphy to demonstrate there was no correlation between age (10 healthy young men 32 ± 2.4 years, and 10 healthy older men 76 ± 1.6 years) and the rate of gastric emptying of food solids, although liquid transit was delayed in the elderly.

However, the apparent negative correlation between the percentage change in gastric emptying times and the weight-to-height ratio and BMI in the present studies may have been influenced by the KFI dose-to-weight ratio as no attempt was made in any of the three studies to titrate the dose to body size; all participants were administered the same dose of KFI (six capsules) regardless of body size.

As measurement of the treatment effect in these *in vivo* studies was confined to the region of the stomach, the change in gastric emptying times raises the question of the mediating effect of existing gastric acidification on the change in the kinetics of gastric emptying in the presence of the kiwifruit protease. Hurwitz et al. (1997), based on a study of 73 men (79 ± 5 years) and 173 women (80 ± 6 years) concluded there was little reason to suspect that gastric acidity diminished as a result of age. Their study used a 2 g quininium resin capsule which released quinine in pH <3.5 which was subsequently measured in the urine. Of the 246 participants, 84% recorded gastric pH <3.5 within two hours of swallowing the resin capsule; participants fasted overnight and no food was consumed prior to, or during the test, only 120 mL of water. Our *in vitro* study (**CHAPTER 3**) demonstrated that 0.022 M HCl with 600 mg of meat powder and 100 mg of KFI resulted in a hydrolysate with a stable pH of 3.1; when pepsin replaced KFI in 0.022 M HCl, hydrolysis of the meat powder was <50% cf >80% with pepsin in 0.074 M HCl. Hurwitz's study did not consider the buffering effect of food on gastric acidity nor did it determine gastric reacidification capacity of the participants, yet the text of the paper states, " *Early studies suggested that gastric acidity declines as people*

age. However, sequelae of achlorhydria are uncommon in older people, making this conventional wisdom unlikely" and in the Conclusion " In contrast to what is commonly stated, nearly 90% of elderly people in this study were able to acidify gastric contents, even in the basal, unstimulated state."

It may be that 'conventional wisdom' is correct and the importance of small changes in gastric acidity and gastric reacidification capacity have been overlooked and may be more relevant to digestion efficiency than basal gastric acidity *per se*. This needs further investigation.

In addition to possible measurement errors, compatibility issues associated with the two data sets were recognised as having the potential to confound the outcome of combining the data in this study. These included the length of treatment being a single dose in the NZ study compared with a multiple dose in the seven days before measurement of treatment in the China study; the use of two different measuring methods (IRMS in the Feasibility study and NDIRS in the China study) and the age and ethnicity differences between the studies. Dietary differences between the two ethnic groups involved in the meta-analysis could be considered a source of errors; however, all the participants were healthy and the baseline ^{13}C was accounted for in the calculation of gastric emptying rate. While every effort was made to ensure these factors did not influence the outcome of the combined data study, the possibility that they may have is acknowledged.

On the assumption our results accurately reflect the effect of treatment, the outcome of the combined study ($n = 22$) indicated that rejecting the null hypothesis that kiwifruit treatment had no effect on gastric emptying, measured by $T_{1/2}$, had only a 2.7% probability of being wrong (**Table 8.5**, P value = 0.027); whereas rejecting the null hypothesis on the basis of the T_{lag} difference had an 6% probability of being wrong (**Table 8.5**, P value = 0.06).

The mean effect of treatment measured by T_{lag} was to delay gastric emptying for the group ($n = 22$) by about seven minutes (8%). However, with a lower confidence limit approaching 0 minutes, it is conceivable the effect was trivial. Likewise, when measured by $T_{1/2}$, the mean effect was to delay gastric emptying by approximately 10 minutes (<8%), with a lower confidence limit again approaching 0 minutes, likewise the effect could be trivial.

The indication that PPI medication may be correlated with a negative response to kiwifruit treatment (accelerates gastric emptying) cannot be substantiated with the data gathered in these *in vivo* trials alone, but the outcome of analysing the data set, excluding the PPI consumers (**Table 8.5**), warrants further investigation when the combined data is analysed with and without PPI consumers as shown in Table 8.5.

The literature confirms that a consequence of PPI medication is to significantly delay the kinetics of gastric emptying (Benini et al., 1996). This is a rarely discussed feature of PPI medication. Our study on the other hand, provides the first evidence that the novel treatment of a protein meal with KFI significantly accelerates gastric emptying for PPI users measured by T_{lag} ($P = 0.036$). Delayed gastric emptying has also been implicated as part of the presentation of acid gastric oesophageal reflux (Hercularno et al., 2004) and dyspeptic symptoms such as heartburn and bloating that have also been associated with gastro oesophageal reflux disease (GERD) (Holtmann, Adam, & Liebrechts, 2004). It has also been reported that in six asymptomatic volunteers and 24 hours monitoring of pH above and below the lower oesophageal sphincter, that seven days of PPI medication decreased gastric acid secretion but not gastric oesophageal reflux (Tamhankar et al., 2004).

Treatment with kiwifruit protease may have important therapeutic implications relating both to GERD and PPI medication. Furthermore the significant effect of KFI treatment in delaying gastric emptying of non-PPI users raises the possibility that

marginally depressed gastric acid secretion may have an important effect on protein digestion efficiency, with longer term implications associated with protein maldigestion and onset of sarcopenia with increasing age.

CHAPTER 9. Discussion

Definitive findings from the three human pilot studies were not obtained but indications from the study outcomes raised new questions, particularly around the need to control for covariates such as the influence on protein digestion of PPIs and *H. pylori* gastric mucosal damage and the influence that inefficient peptic digestion of protein may have on constipation, functional bowel disorders and sarcopenia. This means much larger, better designed and more robust studies are required.

9.1 Outcomes

Multiple outcomes emerging from this compendium of investigative studies, while of a preliminary nature, add to the existing body of knowledge about protein digestion and digestive dysfunction. A gap in the knowledge in relation to kiwifruit and its ameliorating effect on digestive dysfunction raised the question: What is the mode of action? The initial *in vitro* study that manipulated hydrogen ion and protease concentration to elucidate the contribution of kiwifruit protease (KFI) on protein hydrolysis provided, for the first time, strong evidence that (kiwifruit) protease substantially increased protein digestion efficiency *in vitro*, but only when gastric conditions were sub-optimal for peptic hydrolysis (**CHAPTER 3**).

This finding led to the hypothesis that the mode of action by which kiwifruit relieves a variety of symptoms (collectively termed digestive dysfunction) is enhanced protein digestion by the kiwifruit protease in situations where elevated fasting gastric pH or gastric re-acidification insufficiency is the cause of diminished gastric-protein digestion efficiency.

A subsequent literature review of methods appropriate for measuring gastric protein digestion efficiency (**CHAPTER 4**) failed to find any previous intra-individual studies on the subject or, consequently, a suitable measurement methodology. Of the

methods capable of measuring the rate at which a meal exited the stomach (gastric emptying), only the ^{13}C -octanoic acid breath test (^{13}C -OABT) met the criteria for affordability and participant safety, comfort and ongoing compliance. In a comprehensive literature search no previous studies were found where breath testing had been used to measure protein digestion efficiency. Work of other researchers (Low, 1990; Nishi et al., 2001) referred to the controlling influence of partially hydrolysed proteins on gastric emptying and this provided the inspiration to investigate the use of the ^{13}C -octanoic acid breath test with a protein-dominant meal for measuring protein digestion efficiency (**CHAPTER 5** and **CHAPTER 6**). Three subsequent small *in vivo* studies provided the first evidence of the likely existence of three distinct subgroup-gastric emptying responses to consumption of a protein dense meal with kiwifruit compared to the same meal without kiwifruit, as identified by the magnitude of the differences in gastric emptying times.

Results indicated that kiwifruit delayed gastric emptying for the 42% of triallists; but for a smaller subgroup (28%), treatment accelerated gastric emptying; while for a third subgroup (30%) treatment had a minimal effect. This finding was confirmed by the outcome of the third *in vivo* study (**CHAPTER 7**). An explanation of how kiwifruit would have this effect is beyond the scope of this study due to the limited data of the gastric function and health of the participants. However it is **speculated** that amongst the participants in the combined studies there were three distinct groups that could be identified by their fasting gastric pH and/or their gastric re-acidification capacity. The first group, those who recorded less than a 10% T_{lag} or $T_{1/2}$ response to the KFI treatment, may have had relatively normal gastric physiology that maintained fasting gastric pH in the normal range, resulting in normal gastric digestion of protein and therefore recorded relatively normal gastric emptying kinetics. The second group may have been hypochlorhydric due to gastritis impairing the gastric function and resulting

in elevated fasting gastric pH (not uncommon in the elderly). The third group may have been achlorhydric due to autoimmune disease or exposure to bacterial infection (*H. pylori*), medications (PPIs), heavy metals, nicotine or alcohol.

The variation in intra-individual responses in the dynamics of gastric emptying raised the question about what differentiated the relatively homogeneous groups of participants tested. For the six participants consuming proton pump inhibiting medication the time to maximum appearance of $^{13}\text{CO}_2$ on the breath consistently decreased when kiwifruit protease was added to the meal. While not proven in the course of these studies, there is some rationale for speculating that the inter-individual responses were a reflection of either the fasting gastric pH or the gastric re-acidification potential of the individuals. This is supported by (1) the findings of the *in vitro* study (**CHAPTER 3**); (2) the fact that the aim of PPI medication is to neutralize gastric acidity; (3) the participants in the New Zealand studies (**CHAPTER 5** and **6**) were >60 years of age and therefore more likely to have elevated gastric pH than a younger, healthy cohort; (4) the participants in the China study (**CHAPTER 7**) presented as having chronic digestive dysfunction, with an expectation this may have been associated with an existing *H. pylori* infection or gastric tissue damage as a result of prior infection.

If indeed the existence of three distinct response groups to kiwifruit treatment are confirmed in future studies, the status of the participant's fasting gastric pH and gastric re-acidification capacity could be measured to determine if this is a contributing factor.

Proteolytic activity of kiwifruit is well documented in the literature (Baker et al., 1980; Boland & Hardman, 1973) and actinidin, the generic name of the kiwifruit protease, has been extracted and refined for commercial use since the late 1970s. Although commercially refined actinidin has different substrate specificity than

commercially available papain and bromelain, it has similar uses. As the cost of extraction of actinidin exceeds that of the other two, it is ultimately more costly and therefore, other than for very substrate specific functions, its commercial use is limited. In contrast, Kiwi Crush TM, more by chance than design, was the first commercial product to incorporate unrefined, active kiwifruit protease as an ingredient in a concentration suitable for human consumption. Subsequent consumer adoption of Kiwi Crush for its beneficial effects on digestion produced the impetus for this thesis which has attempted to identify the specific mode of action that explains the effect.

The literature review (**CHAPTER 2**) identified gaps in the knowledge regarding the mode of action of kiwifruit on digestion. The *in vitro* study, described in CHAPTER 3 was completed in 2008 and was the first work to identify proteolytic activity as the likely mode of action. An *in vitro* study by Kaur et al. (2010) subsequently confirmed kiwifruit increased protein digestion at pH 1.9 but did not demonstrate the sensitivity of protein hydrolysis by kiwifruit to the subtle changes in gastric acidity identified in the 2008 *in vitro* study, as presented in this thesis. In a second *in vitro* study of small intestinal protein digestion at pH 8.0 following gastric digestion at pH 1.9, Kaur et al. (2010) confirmed the findings of CHAPTER 3, 2008 *in vitro* study; that there was no further digestion of beef protein in the small intestine medium following the gastric digestion phase. These independent results strongly indicate that kiwifruit hydrolysis of meat protein is only likely to occur in the gastric medium of the stomach.

The Chapter 3 *in vitro* study demonstrated that the inverse relationship between pH and pepsin activity dictates that digestion of protein diminishes proportionally as gastric pH increases, for example peptic activity is maximum at pH 1.5–2.0 and almost absent at pH >5.5. The study also identified a potential for kiwifruit to increase protein hydrolysis by as much as 150% when gastric acid and pepsin concentrations were sub-

optimal. A comprehensive literature search and consequent monitoring of the literature has found no comparable studies that have investigated, *in vitro* or *in vivo*, the contribution kiwifruit protease can make to protein hydrolysis when peptic hydrolysis is compromised.

9.2 Do PPIs compromise protein digestion?

Sub-optimal concentrations of gastric acid and pepsin for efficient gastric digestion of protein are apparently not considered a critical health risk (Pohl et al., 2008). The basis of this reasoning is that small intestinal proteases have the capacity to compensate for incomplete gastric hydrolysis and can indeed effect complete hydrolysis in the absence of any peptic hydrolysis. The role of proton pump inhibiting medication (PPIs), currently one of the most widely distributed drugs with reported end user sales of \$US24 billion in 2006, is to suppress gastric acid secretion and maintain gastric pH >4.0 for between nine and 15 hours with a single daily dose (Ali et al., 2009). A less reported feature of PPIs is the suppressive effect they have on pepsin secretion in addition to the deactivating effect elevated gastric pH has on peptic activity (Kittang, Aadland, & Schjønby, 1985).

If indeed impaired gastric protein digestion is asymptomatic, then the findings from this study as they relate to PPI medication may be inconsequential. If however there is a cause and effect relationship between delayed gastric emptying, gastro-oesophageal reflux disease (GERD) and functional dyspepsia, as indicated by Sanaka et al. (2010), then the accelerating effect of kiwifruit protease on the kinetics of gastric emptying for PPI users observed in the current study may have clinical implications. It was interesting to note from the meta-analysis (**CHAPTER 8**) that KFI significantly decreased T_{lag} by 15% ($P = 0.36$) and although the effect on $T_{1/2}$ was not significant the reduction from 154 minutes in the control to 146 minutes in the treatment resulted in the

PPI users recording a similar $T_{1/2}$ as the treatment $T_{1/2}$ recorded for the non-PPI users (143 minutes). Could this be viewed as normalizing gastric emptying for both groups? Guan et al. (1996) demonstrated, using a conscious rat study, that peptic-HCl hydrolysis of bovine serum albumin increased CCK concentration and pancreatic secretion of proteases more than three-fold compared to the same proteins entering the duodenum undigested. They concluded that "*an important role of gastric pre-digestion of protein may be to render them recognizable as protein substrate by the negative feedback mechanism controlling CCK release*" (Guan & Green, 1996). Although this was not a human *in vivo* study, it may indicate that delayed gastric emptying results from undigested protein molecules being unrecognised as a protein substrate by duodenal receptors and therefore failing to stimulate CCK release and pancreatic secretion. Furthermore, the explanation for accelerated gastric emptying resulting from KFI addition to a protein meal for PPI users was a reflection of enhanced gastric protein digestion. This may be driven by recognition of the hydrolysed protein profile of the chyme entering the duodenum by the receptors that stimulate CCK release, and increased pancreatic secretion that result in more efficient post-gastric protein hydrolysis. Nishi et al. (2001) concluded from their rat study, "*peptic hydrolysates of commonly ingested dietary proteins stimulate CCK release via trypsin-independent direct sensing by intestinal mucosal cells.*" (Nishi et al., 2001).

9.3 Stable isotopes to measure protein digestion efficiency

The literature review conducted before the *in vivo* studies failed to identify an existing cost effective, non-invasive method of measuring gastric digestion efficiency of protein *in vivo* that ensured trial participant safety and compliance. Blood serum concentrations of pepsinogen (PG) I/II have been used by others as a measure of potential gastric digestion efficiency based on the inverse relationship between PG I/II

ratio and gastric pH (Miyamoto et al., 2008); however, the method is possibly a more reliable indicator of gastric acidity rather than a measure of protein digestion efficiency (Peitz et al., 2011). Other methods of measuring protein digestion efficiency (detailed in **CHAPTER 4**) were considered but rejected for various reasons.

This thesis reports, for the first time, the use of the stable isotope breath test in conjunction with a protein dense meal appeared to be a reliable but indirect method to measure gastric protein digestion efficiency, but validation was not possible because a 'gold standard' of measurement is not available. What has been shown is simply a measure of the effect the presence of kiwifruit had on the kinetics of gastric emptying. However, if the method does prove to be a reliable predictor of protein digestion efficiency, it may be possible to establish predictive indices for clinical diagnoses of other conditions, using the same technique. While the evidence thus far is encouraging, more comprehensive trials will be required to confirm protein digestion efficiency is indeed being measured.

9.4 Subgroups

Identifying the three subgroups in terms of the response to KFI treatment was a feature of all three *in vivo* trials. Positive and negative responses have not been discussed in the literature reviewed, and this raises the question around the mediators of inter-individual variability which may be intrinsic (e.g. genetic and gastric damage) or extrinsic (medication). If confirmed in more comprehensive trials, this will make a contribution to the current understanding and may provide the future basis for a sarcopenic risk assessment model which includes nutrigenomic and metabolomic factors.

The range of percentage change in T_{lag} due to treatment from all three *in vivo* studies ($n = 29$), including that for the outlier in the China study, was between a

minimum of negative 50% and a maximum of positive 50% (**Figure 9.1**) with 16 positive responders and 13 negative responders. For the individuals at the extremes, this is a huge effect.

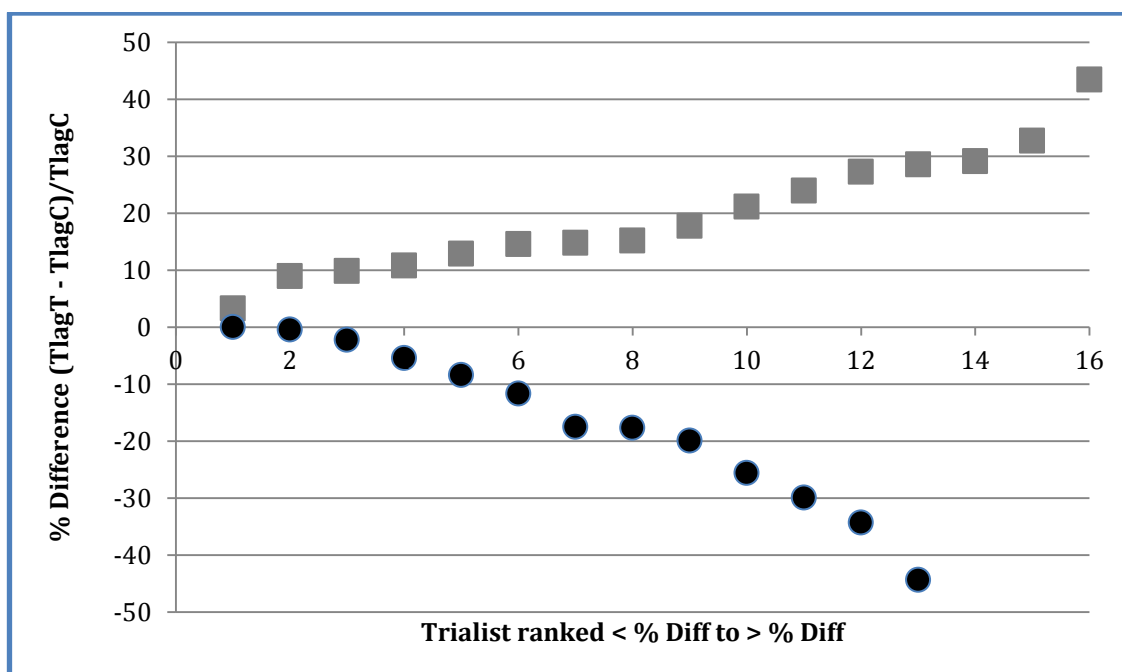


Figure 9.1 T_{lag} percentage difference due to treatment of all triallists ($n = 29$) from all three *in vivo* studies ranked from least to greatest effect for positive responders (delayed gastric emptying, $n = 16$) and negative responders (accelerated gastric emptying, $n = 13$).

The suggestion that participants in these trials demonstrating <10% response to treatment be considered non-responders on the basis that their gastric digestion was likely to be normal is purely an arbitrary ranking for this particular population, which can only be ratified with more comprehensive data collected from a young healthy population known to have optimal gastric digestion.

9.5 Functional Bowel Disorder

The changes in gastric emptying dynamics may be associated with the relief of symptoms of digestive dysfunction as reported by regular kiwifruit consumers particularly those with digestive problems. Functional bowel disorder (FBD) is a general term that describes an unspecified problem associated with an occasional

malfunction of the gastrointestinal system despite all organs appearing to be normal (Thompson et al., 1999). The malfunction can result in a variety of symptoms usually associated with pain or a sense of urgency to void the bowels. Upper gastro-intestinal symptoms of FBD (Longstreth et al., 2006) include nausea, bloating and stomach pain, while lower GI symptoms can include constipation, diarrhoea and pain. Both irritable bowel syndrome and functional dyspepsia are included under the general heading of FBD.

9.6 Constipation

If kiwifruit protease only functions in the stomach, the next question is: How does it relieve constipation? If the proposition that the protease is the active ingredient, the stomach is the active site and enhanced protein hydrolysis is the mode of action, it follows that some forms of constipation must result from incomplete protein digestion. The common perception that constipation results from insufficient dietary fibre and/or a lack of fluids does not explain the constipating effects of morphine-based analgesics, antibiotics and other medications. Nor does it explain how constipation from these causes is relieved by kiwifruit (Wyeth, 2011). It is beyond the scope of this thesis to investigate the mechanism, but what is known is that gut microbiota have a major influence not only on gastrointestinal digestion but also on whole body health. Guarner et al. (Guarner & Malagelada, 2003) concluded "*Gut flora might also be an essential factor in certain pathological disorders, including multisystem organ failure, colon cancer and inflammatory bowel diseases*".

Speculating now, based on the literature: Gut flora reside predominantly in the large intestine or colon (Posserud, Stotzer, Björnsson, Abrahamsson, & Simrén, 2007) but can migrate into the small intestine if conditions adverse to health prevail (Williams & McColl, 2006). The composition of the gut bacteria population (Musso, Gambino, &

Cassader, 2010) is determined by the composition of the food that is presented to the colon. That composition is determined by the quantity, quality and nutritional composition of the diet. What appears to be overlooked, however, is the effect that digestion has on the composition of the chyme deposited in the colon. Dietary fibre cannot be digested by the chemical processes of digestion (Montagne, Pluske, & Hampson, 2003) but provides the feedstock for some forms of bacteria in the colon that have the capability to convert it to beneficial fatty acids. These fatty acids (Henningsson, Bjirck, & Nyman, 2001), mainly butyrate, are subsequently absorbed through the epithelium of the colon and transported in the blood to the liver for on-processing for the benefit of the host. It is postulated that the higher the dietary fibre content of the chyme entering the colon, the more this class of so-called 'friendly' bacteria will flourish. However, sub-optimal peptic digestion of protein increases reliance on pancreatic protease hydrolysis to compensate. Sub-optimal pancreatic hydrolysis will result in chyme high in undigested protein entering the colon, and this will favour the proliferation of a different bacterial population to those flourishing on dietary fibre. If the protein supply is continuous, the protein-digesting bacteria will flourish and the population will expand (MacFarlane, Cummings, & Allison, 1986), but to do so it will either do it at the expense of the fibre consuming, or similar, populations or it will start to migrate into the small intestine. Products of bacterial colonic fermentation of proteins (Woodmansey, 2007) include N-nitroso compounds and sulphides as well as faecal ammonia and urinary phenolic compounds. As the protein content of the diet is increased the concentration of these products increases. Epidemiological evidence indicates high meat consumption increases the risk of colorectal cancer (Hughes et al., 2000). If carcinogenesis is promoted by products from the colonic bacterial fermentation of protein, the question is: Is the high protein diet *per se* the cause, or is it impaired gastric and pancreatic hydrolysis of the protein in the diet?

While the association between diet, digestion and bacterial fermentation is complex, it may provide the explanation for why kiwifruit protease relieves constipation. If gastric protein digestion is impaired and this is mitigated by kiwifruit in the manner discussed in this thesis, enhanced protein hydrolysis will ensure increased uptake of peptides and amino acids in the small intestine and reduced concentrations of undigested protein in the chyme entering the colon. This change in nutrient composition of the chyme will be detrimental to the protein-fermenting bacterial population which will diminish through lack of protein, taking the pressure off the 'friendly bacteria' and effectively returning the gut to normal homeostasis and negating the factors that induced the constipated state.

Alternatively, it is known that small intestine and colonic peristaltic motility is initiated by acetylcholine released from the intrinsic primary afferent neurons (IPAN's) via the 5-hydroxytryptamine (5-HT) mechanism in response to serotonin released from the enterochromaffin cells as a result of mucosal stretch or chemical activation (Kordasti, Sjövall, Lundgren, & Svensson, 2004). Serotonin is synthesized from the essential amino acid tryptophan. Kiwifruit has a relatively high concentration of serotonin (3-30 mg/kg) as does papaya and pineapple which, like kiwifruit, both contain protease. Kiwifruit also contains a relatively high concentration of tryptophan (45 mg/100 g). This thesis has not investigated the relationship between the components of kiwifruit other than the protease and the effect of the fruit on constipation, but the possibility exists that there may be an association and these other bioactive chemicals in kiwifruit may have an effect.

9.7 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS), another functional bowel disorder, is reportedly relieved by consumption of kiwifruit (Vital Foods survey). The lumen of the small

intestine is exposed to protease damage from uncontrolled activation of pancreatic proteases (N. Vergnolle, 2005). To balance the requirement for protease activity with lumen protection, protease activation is controlled by protease inhibiting receptors referred to as protease activated receptors (PARs). PARs operate when the terminal cells are hydrolysed by intracellular signaling which activates chloride secretion and prevents further activation of inert protease. As well as pancreatic and brush border proteases, gut micro flora and invasive bacteria can also produce proteases (Woodmansey, 2007). Acute activation of PARs induces inflammation of the luminal tissues. Sub-optimal peptic hydrolysis of protein will result in over expression of pancreatic protease secretion to compensate, and trypsin is a known PARs activator (N. Vergnolle, 2009). Could there be an association between over expressed trypsin, acute PARs activation, induced tissue inflammation and IBS symptoms?

9.8 Sarcopenia

Sarcopenia, the chronic loss of muscle mass generally associated with ageing, is discussed in detail in CHAPTER 4 (pp 11–12). Age *per se* is not recognised as a factor that causes declining gastric acidity (Lovat, 1996), but damage to the gastric mucosa appears to accumulate with time, and the ability of the mechanism to repair itself diminishes with age (Hopkins, Sharp, & Macfarlane, 2001). The elderly also consume more medication than younger adults (Gallagher & O'Mahony, 2009), and some of these have a damaging effect on the gastric mucosa (Wallace, Keenan, & Granger, 1990) while others suppress acid and enzyme secretion. *Helicobacter pylori* infection is also more prevalent in the elderly than in younger adults (Adamu et al., 2011), possibly as high as 30% of people over 65 in Western countries and higher still in Asian countries (R.E. & Ng, 1995).

As a consequence of all these factors (Delafuente, 2003) elevated gastric pH is thought to be common among the elderly. The effect of elevated gastric acidity, as reported in this thesis, results in sub-optimal peptic hydrolysis of protein which possibly accounts for the delayed gastric emptying, also a common outcome of ageing. The participants in the in vivo studies were between 40 and 80 years old and most either had FBD or were on medication for age-associated conditions. All responded to the kiwifruit treatment; 30% had only a $\leq 10\%$ response but 70% had between a 10% and 45% response.

If this level of response is confirmed in more substantial trials to be a measure of impaired protein digestion efficiency, it may also indicate that 70% of people are in a negative protein balance which may be a contributing factor to sarcopenia. If the test method proves to be an accurate measure of protein digestion efficiency in these more substantial trials, it may be possible to develop a simple non-invasive method of regularly testing an individual's risk of sarcopenia.

Sarcopenia is an indication that protein synthesis is failing to compensate for protein catabolism or autophagy (Hands, Proud, & Wytenbach, 2009). The activity of the signaling protein rapamycin (mTOR) which is suppressed when amino acid supply is restricted links aging with protein synthesis (Caron et al., 2010; Hands, Proud, & Wytenbach, 2009) so could be part of the explanation of sarcopenia.

9.9 *Helicobacter pylori*

Finally, a role for kiwifruit protease in the treatment of those with *H. pylori* induced gastric atrophy should be considered. The asymptomatic nature of *H. pylori* infection means that it can go undetected for many years and is often only identified in a peripheral diagnosis for functional bowel disorder (Tougas, 2000). Untreated chronic infection can result in atrophy of the gastric glandular tissue (Anderson et al., 2008). If

this occurs in the distal region of the stomach it can result in achlorhydria. Infection can be eliminated with antibiotic treatment (Kusters, van Vliet, & Kuipers, 2006) although resistance to treatment has been observed, and treatment will not prevent reinfection. Elimination of the infection can result in the partial recovery of the glandular tissue and the extent of recovery appears to be age dependent. Partial recovery may not provide sufficient parietal and chief cells to achieve conditions conducive to optimal peptic hydrolysis and, as a result, most treated patients develop GERD and are prescribed PPI medication. If GERD, which is associated with delayed gastric emptying, results from sub-optimal peptic hydrolysis, this study indicates kiwifruit protease may well improve protein hydrolysis efficiency, accelerate gastric emptying and relieve the associated discomfort.

9.10 Future Studies and Methodologies

Small sample size and failure to clearly identify in advance covariate factors that may have influenced the results of the *in vivo* human dosing trials in this body of work, mean that the outcomes indicated by the results, at this stage, can be little more than speculation. However, results from the *in vitro* study, clinical studies by other researchers referred to in the literature reviewed, and anecdotal reports all add credence to the legitimacy of this speculation and lend support to the need for further studies.

Future studies could be designed to determine the influence of impaired gastric digestion of protein on symptoms of digestive dysfunction and whether these symptoms are indicative of impending health syndromes such as sarcopenia, or disease conditions such as Type 2 Diabetes mellitus.

Validation of the change in the kinetics of gastric emptying of a protein dominant meal with and without a protease supplement, measured using the ¹³C-octanoic acid breath test, could provide a new diagnostic tool for clinicians to determine

the protein digestion efficiency of individuals. This may have significant implications for prescribing individual dietary advice to restrict the onset of some degenerative conditions associated with ageing. The importance of this work lies in the anticipated expansion of the ratio of >65 year olds to the general population over the ensuing 20 years and the implications this could have for the health care system. Extending the independence of this group of people by improving their health status will help to relieve financial pressure on the health and welfare systems over this period.

9.11 Conclusion

Identifying a mode of action to explain the effects of kiwifruit on the human digestion process was the original aim of this chronological sequence of studies. What has emerged from this body of work is evidence that the protease content of the fruit is the likely active ingredient; that the stomach is the likely site of activity and that the effect is likely to be associated with existing gastric protein digestion inefficiency due to sub-optimal peptic hydrolysis.

Validation of the findings of this study will add considerably to the body of knowledge and support the proposal that the effect of kiwifruit on digestion is associated with gastric protein-digestion efficiency and this may reflect an individual's gastric-acidification capability. Further studies would then be warranted to determine if an inverse correlation exists between gastric protein digestion efficiency, the relief of digestive discomfort associated with functional bowel disorder and, long term, the progression of sarcopenia.

In light of the increasing prevalence of digestive dysfunction, conventional wisdom that efficient peptic hydrolysis is incidental to good health may warrant reappraisal.

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APPENDIX 1

PARTICIPANTS INFORMATION SHEET

Protein digestion efficiency measured by gastric emptying dynamics

An Invitation

You are cordially invited to participate in a research study that is designed to find out how well active older people digest protein. If you choose to participate it will be on a purely voluntary basis and you will be free to withdraw at any time without any adverse consequences. Mr Bruce Donaldson is conducting the research as part of his studies for a PhD in Nutrition and the findings from the study will be published as part of his doctoral thesis. Throughout the research, he will be closely supervised by Dr. Elaine Rush – Professor of Nutrition at Auckland University of Technology.

What is the purpose of the research?

Protein is an essential part of the diet as it is required by the body to maintain the immune system and repair and replace body cells as they age. But first the protein in the food we eat has to be broken down, by the process of digestion, into its component parts which are called amino acids and it is these amino acids that the body uses to build new cells, muscles and other body structures. The first stage of digestion is a chemical reaction between the protein in the food and the acid and enzyme in the stomach. How well this digestive process functions is thought to have an influence on many aspects of our health and physical strength. Because there is a tendency to lose muscle strength with age it is important to be able to digest food and absorb nutrients well to maintain muscle and bone health.

An enzyme-rich extract from kiwifruit has been shown to improve protein digestion in situations where gastric acidity is diminished. The purpose of this study is to see if supplementing the diet with this kiwifruit extract can enhance protein digestion naturally. From this study we hope to be able to develop a non-invasive method of testing a person's ability to digest protein to determine if they may be at risk of

becoming protein malnourished. If this initial study shows an effect of kiwifruit extract the information collected in the current study will be incorporated into a larger study.

How was I chosen for this invitation?

As protein digestion efficiency appears to diminish with age the researchers are seeking to recruit people for the study who are in the 50+ age group and known to be maintaining an active life style. We feel that members of AUT's "Never Too Old" group have the ideal profile required for this study and as a member of that group we hope you will accept the invitation to participate. There are however some conditions that will preclude entry to the study. If you have had gastric bypass surgery or are fitted with a gastric lap band you will not be able to participate; if you are currently using protease supplement medication you will not be able to participate and finally if you are not engaged in a physical activity programme that requires at least 30 minutes of exercise three times a week, you will not be able to participate in the study

What will happen in the research?

You will be required to visit the metabolic research lab at the AUT North Shore Campus on two separate occasions approximately a week apart. You will be required to refrain from eating anything after 10 pm the night before as you will be given breakfast at the lab. You may drink water only. Each test will take three and a half hours and during that time you will be asked to provide a breath sample every 15 minutes. We will provide a comfortable armchair for you to sit in during the test and magazines to read and videos to watch, or you may like to bring a book. We will provide water for you to drink and there are toilet facilities close by. If you would like to bring a partner for company or to participate in the trial you are welcome to do so.

The breakfast will consist of an omelette labelled with a substance that can be detected on the breath almost immediately after it has been digested (for more information refer to the Technical Supplement attached to this Information Sheet)

The breath sampling simply requires you to blow into a special breath collection bag that has a straw attached. A fresh bag and straw are prepared for each sample. A total of 14 samples will be collected over the three and a half hours of the trial.

What are the discomforts and risks?

There are no known risks associated with this type of study. The substance used to track the changes in the breath test is safe and routinely used with children and pregnant women. The kiwifruit extract has also undergone extensive tests, which have shown it to be very safe and it has been used extensively during pregnancy.

It will be necessary to record your weight, height and waist measurements, as these are required in some of the data calculations. We will also measure your blood pressure and grip strength, as these may prove to be factors influenced by protein metabolism. You will also be asked to provide information on any medication you may be taking and to provide information about any digestive tract issues you may have such as hiatus hernia, dyspepsia, pernicious anaemia, Crohn's disease, irritable bowel syndrome or similar as these may also have a bearing on your protein digestion efficiency.

The only discomfort you will experience will be having to sit around for the three and a half hours but you will be able to stand up and move round the room. However if for any reason you feel uncomfortable you will be free to leave at any time. In which case you may care to reschedule or withdraw from the trial.

If for any reason you feel unwell during the trial and require medical attention there is an AUT medical centre close at hand and a qualified medical person can be called.

What are the benefits?

As people get older they tend to lose not only the amount of muscle in their arms and legs and body support but also the strength of that muscle. In later life this loss of muscle leads to instability and can result in falls, fractures and a loss of mobility and independence.

This study may identify a method of determining when people are at risk from chronic protein malnutrition and may also point the way for further research to develop a remedy.

We are unable to reward you for your time in this study but will happily reimburse you for normal travel and parking costs associated with getting to and from the campus.

What compensation is available in the event of injury or negligence?

In the unlikely event of you experiencing physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

All participants will be allocated a number and only the number will be referred to in the data analysis. Only the immediate researchers will know the relationship between numbers and names and this information will be kept under lock and key as will all the baseline data collected for the study. By law the information is retained for 6 years after completion of the trial and then destroyed. The same applies if the data from this study is incorporated with data from a subsequent study. All reference to the data in reports, journal articles and conference presentations will be summary data for the group so that no individual will be identifiable in any way.

What opportunity do I have to consider this invitation?

You are welcome to contact the researcher or the project supervisor or your health care professional to discuss any aspect of the study. If you decide you would like to participate it would be appreciated if you would contact the researcher as soon as possible to arrange a time schedule that is acceptable to you and the researcher.

How do I agree to participate in the study?

To participate in the study it will be necessary for you to complete the Consent Form attached to this document and return it to the Researcher named on the form.

Will I receive feedback on the results of this study?

Yes. Your individual results and the group results will be made available to you provided you tick the appropriate box in the Consent Form.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified to the Project Supervisor, Professor Elaine Rush, Ph: 09 921 9999 Ext: 8091, Email: elaine.rush@aut.ac.nz

Any concerns regarding the conduct of the research should be notified to the Executive Secretary AUTEC, Madeline Banda, Ph: 09 921 9999 Ext: 8044 Email: madeline.banda@aut.ac.nz

Who do I contact for further information about this research?

Contact either the Researcher or the Project Supervisor.

Researcher's Contact Detail;

Bruce Donaldson, P.O. Box 53-163 Auckland; Ph: 09 921 9999 Ext: 7557;
Mbl: 027 580 8838; Email: bruce.donaldson@aut.ac.nz

Project Supervisor's Contact details:

Professor Elaine Rush Ph: 09 921 9999 Ext: 8091; Email:
elaine.rush@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee
on

AUTEC Reference number: 09/284

APPENDIX 2

TECHNICAL SUPPLEMENT

Protein digestion efficiency measured by gastric emptying dynamics

The breakfast you will be offered will consist of an omelette made from one whole egg plus three egg whites. Mixed with the egg yolk will be a small amount of octanoic acid.

Octanoic acid is a common component of food and has been chosen because it is rapidly absorbed from the small intestine, carried in the blood to the liver where it is fully oxidised into carbon dioxide, which is then transported in the blood to the lungs and expelled.

The Octanoic acid used in this study will contain a known amount of the stable isotope 13-carbon also denoted as ^{13}C . A stable isotope is one that does not give off radiation so it is safe and harmless unlike a radioactive isotope. Carbon has two stable isotopes ^{13}C and ^{12}C . Between them they make up most of the carbon in nature although ^{12}C is far more plentiful than ^{13}C .

An isotope simply describes a feature of the atomic structure of an element. An atom is composed of three main components, electrons, protons and neutrons. Whilst all atoms of an element have the same number of protons there can be a variation in the number of neutrons and the isotope label describes this difference.

Most stable carbon atoms in nature contain 12 neutrons but about 1 in every 1,000 contains 13. There is practically no difference between the properties of ^{12}C and ^{13}C other than their weight with ^{13}C being slightly heavier than ^{12}C and this is the important feature for this study as the weight difference enables the isotopes to be separated in much the same way as apples of different size can be separated on a fruit grader. By measuring the ratio of $^{13}\text{C}/^{12}\text{C}$ naturally occurring in your breath before, a meal and for

3 hours after the meal, we can determine a number of characteristics about how efficiently your stomach is digesting the protein in the meal you have eaten. The second time you visit the lab you will have an identical meal plus a dietary supplement made from kiwifruit. What we are trying to determine is if the supplement improves your protein digestion efficiency and we can do that by comparing the breath samples collected from each of the two tests.

If you would like to discuss any aspect of this study please contact the Researcher:-

Bruce Donaldson, P.O. Box 53-163 Auckland; Ph: 09 921 9999 Ext: 7557;
Mbl: 027 580 8838; Email: bruce.donaldson@aut.ac.nz

APPENDIX 3

CONSENT FORM

Approved by the Auckland University of Technology Ethics Committee on

AUTEC Reference number 0928

Note: The Participant should retain a copy of this form.

*Project title: **Protein digestion efficiency measured by gastric emptying dynamics***

Supervisor:

Professor Elaine Rush

*Researcher: **Bruce Donaldson***

- ☐ I have read and understood the information provided about this research project in the Information Sheet dated 14 December 2009.
- ☐ I have had an opportunity to ask questions and to have them answered.
- ☐ I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way.
- ☐ I have not had gastric bypass surgery and I do not have a gastric lap band fitted; I am not taking any medication containing a protease enzyme; I do engage in physical activity for 30 minutes or more at least 3 times per week.
- ☐ I agree to have my weight, height, blood pressure, grip strength and waist measurements recorded for this study.
- ☐ I am aware that the data from this study may be amalgamated with data from a subsequent study and I have no objection to the data being published provided my privacy is protected.
- ☐ I agree to take part in this research.
- ☐ I wish to receive a copy of the report from the research (tick one): Yes ☐ No ☐

Participant's signature:

Participant's name:

Participant's Contact Details (if appropriate):

.....

Date:

Confidential Research Number :

APPENDIX 4

PROTOCOL SHEET

PROTOCOL SHEET					
Gastric Emptying Study AUTEC#09/28 4	<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Subject's Number</div> _____				
	<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Todays Date</div> _____				
	<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Subject's Age</div> _____				
	<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Subject's Gender</div> _____				
<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Health Status</div>					
Medications					
Exercise regime					
Smoking					
Other					
<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Body Dimensions</div>					
	Reading 1	Reading 2	Reading 3	Average	
Weight kg					kg
Height cm					cm
Waist					cm
Grip Right					kg
Grip left					kg
<div style="background-color: #cccccc; padding: 2px 5px; display: inline-block;">Blood Pressure / Resting Pulse</div>					
	Reading 1	Reading 2	Reading 3	Average	
Systolic					mmHg
Diastolic					mmHg
Pulse					bp m

Recordings		
Time	Event	Sample ID
	1st pre meal breath sample	
	2nd premeal breath sample	
	Commence meal	
	Complete meal	
	1st post meal breath sample	
	2nd post meal breath sample	
	3rd post meal breath sample	
	4th post meal breath sample	
	5th post meal breath sample	
	6th post meal breath sample	
	7th post meal breath sample	
	8th post meal breath sample	
	9th post meal breath sample	
<div>Omelette preparation notes</div>		
<div> <div> 13C-Octoanoic Acid dose mg </div> <div> Batch details </div> </div>		