

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Prediction of cellular ATP generation from foods
in the adult human - application to
developing specialist weight-loss foods**

**A thesis presented in partial fulfilment
of the requirements for the degree of**

**Doctor of Philosophy
in
Nutritional Science**

**at Massey University, Palmerston North,
New Zealand.**

Leah Theresa Coles

2010

Abstract

For the accurate prediction of the potential 'available energy' of a food at the cellular level (i.e. ATP generation from food) it is necessary to be able to predict both the quantity and location of uptake (upper-tract or colon) for each energy-yielding nutrient. The objective was to develop a valid model ('Combined Model') for predicting the (potential) ATP available to the body from absorbed nutrients across the total digestive tract. The model was intended for the adult human under conditions where energy intake \leq energy expenditure and all absorbed nutrients are catabolised. The development of the model involved two parts: (i) the experimental development of a dual *in vivo* – *in vitro* digestibility assay ('dual digestibility assay') to predict human upper-tract nutrient digestibility, as modelled by the rat upper digestive tract, and colonic digestibility, as predicted by fermenting rat ileal digesta in an *in vitro* digestion system containing human faecal bacteria; and (ii) the development of a series of mathematical equations to predict the net ATP yielded during the post-absorptive catabolism of each absorbed nutrient at the cellular level.

A strong correlation ($r=0.953$, $P=0.047$) was found between total tract organic matter digestibility (OMD), as predicted with the newly developed dual *in vivo* – *in vitro* digestibility assay and with that determined in a metabolic study with humans for four mixed diets ranging considerably in nutrient content. There were no statistically significant ($P>0.05$) differences for mean OMD between the predicted and determined values for any of the diets.

The Combined Model (dual *in vivo* – *in vitro* digestibility assay + stoichiometric predictive equations) was applied to three meal replacement formulations and was successfully able to differentiate between the diets in terms of both energy digestibility and predicted ATP yields. When the energy content of each diet was compared to that of a baseline food (dextrin), some metabolisable energy (ME) models gave considerably different ratios compared to that predicted by the Combined Model. By way of example, for Diet C a ratio of 0.96 (Atwater and FDA models) was found

versus 0.75 (Combined Model). Thus, the model has practical application for predicting dietary available energy content, particularly in the research and development of specialised weight-loss foods, where it may be more accurate than some current ME models. Uniquely, the Combined Model is able to define a food in terms of ATP content (mol ATP / g food) using recent estimates of cellular P/O ratios and therefore, directly relates dietary energy intake to the quantity and form (ATP) of energy ultimately delivered at the cellular level.

Acknowledgements

The work reported in this thesis was undertaken in collaboration with the Riddet Institute (Massey University, New Zealand) under the chief supervision of Dist. Prof. Paul Moughan and the co-supervision of Dr. Alison Darragh (Fonterra Research Centre) and supported by the Fonterra Co-operative Group Ltd. and the New Zealand Government as part of a New Zealand Foundation of Research Sciences and Technology Project (DRIX0401). All experimental research described in this thesis has been approved by the Massey University Human and/or Animal Ethics Committee as required (relevant application numbers given in the text).

First and foremost I would like to give all thanks and praise to Allah for blessing me with the opportunity and means to complete my studies.

My heartfelt thanks to Prof. Moughan for his patience, invaluable advice, tireless editing and for giving me the guidance to learn whilst also giving me the independence to grow. I am also extremely grateful for the enthusiasm, guidance and advice of Dr Darragh, particularly during the early stages of my studies.

Thank you also to all the dedicated interns, students and assistants who helped with the experimental work: Mrs. Michele Davie, Ms. Weiwei Shen, Ms. Michaela Eckstein, Ms. Hilary McKinnon and Ms. Joen Lim. I would also like to thank Ms. Maggie Zou and Dr. Shane Rutherford (Riddet Institute), Dr. Ajay Awati (Nutra Ingredient Research Centre, The Netherlands), Dr. Sigurd Boisen (Danish Institute of Agricultural Sciences, Denmark) and Dr. Jaap van Milgen (INRA, France) for valuable advice and many useful discussions.

For analytical analysis and advice I would like to acknowledge Mrs. Fliss Jackson and Ms. Leiza Turnbull (IFNHH, Massey University) and Mrs. Wilhelmina Martin and Mr. Michael Agnew (AgResearch, New Zealand). I am also grateful to Mrs. Chris Booth (IFNHH, Massey University) for advice with the human study and Mrs. Debbie Chesterfield and the staff at SAPU (Massey University) for assistance during the rat

studies. Thanks to the many volunteers who took part in the human study and the *in vitro* work.

Thank you to my family for their continuous support, particularly my mother for always encouraging me and my daughter for making me smile. Finally, I am eternally grateful to my husband, without whom I never would have made it through my candidature. I cannot express in words the appreciation I have for his editorial assistance and for his help with everything from braving hungry rats to accompanying me during late night lab sessions. Mostly, I would like to thank him for his patience and for being there for me.

Table of contents

Abstract	i
Acknowledgements.....	iii
Table of contents	v
List of figures	xii
List of tables	xiv
Abbreviations	xviii
Preface	xxii
CHAPTER I - Review of the literature	1
Introduction	2
I. Energy requirements and availability	3
1. Components of energy expenditure.....	3
2. Methods of determining energy expenditure.....	6
II. Energy balance.....	9
1. Introduction	9
2. Terminology	10
III. Energy intake	15
1. Sources of dietary energy intake	15
2. Regulation of dietary energy intake	21
IV. Energy values of foods	22
1. Units of energy.....	22
2. Energy conversion systems.....	22
V. Energy utilisation	31
1. Overview of digestion in man	31
2. Upper-tract digestion, absorption and transport.....	31
3. The hindgut.....	36
VI. Postprandial metabolic fate of dietary energy	43
1. ATP	43
2. Catabolism of absorbed substrates	45

3.	Composition of fuel mix oxidised	52
4.	Relation of dietary intake to body weight maintenance and obesity	53
VII.	Predicting nutrient uptake.....	55
1.	Digestibility	55
2.	Methods to predict ileal digestibility.....	59
3.	Methods to predict the uptake of energy from the hindgut	72
VIII.	Conclusions and justifications of the experimental work	73
	Literature cited	77

CHAPTER II - Specific review of methodologies: <i>In vitro</i> digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals			115
	Abstract.....		116
	Introduction.....		117
I.	The need for <i>in vitro</i> hindgut digestion / fermentation methods.....		118
II.	Application and limitations of <i>in vitro</i> hindgut digestion / fermentation methods.....		119
III.	The monogastric hindgut.....		121
1.	Hindgut physiology		121
2.	Substrates for fermentation		121
3.	Products of fermentation		123
IV.	<i>In vitro</i> hindgut digestion and fermentation methods.....		126
1.	Introduction		126
2.	Types of <i>in vitro</i> hindgut digestion / fermentation methods.....		127
3.	Failings of batch <i>in vitro</i> hindgut fermentation methods		135
V.	Design considerations for a batch <i>in vitro</i> digestion / fermentation method.....		138
1.	Substrate.....		138
2.	Inoculum		140
3.	Nutritive medium		140

4.	Buffer	141
5.	Stirring.....	141
6.	Fluid surface area / volume ratio.....	141
7.	Measurements.....	142
8.	Incubation time.....	142
VI.	The gas production technique	143
VII.	Merits and shortcomings of <i>in vitro</i> digestion / fermentation methods	147
VIII.	Conclusion.....	150
	Literature cited	151

CHAPTER III - Predicted apparent digestion of energy-yielding nutrients differs

between the upper and lower digestive tract in rats and humans	165
Abstract.....	166
Introduction	167
Materials and methods.....	169
Human balance study.....	169
Rat Study	171
Chemical analyses	173
Calculations	175
Statistical analysis	177
Results.....	179
Human balance study.....	179
Rat ileal digestibility study	181
Discussion	183
Conclusion.....	191
Literature cited	192
Appendix to Chapter 3.....	197

CHAPTER IV - The 'Combined Model'

Introduction	204
Description of model	205

Discussion	208
Conclusion.....	210
Literature cited	211

CHAPTER V - Influence of assay conditions on the *in vitro* hindgut digestibility

of dry matter	215
Abstract.....	216
Introduction	217
Materials and methods.....	219
<i>In vitro</i> hindgut digestion methods.....	219
Inoculum	220
Substrates	221
Experimental design.....	223
Chemical analyses	225
Calculations	226
Statistical analyses	227
Results	228
pH.....	228
Enzyme / inoculum concentration.....	230
Duration of incubation.....	231
Mixing of materials during incubation.....	233
Discussion	234
Adaptation of donors.....	235
Boisen assay	235
pH.....	235
Enzyme / inoculum concentration.....	236
Duration of incubation.....	237
Mixing of materials during incubation.....	237
Repeatability	238
Comparison of methods.....	240
Conclusion.....	243

Literature cited	244
CHAPTER VI - Optimisation of inoculum concentration and incubation duration for an <i>in vitro</i> hindgut digestibility assay	247
Abstract.....	248
Introduction	249
Materials and methods.....	251
Experiment A - concentration of the inoculum	251
Experiment B - duration of incubation	255
Chemical analyses	256
Calculations	259
Statistical analysis	260
Results.....	262
Experiment A - concentration of inoculum.....	264
Experiment B - duration of incubation	267
Discussion	270
Substrate for <i>in vitro</i> hindgut digestibility	270
Experiment A - concentration of inoculum.....	270
Experiment B - duration of incubation	273
Conclusion.....	275
Literature cited	276
CHAPTER VII - Validation of a dual <i>in vivo</i> – <i>in vitro</i> assay for predicting the digestibility of dietary energy in humans	279
Abstract.....	280
Introduction	281
Materials and methods.....	283
Human balance study.....	283
Rat study	285
<i>In vitro</i> fermentation method	286

Chemical analyses	287
Calculations	289
Statistical analysis	291
Results	292
Human balance study	292
Dual <i>in vivo</i> - <i>in vitro</i> digestibility assay	294
Discussion	296
Comparison of total tract OMD: human <i>in vivo</i> OMD and predicted OMD from the dual digestibility assay	298
Conclusion.....	302
Literature cited	303
CHAPTER VIII - A model to predict the ATP equivalents of macronutrients absorbed from food	307
Abstract.....	308
Introduction	309
Experimental methods	311
Model overview	311
ATP yield (ATP_a).....	312
Prediction of uptake of substrates using a dual <i>in vivo</i> / <i>in vitro</i> digestibility assay	322
Discussion	327
Energetic efficiency.....	327
Urea recycling	329
Extent of gluconeogenesis from amino acids	329
Net ATP yield (ATP_{net}).....	330
ATP costs (ATP_d and ATP_t)	332
Accuracy of ATP_{net}	334
P/O ratios	335
Conclusion.....	337
Literature cited	338

CHAPTER IX - Available energy content of three meal replacement formulations determined using a model to predict the cellular ATP yield of absorbed macronutrients in the adult human	343
Abstract.....	344
Introduction	345
Materials and methods.....	347
Diets	347
<i>In vivo</i> digestibility assay	350
<i>In vitro</i> hindgut digestibility assay.....	351
Chemical analyses	351
Calculations	353
Statistical analyses	358
Results.....	359
Animal Study	359
Upper-tract nutrient digestibility.....	359
<i>In vitro</i> hindgut OM digestibility and SCFA production	362
Predicted ATP gains and costs	363
Discussion	365
Total tract organic matter digestibility	366
Predicted ATP yields.....	366
Conclusion.....	373
Literature cited	374
CHAPTER X - General discussion and conclusions	379
Recommendations for future research	390
Literature cited	392

List of figures

CHAPTER I

Figure 1. Overview of the flow of energy through the body.....	10
Figure 2. The pathways of fermentation in the hindgut leading to the production of short chain fatty acids.....	38
Figure 3. Alcohol metabolism pathways.....	50
Figure 4. A futile cycle during ethanol oxidation.....	51
Figure 5. Collection of ileal digestion in humans using naso-intestinal intubation.	62
Figure 6. Ileo-rectal anastomosis without isolation of the large intestine (top figure) or with isolation of the large intestine by a T-cannula (bottom figure)	70

CHAPTER III

Figure 1. Ratio of predicted colonic loss : predicted total tract loss of NSP (A) and the ratio of predicted colonic loss : predicted upper-tract uptake of Protein (B) and Gross Energy (C).	189
--	-----

CHAPTER IV

Figure 1. Schematic diagram of a Combined Model of digestion (combination of <i>in vivo</i> and <i>in vitro</i> digestion assays and stoichiometric chemical relationships) over the total tract, showing inputs and outputs at each stage.....	207
--	-----

CHAPTER VI

Figure 1. Mean ($n=3$) hindgut dry matter digestibility versus inoculum concentration for the: (a) Barry method, Low Fibre Diet; (b) Edwards method, Low Fibre Diet; (c) Barry method, High Fibre Diet and (d) Edwards method, High Fibre Diet.	266
---	-----

CHAPTER VII

Figure 1. Flow of OM in human digestive system and dual <i>in vivo</i> - <i>in vitro</i> digestibility assay over the total tract, showing inputs and outputs at each stage.	297
--	-----

Figure 2. Total tract OMD (%) as determined using the dual digestibility assay (OMD _{dual}) versus OMD from a human balance study (OMD _{human}) for four mixed human diets.	299
Figure 3. Total tract OMD (%) as determined using the dual digestibility assay (OMD _{dual}) versus OMD from a human balance study (OMD _{human}) for four mixed human diets from the present study and three additional mixed human diets (Zou <i>et al.</i> 2007).	301

CHAPTER VIII

Figure 1. Schematic diagram of a Combined Model of digestion (combination of <i>in vivo</i> and <i>in vitro</i> digestion assays and stoichiometric chemical relationships) over the total tract, showing inputs and outputs at each stage.....	323
Figure 2. Overview of model.....	331

List of tables

CHAPTER I

Table 1 Models predicting the metabolisable energy values (kJ) of diets	24
--	----

CHAPTER II

Table 1 A summary of batch <i>in vitro</i> fermentation methods proposed for studying digestion and fermentation in the human large intestine	130
--	-----

Table 2 Combinations of sources of ileal digesta and inoculum for published <i>in vitro</i> hindgut digestion / fermentation assays.....	139
---	-----

CHAPTER III

Table 1 Participants' characteristics	169
--	-----

Table 2 Daily intakes and faecal and urinary excretions of gross energy, and determined apparent faecal energy digestibility and metabolisable energy	179
--	-----

Table 3 Apparent faecal digestibility of nutrients in the experimental diets.....	180
--	-----

Table 4 Daily food and nutrient intakes for the growing rats	181
---	-----

Table 5 Apparent ileal nutrient digestibility for the four human diets fed to growing rats	182
---	-----

Table 6 Actual daily intakes and faecal excretions and derived values (predicted) for human ileal output, upper-tract uptake and colonic loss for several dietary components	186
---	-----

APPENDIX TO CHAPTER III

Table A1 Food ingredients and foods included in the human diets.....	197
---	-----

Table A2 Formulation of the rat diets	199
--	-----

Table A3 Determined chemical composition of the four experimental diets given to the women and used as a base material for diets given to growing rats	200
---	-----

CHAPTER V

Table 1 Determined chemical composition of the three substrates	222
Table 2 <i>In vitro</i> hindgut digestion methodologies and the levels at which experimental variables were tested	224
Table 3 Effect of pH on mean dry matter digestibility for two <i>in vitro</i> methods and three experimental diets.....	229
Table 4 Effect of concentration of inoculum on mean dry matter digestibility for each of the two <i>in vitro</i> methods and three experimental diets.....	230
Table 5 Effect of duration of incubation on mean dry matter digestibility for two <i>in vitro</i> methods and three experimental diets.....	232
Table 6 Effect of mixing during incubation on mean dry matter digestibility for two <i>in vitro</i> methods and three experimental diets	233
Table 7 Comparison of mean dry matter digestibility for three diets on different days for two <i>in vitro</i> methods using standard protocols.....	239
Table 8 Comparison of mean dry matter digestibility for two <i>in vitro</i> methods using standard protocols with the same batch of faeces used for the inocula and three experimental diets.....	240

CHAPTER VI

Table 1 Inoculum concentrations tested ¹ for two <i>in vitro</i> hindgut digestion methodologies	252
Table 2 Ingredient compositions of four experimental diets (g/kg diet) (as weighed prior to freeze drying)	253
Table 3 Determined chemical composition of four experimental diets.....	257
Table 4 Determined chemical composition of the chicken and rat ileal digesta	263
Table 5 Effect of concentration of inoculum on the mean dry matter digestibility of ileal digesta for two diets for each of the two <i>in vitro</i> hindgut digestibility methods	264
Table 6 Mean dry matter digestibility and organic matter digestibility for blanks containing inoculum alone (160 g/L) at three incubation durations using the modified Edwards method	267

Table 7 Effect of three incubation durations on substrate organic matter digestibility after <i>in vitro</i> digestion (Edwards method) for four experimental diets	268
--	-----

CHAPTER VII

Table 1 Mean daily intake, faecal excretion and faecal digestibility of dry matter and organic matter and faecal bulking for four diets consumed by the subjects	293
---	-----

Table 2 Mean ileal (rat) and large intestinal (<i>in vitro</i>) organic matter digestibility for the four experimental diets	294
---	-----

Table 3 Comparison of mean predicted (determined using dual <i>in vivo</i> - <i>in vitro</i> digestibility assay) and actual (human <i>in vivo</i> balance study) total tract organic matter digestibility for the four experimental diets	295
---	-----

CHAPTER VIII

Table 1 ATP yields (ATPa) of glucose, amino acids, fatty acids, SCFA and ethanol and the costs associated with their digestion (ATPd) and absorption / transport (ATPt)	314
--	-----

Table 2 ATP yields from the catabolism of amino acids	316
--	-----

Table 3 ATP yields from catabolism of glycerol, short chain fatty acids and fatty acids	318
--	-----

Table 4 Gross Energy, Physiological Energy and Energetic Efficiency values of selected nutrients	328
---	-----

CHAPTER IX

Table 1 Ingredient composition (g/kg air-dry weight) of the three experimental diets	348
---	-----

Table 2 Determined chemical composition of the three experimental diets (per kg dry matter)	349
--	-----

Table 3 ATP yields from the catabolism of amino acids for selected food proteins	357
---	-----

Table 4 Mean ileal excretion (g or MJ per kg digesta DM) and apparent ileal digestibility (%) of gross energy, crude protein, fat, total carbohydrate, starch, sugar, DM and OM in the growing rat fed three diets	360
---	-----

Table 5 Mean ileal excretion (g/kg digesta dry matter) and apparent ileal digestibility (%) of fatty acids in the growing rat fed three diets	361
Table 6 Mean hindgut organic matter digestibility (OMD) (%) for three experimental diets as determined using the hindgut <i>in vitro</i> digestibility assay and predicted total tract OMD.....	362
Table 7 Mean short chain fatty acid production (μmol per bottle) of the three ileal substrates during <i>in vitro</i> hindgut fermentation	363
Table 8 Predicted ATP yields (ATP_a) (mol/kg DM diet) for three meal replacement formulations.....	364
Table 9 Models used to predict ME of the three experimental diets (kJ/g dry diet)	369
Table 10 Available energy and metabolisable energy (ME) based on factorial and empirical models expressed as kJ/g dry diet and kJ/kJ gross energy (GE)	370
Table 11 Available energy and Metabolisable Energy (ME) of dextrin and the ratio of energy meal replacement formulation : energy dextrin	372

Abbreviations

ΔG	Free Energy
AA	Amino Acid
AA_d	Amino Acids Present In The Diet
AA_i	Amino Acids Present In The Ileal Digesta
ADH	Alcohol Dehydrogenase
ADP	Adenosine Diphosphate
Ala	Alanine
AMG	Amyloglucosidase
AMP	Adenosine Monophosphate
ANOVA	Analysis Of Variance
AOAC	Association Of Analytical Chemists
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
ATP	Adenosine Triphosphate
ATP_a	Available ATP (ATP Yield)
ATP_{AA}	ATP Yield From Amino Acids
ATP_d	ATP Cost Of Digestion
ATP_{FA}	ATP Yield From Fatty Acids
ATP_{net}	Net ATP Yield
ATP_{SCFA}	ATP Yield From Short Chain Fatty Acids
ATP_{ST+SU}	ATP Yield From Starch And Sugars
ATP_t	ATP Cost Of Absorption / Transportation
BMR	Basal Metabolic Rate
BW	Body Weight
CHO	Carbohydrate
CoA	Coenzyme A
CV	Coefficient Of Variation
Cys	Cysteine
dE	Digestibility Of Energy
DE	Digestible Energy
DF	Dietary Fibre

dHE	Increment Of Heat Energy
DIT	Dietary Induced Thermogenesis
DM	Dry Matter
DMD	Dry Matter Digestibility
ER	Endoplasmic Reticulum
EtOH	Ethanol
FA	Fatty Acid
FA_d	Fatty Acids Present In The Diet
FADH₂	Flavin Adenine Dinucleotide H ₂
FA_i	Fatty Acids Present In The Ileal Digesta
FAO	Food And Agriculture Organization
FDA	Food And Drug Administration
FE	Faecal Energy
FFA	Free Fatty Acid
FID	Flame Ionisation Detector
GaE	Gaseous Energy
GC	Gas Chromatography
GE	Gross Energy
GI	Gastrointestinal
GL	Glucose
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
GP	Glycerol Phosphate
GTP	Guanosine Triphosphate
GY	Glycerol
HE	Heat Energy
HF	High Fibre
His	Histidine
IE	Intake Energy
Ile	Isoleucine
Leu	Leucine
LF	Low Fibre
LPL	Lipoprotein Lipase

Lys	Lysine
ME	Metabolisable Energy
MEOS	Microsomal Ethanol Oxidising System
Met	Methionine
MF	Mixed Fibre
NADH	Nicotinamide Adenine Dinucleotide H
NADPH	Nicotinamide Adenine Dinucleotide Phosphate H
N_d	Nitrogen Present In The Diet
NDF	Neutral Detergent Fibre
NE	Net Energy
NEAT	Non-Exercise Activity Thermogenesis
NEFA	Non-Esterfied Fatty Acids
N_i	Nitrogen Present In The Ileal Digesta
NR	Not Reported
NSP	Non-Starch Polysaccharide
OFN	Oxygen-Free Nitrogen
OM	Organic Matter
OMD	Organic Matter Digestibility
OM_{D+F}	Organic Matter That Is Digested And Fermented By The Body
OM_i	Organic Matter Present In The Ileal Digesta
OM_{uf}	Unfermented Organic Matter At The End Of Incubation
P	P-Value (Probability)
PE	Pectin
PEG	Polyethylene Glycol
Phe	Phenylalanine
Pro	Proline
PSP	Phenolsulphonphthalein
PVTC	Post-Valve T-Caecum
r	Correlation Coefficient
RE	Retained Energy
RMR	Resting Metabolic Rate
RS	Resistant Starch
SAPU	Small Animal Production Unit
SCFA	Short Chain Fatty Acid

SE	Standard Error
SE	Surface Energy
SEM	Standard Error Of The Mean
Ser	Serine
SI	Le Système International D'unités
ST	Starch
ST_d	Starch Present In The Diet
ST_i	Starch Present In The Ileal Digesta
SU	Sugars
SU_d	Sugars Present In The Diet
SU_i	Sugars Present In The Ileal Digesta
TAG	Triacylglycerol
TAG_d	Triacylglycerol Present In The Diet
TAG_i	Triacylglycerol Present In The Ileal Digesta
TCA	Tricarboxylic Acid
TEE	Total Energy Expenditure
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UC	Unavailable Carbohydrate
UCP	Uncoupling Protein
UE	Urinary Energy
UV	Ultraviolet
Val	Valine
VFA	Volatile Fatty Acid
VLDL	Very Low-Density Lipoprotein
WB	Wheat Bran
WHO	World Health Organization

Preface

After ingestion, the energy-providing nutrients in food (carbohydrate, fats, protein, and for some individuals, ethanol) undergo a series of catabolic reactions in the human digestive tract, and then (primarily) in hepatocytes to release energy from their chemical bonds. This energy then becomes available to the body, primarily in the form of ATP (the universal currency of chemical energy in the body) and is subsequently converted into other forms of energy such as mechanical energy, thermic energy and so on. However, not all of the energy present in ingested food is ultimately converted to ATP due to the energy requirements involved with the digestion, absorption and intermediary metabolism of food, which vary with the type of food and the nutrients ingested. Some energy is also lost through the heat of fermentation of undigested dietary material entering the large intestine. Furthermore, nutrients vary in their degree of digestibility and absorption (i.e. uptake from the gut) and the efficiency by which they yield energy that is ultimately useful to the body (net ATP gains), with the energy made available to the body via short chain fatty acids from nutrients fermented in the hindgut being less than that obtained from direct nutrient uptake in the upper-tract. For the accurate prediction of the potential 'available energy' (ATP) at the cellular level it is therefore important to be able to predict both the quantity and location of uptake (upper-tract or colon) for each nutrient. The use of metabolisable energy (ME) systems (e.g. Atwater system), as commonly used for food labelling purposes, may not be the most appropriate or accurate means of predicting the useful energy at the cellular level because amongst other weaknesses, ME systems do not account for the unique features of each diet, such as differences in digestibility or inter-nutrient interactions that may affect nutrient assimilation. A valid alternative means needs to be found to model and predict the available energy content of a food for the research and development of foods required to deliver a specific quantity of energy to the body at the cellular level, such as those specifically designed for weight-loss. The need for such foods is growing in importance due to the rapid increase in overweight and obese persons in recent years.

CHAPTER I

Review of the literature

Introduction

Humans meet all of their energy requirements for normal physiological and metabolic function through dietary intake (ingested energy). The difference between ingested energy and the sum of energy losses from the body (i.e. faecal, gaseous, urinary surface and heat energy losses), provides the energy (retained energy) required for basal energy needs and physical work. When retained energy exceeds energy requirements the excess energy is stored as body fat, whereas a shortfall in retained energy relative to the body's energy requirements necessitates using body fat stores. The anatomical distribution and levels of body fat vary with gender and race (Blaak, 2001; Lovejoy *et al.*, 1996; Weinsier *et al.*, 2001). In particular, women have a higher percentage of body fat and store more fat in the gluteal-femoral area than men who store more body fat in the visceral (abdominal) area (Blaak, 2001). The adipocytes (fat cells in adipose tissue) are capable of storing large amounts of energy (33.5 kJ/g) (Flatt, 1995b) and the human body's capacity for increasing body fat stores as required is virtually unlimited. As a result, body fat stores can become excessive over time such that an individual may be classified as overweight (BMI of $\geq 25 \text{ kg/m}^2$) or obese (BMI of $\geq 30 \text{ kg/m}^2$). In New Zealand there has been a considerable rise in overweight and obese individuals over the past decade (36.3% of the adult population were overweight and 26.5% obese, as of 2007) (Ministry of Health, 2008). With similar statistics in other affluent societies the World Health Organisation has described the prevalence of obesity as a global epidemic (WHO, 2000). Despite the effects of physical exercise, the adjustment of food intake remains the primary means of maintaining a constant body weight (Flatt, 1987, 1993). Manipulation of the diet through the development of new foods specifically designed for weight-loss may be one means of counteracting obesity. The valid and accurate prediction of the amount of energy a given food delivers to the body at the cellular level is required to assess the likely efficacy of such a dietary intervention.

I. Energy requirements and availability

1. Components of energy expenditure

Total energy expenditure (TEE) refers to “the energy spent, on average, in a 24-hour period by an individual or a group of individuals. By definition, it reflects the average amount of energy spent in a typical day, but it is not the exact amount of energy spent each and every day” (FAO/WHO/UNU, 2001). The three components of TEE are: the basal energy expenditure (or basal metabolic rate (BMR)), the thermic effect of physical activity (activity thermogenesis) and the thermic effect of food (dietary-induced thermogenesis). The contribution of each of these components is estimated to be approximately 60%, 30% and 10% of TEE, respectively in individuals with sedentary occupations (Levine, 2005; Westerterp, 1998). There may also be energy costs relating to thermogenesis from cold, the action of drugs, hormones, emotions etc., but these are relatively minor compared to the abovementioned variables (FAO, 2003; Levine, 2005).

The average TEE varies considerably from one individual to another as confirmed by studies using the doubly labelled water (DLW) technique (Black *et al.*, 1996; Coward, 1998; Ravussin *et al.*, 1991). Whilst the basal metabolic rate is typically the largest component of TEE, for most adults it does not vary considerably among individuals of a given gender and age (FAO/WHO/UNU, 2001). Physical activity and body weight therefore account for most of the variation in TEE seen in populations with different lifestyles (James and Schofield, 1990). Individuals undergoing growth have increased energy requirements, as do women who are pregnant or lactating. These special circumstances are not considered here and energy requirements are based on that of a healthy, well-nourished adult in a normal physiological state.

1.1 Basal metabolism

Basal energy refers to the energy the body requires to do nothing more than sustain life, including the energy required for digestive, metabolic, respiratory, cardiac and

brain functions. It does not include the energy required for movement or work, growth, lactation or reproduction. Basal energy is independent of dietary intake and it is defined clinically as the energy the body uses while at rest (but not sleeping) in the post-absorptive state (12 – 14 h after a meal), at sexual repose in a neutrally temperate environment (26 – 30°C) (Shetty, 2005).

The BMR (also called the average daily metabolic rate (ADMR)) refers to the basal energy used over a given period of time (routinely 24 h). The BMR of an individual is measured under standard conditions such that it is possible to measure the minimum rate of heat production free from the effects of dietary intake and extreme physical environments (Blaxter, 1989). According to Henry (2005) the four conditions which must be met to achieve this aim are:

1. The subject should be completely rested, both before and during the measurements. They should be lying down and fully awake.
2. The subjects should be fasted for at least 10 – 12 h before the measurements are taken.
3. The environment in which the measurements are taken should be thermo-neutral (22 – 26°C) so that there is no thermoregulatory effect on heat production.
4. The subject should be free from emotional stress and familiar with the apparatus used.

The various means of determining BMR are discussed in detail in Section I, 2.4 of this review.

Closely related to the BMR, is the Resting Metabolic Rate (RMR). Measurement of the RMR does not need to take place immediately upon waking and the subject normally travels to the testing site and therefore has a slightly elevated heart rate compared with the BMR measure. The less stringent measurement conditions of RMR compared to BMR mean that RMR is typically around 5 - 10% higher than BMR, although recent evidence has shown that given a 10 - 15 min rest prior to measurement of RMR there is little difference between RMR and BMR (Levine, 2005). The main determinants of

BMR (and RMR) are age, gender, body size and body composition. There is no correlation between BMR and the aerobic fitness level of an individual when body weight is maintained (Broeder *et al.*, 1992; Westerterp, 1998), however, there is evidence that higher fat-free body mass, as may be gained with weightlifting, increases the RMR (Smith *et al.*, 1997).

1.2 Physical activity

The contribution of activity thermogenesis to TEE is the most variable component of TEE (FAO/WHO/UNU, 2001) and can be divided into exercise thermogenesis and non-exercise activity thermogenesis (NEAT) (Levine, 2005). Exercise thermogenesis refers to the energy expended as part of sport and other purposeful exercise, whilst NEAT can be described as 'energy expenditure of spontaneous physical activity' i.e. physical work (Levine, 2005). For the minority of individuals who undertake formal exercise, the contribution of exercise thermogenesis is around 10% of TEE. Otherwise, NEAT is the sole contributor of activity thermogenesis to TEE through the energy costs of moving around as part of daily life, fidgeting, spontaneous muscle contraction and maintaining upright posture (Levine, 2005). Due to the difficulty in quantifying activity thermogenesis experimentally it is common to employ factorial equations (FAO/WHO/UNU, 2001) as discussed further in Section 1, 2, 2.4 of this review.

1.3 Metabolic response to food

There is an energy cost associated with the ingestion, digestion, absorption and assimilation of nutrients as a result of meal feeding. These processes use oxygen and produce heat and are collectively referred to as dietary-induced thermogenesis (DIT), the thermic effect of feeding (TEF) or the Specific Dynamic Action (SDA). The exact causes of DIT are still not entirely understood (McCue, 2006) and although it is common to estimate the DIT of a food as 10% of the energy content of the food (Westerterp, 1998), it is difficult to quantify DIT as there are differences in the DIT observed across the three primary macronutrients. Fat has been shown to have the lowest effect on DIT due to fat's lower processing requirement, when compared to protein, which exhibits the greatest effect (Prentice, 1995, 1998; Thomas *et al.*, 1992).

Alcohol has been observed to give a similar response to carbohydrate on DIT when measured with whole-body calorimetry (Westerterp *et al.*, 1999). These differences in DIT were observed when feeding subjects a pure dose of one particular macronutrient. For mixed diets, more recent studies have found no detectable difference in DIT using whole-body calorimetry over 24 h when feeding subjects diets ranging from 7 – 79% fat, with the remainder of the diet composed of 14 – 16% protein and the rest from carbohydrate (Shetty *et al.*, 1994). It is still unknown if a high protein diet might give different results, but for mixed diets over a wide range of fat and carbohydrate intakes and protein intake at ‘normal’ levels, it has been concluded that the impact of the diet composition on DIT can effectively be ignored when determining energy requirements (Prentice, 2005). This point remains controversial.

Aerobic exercise and anaerobic weight training are also known to increase DIT following ingestion of a carbohydrate meal when the exercise is of sufficient intensity to deplete muscle glycogen (Denzer and Young, 2003; Nichols *et al.*, 1988).

2. Methods of determining energy expenditure

2.1 Direct and indirect calorimetry

Direct calorimetry is the most accurate means of determining energy expenditure and works on the principle of measuring heat generated by a subject, whilst indirect calorimetry works on the principle of heat dissipation of a subject, which over a long period of time will be very similar to or will equal the rate of heat generation (McLean and Tobin, 2008). The heat dissipated is predicted by measuring the production and consumption of materials used for metabolism and then appropriately converting these into energy equivalents (Shetty, 2005). Most commonly, indirect calorimeters use respiratory gas exchange and measure the consumption of O₂ either on its own or in conjunction with the expiration of CO₂. The production of methane and excretion of urinary nitrogen may also be measured. Determination of the quantities of O₂ consumption and (if measured) CO₂ production and urinary nitrogen excretion enables

energy expenditure to be predicted (Blaxter, 1989) based on the stoichiometric equations for the oxidation of carbohydrate, fat and protein.

A degree of error is introduced when translating O₂ consumption (ml or litres of O₂) into BMR energy equivalents (kJ or MJ). This is due to a number of assumptions made using predictive equations, which are discussed in detail by Shetty (2005), who concludes that it may be expected that a difference of around 5% in BMR due to methodology alone may be observed between individuals or groups unless such assumptions are consistent.

2.2 Doubly labelled water

The doubly labelled water (DLW) technique is an isotope-based technique that involves introducing 2 heavy isotopic tracers into the body water (1 of hydrogen and 1 of oxygen) to measure the behaviour of hydrogen and oxygen in the body. The theory of the technique is based on the principle that both oxygen turnover and hydrogen turnover in the body are dominated by the flow of water through the body, but that oxygen turnover is additionally dominated by inspired O₂ and exhaled CO₂. Therefore, the difference between hydrogen and oxygen turnover provides a measure of the carbon dioxide expired, and therefore the energy expended (Speakman, 1998). The most important development that was brought about by the method is that it allows measurement in free-living subjects undertaking regular daily activities. Whilst the DLW technique is considered sufficiently accurate for determining TEE in groups of subjects, the method is not yet refined enough to provide estimates of TEE in individual subjects (Speakman, 1998) with repeated measurements having variation of approximately 6 - 10% (Schoeller and Hnilicka, 1996). Other barriers to the use of the DLW method include the high cost, and more recently, a shortage of oxygen-18 isotope (North American Association for the Study of Obesity, 2000).

2.3 Flex-heart rate method

The flex-heart rate method has also been shown to be a valid means of predicting TEE in free-living subjects (FAO/WHO/UNU, 2001; Leonard, 2003). The method is based

on the linear relationship that exists between heart-rate and energy expenditure and is measured beat-by-beat with a chest strap wirelessly connected to a data logger. The heart rate is calibrated against rate of O₂ consumption to estimate energy expenditure (Rennie *et al.*, 2001). The main advantages of the flex-heart rate method over methods such as the DLW technique are that it is inexpensive and non-invasive, whilst still maintaining highly reproducible results (7 - 10% error) within subjects (Strath *et al.*, 2000).

2.4 Predictive equations

Factorial equations provide a quick, low-cost means of estimating BMR and TEE without the requirement for laboratory measurement. The contribution of activity thermogenesis is estimated by determining an individual's physical activity level (PAL), defined as "the total energy required over 24 hours divided by the basal metabolic rate over 24 hours" (James and Schofield, 1990). Tables providing calculated TEE values (BMR x PAL) are readily available along with tables giving estimated BMR values based on gender, age and body weight (FAO/WHO/UNU, 2001). Factorial equations for BMR are able to predict the BMR with reasonable accuracy (coefficient of variation of 8%) (Shetty, 2005). However, there are a number of limitations and criticisms of the underlying data, including shortcomings and availability of data for certain population groups (age, geographical location and ethnicity) and the divergence from standard measurement practices (Henry, 2005).

II. Energy balance

1. Introduction

Intra-individual differences in daily food intake vary by $\pm 23\%$ (Schrauwen *et al.*, 1998), yet when taken over the period of a year the difference is seldom greater than 1 – 2% (Flatt, 2001). The intake of dietary energy and physical activity vary considerably on a daily basis, yet despite these short-term variances, body weight maintenance (energy balance), still occurs (Flatt, 1995a). Energy balance, defined simply, is the difference between energy intake and energy expenditure. In an individual where energy intake is balanced by the energy expenditure, the energy balance is zero and a stable body weight is maintained. A positive energy balance occurs when dietary energy is consumed in excess of energy requirements with the excess energy stored as fat. Conversely, a negative energy balance occurs when energy intake is not sufficient to meet energy requirements and fat stores are used.

The full energy balance equation (National Research Council, 1981) is given as:

$$IE = FE + GaE + UE + SE + HE + RE \quad (Eq. 1)$$

Dietary energy enters the body as ingested energy (IE). Energy losses from the body consist of faecal energy (FE), gaseous energy (GaE), urinary energy (UE), surface energy (SE) and heat energy (HE) losses. The energy that is not lost but available for physiological processes is deemed retained energy (RE) (Eq. 2). All retained energy is available for metabolism in healthy adult (i.e. not growing) men, and women who additionally are not lactating or pregnant. Excess RE is retained as fat stores.

$$RE = IE - (FE + GaE + UE + SE + HE) \quad (Eq. 2)$$

2. Terminology

2.1 Ingested energy (IE) or gross energy (GE)

Ingested energy (IE) (also called gross energy (GE), the heat of combustion, intake energy, energy intake and combustible energy) refers to the maximum energy of a food (Elia and Cummings, 2007). It is determined as the heat released during complete combustion to CO₂ and H₂O in the presence of O₂ in a bomb calorimeter (AOAC, 2000). Whilst theoretically the amount of energy released when macronutrients are fully oxidised in the body is the same as that in the bomb calorimeter, the reality is that not all energy contained in ingested food is available to the tissues due to a number of energy losses. Depending on the energy losses that are accounted for, the energy content of a food may be described as digestible energy (DE), metabolisable energy (ME) or net metabolisable energy (NME), among other definitions. The differences between these energy systems are illustrated in **Figure 1**.

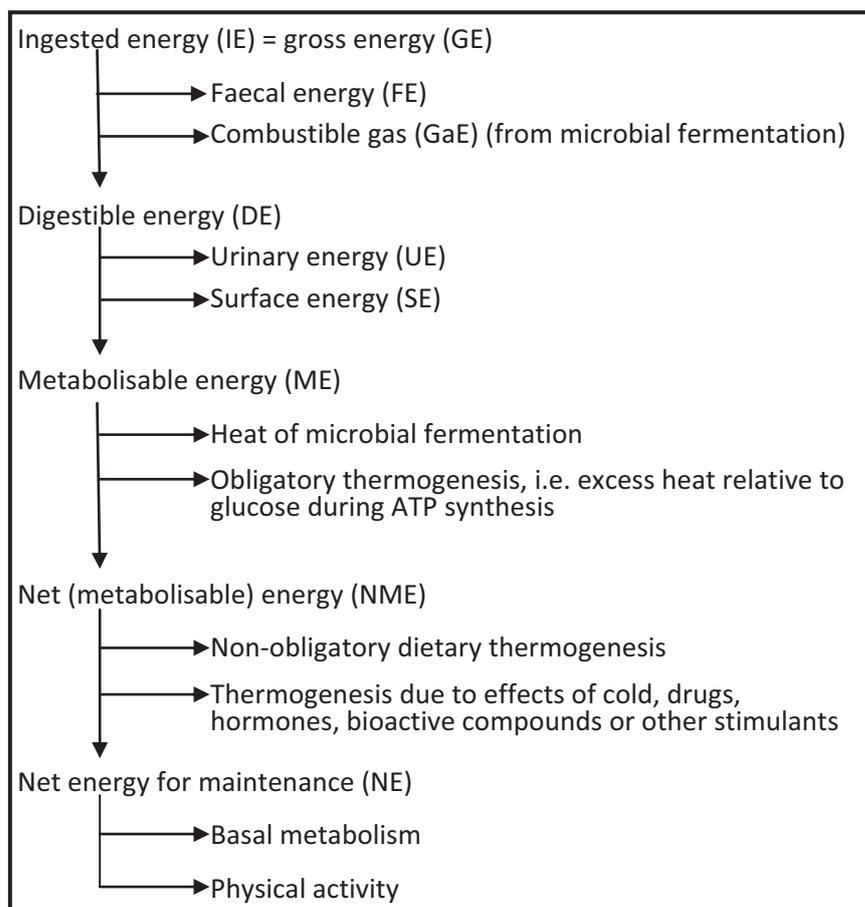


Figure 1. Overview of the flow of energy through the body. From FAO (2003).

2.2 Digestible energy (DE)

The digestible energy (DE) content of a food (Eq. 3) refers to the energy available after accounting for faecal energy (FE) and gaseous energy (GaE) losses and accounts for the fact that not all ingested energy is absorbed in the small intestine (FAO, 2003). In other words, DE refers to the difference in GE present in the ingested food and that present in the faeces and combustible gases (e.g. H₂ and CH₄ in flatus and breath) lost from the body. It should be noted that DE is an 'apparent' energy value because other material aside from undigested dietary material enters the hindgut and may be lost in the faeces. This endogenous material includes approximately 2 - 3 g/d of mucus, along with desquamated cells from both the hindgut and the small intestine (Elia and Cummings, 2007).

$$DE = IE - FE - GaE \quad (\text{Eq. 3})$$

In the case of macronutrients that are completely absorbed in the upper-tract (digestibility of 1.0), the IE = DE. This applies to nutrients such as glucose, fructose, lactose, sucrose and (non-resistant) starch. Conversely, for those nutrients that are not absorbed at all in either the upper-tract or hindgut the digestibility and DE for that particular nutrient are zero.

2.3 Faecal energy (FE)

Faecal energy (FE) is that energy that is lost from the body to the faeces including undigested and unfermented dietary material, endogenous material (e.g. sloughed epithelial cells, mucus etc) and bacterial mass. The energy lost to the faeces is highly dependent on the diet and increases with increasing intake of dietary fibre (Baer *et al.*, 1997; Calloway and Kretsch, 1978; Farrell *et al.*, 1978; Kelsay *et al.*, 1978; Southgate and Durnin, 1970). Assuming 'dietary fibre' (defined here as undigested carbohydrate reaching the colon) has an energy value of 17 kJ/g and that 70% of this is fermented in the colon, of which approximately 60% is transformed into short chain fatty acids (SCFA) and 5% into gaseous energy, the energy loss to the faeces is estimated at 8.7 kJ/g (Elia and Cummings, 2007). This value is composed of 5.1 kJ/g of carbohydrate

entering the hindgut and not being fermented and a further 3.6 kJ/g of products of fermentation (primarily bacterial matter) being lost to the faeces.

2.4 Gaseous energy (GE)

The small portion of SCFA produced as a result of bacterial fermentation in the colon are not absorbed but are expelled in the flatus. Combustible gases (H₂, CH₄ etc.) are also an energy loss to the body, and also are mostly lost via the flatus, although variable quantities are also lost in the breath. Losses of CH₄ and H₂ are, however, relatively small and estimated at less than 1 litre / day in healthy subjects when determined using whole body calorimetry (King *et al.*, 1998; Poppitt *et al.*, 1996). The gaseous losses correspond to around 3 – 5%, or 0.5 – 0.85 kJ/g of the GE of fermentable carbohydrate entering the colon (Elia and Cummings, 2007).

2.5 Urinary energy (UE)

The majority of the energy that it absorbed from the digestive tract is available for metabolism. However, some of it is lost in the urine as nitrogenous waste compounds formed as a by-product of protein metabolism, with urea (22.5 kJ/g N) accounting for around 80 – 90% of the urinary nitrogen excreted in mammals (Hawk, 1965). The higher energetic content of the non-specific nitrogen (NSN) fraction of the urine (e.g. creatinine 56 kJ/g N, amino acids 140 kJ/g N) (Birkett and de Lange, 2001b) increases the overall energetic cost of urinary excretion to between 29 and 31 kJ/g urinary N (Blaxter, 1989; Buttery and Boorman, 1976; Emmans, 1994; Whittemore, 1983, 1997). The energy cost of urinary nitrogen excretion varies with dietary composition.

2.6 Surface energy (SE)

Surface energy losses include desquamated cells, hair loss and perspiration (Elia and Cummings, 2007). These losses are small and considered negligible in normal healthy individuals (Elia and Cummings, 2007; Livesey, 2001).

2.7 Metabolisable energy (ME)

Metabolisable energy (ME) may be defined as the useable energy available to the body after accounting for faecal, gaseous, urinary and surface energy losses (Eq. 4) (Elia and Cummings, 2007). It may also be described as ‘the amount of energy available for total (whole-body) heat production at nitrogen and energy balance’ (Livesey, 2001). By definition, ME does not include the heat energy lost as a result of the heat increment of dietary intake, such that:

$$ME = IE - FE - GaE - UE - SE \quad (Eq. 4)$$

2.8 Heat energy (HE)

Not all metabolisable energy is ultimately available for cellular ATP production due to the energy required for the digestion, absorption and intermediary metabolism of ingested food and its nutrients and this energy is released in the form of heat (FAO, 2003).

2.9 Net metabolisable energy (NME)

Net metabolisable energy (NME) differs from ME in that it accounts for the heat released during metabolism (dHE) - both as the heat of fermentation and as a result of dietary-induced thermogenesis (FAO/WHO/UNU, 2001). As such, NME may be termed ‘available energy’ because this represents the energy available to cells (as ATP) from dietary intake (IE) after accounting for all processes relating to the intake of food (FE, GaE, UE, SE, dHE) (Eq. 5).

$$NME = IE - (FE + GaE + UE + SE + dHE) \quad (Eq. 5)$$

Other processes not related to the intake of food are not included in NME, including non-obligatory thermogenesis and thermogenesis induced by cold and the action of drugs, hormones, bioactive compounds or other stimulants (FAO, 2003). The inclusion of these other non-dietary related processes is known as net energy (NE).

2.10 Net energy (NE)

The term 'net energy', as commonly used in animal nutrition, should not be confused with 'net energy for maintenance' (NE) as is used in human studies, nor with NME (see Section II, 2.9), which although is derived from the concept of NE, has quite a different meaning (Livesey, 2001). Whilst NME only includes the thermogenesis as a result of dietary intake, NE encompasses heat lost due to other factors, including cold, and the action of hormones, drugs, emotions, bioactive compounds and other stimulants (FAO/WHO/UNU, 2001). Net energy for maintenance, therefore, is the energy available to the body for metabolism, growth, pregnancy and maintenance.

The various systems for describing dietary energy (GE, DE, ME and NME), including their strengths and shortcomings are discussed later in detail in relation to the energy value of foods (SectionIV).

III. Energy intake

1. Sources of dietary energy intake

Humans meet all of their energy requirements for normal physiological and metabolic function through dietary intake. The principal energy-providing nutrients in humans are carbohydrates, fats and proteins, and for some individuals, ethanol (FAO/WHO/UNU, 2001). Starch, sugars and triacylglycerols in particular, are the main sources of fuel, with the latter contributing significantly to energy supply in affluent populations (Flatt, 2001). Once consumed, the ingested macronutrients undergo catabolic reactions that release energy from their chemical bonds and this energy then becomes available to the body.

1.1 Carbohydrate

1.1.1 Presence in the diet

Carbohydrates (CHO) are the largest contributor to dietary energy in adult humans, making up 40 – 80% of the diet (Mathers and Wolever, 2009). This energy originates almost entirely from plant foods (fruits, vegetables, cereals, grains, legumes etc), except in the case where milk or milk products containing lactose are consumed (Mathers and Wolever, 2009). The starches contained in grains and edible tubers are the most abundant forms of carbohydrates for most of the population, except in affluent societies where sucrose provides almost as much energy as starch (Danforth, 1985).

Carbohydrates may be classified into three groups according to their degree of polymerisation: sugars, oligosaccharides and polysaccharides (FAO/WHO, 1998). Sugars consist of 1 – 2 monosaccharide units and include monosaccharides (glucose, galactose and fructose) and disaccharides (sucrose, lactose, lactulose, trehalose) (FAO/WHO, 1998; Williams *et al.*, 2001). Within the group of oligosaccharides (3 – 9 monosaccharide units), malto-oligosaccharides (e.g. maltodextrins) form a specific group separate from the other oligosaccharides (raffinose, stachyose, fructo-oligosaccharides) (FAO/WHO, 1998; Livesey, 1992). The polysaccharides (degree of

polymerisation greater than nine) are divided into starches (amylose, amylopectin, modified starches) and non-starch polysaccharides (NSP) (cellulose, hemicellulose, pectins, hydrocolloids) (FAO/WHO, 1998; Mathers and Wolever, 2009). Additionally, the polyols (alditols, sugar alcohols) are mono- and disaccharides in which the aldose and ketose functional groups have been reduced to hydroxyl groups (FAO/WHO, 1998). Polyols are commonly added to processed foods as low-calorie sweeteners and include sorbitol, mannitol, xylitol, lactitol, maltitol, Isomalt and others (FAO/WHO, 1998; Livesey, 1992).

Free monosaccharides, consisting mainly of glucose, fructose and galactose are not commonly found in significant quantities in the diet (Livesey, 1984), although free glucose and fructose are found in honey and certain fruits (Witczak, 2008) and processed foods may have added monosaccharides. Ribose may also be present in some foods but its contribution to dietary energy is negligible (Livesey, 1984). Polysaccharides and disaccharides (two monosaccharides joined together by covalent bonds) therefore constitute the most prevalent and nutritionally important sources of CHO in the diet (Gropper *et al.*, 2008).

1.1.2 *Methods of chemical analysis*

Total carbohydrate (Eq. 6) refers to all CHO present in the food and may be analysed (i) directly as the sum of mono-, di-, oligo- and polysaccharides, including dietary fibre (FAO, 2003); or (ii) calculated as 'total carbohydrate by difference' according to the following equation:

$$\text{Total CHO by difference} = 100 - (\text{weight in grams} [\text{protein} + \text{fat} + \text{water} + \text{ash} + \text{alcohol}] \text{ in } 100 \text{ g of food}) \quad (\text{Eq. 6})$$

Calculation of total carbohydrate by difference is widely used for food labelling purposes but lacks the accuracy required for scientific and research applications. This shortcoming is largely due to the fact that the calculated value for total CHO is entirely dependent on the accuracy of the analysis of the other components (protein, fat, water, ash and alcohol) and also because other components, such as organic acids,

that are not strictly CHO are also included as CHO (FAO, 2003; Merrill and Watt, 1973). Total CHO includes both 'available carbohydrates' and 'unavailable carbohydrates', and for most purposes it is preferable to separately determine these two portions.

Available carbohydrate refers to CHO which is digested by human enzymes, absorbed in the upper-tract and becomes available for intermediary metabolism. When calculated by difference, available carbohydrate is total carbohydrate less dietary fibre and when analysed directly it is calculated as the sum of mono-, di-, oligo- and polysaccharides, excluding dietary fibre. Available carbohydrate may be expressed as the weight of CHO (anhydrous forms) or as the monosaccharide equivalent (hydrous forms, including water) (FAO, 2003).

'Unavailable carbohydrate' or 'dietary fibre' (DF) is the portion of CHO that escapes digestion and absorption in the upper-tract and becomes available for bacterial fermentation in the large intestine (see Section 3 for a detailed discussion on hindgut fermentation). Dietary fibre is a broad term and depending on the analytical assay used, encompasses a selection of the following: cellulose, hemicellulose, lignin, pectin, resistant starch, non-pectin soluble and non-specific residue (Monro and Burlingame, 1996). Cellulose is the main polysaccharide in the plant cell-wall, composed of several hundred to over ten thousand $\beta(1\rightarrow4)$ D-glucose linkages (Fuller, 2004). Hemicelluloses (which have no chemical relationship with cellulose) are short chain, partially soluble polysaccharides found in plant cell-walls composed of a variety of sugars (e.g. arabinose, galactose, glucose, manose, xylose) and uronic type acids (e.g. glucuronic acid, galacturonic acid) (Faithfull, 2002). Lignin is a highly insoluble, small polymer, sometimes found in cereals and may, under some definitions, be included as DF (Cummings, 1996). Although lignin is not a carbohydrate, it may be derived from some plant cell-walls (Cummings, 1996). Resistant starch, consisting mostly of amylose, refers to that portion of dietary starch which escapes enzymic digestion in the small intestine, often due to being trapped inside intact cell walls or other plant cell structures (Williams *et al.*, 2001). There are four classifications of resistant starch: RS₁ – physically enclosed starch; RS₂ – raw starch granules; RS₃ – retrograded amylose; and RS₄ – chemically modified starch (Sajilata *et al.*, 2006).

The most common assays used for determining DF in human foods are the AOAC (2000) enzymatic, gravimetric method 'Prosky' (985.29); the enzymatic, chemical method of Englyst and Cummings (Englyst and Cummings, 1988); and the enzymatic, chemical method of Theander and Åman (1982). These methods for determining DF and others are summarised by DeVries *et al.* (1999). The accuracy of the DF determination method used has importance not only for DF determination, but also for total and available CHO by difference.

The term 'neutral detergent fibre' (NDF) encompasses the plant cell-wall constituents cellulose, hemicellulose, lignin, cutin, NDF-insoluble tannin and ash, whilst 'acid detergent fibre' (ADF) is determined as NDF less the hemicellulose fraction (Faithfull, 2002; Fuller, 2004). The determination of NDF was originally based on the method of (Van Soest and Wine, 1967) and has since undergone a number of modifications (Faithfull, 2002). Whilst ADF and NDF are commonly used for defining the composition of animal feeds (where NDF is considered the only fibre determination method suitable for non-ruminants) (Faithfull, 2002), these measures are rarely used nowadays for defining the DF content human foods.

1.2 Fat

1.2.1 Presence in the diet

The majority of dietary lipid (fat) intake is as triacylglycerol (TAG) (FAO/WHO, 1994). Each TAG consists of three fatty acids esterified to a glycerol molecule backbone (Flatt, 1995b). Cholesterol and phospholipids are also present in the diet, but their contribution to energy intake is negligible (FAO, 2003; Livesey, 1984).

1.2.2 Methods of chemical analysis

The standard method for the determination of fat is the use of a gravimetric method to determine 'crude fat' with fat and fatty acids extracted from food by hydrolytic methods (AOAC, 2000). Prior to gravimetric analysis, most samples (except those that are 100% fat) undergo either Soxhlet extraction (foods low in fat), acid hydrolysis

(most foods) or alkaline hydrolysis (dairy products) (Nielson, 2003). The Soxhlet method and the Mojonnier method are both common laboratory gravimetric methods which involve solvent extraction of the fat and drying the extracted fat to a constant weight (Nielson, 2003). The gravimetric approach is considered adequate for fat evaluation purposes, however, the preferred method for energy evaluation purposes, is for fats to be analysed as fatty acids and expressed as triacylglycerol equivalents (FAO, 2003). The advantage of the latter approach, which may be undertaken using instrumental methods using the principles of infrared, nuclear magnetic resonance or specific gravity (e.g. Foss-Let Method) (Nielson, 2003), is that waxes and the phosphate content of phospholipids are not measured, neither of which have an energy value (James *et al.*, 1986).

1.3 Protein

1.3.1 Presence in the diet

Although carbohydrates and fats are the primary sources of dietary energy for most of the population, protein is also an important contributor to energy intake, particularly for individuals with low total energy intake (FAO/WHO/UNU, 2001). Proteins consist of two or more polypeptide chains – polymerised L- α -amino acids linked by peptide bonds (Fukagawa and Yu, 2009). The average Western diet provides approximately 100 g/d of protein (Ganapathy *et al.*, 2006). Additionally, significant amounts of endogenous salivary and gastrointestinal (GI) secretions enter the intestinal lumen to be potentially digested and absorbed. The extent of endogenous N flow in the GI tract is not known with accuracy. However, total endogenous protein entering the human small intestine has been given as 70 - 100 g/d (FAO/WHO/UNU, 2002b), whilst that entering the large intestine has been given as 7 – 13 g/d (Cummings, 1996). The sources of dietary protein in the human diet are numerous, each varying in amino acid composition and correspondingly, the energy delivered per gram of protein (1981; FAO, 2003). Protein is also the main source of dietary nitrogen in humans, although other sources are also present in smaller quantities e.g. free amino acids, nucleotides and creatine (FAO/WHO/UNU, 2002a).

1.3.2 *Methods of chemical analysis*

There is no AOAC method for the determination of protein and instead the determination of the N content of foods using the Kjeldahl method (or similar) is the standard practice (AOAC, 2000). Based on the assumption that the N content of a protein is approximately 16%, a general conversion factor of $N \times 6.25$ is applied to the determined N value to estimate the protein content. This approach has two major shortcomings. Firstly, as already mentioned, not all N in foods is in protein. Secondly, the N content (on a percentage weight basis) varies for each specific amino acid, meaning that the N content of proteins varies from 13 – 19%. Use of the general factor ($N \times 6.25$) for the conversion of N to the protein content assumes that all proteins have a fixed N content and this may result in errors of between -2% to +9% (FAO, 2003). In foods where the protein content is around 15% this would equate to a small difference in total dietary energy of approximately 1% (FAO, 2003). Determining the protein content through amino acid analysis is the preferable method, and the FAO have stated that this is particularly important in the case of novel foods and for foods specifically designed for specialised dietary requirements (FAO, 2003).

1.4 Alcohol

1.4.1 *Presence in the diet*

Alcohol (ethanol or ethyl alcohol) is consumed by a considerable proportion of the population. In New Zealand, 85% of adults consume at least one alcoholic beverage a year and 7% consume alcohol daily (Ministry of Health, 2009). In the United States, approximately 64% of individuals drink alcohol, and 14% are regular drinkers (at least 12 drinks a year) (Pleis *et al.*, 2009). For many individuals, therefore, alcohol constitutes a quantitatively important energy source and so its digestive and metabolic fate will be considered here along with the major macronutrients.

2. Regulation of dietary energy intake

Satiety is defined as the state in which further eating is inhibited and occurs as a consequence of having eaten (Slavin, 2005). Macronutrients vary in their ability to satiate with protein being the most satiating and fat being the least satiating (Westerterp-Platenga *et al.*, 2009). In particular, it has been observed that diets high in protein (20 - 30% of energy intake when consumed in energy balance) and/or high in DF increase satiety and reduce post-meal hunger (Howarth *et al.*, 2001; Paddon-Jones *et al.*, 2008; Slavin, 2005; Veldhorst *et al.*, 2008). Protein-induced satiety may be mainly due to oxidation of amino acids in excess of that required for protein synthesis, which increases blood or plasma amino acid levels and may serve as a satiety signal for a food-regulating mechanism to depress hunger (Westerterp-Platenga *et al.*, 2009). The satiety imparted by DF is due to a number of factors that are largely a product of its bulking and viscosity properties (Burton-Freeman, 2000). Foods rich in DF reduce the rate of gastric emptying and demand increased effort and/or time for mastication which results in a decreased rate of ingestion and increases the secretion of saliva and gastric juice (resulting in an expansion of the stomach) (Howarth *et al.*, 2001; Slavin, 2005). The satiating effect of foods has particular relevance for foods specifically designed for weight-loss (which are often high in protein and/or DF for their satiating effect) because the greater the satiating effect of a meal, the longer post-meal hunger is depressed for. As such, the aim of weight-loss foods is generally to provide the lowest 'available energy' (ATP) per gram or per unit satiety. An accurate means of predicting the minimal 'available energy' a food delivers *in vivo* may be considered the 'Holy Grail' when defining the energy content of foods.

IV. Energy values of foods

1. Units of energy

The SI unit for measuring energy is the Joule (J), defined as 1 Joule is expended to move an object of mass of 1 kg a distance of 1 m with a force of 1 Newton. In the case of human energetics, kilojoules (kJ) (10^3 Joules) or megajoules (MJ) (10^6 Joules) are the common units of measure. The calorie is an outdated, but still common unit of measure. Conversion factors between kJ and kcal are: 1 kJ = 0.239 kcal; and 1 kcal = 4.184 kJ.

2. Energy conversion systems

The determination of the energy content of food depends not only on the accuracy of analytical methods to determine the individual energy-contributing nutrients (see Section III of this review), but also the conversion of the quantity of these nutrients into energy equivalents to allow the calculation of the total energy content of the food. There are a number of ways to express the energy value of a food, including GE, DE, ME and NME, as already described in Section II of this review.

At the most basic level, a food may be described in terms of its GE content. Determination of the GE content of a food is undertaken in a bomb calorimeter and is straightforward and cost effective. Additionally, the results are highly reproducible (Swift, 1957). However, the GE values of food components assume that all proteins have the same amino acid profile, and that all lipids have the same fatty acid profile, which they do not (Boisen and Verstegen, 2000; van Milgen, 2002). The usefulness of GE values is further limited because not all ingested nutrients are absorbed and dietary-induced heat losses and urinary, faecal and gaseous energy losses are not accounted for. Although expression of the energy value in terms of DE takes into account the digestibility of the food/diet, the other shortfalls present in the GE system persist making these two systems (DE and GE) of little practical use for scientists and consumers of food products.

In practice, and for the purposes of food labelling, the energy values of foods are most commonly given in terms of ME. A number of ME systems (**Table 1**) have been developed, as reviewed in detail by Livesey (1995) and more recently by Zou *et al.* (2007). The two means of predicting ME are factorial equations and empirical equations.

Table 1Models predicting the metabolisable energy values (kJ) of diets^{1,2}

Reference and year	Model	Notes
Empirical models		
1958: Levy <i>et al.</i> (1958)	$ME = 0.976E - 33.3N - 250$	Overestimates ME at high UC intake
1959: Miller and Payne (1959)	$ME = 0.95E - 31.4N$	Overestimates ME at high UC intake
1975: Southgate (1975)	$ME = 0.977E - 16.7UC - 27.6N$	Underestimates ME at high UC intake
1984: Miller and Judd (1984)	$ME = (0.95E - UC\%) - 31.4N$	Underestimates ME, especially at high UC intake
1990: Livesey (1990)	$ME = 0.978E - (17.2UC \times S) - 30N$	No bias; computation of S is complex ³
1991: Livesey (1991)	$ME = 0.97E - 56UC^{0.6} - 30N$	No bias
1991: Livesey (1991)	$ME = 0.96E - 8.4UC - 30N$	No bias
1993: FDA (1993)	$ME = E - 5.23P$	Always overestimates ME, more so with diets high in UC
1993: Brown <i>et al.</i> (1993)	$ME = 0.96E - 10.5UC - 50N$	Alternative formula to Livesey (1991) that deducts known energy losses in metabolism
Factorial models		
1902: Rubner (1902)	$ME = 17.2P + 38.9F + 17.2C$	Always overestimates ME
1910: Atwater (1910)	$ME = 16.7P + 37.6F + 16.7C$	Overestimates ME at high UC intake
1945: Medical Research Council (1945)	$ME = 16.7P + 37.6F + 17.5C_{st}$	Underestimates ME at high UC intake

1972: McCance and Widdowson (1972)	$ME = 16.7P + 37.6F + 15.7C_m$	Underestimates ME at high UC intake
1990: Livesey (1990)	$ME = 16.7P + 37.6F + 15.7C_m + 8.4U$	Zero bias with increasing UC intake
1992: European Council (1992)	$ME = 16.7P + 37.6F + 16.7(C-DF)$	Underestimates ME at high UC intake
1993: FDA (1993)	$ME = 16.7P + 37.6F + 16.7(C-isDF)$	No bias expected unless diet high in resistant starch

¹ From Livesey (1995)

² kJ = 4.184 kcal; ME (kJ), metabolisable energy; E (kJ), gross energy; N (g), dietary nitrogen; UC (g), unavailable carbohydrate; UC%, unavailable carbohydrate as a percentage of the dry weight of food; $UC^{0.6}$ ($g^{0.6}$); P (g), protein; F (g), fat; C (g), carbohydrate by difference; DF (g), dietary fibre; isDF (g), insoluble dietary fibre; C_{st} (g) carbohydrate determined directly and expressed as equivalent starch weight; C_m , carbohydrate determined directly and expressed as equivalent weight of monosaccharide; FDA, Food and Drug Administration

³ $S = 1/(2.6\alpha + 6.2\beta - 0.96)$ where α (a fraction) is the fermentability of the unavailable carbohydrate and β (a fraction) is the intake of unavailable carbohydrate as E from UC alone as a fraction of total E in the diet.

Factorial approaches are based on the quantities of protein, fat, carbohydrate and sometimes unavailable carbohydrate (dietary fibre), which are assigned energy values based on the apparent digestibility (faecal losses) and metabolisability (urinary losses) and corrected for both of these components (Atwater, 1910; Miller and Payne, 1959). Empirical models are based on the determined GE content of the food and the excretion of unavailable carbohydrate and nitrogen.

2.1 Empirical ME systems

Empirical models are preferable to factorial systems from a scientific perspective, but in most cases the proposed models have severe limitations (Livesey, 1995). Empirical ME models developed in the 1950's by Levy *et al.* (Levy *et al.*, 1958) and Miller and Payne (1959) were solely based on the GE and N content of the food. Both these systems were based on diets with a limited range of unavailable CHO and have consequently been shown to overestimate the ME content of foods high in unavailable carbohydrate (UC) (Livesey, 1995). With the advent of analytical techniques to measure dietary fibre (undigested carbohydrate), an account of UC was added to the more recent methods of Southgate (1975), Miller and Judd (1984) and Livesey (1991). The Southgate method, like its predecessors, does not perform well at high intakes of UC, in this case overestimating ME (Livesey, 1990). The model of Miller and Judd (1984) lacks application to mixed diets because it was based on a limited data set of four diets, two of which were extreme in that they were very high in UC from whole-grain cereals (Livesey, 1991). Furthermore, this model underestimates the available energy, particularly so as the UC content of diets increases (Livesey, 1990). The empirical model proposed by the FDA (1993) does not correct for any faecal energy loss but incorporates a factor of 5.23 x total protein intake to account for energy lost in the urine. This correction factor was proposed by Atwater (1910), but on the basis of digestible (not total) protein intake, which means that the FDA model will always overestimate ME (Livesey, 1995).

2.2 Factorial ME systems

2.2.1 *Atwater general system*

Prior to the introduction of any empirical ME methods, the seminal work of W.O. Atwater and colleagues in the nineteenth century introduced the first factorial ME system (Atwater and Woods, 1896). The 'Atwater general factor system', as it is known as, was based on general factors for protein (16.7 kJ/g), fat (37.4 kJ/g) and carbohydrate (16.7 kJ/g), which were corrected for losses of energy in digestion and absorption and the loss of urea in urine. A value for alcohol (28.9 kJ/g) was also provided by the Atwater general system (Atwater and Benedict, 1902). When the Atwater general system was developed, carbohydrate was determined by difference (the analytical shortcomings of which are discussed earlier in Section III of this review), which overestimates energy available from mixed Western diets (Livesey, 1990). The system was not intended to be used for diets high in UC as no account of UC was incorporated (Livesey, 1995). The main criticism of the Atwater general system, however, is that it assumes constant energy values for carbohydrate, fat and protein regardless of the food in which they are found.

2.2.2 *The modified Atwater general factor system*

Modifications to the general system over the last century have included dividing carbohydrate into available and unavailable CHO (see Section III of this review for definitions) and assigning values to these portions. A value for available CHO expressed as monosaccharide (15.7 kJ/g) was added in the 1970's (Southgate and Durnin, 1970) and this change appeared in McCance and Widdowson's 'The Composition of Foods' and was subsequently adopted as part of the British Food Tables system (Holland *et al.*, 1991). Separately, a value of 8.4 kJ/g for unavailable carbohydrate (DF) was added more recently to the Atwater general factor system (Brown *et al.*, 1998; FAO/WHO, 1998) resulting in zero bias with increasing UC intake (Livesey, 1995). Livesey (1990) brought together the values of 15.7 kJ/g for CHO monosaccharide and 8.4 kJ/g for UC in his modified version of the Atwater model. The European Council system (European Council Directive, 1990) determines carbohydrate

by difference minus UC content, which underestimates ME (Livesey, 1995). In the United States, the FDA system (1993) determines CHO by difference less insoluble DF resulting in some small bias when the diet is high in resistant starch. Specific factors for organic acids (13 kJ/g) (Codex Alimentarius, 2001), polyols (10 kJ/g) and specific polyols and other organic acids are other additions included in the modified version of the general system (Livesey, 2001). The modified Atwater approach has been shown to be suitable for application with diets high in UC, but not when food intake is spontaneously reduced (Brown *et al.*, 1998; Zou *et al.*, 2007).

2.2.3 *The Atwater specific factor system*

The Atwater specific factor system was introduced by Merrill and Watt (1955) as a series of tables as a modification of the Atwater general system to account for the differences in both the heats of combustion and digestibility of carbohydrate, protein and fats in different foods. The specific Atwater system also uses “Jones factors” (Jones, 1941), which use specific factors for converting N content into protein content (rather than the generic $6.25 \times N$) (FAO, 2003). When compared to the general factor system, the specific factor system gave ME values around 2% lower when excluding foods giving extreme differences (20 - 38% difference between the two systems) (FAO, 2003). The specific factor system is used wherever possible in the U.S. for food labelling and is considered to be far superior to the general factor system, but not necessarily so compared to the modified general system (Livesey, 2001). The specific factor system does not account for UC and it has been suggested that it is independent of the effects of UC (Livesey, 1990; Mathews, 1995). The specific factor system is regarded as the gold standard in the U.S. (Brown *et al.*, 1998), but the fact that it does not include specific values for nutrients other than protein, CHO and fat make it in some ways inferior to the Atwater modified general system (FAO, 2003). The main criticism of the specific factor system is its complexity - each food must be located in the food tables and the specific factors applied for that food (Livesey, 2001).

2.3 Summary of shortcomings of ME systems

A full discussion of the advantages and disadvantages of the various ME models is given by Livesey (1995). In summary, the empirical models generally do not account well for UC (e.g. Levy *et al.* (1958), Miller and Payne (1959), Southgate (1975), Miller and Judd (1984) and FDA (1993) models) or are too complex for routine use and/or not suitable for whole diets (e.g. two of the models proposed by Livesey (1990; 1991)). The factorial models often do not account for UC at all (e.g. Rubner (1902), Atwater (1910), Medical Research Council (1945) and McCance and Widdowson (1946) models) and almost always assume a constant heat of combustion for each macronutrient (e.g. all factorial models given in Table 1). The general factor systems, which include the Atwater general system (Atwater, 1910), the European Council system (European Council Directive, 1990) and the FDA general system (FDA, 1993), show increasing bias with increasing NSP intake (Brown *et al.*, 1998). The specific factor system, however, is relatively complex and may not be applicable to novel foods. All ME systems have one major shortcoming from a scientific point of view, however, in that they provide only approximate estimates of the availability of energy to the body for intermediate metabolism (Livesey, 2001).

2.4 Expressing dietary energy as net metabolisable ('available') energy

Expression of the energy content of foods in terms of NME remedies the primary issues associated with ME systems. The energy lost from the body as the heat of fermentation and DIT is not available to generate ATP. The NME system accounts for this fact (unlike ME systems) and thereby predicts the 'available energy' content of a food. The NME definition is based on the fact that nutrients replace themselves in proportion to NME values or ATP equivalents (Blaxter, 1989; Livesey, 2001), rather than in proportion to their ME values or heat equivalents (Rubner, 1902). As such, NME accounts for the efficiency of energy utilisation in metabolism, relative to glucose (Elia and Cummings, 2007; Livesey, 2001). The NME values for the relevant food components are: available carbohydrate expressed as monosaccharide (15.7 kJ/g), protein (13.3 kJ/g), fat (36.6 kJ/g), 6.2 kJ/g for DF in mixed diets, 8.0 kJ/g for

fermentable DF and 26.4 kJ/g for alcohol (Warwick, 2005). For most food components (except glucose), NME values are lower than ME values, particularly for foods high in DF (25% decrease for DF in mixed diets and 27% decrease for fermentable DF), protein (24% decrease) and some novel food components (FAO, 2003). The validity of NME has been confirmed using 24 h calorimetry in humans and by calculation of 'high-energy' bond yields with comparable results (Livesey, 2001). Although there has been some debate in the literature regarding the use of NME for food labelling purposes (Livesey, 2001; MacLean, 2005; Warwick, 2005; Warwick and Baines, 2000), even opponents to the NME system agree that it better reflects the available energy of a food (Warwick, 2005). It has been stated by the FAO that "NME represents the biological ATP-generating potential and, as such, the maximum potential of individual food components and foods to meet energy requirements that require ATP; thus, NME represents a potential improvement in the description of food energy, especially when individual foods are to be compared" (FAO, 2003).

The application of NME has particular importance for novel foods and those foods high in DF, resistant starch and protein (FAO, 2003; Livesey, 2001). Foods that are specifically formulated for body weight-loss contain high concentrations of one or more of these ingredients for satiety, maintaining a low energy content and for imparting desirable functional attributes on the food. For this reason, and also because weight-loss food products are designed to deliver a specific quantity of energy, expressing the dietary energy content in terms of 'available energy' would seem to be preferable.

It should be noted that although the terms "available" and "digestible" are often used interchangeably, not all nutrients that are digested and absorbed become available for metabolism (e.g. lysine, (Moughan, 2003)). The term "available", will therefore be used in the present work to describe the release and uptake of nutrients in a chemical form that can be potentially used for yielding ATP via the body's metabolic processes.

V. Energy utilisation

1. Overview of digestion in man

Man is a simple-stomached animal species and the majority of nutrient digestion and absorption occurs prior to the terminal ileum (i.e. in the upper-tract). Any material that escapes upper-tract digestion enters the colon and is a potential substrate for fermentation by the bacteria populating the hindgut. Unfermented material is excreted in the faeces along with methane, H₂ and CO₂ gas, epithelial cells, mucus and considerable quantities of bacterial biomass (Cummings, 1996). Collectively, these faecal components total around 25 g/d of dry matter for a typical Western diet (Stephen and Cummings, 1980a). The digestive process, from mouth to anus, typically takes about 60 h in Western subjects, of which 4 – 6 h is mouth to caecum transit time (Cummings, 1996; Cummings *et al.*, 1992). The large intestinal transit time is therefore around 54 h, although 24 – 48 h has been reported in Africans (Burkitt *et al.*, 1972).

Quantitatively, the energy gained via upper-tract digestion is by far the most important with hindgut fermentation contributing only 3 – 11% to the body's maintenance energy needs for individuals consuming mixed Western diets (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984). The processes by which each class of nutrients (carbohydrate, protein, fat and alcohol) and dietary fibre are dealt with by the body to eventually yield useful energy for metabolic processes will now be discussed.

2. Upper-tract digestion, absorption and transport

2.1 Carbohydrates

2.1.1 Digestion

Mammalian enzymes in the small intestine, except for β -galactosidase, are only able to degrade α -glycosidic linkages leaving β -glycosidic linkages in NSP intact (Livesey, 1992). Both disaccharides and digestible polysaccharides (non-resistant starch) must first be

hydrolysed to their constituent monosaccharide equivalents to allow absorption from the digestive tract. Digestible polysaccharides are firstly digested primarily to the disaccharides maltose, glucose and isomaltose in the mouth (minimal)/ stomach and duodenum by salivary and pancreatic α -amylase respectively (Smith and Morton, 2001). These disaccharides then undergo digestion in the upper small intestine in the same way as ingested disaccharides. Specifically, disaccharide digestion occurs in the microvilli of the intestinal mucosal cells (brush border), not in the intestinal lumen, where the enzymes lactase and sucrase act on lactose (to produce one galactose and one glucose) and sucrose (to produce one glucose and one fructose), respectively (Smith and Morton, 2001). Additionally, the action of maltase and isomaltase both yield two glucose units from the breakdown of maltose and isomaltose, respectively.

Although 95% of CHO in the human diet are digested and absorbed within the small intestine (Mathers and Wolever, 2009), some (mostly oligosaccharides and polysaccharides) are resistant to human digestive enzymes and are collectively termed 'dietary fibre' (Cummings, 1996). Specifically, oligosaccharides (except maltodextrins) and NSP are largely unabsorbed in the small intestine (Mathers and Wolever, 2009).

Whilst their presence in the diet is small compared to the oligosaccharides and polysaccharides, polyols are also all largely indigestible, as are all the monosaccharides and disaccharides except for glucose, galactose and fructose, lactose, maltose and sucrose (Livesey, 1992; Mathers and Wolever, 2009). An example of one such indigestible sugar is lactulose (Livesey, 1992; Saunders and Wiggins, 1981).

2.1.2 *Absorption and transport*

The rate of absorption of digested CHO needs to be rapid in order to avoid large quantities of unabsorbed CHO entering the hindgut where it may be fermented with unpleasant side-effects (Flatt, 1995b). Approximately 60% of the glucose available for absorption in the gut is believed to be absorbed and transported from the enterocyte into circulation, while approximately 25% diffuses through the basolateral membrane into circulation and the remaining 15% leaks back across the brush border into the intestinal lumen (Groff and Gropper, 2000). Glucose and galactose are absorbed by

both passive diffusion and active transport systems and transported to the liver requiring 0.5 mol ATP / mol glucose or galactose (Blaxter, 1989; Flatt and Tremblay, 1998). A specific carrier, known as SGLTI is also involved and both glucose and galactose must attach to Na⁺ before SGLTI can attach to the sugars, a process relying on the Na⁺/K⁺-ATPase pump (Smith and Morton, 2001). Fructose does not bind to SGLTI and is absorbed more slowly than glucose or galactose, but faster than polyols such as sorbitol and xylitol which use passive diffusion (Gropper *et al.*, 2008). Fructose absorption is thought to occur by passive carrier-mediated facilitated diffusion, involving a specific hexose transporter named GLUT5 (Burant *et al.*, 1992), which can only occur down a concentration gradient. However, the uptake of fructose against this gradient implies that at least some absorption occurs via active transport (Macrae and Neudoerffer, 1972). In 60% of adults the absorption of fructose is limited and there is evidence to suggest that the presence of glucose aids the absorption of fructose (Truswell *et al.*, 1988).

Absorbed monosaccharides are transported to the liver where the majority of fructose and galactose are taken up into liver cells by specific receptors and facilitated transport and then metabolised to glycogen via glucose derivatives (glycolysis) (Mathers and Wolever, 2009). Glucose is also metabolised in the liver, but not as completely as for fructose and galactose and also enters other cells, such as muscle, skeletal, kidney and adipose tissue, by facilitated transport (Gropper *et al.*, 2008).

2.2 Fats

2.2.1 Digestion

The process of TAG digestion begins in the stomach with the addition of lingual lipase produced in the mouth which acts on medium- and short-chain length fatty acids. To enable gastric digestion to occur, the fats are emulsified by the physical action of the stomach and the addition of bile salts when the partially hydrolysed emulsion enters the duodenum. Pancreatic lipase then takes over TAG digestion, yielding a wide range of diacylglycerols, monoacylglycerols and free fatty acids (FFA) as well as a small amount of free glycerol.

2.2.2 *Absorption and transport*

Partially digested lipids are combined with bile salts to form micelles (Morgan and Hoffman, 1971). The lipid content of the micelles diffuses into the brush border of the enterocytes in the distal duodenum and jejunum aided by a concentration gradient (FAO/WHO, 1994). After absorption into enterocytes, the lipid components of longer chain fatty acids (10 to 12 carbon atoms) are reformed into TAG, requiring activation (i.e. conversion to a compound that can enter the TCA cycle) by coupling to coenzyme-A (Morgan and Hoffman, 1971; Tso and Balint, 1986). The resynthesised TAG are formed into lipoproteins (chylomicrons) in the enterocyte's endoplasmic reticulum and are released into the lymphatic circulation and then the portal blood (Gropper *et al.*, 2008). Short chain fatty acids (fewer than 10 to 12 carbon atoms) pass directly into the portal blood without being resynthesised, although the portal route may be an absorptive route for long chain FA as well (McDonald *et al.*, 1980). The role of lipoproteins is to transport lipids between tissues (Groff and Gropper, 2000). Other lipoproteins, such as very low-density lipoproteins (VLDL) are formed in the liver from endogenous lipid and are also circulated in the bloodstream (Gropper *et al.*, 2008). Circulating lipoproteins are acted on by the enzyme lipoprotein lipase (LPL) to release FFA and diacylglycerols, which are rapidly absorbed by adipose tissue cells (Flatt, 1995b; Gropper *et al.*, 2008). Only a small percentage of hydrolysed lipoproteins reach the body's pool of albumin-bound fatty acids with dietary fat largely targeted for adipose tissue (Flatt, 1995b; Griffiths *et al.*, 1994).

2.3 **Protein**

2.3.1 *Digestion*

Hydrochloric acid first denatures the structure of the protein in the stomach and then pepsin attacks peptide bonds, favouring aromatic amino acids (Smith and Morton, 2001). This produces large polypeptides, some oligopeptides and free amino acids. In the small intestine, including the ileum, a number of peptidases are produced to act specifically on certain peptide bonds, depending on the adjacent amino acid in the

polypeptide chain. The end products of protein digestion are free amino acids and peptides (consisting mainly of di- and tripeptides).

2.3.2 Absorption and transport

The majority of N absorption occurs in the proximal intestine (Gausserès *et al.*, 1996; Mahé *et al.*, 1996; Nixon and Mawer, 1970), although the distal ileum may also be an important site of absorption for di- and tri-peptides in particular (Erickson *et al.*, 1995). Some amino acids are not absorbed into the tissues beyond the intestines and released into circulation, but rather, are used by intestinal cells for energy and to synthesise other compounds e.g. glutamine is an important energy source for intestinal cells (Matthews *et al.*, 1993).

The majority of the products of protein digestion, however, are absorbed, primarily in the upper small intestine (although sites of maximal absorption vary between amino acids) via a number of ATP-requiring carrier systems (Matthews *et al.*, 1971). There is evidence for some degree of competition for uptake between amino acids requiring the same carrier and peptides also compete for carriers for absorption (Groff and Gropper, 2000). Generally, branched-chain amino acids are absorbed faster than smaller amino acids and peptides appear to be absorbed faster than the equivalent mix of free amino acids (Abidi *et al.*, 1967). The absorption of peptides accounts for 67% of the absorbed amino acids and the remainder are absorbed as free amino acids (Zaloga, 1990). The majority of amino acids are absorbed intact across the brush-border membrane into the enterocytes as small peptides (di- and tripeptides) where they are hydrolysed by cytoplasmic proteases (Ganapathy *et al.*, 2006). The absorption process is facilitated by specific peptide transport systems (Ganapathy *et al.*, 2006). Absorbed amino acids traverse the basolateral membrane of the enterocytes, mostly as free amino acids, into the interstitial fluid and the portal vein, primarily through diffusion and sodium-independent transport (Gropper *et al.*, 2005). Peptides that are resistant to cytoplasmic proteases are transported intact across the basolateral membrane, but this route is minor compared to the transport of the bulk of the products of protein digestion (Ganapathy *et al.*, 2006). The transport of amino acids

into hepatocytes uses carrier systems similar to those found in the intestinal basolateral membrane (Gropper *et al.*, 2008).

2.4 Alcohol

2.4.1 Digestion

Approximately 5 – 10% of ingested ethanol is excreted unaltered, primarily in the expired breath and urine (Rubin and Strayer, 2008).

2.4.2 Absorption and transport

Ethanol is absorbed unchanged by the intestinal mucosa via simple diffusion (Bode and Bode, 1997). The absorption process can potentially take place along the entire length of the digestive tract (Pawan, 1972), but the majority of ethanol is absorbed in the stomach (10 – 30%) and the upper small intestine (approximately 80%) (Levitt *et al.*, 1997). The rate of absorption is affected by a number of factors. Higher concentrations of ingested alcohol increase the rate of absorption by the mucosa, with alcohol concentrations of 10 - 20% being the most rapidly absorbed (Bode and Bode, 1997; Pawan, 1972). The presence of food in the stomach reduces the rate of absorption (Bode and Bode, 1997). The type of beverage also effects the rate of absorption e.g. whisky is absorbed more rapidly than the equivalent amount of alcohol in beer (Bode and Bode, 1997). Other factors affecting alcohol absorption include the rate of gastric emptying, the permeability of the gastric and intestinal tissues, the types of spices in foods, certain medications and the drinker's gender and body temperature (Bode and Bode, 1997; Bode, 1980; Pawan, 1972). Ethanol is absorbed intact into the portal vein and transported unaltered, primarily to the liver. Other tissues, such as the kidney, muscle, lung, intestine and brain are also minor sites of metabolism (Mezey, 1985; Pawan, 1972).

3. The hindgut

There is evidence to suggest that microbial activity in the small intestine exists (Bach Knudsen and Jensen, 1991; Dierick *et al.*, 1986; Jørgensen and Jensen, 1994), although

compared to that occurring in the large intestine, the effect on total tract digestibility may be considered minimal. The large intestine (caecum and colon) is heavily populated with bacteria, as evidenced by colonic microbial counts of 10^{10} to 10^{11} colony-forming units per g wet weight (Hill, 1995). The colonic microflora is comprised of mostly anaerobic bacteria, at least one hundred times greater in number than facultative organisms (Williams *et al.*, 2001). Individual variation in the makeup of the large intestinal microflora may be at least partly attributed to the composition of the diet, especially the fermentable fraction (Djouzi and Andrieux, 1997). This realisation has led to a great deal of interest in prebiotics (indigestible food ingredients that stimulate bacterial activity) and probiotics (introduction of beneficial bacteria) as food sources that may be able to beneficially influence the health of the hindgut ecosystem (Marteau and Boutron-Ruault, 2002). The absorption of amino acids and di- and tripeptides by the hindgut is not likely to contribute significantly towards the energy supply in humans (Darragh *et al.*, 1994; Just *et al.*, 1981). This is supported by Hume *et al.* (1993) who state that amino acids produced as a by-product of bacterial protein synthesis do not accumulate in the mammalian hindgut, due to the action of microbial deaminases.

3.1 Hindgut fermentation

The process of anaerobic breakdown of material entering the hindgut by the large intestinal microflora is known as fermentation (**Figure 2**), and it is by this process that the human host is able to benefit from the energy that would otherwise be lost due to the indigestibility of dietary material in the upper digestive tract (Cummings, 1987).

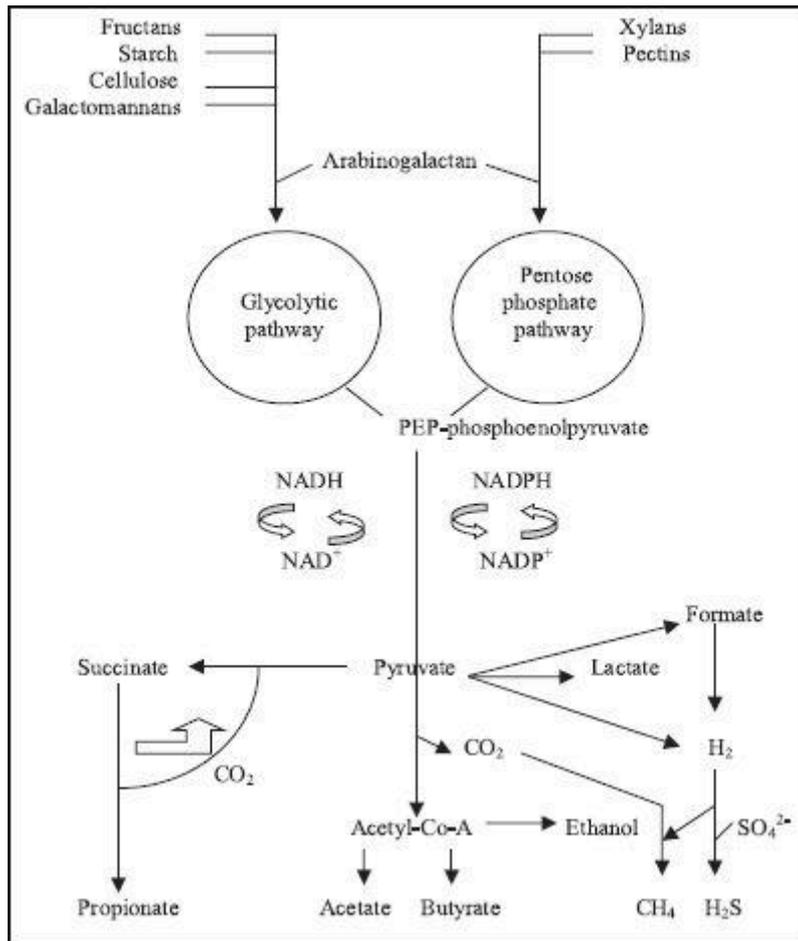


Figure 2. The pathways of fermentation in the hindgut leading to the production of short chain fatty acids. From Macfarlane & Gibson (1997).

3.1.1 Substrates for hindgut fermentation

Undigested CHO is quantitatively the most important substrate for hindgut fermentation (Mathers and Annison, 1993). Of the estimated 25 – 80 g of material entering the colon each day, approximately 20 – 60 g is undigested dietary CHO (of which resistant starch contributes 5 – 10 g) and 1 - 12 g is undigested dietary protein (Cummings, 1996; Cummings and Englyst, 1987; Cummings *et al.*, 1989). Additionally, 4 – 12 g of endogenous protein (e.g. pancreatic enzymes and other secretions), 0.5 g of urea and nitrate, around 3 – 5 g of mucus and unknown quantities of sloughed epithelial cells also contribute to the pool of potential substrates for colonic fermentation (Cummings, 1996).

As discussed earlier, there is a wide range of undigested carbohydrates that enter the colon, including polysaccharides, oligosaccharides, polyols and even some mono- and disaccharides (Livesey, 1992; Mathers and Wolever, 2009; Williams *et al.*, 2001). The degree to which each of these carbohydrates is fermented in the hindgut varies, although a generic hindgut apparent digestibility value of 0.7 for unavailable carbohydrate (DF) is applied to mixed human diets, which is the same as fermentability (Livesey, 1995, 2001). Polyols and oligosaccharides are generally all completely fermented (Livesey, 1992). Some oligosaccharides are largely fermented in the terminal ileum and proximal colon, being unavailable for fermentation in the distal colon (Houdijk, 1998). Fermentability of resistant starch is variable, ranging from 30 - 70% (Behall and Howe, 1995, 1996; Cummings *et al.*, 1996; Ranhotra *et al.*, 1991; Ranhotra *et al.*, 1996). The NSP fraction of DF is the most variable when considering fermentability. For example, pectin is readily fermentable (Holloway *et al.*, 1983), whilst the faecal digestibility of wheat bran DF in humans was observed by Nyman *et al.* (1986) to be 34% and by Chen *et al.* (1998) to be 56%. The fermentability of gums varies, ranging from poorly fermented (e.g. gum karaya) (Davies, 1990) to highly fermentable (e.g. guar gum and gum arabic) (McLean *et al.*, 1983; Nyman *et al.*, 1986). The classification of a DF as “soluble” or “insoluble” is not a reliable indicator of its ability to be fermented, as illustrated by the gums, which are all considered to be soluble DF (Livesey, 1995). For mixed human diets there is a consensus that around 50% of the gross energy in fermentable CHO becomes available (Livesey, 1995).

3.1.2 Products of hindgut fermentation

One of the most important products of hindgut fermentation is the energy that is used by the colonic bacteria for its own growth and maintaining cellular function, the former resulting in increased biomass and faecal bulk (Cummings, 1987, 1996). From the perspective of the human host, the only major products of the hindgut fermentation of both carbohydrate and protein that contribute to energy supply are short chain fatty acids (SCFA) (organic fatty acids containing 1 – 6 carbon atoms), primarily acetic, propionic and butyric acids (Cook and Sellin, 1998; Mathers and Wolever, 2009). The fermentation of protein also produces branched-chain fatty acids

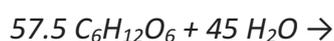
(isobutyrate and isovalerate), valerate, formate, caproate, ammonia, amines and phenols (Cummings, 1996; Macfarlane *et al.*, 1992), although the concentration of the branched-chain fatty acids is small relative to total SCFA production (5 – 20 mol / 100 mol total SCFA) (Cook and Sellin, 1998; Williams *et al.*, 2001). Total daily SCFA production is in the range of 100 – 200 mmol (Cook and Sellin, 1998).

Hydrogen gas, carbon dioxide gas and indirectly, methane gas (Cummings, 1996; Williams *et al.*, 2001), as well as heat (Topping and Clifton, 2001) are also major products of hindgut fermentation. All such gas produced in the large intestine is excreted in the flatus and the breath, although CO₂ from fermentation is a small contributor to breath CO₂ (Cummings, 1996). The presence of H₂ gas in exhaled breath was once thought to be a reliable indicator of fermentative activity in the large intestine, but it is now apparent that this is not the case (Christl *et al.*, 1992). The production of H₂ gas is unique to fermentation and aside from being used to increase bacterial biomass, it may be disposed of in a number of additional ways. Hydrogen gas may be used by individuals harbouring methanogenic bacteria (30 - 50% of the population) to reduce CO₂ to methane (Cummings, 1996), or in the case of non-methane producers who carry large numbers of sulphate-reducing bacteria, to reduce SO₄ to sulphides, including H₂S (Cook and Sellin, 1998; Cummings, 1996). Acetogenic bacteria also consume H₂ in the process of reducing CO₂ to acetic acid (Cook and Sellin, 1998; Cummings, 1996). Lactic acid, the predominant organic acid found in the upper digestive tract (Serena *et al.*, 2007), is also formed in the hindgut as an intermediate product of starch fermentation (Macfarlane and Englyst, 1986), consuming H₂, but only accumulating when SCFA production is inhibited and the colonic environment is at a pH lower than pH 5.5 (Soergel, 1994). Since lactic acid produced in the hindgut generally does not accumulate in the colon, its fate, like ethanol and succinate (also products of fermentation), is as a substrate for other bacterial species, rather than being absorbed by the bowel for use by the host.

The production of SCFA causes a drop in pH in the colon with pH increasing in more distal regions of the colon, such that the caecum (although of a small size in man) is the most acid region, followed by the proximal colon (also acidic) and then the sigmoid

colon which maintains a near neutral pH (Cook and Sellin, 1998; Cummings, 1996). The right-hand (proximal) side of the colon is an active site of CHO fermentation and SCFA production, whilst the sigmoid region is depleted of CHO as a bacterial food source, so protein fermentation and the accumulation of branched-chain fatty acids, phenols and amines dominates along with a shift in the type of microflora to more methanogenic and sulphate-reducing bacteria (Cummings, 1996). The production of SCFA may shift distally depending on the availability of CHO and colonic transit time (Cook and Sellin, 1998).

The general equation for the fermentation of CHO is given by Ewing and Cole (1994) as:



which suggests that theoretical molar ratios of acetic acid: propionic acid: butyric acid are 65 : 20 : 15. The direct measurement of SCFA in the human hindgut is difficult, since virtually all of the SCFA produced are rapidly absorbed in the colon (Elia, 1995b; Ruppin *et al.*, 1980). Experimental findings in healthy humans are therefore based on autopsy from sudden death victims (Cummings *et al.*, 1987) or after sampling by enema (Cummings, 1996; Weaver *et al.*, 1988). Experimental findings and theoretical estimates both support molar ratios of approximately 60 : 20 : 18 for acetic : propionic : butyric acid in humans (Cummings and Macfarlane, 1997). It is well known, however, that the ratios vary depending on diet, region of the colon and other factors (Cook and Sellin, 1998). For example, starch as a fermentative substrate, yields a greater proportion of butyric acid than other carbohydrates (Cummings, 1996). The molar ratio of 60 : 20 : 18 given above equates to a yield of 63 g SCFA per 100 g of CHO fermented (Cummings and Macfarlane, 1997), similar to the value of 61 g SCFA per 100 g reported by Livesey and Elia (1995).

3.1.3 Absorption and transport of SCFA

Short chain fatty acids are the only product of hindgut fermentation available as an energy source for the host. Short chain fatty acids are absorbed rapidly by the colonic mucosa and become available for intermediary metabolism in the body (Cummings, 1981). There is no evidence for active transport of SCFA (Henning and Hird, 1972; McNeil *et al.*, 1978) and the absorption of SCFA is thought to proceed by either non-ionic diffusion of protonated SCFA (accounting for approximately 60% of total SCFA absorption); or cellular uptake by ionic diffusion of the sodium or potassium salt of the SCFA (Ruppin *et al.*, 1980). The relative proportion of protonated or ionised SCFA is determined by the pH in the colon (Cook and Sellin, 1998).

VI. Postprandial metabolic fate of dietary energy

1. ATP

Once nutrients have been absorbed the energy contained within these substrates must be released. This is achieved through cleaving the covalent bonds of the absorbed molecules so that the bonding forces “relax” and their energy is released. This energy can then be used to form the phosphate anhydride bonds of ATP (adenosine triphosphate), from ADP (adenosine diphosphate). ATP can be subsequently hydrolysed to release energy as required, which in almost all cases, involves the enzymatic hydrolysis of the β - and γ -phosphates in ATP, producing ADP and a free phosphate group. The free energy (ΔG) for the hydrolysis of ATP is normally given as 30.5 kJ / mol ATP (7.3 kcal / mol ATP) under standard conditions (Karp, 2009). However, *in vivo* conditions may not be representative of standard conditions, leaving some possible degree of error in this value. The free hydrolysis of ATP under typical cellular conditions, and in muscle, is approximately -59.8 kJ/mol (-14.3 kcal/mol), which is more indicative of the actual energy available (Flatt and Tremblay, 1998; Stryer *et al.*, 2002). ATP is an intermediate compound with a small pool size, that is constantly and rapidly being used for metabolic or external work, and as such, it does not accumulate in cells (Elia and Cummings, 2007). Although ATP may be considered as the universal energy currency in the body, there are also other intermediate phosphorylated compounds that play a role. These include phosphoenolpyruvate, 1,3-biphosphoglycerate, phosphocreatine, glucose 1-phosphate, adenosine monophosphate (AMP) and glucose 6-phosphate.

The primary means by which ATP is formed from ADP is via oxidative phosphorylation (in the mitochondrion) using energy from the flow of electrons generated by the oxidation of the substrate. These electrons are passed sequentially through a series of intermediate compounds, ultimately to O₂, which is reduced to H₂O – a process referred to as the electron transport chain (respiratory chain). The specific process by which each of the energy yielding substrates is catabolised is dealt with in the remainder of this section of the review.

The cellular P/O ratio describes the ATP produced per oxygen atom reduced by the electron transport chain during oxidative phosphorylation. Traditionally, it was assumed that cellular P/O ratios were integers (3 for NADH and 2 for FADH₂) (Hinkle, 2005). More recently, chemiosmotic theory has led to the proposition of non-integer values (2.31 for NADH and 1.38 for FADH₂). The basis for these new modern ratios is discussed in detail by Brand (2005). The possibility remains, however, that these theoretical cellular P/O ratios may still be higher than what occurs *in vivo* in mitochondria as these ratios do not fully account for slip reactions, proton leakage and the activity of uncoupling proteins.

Slip may occur when the proton pumps are not perfectly coupled, resulting in fewer protons pumped across the mitochondrial membrane and therefore less ATP synthesised, effectively lowering the cellular P/O ratio (Brand, 2005). Whilst it would seem that slip does not occur under normal physiological cell conditions, some uncertainty remains (Brand, 2005; Brown, 1992; Kadenbach, 2003; Murphy, 1989).

Proton leakage (where protons leak back across the coupling membrane and are therefore unavailable for ATP synthesis), is more widely accepted. A number of *in vitro* studies have demonstrated that proton leakage occurs in both isolated mitochondria and intact cells, accounting for around 20% of the total cellular respiration rate, dependent largely on the species and type of cell (Brand, 2005). It is estimated that only 60% of an hepatocyte's total oxygen consumption is used for ATP synthesis, effectively lowering the cellular P/O ratio considerably (Brand, 2005). Again, it is uncertain if *in vitro* observations are completely transferable to the *in vivo* situation and to what extent proton leakage should be accounted for when predicting the cellular P/O ratio (Marcinek *et al.*, 2004; Rolfe *et al.*, 1999).

The specialised uncoupling proteins (UCP) present in some mitochondria, most likely used for transporting anionic fatty acids, may create proton permeability by allowing H⁺ pumped out of the mitochondria by electron transport to re-enter (Groff and Gropper, 2000; Hinkle, 2005). However, they are not seen as affecting mechanistic P/O ratios, at least in liver and heart (Hinkle, 2005). In summary, corrections to the

cellular P/O ratios on account of uncoupling, including slip, proton leakage and uncoupling proteins appear to be small or of limited relevance (Hinkle, 2005), although there remains a need to confirm the extent of proton leakage *in vivo*.

2. Catabolism of absorbed substrates

2.1 Catabolism of carbohydrates

Catabolism of available glucose proceeds via aerobic glycolysis using either the glutamate-aspartate shuttle (the dominant shuttle system in the liver), or the more costly glycerol phosphate (α GP) shuttle (Houston, 2006). Each glucose molecule catabolised uses 2 ATP and generates 4 ATP and 2 NADH (Stryer *et al.*, 2002). Two pyruvate are also produced and each enters the tricarboxylic acid (TCA) cycle to yield 4 NADH, 1 FADH₂ and 1 GTP (Stryer *et al.*, 2002). Excess glucose is stored by conversion to glycogen (requiring 2 ATP for each mol of glucose converted) and stored primarily in the liver and muscle (Flatt, 1995b).

The heats of combustion (routinely measured by bomb calorimetry) and the ATP and NADH produced in the cytoplasm and mitochondria and the mitochondrial FADH₂ produced during oxidation are the same for glucose, fructose and galactose (Livesey, 1984).

It is critical that glucose availability in the body is maintained at all times to supply the nervous system and prevent hypoglycaemia. The body's glycogen stores in the liver (\approx 120 g) and muscle (\approx 80 – 380 g) are rapidly replenished after a meal of carbohydrate to help ensure a constant supply of glucose (\geq 5 g glucose / h) (Flatt, 1995b). Glycogen stores are limited to around 200 – 500 g depending on body size and CHO intake (Flatt, 1995b). It is estimated that one quarter to one third of ingested CHO is converted to glycogen in the liver, one third to one half to glycogen in the muscle and the remainder (approximately one third) oxidised during postprandial hours (Flatt, 1995b). If dietary intake fails to adequately replenish glycogen stores the oxidation of glucose decreases rapidly (Flatt, 1995a). A decrease in blood glucose levels initiates the release of

glucagon by the liver, which in turn activates phosphorylase to degrade glycogen to release glucose and increases gluconeogenesis from amino acids and lactate (DeFronzo and Ferrannini, 1987; McGarry *et al.*, 1987). Once blood glucose levels are sufficiently high, insulin signals the liver to restrain the release of hepatic glucose (Cahill, 1971).

There is a common misconception that carbohydrates are readily turned to fat and that the high cost of this process (lipogenesis) is responsible for the weight reduction observed on high carbohydrate diets (Flatt, 1995b). The consumption of carbohydrate does, however, reduce the need to use fat as a fuel because after consumption of a meal containing both carbohydrate and fat, the body has a preference to use carbohydrate as a fuel. As such, carbohydrate intake influences the rate of fat oxidation, although fat intake does not influence the rate of carbohydrate oxidation (Flatt *et al.*, 1985).

2.2 Catabolism of fats

Each TAG yields three fatty acids (FA) and one molecule of glycerol when completely hydrolysed (van Milgen, 2002). The catabolism of FA occurs via β -oxidation in the mitochondrion and ATP yields are dependent on: (i) the number of C atoms in the FA (ii) the number of double bonds in the FA, and (iii) whether the number of C atoms is odd or even (van Milgen, 2002). Fatty acids are iteratively reduced through consecutive cycles of β -oxidation. Each cycle produces 1 mole each of NADH, FADH₂ and acetyl-CoA and shortens the C chain by two C atoms (Stryer *et al.*, 2002). For every acetyl-CoA generated through β -oxidation a further 3 NADH, 1 FADH₂ and 1 GTP are yielded via the TCA cycle (Brand, 2005). Fatty acids containing an even number of C atoms produce one extra acetyl-CoA during β -oxidation than those FA of an odd C chain length (van Milgen, 2002).

Unsaturated FA must first be converted into suitable intermediate forms before undergoing β -oxidation. This occurs by the action of either an isomerase (for odd numbered double bonds) or a reductase and an isomerase (for even numbered double bonds) (Roskoski and Roskoski, 1996). The details of these pathways will not be discussed here, but in summary the result is 1 less FADH₂ being generated for each

double bond present and in addition, 1 less NADPH being generated for each even numbered double bond (van Milgen, 2002).

Glycerol accounts for approximately 5% of the energy content of TAG (Flatt, 1995b). Catabolism of glycerol proceeds via one of two pathways which vary in ATP yields. The primary pathway produces acetyl-CoA, which then enters the TCA cycle (van Milgen, 2002). The secondary pathway proceeds via gluconeogenesis (van Milgen, 2002) and under normal physiological conditions (i.e. not in a state of starvation) the contribution of glycerol to gluconeogenesis is relatively minor compared to the other major precursors of gluconeogenesis (alanine, glutamine and lactate). Even after a 17 h fast, only around 10% of the total glucose was produced from glycerol as a result of gluconeogenesis in both lean and obese humans (Chevalier *et al.*, 2006). In fasted (16 h - 18 h) dogs, glycerol was estimated to be responsible for only 5% of total carbon used for gluconeogenesis (Rolfe and Brown, 1997).

Spontaneous mechanisms exist in humans to effectively balance the intake of protein and carbohydrate against the oxidation of these fuels by the body, but the same is not true of fat, possibly because of the important functional role of amino acids and to ensure a constant supply of glucose to the brain (Flatt, 1995a). Once fat has been stored in adipose tissue, increased fat oxidation is the only means of reducing body fat stores (except for liposuction) (Flatt, 2001). The amount of fat oxidation is limited to the difference between total energy expenditure and the energy intake from other fuels (carbohydrate + protein + alcohol), rather than the amount of fat consumed on a given day (Flatt, 1991). Whilst carbohydrate intake induces carbohydrate oxidation and protein intake increases amino acid oxidation (Garlick *et al.*, 1980), the intake of fat only marginally increases the postprandial rate of fat oxidation (Flatt, 1995b). This is thought to be due to the slow rate of absorption of fat from the digestive tract and the fact that the fat in chylomicrons is targeted for storage in adipose tissue so that only small amounts of free fatty acids reach other cells (Flatt, 1995b). The result is that even after consumption of a high fat meal, the rate of fat oxidation does not increase markedly (Flatt, 1995b). Furthermore, when carbohydrate intake is high, carbohydrate oxidation is increased to limit excessive glycogen stores, thereby reducing fat oxidation

and increasing fat storage to an even greater extent (Flatt, 1988, 1993). As such, reduced carbohydrate intake, coupled with moderate fat intake, is essential to induce a rapid rate of fat oxidation (Flatt, 2001).

2.3 Catabolism of protein

The liver is primarily responsible for dietary amino acid (AA) metabolism (50 - 60% of absorbed amino acids), with other tissues and organs (skeletal muscle, neural tissue, kidney tissue) also contributing on a smaller scale (Gropper *et al.*, 2005). Specifically, the skeletal muscle is the major site of catabolism for dietary branched chain amino acids (Shimomura *et al.*, 2006). The majority (57%) of amino acids arriving at the liver are subsequently catabolised there (Gropper *et al.*, 2005). The remainder are either released into the systemic circulation (23%) or are used for the synthesis of proteins and nitrogen-containing compounds (20%) (Berthold *et al.*, 1995). The AA catabolised by the liver, may either be oxidised (90%) (Lindsay, 1976), or in the case of glucogenic AA, used for gluconeogenesis, producing 0.5 mol glucose per AA in most cases (van Milgen, 2002). In the case of the oxidative pathway, the ATP yield varies depending on the AA oxidised, and in the case of the gluconeogenic pathway, the ATP required to produce glucose also varies considerably depending on the AA (van Milgen, 2002). Although Livesey (1984) suggests that the choice of catabolic pathway may be an important consideration for ATP yields from dietary protein, calculations and model simulations at the whole animal level by Birkett and de Lange (2001a) showed little difference in overall ATP yields for the two catabolic pathways for AA (providing glucose is non-limiting).

The removal of the amino group, either by transamination or deamination is the first step in amino acid catabolism (Gropper *et al.*, 2008). Transamination transfers the amino group to an amino acid carbon skeleton or an amino acid without an amino group (α -keto acid), while deamination simply removes the amino group, with no direct transfer involved (Gropper *et al.*, 2008). Deamination reactions produce ammonia that is disposed of through the urea cycle (Hinwood, 1992). The urea cycle takes place in the liver and consumes 4 ATP for each molecule of urea produced from one molecule of ammonia (Blaxter, 1989). Urea is typically excreted as a component

of urine and constitutes around 80% of total urinary nitrogen (Hawk, 1965). However, it is estimated that about 25% of urea is recycled in that it is released into the intestinal lumen and subsequently degraded by bacteria to ammonia (Groff and Gropper, 2000; Moran and Jackson, 1990; Walser and Bodenlos, 1959). The carbon skeleton remaining from amino acid catabolism can be used for energy directly or converted to glucose (in the case of glucogenic amino acids) or ketone bodies (in the case of ketogenic amino acids) (D'Mello, 2003).

The body is able to maintain constant protein levels and this is independent of the ratio of fat to carbohydrate in the diet. Even small changes in the body's protein content can cause a significant adjustment of AA oxidation rates to protein intake (Flatt, 1995a).

2.4 Catabolism of alcohol

There are two major pathways for the metabolism of ethanol (**Figure 3**): the traditionally described alcohol dehydrogenase (ADH) pathway and the microsomal ethanol oxidising system (MEOS) pathway (Buemann and Astrup, 2001; Lands and Zakhari, 1991).

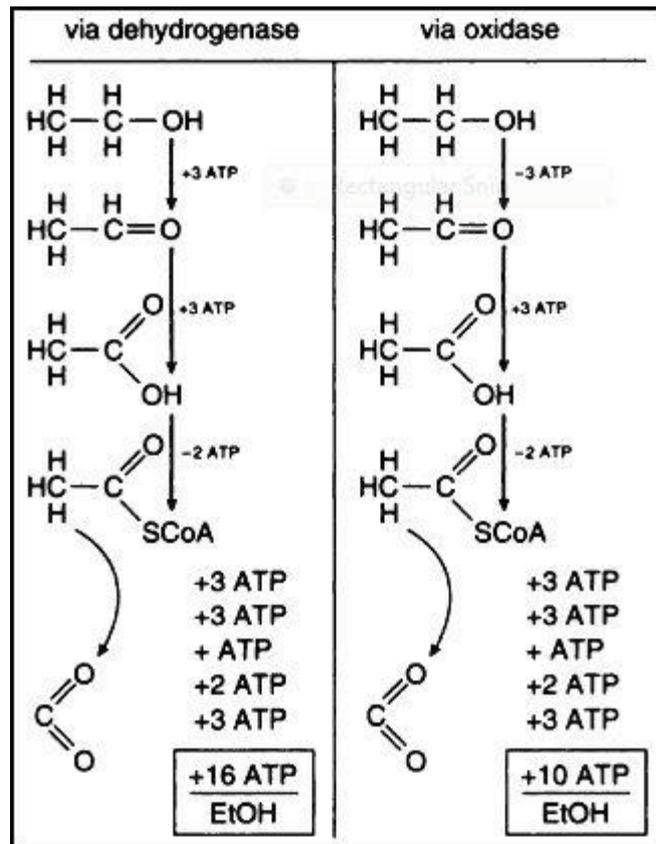


Figure 3. Alcohol metabolism pathways. From Lands and Zakhari (1991).

The ADH pathway is considered the most important for individuals with moderate ethanol intake, whilst the MEOS pathway may be induced in chronic alcoholics (Bamworth, 2005; Lieber, 1988).

Upon arrival at the liver, ethanol is first degraded to acetaldehyde (Gropper *et al.*, 2008). In the case of the ADH pathway, this step results in the production of 1 NADH (a gain of 3 ATP) and in the case of the MEOS pathway it is at a cost of 3 ATP due to the reduction of NADPH to NADH⁺ (Buemann and Astrup, 2001; Lands and Zakhari, 1991). Further details of the processes involved in the consumption of these 3 ATP by the MEOS pathway are given by Lieber (1988). Overall, there is a net loss of 6 ATP for the MEOS pathway relative to the ADH pathway (Lands and Zakhari, 1991; Lieber, 1988).

After ethanol is degraded to acetaldehyde (via the ADH or MEOS pathway), the remainder of the metabolism of ethanol continues as follows: acetaldehyde is degraded to acetate, producing 1 NADH (a gain of 3 ATP), which then enters normal metabolic pools, largely outside the liver, and is activated to acetyl-CoA (at a cost of 2 ATP), which enters the TCA cycle producing 3 NADH (9 ATP), 1 FADH₂ (2 ATP) and 1 GTP (1 ATP) (Bamworth, 2005; Groff and Gropper, 2000; Lands and Zakhari, 1991).

Lands and Zakhari (1991) proposed an unregulated futile cycle involving the first step of ethanol oxidation and capable of producing an abnormally high degree of thermogenesis (**Figure 4**) (Buemann and Astrup, 2001). They suggest that the 3 ATP consumed during the irreversible oxidation of ethanol to acetaldehyde by MEOS and the subsequent reduction of acetaldehyde to ethanol (also consuming 3 ATP) by ADH would result in a net loss of energy since the energy consumed by two to three futile cycles would be more than the ATP gained from the ingested ethanol (Lands and Zakhari, 1991).

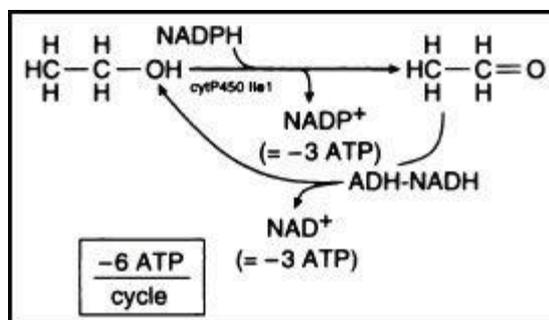


Figure 4. A futile cycle during ethanol oxidation. From Lands and Zakhari (1991).

The significance of such a futile cycle has been questioned at moderate intakes of alcohol when MEOS is the minor pathway for ethanol oxidation (Lieber, 1991), but may play a role in individuals with high alcohol intakes (Lieber and DeCarli, 1968), who may also be prone to lower cellular energy efficiency owing to mitochondrial damage (Lands, 1995).

The intake of alcohol also influences the oxidation of other fuel sources. Approximately 80% of the energy consumed as alcohol is used for ATP resynthesis and

this has the effect of reducing fat oxidation, but not carbohydrate oxidation (Sonko *et al.*, 1994; Suter *et al.*, 1992).

2.5 Catabolism of SCFA produced from hindgut fermentation

Propionic acid is transported to the liver where it may be converted to acetyl-CoA (minor pathway) or glucose (major pathway) (McDonald *et al.*, 1995). Acetic acid and butyric acid are both rapidly converted to acetyl-CoA (Birkett and de Lange, 2001a). Acetic acid is largely metabolised by the liver (50 – 70%) (Hijova and Chmelarova, 2007), where it is used in the synthesis of long chain fatty acids, glutamate, glutamine and hydroxybutyrate (Reilly and Rombeau, 1993). Acetic acid may also be utilised by the peripheral tissues (Pomare *et al.*, 1985), converted to butyric acid by colonic bacteria (Duncan *et al.*, 2002) or used for energy by the muscles (Hijova and Chmelarova, 2007). Butyric acid is the preferential energy source for colonocytes and is largely (70 – 90% of butyric acid absorbed) metabolised by the colonic epithelium (Basson *et al.*, 2000; Della Ragione *et al.*, 2001; Zoran *et al.*, 1997). Butyric acid is an important energy source for these cells, accounting for 60 - 70% of their maintenance energy needs (Cummings, 1996; Smith and German, 1995). Residual butyric acid is transported via the portal vein to the liver for hepatic gluconeogenesis (Cummings, 1987; Hijova and Chmelarova, 2007).

3. Composition of fuel mix oxidised

Humans consume diets that are composed of widely varying mixes of fuels and the body is able, within limits (protein \geq 10% and fatty acids \geq 1% of energy supply), to accommodate these differences (Flatt, 1995a). This is possible because ATP can be regenerated from the metabolic intermediates of all three classes of macronutrients in an interchangeable manner, but only in such a way as to minimise changes in body protein content and maintain the concentration of glycogen within certain limits (Abbott *et al.*, 1988; Flatt, 1995a). Between meals and bouts of physical exertion the mix of fuel oxidised is determined by the size of the body's protein pools and adipose tissue fat reserves and the degree of repletion of glycogen stores (Flatt, 1995a) and is

largely controlled by substrate and hormonal changes in the body's circulatory system (Cahill, 1971; Flatt and Blackburn, 1974). The intake of each fuel source may or may not influence the rate of oxidation of the other fuel sources. This is evidenced by the influence of alcohol and carbohydrate intake on fat oxidation and the lack of effect of both fat intake on carbohydrate oxidation and carbohydrate and fat intake on amino acid oxidation (Flatt, 1995b; Flatt *et al.*, 1985; Suter *et al.*, 1992).

4. Relation of dietary intake to body weight maintenance and obesity

It is only when the mix of fuels consumed in the diet is equal to the mix of fuels used by the body that weight maintenance is achieved (Flatt, 2001). This requires storing most of the absorbed glucose and fat for later use, achieved via glycogen storage in the liver and muscle (4.2 kJ/g) and fat storage in the adipose tissue (33.5 kJ/g) (Flatt, 1995b). Although the body normally spontaneously operates below maximal glycogen storage levels, the maximum storage capacity is relatively limited, unlike body fat stores which have a virtually unlimited capacity, some 50 - 200 times larger than glycogen stores (Flatt, 1995a, 1995b). As such, whilst changes in glycogen stores are unlikely to have a noticeable effect on body weight, the same is not true for changes in body fat (Flatt, 1995b). When fat stores become excessive an individual may become overweight (BMI of $\geq 25 \text{ kg/m}^2$) or obese (BMI of $\geq 30 \text{ kg/m}^2$).

The reasons for the high prevalence of obesity in affluent societies in recent times are not well understood (Heitman and Garby, 1999), but may be at least partly attributed to a reduction in physical activity, ready access to foods high in fat, the tendency to consume foods prepared outside the home and other factors relating to the palatability of foods consumed and the dietary variety consumed within food groups (Flatt, 1995a; Hill *et al.*, 2000; Lissner and Heitmann, 1995; McCrory *et al.*, 2000). The role of dietary fat intake in the incidence of obesity has been challenged (McCrory *et al.*, 2000). Evidence suggests that the weight losses of 2 – 4 kg elicited by *ab libitum* low-fat diets are too small for dietary fat intake alone to be the major cause of obesity

(Willett, 1998). Furthermore, epidemiological studies show only a weak correlation between dietary fat intake and the degree of adiposity in humans and dietary fat intake has remained essentially stable over a period of time when the rate of obesity has risen dramatically (Dreon *et al.*, 1988; Drewnowski *et al.*, 1992; Miller *et al.*, 1990; Romieu *et al.*, 1988). It was once thought that obese individuals had unusually low rates of basal energy expenditure. This theory too has been dismissed because it is well known that BMR is highly correlated with lean body mass, which is higher in obese subjects because fat deposition is accompanied by an increase in lean body mass (Prentice *et al.*, 1986; Schoeller and Fjeld, 1991). Obese subjects therefore have increased, not decreased, basal energy requirements (Flatt, 1995a).

Physical activity appears effective in maintaining or reducing body weight and is more effective in inducing fat oxidation than carbohydrate (glucose) oxidation (Bouchard *et al.*, 1993; Flatt, 1988, 1993). Exercise increases substrate oxidation in muscle and this uses both fatty acids and glucose (Flatt, 1995b). During exercise muscle glycogen is the primary fuel source for muscles, which has an effect on substrate oxidation not only during, but also after, the period of exercise due to a depletion in body glycogen stores (Bouchard *et al.*, 1993). Fatty acids are the primary energy source in muscles during the postabsorptive period (Elia, 1995a).

Despite the effects of physical exercise, the adjustment of food intake remains the primary means of maintaining a constant body weight (Flatt, 1987, 1993). There exists therefore, an opportunity to reduce, or at least minimise, the prevalence of obesity through manipulation of the diet.

VII. Predicting nutrient uptake

1. Digestibility

Despite the high efficiency of the human digestive system, some food escapes digestion in the upper-tract and also in some cases, fermentation in the hindgut. The degree to which ingested food is absorbed (digestibility) is indirectly calculated as the difference between intake and losses in the digestive system, as expressed as a fraction of intake (Livesey, 1990). Digestibility may be expressed as either a percentage of intake or as a coefficient of digestibility. For example, a digestibility of 90% is analogous to a coefficient of digestibility of 0.9 and implies that 10% of ingested food was unabsorbed (i.e. 90% of material disappeared during transit). The prediction of the digestibility of energy for a given food is of paramount importance because if a nutrient is not absorbed, it cannot be transported to the site of metabolism and used to generate ATP. The WHO have stated that the accurate determination of digestibility of a food is one of the key areas for better defining the available energy (FAO/WHO/UNU, 2001).

1.1 Terminology

1.1.1 *Faecal and ileal digestibility*

In the past, it was common to determine digestibility at the faecal level (faecal digestibility or total tract digestibility) by subtracting the energy found in the faeces from that in the ingested food. This approach has a number of shortcomings, particularly in the case of the absorption of AA. Firstly, faecal digestibility fails to separate the uptake of energy in the upper-tract and the hindgut, which is pertinent because the energy (per unit food component) liberated by enzymic digestion in the upper-tract is much greater than the energy from SCFA absorbed in the hindgut. For example, the NME of available CHO as monosaccharide is 15.7 kJ/g, whilst the value is only 6.2 kJ/g for UC in mixed diets (Warwick, 2005). Secondly, the hindgut is an active site of microbial N metabolism where the products of protein digestion are fermented

by bacteria and *de novo* synthesis of microbial protein occurs (Low, 1980). This affects not only the quantity of N appearing in the faeces through increased biomass, but also alters the faecal AA profile (Fuller and Tomé, 2005). Finally, the composition of the diet may affect the degree of endogenous losses in both the upper-tract and the hindgut (refer Section VII, 1.1.2). Diets containing UC consumed at moderate levels cause faecal energy losses as fat and protein in amounts in excess of what would be expected solely from the fermentation of the UC (Livesey, 1992). For these reasons, there is consensus that protein digestibility should be measured from the mouth to the terminal ileum (i.e. ileal digestibility) (Darragh and Hodgkinson, 2000; Fuller and Tomé, 2005; Mosenthin, 2002; Moughan, 2003; Sauer and Ozimek, 1986). Faecal carbohydrate, unlike protein and fat, is mostly of dietary origin. With the exception of unavailable CHO, most nutrients are primarily absorbed in the upper-tract, so it follows that the digestibility of energy for all such nutrients (protein, fat, digestible starch and sugars) should be determined up until the terminal ileum in order to accurately predict energy absorption in the upper-tract (Birkett and de Lange, 2001a; Jørgensen *et al.*, 1992). To predict uptake across the entire digestive tract (i.e. total available energy) there is also a need to separately predict the uptake of energy in the large intestine, ideally determined according to the absorption of SCFA by the hindgut.

1.1.2 *Apparent and true digestibility*

Ileal effluent and faeces both contain not only undigested dietary material, but also protein and fat of endogenous and/or microbial origin. The contribution of biomass to faecal dry matter is approximately 55% for typical Western diets (Stephen and Cummings, 1980b). Microbial N and fat make up to 60 % and 70% of faecal N and fat in humans, respectively (Stephen and Cummings, 1980b).

Endogenous losses into the gut can be separated into basal losses and specific losses. Basal (minimal losses) refer to those losses that are inevitably lost regardless of the diet consumed and specific losses are those that are affected by dietary factors (Darragh and Hodgkinson, 2000; Nyachoti *et al.*, 1997; Stein *et al.*, 2007). When apparent digestibility values are corrected for basal and specific endogenous losses,

'true digestibility' (sometimes also called 'real digestibility') values are obtained (de Lange *et al.*, 1990; Mariotti *et al.*, 2001). Additionally, the correction of apparent digestibility values to account only for basal endogenous losses may be referred to as 'standardised digestibility' (Fuller and Tomé, 2005; Stein *et al.*, 2007). Expressing digestibility as true digestibility allows endogenous losses of the nutrient to be taken into account (Mosenthin, 2002). However, because specific endogenous losses do not exist independent of the diet in either the upper-tract (Bartelt *et al.*, 1999; Boisen and Moughan, 1996; Duran-Montge *et al.*, 2007; Libao-Mercado *et al.*, 2006) or the hindgut (Wang *et al.*, 2002), diet composition is an important variable in determining true digestibility.

1.2 Typical digestibility values and nutrient interactions

Livesey (1990) reviewed apparent faecal digestibilities of gross energy from a wide range of human diets, as found in the literature (Calloway and Kretsch, 1978; Farrell *et al.*, 1978; Göransson and Forsum, 1987; Göransson *et al.*, 1983; Judd, 1982; Kelsay *et al.*, 1978; Southgate and Durnin, 1970; Stevens *et al.*, 1987; Wisker *et al.*, 1988). Digestibility coefficients ranged from 0.82 for barley (Judd, 1982) to 0.97 for a fibre-free egg protein diet (Calloway and Kretsch, 1978). Extrapolating the data for zero UC gave a mean apparent digestibility of gross energy of 0.978 (Livesey, 1990). Furthermore, UC (quantity and source) was found to be a key determinant for the apparent faecal digestibility of dietary energy (Livesey, 1990).

It is well known that the presence of UC may reduce the apparent digestibility of energy, protein and fat at the ileal and faecal level (Baer *et al.*, 1997; Calloway and Kretsch, 1978; Farrell *et al.*, 1978; Kelsay *et al.*, 1978; Livesey, 1995; Southgate and Durnin, 1970). The majority of the increase in energy present in faeces with higher levels of UC intake is due to the increase in faecal protein, and less so, fat from the increased faecal excretion of biomass from the fermentation of fermentable UC (Livesey, 1990, 1995), rather than from increased endogenous losses in the upper-tract (Livesey, 1990, 1991, 1992). Although the presence of UC may increase endogenous protein losses from the ileum, this phenomenon should not be assumed to be present

across all diets (Livesey *et al.*, 1995b). The decrease in apparent ileal digestibility of protein upon addition of various sources of UC (e.g. oat bran, cassava leaf meal, sugar beet pulp and wheat bran) has also been well documented in pigs (Bach Knudsen *et al.*, 1993; Graham *et al.*, 1986; Phuc and Lindberg, 2000; Wang *et al.*, 2002). However, the application of these observations to humans should be made with care due to the higher level of UC found in animal feedstuffs (Deglaire, 2008).

Livesey (1990) also reported apparent faecal N coefficients of digestibility of between 0.69 – 0.93 in his review. Although protein digestibility varies and is largely complicated by the fact that there are endogenous and microbial protein losses from the gut, dietary N in human diets is usually absorbed at levels exceeding 70% (FAO/WHO, 1991).

Differences in the faecal digestibility of fat in humans were observed by Atwater more than a century ago (Atwater and Bryant, 1900; Merrill and Watt, 1973). Faecal fat losses are small compared to faecal protein losses, and depend on the amount (Apgar *et al.*, 1987; Kasper, 1970) and sometimes the type of ingested fat (Chen *et al.*, 1987; De Schrijver *et al.*, 1992). In contrast to protein digestibility, the effect of DF on apparent fat digestibility seems to be related to an interference with the digestion and absorption of dietary fat in the upper-tract, rather than to the fermentation of DF (Vahouny and Cassidy, 1987). Fatty acids entering the hindgut are readily hydrolysed by microbial action in pigs, indicating that faecal fat is largely of microbial origin (Bayley and Lewis, 1965; Jørgensen *et al.*, 1992). This is supported by studies in pigs where the ileal digestibility and faecal digestibility of fat was observed to be similar (Jørgensen *et al.*, 2000; Jørgensen *et al.*, 1992). Saturated fatty acids may have a lower ileal digestibility than unsaturated fatty acids (Jørgensen *et al.*, 1992, 1993; Øverland *et al.*, 1994). The dietary fat level, the fatty acid chain length, interactions among fatty acids, the source of the fat (which influences the extent to which the TAG are hydrolysed) and the level of endogenous fatty acids all influence the apparent digestibility of fatty acids (Jørgensen *et al.*, 2000). These factors must be taken into account when using apparent, as opposed to true digestibilities, particularly at low levels of fat intake where the proportional effect of endogenous fatty acids present in

ileal digesta will have a larger effect (Jørgensen *et al.*, 1992, 1993). The dietary source of fat and dietary fat level has been observed to influence the ileal and faecal digestibility of protein. Li and Sauer (1994) recorded a significant increase in apparent ileal AA digestibilities upon increasing the level of canola oil in the diet. Similar effects were seen on the apparent faecal digestibility of protein when growing pigs were fed diets containing 4, 8 and 16% rapeseed oil (Jørgensen *et al.*, 1996a).

Resistant starch by definition is indigestible in the upper-tract and has been observed to have a large intestinal digestibility ranging from 30 – 70% (Behall and Howe, 1995, 1996; Cummings *et al.*, 1996; Ranhotra *et al.*, 1991; Ranhotra *et al.*, 1996). Digestible starch has a total tract digestibility of close to 100%, with the majority of digestion (96 - 100%) occurring prior to the terminal ileum for most ingredients (Bakker, 1996; Jørgensen *et al.*, 2000; Jørgensen *et al.*, 1996b). Lower ileal starch digestibilities have been observed for some ingredients, but never less than 95% (Bakker, 1996; Jørgensen *et al.*, 1996b). The ileal digestibility of sugars is generally also close to 100%, and all sugars are completely digested over the total tract (Jørgensen *et al.*, 2000; Livesey, 1990; Noblet *et al.*, 1989; Noblet *et al.*, 1994; Noblet and Perez, 1993). Starch and sugars have no effect on the digestibility of other nutrients (Jørgensen *et al.*, 1996b).

From the above discussion, it is clear that the digestibility of energy in human diets differs widely and is influenced by the specific interactions between nutrients for a particular diet. It is not possible, therefore, to rely on a diet's determined chemical composition data and generic digestibility factors if an accurate assessment of digestibility is required. Each diet needs to be experimentally examined to predict the actual digestibility *in vivo*. The following section deals with experimental methods that are intended to fulfil this purpose.

2. Methods to predict ileal digestibility

The classic means of determining digestibility in humans is through human balance studies, whereby absorbed energy is considered to be the difference in ingested energy and that excreted in the faeces (and sometimes urine, i.e. total energy

balance). Balance studies are usually conducted over a number of days or weeks under controlled conditions. The data obtained have limited application, however, due to the shortcomings of apparent faecal digestibility data, as already discussed. For the determination of ileal digestibility, which is considered to be a more accurate representation of digestibility for some nutrients, intestinal fluid (ileal digesta) is collected from the terminal ileum. Ileal digesta can be collected directly from conscious humans, either via naso-intestinal intubation or from subjects (ileostomates) fitted with an ileostomy bag, or alternatively, from an animal considered to be a valid model for human digestion. Each of these approaches is discussed further in the remainder of this chapter. *In vitro* upper-tract digestibility assays (which use a series of digestive enzymes to mimic digestion) (Boisen and Eggum, 1991; Boisen and Fernández, 1995, 1997; Lebet *et al.*, 1998) will not be discussed as part of this review because such assays are considered unlikely to be representative of *in vivo* conditions over the wide range of human diets consumed and have a number of shortcomings (e.g. the lack of endogenous material).

2.1 Ileal digesta collection methods in humans

2.1.1 Ileostomy studies

Ileal digesta is commonly sampled from adult human subjects who have had the entire large intestine surgically removed (total colectomy) for medical reasons (usually ulcerative colitis) and have been fitted with a plastic stoma bag attached to the outer abdominal wall (Moughan *et al.*, 2005). In these subjects, effluent from the upper GI tract empties via a stoma into the ostomy bag, thus allowing total collection of ileal digesta (Moughan *et al.*, 2005). An important consideration when using ileostomates is that, ideally, the surgery to remove the large intestine should have been performed recently, due to rapid microbial colonisation around the terminal ileum (Dowsett *et al.*, 1990). The total microbial count in ileal effluent may be up to eighty times that of ileal effluent in 'intact' subjects (Gorbach *et al.*, 1967). Administering a 2-day course of antibiotics prior to the study period may help minimise bacterial growth post-surgery but appears to have minimal effect on ileal DM and AA flow (Fuller *et al.*, 1994). Other questions surrounding the true physiology of ileostomised subjects have been raised,

such as the effect of the disease for which the colectomy was prescribed on the function of the digestive tract, and the effect of the colectomy on other physiological and metabolic processes (Christl and Scheppach, 1997).

The use of human ileostomates for routine digestibility studies is largely impractical, is financially and logistically demanding and requires many willing subjects to consume diets which are often less palatable than regular human diets. Furthermore, the use of human ileostomates for such studies is becoming less acceptable from an ethical perspective (Rowan *et al.*, 1994). Despite its limitations, the ileostomy method has been used in a large number of human *in vivo* digestibility studies (Deglaire *et al.*, 2009; Fuller *et al.*, 1994; Holloway and Tasman-Jones, 1978; Holloway *et al.*, 1983; McBurney and Thompson, 1989; Moughan *et al.*, 2005; Rowan *et al.*, 1994; Sandberg *et al.*, 1981; Sandstrom *et al.*, 1986).

2.1.2 *Naso-intestinal intubation*

Unlike ileostomy studies, the naso-intestinal intubation method (or the naso-ileal intubation method) (Mahé *et al.*, 1992; Modigliani *et al.*, 1973; Schedl and Clifton, 1961) allows ileal digesta to be sampled from 'intact' human subjects possessing a normal and complete digestive tract. The method involves inserting a triple-lumen polyvinyl chloride (PVC) tube (**Figure 5**) through the nose of conscious subjects. The tube progressively travels along the digestive tract to the terminal ileum with the assistance of peristaltic movements and a terminal inflatable balloon containing mercury. Ileal digesta is collected in a continuous manner, usually for 8 h postprandially to allow for complete passage of the food, by siphoning or slight aspiration through the distal opening of the tube (Deglaire, 2008).

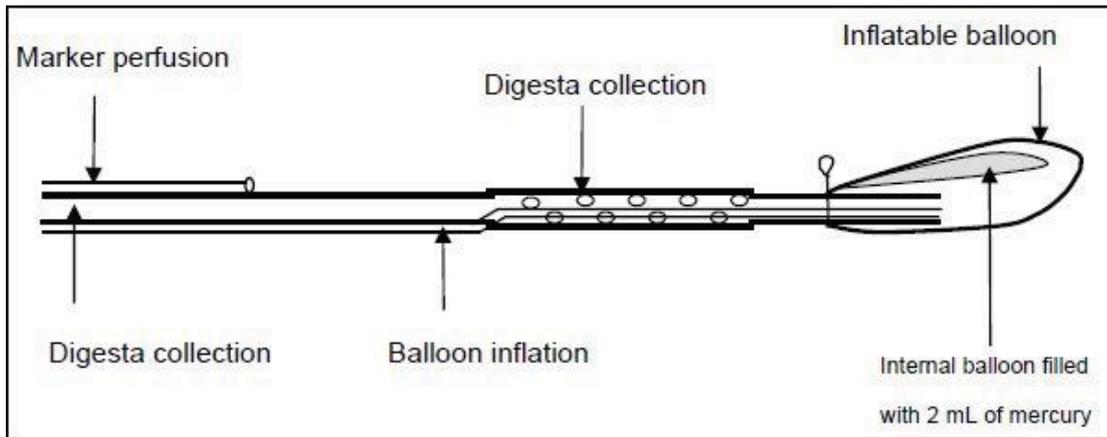


Figure 5. Collection of ileal digestion in humans using naso-intestinal intubation. From Deglaire (2008).

To predict digestibility, a non-absorbable marker (i.e. PEG-4000) is added to the ingested food and analysed for in digesta sampled through the first tube lumen. A second non-absorbable marker (i.e. phenolsulphonphthalein, PSP) is continuously infused above the site of sampling and simultaneously sampled (i.e. at the same time as ileal digesta through the first tube lumen) through the second tube lumen to determine the intestinal fluid flow rate. The third tube lumen is used to inflate the terminal balloon to aid progression of the tube to the terminal ileum once it has reached the stomach.

The method has not yet been fully validated using controlled studies, partly because it is difficult to externally validate the method due to the lack of a suitable external control (Deglaire, 2008). Although the amount of resistant starch in ileal digesta sampled from ileostomates has been compared with that sampled using the intubation technique (Langkilde *et al.*, 1994), the higher quantities of starch (22%) in the ileostomates compared to intubated subjects may have arisen from a higher intestinal flow rate with the intubation method and/or a greater degree of starch degradation with the ileostomy study (Champ *et al.*, 2003). As such, it is uncertain if the difference in recovery rates was a result of an underestimation of the ileostomy method or an overestimation of the intubation method, or both. Noah *et al.* (1998) compared resistant starch in ileal digesta from T-cannulated pigs and intubated pigs, with the

tube inserted 40 cm proximal to the ileo-caecal valve. Lower quantities of resistant starch were seen in the ileal digesta sampled from the intubated pigs, although qualitatively, the makeup of the ileal digesta was comparable (Noah *et al.*, 1998).

A critical assumption is that a representative sample of ileal digesta is obtained with the intubation technique. Deglaire (2008) conducted a review of data obtained from five separate naso-intestinal intubation studies ($n = 75$ adults, 9 protein-based test meals) conducted by the same laboratory over a period of more than 4 years, using the same methodologies, but different experimenters. Based on estimated flow rates of the PEG marker it was found that a large portion of the ileal digesta was captured by the sampling tube (>50%) and that intestinal fluid flow rates were accurate (Deglaire, 2008). The method was also found to be reproducible with a CV for integrated PEG flows among studies of 3% (Deglaire, 2008).

There are reports in the literature that intubation delays gastric emptying (Fone *et al.*, 1991; Medhus *et al.*, 1999), as well as evidence of no such effect (Longstreth *et al.*, 1975; Muller-Lissner *et al.*, 1982). Whilst it is uncertain what effect intubation may have on GI transit times, the transit rate of food has been shown to have no more than a minimal effect on overall protein digestibility (Gaudichon *et al.*, 1999; Huge *et al.*, 1995; Mariotti, 2000).

Overall, the method appears to be a valid means of determining digestibility in humans given that samples of ileal digesta are representative and accurate and ileal digestibility values are in line with those expected based on the general body of scientific knowledge (Gaudichon *et al.*, 1999; Mariotti, 2000; Mariotti *et al.*, 2001; Mariotti, 2002; Noah *et al.*, 1998). It does remain, however, for the method to be formally validated and as with ileostomy studies, the method requires a number of willing subjects, demands considerable resources and ethical considerations mean that it is unlikely to be suitable for the routine evaluation of foods. Other significant drawbacks include the fact that studies must be acute and only purified diets can be used.

2.2 Ileal collection from animal models

Studies with humans demand many financial resources, are complicated, labour intensive and require a large number of willing subjects. Given these limitations, digestibility studies using animal models, most commonly the rat or pig, are frequently used as an alternative (Livesey, 1990; Livesey *et al.*, 1995a). The rat, the pig and man are all simple-stomached omnivores with similar gastrointestinal anatomy and physiology (Kararli, 1995; Moughan and Rowan, 1989), including a small intestinal transit time of 3 - 4 h (DeSesso and Jacobson, 2001; Wilfart *et al.*, 2007). These similarities allow for the two animal species to be used as models for digestibility studies with a wide range of human diets, including single foods as the sole source of nutrition.

2.2.1 *The growing pig*

Compared to man, there are remarkably close similarities in the digestive system and accompanying metabolic processes of the pig - more so than for any other non-primate species (Patterson *et al.*, 2008). The growing pig, a meal-eating omnivore, has been proposed as a suitable model for human digestibility studies and is widely used for this purpose, particularly in relation to protein digestibility (Bergen, 2007; Darragh and Hodgkinson, 2000; Moughan, 2005; Moughan *et al.*, 1992; Moughan *et al.*, 1994). Although the length of the pig intestines (both small and large) is considerably greater than for humans, on a length per kg bodyweight basis, both species measure approximately 0.1 m intestine / kg body weight (Emmans and Kyriazakis, 1999).

Faecal gross energy digestibility coefficients for the growing pig (0.94) and man (0.93) were observed to be in good agreement for a meat-vegetable-cereal-dairy product based diet (Rowan *et al.*, 1994). Apparent faecal N and AA digestibility between piglets (97.5%) and infants fed milk formula (94.5%) was also comparable (Darragh and Moughan, 1995). Similarly, Forsum *et al.* (1981) found good agreement in N digestibility at the faecal level between the species for vegetable and vegetable-animal protein diets.

True ileal digestibility coefficients have also been reported to be similar for most AA in studies comparing human ileostomates and ileostomised pigs (Rowan *et al.*, 1994). The correlation ($r = 0.83$) between true ileal AA digestibility using ileal digesta sampled from humans through a naso-ileal tube and that collected from cannulated pigs was statistically significant ($P < 0.05$) for a number of mixed diets containing protein of vegetable or animal origin (Deglaire *et al.*, 2009). Indirect evidence across independent studies also showed close agreement between pig and human true ileal N and AA digestibility values when soya protein or casein was the test protein (Fuller and Tomé, 2005). In general, the use of the growing pig as a model animal for upper-tract protein digestibility in adult humans is widely accepted (Deglaire *et al.*, 2009; Moughan and Rowan, 1989; Rowan *et al.*, 1994).

2.2.2 *The growing rat*

The rat is the most commonly used animal model for studying digestibility in humans and rat balance studies have been recommended by the FAO for studying protein digestibility in man (FAO/WHO, 1991). The rat is also considered a suitable animal model for predicting the energy values of NSP isolates (British Nutrition Foundation, 1990; Livesey, 1990), a conclusion reached in response to favourable results from the study of Nyman *et al.* (1986).

Although the pig enjoys wide acceptance as an animal model due to the aforementioned anatomical and physiological similarities with man, the rat is also a useful, less labour intensive alternative (Rutherfurd and Moughan, 2003). The main advantages of the rat compared to the pig are the low cost, relatively simple research facilities and the lack of surgery and veterinary expertise required (Bach Knudsen *et al.*, 1994; Rutherfurd and Moughan, 2003). This allows large numbers of animals to be studied and the rapid availability of results. The method of collection of ileal digesta in the rat is straightforward and for this reason and others, the rat may be considered a more acceptable animal model than the pig.

The rat may practice coprophagy (as may the pig, albeit with less frequency) and this needs to be prevented to ensure valid data are obtained (Patterson *et al.*, 2008). The

primary digestive differences between humans and the rat relate to the hindgut. Comparative studies by Van Soest (1983) indicate that fermentation of DF in man is less extensive than in the pig, but more extensive than in the rat. Nyman *et al.* (1986) observed good agreement between rat and man for the apparent faecal digestibility of a range of DF sources (cabbage, wheat bran, apple, guar gum and carrot). However, these findings were in contrast to those seen by Bach Knudsen *et al.* (1994) who found poor correlation between the apparent faecal digestibility of NSP in rats and humans with mixed diets, and also questioned the methodology of the study by Nyman *et al.* (1986) in that there were important differences in the way the rat and human balance trials were undertaken. Comparative studies between the rat and man undertaken by Wisker *et al.* (1997; 1996) gave varied results. Poor inter-species agreement was seen for a low DF diet, a diet containing barley DF at high protein intakes and diets containing NSP from fruits and vegetables and citrus fibre (Wisker *et al.*, 1997; Wisker *et al.*, 1996). Conversely, good agreement was seen for diets containing NSP from barley DF at low protein intake and fine or wholemeal rye bread (Wisker *et al.*, 1997; Wisker *et al.*, 1996).

The apparent faecal digestibility of N has been observed to be some 14% higher in the rat than in man (Bodwell *et al.*, 1980) and Ritchey and Taper (1981) found a poor correlation ($r = 0.17$) between the apparent faecal digestibility of nitrogen in the rat and man. Similarly, Bach Knudsen *et al.* (1994) found a statistically significant ($P < 0.05$) difference in apparent protein digestibility across the species for two diets (high protein and low protein) containing barley DF. However, in the same set of studies, Bach Knudsen *et al.* (1994) found no statistically significant ($P > 0.05$) difference between the species in apparent protein digestibility for two other diets (one rich in fruits and vegetables and the other with added citrus fruit DF). Likewise, Rich *et al.* (1980) tested a wide variety of foods in both man and the rat and concluded that the degree of apparent protein digestibility is the same in both species. There may be more consistent agreement between the species after adjusting for endogenous losses. Deglaire (2008) concluded from a review of published rat digestibility studies (Bodwell *et al.*, 1980; Forsum *et al.*, 1981; Wisker *et al.*, 1996) that true N faecal digestibility values were on average only 3% higher in the rat than in humans.

Furthermore, Forsum *et al.* (1981) saw better agreement in the true N digestibility between man and the rat than between man and the pig for two mixed diets (vegetable or vegetable-animal proteins) and a diet with egg white protein. It is to be noted, however, that the three species studied did not receive identical diets and this may have influenced the outcomes of the study (Rowan *et al.*, 1994). Based on the studies of Forsum *et al.* (1981), there may also be differences in the way the rat digests protein compared to humans because there was better agreement for the vegetable-animal protein diet than the vegetable protein diet, which gave lower true N digestibility values in man than for the rat.

Faecal fat losses in both man and the rat are minimal and Bach Knudsen *et al.* (1994) found no difference in apparent faecal fat digestibility between the two species. Whilst higher fat intakes have been shown to increase faecal fat excretion in both humans and rats (Apgar *et al.*, 1987; Walker *et al.*, 1973), in most cases no effect on the apparent digestibility of fat has been observed (De Schrijver *et al.*, 1992; Walker *et al.*, 1973). The presence of DF may decrease the apparent faecal fat digestibility through increased faecal fat losses in both species, particularly for foods containing DF that may form viscous solutions (Mokady, 1973; Southgate and Durnin, 1970).

In general, the apparent faecal digestibility of energy appears comparable in both humans and the rat. Bach Knudsen *et al.* (1994) found that in most cases there is good agreement between the digestibility coefficients of energy in man and the rat: 0.941 - 0.950 in man versus 0.933 – 0.952 in the rat (low DF control diets) and 0.897 - 0.931 in man and 0.865 - 0.920 in the rat (high DF diets). Similarly, Wisker *et al.* (1996) saw comparable results between the species for the faecal digestibility of energy for a low fibre diet (94.7 vs. 95.0%) and diets containing fine (91.2 vs. 92.5%) or coarse (91.6 vs. 91.7%) wholemeal rye bread in man and the rat, respectively. A statistically significant ($P < 0.01$) difference in digestible energy between the two species was found for diets containing high levels of either fruits and vegetables (0.032 absolute units) or citrus fibre (0.025 absolute units) (Bach Knudsen *et al.*, 1994).

The use of the rat to predict ileal digestibility avoids issues associated with the interspecies differences in hindgut fermentation. Controlled comparative studies measuring ileal digestibility in rats and humans have not, to our knowledge, been undertaken. However, the growing pig is widely accepted as a valid model animal for upper-tract protein digestibility in adult humans (Deglaire *et al.*, 2009; Moughan and Rowan, 1989; Rowan *et al.*, 1994) and there is good agreement between ileal protein digestibility in the rat and the pig over a very wide range of diets and protein sources (Donkoh *et al.*, 1994b; Moughan *et al.*, 1984; Moughan *et al.*, 1987; Picard *et al.*, 1984; Rutherfurd and Moughan, 2003; Smith *et al.*, 1987). Ileal AA digestibility coefficients have been shown to be similar for most AA in both man and the rat (Fuller and Tomé, 2005). However, there is evidence to indicate that the rat may be better able to digest some proteins that are poorly digested in humans, such as rapeseed protein (Bos *et al.*, 2007; Boutry *et al.*, 2008). The maintenance protein requirements of adult humans are relatively higher than those in the growing rat (Ritchey and Taper, 1981). However, the growing rat may be a better model for the adult human than the adult rat based on a correlation of the biological values of proteins between the species (growing rat, $r = 0.92$; adult rat, $r = 0.67$) (Mitchell, 1954).

The rat has been used to study ileal protein digestibility for a range of diets, including blood meal (Pearson *et al.*, 1999), meat and bone meal (Donkoh *et al.*, 1994b; Skilton *et al.*, 1991), barley (Moughan *et al.*, 1987; Smith *et al.*, 1987; van Wijk *et al.*, 1998), casein (James *et al.*, 2002; Rutherfurd and Moughan, 2003), enzymically hydrolysed casein (Butts *et al.*, 2002; James *et al.*, 2002; Rutherfurd and Moughan, 2003), fish (Butts *et al.*, 2002), lactalbumin (Butts *et al.*, 2002), soy protein isolate (Butts *et al.*, 2002; Rutherfurd and Moughan, 2003), oats (Smith *et al.*, 1987), soy protein concentrate (Rutherfurd and Moughan, 2003) maize (Smith *et al.*, 1987), wheat (Smith *et al.*, 1987), wheat gluten (Butts *et al.*, 2002) and triticale (Smith *et al.*, 1987).

2.3 Ileal digesta collection methods in animal models

2.3.1 Terminal anaesthesia

In the case of both the rat and pig, ileal digestibility is predicted through the collection of ileal effluent from the last 10 - 20 cm of the ileum. Sampling after 3 - 4 h is considered optimal in the growing rat (Butts *et al.*, 2002) and may occur after a single meal fed for 3 h, or after the last meal in the case of a 'frequent feeding' regimen. Frequent feeding generally involves limited access (10 min) to food every hour for 4 - 8 h of the day. Such a regimen over the course of the trial (routinely 10 - 14 days) trains the rats to consistently eat during meal times and ensures a constant flow of digesta and a more representative sample at the terminal ileum on the day of slaughter (Hodgkinson *et al.*, 2003; James *et al.*, 2002; Rutherford and Moughan, 2003). The impracticality of fitting cannulae to rats necessitates sacrificing the animal (the 'slaughter' method) and decapitation immediately following death to cease neural stimulation, thereby minimising any disturbance to the digestive tract prior to digesta collection. The disadvantages of the slaughter method are that a dietary marker is required as total ileal digesta collection is not possible and repeated observations on the same animal cannot be performed, reducing the variability and increasing the number of animals required. However, multiple measurements can be taken along different parts of the digestive tract, which is not possible with cannulated pigs. Concerns about whether a single sample is representative of the entire feeding cycle were addressed by Donkoh *et al.* (1994a) who found no difference in ileal protein digestibility in pigs sampled under anaesthesia prior to death (9 h after a meal) or those fitted with a T-cannula (10 h after a meal).

Similar to studies with the growing rat, ileal digesta from the growing pig may be collected using the slaughter method, with collection taking place under anaesthesia prior to death and up to 9 h after a single meal (Nyachoti *et al.*, 1997). More common, however, is the fitting of a cannula to the terminal ileum to allow repeated measurements over the whole feeding cycle.

2.3.2 Cannulation methods

There are a number of cannulation techniques that have been used with pigs including the simple T-cannula (Livingstone *et al.*, 1977), the re-entrant cannula (Cunningham, 1962; Darcy *et al.*, 1980), the popular PVTC (post-valve T-caecum) cannula (van Leeuwen *et al.*, 1991) and the steered ileal-caecal valve cannula (Mroz *et al.* (1996).

2.3.3 Ileo-rectal anastomosis

The most invasive of the ileal digesta collection methods used with pigs is ileo-rectal anastomosis (Fuller and Livingstone, 1982). The method (**Figure 6**) involves diverting the terminal ileum directly to the rectum to allow digesta collection from the anus and isolating the large intestine, with or without a T-cannula inserted in the large intestine to remove residual colonic material (Green *et al.*, 1987).

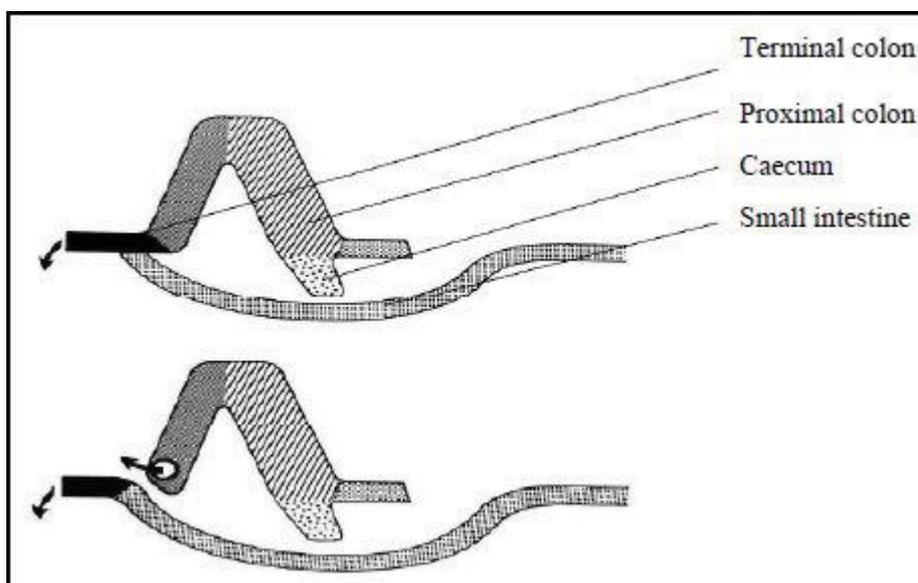


Figure 6. Ileo-rectal anastomosis without isolation of the large intestine (top figure) or with isolation of the large intestine by a T-cannula (bottom figure). From Sauer *et al.* (1989).

Although the details, advantages and shortcomings of ileo-rectal anastomosis and the various cannulation techniques will not be discussed here, a number of such reviews have been undertaken by other workers (Deglaire, 2008; Hodgkinson and Moughan, 2000; Kohler *et al.*, 1991; Kohler *et al.*, 1990). In summary, however, compared to the slaughter method all require veterinary and surgical intervention, varying post-surgery recovery time and the possibility of altered physiology affecting digestibility. Furthermore, the use of surgically altered pigs may be less ethically acceptable compared to the use of the slaughter method or the use of laboratory animals such as the rat. Pigs also demand a greater degree of animal husbandry and increased food, housing and labour costs compared to digestibility studies with rats.

2.4 Dietary markers

Dietary markers are non-absorbable compounds added to the ingested diet to allow calculation of ileal digestibility of the diet when total ileal digesta collection is not possible. Apparent ileal digestibility is calculated using the ratio of the nutrient to the marker in the diet ($(\text{Nutrient/Marker})_{\text{diet}}$) and the ratio of the nutrient to the marker in the ileal digesta ($(\text{Nutrient/Marker})_{\text{ileal}}$), where the ratios are expressed on a DM basis (e.g. mg/g DM diet and mg/g DM ileal digesta) and the overall equation (Eq. 7) is as follows:

Apparent ileal digestibility of nutrient

$$= \frac{(\text{Nutrient/Marker})_{\text{diet}} - (\text{Nutrient/Marker})_{\text{ileal}}}{(\text{Nutrient/Marker})_{\text{diet}}} \times \frac{100}{1} \quad (\text{Eq. 7})$$

Markers need to have certain inherent properties to be of practical use in digestibility studies. Specifically, a marker should not be digested or absorbed in the GI tract (it should be inert). The method for its analysis in samples should be both sensitive and specific, it should have the same dissolving properties as the substance being studied and it should be distributed homogeneously within the intestinal lumen (Kozloski, 1998; Modigliani *et al.*, 1973). No known substance fits all of these criteria. However, chromic oxide and titanium dioxide largely conform to these requirements and have been widely used in animal digestibility studies. Of the two, TiO₂ has been suggested as a more suitable marker based on lower standard errors for apparent ileal N

digestibility and higher faecal recovery rates (98% for TiO₂ vs. 75% for CrO₃) (Jagger *et al.*, 1992). There have also been reports of CrO₃ being carcinogenic (Jagger *et al.*, 1992; Peddie *et al.*, 1982). When using TiO₂ as a marker, its presence in samples is routinely determined using the method of Short *et al.* (1996).

Although acid-insoluble ash or diatomaceous earth (Celite®) has also been proposed as a reliable marker for both human and animal digestibility studies (Kavanagh *et al.*, 2001; Rowan *et al.*, 1991), the size of the digesta sample required for analytical determination (1.5 – 2 g) is a drawback (Rowan *et al.*, 1993). Phenolsulphonphthalein (phenol red) or polyethylene glycol (PEG)-4000 are liquid phase markers commonly used in human protein digestion studies (Bos *et al.*, 2007; Gaudichon *et al.*, 2002; Gaudichon *et al.*, 1999; Gausserès *et al.*, 1996; Mariotti, 2002) and are considered suitable for use with naso-intestinal intubation (Modigliani *et al.*, 1973).

3. Methods to predict the uptake of energy from the hindgut

Whilst the focus of this review of the literature has so far focused on the need and methods to predict the uptake of energy from the upper-tract, methods to predict uptake of energy from the hindgut (estimated to contribute 3 – 11% to the body's maintenance energy needs for individuals consuming a Western diet (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984)) are also needed. The production of SCFA in the hindgut is difficult to quantify using human studies and no suitable animal model exists. *In vitro* hindgut digestibility assays offer a routine means of assessment to study hindgut digestibility whilst taking into account complex dietary factors by incubating the substrate under study with live faecal inoculum. A comprehensive review of *in vitro* digestion and fermentation methods to predict the uptake of energy from the hindgut is covered as a separate addendum to this literature review in the form of a published review (Chapter II).

VIII. Conclusions and justifications of the experimental work

Obesity has reached very high levels in recent years, placing a large economic and social burden on affluent societies in particular. Although increased physical activity is recognised as one way to combat the problem, controlled dietary intake remains the primary means of managing obesity. The food industry plays an important role in the provision of foods that are targeted at individuals seeking to reduce body weight. The general objective when developing these weight-loss foods is to minimise the energy delivered to the body (i.e. available energy, ATP) per gram of food, whilst (ideally) imparting maximum satiety. This is often achieved through the addition of high levels of dietary fibre and/or protein or the addition of novel ingredients. However, the energy content of foods containing these very same components, are known to be poorly predicted by popular energy conversion systems (Livesey, 1995, 2001; Zou *et al.*, 2007). Current methods of comparing the energy content of foods generally rely on ME systems and these do not accurately describe the energy available at the cellular level (ATP). Furthermore, the digestion of foods in humans is a complex process with digestibility of the energy-yielding nutrients being affected by numerous factors and the use of generic calculation factors to describe the energy provided by each nutrient, does not account for the specific digestibility of a diet. The accurate determination of digestibility is particularly important for novel foods as these are often formulated to have reduced nutrient digestibility that may be significantly different from what is expected based on determined chemical composition alone.

To more accurately predict the actual energy a given food delivers in terms of ATP, there is a requirement for a routine means of (i) predicting the uptake of all energy-yielding components of food (mainly sugars, starch, fats, protein, alcohol) as a result of upper-tract digestion; (ii) predicting the production of SCFA in the hindgut; and (iii) predicting the available energy (mol of ATP) as a result of the post-absorptive catabolism of known quantities of absorbed nutrients and SCFA (as predicted by (i) and

(ii), respectively). For application to weight-loss foods the model should be specific to adults in the catabolic state (i.e. sub-maintenance energy requirements).

A model is put forward which combines a dual *in vivo* – *in vitro* nutrient digestibility assay ('dual digestibility assay') to predict the uptake of energy across the total tract, with a series of mathematical predictive equations to translate absorbed energy across the total tract into available energy (ATP) at the cellular level. The dual digestibility assay would use an ileal assay to predict upper-tract digestibility, and a hindgut *in vitro* fermentation assay to predict SCFA production in the human large intestine. The mathematical predictive equations would use known stoichiometric relationships between nutrients and catabolic ATP yields, based on current (i.e. non-integral) estimates of cellular P/O ratios.

The ethical, financial and scientific shortcomings of routinely determining the ileal digestibility of foods with humans directly using either the ileostomy or intubation method necessitates the use of a valid animal model. Although the growing pig is widely used as a model for human upper-tract digestion, the growing rat is also accepted as a valid model and has a number of advantages over the pig (less labour intensive, lower cost, relatively simply research facilities and ileal digesta collection method, rapid availability of results, no requirements for surgery and veterinary expertise).

The impracticality of determining SCFA production or organic matter loss directly in the human colon and the lack of a suitable animal model has led to the development of several hindgut *in vitro* digestion and fermentation assays. However, all published hindgut *in vitro* fermentation assays fail to: (i) provide justification for and optimisation of the values of assay variables (e.g. pH, duration of incubation, inoculum concentration); (ii) validate the method with human *in vivo* data from controlled studies; and (iii) demonstrate application of the method for a range of fermentative substrates, as are present in mixed human diets. Due to these failings, no current *in vitro* fermentation assay, to the authors' knowledge, has demonstrated the ability to

do more than simply rank substrates according to digestibility. For the purposes of predicting the quantity of available energy gained from hindgut fermentation, however, accurate absolute data are required.

A few workers (see Table 2, Chapter II) have explored the concept of a dual *in vivo* – *in vitro* digestibility assay for modelling human digestion using ileal digesta from humans, pigs or rats as a substrate for hindgut *in vitro* fermentation with inoculum sourced from human faeces or faeces or caecal / colonic contents from the pig or rat. However, the shortcomings of presently available *in vitro* fermentation assays in general (as discussed above) also apply to the *in vitro* fermentation assays used in these studies, thereby making these dual *in vivo* – *in vitro* digestibility assays unsuitable for predicting the quantitative uptake of available energy across the total tract in humans.

The key objectives of the experimental work described in this dissertation were as follows:

- To compare apparent human faecal nutrient digestibility data with apparent ileal nutrient digestibility data (using the growing rat as an animal model for human upper-tract digestion) for a range of mixed human diets to determine the effect of the source and level of DF on nutrient digestibility in the upper-tract and hindgut
- To identify key assay variables of selected *in vitro* hindgut dry matter digestibility assays for subsequent optimisation.
- To optimise the values of identified key assay variables in an *in vitro* hindgut dry matter digestibility assay using ileal digesta pertaining to a mixed human diet as the substrate. This was undertaken with the objective of developing an *in vitro* hindgut digestibility assay that is able to make accurate and valid predictions of nutrient digestibility when compared to human *in vivo* nutrient digestibility data.
- To validate the dual *in vivo* – *in vitro* digestibility assay (using the growing rat as

an animal model for human upper-tract digestion and an optimised *in vitro* hindgut fermentation method using human faecal inoculum) against human *in vivo* nutrient digestibility data for a range of mixed human diets to verify that the *in vitro* hindgut assay predicts absolute nutrient digestibility data (as opposed to giving a simple ranking of digestibilities between diets).

- To develop a series of predictive mathematical equations, based on non-integral cellular P/O ratios, for translating quantities of absorbed nutrients in the upper-tract and SCFA in the hindgut, into available energy at the cellular level such that assimilation of a food may be described in terms of mol ATP / g dietary intake.
- To demonstrate the practical application of the overall 'Combined Model' (dual digestibility assay + mathematical predictive equations) to predict the available energy content of commercially available meal replacement formulations.

Literature cited

- Abbott, W. G. H., Howard, B. V., Christin, L., Freymond, D., Lillioja, S., Boyce, V. L., Anderson, T. E., Bogardus, C. & Ravussin, E. (1988). Short-term energy balance: relationship with protein, carbohydrate, and fat balances. *American Journal of Physiology*, 255, E332-337.
- Abidi, S. A., Gray, S. & Menden, E. (1967). The kinetics of amino acid absorption and alteration of plasma composition of free amino acids after intestinal perfusion of amino acid mixtures. *American Journal of Clinical Nutrition*, 20, 24-33.
- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Apgar, J. L., Shively, C. A. & Tarka, S. (1987). Digestibility of cocoa butter and corn oil and their influence on fatty acid distribution in rats. *Journal of Nutrition*, 117, 660-665.
- Atwater, W. O. (1910). Principles of nutrition and nutritive values of food. *United States Farmers' Bulletin*, 142.
- Atwater, W. O. & Benedict, F. G. (1902). Experiments on the metabolism of matter and energy in the human body, 1898–1900. In *US Office of Experiment Stations Bulletin No 109*. Washington, DC: Government Printing Office.
- Atwater, W. O. & Bryant, A. P. (1900). Availability and fuel value of food materials. *Storrs Agr Exp Sta 12th Annl Rept for 1899*, 73-110.
- Atwater, W. O. & Woods, C. D. (1896). The chemical composition of American food materials. In *US Official Experiment Stations, Experiment Station Bulletin No 28*. Washington, DC.
- Bach Knudsen, K. E. & Jensen, B. B. (1991). *Effect of source and level of dietary fibre on microbial fermentation in the large intestine of pigs*. Paper presented at the Proceedings of the Vth International Symposium on Digestive Physiology in Pigs, Wageningen, The Netherlands.

-
- Bach Knudsen, K. E., Jensen, B. B. & Hansen, I. (1993). Digestion of polysaccharides and other major components in the small and large intestine of pigs fed on diets consisting of oat fractions rich in β -D-glucan. *British Journal of Nutrition*, 70, 537–556.
- Bach Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, W. & Eggum, B. O. (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat. *British Journal of Nutrition*, 71, 471-487.
- Baer, D. J., Rumpler, W. V., Miles, C. W. & Fahey Jr., G. C. (1997). Dietary fiber decreases the metabolizable energy content and nutrient digestibility of mixed diets fed to humans. *Journal of Nutrition*, 127, 579-586.
- Bakker, G. (1996). *Interaction between carbohydrates and fat in pigs*. PhD Thesis, Wageningen Agricultural University, Wageningen, Netherlands.
- Bamworth, C. W. (2005). Beer, carbohydrates and diet. *Journal of the Institute of Brewing*, 111(3), 259-264.
- Bartelt, J., Drochner, W., Gotz, K. P., Szakacz, J., Ceresnakova, Z. & Sommer, A. (1999). Determination of endogenous nitrogen associated with bacteria in ileal digesta of pigs receiving cereal-based diets with or without fish meal and various fibre supplements by using a simple ^{15}N -dilution technique. *Journal of Animal Feed Science*, 8(3), 425-440.
- Basson, M., Liu, Y. W., Hanly, A. M., Emenecker, N. J., Shenoy, S. G. & Gould Rothberg, B. E. (2000). Identification and comparative analysis of human short chain fatty acid response genes. *Journal of Gastrointestinal Surgery*, 4, 501-502.
- Bayley, H. S. & Lewis, D. (1965). The use of fats in pig feeding. I. Pig faecal fat not of immediate dietary origin. *Journal of Agricultural Science*, 64, 367-372.
- Behall, K. M. & Howe, J. C. (1995). Contribution of fiber and resistant starch to metabolizable energy. *American Journal of Clinical Nutrition*, 62(Suppl), 1158S–1160S.
- Behall, K. M. & Howe, J. C. (1996). Resistant starch as energy. *Journal of the American College of Nutrition*, 15(3), 248–253.

-
- Bergen, W. G. (2007). Contribution of research with farm animals to protein metabolism concepts: A historical perspective. *Journal of Nutrition*, 137(3), 706-710.
- Berthold, H., Jahoor, F., Klein, P. D. & Reeds, P. J. (1995). Estimates of the effect of feeding on whole-body protein degradation in women vary with the amino acid used as tracer. *Journal of Nutrition*, 125, 2516-2527.
- Birkett, S. & de Lange, K. (2001a). A computational framework for a nutrient flow representation of energy utilization by growing monogastric animals. *British Journal of Nutrition*, 86, 661-674.
- Birkett, S. & de Lange, K. (2001b). Limitations of conventional models and a conceptual framework for a nutrient flow representation of energy utilization by animals. *British Journal of Nutrition*, 86, 647-659.
- Blaak, E. (2001). Gender differences in fat metabolism. *Current Opinion in Clinical Nutrition & Metabolic Care*, 4(6), 499-502.
- Black, A., Coward, W., Cole, T. & Prentice, A. (1996). Human energy expenditure in affluent societies: an analysis of 574 doubly labelled water measurements. *European Journal of Clinical Nutrition*, 50, 72-92.
- Blaxter, K. L. (1989). *Energy Metabolism in Animals and Man*. Cambridge, United Kingdom: Cambridge University Press.
- Bode, C. & Bode, J. C. (1997). Alcohol's role in gastrointestinal tract disorders. *Alcohol Health and Research World*, 21(1), 76-83.
- Bode, J. C. (1980). Alcohol and the gastrointestinal tract. *Advances in Internal Medicine and Pediatrics*, 45, 1-75.
- Bodwell, C. E., Satterlee, L. D. & Hackler, L. R. (1980). Protein digestibility of the same protein preparations by human and rat assays and by *in vitro* enzymic digestion methods. *American Journal of Clinical Nutrition*, 33(3), 677-686.
- Boisen, S. & Eggum, B. O. (1991). Critical evaluation of *in vitro* methods for estimating digestibility in simple-stomached animals. *Nutrition Research Reviews*, 4, 141-162.

-
- Boisen, S. & Fernández, J. A. (1995). Prediction of the apparent ileal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by *in vitro* analyses. *Animal Feed Science and Technology*, 51, 29-43.
- Boisen, S. & Fernández, J. A. (1997). Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. *Animal Feed Science and Technology*, 68, 277-286.
- Boisen, S. & Moughan, P. J. (1996). Dietary influences on endogenous ileal protein and amino acid loss in the pig - A review. *Acta Agriculturae Scandinavica, Section A - Animal Science*, 46, 154-164.
- Boisen, S. & Verstegen, M. W. A. (2000). Developments in the measurement of the energy content of feeds and energy utilisation in animals. In P. J. Moughan, M. W. A. Verstegen & M. I. Visser-Reyneveld (Eds.), *Feed Evaluation, Principles and Practice* (pp. 57-76). Wageningen: Wageningen Pers.
- Bos, C., Airinei, G., Mariotti, F., Benamouzig, R., Berot, S., Evrard, J., Fenart, E., Tome, D. & Gaudichon, C. (2007). The poor digestibility of rapeseed protein is balanced by its very high metabolic utilization in humans. *Journal of Nutrition*, 137(3), 594-600.
- Bouchard, C., Deprés, J. P. & Tremblay, A. (1993). Exercise and obesity. *Obesity Research*, 1, 133-147.
- Boutry, C., Chevalier, L., Fouillet, H., Blachier, F., Gaudichon, C., Tomé, D. & Bos, C. (2008). Rapeseed and milk protein induce different patterns of dietary N tissue accretion despite a similar postprandial retention in rats. *The FASEB Journal*, 22(Abs 312.2).
- Brand, M. D. (2005). *The efficiency and plasticity of mitochondrial energy transduction*. Paper presented at the Keilin Memorial Lecture, Glasgow.
- British Nutrition Foundation. (1990). *Complex Carbohydrates in Foods*. London, U.K.: Chapman and Hall.
- Broeder, C., Burrhus, K., Svanevik, L. & Wilmore, J. (1992). The effects of aerobic fitness on resting metabolic rate. *American Journal of Clinical Nutrition*, 55(4), 795-801.

-
- Brown, G. C. (1992). The leaks and slips of bioenergetic membranes. *FASEB Journal*, *6*, 2961-2965.
- Brown, J., Livesey, G., Roe, M., Faulks, R., Poppitt, S., Wilkinson, J. & Elia, M. (1998). Metabolizable energy of high non-starch polysaccharide-maintenance and weight-reducing diets in men: experimental appraisal of assessment systems. *Journal of Nutrition*, *128*, 986-995.
- Buemann, B. & Astrup, A. (2001). How does the body deal with energy from alcohol? *Nutrition*, *17*, 638-641.
- Burant, B., Takeda, J., Brot-Laroche, E., Bell, G. & Davidson, N. (1992). Fructose transporter in human spermatozoa and small intestine is GLUT5. *Journal of Biological Chemistry*, *267*, 14523-14526.
- Burkitt, D. P., Walker, A. R. P. & Painter, N. S. (1972). Effect of dietary fibre on stools and transit times, and its role in the causation of disease. *Lancet*, *2*(7792), 1408-1412.
- Burton-Freeman, B. (2000). Dietary fiber and energy regulation. *Journal of Nutrition*, *130*(suppl), 272S-275.
- Buttery, P. J. & Boorman, K. N. (1976). The energetic efficiency of amino acid metabolism. In D. J. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale & H. Swan (Eds.), *Protein Metabolism and Nutrition*, EAAP Publication no 16 (pp. 197-206). London: Butterworths.
- Butts, C. A., James, K. A., Koolard, J. P., Booth, C. L., Donaldson, H. E., Scott, M. F. & Moughan, P. J. (2002). The effect of digesta sampling time and dietary protein source on ileal nitrogen digestibility for the growing rat. *Journal of the Science of Food and Agriculture*, *82*, 343-350.
- Cahill, J., G.F. . (1971). Physiology of insulin in man. *Diabetes*, *20*, 785-799.
- Calloway, D. H. & Kretsch, M. J. (1978). Protein and energy utilization in men given a rural Guatemalan diet and egg formulas with and without added oat bran. *American Journal of Clinical Nutrition*, *31*, 1118-1126.

-
- Champ, M., Langkilde, A. M., Brouns, F., Kettlitz, B. & Le Bail-Collet, Y. (2003). Advances in dietary fibre characterisation. 2. Consumption, chemistry, physiology and measurement of resistant starch; implications for health and food labelling. *Nutrition Research Reviews*, *16*(2), 143-161.
- Chen, H.-L., Haack, V. S., Janecky, C. W., Vollendorf, N. W. & Marlett, J. A. (1998). Mechanisms by which wheat bran and oat bran increase stool weight in humans. *American Journal of Clinical Nutrition*, *68*, 711-719.
- Chen, I. S., Hotta, S. S., Ikeda, I., Cassidy, M. M., Sheppard, A. J. & Vahouny, G. V. (1987). Digestion, absorption and effects on cholesterol absorption of menhaden oil, fish oil concentrate and corn oil by rats. *Journal of Nutrition*, *117*, 1676-1680.
- Chevalier, S., Burgess, S. C., Malloy, C. R., Gougeon, R. & Marliss, E. B. (2006). The greater contribution of gluconeogenesis to glucose production in obesity is related to increased whole-body protein catabolism. *Diabetes*, *55*(3), 675-681.
- Christl, S. U., Murgatroyd, P. R., Gibson, G. R. & Cummings, J. H. (1992). Production, metabolism and excretion of hydrogen in the large intestine. *Gastroenterology*, *102*, 1269-1277.
- Christl, S. U. & Scheppach, W. (1997). Metabolic consequences of total colectomy. *Scandinavian Journal of Gastroenterology - Supplement*, *222*, 20-24.
- Codex Alimentarius. (2001). Codex Guidelines on Nutrition Labelling CAC/GL 2-1985 (Rev. 1 – 1993).
- Cook, S. I. & Sellin, J. H. (1998). Review article: short chain fatty acids in health and disease. *Alimentary Pharmacology & Therapeutics*, *12*(6), 499–507.
- Coward, W. (1998). Contributions of the doubly labelled water method to studies of energy balance in the Third World. *American Journal of Clinical Nutrition*, *68* (suppl.), 962S-969S.
- Cummings, J. H. (1981). Short chain fatty acids in the human colon. *Gut*, *22*, 763-779.
- Cummings, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet*, *321*(8335), 1206-1209.
- Cummings, J. H. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*, *28*, 1221-1227.

-
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H., Beatty, E. R., Kingman, S. M., Bingham, S. A. & Englyst, H. N. (1996). Digestion and physiological properties of resistant starch in the human large bowel. *British Journal of Nutrition*, *75*, 733-747.
- Cummings, J. H., Bingham, S. A., Heaton, K. W. & Eastwood, M. A. (1992). Fecal weight, colon cancer risk and dietary intake of non-starch polysaccharides (dietary fiber). *Gastroenterology*, *103*, 1783-1789.
- Cummings, J. H. & Englyst, H. N. (1987). Fermentation in the large intestine and the available substrates. *American Journal of Clinical Nutrition*, *45*, 1243-1255.
- Cummings, J. H., Gibson, G. & Macfarlane, G. T. (1989). Quantitative estimates of fermentation in the hind gut of man. *Acta Veterinaria Scandinavica Supplementum*, *86*, 76-82.
- Cummings, J. H. & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, *70*, 443-459.
- Cummings, J. H. & Macfarlane, G. T. (1997). Role of intestinal bacteria in nutrient metabolism. *Journal of Parenteral and Enteral Nutrition*, *21*(6), 357-365.
- Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P. E. & Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*, *28*, 1221-1227.
- Cunningham, H. M. (1962). Note on a re-entrant fistula for digestion studies with pigs. *Canadian Journal of Animal Science*, *42*, 112-113.
- D'Mello, J. P. F. (2003). An outline of pathways in amino acid metabolism. In J. P. F. D'Mello (Ed.), *Amino Acids in Animal Nutrition* (2nd ed., pp. 71-86). Cambridge, MA: CABI.
- Danforth, J., E. . (1985). Diet and obesity. *American Journal of Clinical Nutrition*, *41*, 1132-1145.

-
- Darcy, B., Laplace, J. P. & Villiers, P. A. (1980). Digestion in the pig small intestine. 2. Comparative kinetics of the passage of digesta according to the mode of fistulation, ileocecal or post-valvular ileocolic, in various feeding conditions. *Annales de Zootechnie*, 29(2), 147-177.
- Darragh, A. J., Cranwell, P. D. & Moughan, P. J. (1994). Absorption of lysine and methionine from the proximal colon of the piglet. *British Journal of Nutrition*, 75(1), 739-752.
- Darragh, A. J. & Hodgkinson, S. M. (2000). Quantifying the digestibility of dietary protein. *Journal of Nutrition*, 130, 1850S-1856S.
- Darragh, A. J. & Moughan, P. J. (1995). The three-week-old piglet as a model for studying protein digestion in human infants. *Journal of Pediatric Gastroenterology and Nutrition*, 21, 387-393.
- Davies, I. R. (1990). *The Food Energy Values of Unavailable Carbohydrates*. Norwich, UK: University of East Anglia.
- de Lange, C. F. M., Souffrant, W. B. & Sauer, W. C. (1990). Real ileal protein and amino acid digestibilities in feedstuffs for growing pigs as determined with the ¹⁵N-Isotope Dilution Technique. *Journal of Animal Science*, 68(2), 409-418.
- De Schrijver, R., Fremaut, D. & Verheyen, A. (1992). Cholesterol-lowering effects and utilization of protein, lipid, fiber and energy in rats fed unprocessed and baked oat bran. *Journal of Nutrition*, 122, 1318 -1324.
- DeFronzo, R. A. & Ferrannini, E. (1987). Regulation of hepatic glucose metabolism in humans. *Diabetes/Metabolism Reviews*, 3, 415-459.
- Deglaire, A. (2008). *Gut endogenous protein flows and postprandial metabolic utilization of dietary amino acids in simple-stomached animals and humans*. PhD thesis, Massey University, Palmerston North, New Zealand.
- Deglaire, A., Bos, C., Tomé, D. & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- Della Ragione, F., Criniti, V., Della Pietra, V., Borriello, A., Oliva, A., Indaco, S., Yamamoto, T. & Zappia, V. (2001). Genes modulated by histone acylation as new effectors of butyrate activity. *FEBS Letters*, 499, 199-204.

-
- Denzer, C. M. & Young, J. C. (2003). The effect of resistance exercise on the thermic effect of food. *International Journal of Sport Nutrition and Exercise Metabolism*, 13(3), 396-402.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39, 209-228.
- DeVries, J. W., Prosky, L., Li, B. & Cho, S. (1999). A Historical Perspective on Defining Dietary Fiber. *Cereal Foods World*, 44(5), 367-369.
- Dierick, N. A., Vervaeke, I. J., Decuyper, J. A. & Hendrickx, H. K. (1986). Influence of the gut flora and of some growth-promoting feed additives on nitrogen metabolism in pigs. II. Studies *in vivo*. *Livestock Production Science*, 14(2), 177-193.
- Djouzi, Z. & Andrieux, C. (1997). Compared effects of three oligosaccharides on metabolism of intestinal microflora in rats inoculated with a human faecal flora. *British Journal of Nutrition*, 78, 313-324.
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994a). Comparison of the slaughter method and simple T-piece cannulation of the terminal ileum for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, 49(1-2), 43-56.
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994b). The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, 49, 57-71.
- Dowsett, J., Gibney, M. J. & Kennedy, N. P. (1990). Bacterial fermentation occurs in the terminal ileum of ileostomates. *Proceedings of the Nutrition Society*, 49, 110A. (abs.).
- Dreon, D. M., Frey-Hewitt, B., Ellsworth, N., Williams, P. T., Terry, R. B. & Wood, P. D. (1988). Dietary fat: carbohydrate ratio and obesity in middle-aged men. *American Journal of Clinical Nutrition*, 47, 995-1000.
- Drewnowski, A., Kurth, C., Holden-Wiltse, I. & Saari, J. (1992). Food preferences in human obesity: carbohydrates versus fats. *Appetite*, 18, 207-221.

-
- Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E. & Flint, H. J. (2002). Acetate utilization and butyryl coenzyme A (CoA): Acetate CoA transferase in butyrate-producing bacteria from the human large intestine. *Applied and Environmental Microbiology*, *68*(10), 5186-5190.
- Duran-Montge, P., Lizardo, R., Torrallardona, D. & Esteve-Garcia, E. (2007). Fat and fatty acid digestibility of different fat sources in growing pigs. *Livestock Science*, *109*(1-3), 66-69.
- Elia, M. (1995a). General integration and regulation of metabolism at the organ level. *Proceedings of the Nutrition Society*, *54*, 213-232.
- Elia, M. (1995b). Insights into energy requirements in disease. *Public Health Nutrition*, *8*, 1037-1052.
- Elia, M. & Cummings, J. H. (2007). Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *European Journal of Clinical Nutrition*, *61* S40-S74.
- Emmans, G. C. (1994). Effective energy: a concept of energy utilization applied across species. *British Journal of Nutrition*, *71*, 801-821.
- Emmans, G. C. & Kyriazakis, I. (1999). Growth and body composition. In I. Kyriazakis (Ed.), *A Quantitative Biology of the Pig* (pp. 181-198). Oxon, UK: CAB International.
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, *71*, 808-814.
- Erickson, R. H., Gum, J. R., Lindstrom, M. M., Mckean, D. & Kim, Y. S. (1995). Regional expression and dietary regulation of rat small intestinal peptide and amino acid transporter messenger-RNAs. *Biochemical and Biophysical Research Communications*, *216*(1), 249-257.
- European Council Directive. (1990). Nutritional labelling of foodstuffs. *Official Journal of the European Community*, *33*, 176.
- Ewing, W. N. & Cole, D. J. A. (1994). *The Living Gut: An Introduction to Micro-organisms in Nutrition*. Dungannon, Ireland: Context.

-
- Faithfull, N. T. (2002). *Methods in Agricultural Chemical Analysis: A Practical Handbook*. Oxon, UK: CABI Publishing.
- FAO. (1981). *Amino-acid content of foods and biological data on proteins*. Rome, Italy: Food Policy and Food Science Service Nutrition Division, FAO.
- FAO. (2003). *Food energy - methods of analysis and conversion factors*. Rome: FAO.
- FAO/WHO. (1991). *Protein quality evaluation in human diets. Report of a Joint FAO/WHO Expert Consultation*. Geneva: Food and Agriculture Organization of the United Nations.
- FAO/WHO. (1994). *Fats and oils in human nutrition. Report of a Joint Expert Consultation*. Rome: FAO Food and Nutrition.
- FAO/WHO. (1998). *Carbohydrates in human nutrition. Report of a Joint FAO/WHO Expert Consultation. FAO Food and Nutrition Paper No. 66*. Rome.
- FAO/WHO/UNU. (2001). *Human energy requirements (No. 1)*. Rome: FAO/WHO/UNU.
- FAO/WHO/UNU. (2002a). *Joint FAO/WHO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition*. Geneva, Switzerland: WHO.
- FAO/WHO/UNU. (2002b). *Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation*. Geneva, Switzerland.
- Farrell, D. J., Girlic, L. & Arthur, J. (1978). Effects of dietary fibre on the apparent digestibility of major food components and on blood lipids in men. *Australian Journal of Experimental Biology and Medical Science*, 56, 469-479.
- FDA. (1993). Nutritional labeling of food regulations. Title 21 of the Code of Federal Regulations (21CF). *Federal Register*, 53, 2175.
- Flatt, J.-P. (1987). Dietary fat, carbohydrate balance, and weight maintenance: effects of exercise. *American Journal of Clinical Nutrition*, 45, 296-306.
- Flatt, J.-P. (1988). Importance of nutrient balance in body weight regulation. *Diabetes/Metabolism Reviews*, 4, 471-481.
- Flatt, J.-P. (1991). Opposite effects of variations in food intake on carbohydrate and fat oxidation in ad libitum fed mice. *Journal of Nutritional Biochemistry*, 2, 186-192.
- Flatt, J.-P. (1993). Dietary fat, carbohydrate balance, and weight maintenance. *Annals of the New York Academy of Sciences*, 683, 122-140.

-
- Flatt, J.-P. (1995a). Body composition, respiratory quotient, and weight maintenance. *American Journal of Clinical Nutrition*, *62*, 1107S-1117S.
- Flatt, J.-P. (1995b). Use and storage of carbohydrate and fat. *American Journal of Clinical Nutrition*, *61*, 952S-959S.
- Flatt, J.-P. (2001). Macronutrient composition and food selection. *Obesity Research*, *9*, 256S-262S.
- Flatt, J.-P. & Blackburn, G. L. (1974). The metabolic fuel regulatory system: implications for protein sparing therapies during caloric deprivation and disease. *American Journal of Clinical Nutrition*, *27*, 175-187.
- Flatt, J.-P., Ravussin, E., Acheson, K. J. & Jéquier, E. (1985). Effects of dietary fat on postprandial substrate oxidation and on carbohydrate and fat balances. *Journal of Clinical Investigation*, *76*, 1019-1024.
- Flatt, J.-P. & Tremblay, A. (1998). Energy expenditure and substrate oxidation. In G. A. Bray, C. Bouchard & W. P. T. James (Eds.), *Handbook of Obesity* (1st ed., pp. 513-537). New York: Marcel Dekker.
- Fone, D. R., Horowitz, M., Heddle, R., Maddox, A. F., Collins, P. J., Read, N. W. & Dent, J. (1991). Comparative effects of duodenal and ileal intubation on gastric emptying and postprandial antral, pyloric, and duodenal motility. *Scandinavian Journal of Gastroenterology*, *26*(1), 16-22.
- Forsum, E., Goranzon, H., Rundgren, M., Thilen, M. & Hambraeus, L. (1981). Protein evaluation of mixed diets. Comparative study in man and in the pig and rat of vegetable-animal and vegetable protein diets. *Annals of Nutrition and Metabolism*, *25*(3), 137-150.
- Fukagawa, N. K. & Yu, Y.-M. (2009). Nutrition and metabolism of proteins and amino acids. In M. J. Gibney, S. A. Lanham-New & A. Cassidy (Eds.), *Introduction to Human Nutrition* (pp. 49-73). Chichester, UK: Wiley-Blackwell.
- Fuller, M. F. (2004). *The Encyclopedia of Farm Animal Nutrition*. Oxon, UK: CABI Publishing.
- Fuller, M. F. & Livingstone, R. M. (1982). *Annual report of studies in animal nutrition and allied sciences*. Aberdeen, Scotland: Rowett Research Institute.

-
- Fuller, M. F., Milne, A., Harris, C. I., Reid, T. M. S. & Keenan, R. (1994). Amino acid losses in ileostomy fluid on a protein-free diet. *American Journal of Clinical Nutrition*, 59, 70-73.
- Fuller, M. F. & Tomé, D. (2005). *In vivo* determination of amino acid bioavailability in humans and model animals. *Journal of AOAC International*, 88(3), 923-934.
- Ganapathy, V., Gupta, N. & Martindale, R. G. (2006). Protein Digestion and Absorption. In L. R. Johnson, K. E. Barrett, F. K. Ghishan, J. L. Merchant, H. M. Said & J. D. Wood (Eds.), *Physiology of the Gastrointestinal Tract* (4th ed., Vol. 1, pp. 2080). Burlington, MA: Academic Press.
- Garlick, P. J., Clugston, G. A., Swick, R. W. & Waterlow, J. C. (1980). Diurnal pattern of protein and energy metabolism in man. *American Journal of Clinical Nutrition*, 33, 1983-1986.
- Gaudichon, C., Bos, C., Morens, C., Petzke, K. J., Mariotti, F., Everwand, J., Benamouzig, R., Daré, S., Tomé, D. & Metges, C. C. (2002). Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. *Gastroenterology*, 123(1), 50-59.
- Gaudichon, C., Mahé, S., Benamouzig, R., Luengo, C., Fouillet, H., Daré, S., van Oycke, M., Ferrière, F., Rautureau, J. & Tomé, D. (1999). Net postprandial utilization of ¹⁵N-labeled milk protein nitrogen is influenced by diet composition in humans. *Journal of Nutrition*, 129(4), 890-894.
- Gausserès, N., Mahé, S., Benamouzig, R., Luengo, C., Drouet, H., Rautureau, J. & Tomé, D. (1996). The gastro-ileal digestion of ¹⁵N-labelled pea nitrogen in adult humans. *British Journal of Nutrition*, 76(1), 75-85.
- Gorbach, S. L., Nahas, L., Weinstein, L., Levitan, R. & Patterson, J. F. (1967). Studies of intestinal microflora. IV. The microflora of ileostomy effluent: a unique microbial ecology. *Gastroenterology*, 53, 874-880.
- Graham, H., Hesselman, K. & Åman, P. (1986). The influence of wheat bran and sugar beet pulp on the digestibility of dietary components in a cereal-based pig diet. *Journal of Nutrition*, 116, 242-251.

-
- Green, S., Bertrand, S. L., Duron, M. J. C. & Maillard, R. A. (1987). Digestibility of amino acids in maize, wheat and barley meal, measured in pigs with ileorectal anastomosis and isolation of the large intestine. *Journal of the Science of Food and Agriculture*, 41(1), 29-43.
- Griffiths, A. I., Humphreys, S. M., Clark, M. L., Fielding, B. A. & Frayn, K. N. (1994). Immediate metabolic availability of dietary fat in combination with carbohydrate. *American Journal of Clinical Nutrition*, 59, 53-59.
- Groff, J. L. & Gropper, S. S. (2000). *Advanced Nutrition and Human Metabolism* (3rd ed.). Belmont, CA, USA: Wadsworth.
- Gropper, S. S., Smith, J. L. & Groff, J. L. (2005). *Advanced Nutrition and Human Metabolism* (4th ed.). Belmont, CA: Cengage learning.
- Gropper, S. S., Smith, J. L. & Groff, J. L. (2008). *Advanced Nutrition and Human Metabolism* (5th ed.). Belmont, CA: Wadsworth.
- Göranzon, H. & Forsum, E. (1987). Metabolizable energy in humans in two diets. Calculation and analysis. *Journal of Nutrition*, 117, 267-273.
- Göranzon, H., Forsum, E. & Thilén, M. (1983). Calculation and determination of metabolizable energy in mixed diets to humans. *American Journal of Clinical Nutrition*, 38, 954-963.
- Hawk, P. B. (1965). Urine: quantitative analysis. In B. L. Oser (Ed.), *Physiological Chemistry* (14th ed., pp. 1206–1215). New York, NY: McGraw-Hill.
- Heitman, B. L. & Garby, L. (1999). Patterns of long-term weight changes in overweight developing Danish men and women aged between 30 and 60 years. *International Journal of Obesity and Related Metabolic Disorders*, 23, 1074-1078.
- Henning, S. J. & Hird, F. J. R. (1972). Transport of acetate and butyrate in the hind-gut of rabbits. *Biochemistry Journal*, 130, 791-796.
- Henry, C. J. K. (2005). Basal metabolic rate studies in humans: measurement and development of new equations. *Public Health Nutrition*, 8(7A), 1133-1152.
- Hijova, E. & Chmelarova, A. (2007). Short chain fatty acids and colonic health. *Bratislavske Lekarske Listy*, 108(8), 354-358.

-
- Hill, J. O., Melanson, E. L. & Wyatt, H. T. (2000). Dietary fat intake and regulation of energy balance: implications for obesity. *Journal of Nutrition*, 130, 284S-286S.
- Hill, M. J. (1995). Bacterial fermentation of complex carbohydrate in the human colon. *European Journal of Cancer Prevention*, 4, 353-358.
- Hinkle, P. C. (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta*, 1706, 1-11.
- Hinwood, B. G. (1992). *A textbook of science for the health professionals* (2nd ed.). Cheltenham, UK: Nelson Thornes.
- Hodgkinson, S. M. & Moughan, P. J. (2000). Amino acids - The collection of ileal digesta and characterisation of the endogenous component. In P. J. Moughan, M. W. A. Verstegen & M. I. Visser-Reyneveld (Eds.), *Feed Evaluation: Principles and Practice* (pp. 105-124). Wageningen, The Netherlands: Wageningen press.
- Hodgkinson, S. M., Souffrant, W. B. & Moughan, P. J. (2003). Comparison of the enzyme-hydrolyzed casein, guanidination, and isotope dilution methods for determining ileal endogenous protein flow in the growing rat and pig. *Journal of Animal Science*, 81(10), 2525-2534.
- Holland, B., Welch, A. A., Unwin, I. D., Buss, D. H., Paul, A. A. & Southgate, D. A. T. (1991). *McCance and Widdowson's The Composition of Foods* (5th ed.). Cambridge, United Kingdom: Royal Society of Chemistry, Ministry of Agriculture, Fisheries and Food.
- Holloway, W. D. & Tasman-Jones, C. (1978). Digestion of certain fractions of dietary fiber in humans. *American Journal of Clinical Nutrition*, 31, 927-930.
- Holloway, W. D., Tasman-Jones, C. & Mather, K. (1983). Pectin digestion in humans. *American Journal of Clinical Nutrition*, 37, 253-255.
- Houdijk, J. (1998). *Effects of non-digestible oligosaccharides in young pig diets*. PhD thesis, Wageningen Agricultural University, Wageningen.
- Houston, M. E. (2006). *Biochemistry primer for exercise science* (3rd ed.). Champaign, IL: Human Kinetics.
- Howarth, N. C., Saltzman, E. & Roberts, S. B. (2001). Dietary fiber and weight regulation. *Nutrition Reviews*, 59(5), 129-139.

-
- Huge, A., Weber, E. & Ehrlein, H. J. (1995). Effects of enteral feedback inhibition on motility, luminal flow, and absorption of nutrients in proximal gut of minipigs. *Digestive Diseases and Sciences*, 40(5), 1024-1034.
- Hume, I. D., Karasov, W. H. & Darken, B. W. (1993). Acetate, butyrate and proline uptake in the caecum and colon of prairie voles (*Microtus ochrogaster*). *Journal of Experimental Biology*, 176, 285-297.
- Jagger, S., Wiseman, J., Cole, D. J. & Craigon, J. (1992). Evaluation of inert markers for the determination of ileal and faecal apparent digestibility values in the pig. *British Journal of Nutrition*, 68(3), 729-739.
- James, K. A., Butts, C. A., Koolaard, J. P., Donaldson, H. E., Scott, M. F. & Moughan, P. J. (2002). The effect of feeding regimen on apparent and true ileal nitrogen digestibility for rats fed diets containing different sources of protein. *Journal of the Science of Food and Agriculture*, 82, 1050-1060.
- James, K. A. C., Body, D. R. & Smith, W. C. (1986). A nutritional evaluation of orange roughy (*Hoplostethus atlanticus*) using growing pigs. *New Zealand Journal of Technology*, 2(4), 219-223.
- James, W. P. T. & Schofield, E. C. (1990). *Human energy requirements. A manual for planners and nutritionists*. Oxford, UK.: Oxford Medical Publications under arrangement with FAO.
- Jones, D. B. (1941). *Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins*. Washington, DC: United States Department of Agriculture.
- Jørgensen, H., Gabert, V. M., Hedemann, M. S. & Jensen, S. K. (2000). Digestion of Fat Does Not Differ in Growing Pigs Fed Diets Containing Fish Oil, Rapeseed Oil or Coconut Oil. *Journal of Nutrition*, 130(4), 852-857.
- Jørgensen, H., Jakobsen, K. & Eggum, B. O. (1992). The influence of different protein, fat and mineral levels on the digestibility of fat and fatty acids measured at the terminal ileum and in feces of growing pigs. *Acta Agriculturae Scandinavica, Section A - Animal Science*, 42, 177-184.

-
- Jørgensen, H., Jakobsen, K. & Eggum, B. O. (1993). Determination of endogenous fat and fatty acids at the terminal ileum and in faeces in growing pigs. *Acta Agriculturae Scandinavica, Section A - Animal Science*, 43, 101-106.
- Jørgensen, H. & Jensen, B. B. (1994). *The effect of dietary fiber on digestibility, microbial activity, and microbial gas production in various regions of the gastrointestinal tract of pigs*. Paper presented at the VIth International Symposium on Digestive Physiology in Pigs, Germany.
- Jørgensen, H., Jensen, S. K. & Eggum, B. O. (1996a). The influence of rapeseed oil on digestibility, energy metabolism and tissue fatty acid composition in pigs. *Acta Agriculturae Scandinavica, Section A - Animal Science*, 46, 65-75.
- Jørgensen, H., Zhao, X.-Q. & Eggum, B. O. (1996b). The influence of dietary fibre and environmental temperature on the development of the gastrointestinal tract, digestibility, degree of fermentation in the hind-gut and energy metabolism in pigs. *British Journal of Nutrition*, 75, 365-378.
- Judd, P. A. (1982). The effects of high intakes of barley on gastrointestinal function and apparent digestibility of dry matter, nitrogen and fat in human volunteers *Journal of Plant Foods*, 4, 79-88.
- Just, A., Jørgensen, H. & Fernández, J. A. (1981). The digestive capacity of the cecumcolon and the value of the nitrogen absorbed from the hindgut for protein synthesis in pigs. *British Journal of Nutrition*, 46(1), 209-219.
- Kadenbach, B. (2003). Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochimica et Biophysica Acta*, 1604(2), 77-94.
- Kararli, T. T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharmaceutics & Drug Disposition*, 16, 351 - 380.
- Karp, G. (2009). *Cell and Molecular Biology: Concepts and Experiments* (6th ed.). Hoboken, NJ: John Wiley and Sons.
- Kasper, H. (1970). Fecal fat excretion, diarrhea, and subjective complaints with highly dosed oral fat intake. *Digestion*, 3, 321-330.

-
- Kavanagh, S., Lynch, P. B., O'Mara, F. & Caffrey, P. J. (2001). A comparison of total collection and marker technique for the measurement of apparent digestibility of diets for growing pigs. *Animal Feed Science and Technology*, 89(1-2), 49-58.
- Kelsay, J. L., Behall, K. M. & Prather, E. S. (1978). Effect of fiber from fruits and vegetables on metabolic responses of human subjects I. Bowel transit time, number of defecations, fecal weight, urinary excretions of energy and nitrogen and apparent digestibilities of energy, nitrogen, and fat. *American Journal of Clinical Nutrition*, 31, 1149-1153.
- King, T. S., Elia, M. & Hunter, J. O. (1998). Abnormal colonic fermentation in irritable bowel syndrome. *Lancet*, 352, 1187-1189.
- Kohler, T., Denhartog, L. A., Huisman, J., Mosenthin, R. & Verstegen, M. W. A. (1991). Digestibility measurements in pigs by using post-valve T-caecum cannulation or end-to-side ileo-rectal anastomosis. *Journal of Animal Physiology and Animal Nutrition*, 66(5), 278-286.
- Kohler, T., Huisman, J., Denhartog, L. A. & Mosenthin, R. (1990). Comparison of different digesta collection methods to determine the apparent digestibilities of the nutrients at the terminal ileum in pigs. *Journal of the Science of Food and Agriculture*, 53(4), 465-475.
- Kozloski, G. V., Flores, E. M. D. & Martins, A. F. (1998). Use of chromium oxide in digestibility studies: Variations of the results as a function of the measurement method. *Journal of the Science of Food and Agriculture*, 76(3), 373-376.
- Lands, W. E. M. (1995). Alcohol and energy intake. *American Journal of Clinical Nutrition*, 62, 1101S - 1106S.
- Lands, W. E. M. & Zakhari, S. (1991). The case of the missing calories. *American Journal of Clinical Nutrition*, 54, 47-48.

-
- Langkilde, A. M., Andersson, H., Faisant, N. & Champ, M. (1994). A comparison between the intubation technique and the ileostomy model for *in vivo* measurement of RS. In N. G. Asp, J. M. M. van Amelsvoort, J. G. Hautvast & A. J. (Eds.), *Proceedings of the Concluding Plenary Meeting of EURESTA* (pp. 28-30). Den Haag, The Netherlands: CIP-Data Koninklijke Bibliotheek.
- Lebet, V., Arrigoni, E. & Amado, R. (1998). Digestion procedure using mammalian enzymes to obtain substrates for *in vitro* fermentation studies. *Lebensmittel-Wissenschaft und-Technologie*, *31*, 509-515.
- Leonard, W. R. (2003). Measuring human energy expenditure: what have we learned from the flex-heart rate method? *American Journal of Human Biology*, *15*(4), 479-489.
- Levine, J. A. (2005). Measurement of energy expenditure. *Public Health Nutrition*, *8*(7A), 1123-1132.
- Levitt, M. D., Li, R., Demaster, E. G., Elson, M., Furne, J. & Levitt, D. G. (1997). Use of measurements of ethanol absorption from stomach and intestine to assess ethanol metabolism. *American Journal of Physiology*, *273*, G951-G957.
- Levy, L. M., Bernstein, L. M. & Grossman, M. I. (1958). *The calorie content of urine of human beings and the estimation of the metabolizable energy of human foodstuffs* (No. 26). Denver, CO: US Army Medical Research and Nutrition Laboratory.
- Li, S. & Sauer, W. C. (1994). The effect of dietary fat content on amino acid digestibility in young pigs. *Journal of Animal Science*, *72*, 1737-1743.
- Libao-Mercado, A. J., Yin, Y., van Eys, J. & de Lange, C. F. M. (2006). True ileal amino acid digestibility and endogenous ileal amino acid losses in growing pigs fed wheat shorts- or casein-based diets. *Journal of Animal Science*, *84*(6), 1351-1361.
- Lieber, C. S. (1988). The influence of alcohol on nutritional status. *Nutrition Reviews*, *46*, 241-254.
- Lieber, C. S. (1991). Hepatic, metabolic and toxic effects of ethanol: 1991 update. Alcohol, liver and nutrition. *Alcoholism, Clinical and Experimental Research*, *15*(4), 573-592.

-
- Lieber, C. S. & DeCarli, L. M. (1968). Ethanol oxidation by hepatic microsomes: adaptive increase after ethanol feeding. *Science*, *162*, 917-918.
- Lindsay, D. B. (1976). Amino acids as sources of energy. In D. J. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale & H. Swan (Eds.), *Protein Metabolism and Nutrition, EAAP Publication no 16* (pp. 183-195). London: Butterworths.
- Lissner, L. & Heitmann, B. L. (1995). Dietary fat and obesity: evidence from epidemiology. *European Journal of Clinical Nutrition*, *49*, 79-90.
- Livesey, G. (1984). The energy equivalents of ATP and the energy values of food proteins and fats. *British Journal of Nutrition*, *51*, 15-28.
- Livesey, G. (1990). Energy values of unavailable carbohydrate and diets: an inquiry and analysis. *American Journal of Clinical Nutrition*, *51*, 617-637.
- Livesey, G. (1991). Calculating the energy value of foods - towards new empirical formulas based on diets with varied intakes of unavailable complex carbohydrates. *European Journal of Clinical Nutrition*, *45*, 1-12.
- Livesey, G. (1992). The energy values of dietary fibre and sugar alcohols for man. *Nutrition Research Reviews*, *5*, 61-84.
- Livesey, G. (1995). Metabolizable energy of macronutrients. *American Journal of Clinical Nutrition*, *62*, 1135S-1142S.
- Livesey, G. (2001). A perspective on food energy standards for nutrition labelling. *British Journal of Nutrition*, *85*, 271-287.
- Livesey, G. & Elia, M. (1995). Short chain fatty acids as an energy source in the colon: metabolism and clinical implications. In J. H. Cummings, J. L. Rombeau & T. Sakata (Eds.), *Physiological and Clinical Aspects of Short Chain Fatty Acids* (pp. 427-481). Cambridge, United Kingdom: Cambridge University Press.
- Livesey, G., Smith, T., Eggum, B. O., Tetens, H., Nyman, M. & Roberfroid, M. (1995a). Determination of digestible energy values and fermentabilities of dietary fibre supplements: a European interlaboratory study *in vivo*. *British Journal of Nutrition*, *74*, 289-302.

-
- Livesey, G., Wilkinson, J. A., Roe, M., Faulks, R., Clark, S., Brown, J. C., Kennedy, H. & Elia, M. (1995b). Influence of the physical form of barley grain on the digestion of its starch in the human small intestine and implications for health. *American Journal of Clinical Nutrition*, 61(1), 75-81.
- Livingstone, R. M., Fowler, V. R., White, F. & Wenham, G. (1977). Annealed glass cannulae for use in digestion studies with pigs. *The Veterinary Record*, 101, 368.
- Longstreth, G. F., Malagelada, J. R. & Go, V. L. W. (1975). Gastric response to a transpyloric duodenal tube. *Gut*, 16(10), 777-780.
- Lovejoy, J. C., de la Bretonne, J. A., Klemperer, M. & Tulley, R. (1996). Abdominal fat distribution and metabolic risk factors: Effects of race. *Metabolism*, 45(9), 1119-1124.
- Low, A. G. (1980). Nutrient absorption in pigs. *Journal of the Science of Food and Agriculture*, 31(11), 1087-1130.
- Macfarlane, G. T. & Englyst, H. N. (1986). Starch utilization by the human large intestinal microflora. *Journal of Applied Bacteriology*, 60, 195-201.
- Macfarlane, G. T. & Gibson, G. (1997). Carbohydrate fermentation, energy transduction and gas metabolism in the human large intestine. In R. I. Mackie & B. A. White (Eds.), *Ecology and Physiology of Gastrointestinal Microbes: Gastrointestinal Ecosystems and Fermentations* (pp. 269-318). New York: Chapman and Hall.
- Macfarlane, G. T., Gibson, G. R., Beatty, E. & Cummings, J. H. (1992). Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. *FEMS Microbiology Ecology*, 101, 81-88.
- MacLean, W. C., Jr. (2005). Use of the net metabolizable energy values for labelling of infant formulas and foods - potential issues. *Journal of Food Composition and Analysis*, 18, 223-239.
- Macrae, A. R. & Neudoerffer, T. S. (1972). Support for the existence of an active transport mechanism of fructose in the rat. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 288(1), 137-144.

-
- Mahé, S., Huneau, J. F., Marteau, P., Thuillier, F. & Tomé, D. (1992). Gastroileal nitrogen and electrolyte movements after bovine-milk ingestion in humans. *American Journal of Clinical Nutrition*, 56(2), 410-416.
- Mahé, S., Roos, N., Benamouzig, R., Davin, L., Luengo, C., Gagnon, L., Gausserès, N., Huneau, J. F., Rautureau, N. & Tomé, D. (1996). Gastrojejunal kinetics and the digestion of ¹⁵N-beta-lactoglobulin and casein in humans: The influence of the nature and quantity of the protein. *American Journal of Clinical Nutrition*, 63(4), 546-552.
- Marcinek, D. J., Schenkman, K. A., Ciesielski, W. A. & Conley, K. E. (2004). Mitochondrial uncoupling *in vivo* in mouse skeletal muscle. *American Journal of Physiology*, 286, C457-C463.
- Mariotti, F., Mahé, S., Luengo, C., Benamouzig, R. & Tomé, D. (2000). Postprandial modulation of dietary and whole-body nitrogen utilization by carbohydrates in humans. *American Journal of Clinical Nutrition*, 72(4), 954-962.
- Mariotti, F., Pueyo, M. E., Tomé, D., Bérot, S., Benamouzig, R. & Mahé, S. (2001). The influence of the albumin fraction on the bioavailability and postprandial utilization of pea protein given selectively to humans. *Journal of Nutrition*, 131(6), 1706-1713.
- Mariotti, F., Pueyo, M. E., Tomé, D. & Mahé, S. (2002). The bioavailability and postprandial utilisation of sweet lupin (*Lupinus albus*)-flour protein is similar to that of purified soyabean protein in human subjects: A study using intrinsically ¹⁵N-labelled proteins. *British Journal of Nutrition*, 87(4), 315-323.
- Marteau, P. & Boutron-Ruault, M. C. (2002). Nutritional advantages of probiotics and prebiotics. *British Journal of Nutrition*, 87, S153-157.
- Mathers, J. C. & Annison, E. F. (1993). Stoichiometry of polysaccharide fermentation in the large intestine. In S. Samman & G. Annison (Eds.), *Dietary Fibre and Beyond – Australian Perspectives* (Vol. 1, pp. 123–135). Perth, Australia: Nutrition Society of Australia Occasional Publications.
- Mathers, J. C. & Wolever, T. M. S. (2009). Digestion and Metabolism of Carbohydrates. In M. J. Gibney, S. A. Lanham-New & A. Cassidy (Eds.), *Introduction to Human Nutrition* (pp. 74-121). Chichester, UK: Wiley-Blackwell.

-
- Mathews, R. H. (1995). Nutrient databank perspectives on energy. *American Journal of Clinical Nutrition*, 62, 1147S-1150S.
- Matthews, D. E., Marano, M. A. & Campbell, R. G. (1993). Splanchnic bed utilization of glutamine and glutamic acid in humans. *American Journal of Physiology*, 264, E848-854.
- Matthews, D. M., Crampton, R. F. & Lis, M. T. (1971). Sites of maximal intestinal absorptive capacity for amino acids and peptides: evidence for an independent peptide uptake system or systems. *Journal of Clinical Pathology*, 29(9), 882-883.
- McBurney, M. I. & Thompson, L. U. (1989). Dietary fiber and energy balance: integration of the human ileostomy and *in vitro* fermentation models. *Animal Feed Science and Technology*, 23, 261-275.
- McBurney, M. I., Thompson, L. U. & Jenkins, D. J. A. (1987). Colonic fermentation of some breads and its implications for energy availability in man. *Nutrition Research*, 7, 1229-1241.
- McCance, R. A. & Widdowson, E. H. (1946). *The Composition of Foods* (2nd ed.). London: Medical Research Council.
- McCrary, M. A., Fuss, P. J., Saltzman, E. & Roberts, S. B. (2000). Dietary determinants of energy intake and weight regulation in healthy adults. *Journal of Nutrition*, 130, 276S-279S.
- McCue, M. D. (2006). Specific dynamic action: A century of investigation. *Comparative Biochemistry and Physiology*, 144, 381-394.
- McDonald, G. B., Saunders, D. R., Weidman, M. & Fisher, L. (1980). Portal venous transport of long chain fatty acids absorbed from rat intestine. *American Journal of Physiology*, 239, G141-G150.
- McDonald, P., Greenhalgh, J. F. D., Edwards, R. A. & Morgan, C. A. (1995). *Animal Nutrition* (5th ed.). New York: Longmans.
- McGarry, J. D., Kuwajima, M., Newgard, C. B. & Foster, D. W. (1987). From dietary glucose to liver glycogen: the full circle round. *Annual Review of Nutrition*, 7, 51-73.

-
- McLean, J. A. & Tobin, G. (2008). *Animal and Human Calorimetry*. New York: Cambridge University Press.
- McLean, R. A. H., Eastwood, M. A., Brydon, W. G., Anderson, J. R. & Anderson, D. M. W. (1983). A study of the effects of dietary gum arabic in humans. *American Journal of Clinical Nutrition*, 37, 368-375.
- McNeil, N. I. (1984). The contribution of the large intestine to energy supplies in man. *American Journal of Clinical Nutrition*, 39, 338-342.
- McNeil, N. I., Cummings, J. H. & James, W. P. T. (1978). Short-chain fatty acid absorption by the human large intestine. *Gut*, 19, 819-822.
- Medhus, A. W., Sandstad, O., Bredesen, J. & Husebye, E. (1999). Delay of gastric emptying by duodenal intubation: Sensitive measurement of gastric emptying by the paracetamol absorption test. *Alimentary Pharmacology and Therapeutics*, 13(5), 609-620.
- Medical Research Council. (1945). *Nutritive Value of Wartime Foods*. London: His Majesty's Stationery Office.
- Merrill, A. L. & Watt, B. K. (1955). *Energy Value of Foods, Basis and Derivation*. Washington, DC: United States Department of Agriculture
- Merrill, A. L. & Watt, B. K. (1973). Energy value of foods: basis and derivation. In *Agriculture Handbook No 74*. Washington, DC: Agricultural Research Service, United States Department of Agriculture.
- Mezey, E. (1985). Effect of ethanol on intestinal morphology, metabolism, and function. In H. K. Seitz & B. Kommerell (Eds.), *Alcohol-Related Diseases in Gastroenterology* (pp. 342-360). New York: Springer-Verlag.
- Miller, D. S. & Judd, P. A. (1984). The metabolizable energy value of foods. *Journal of the Science of Food and Agriculture*, 35, 111-116.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, 13, 501-508.
- Miller, W. C., Lindeman, A. K., Wallace, I. & Niederpruem, M. (1990). Diet composition, energy intake, and exercise in relation to body fat in men and women. *American Journal of Clinical Nutrition*, 52, 426-430.

-
- Ministry of Health. (2008). *A Portrait of Health: Key results of the 2006/07 New Zealand Health Survey*. Wellington, New Zealand: Ministry of Health.
- Ministry of Health. (2009). *Alcohol Use in New Zealand: Key results of the 2007/08 New Zealand Alcohol and Drug Use Survey*. Wellington, New Zealand: Ministry of Health.
- Mitchell, H. H. (1954). The dependence of the biological value of proteins and amino acid interrelationships. In V. K. Nehring (Ed.), *Die Bewertung der Futterstoffe und andere Probleme der Tierernahrung*. Berlin: Deutsche Akademie der Landwirtschaftswissenschaften.
- Modigliani, R., Rambaud, J. C. & Bernier, J. J. (1973). The method of intraluminal perfusion of the human small intestine. I. Principle and technique. *Digestion*, 9(2), 176-192.
- Mokady, S. (1973). Effect of dietary pectin and algin on blood cholesterol level in growing rats fed a cholesterol-free diet. *Nutrition and Metabolism*, 15, 290-294.
- Monro, J. & Burlingame, B. (1996). Carbohydrates and related food components: INFOODS tag names, meanings, and uses. *Journal of Food Composition and Analysis*, 9, 100-118.
- Moran, B. J. & Jackson, A. A. (1990). ¹⁵N-urea metabolism in the functioning human colon: luminal hydrolysis and mucosal permeability. *Gut*, 31, 454-457.
- Morgan, R. G. & Hoffman, N. E. (1971). The interaction of lipase, lipase cofactor and bile salts in triglyceride hydrolysis. *Biochimica et Biophysica Acta*, 248(1), 143-148.
- Mosenthin, R. (2002). Current concepts of protein digestion and absorption in the pig - review. *Veterinarija ir Zootechnika*, 20(42), 30-38.
- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16, 127-141.
- Moughan, P. J. (2005). Dietary protein quality in humans - An overview. *Journal of AOAC International*, 88(3), 874-876.

-
- Moughan, P. J., Birtles, M. J., Cranwell, P. D., Smith, W. C. & Pedraza, M. (1992). The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. *World Review of Nutrition and Dietetics*, 67, 40-113.
- Moughan, P. J., Butts, C. A., van Wijk, H., Rowan, A. M. & Reynolds, G. W. (2005). An acute ileal amino acid digestibility assay is a valid procedure for use in human ileostomates. *Journal of Nutrition*, 135(3), 404-409.
- Moughan, P. J., Cranwell, P. D., Darragh, A. J. & Rowan, A. M. (1994). The domestic pig as a model animal for studying digestion in humans. In W. B. Souffrant & H. Hagemester (Eds.), *Proc VIth Int Symp on Digestive Physiology in Pigs* (Vol. 80, pp. 389–396). Bad Doberan, Germany: EAAP Publ.
- Moughan, P. J. & Rowan, A. M. (1989). The pig as a model animal for human nutrition research. *Proceedings of the Nutrition Society of New Zealand*, 14, 116-123.
- Moughan, P. J., Smith, W. C. & James, K. A. C. (1984). Preliminary observations on the use of the rat as a model for the pig in determination of apparent digestibility of dietary proteins. *New Zealand Journal of Agricultural Research*, 27, 509-512.
- Moughan, P. J., Smith, W. C. & Kies, A. K. (1987). Comparison of the ileal digestibility of amino acids in ground barley for the growing rat and pig. *New Zealand Journal of Agricultural Research*, 30, 59-66.
- Mroz, Z., Bakker, G. C. M., Jongbloed, A. W., Dekker, R. A., Jongbloed, R. & vanBeers, A. (1996). Apparent digestibility of nutrients in diets with different energy density, as estimated by direct and marker methods for pigs with or without ileocecal cannulas. *Journal of Animal Science*, 74(2), 403-412.
- Muller-Lissner, S. A., Fimmel, C. J., Will, N., Muller-Duysing, W., Heinzl, F. & Blum, A. L. (1982). Effect of gastric and transpyloric tubes on gastric emptying and duodenogastric reflux. *Gastroenterology*, 83(6), 1276-1279.
- Murphy, M. P. (1989). Slip and leak in mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta*, 977, 123-141.
- National Research Council. (1981). *Nutritional energetics of domestic animals: and glossary of terms, 2nd revision*. Washington, DC: National Academy Press.

-
- Nichols, J., Ross, S. & Patterson, P. (1988). Thermic effect of food at rest and following swim exercise in trained college men and women. *Annals of Nutrition and Metabolism*, 32, 215-219.
- Nielson, S. S. (2003). *Food Analysis* (3 ed.). New York: Birkhäuser.
- Nixon, S. E. & Mawer, G. E. (1970). The digestion and absorption of protein in man. 2. The form in which digested protein is absorbed. *British Journal of Nutrition*, 24(1), 241-258.
- Noah, L., Guillon, F., Bouchet, B., Buleon, A., Molis, C., Gratas, M. & Champ, M. (1998). Digestion of carbohydrate from white beans (*Phaseolus vulgaris* L.) in healthy humans. *Journal of Nutrition*, 128(6), 977-985.
- Noblet, J., Fortune, H., Dubois, S. & Henry, Y. (1989). *Nouvelles Bases d'Estimation des Teneurs en Energie Digestible, Metabolisable et Nette des Aliments pour le Porc (New Basis for Estimation of Digestible and Metabolizable Net Energy in Swine Feeds)*. Saint Gilles: INRA.
- Noblet, J., Fortune, H., Shi, X. S. & Dubois, S. (1994). Prediction of net energy values of feeds for growing pigs. *Journal of Animal Science*, 72, 344–354.
- Noblet, J. & Perez, J. M. (1993). Prediction of digestibility of nutrients and energy values of pig diets from chemical analysis. *Journal of Animal Science*, 71, 3389-3398.
- North American Association for the Study of Obesity. (2000). Report on the supply and demand of ¹⁸O-enriched water. Retrieved 13 October 2010, from <http://www.obesity.org/about/19990121.asp>
- Nyachoti, C. M., deLange, C. F. M., McBride, B. W. & Schulze, H. (1997). Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: A review. *Canadian Journal of Animal Science*, 71(1), 149-163.
- Nyman, M., Asp, N. G., Cummings, J. H. & Wiggins, H. (1986). Fermentation of dietary fibre in the intestinal tract: comparison between man and rat. *British Journal of Nutrition*, 55, 487-496.
- Øverland, M., Mroz, Z. & Sundstøl, F. (1994). Effect of lecithin on the apparent ileal and overall digestibility of crude fat and fatty acids in pigs. *Journal of Animal Science*, 72, 2022-2028.

-
- Paddon-Jones, D., Westman, E., Mattes, R. D., Wolfe, R. R., Astrup, A. & Westerterp-Plantenga, M. (2008). Protein, weight management, and satiety. *American Journal of Clinical Nutrition*, 87, 1558S-1561S.
- Patterson, J. K., Lei, X. G. & Miller, D. D. (2008). The pig as an experimental model for elucidating the mechanisms governing dietary influence on mineral absorption. *Experimental Biology and Medicine*, 233(6), 651-664.
- Pawan, G. L. S. (1972). Metabolism of alcohol (ethanol) in man. *Proceedings of the Nutrition Society* 31, 83-89.
- Pearson, G., Moughan, P. J., Dong, G. Z. & Morel, P. C. H. (1999). Protein quality in blood meal. I. The rat as a model animal for determining apparent ileal amino acid digestibility in the growing pig. *Animal Feed Science and Technology*, 79, 301-307.
- Peddie, J., Dewar, W. A., Gilbert, A. B. & Waddington, D. (1982). The use of titanium dioxide for determining apparent digestibility in mature domestic fowls (*Gallus domesticus*). *Journal of Agricultural Science*, 99 (Aug), 233-236.
- Phuc, B. H. N. & Lindberg, J. E. (2000). Ileal and total tract digestibility in growing pigs given cassava root meal diets with inclusion of cassava leaves, leucaena leaves and groundnut foliage. *Animal Science*, 71, 301-308.
- Picard, M., Bertrand, S., Duron, M. & Maillard, R. (1984). *Comparative digestibility of amino acids using 5 animal models: intact cockerel, caectomised cockerels, rat deprived of large intestine, piglet with an ileo-caecal cannulation, piglet with an ileo-rectal shunt*. Paper presented at the Proceedings of the IVth European Symposium of Poultry Nutrition, Tours, France.
- Pleis, J. R., Lucas, J. W. & Ward, B. W. (2009). Summary health statistics for U.S. adults: National Health Interview Survey, 2008. *Vital and Health Statistics*, 10(242).
- Pomare, E. W., Branch, W. J. & Cummings, J. H. (1985). Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood. *Journal of Clinical Investigation*, 75, 1448-1458.

-
- Poppitt, S. D., Livesey, G., Faulks, R. M., Roe, M., Prentice, A. M. & Elia, M. (1996). Circadian patterns of total 24-h hydrogen and methane excretion in humans ingesting nonstarch polysaccharide (NSP) diets and the implications for indirect calorimetric and D2 18O methodologies. *European Journal of Clinical Nutrition*, 50, 524-534.
- Prentice, A. M. (1995). Are all calories equal? In R. Cottrell (Ed.), *Weight Control: The Current Perspective* (pp. 8-33). London: Chapman & Hall.
- Prentice, A. M. (1998). Manipulation of dietary fat and energy density: effect on substrate flux and food intake. *American Journal of Clinical Nutrition*, 67, 535S-541S.
- Prentice, A. M. (2005). Macronutrients as sources of food energy. *Public Health Nutrition*, 8(7A), 932-939.
- Prentice, A. M., Black, A. E., Coward, W. A., Davies, H. L., Goldberg, G. R., Murgatroyd, P. R., Ashford, J., Sawyer, M. & Whitehead, R. G. (1986). High levels of energy expenditure in obese women. *British Medical Journal (Clinical Research Education)*, 292, 983-987.
- Ranhotra, G. S., Gelroth, J. A., Astroth, K. & Eisenbraun, G. J. (1991). Effect of resistant starch on intestinal responses in rats. *Cereal Chemistry*, 68(2), 130-132.
- Ranhotra, G. S., Gelroth, J. A. & Glaser, B. K. (1996). Energy value of resistant starch. *Journal of Food Science*, 61(2), 453-455.
- Ravussin, E., Harper, I. T., Rising, R. & Bogardus, C. (1991). Energy expenditure by doubly labelled water: Validation in lean and obese subjects. *American Journal of Physiology*, 261, E402-E409.
- Reilly, K. J. & Rombeau, J. L. (1993). Metabolism and potential clinical applications of short-chain fatty acids. *Clinical Nutrition*, 12, S97-105.
- Rennie, K. L., Hennings, S. J., Mitchell, J. & Wareham, N. J. (2001). Estimating energy expenditure by heart-rate monitoring without using individual calibration. *Medicine and Science in Sports and Exercise*, 33(6), 939-945.
- Rich, N., Satterlee, L. D. & Smith, J. L. (1980). A comparison of *in vivo* apparent digestibility as determined using human and rat pancreatins and commercially available proteases. *Nutrition Reports International*, 21, 285-299.

-
- Ritchey, S. J. & Taper, L. J. (1981). Estimating protein digestibility for humans from rat assays. In C. E. Bodwell, J. S. Adkins & D. T. Hopkins (Eds.), *Protein Quality in Humans: Assessment and In vitro Estimation* (pp. 306-315). Westport, CT: AVI Pub. Co.
- Rolfe, D. F. S. & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological Reviews*, 77(3), 731-759.
- Rolfe, D. F. S., Newman, J. M. B., Buckingham, J. A., Clark, M. G. & Brand, M. D. (1999). Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver to SMR. *American Journal of Physiology*, 276, C692-C699.
- Romieu, I., Willett, W. C., Stampfer, M. J., Colditz, G. A., Sampson, L., Rosner, B., Hennekens, C. H. & Speizer, F. E. (1988). Energy intake and other determinants of relative weight. *American Journal of Clinical Nutrition*, 47, 406-412.
- Roskoski, R. & Roskoski, R., Jr. (1996). *Biochemistry* (1st ed.). Philadelphia, PA: W.B. Saunders Co.
- Rowan, A. M., Moughan, P. J. & Wilson, M. N. (1991). Acid insoluble ash as a marker compound for use in digestibility studies with humans. *Journal of the Science of Food and Agriculture*, 54(2), 269-274.
- Rowan, A. M., Moughan, P. J. & Wilson, M. N. (1993). Endogenous amino acid flow at the terminal ileum of adult humans determined following the ingestion of a single protein-free meal. *Journal of the Science of Food and Agriculture*, 61(4), 439-442.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K. & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71, 29-42.
- Rubin, R. & Strayer, D. S. (2008). *Rubin's Pathology: Clinicopathologic Foundations of Medicine* (5th ed.). Philadelphia: Lippincott Williams and Wilkins.
- Rubner, M. (1902). In R. T. J. Joy (Ed.), *The Law of Energy Consumption in Nutrition* (pp. 7-57). Washington, DC: US Army Research Institute.

-
- Ruppin, H., Bar-Meir, S., Soergel, K. H., Wood, C. M. & Schmitt, M. G., Jr. (1980). Absorption of short-chain fatty acids by the colon. *Gastroenterology*, *78*, 1500-1517.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, *87*, 292-300.
- Sajilata, M. G., Rekha, S. S. & Pushpa, R. K. (2006). Resistant Starch - A Review. *Comprehensive Reviews in Food Science and Food Safety*, *5*, 1-17.
- Sandberg, A.-S., Andersson, H., Hallgren, B., Hasselblad, K. & Isaksson, B. (1981). Experimental model for *in vivo* determination of dietary fibre and its effects on the absorption of nutrients in the small intestine. *British Journal of Nutrition*, *45*, 283-294.
- Sandstrom, B., Andersson, H., Kivisto, B. & Sandberg, A.-S. (1986). Apparent small intestinal absorption of nitrogen and minerals from soy and meat protein based diets. *Journal of Nutrition*, *116*, 2209-2218.
- Sauer, W. C., Dugan, M. E. R., de Lange, C. F. M., Imbeah, M. & Mosenthin, R. (1989). Considerations in methodology for the determination of amino acid digestibilities in feedstuffs for pigs. In M. Friedman (Ed.), *Absorption and Utilization of Amino Acids* (Vol. III, pp. 217-230). Florida: CRC Press Inc.
- Sauer, W. C. & Ozimek, L. (1986). Digestibility of amino acids in swine: Results and their practical applications. A review. *Livestock Production Science*, *15*, 367-388.
- Saunders, D. R. & Wiggins, H. S. (1981). Conservation of mannitol, lactulose, and raffinose by the human colon. *American Journal of Physiology*, *21*, G397-G402.
- Schedl, H. P. & Clifton, J. A. (1961). Kinetics of intestinal absorption in man: Normal subjects and patients with sprue *Journal of Clinical Investigation*, *40*, 1079-1080.
- Schoeller, D. A. & Fjeld, C. R. (1991). Human energy metabolism: what have we learned from the doubly labeled water method. *Annual Review of Nutrition*, *11*, 355-373.

-
- Schoeller, D. A. & Hnilicka, J. M. (1996). Reliability of the doubly-labeled water method for the measurement of total daily energy expenditure in freelifing subjects. *Journal of Nutrition*, 126, S348–354.
- Schrauwen, P., van Marken Lichtenbelt, W. D., Saris, W. H. M. & Westerterp, K. R. (1998). Fat balance in obese subjects: role of glycogen stores. *American Journal of Physiology*, 274, E1-7.
- Serena, A., Jørgensen, H. & Bach Knudsen, K. E. (2007). The absorption of lactic acid is more synchronized with the absorption of glucose than with the absorption of short-chain fatty acids - A study with sows fed diets varying in dietary fibre. *Livestock Science*, 109(1-3), 118-121.
- Shetty, P. S. (2005). Energy requirements of adults. *Public Health Nutrition*, 8(7A), 994-1009.
- Shetty, P. S., Prentice, A. M., Goldberg, G. R., Murgatroyd, P. R., McKenna, A. P. M., Stubbs, R. J. & Volschenk, P. A. (1994). Alterations in fuel selection and voluntary food intake in response to isoenergetic manipulation of glycogen stores in humans. *American Journal of Clinical Nutrition*, 60, 534-543.
- Shimomura, Y., Yamamoto, Y., Bajotto, G., Sato, J., Murakami, T., Shimomura, N., Kobayashi, H. & Mawatari, K. (2006). Nutraceutical effects of branched-chain amino acids on skeletal muscle. *Journal of Nutrition*, 136(2), 529S-532.
- Short, F. J., Gorton, P., Wiseman, J. & Boorman, K. N. (1996). Determination of titanium oxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, 59, 215-221.
- Skilton, G. A., Smith, W. C. & Moughan, P. J. (1991). The ileal digestibility of nitrogen and amino acids in meat and bone meals determined using a rat assay. *Animal Feed Science and Technology*, 34, 111-126.
- Slavin, J. L. (2005). Dietary fiber and body weight. *Nutrition*, 21, 411-418.
- Smith, D. A., Dollman, J., Withers, R. T., Brinkman, M., Keeves, J. P. & Clark, D. G. (1997). Relationship between maximum aerobic power and resting metabolic rate in young adult women. *Journal of Applied Physiology*, 82(1), 156-163.
- Smith, J. G. & German, J. B. (1995). Molecular and genetic effects of dietary derived butyric acid. *Food Technology*, 49(87-90).

-
- Smith, M. E. & Morton, D. (2001). *The Digestive System*. Philadelphia, PA: Elsevier Health Sciences.
- Smith, W. C., Moughan, P. J., Pearson, G. & James, K. A. C. (1987). Comparative bioavailable energy values of five ground cereal grains measured with growing rats and pigs. *Animal Feed Science and Technology*, 18, 143-150.
- Soergel, K. H. (1994). Colonic fermentation: metabolic and clinical implications. *Clinical Investigator*, 72, 742-748.
- Sonko, B. J., Prentice, A. M., Murgatroyd, P. R., Goldberg, G. R., Van de Ven, M. L. H. M. & Coward, W. A. (1994). Effect of alcohol on postmeal fat storage. *American Journal of Clinical Nutrition*, 59, 619-625.
- Southgate, D. A. & Durnin, J. V. (1970). Calorie conversion factors. An experimental reassessment of the factors used in the calculation of the energy value of human diets. *British Journal of Nutrition*, 24(2), 517-535.
- Southgate, D. A. T. (1975). Fiber and other unavailable carbohydrates and energy effects in the diet. In P. L. White & N. Selvey (Eds.), *Proceedings of the Western Hemisphere Nutrition Congress IV* (pp. 51-55). Acton, MA: Publishing Science Group Inc.
- Speakman, J. R. (1998). The history and theory of the doubly labeled water technique. *American Journal of Clinical Nutrition*, 68(4), 932S-938.
- Stein, H. H., Sève, B., Fuller, M. F., Moughan, P. J. & de Lange, C. F. M. (2007). Invited review: Amino acid bioavailability and digestibility in pig feed ingredients: Terminology and application. *Journal of Animal Science*, 85(1), 172-180.
- Stephen, A. M. & Cummings, J. H. (1980a). Mechanism of action of dietary fibre in the human colon. *Nature*, 284, 283-284.
- Stephen, A. M. & Cummings, J. H. (1980b). The microbial contribution to human faecal mass. *Journal of Medical Microbiology*, 13(1), 45-56.
- Stevens, J., Levitsky, D. A., Van Soest, P. J., Roberts, J. B., Kalkwarf, H. J. & Roe, A. D. (1987). Effect of psyllium gum and wheat bran on spontaneous energy intake. *American Journal of Clinical Nutrition*, 46, 812-817.

-
- Strath, S. J., Swartz, A. M., Bassett, D. R., Jr., O'Brien, W. L., King, G. A. & Ainsworth, B. E. (2000). Evaluation of heart rate as a method for assessing moderate intensity physical activity. *Medicine and Science in Sports and Exercise*, 32(9), S465-S470.
- Stryer, L., Berg, J. M. & Tymoczko, J. L. (2002). *Biochemistry* (5th ed.). New York: W.H. Freeman and Co. Ltd.
- Suter, P. M., Schutz, Y. & Je'quier, E. (1992). The effect of ethanol on fat storage in healthy subjects. *New England Journal of Medicine*, 326, 983-987.
- Swift, W. (1957). The nutritive evaluation of forages. *Nutrition Reviews*, 15, 240-243.
- Thomas, C. D., Peters, J. C., Reed, G. W., Abumrad, N. N., Sun, M. & Hill, J. O. (1992). Nutrient balance and energy expenditure during *ad libitum* feeding of high-fat and high-carbohydrate diets in humans. *American Journal of Clinical Nutrition*, 55, 934-942.
- Topping, D. L. & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews*, 81, 1031-1064.
- Truswell, A. S., Seach, J. M. & Thorburn, A. W. (1988). Incomplete absorption of pure fructose in healthy subjects and the facilitating effect of glucose. *American Journal of Clinical Nutrition*, 48, 1424-1430.
- Tso, P. & Balint, J. A. (1986). Formation and transport of chylomicrons by enterocytes to the lymphatics. *American Journal of Physiology*, 250, G715-G726.
- Vahouny, G. V. & Cassidy, M. M. (1987). Effect of dietary fiber on intestinal absorption of lipids. In G. A. Spiller (Ed.), *CRC Handbook of Dietary Fiber in Human Nutrition* (pp. 121-128). Boca Raton: CRC Press.
- van Leeuwen, P., van Kleef, D. J., van Kempen, G. J. M., Huisman, J. & Verstegen, M. W. A. (1991). The post valve T-caecum cannulation technique in pigs applicated to determine the digestibility of amino acid in maize, groundnut and sunflower meal. *Journal of Animal Physiology and Animal Nutrition* 65(3-4), 183-193.
- van Milgen, J. (2002). Modeling biochemical aspects of energy metabolism in mammals. *Journal of Nutrition*, 132, 3195-3202.

-
- Van Soest, P. J., Jeraci, J., Foose, T., Wrick, K. & Ehle, F. (1983). Comparative fermentation of fibre in man and other animals. In G. Wallace & L. Bell (Eds.), *Fibre in Human and Animal Nutrition: Proceedings of Dietary Fibre in Human and Animal Nutrition Symposium* (pp. 75-80). Palmerston North, New Zealand: The Royal Society of New Zealand.
- Van Soest, P. J. & Wine, R. H. (1967). Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. *Journal of AOAC International*, *50*, 50-55.
- van Wijk, H. J., Moughan, P. J., Hodgkinson, S. M., Jansen, P. P. & Pearson, G. (1998). Variation in apparent and true ileal amino acid digestibility in barley using a rat model. *Animal Feed Science and Technology*, *76*, 9-22.
- Veldhorst, M., Smeets, A., Soenen, S., Hochstenbach-Waelen, A., Hursel, R., Diepvens, K., Lejeune, M., Luscombe-Marsh, N. & Westerterp-Plantenga, M. (2008). Protein-induced satiety: Effects and mechanisms of different proteins. *Physiology & Behavior*, *94*, 300-307.
- Walker, B. E., Kelleher, J., Davies, T., Smith, C. L. & Losowsky, M. S. (1973). Influence of dietary fat on fecal fat. *Gastroenterology*, *64*, 233-239.
- Walser, M. & Bodenlos, L. J. (1959). Urea metabolism in man. *Journal of Clinical Investigation*, *38*, 1617-1626.
- Wang, J. F., Jensen, B. B., Jørgensen, H., Li, D. F. & Lindberg, J. E. (2002). Ileal and total tract digestibility, and protein and fat balance in pigs fed rice with addition of potato starch, sugar beet pulp or wheat bran. *Animal Feed Science and Technology*, *102*(1-4), 125-136.
- Warwick, P. M. (2005). Expression of energy: commentary on the case for net metabolizable energy. *Journal of Food Composition and Analysis*, *18*, 241-247.
- Warwick, P. M. & Baines, J. (2000). Point of view: energy factors for food labelling and other purposes should be derived in a consistent fashion for all food components. *British Journal of Nutrition*, *84*, 897-902.

-
- Weaver, G. A., Krauser, J. A., Miller, T. L. & Wolin, M. J. (1988). Short chain fatty acid distributions of enema samples from a sigmoidoscopy population: an association of high acetate and low butyrate ratios with adenomatous polyps and colon cancer. *Gut*, 29, 1539-1543.
- Weinsier, R. L., Hunter, G. R., Gower, B. A., Schutz, Y., Darnell, B. E. & Zuckerman, P. A. (2001). Body fat distribution in white and black women: different patterns of intraabdominal and subcutaneous abdominal adipose tissue utilization with weight loss. *American Journal of Clinical Nutrition*, 74(5), 631-636.
- Westerterp-Platenga, M. S., Nieuwenhuizen, A., Tomé, D., Soenen, S. & Westerterp, K. R. (2009). Dietary protein, weight loss, and weight maintenance. *Annual Review of Nutrition*, 29, 21-41.
- Westerterp, K. R. (1998). Alterations in energy balance with exercise. *American Journal of Clinical Nutrition*, 68(4), 970S-974S.
- Westerterp, K. R., Prentice, A. M. & Jéquier, E. (1999). Alcohol and body weight. In I. Macdonald (Ed.), *Health Issues Related to Alcohol Consumption* (pp. 103-123). Oxford: Blackwell Science.
- Whittemore, C. T. (1983). Development of recommended energy and protein allowances for growing pigs. *Agricultural Systems* 11, 159-186.
- Whittemore, C. T. (1997). An analysis of methods for the utilisation of net energy concepts to improve the accuracy of feed evaluation in diets for pigs. *Animal Feed Science and Technology*, 68, 89-99.
- WHO. (2000). *Obesity: Preventing and managing the global epidemic*. Geneva: World Health Organization.
- Wilfart, A., Montagne, L., Simmins, H., Noblet, J. & van Milgen, J. (2007). Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. *Livestock Science*, 109(1-3), 27-29.
- Willett, W. C. (1998). Dietary fat and obesity: an unconvincing relation. *American Journal of Clinical Nutrition*, 68, 1149-1150.
- Williams, B. A., Verstegen, M. W. A. & Tamminga, S. (2001). Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutrition Research Reviews*, 14, 207-227.

-
- Wisker, E., Bach Knudsen, K. E., Daniel, M., Eggum, B. O. & Feldheim, W. (1997). Energy values of non-starch polysaccharides: comparative studies in humans and rats. *Journal of Nutrition*, *127*, 108-116.
- Wisker, E., Bach Knudsen, K. E., Daniel, M., Feldheim, W. & Eggum, B. O. (1996). Digestibilities of energy, protein, fat and nonstarch polysaccharides in a low fiber diet and diets containing coarse or fine whole meal rye are comparable in rats and humans. *Journal of Nutrition*, *126*(2), 481-488.
- Wisker, E., Maltz, A. & Feldheim, W. (1988). Metabolizable energy of diets low or high in dietary fiber from cereals when eaten by humans. *Journal of Nutrition*, *118*, 945-952.
- Witczak, Z. J. (2008). Monosaccharides: occurrence, significance, and properties. *Glycoscience*, *4*, 815-840.
- Zaloga, G. P. (1990). Physiological effects of peptide-based enteral formulas. *Nutrition in Clinical Practice*, *5*, 231-237.
- Zoran, D., Barhoumi, R., Burghardt, R., Chapkin, R. & Lupton, J. (1997). Diet and carcinogen alter luminal butyrate concentrations and intracellular pH in isolated rat colonocytes. *Nutrition and Cancer*, *27*, 222-230.
- Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, *86*, 1649-1656.

CHAPTER II

Specific review of methodologies:

***In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals**

This chapter, in the form of a published review, extends the review of literature (Chapter I) and deals specifically with the use of in vitro digestion and fermentation methods to predict the uptake of energy from the hindgut of humans and other simple-stomached animals.

Published in: Coles, L. T., Moughan, P. J., & Darragh, A. J. (2005). In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Animal Feed Science and Technology, 123, 421 – 444.

Abstract

For the purposes of food evaluation, *in vitro* digestion / fermentation methods are ethically superior, faster, and less expensive than *in vivo* techniques, whilst still offering a degree of animal-food interaction that pure chemical analysis lacks. One such *in vitro* fermentation method is the *in vitro* gas production technique, which utilises the relationship between degradation and fermentative gas production to evaluate the nutritional parameters of foodstuffs. Several different methodologies have been proposed for the gas production technique, each varying in its complexity, shortcomings and benefits. Although historically the gas production technique has been used almost exclusively with ruminants, it may also be of value for the nutritive evaluation of foods for man and other monogastric animals. Among the benefits of the technique are: being able to run large batches simultaneously at a low cost, the ability to measure the fermentation kinetics of the soluble as well as the insoluble fraction of food, and the ability to easily make relative comparisons among different foodstuffs.

The present contribution reviews the *in vitro* gas production technique and *in vitro* hindgut digestion assays generally, for their application in predicting *in vitro* hindgut digestion and fermentation in humans and monogastric farm animals. It is concluded that currently available *in vitro* digestion methods of relevance to human food evaluation lack standardisation, *in vivo* validation and justification to support their specific methodology, and have not been tested with a wide range of fermentative substrates.

Introduction

Historically, and with regard to evaluating the digestibility and availability of dietary energy in humans, *in vitro* digestion methods have focused primarily on upper-tract digestion, the hindgut being considered of little nutritional significance (Cummings, 1983; Dobbins and Binder, 1981; Ramakrishna *et al.*, 1990). However, the need for accurate *in vitro* methods to study digestion and fermentation in the hindgut of humans has become increasingly apparent given the recently recognised role the hindgut has in nutrition and gut health (Cummings, 1996; Williams *et al.*, 2001c). This review addresses *in vitro* methods, including the *in vitro* gas production technique, for the study of hindgut digestion and fermentation in humans, and where appropriate as a means of comparison, in monogastric farm animals. The need for and purpose of *in vitro* digestion methods is discussed. The design features of batch *in vitro* fermentation methods are outlined along with a discussion of the criteria that a sound method should fulfil and a critical assessment of current *in vitro* hindgut digestion / fermentation methods.

I. The need for *in vitro* hindgut digestion / fermentation methods

In vitro hindgut digestion / fermentation methods for humans have been developed predominantly for pathophysiological studies, and specifically as a tool for greater understanding of colon cancer, its dietary causes, and possible prevention. The role of dietary fibre (DF) and its fermentation has been of considerable interest.

In addition to its physiological roles in the maintenance of health and gut function, the dietary fibre component of food has a nutritional role. For humans on a typical Western diet low in dietary fibre, hindgut fermentation contributes around 3 - 11% of maintenance energy needs (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984), while for pigs, the value is more significant and may range from 7 – 40% (Breves *et al.*, 1993; Freeman *et al.*, 1993; Yen *et al.*, 1991).

As diets rich in dietary fibre become more commonplace, a suitable means of studying and describing their fate in the digestive system is necessary. It is difficult and expensive to study nutrient digestion and fermentation in the human hindgut directly and there are important differences in hindgut function among different species of simple-stomached animals making the choice of an *in vivo* model difficult. Thus, there is an important role for *in vitro* models.

II. Application and limitations of *in vitro* hindgut digestion / fermentation methods

Simulation of the gastrointestinal (GI) tract function of any animal species presents a great challenge (Moughan, 1999). The complex microbial environment of the large intestine makes this task especially demanding when attempting to develop a rapid and simple hindgut *in vitro* digestion method.

In assessing an assay it needs to be asked what the purpose of developing such a method is, in terms of the context in which it will be used. Specifically, there is a need to determine whether the primary concern is repeatability, rapid turnaround of results and relative accuracy (i.e. ranking) or absolute accuracy. Whilst the latter consideration is of considerable value to researchers, the former address the primary aim of a routine laboratory *in vitro* digestion method for testing feedstuffs and foods. The ideal *in vitro* digestion method would have the ability to provide highly accurate results in a short space of time. However, in reality it must be accepted that at present, it is assumed that a degree of accuracy is often sacrificed for rapid results and emphasis is placed on the assay's ability to rank the digestibility of foods relative to one another. Any *in vitro* method is inevitably going to fail to match the accuracy that may be achieved by actually studying a food *in vivo* (Fuller, 1991). Specifically, it is not possible to simulate the influx of endogenous compounds to the digestive tract and their subsequent digestion and absorption and it is difficult to replicate the effect of anti-nutritional factors and subtle interactions between the host, the food and the bacteria present in the digestive tract.

That *in vitro* methods have limitations and drawbacks, however, does not discount them from being a valuable research tool. Even *in vivo* digestibility methods have their own set of shortcomings and difficulties depending on how outputs are classified. Also, the accuracy of the *in vivo* method itself will impact on the benchmark for *in vitro* results. The availability of accurate *in vivo* data is crucial for critical evaluation of any potential *in vitro* method.

Whilst they may lack the accuracy of more complex *in vitro* digestion / fermentation methods, the simplest *in vitro* methods are generally those suited to a routine laboratory application. This is partly because, by their very nature, they are easier to carry out in any standard laboratory without the need for specialised equipment or materials and specially trained staff. Additionally, the more complex a method, the greater the possibility that errors are introduced into the assay through multiple steps in methodology and through operator error and laboratory differences. Thus, repeatability both at an intra-laboratory and inter-laboratory level becomes important. If a method is to be used for standard evaluation purposes in different parts of the world where comparisons need to be made, it is essential that both repeatability and reproducibility are assured.

III. The monogastric hindgut

To understand the rationale underlying an *in vitro* hindgut digestion / fermentation method and to identify possible merits and shortcomings, it is of value to briefly discuss large intestinal physiology and the fermentation process.

1. Hindgut physiology

Anatomically, the human large intestine is devoid of the well developed caecum present in other monogastric animals such as the rat, and to an even greater extent, the pig, which also exhibits extensive sacculatation and elongation of the large intestine. However, a common feature of simple-stomached animals is that, in contrast to ruminants, they ferment their food in the large intestine after digestion has occurred in the small intestine, rather than relying on forestomach fermentation. Like the rumen, the large intestine is essentially a fermenting chamber where material is broken down by bacteria numbering approximately 10^{10} - 10^{12} /g gut contents in humans (Macfarlane and Cummings, 1991). The profile of the large intestinal microflora is diverse and although the human tract contains several hundred species, there are some 30 – 40 anaerobic bacterial species that constitute 99% of the total microbial population present, the rest consisting of other facultative organisms (Drasar and Hill, 1974; Finegold and Sutter, 1978; Finegold *et al.*, 1983).

By the time undigested food reaches the hindgut it will have been in the human body for around two to six hours. This length of time is relatively short when compared to the 20 - 80 hours, on average, that food will reside in the adult human colon (Burkitt *et al.*, 1972; Cummings *et al.*, 1992; Williams *et al.*, 2001c).

2. Substrates for fermentation

The large intestine is constantly supplied with material consisting of undigested dietary components and other materials of endogenous origin, such as the host's enzymes, mucus and desquamated gut mucosal cells. A large part of the undigested diet at the

end of the ileum will comprise the non-starch polysaccharides (NSP), a major component of dietary fibre. Dietary fibre is a term that has historically been open to interpretation and subject to a raft of definitions. Cummings (1996) provides valuable insight and argues that the most appropriate view is that dietary fibre is simply plant cell wall material, and should be measured as total NSP. It is not uncommon, however, for the definition of DF to include the term 'non-digestible' and the regulatory food labelling authorities in many countries include not only NSP, but also lignin, oligosaccharides and starch resistant to host enzymes, termed resistant starch (RS), to be classified as DF. For the purposes of this review, DF is defined as the “non-digestible part of plant food”.

The principal dietary substrates available for hindgut fermentation are carbohydrates, and it is this group of substrates that limits colonic microflora growth (Macfarlane and Cummings, 1991). Of the carbohydrates, and also overall, the most significant contributors to the flow of material entering the human large intestine are NSP and RS. A Western diet provides 10 – 25 g/day of NSP (which includes cellulose, hemicellulose, pectins and gums) to the large intestine, and from 5 – 35 g/day of RS (Cummings, 1996). Other carbohydrates include oligosaccharides, sugars, sugar alcohols and synthetic carbohydrates. Sugars and sugar alcohols collectively contribute 2 - 5 g/day, while for oligosaccharides the amount is 2 - 8 g/day (Cummings, 1996).

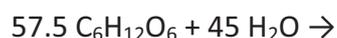
While carbohydrates are quantitatively the most significant, they are not the only substrates available for colonic fermentation. Some dietary protein (1 - 12 g/day) also escapes digestion, as do some lipids. In addition to dietary sources, any gut endogenous proteins, lipids, bile and mucins that reach the hindgut (4 - 8 g/day) are also potential substrates for fermentation in the hindgut (Cummings, 1996). In all these cases, rapid fermentation occurs.

Lignin is a small and highly insoluble polymer, and whilst not a carbohydrate it is present in the cell walls of some plants. Lignin is neither digestible nor fermentable

and is thought to inhibit the fermentation of associated proteins and carbohydrates (Cummings, 1996).

3. Products of fermentation

Bacteria in the hindgut produce enzymes to digest fermentable substrates entering the large intestine. Anaerobic fermentation enables bacteria to use the products of this process to supply their own energy needs for maintenance and growth, thus increasing their biomass. The anaerobic fermentation of carbohydrates, quantitatively the most important process in the hindgut, can be described by the following equation (Ewing and Cole, 1994):



The primary products of fermentation in the hindgut are the short chain fatty acids (SCFA) n-butyrate, propionate and acetate, and gaseous hydrogen and carbon dioxide. Proportionally, in both humans and pigs, acetate is the predominant SCFA, followed by propionate, and then butyrate (Breves and Stuck, 1995). However, molar ratios of the different SCFA vary depending on the particular substrate, as does the rate of fermentation (Salvador *et al.*, 1993). The SCFA are well absorbed (around 95%) but have different fates. Acetate reaches muscle tissue where it contributes to the energy supply and whilst propionate is processed by the liver, little more about its fate has been studied in man (Cummings, 1996). Butyrate is utilised directly by the colonic epithelium, which derives 60 - 70% of its energy from the products of bacterial fermentation (Roediger, 1980, 1989). Hydrogen gas is either exhaled from the body via the lungs, voided as flatus or is metabolised further to methane, sulphide and acetate (Cummings, 1996) by methanogenic, sulphate-reducing and acetogenic microbial species. However, not all individuals possess methanogenic bacteria in their GI tract and the reasons for this and the consequences of it to the colonic ecosystem are not fully understood (Macfarlane and Cummings, 1991).

In the case of proteins, peptides and amino acids, these are rapidly fermented to produce ammonia, amines, phenols and branched chain fatty acids, all of which are products unique to protein breakdown, in addition to the other products common to carbohydrate fermentation (Cummings, 1996). Unused products from the bacterial fermentation of nitrogenous compounds, namely ammonia, amines, phenols and branched chain fatty acids, may enter the bloodstream, and with the exception of the branched chain fatty acids, are also largely excreted in the urine and faeces (Cummings, 1996). There are minimal quantities of free amino acids in human ileal effluent (Chako and Cummings, 1988), yet a wide range of amino acid fermenting species populate the hindgut. Some bacterial species in the hindgut are able to utilise peptides and free amino acids directly (Payne, 1975). However, the amino acids available to the majority of these amino acid fermenting species must result from the hydrolysis of peptides by proteases and peptidases. The hydrolysis reaction releases the peptides and amino acids that then become available for assimilation and further metabolism, including fermentation, by the bacteria. Colonic bacteria are therefore able to release amino acids via hydrolysis, as well as having their own mechanisms for amino acid synthesis. Moughan (2003) has reviewed the evidence for amino acids being available to the host as a result of hindgut hydrolysis. It appears that the absorption of intact amino acids or peptides from the large intestine is of little nutritional significance to the host. Significant amounts of ammonia may be absorbed, and under most conditions these are of minor nutritional significance, although they may have a toxic effect (Clinton *et al.*, 1988).

Although SCFA are the only energy-yielding products of fermentation available to the host, only a small quantity become available, with the majority of SCFA being used by the hindgut bacteria to fuel protein synthesis, resulting in an increase in microbial biomass. The increased biomass is expelled in the faeces along with the non-absorbed SCFA and any carbohydrates which are in excess of the fermentative capacity.

End products of fermentation differ according to each genus of bacteria (Macfarlane *et al.*, 1995) and the relative proportion and amounts of each of the SCFA produced are

dependent on the substrate passing through the hindgut and substrate transit time. Many of the health effects of dietary fibre are due to its fermentation in the large intestine (Van Loo *et al.*, 1999), and consequently, there is significant interest in *in vitro* models to study the SCFA profile resulting from the bacterial fermentation of different foods.

IV. *In vitro* hindgut digestion and fermentation methods

1. Introduction

Continuous, semi-continuous and batch (static) systems are the three main types of *in vitro* models used for hindgut studies in man. These systems remove fermentation products continuously, intermittently, or not at all. Karppinen (2003) has recently presented a brief discussion of batch *in vitro* fermentation methods proposed for studying the human large intestine, as have others (Barry *et al.*, 1995; Edwards *et al.*, 1996). Earlier, Rumney *et al.* (1992) undertook an in-depth review, but as applied to semi-continuous and continuous *in vitro* fermentation methods for humans. There are a number of detailed critical reviews of *in vitro* digestibility assays as applied to simple-stomached farm animals (Boisen, 2000; Boisen and Eggum, 1991; Fuller, 1991; Moughan, 1999; Van Kempen *et al.*, 2004).

Simulation of hindgut digestion, *in vitro*, requires the presence of hindgut microbial enzymes to digest the substrate. These enzymes are provided either as purified enzymes commercially available “off-the-shelf” or directly as the result of enzyme production by live microbes present in the *in vitro* system. Purified enzymes usually consist of a selection of either individual or mixed digestive enzymes that are known to be produced by bacteria in the hindgut (Boisen and Fernández, 1997). Live microbes are supplied as an inoculum prepared from freshly voided human faeces or caecal / colonic contents. It is important to note that although both purified enzymes and live microbes have the potential to simulate digestion in the hindgut, only live microbes are able to ferment a substrate. This is because enzymes alone are unable to simulate the fermentation process, which involves bacteria taking up the simpler molecules resulting from digestive breakdown by bacterial enzymes and synthesising these into energy yielding and gaseous byproducts. While the use of purified enzymes has been well explored for *in vitro* hindgut digestion methods in pigs and cattle, this is not so for humans. Consequently, *in vitro* hindgut digestion methods for human studies have

generally used live microbes as the enzyme source.

2. Types of *in vitro* hindgut digestion / fermentation methods

2.1 Semi-continuous and continuous systems

The *in vitro* digestion methods most suited to studying microbial ecology of the large intestine, as opposed to the digestive fates of foods, are the semi-continuous and continuous methods.

A specific group of semi-continuous and continuous *in vitro* methods are the dynamic *in vitro* hindgut models, which use either live microbes, purified enzymes or both. These models are multi-compartmental, computer-controlled systems that are capable of dynamically interacting with the substrate by adjusting pH, temperature and other assay parameters as substrate passes through the system. The Dutch TIM-2 (TNO gastro-Intestinal Model-2) (Minekus *et al.*, 1999) uses a series of linked glass chambers with flexible internal walls. Physiological temperature is maintained and the peristaltic movements and mixing of the large intestine are simulated by periodically pumping water into the space between the glass and the flexible wall and by computer-controlled contractions exerted on the wall, respectively. Products of fermentation are removed from the anaerobic system using hollow fibre membranes and the system is able to simulate digestion in a variety of animals, including pigs, chickens, humans and others. In combination with the corresponding upper-tract dynamic *in vitro* model, TIM-1, the system aims to simulate digestion in the entire digestive tract (Havenaar and Minekus, 1996).

As with other semi-continuous or continuous *in vitro* digestion systems, dynamic systems often have their application focused on large intestinal microbial ecology, rather than hindgut digestion. Furthermore, semi-continuous and continuous systems do not lend themselves easily to routine evaluation in any standard laboratory and in many cases require specialised equipment and computer systems. Consequently,

batch *in vitro* digestion systems will be dealt with in more detail in this review.

2.2 Batch systems

Batch systems are simpler and considerably easier to run and are the method of choice for routinely studying the products of fermentation in the large intestine and for food evaluation purposes. In contrast to semi-continuous and continuous systems, the typical incubation time for batch *in vitro* systems is 24 h.

Batch *in vitro* hindgut digestion methods typically use a faecal slurry from faecal samples, a buffer and sometimes a nutritive solution and / or a redox agent. The substrate is inoculated with the faecal slurry and incubated. The degree of fermentation is usually measured in terms of NSP degradation, SCFA or gas production, or dry matter (DM) / organic matter (OM) disappearance. The gas production technique is a special type of batch *in vitro* fermentation system and will be discussed separately in more detail later.

The use of purified enzymes as an alternative to a faecal slurry is an area that warrants investigation for humans given its success in feed evaluation for pigs. The concept of using purified enzymes in porcine assays evolved over a long period of time after a progression away from using biological inocula. Initially, the seminal research of Tilley *et al.* (1963) with rumen fluid was applied to pigs by using rumen fluid (Vervaeke *et al.*, 1979; Vervaeke *et al.*, 1989) and more logically, the intestinal fluids of pigs (Ehle *et al.*, 1982; Furuya *et al.*, 1979). Following on from this, Löwgren *et al.* (1989) compared the effectiveness of inoculum from the pig duodenum, ileum or faeces, and in subsequent studies Graham *et al.* (1989) showed that a 48 h incubation with ileal or faecal inoculum could be used to predict the faecal apparent digestibility of dietary fibre in the pig.

Van der Meer *et al.* (1992) developed a total tract *in vitro* method for predicting OM digestibility using consecutive incubations with pepsin / pancreatin and cellulase to simulate the stomach / small intestine and hindgut, respectively. Whilst a high correlation was found between pig *in vivo* / *in vitro* digestibility results using the

method, the single enzymatic preparation to simulate the hindgut would seem to be an oversimplification. Boisen *et al.* (1997) later refined an earlier assay (Boisen and Fernández, 1991) for predicting total tract digestibility, which included an enzymatic step to simulate hindgut digestion after consecutive pepsin and pancreatin incubations. Rather than using cellulase for the hindgut enzymes, Boisen *et al.* (1997) introduced a complex enzymatic preparation known as Viscozyme® into their *in vitro* method to predict total tract dry matter digestibility. Viscozyme® is a multi-enzyme complex containing a wide range of carbohydrases including arabinase, cellulase, β -glucanase, hemicellulase, pectinase and xylanase. The method of Boisen *et al.* (1997) has been the subject of a number of *in vivo* / *in vitro* digestibility comparisons for several monogastric species. Specifically, rat faecal digestibility using oats (Pettersson *et al.*, 1996), and pig apparent faecal energy and OM digestibility using hulled and hulless barleys (Beames *et al.*, 1996) have been found to correlate well with the *in vitro* results. In a study similar to that conducted by Beames *et al.* (1996) and involving feeding barley to pigs, Chen (1997) found that *in vitro* DM estimates had a low correlation with the *in vivo* apparent digestibility of energy, although *in vitro* / *in vivo* digestibilities with wheat-milling byproducts were highly correlated. The method of Boisen *et al.* (1997) has been shown to be highly repeatable (Chen, 1997), and has subsequently been officially adopted in Denmark for the practical energy evaluation of mixed diets for pigs (Spanghero and Volpelli, 1999). No such parallel exists for humans and consequently, there is a lack of a standard *in vitro* hindgut digestion method for the evaluation of foods. This is not to say, however, that there has been an absence of *in vitro* hindgut digestion methods proposed for humans, as there are a large number available that use live microbes. **Table 1** provides an extensive list of such batch *in vitro* models proposed in relatively recent times. Barry *et al.* (1995) have provided a reference point for earlier examples.

Table 1

A summary of batch *in vitro* fermentation methods proposed for studying digestion and fermentation in the human large intestine

Literature reference	Fermentative substrate	Inoculum source	Parameters measured	Incubation duration, <i>h</i>
Wang <i>et al.</i> (2004)	Animal protein by itself or with addition of: potato starch, sugar beet pulp, wheat bran	Pig faeces	NSP, SCFA, starch	48
Goñi <i>et al.</i> (2002)	Seaweed	Rat caecal contents	Protein	24
Langkilde <i>et al.</i> (2002)	Banana flour	Human faeces	SCFA, RS, pH, H ₂ , CH ₄	24
Granito <i>et al.</i> (2001)	Haricot bean fibre	Human faeces	SCFA, pH, gas production	24
Birkett <i>et al.</i> (2000)	Human ileal effluent from high or low, mixed starch diet	Human faeces	SCFA, pH, NH ₃ , glucose	48
Fernandes <i>et al.</i> (2000)	Lactulose, rhamnose, guar gum, cornstarch	Human faeces	SCFA	24
Karppinen <i>et al.</i> (2000)	Brans (rye, wheat, oat), inulin	Human faeces	SCFA, pH, gas production, neutral sugars	24

Literature reference	Fermentative substrate	Inoculum source	Parameters measured	Incubation duration, <i>h</i>
Monsma <i>et al.</i> (2000)	Oat bran, wheat bran	Rat caecal contents	SCFA, neutral and amino sugars	96
Christensen <i>et al.</i> (1999)	Wheat flour, wheat bread, oat bran	Pig faeces	SCFA	72
Goñi <i>et al.</i> (1998)	Commercial purified dietary fibre supplements	Rat caecal contents	SCFA, DM, gas production	24
Hoebler <i>et al.</i> (1998)	Wheat bran, barley bran, beet fibre	Human faeces	SCFA, neutral sugars	24
Lebet <i>et al.</i> (1998b)	Pea hulls, apple pomace, celery cell walls, oat bran concentrate	Human faeces	SCFA, pH, H ₂ , neutral sugars, gas production	24
Wisker <i>et al.</i> (1998)	NSP (mixed fibre), citrus concentrate, wholemeal bread	Human faeces	NSP, neutral sugars	48

Literature reference	Fermentative substrate	Inoculum source	Parameters measured	Incubation duration, <i>h</i>
Casterline Jr. <i>et al.</i> (1997)	Brans (wheat, oat, corn), fruit and vegetable fibres (pea, apple, pear, fig), β -glucan, pectin, starch, soy fibre	Human faeces	SCFA	48
Fardet <i>et al.</i> (1997)	Beet fibre, barley bran	Human faeces	SCFA, neutral and acidic sugars	24
Stevenson <i>et al.</i> (1997)	Pectin, ispaghula, cornstarch	Rat caecal contents	SCFA, protein production	24
Bourquin <i>et al.</i> (1996)	Oat fibre, wheat bran, corn bran, xanthan gum, gum karaya, guar gum, gum arabic, soy fibre, citrus pectin	Human faeces	SCFA, OM, potential water holding capacity	24
Edwards <i>et al.</i> (1996)	Pregelatinised potato starch, raw potato starch, retrograded amylose, glassy pea starch	Human faeces	SCFA, pH, RS	24

Literature reference	Fermentative substrate	Inoculum source	Parameters measured	Incubation duration, <i>h</i>
Barry <i>et al.</i> (1995)	Cellulose, sugarbeet fibre, soybean fibre, maize bran, pectin	Human faeces	SCFA, NSP, pH	24
Guillon <i>et al.</i> (1995)	Pea fibre, apple fibre	Human faeces	SCFA, pH	24
Monsma <i>et al.</i> (1995)	Canned peas, psyllium seed husk fibre	Rat caecal contents/ faeces	SCFA	96
Silvester <i>et al.</i> (1995)	Ileal effluent containing resistant Starch	Human faeces	SCFA, NH ₃	24
Sunvold <i>et al.</i> (1995)	Cellulose, beet pulp, citrus pulp, citrus pectin	Cattle ruminal fluid, faeces from: cat, dog, horse, human, pig	SCFA, OM	48
Daly <i>et al.</i> (1993)	Xanthan gum	Human faeces	SCFA, pH, H ₂	24
McBurney <i>et al.</i> (1993)	Pea fibre	Human faeces	SCFA	24
Salvador <i>et al.</i> (1993)	Wheat bran, sugar beet, maize, pea hulls, cocoa	Human faeces	SCFA	24
Bourquin <i>et al.</i> (1992)	Oat bran, wheat bran, corn fibre	Human faeces	SCFA, DM	48

Literature reference	Fermentative substrate	Inoculum source	Parameters measured	Incubation duration, <i>h</i>
Guillon <i>et al.</i> (1992)	Sugar beet fibre	Human faeces	SCFA, pH	24
Titgemeyer <i>et al.</i> (1991)	Oat fibre, corn bran, soy fibre, sugarbeet fibre, pea fibre, gum arabic, guar gum, apple pectin, citrus pectin	Human faeces	SCFA, neutral sugars, uronic acid	48
Adiotomre <i>et al.</i> (1990)	Wheat bran, pectin, guar gum, gum arabic, carboxymethylcellulose, gellan, tragacanth gum, xanthan gum, gum karaya	Human faeces	SCFA, pH	24
Vince <i>et al.</i> (1990)	Lactulose, pectin, arabinogalactan, cellulose	Human faeces	SCFA, NH ₃	48

DM, dry matter; NSP, non-starch polysaccharides; OM, organic matter; RS, resistant starch; SCFA, short chain fatty acids

3. Failings of batch *in vitro* hindgut fermentation methods

In addition to the basic design of all batch *in vitro* fermentation methods reported in the literature for human studies being similar, and almost without exception, the methods all fail in one or more of the following ways:

a. A lack of explanation as to the rationale for choosing the parameters and conditions selected

Every method differs at least slightly in one way or another, yet few explanations are given as to why the workers have selected the particular parameters. Such lack of justification adds little credibility to the methodology.

b. A lack of inter-laboratory investigation to test or standardise the method

To enable results to be compared and for any *in vitro* method to be considered robust and repeatable, the method needs to prove that it can produce the same results, within reasonable limits of statistical variation, at multiple laboratories. If it cannot, there is little chance that it can be used as a standard method on a global scale. Generally, there is a lack of inter-laboratory testing.

*c. A lack of validation with *in vivo* data*

Surprisingly, few papers describing the development of *in vitro* fermentation methods include a discussion on validation. The method of Barry *et al.* (1995) is widely used and has been tested on an inter-laboratory scale, yet validation tests used rats as the *in vivo* model for NSP degradation (Nyman *et al.*, 1986), rather than humans. There is uncertainty as to whether rats can be used as a suitable predictor for digestion in the human large intestine due to differences in hindgut anatomy and physiology, and in addition, several issues regarding the suitability of the study by Nyman *et al.* (1986), as a comparative baseline, have been raised (Bach Knudsen *et al.*, 1994).

Other methods to have been subjected to validation are those of Christensen *et al.* (1999) and McBurney *et al.* (1993). Here pig ileal digesta was used as the fermentable substrate and pig faecal inocula (Christensen *et al.*, 1999) or human faecal inocula (McBurney and Sauer, 1993) for the provision of fermentation capability. In both cases, validation was carried out using pigs, rather than humans, despite the method's application being for the latter.

A lack of relevant *in vivo* comparison and validation is perhaps not surprising given the expense and time-consuming nature of conducting human balance studies. Notable exceptions are the large studies undertaken by Daniel *et al.* (1997) and Wisker *et al.* (1998) that compared NSP degradation *in vivo* for mixed diets in man, with results from an *in vitro* fermentation method adapted from one proposed some time ago by Goering *et al.* (1970) for ruminant studies. Whilst a general relationship existed between the *in vitro* and *in vivo* results, the degree of relationship improved as the fermentability of the substrate increased. Bourquin *et al.* (1996) also compared their *in vitro* results with human *in vivo* results and found that substrates which were highly fermentable *in vitro*, were of a similar nature *in vivo*. This observation is not entirely unexpected given that highly fermentable substrates are easily and almost completely degraded both *in vitro* and *in vivo*, thus it is less likely that significant overestimation or underestimation of the fermentability of the substrate will occur *in vitro*. Furthermore, the enzymatic preparation of DF residues may cause the fermentability of substrates to be overestimated *in vitro* since the structural properties of the cell wall may become compromised in a manner that would not occur *in vivo*, thus making the DF more susceptible to *in vitro* bacterial degradation (Wisker *et al.*, 1998). The effect of such pre-treatment is likely to be less important for the substrates which are already highly fermentable.

Although these comparisons are encouraging, there is still the need for a comprehensive, well-controlled and methodologically relevant validation of potential *in vitro* assays with data obtained from *in vivo* human studies.

d. Lack of testing with a range of substrates

Most *in vitro* methods have focused on NSP degradation when studying hindgut fermentation in man. It is now well accepted that NSP are not the only substrates available for fermentation in the human hindgut (Cummings, 1996) and a robust *in vitro* method should also aim to predict the fermentation of other materials entering the hindgut, including protein, fats, and other carbohydrates such as resistant starch, sugars (including sugar alcohols) and oligosaccharides.

V. Design considerations for a batch *in vitro* digestion / fermentation method

When developing any *in vitro* digestion method, along with cost and practicality, the objective is to have an *in vitro* method that is representative, to a certain extent, of *in vivo* events. For this reason, it is imperative to constantly refer back to the species in question so that simulated physiological conditions such as pH, temperature, and incubation time are appropriate. Hindgut *in vitro* fermentation methods also need to provide a suitable environment for the functioning of the hindgut bacterial species present in the inoculum. The components of all batch *in vitro* fermentation methods developed to date are essentially the same, but the way in which they are applied differs. Of all the possible factors that differ, the most important to consider are the inoculum concentration, the incubation time, the presence and type of buffer, the fluid surface / volume ratio, the form of the substrate and the output ultimately measured.

1. Substrate

Monogastric species ferment their food after upper-tract digestion, which means that the substrate that is available for fermentation is not the food as eaten. Rather, the substrate presented to the hindgut is the components of the food that are undigested by the host's digestive enzymes. Therefore, it is unsuitable to test the food as eaten directly, since that would expose the inocula to food components not normally available for fermentation.

Appreciating this, it is common for workers to pre-digest the food, or only test those components of specific interest, for example, by using dietary fibre isolates or resistant starch as the substrate. Some workers have taken this a step further, and used ileal digesta as the substrate. Although the use of ileal digesta does increase the complexity of the assay, the substrate in this case is in the form that it would actually arrive at the ileo-caecal junction *in vivo* (Monsma and Marlett, 1996). While dietary fibres may be resistant to host enzymatic breakdown, they still undergo certain

physical changes in the upper-tract (Åman *et al.*, 1994; Salvador *et al.*, 1993). Combinations of ileal digesta and various inocula that have been investigated are given in **Table 2**.

Table 2

Combinations of sources of ileal digesta and inoculum for published *in vitro* hindgut digestion / fermentation assays

Substrate	Inoculum source	Literature reference
Human ileal digesta	Human faeces	McBurney <i>et al.</i> (1989)
		Silvester <i>et al.</i> (1995)
		Fernandes <i>et al.</i> (2000)
		Birkett <i>et al.</i> (2000)
		Langkilde <i>et al.</i> (2002)
Pig ileal digesta	Human faeces	McBurney <i>et al.</i> (1993)
		Fardet <i>et al.</i> (1997)
		Hoebler <i>et al.</i> (1998)
	Rat caecal contents	Monsma <i>et al.</i> (2000)
	Pig faeces	Christensen <i>et al.</i> (1999)
	Wang <i>et al.</i> (2004)	
	Pig caecal contents	Fondevilla <i>et al.</i> (2002)
Rat ileal digesta	Rat caecal contents/faeces	Monro <i>et al.</i> (1996)
		Bullock <i>et al.</i> (1999)

2. Inoculum

The inoculum provides the bacteria necessary to digest and ferment the substrate. Whilst for species such as the pig, caecal contents for use in an *in vitro* fermentation system may be obtained, it is impractical to obtain human colonic contents and so faeces are routinely used in the inoculum. It is essential to use freshly voided faeces that have been collected and processed under anaerobic conditions to preserve the viability of the microbial population. A discussion of the validity of using faeces, as opposed to colonic contents, is presented in Section VI of this review.

Inoculum donors are healthy adult subjects, who have not taken antibiotics for at least 3 months prior and are consuming their normal Western diet. It has been shown that adapting donors to the substrate to be tested does not affect *in vitro* results (Barry *et al.*, 1995; Daniel *et al.*, 1997), although some workers have chosen to use adapted donors (Titgemeyer *et al.*, 1991; Wisker *et al.*, 2000). Most methods use at least three donors to provide faecal material for the pooled inocula, although five or more are recommended to help reduce the effect of biological variation between individual donors (Edwards *et al.*, 1996). Some workers also limit the donors to being methane non-producers (Granito *et al.*, 2001; Lebet *et al.*, 1998b) to reduce variation in the inoculum.

The ratio of inoculum to substrate has been found to be an important factor and proportionally more SCFA are produced after 24 h when less polysaccharide has been added to the fermentation system (Stevenson *et al.*, 1997). It has been hypothesised by Stevenson *et al.* (1997) that the reason for this is related to access to the substrate and that any more than 10 g/l of substrate may overload the system.

3. Nutritive medium

The nutritive medium contains a nitrogen source and other essential minerals. One advantage of using a high concentration of faecal inoculum, is that such nutritive

components are naturally present and do not need to be added to the incubation vessel (Edwards *et al.*, 1996).

4. Buffer

Without the inclusion of a buffer in the *in vitro* fermentation system, the acidic nature of SCFA produced during fermentation causes the pH to rapidly drop below the pH 6 – 7 range normally observed in the colon (McBurney and Van Soest, 1991). *In vivo*, SCFA are rapidly absorbed across the colon wall, thereby preventing the colonic contents from becoming too acidic (McBurney and Van Soest, 1991). In the closed environment of the batch *in vitro* system, however, no such removal mechanism operates. A buffer (either carbonate, phosphate, or both) is therefore usually included in the fermentation vessel. The phosphate-only buffers have the advantage that inoculum preparation under CO₂ is not required to preserve the effectiveness of the buffer (Edwards *et al.*, 1996), thereby allowing oxygen-free N₂ to be used as an alternative means for ensuring an anaerobic environment.

5. Stirring

To mimic the peristaltic action of the colon, stirring is often included in the methodology. This also prevents the substrate from settling at the bottom of the incubation vessel or being poorly dispersed, thereby becoming less accessible for fermentation (Edwards *et al.*, 1996). However, it has been found that shaking has no effect on fermentation or the bacterial mass increase (Stevenson *et al.*, 1997).

6. Fluid surface area / volume ratio

The surface area / volume ratio of fluid in the incubation vessel may influence fermentation results. A surface area / volume ratio of 1:1 has been shown to allow more fermentation than a ratio of 1:4 when samples of pectin and starch were fermented *in vitro* (Stevenson *et al.*, 1997). This is most likely to be associated with

the increased surface area available for gaseous exchange at the liquid-gas interface to remove the gaseous fermentation products.

7. Measurements

To assess the fermentability there are at least two options – measure metabolite production or substrate disappearance. Measuring SCFA production, which is the primary indicator of fermentability falls into the first category. In the second category are methods that measure the losses of DM, OM, gross energy or the loss of specific components such as NSP, RS, protein, carbohydrate or other fermentable substrates. Measurements may be taken at the end of the incubation period, or at stages during the incubation to study the kinetics of the fermentation. The latter necessitates multiple samples since there are multiple end points for each substrate tested.

8. Incubation time

The majority of methods use a 24 h incubation period, which is convenient in the laboratory. Whilst 24 h is shorter than the average transit time through the entire human large intestine, it has been shown to be comparable to the average transit time of material through the proximal colon (Metcalf *et al.*, 1987), which is considered to be the main fermentation chamber in humans (Edwards *et al.*, 1996). Wisker *et al.* (1998) found a good relationship between *in vitro* and *in vivo* NSP degradation after 24 h of fermentation, but not after 48 h. Although Monsma *et al.* (1996) argue that 96 h is necessary to properly characterise the fermentation pattern of carbohydrates *in vitro*, incubations greater than 24 h may be affected by product inhibition that results in a declining microbial population and the death of bacteria (Brøbech Mortensen *et al.*, 1991; Vince *et al.*, 1976).

VI. The gas production technique

The *in vitro* gas production technique can be regarded as a specific example of a batch *in vitro* method used for fermentation studies and as with other batch *in vitro* methods, facilitates the necessary anaerobic environment by preparation of the inoculum under strictly anaerobic conditions and fermentation in a closed system. The technique is similar in its setup to other batch *in vitro* methods, with the only differences being that the fermentation vessel may be either a syringe, flask or bottle, and that a means of recording the pressure in the fermentation vessel is employed, such as an attached pressure transducer. In the case of the automated versions, the pressure readings are recorded automatically by a computer system. Whilst SCFA and degraded substrates may be analysed for at the conclusion of the assay, in terms of methodology, it is the gas evolved as a result of fermentation that is the primary measurement, rather than the disappearance of fermentable substrate. The gas may be measured either at a single endpoint, or alternatively at various stages during the fermentation to study the kinetics of the entire fermentation period. The cumulative gas production technique (Theodorou *et al.*, 1994) is an example of the latter. The gas that is measured is not only that produced directly as a result of microbial fermentation (hydrogen, methane and carbon dioxide), but also the carbon dioxide that is liberated indirectly as a result of the SCFA produced reacting with the bicarbonate buffer that is used in gas production methods. Although it is not possible to separately measure the gas originating via the different pathways, both are produced either directly or indirectly as a result of the microbial fermentation of the substrate and are therefore indicative of fermentative activity.

The gas production technique is well-established in the area of ruminant feed evaluation, and a great deal of research using the technique in this context has been undertaken over a long period of time, dating back over three decades. Despite this wealth of knowledge, the use of the gas production technique in monogastric feed evaluation is less common and has only emerged relatively recently. Like its ruminant

counterpart, the method measures the volume of gas that is produced (or the pressure that this gas exerts) as a result of fermentative degradation of a substrate.

While some studies have investigated using the technique for equine application (Lowman *et al.*, 1999; Moore-Colyer and Longland, 2001; Murray *et al.*, 2003), and less commonly with rabbits (Calabró *et al.*, 1999), the majority of studies have been applied with pigs.

Ahrens *et al.* (1991) modified the method of Menke *et al.* (1979) for use with pigs, using caecal contents as the inoculum and centrifuged, freeze dried caecal contents as the substrate. The ruminant method was modified by using smaller syringes and corresponding smaller quantities of buffer and inoculum. The fermentation of pectin and lactitol were investigated *in vitro* by being directly added to the caecal substrate and compared with *in vivo* sampling of caecal contents following diets containing corresponding amounts of pectin and lactitol. Good agreement was found for a 24 h *in vitro* incubation and *in vivo* measures for pH, ammonia and total volatile fatty acids (VFA).

It was not long after, that the research group at Wageningen University began applying the cumulative gas production technique (Theodorou *et al.*, 1994) that they had been using with ruminants, to monogastric animals. Both poultry (Williams *et al.*, 1997b) and pigs were initially investigated (Williams *et al.*, 1997a) and since then, the group has undertaken numerous studies using the technique with pigs to investigate differences between feedstuffs (Bosch *et al.*, 2002; Van Laar *et al.*, 2000; Van Laar *et al.*, 2002; Williams *et al.*, 2003; Williams *et al.*, 2001a; Williams *et al.*, 2001b; Williams *et al.*, 2000), unweaned and adult animals (Bauer *et al.*, 2001) and also the effect of enzymatic pre-treatment on the fermentation kinetics of feedstuffs (Bauer *et al.*, 2003).

Fondevilla *et al.* (2002) have used the same technique to compare Iberian and Landrace pigs fed acorns using ileal digesta as the substrate and caecal contents as the

inoculum. Becker *et al.* (2003) recently investigated potential roughages for pregnant sows using an adaptation of the fully automated version proposed by Cone *et al.* (1996). An adaptation of the classic syringe method proposed by Menke *et al.* (1988) was used by Boudry *et al.* (2003) to investigate the effect of *in vitro* pre-digestion on fermentability of feedstuffs for pigs, whilst the fermentation of oligosaccharides in pigs has also been studied using the gas production technique (Smiricky-Tjardes *et al.*, 2003a; Smiricky-Tjardes *et al.*, 2003b), but only with a single endpoint using a fluid displacement version.

Campbell *et al.* (1997) used a fluid displacement version to study the fermentation of psyllium, methylcellulose, pectin and Solka Floc® with human faecal inocula. Aside from the latter study, there is an absence of literature on the application of the gas production technique with humans.

For simple-stomached animals it is common, for logistical reasons, to use faecal inocula for the gas production technique rather than caecal or colonic contents. Even for ruminants, the use of faeces over rumen fluid has the same logistical advantages. It is well established, however, that rumen fluid gives a different fermentation profile to faeces, and so mathematical models have been developed for ruminants to account for differences, such as a longer lag time (France *et al.*, 2000). These mathematical models are promising and a case for developing similar models for monogastric animals is supported by the studies of Monsma *et al.* (1995), who found significant differences between *in vitro* fermentation profiles for rat caecal and faecal inocula. Specifically a longer lag-phase was observed with faecal inocula (Monsma and Marlett, 1996), which is somewhat analogous to the lag-phase phenomenon that has been observed with rumen fluid versus cattle faeces when using the gas production technique (Mauricio *et al.*, 1997). Monsma *et al.* (1995) argue that the same differences between the use of colonic contents and faeces exist in humans and so faecal inocula will not necessarily provide an accurate representation of *in vivo* fermentation in the colon. In both pigs and humans, it has been found that the profile of colonic bacteria varies according to whether samples are taken from the

proximal, transverse or distal colon (Williams *et al.*, 1997a). Despite this, however, Williams *et al.* (1997a) believe that faecal microbial inocula are indicative of the colonic microflora. The approach of Edwards *et al.* (1996), of using a faecal inoculum with a high concentration of microbes, to prevent the initial lag time that is often evident when using faeces as opposed to caecal contents, may be another means of overcoming the differences due to the inoculum sources. Consideration needs to be given to such alternatives, as it remains difficult to get representative human colonic samples.

VII. Merits and shortcomings of *in vitro* digestion / fermentation methods

In vitro digestion methods that use purified enzymes have the advantage of being generally more reproducible than those that use live microbes, since there is very little variation in the standardised enzyme preparations that are used (Moughan, 1999). It is also easier to handle purified enzymes than faecal samples, and donors do not need to be sourced. Among the disadvantages, is that the selection of enzymes chosen is not necessarily representative of the enzymes produced by the hundreds of bacterial species known to inhabit the hindgut. Additionally, the lack of microbes *per se* means that the dynamic interactions occurring between the substrate and the microbes are not represented. Such methods determine hindgut hydrolysis (digestion) rather than fermentation.

Of the methods detailed in the literature that use purified enzymes, that presented by Boisen *et al.* (1997) for pigs, shows the most promise and has the advantage that it has been developed specifically in combination with an upper-tract method to provide a more suitable substrate for the hindgut step. A similar total tract *in vitro* method for human studies has been developed by Lebet *et al.* (1998a) which employs an enzymatic *in vitro* upper-tract method similar to that of Aura *et al.* (1999). Like the approach of Aura *et al.* (1999), dialysis is included, which makes the simulated upper tract component of the method more complicated than that of Boisen *et al.* (1997). The corresponding hindgut step of the method proposed by Lebet *et al.* (1998b), uses live microbes and is essentially the same as that proposed by Barry *et al.* (1995).

Of the methods available for humans that use live microbes, that of Barry *et al.* (1995) has the advantage that it has been tested on an inter-laboratory scale. Another method that uses live microbes presented by Edwards *et al.* (1996) for the fermentation of resistant starch has received some positive feedback, as well as also having been tested in an inter-laboratory study. The main advantage of this latter method is its simplicity, since it requires no nutritive medium and only employs a

simple phosphate buffer, thus negating the need for continuous bubbling with CO₂ during inoculum preparation. The system has been validated however, only for RS.

The gas production technique has some unique benefits over and above other *in vitro* methods that use live microbes. Firstly, the gas production technique allows quick comparisons between fermentative substrates to be made easily. This is because the amount of gas produced can be measured readily, and thus a direct comparison between the amounts of gas produced allows for a non-quantitative estimate of the fermentability of different substrates to be made. Gas production curves allow the fermentation kinetics of substrates to be compared, and the relative fermentability of substrates according to the gas produced at any moment in time can be a useful indicator of the comparative fermentability of different substrates at different stages of the incubation. The gas production technique allows for less sample and equipment to be used if multiple end times of fermentation are required. While other hindgut *in vitro* digestion methods necessitate destruction of the sample to determine DM, the gas production technique preserves the sample. If the amount of gas produced at multiple end points, such as 6 h, 12 h, 24 h, 48 h, was desired, only one incubation vessel and one sample of the substrate would be required for each replicate, rather than the four or more required with other *in vitro* methods. The equipment required for the non-automated versions of the method is relatively straightforward and available in most standard laboratories.

Shortcomings of the non-automated version of the gas production technique are that it is labour-intensive and subject to operator-error. For the automated version, the capital investment is a drawback. The automated versions have the advantage that personnel do not need to manually record the volume of gas produced if multiple readings over time are required. The high labour cost and time required for reading measurements with the manual technique inevitably mean that fewer readings are taken than with the automated version, which depending on purpose, may compromise the results. By automatically recording the pressure in the headspace of the incubation vessel, there is the ability to easily make many measurements during

the incubation time period. Still, the higher cost of the automated system means that for small laboratories the investment may be difficult to justify. The gas production technique only provides an indirect measure of hindgut digestion.

VIII. Conclusion

There is agreement, based on a limited number of suitable validation studies, that *in vitro* batch fermentation assays for humans are able to rank substrates in a similar manner to *in vivo* results. It is yet to be demonstrated empirically, however, that the energy provided to humans as a result of fermentation in the hindgut can be accurately predicted *in vitro* by any of the methods. There is a need for such *in vivo* validation studies to be carried out and a sound *in vitro* system to be correspondingly developed and standardised for a wide range of foods.

In the interim, currently available methods such as those described by Barry *et al.* (1995) and Edwards *et al.* (1996) are straightforward and have at least been subjected to some degree of validation. The porcine digestion method proposed by Boisen *et al.* (1997) has been subjected to a number of validation studies and stands out as offering considerable promise for total tract studies. The method, given the practical advantages of using purified enzymes, warrants adaptation to humans.

The gas production technique is useful for readily and simply comparing feedstuffs / foods, species, inter-individual variation and the fermentation kinetics associated with these factors, using a minimum amount of sample. However, as with other *in vitro* methods proposed for studying hindgut digestion and fermentation in simple-stomached animals and humans, the technique is currently limited to relative comparisons. An *in vitro* method is needed which accurately predicts the absolute amount of energy available to the host from any given substrate in a rapid, standardised and reliable manner.

Literature cited

- Adiotomre, J., Eastwood, M. A., Edwards, C. A. & Brydon, W. (1990). Dietary fibre: *in vitro* methods that anticipate nutrition and metabolic activity in humans. *American Journal of Clinical Nutrition*, *52*, 128-134.
- Ahrens, F., Schön, J. & Schmitz, M. (1991). A discontinuous *in vitro* technique for measuring hind gut fermentation in pigs. In M. W. A. Verstegen, J. Huisman & L. A. den Hartog (Eds.), *Digestive Physiology in Pigs EAAP Publication no 54*, (pp. 226–230). Wageningen, The Netherlands: PudocWageningen.
- Åman, P., Zhang, J. X., Hallmans, G. & Lundin, E. (1994). Excretion and degradation of dietary fiber constituents in ileostomy subjects consuming a low fiber diet with and without brewer's spent grain. *Journal of Nutrition*, *124*, 359–363.
- Aura, A. M., Härkönen, H., Fabritius, M. & Poutanen, K. (1999). Development of an *in vitro* enzymatic digestion method for removal of starch and protein and assessment of its performance using rye and wheat breads. *Journal of Cereal Science*, *29*, 139-152.
- Bach Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, W. & Eggum, B. O. (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat. *British Journal of Nutrition*, *71*, 471-487.
- Barry, J.-L., Hoebler, C., Macfarlane, G. T., Mathers, J. C., Reed, K. A., Mortensen, P. B., Nordgaard, I., Rowland, I. R. & Rumney, C. J. (1995). Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *British Journal of Nutrition*, *74*, 303-322.
- Bauer, E., Williams, B. A., Voigt, C., Mosenthin, R. & Verstegen, M. W. A. (2001). Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Animal Science*, *73*, 313-322.
- Bauer, E., Williams, B. A., Voigt, C., Mosenthin, R. & Verstegen, M. W. A. (2003). Impact of mammalian enzyme pretreatment on the fermentability of carbohydrate-rich feedstuffs. *Journal of the Science of Food and Agriculture*, *83*, 207-214.

-
- Beames, R. M., Helm, J. H., Eggum, B. O., Boisen, S., Bach Knudsen, K. E. & Swift, M. L. (1996). A comparison of methods for measuring the nutritive value for pigs of a range of hulled and hulless barley cultivars. *Animal Feed Science and Technology*, *62*, 189-201.
- Becker, P. M., van Gelder, A. H., van Wikselaar, P. G., Jongbloed, A. W. & Cone, J. W. (2003). Carbon balances for *in vitro* digestion and fermentation of potential roughages for pregnant sows. *Animal Feed Science and Technology*, *110*, 159-174.
- Birkett, A. M., Mathers, J. C., Jones, G. P., Walker, K. Z., Roth, M. J. & Muir, J. G. (2000). Changes to the quantity and processing of starchy foods in a Western diet can increase polysaccharides escaping digestion and improve *in vitro* fermentation variables. *British Journal of Nutrition*, *84*, 63-72.
- Boisen, S. (2000). *In vitro* digestibility methods: history and specific approaches. In P. J. Moughan, M. W. A. Verstegen & M. I. Visser-Reyneveld (Eds.), *Feed Evaluation: Principles and Practice*. Wageningen, The Netherlands: Wageningen Pers.
- Boisen, S. & Eggum, B. O. (1991). Critical evaluation of *in vitro* methods for estimating digestibility in simple-stomached animals. *Nutrition Research Reviews*, *4*, 141-162.
- Boisen, S. & Fernández, J. A. (1991). *In vitro* digestibility of energy and amino acids in pig feeds. In M. W. A. Verstegen, J. Huisman & L. A. Den Hartog (Eds.), *Digestive Physiology in the Pig*. Wageningen, The Netherlands: Pudoc.
- Boisen, S. & Fernández, J. A. (1997). Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. *Animal Feed Science and Technology*, *68*, 277-286.
- Bosch, M. W., Williams, B. A., Bos, L. W. & Verstegen, M. W. A. (2002). *In vitro* fermentability of carbohydrates by faecal microflora of pigs at weaning. Paper presented at the Proceedings of the 3rd Rowett/INRA Symposium on Beyond Antimicrobials—The Future of Gut Microbiology, Aberdeen, UK.

-
- Boudry, C., Estrada, F., Schoeling, O., Froidmont, E., Wavreille, J. & Buldgen, A. (2003). *Interest of in vitro predigestion to estimate fermentability of feedstuffs in pig large intestine*. Paper presented at the Proceedings of the 9th International Symposium on Digestive Physiology in Pigs, Banff, AB, Canada.
- Bourquin, L. D., Titgemeyer, E. C. & Fahey Jr., G. C. (1996). Fermentation of various dietary fiber sources by human fecal bacteria. *Nutrition Research*, 16(7), 1119-1131.
- Bourquin, L. D., Titgemeyer, E. C., Garleb, K. A. & Fahey Jr., G. C. (1992). Short-chain fatty acid production and fiber degradation by human colonic bacteria: effects of substrate and cell wall fractionation procedures. *Journal of Nutrition*, 122, 1508-1520.
- Breves, G., Schulze, E., Sallmann, H. P. & Gaden, D. (1993). The application of ¹³C labelled short chain fatty acids to measure acetate and propionate production rates in the large intestines: studies in a pig model. *Zeitschrift fur Gastroenterologie*, 31, 179-182.
- Breves, G. & Stuck, K. (1995). Short-chain fatty acids in the hindgut. In J. H. Cummings, J. L. Rombeau & T. Sakata (Eds.), *Physiological and Clinical Aspects of Short-chain Fatty Acids* (pp. 73-85). Cambridge, UK: Cambridge University Press.
- Brøbech Mortensen, P., Hove, H., Rye Clausen, M. & Holtug, K. (1991). Fermentation to short-chain fatty acids and lactate in human faecal batch cultures intra- and inter-individual variations versus variations caused by changes in fermented saccharides. *Scandinavian Journal of Gastroenterology*, 26, 1285-1294.
- Bullock, N. R. & Norton, G. (1999). Biotechniques to assess the fermentation of resistant starch in the mammalian gastrointestinal tract. *Carbohydrate Polymers*, 38, 225-230.
- Burkitt, D. P., Walker, A. R. P. & Painter, N. S. (1972). Effect of dietary fibre on stools and transit times, and its role in the causation of disease. *Lancet*, 2(7792), 1408-1412.
- Calabró, S., Nizza, A., Pinna, W., Cutrignelli, M. I. & Piccolo, V. (1999). Estimation of digestibility of compound diets for rabbits using the *in vitro* gas production technique. *World Rabbit Science*, 74, 197-201.

-
- Campbell, J. M. & Fahey Jr., G. C. (1997). Psyllium and methylcellulose fermentation properties in relation to insoluble and soluble fiber standards. *Nutrition Research (New York, NY, United States)*, 17, 619-629.
- Casterline Jr., J. L., Oles, C. J. & Ku, Y. (1997). *In vitro* fermentation of various food fiber fractions. *Journal of Agricultural and Food Chemistry*, 45, 2463-2467.
- Chako, A. & Cummings, J. H. (1988). Nitrogen losses from the small bowel: obligatory losses and the effect of physical form. *Gut*, 29, 608-613.
- Chen, J. (1997). *Prediction of the in vivo digestible energy value of barley for the growing pig on the basis of physical and chemical characteristics and in vitro digestible energy*. MAgSci Thesis, Massey University, Palmerston North, New Zealand.
- Christensen, D. N., Bach Knudsen, K. E., Wolstrup, J. & Jensen, B. B. (1999). Integration of ileum cannulated pigs and *in vitro* fermentation to quantify the effect of diet composition on the amount of short-chain fatty acids available for fermentation in the large intestine. *Journal of the Science of Food and Agriculture*, 79, 755-762.
- Clinton, S. K., Bostwick, D. G., Olson, L. M., Mangian, H. J. & Wisek, W. J. (1988). Effects of ammonium acetate and sodium cholate on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced colon carcinogenesis of rats. *Cancer Research*, 48, 3035-3039.
- Cone, J. W., van Gelder, A. H., Visscher, G. J. W. & Oudshoorn, L. (1996). Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. *Animal Feed Science and Technology*, 61, 113-128.
- Cummings, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet*, 321(8335), 1206-1209.
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H., Bingham, S. A., Heaton, K. W. & Eastwood, M. A. (1992). Fecal weight, colon cancer risk and dietary intake of non-starch polysaccharides (dietary fiber). *Gastroenterology*, 103, 1783-1789.

-
- Cummings, J. H. & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, *70*, 443-459.
- Daly, J., Tomlin, J. & Read, N. W. (1993). The effect of feeding xanthan gum on colonic function in man: correlation with *in vitro* determinant of bacterial breakdown. *British Journal of Nutrition*, *69*, 897-902.
- Daniel, M., Wisker, E., Rave, G. & Feldheim, W. (1997). Fermentation in human subjects of nonstarch polysaccharides in mixed diets, but not in barley fiber concentrate, could be predicted by *in vitro* fermentation using human fecal inocula. *Journal of Nutrition*, *127*, 1981-1988.
- Dobbins, J. W. & Binder, H. J. (1981). Pathophysiology of diarrhoea: alterations in fluid and electrolyte transport. *Journal of Clinical Gastroenterology*, *10*, 605-626.
- Drasar, B. S. & Hill, M. J. (1974). *Human Intestinal Flora*. New York: Academic Press.
- Edwards, C. A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J. C., Nagengast, F., Rumney, C. & Quehl, A. (1996). *In vitro* method for quantification of the fermentation of starch by human faecal bacteria. *Journal of the Science of Food and Agriculture*, *71*, 209-217.
- Ehle, F. R., Jeraci, J. L., Roberston, J. B. & Van Soest, P. J. (1982). The influence of dietary fibre on digestibility, rate of passage and gastrointestinal fermentation in pigs. *Journal of Animal Science*, *55*, 1071-1081.
- Ewing, W. N. & Cole, D. J. A. (1994). *The Living Gut: An Introduction to Micro-organisms in Nutrition*. Dungannon, Ireland: Context.
- Fardet, A., Guillon, F., Hoebler, C. & Barry, J. L. (1997). *In vitro* fermentation of beet fibre and barley bran, of their insoluble residues after digestion and of ileal effluents. *Journal of the Science of Food and Agriculture*, *75*, 315-325.
- Fernandes, J., Rao, A. V. & Wolever, T. M. S. (2000). Different substrates and methane producing status affect short-chain fatty acid profiles produced by *in vitro* fermentation of human faeces. *Journal of Nutrition*, *130*, 1932-1936.
- Finegold, S. M. & Sutter, V. L. (1978). Fecal flora in different populations, with special reference to diet. *American Journal of Clinical Nutrition*, *31*, S116-S122.

-
- Finegold, S. M., Sutter, V. L. & Mattisen, G. E. (1983). Normal indigenous intestinal flora. In D. J. Hentges (Ed.), *Human Intestinal Microflora in Health and Disease* (pp. 3–31). New York, NY, USA: Academic Press.
- Fondevilla, M., Morales, J., Pérez, J. F., Barrios-urdaneta, A. & Baucells, M. D. (2002). Microbial caecal fermentation in Iberic or Landrace pigs given acorn/sorghum or maize diets estimated *in vitro* using the gas production technique. *Animal Feed Science and Technology*, *102*, 93-107.
- France, J., Dijkstra, J., Dhanoa, M. S., Lopez, S. & Bannink, A. (2000). Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. *British Journal of Nutrition*, *83*, 143-150.
- Freeman, K., Foy, T., Feste, A. S., Reeds, P. J. & Lifschitz, C. L. (1993). Colonic acetate in the circulating acetate pool of the infant pig. *Paediatric Research*, *34*, 318-322.
- Fuller, M. F. (1991). *In vitro Digestion in Pigs and Poultry*. Slough: Commonwealth Agricultural Bureaux International.
- Furuya, S., Sakamoto, K. & Takahashi, S. (1979). A new *in vitro* method for the estimation of digestibility using intestinal fluid of the pig. *British Journal of Nutrition*, *41*, 511-520.
- Goering, H. K. & Van Soest, P. J. (1970). Forage fibre analyses. In *US Department of Agriculture Handbook* (Vol. 379). Washington, DC, USA: U.S. Government Printing Office.
- Goñi, I., Gudiel-Urbano, M. & Saura-Calixto, F. (2002). *In vitro* determination of digestible and unavailable protein in edible seaweeds. *Journal of the Science of Food and Agriculture*, *82*, 1850-1854.
- Goñi, I. & Martin-Carrón, N. (1998). *In vitro* fermentation and hydration properties of commercial dietary fiber-rich supplements. *Nutrition Research*, *6*, 1077-1089.
- Graham, H., Löwgren, W. & Åman, P. (1989). An *in vitro* method for studying digestion in the pig 2. Comparison with *in vivo* ileal and faecal digestibilities. *British Journal of Nutrition*, *61*, 689-698.

-
- Granito, M., Champ, M., David, A., Bonnet, C. & Guerra, M. (2001). Identification of gas-producing components in different varieties of *Phaseolus vulgaris* by *in vitro* fermentation. *Journal of the Science of Food and Agriculture*, *81*, 543-550.
- Guillon, F., Barry, J.-L. & Thibault, J. F. (1992). Effect of autoclaving sugar-beet fibre on its physico-chemical properties and its *in-vitro* degradation by human faecal flora. *Journal of the Science of Food and Agriculture*, *60*, 69-79.
- Guillon, F., Renard, C., Hospers, J., Thibault, J. F. & Barry, J. L. (1995). Characterisation of residual fibres from fermentation of pea and apple fibres by human colonic bacteria. *Journal of the Science of Food and Agriculture*, *68*, 521-529.
- Havenaar, R. & Minekus, M. (1996). Simulated assimilation. *Dairy Industries International*, *61*(9), 17-21.
- Hoebler, C., Guillon, F., Fardet, A., Cherbut, C. & Barry, J. L. (1998). Gastrointestinal or simulated *in vitro* digestion changes dietary fibre properties and their fermentation. *Journal of the Science of Food and Agriculture*, *77*, 327-333.
- Karppinen, S. (2003). *Dietary fibre components of rye bran and their fermentation in vitro*. Ph.D. Thesis, University of Helsinki, Finland.
- Karppinen, S., Liukkonen, K., Aura, A. M., Forssell, P. & Poutanen, K. (2000). *In vitro* fermentation of polysaccharides of rye, wheat and oat brans and inulin by human faecal bacteria. *Journal of the Science of Food and Agriculture*, *80*, 1469-1476.
- Langkilde, A. M., Champ, M. & Andersson, H. (2002). Effects of high-resistant-starch banana flour (RS₂) on *in vitro* fermentation and the small-bowel excretion of energy, nutrients, and sterols: an ileostomy study. *American Journal of Clinical Nutrition*, *75*, 104-111.
- Lebet, V., Arrigoni, E. & Amado, R. (1998a). Digestion procedure using mammalian enzymes to obtain substrates for *in vitro* fermentation studies. *Lebensmittel-Wissenschaft und-Technologie*, *31*, 509-515.
- Lebet, V., Arrigoni, E. & Amado, R. (1998b). Measurement of fermentation products and substrate disappearance during incubation of dietary fibre sources with human faecal flora. *Lebensmittel-Wissenschaft und-Technologie*, *31*, 473-479.

-
- Löwgren, W., Graham, H. & Åman, P. (1989). An *in vitro* method for studying digestion in the pig 1. Simulating digestion in the different compartments of the intestine. *British Journal of Nutrition*, *61*, 673-687.
- Lowman, R. S., Theodorou, M. K., Hyslop, J. J., Dhanoa, M. S. & Cuddeford, D. (1999). Evaluation of an *in vitro* batch culture technique for estimating the *in vivo* digestibility and digestible energy content of equine feeds using equine faeces as the source of microbial inoculum. *Animal Feed Science and Technology*, *80*, 11-27.
- Macfarlane, G. T. & Cummings, J. H. (1991). The colonic flora, fermentation, and large bowel digestive function. In S. F. Phillips, J. H. Pemberton & R. G. Shorter (Eds.), *The Large Intestine: Physiology, Pathophysiology and Disease* (pp. 51-92). New York, NY, USA: Raven Press.
- Macfarlane, G. T., Gibson, G. R., Drasar, B. S. & Cummings, J. H. (1995). Metabolic significance of the gut microflora. In R. Whitehead (Ed.), *Gastrointestinal Oesophageal Pathology* (Second ed., pp. 249-274). Edinburgh, UK: Churchill Livingstone.
- Mauricio, R. M., Owen, E., Dhanoa, M. S. & Theodorou, M. K. (1997). *Comparison of rumen liquor and faeces from cows as source of microorganisms for the in vitro gas production technique*. Paper presented at the Proceedings of the Occasional Meeting of the British Society of Animal Science on *In vitro* Techniques for Measuring Nutrient Supply to Ruminants, University of Reading, UK.
- McBurney, M. I. & Sauer, W. C. (1993). Fiber and large bowel energy absorption: validation of the integrated ileostomy-fermentation model using pigs. *Journal of Nutrition*, *123*, 721-727.
- McBurney, M. I. & Thompson, L. U. (1989). Dietary fiber and energy balance: integration of the human ileostomy and *in vitro* fermentation models. *Animal Feed Science and Technology*, *23*, 261-275.
- McBurney, M. I., Thompson, L. U. & Jenkins, D. J. A. (1987). Colonic fermentation of some breads and its implications for energy availability in man. *Nutrition Research*, *7*, 1229-1241.

-
- McBurney, M. I. & Van Soest, P. J. (1991). Structure–function relationships. In S. F. Phillips, J. H. Pemberton & R. G. Shorter (Eds.), *The Large Intestine: Physiology, Pathophysiology and Disease* (pp. 37–49). New York, NY, USA: Raven Press.
- McNeil, N. I. (1984). The contribution of the large intestine to energy supplies in man. *American Journal of Clinical Nutrition*, *39*, 338-342.
- Menke, K. H., Raab, L., Salewski, A., H., S., Fritz, D. & Schneider, W. (1979). The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. *Journal of Agricultural Science*, *93*, 217–222.
- Menke, K. H. & Steingass, H. (1988). Estimation of the energetic feed value obtained from chemical analyses and *in vitro* gas production using rumen fluid. *Animal Research and Development*, *28*, 7-55.
- Metcalf, A., Phillips, S. F., Zinsmeister, A. R., MacCarty, R. L., Beart, R. W. & Wolff, B. G. (1987). Simplified assessment of segmental colonic transit. *Gastroenterology*, *92*, 40-47.
- Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G. & Huis in't Veld, J. H. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Applied Microbiology and Biotechnology*, *53*, 108–114.
- Monro, J. & Burlingame, B. (1996). Carbohydrates and related food components: INFOODS tagnames, meanings, and uses. *Journal of Food Composition and Analysis*, *9*, 100-118.
- Monsma, D. J. & Marlett, J. A. (1995). Rat cecal inocula produce different patterns of short-chain fatty acids than fecal inocula in *in vitro* fermentations. *Journal of Nutrition*, *125*, 2463–2470.
- Monsma, D. J. & Marlett, J. A. (1996). Fermentation of carbohydrate in rat ileal excreta is enhanced with cecal inocula compared with fecal inocula. *Journal of Nutrition*, *126*, 554–563.

-
- Monsma, D. J., Thorsen, P. T., Vollendorf, N. W., Crenshaw, T. D. & Marlett, J. A. (2000). *In vitro* fermentation of swine ileal digesta containing oat bran dietary fiber by rat cecal inocula adapted to the test fiber increases propionate production but fermentation of wheat bran ileal digesta does not produce more butyrate. *Journal of Nutrition*, 130, 585-593.
- Moore-Colyer, M. J. S. & Longland, A. C. (2001). *The in vitro digestion of mature grass hay in the presence or absence of added nitrogen and sugar beet pulp by an equine faecal inoculum using the pressure transducer technique*. Paper presented at the Proceedings of the British Society of Animal Science Winter Meeting, Scarborough, UK.
- Moughan, P. J. (1999). *In vitro* techniques for the assessment of the nutritive value of feed grains for pigs: a review. *Australian Journal of Agricultural Research*, 50, 871–879.
- Moughan, P. J. (2003). Amino acid availability: aspects of chemical analysis and bioassay methodology. *Nutrition Research Reviews*, 16, 127-141.
- Murray, J. M. D., Moore-Colyer, M. J. S., Longland, A. C. & Dunnett, C. (2003). *The use of faecal inocula for estimating the in vitro digestibility of horse feeds*. Paper presented at the Proceedings of the British Society of Animal Science Winter Meeting, York, UK.
- Nyman, M., Asp, N. G., Cummings, J. H. & Wiggins, H. (1986). Fermentation of dietary fibre in the intestinal tract: comparison between man and rat. *British Journal of Nutrition*, 55, 487-496.
- Payne, J. W. (1975). Transport of peptides in microorganisms. In D. M. Matthews & J. W. Payne (Eds.), *Peptide Transport in Protein Nutrition, The Frontiers of Biology Series* (Vol. 37, pp. 283–364). Amsterdam, The Netherlands: North Holland Publishing Company.
- Pettersson, Å., Lindberg, J. E., Thomke, S. & Eggum, B. O. (1996). Nutrient digestibility and protein quality of oats differing in chemical composition evaluated in rats and by an *in vitro* technique. *Animal Feed Science and Technology*, 62, 203–213.

-
- Ramakrishna, B. S., Nance, S. H., Roberts-Thomson, I. C. & Roediger, W. E. W. (1990). The effects of enterotoxins and shortchain fatty acids on water and electrolyte fluxes in ileal and colonic loops *in vivo* in the rat. *Digestion*, *45*, 93–101.
- Roediger, W. E. W. (1980). Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut*, *21*, 793–798.
- Roediger, W. E. W. (1989). Short chain fatty acids as metabolic regulators of ion absorption in the colon. *Acta Veterinaria Scandinavica*, *86*, 116–125.
- Rumney, C. J. & Rowland, I. R. (1992). *In vivo* and *in vitro* models of the human colonic flora. *Critical Reviews in Food Science and Nutrition*, *31*, 299–331.
- Salvador, V., Cherbut, C., Barry, J. L., Bertrand, D., Bonnet, C. & Delort-Laval, J. (1993). Sugar composition of dietary fibre and short-chain fatty acid production during *in vitro* fermentation by human bacteria. *British Journal of Nutrition*, *70*, 189–197.
- Silvester, K. R., Englyst, H. N. & Cummings, J. H. (1995). Ileal recovery of starch from whole diets containing resistant starch measured *in vitro* and fermentation of ileal effluent. *American Journal of Clinical Nutrition*, *62*, 403–411.
- Smiricky-Tjardes, M. R., Flickinger, E. A., Grieshop, C. M., Bauer, L. L., Murphy, M. R. & Fahey Jr., G. C. (2003a). *In vitro* fermentation characteristics of selected oligosaccharides by swine fecal microflora. *Journal of Animal Science*, *81*, 2505–2514.
- Smiricky-Tjardes, M. R., Grieshop, C. M., Flickinger, E. A., Bauer, L. L. & Fahey Jr., G. C. (2003b). Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. *Journal of Animal Science*, *81*, 2535–2545.
- Spanghero, M. & Volpelli, L. A. (1999). A comparison of the predictions of digestible energy content of compound feeds for pigs by chemical or *in vitro* analysis. *Animal Feed Science and Technology*, *81*, 151–159.
- Stevenson, A., Buchanan, C. J., Abia, R. & Eastwood, M. A. (1997). A simple *in vitro* fermentation system for polysaccharides - the effects of fermenter fluid surface area/fluid volume ratio and amount of substrate. *Journal of the Science of Food and Agriculture*, *73*, 101–105.

-
- Sunvold, G. D., Hussein, H. S., Fahey Jr., G. C., Merchen, N. R. & Reinhart, G. A. (1995). *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *Journal of Animal Science*, *73*, 3639-3648.
- Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllan, A. B. & France, J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feedstuffs. *Animal Feed Science and Technology*, *48*, 185-197.
- Tilley, J. M. A. & Terry, R. A. (1963). A two-stage technique for the *in vitro* digestion of forage crops. *Journal of the British Grassland Society*, *18*, 104-111.
- Titgemeyer, E. C., Bourquin, L. D., Fahey Jr., G. C. & Garleb, K. A. (1991). Fermentability of various fiber sources by human fecal bacteria *in vitro*. *American Journal of Clinical Nutrition*, *53*, 1418-1424.
- Van der Meer, J. M. & Perez, J. M. (1992). *In vitro* evaluation of European diets for pigs. Prediction of the organic matter digestibility by an enzymic method or by chemical analysis. *Journal of the Science of Food and Agriculture*, *59*, 359-363.
- Van Kempen, T., Peak, S. & Qiao, Y. (2004). *In vitro* digestibility could meet quality control needs. *Feedstuffs*, *23*(February), 11-13.
- Van Laar, H., Tamminga, S., Williams, B. A. & Verstegen, M. W. A. (2000). Fermentation of the endosperm cells walls of monocotyledon and dicotyledon plant species by faecal microbes from pigs. The relationship between cell wall characteristics and fermentability. *Animal Feed Science and Technology*, *88*, 13-30.
- Van Laar, H., Tamminga, S., Williams, B. A., Verstegen, M. W. A. & Schols, H. A. (2002). Fermentation characteristics of polysaccharide fractions extracted from the cell walls of maize endosperm. *Journal of the Science of Food and Agriculture*, *82*, 1369-1375.

-
- Van Loo, J., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G. H., Newton, D., Quigley, M., Roberfroid, M., van Vliet, T. & van den Heuvel, E. (1999). Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project. *British Journal of Nutrition*, *81*, 121–132.
- Vervaeke, I. J., Decuypere, J. A., Dierick, N. A. & Henderickx, H. K. (1979). Quantitative *in vitro* evaluation of the energy metabolism influenced by virginiamycin and spiramycin used as growth promoters in pig nutrition. *Journal of Animal Science*, *49*, 846–856.
- Vervaeke, I. J., Dierick, N. A., Demeyer, D. I. & Decuypere, J. A. (1989). Approach to the energetic importance of fibre digestion in pigs. II. An experimental approach to hindgut digestion. *Animal Feed Science and Technology*, *23*, 169–194.
- Vince, A. J., Down, P. F., Murison, J., Twigg, F. J. & Wrong, O. M. (1976). Generation of ammonia from non-urea sources in a faecal incubation system. *Gastroenterology*, *74*, 313–322.
- Vince, A. J., McNeil, N. I., Wager, J. D. & Wrong, O. M. (1990). The effect of lactulose, pectin, arabinogalactan and cellulose on the production of organic acids and metabolism of ammonia by intestinal bacteria in a faecal incubation system. *British Journal of Nutrition*, *63*, 17–26.
- Wang, J. F., Zhu, Y. H., Li, D. F., Wang, Z. & Jensen, B. B. (2004). *In vitro* fermentation of various fiber and starch sources by pig fecal inocula. *Journal of Animal Science*, *82*, 2615–2622.
- Williams, B. A., Awati, A., Bosch, M. W., Konstantinov, S. R. & Akkermans, A. D. L. (2003). *The effect of dietary fermentable carbohydrates on lactic acid concentration in small intestinal digesta of piglets at ten days after weaning*. Paper presented at the Proceedings of the British Society of Animal Science Winter Meeting, York, UK.
- Williams, B. A., Bosch, M., Houdijk, J. & van de Camp, Y. (1997a). *Differences in potential fermentative capabilities of four sections of porcine digestive tract*. Paper presented at the Proceedings of the 48th EAAP meeting, Vienna, Austria.

-
- Williams, B. A., Bosch, M. W., Noteborn, R. & Verstegen, M. W. A. (2001a). *Changes in GIT digesta VFA as a result of fermentable carbohydrates in piglet diets*. Paper presented at the Proceedings of the British Society of Animal Science Winter Meeting, Scarborough, UK.
- Williams, B. A., Bosch, M. W. & Verstegen, M. W. A. (2001b). *Changes in digesta NH₃ concentration related to fermentable carbohydrates in piglet diets*. Paper presented at the Proceedings of the British Society of Animal Science Winter Meeting, Scarborough, UK.
- Williams, B. A., Tamminga, S. & Verstegen, M. W. A. (2000). *Fermentation kinetics to assess microbial activity of gastrointestinal microflora*. Paper presented at the Proceedings of the Symposium on Gas Production: Fermentation Kinetics for Feed Evaluation and to Assess Microbial Activity, Wageningen, Netherlands.
- Williams, B. A., Van Osch, L. J. M. & Kwakkel, R. P. (1997b). Fermentation characteristics of the caecal contents of broiler chickens fed fine- and coarse particle diets. *British Poultry Science*, 38, S41–S42.
- Williams, B. A., Verstegen, M. W. A. & Tamminga, S. (2001c). Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutrition Research Reviews*, 14, 207–227.
- Wisker, E., Daniel, M., Rave, G. & Feldheim, W. (1998). Fermentation of non-starch polysaccharides in mixed diets and single fibre sources: comparative studies in human subjects and *in vitro*. *British Journal of Nutrition*, 80(253–261).
- Wisker, E., Daniel, M., Rave, G. & Feldheim, W. (2000). Short-chain fatty acids produced *in vitro* from fibre residues obtained from mixed diets containing different breads and in human faeces during the ingestion of the diets. *British Journal of Nutrition*, 84, 31–37.
- Yen, J. T., Nienaber, J. A., Hill, D. A. & Pond, W. G. (1991). Potential contribution of absorbed volatile fatty acids to whole-animal energy requirements in conscious swine. *Journal of Animal Science*, 69, 2001–2012.

CHAPTER III

Predicted apparent digestion of energy-yielding nutrients differs between the upper and lower digestive tract in rats and humans

The objective was to compare the predicted apparent digestibility of energy-yielding nutrients (carbohydrate, protein and fat) in the human upper digestive tract and large bowel separately for four diverse diets containing either a single dietary fibre source (Wheat Bran and Pectin diets) or mixed fibre sources (Low Fibre and High Fibre diets). A human balance study was used to determine apparent faecal digestibility and a rat model was used to predict human apparent ileal energy and nutrient excretion. The study highlighted the importance of quantifying nutrient digestion separately in the upper and lower digestive tracts.

Data published in: Coles, L. T., Moughan, P. J., Awati, A., Darragh, A. J., & Zou, M. L. (2010). Predicted apparent digestion of energy-yielding nutrients differs between the upper and lower digestive tract in rats and humans. *Journal of Nutrition*, 140(3), 469-476.

Abstract

The apparent digestibility of energy-yielding nutrients (carbohydrate, protein and fat) was predicted in the human upper digestive tract and large bowel separately for four diverse diets containing either a single dietary fibre source (Wheat Bran and Pectin diets) or mixed fibre sources (Low Fibre and High Fibre diets). A human balance study was undertaken to determine faecal energy and nutrient excretion and a rat model was used to predict human ileal energy and nutrient excretion. Total tract energy digestibility ranged from 92 (High Fibre Diet) to 96% (Pectin Diet and Low Fibre Diet), while at the ileal level it ranged from 79 – 86% for the High Fibre Diet to the Low Fibre Diet. The predicted upper-tract digestion of starch, sugars, and fat was high with ileal digestibilities exceeding 90% for all diets. Non-starch polysaccharides were poorly digested in the upper-tract for all diets except in the case of the Pectin diet. The daily quantity of protein excreted at the ileal level was between two (High Fibre Diet) and five (Pectin Diet) times higher than that at the faecal level. The large differences observed between faecal and ileal nutrient loss highlight that faecal digestibility data alone provide incomplete information on nutrient loss. There is a need to be able to routinely determine the uptake of energy in the upper and lower digestive tracts separately.

Introduction

Nutrient and energy digestibility are important measures for describing diets and whenever practical, digestibility data should be specific to the diet or dietary component in question. This is preferable to relying on generic digestibility values because of the many factors known to affect digestibility, often specific to a particular diet. Human balance studies are the traditional means of quantifying the digestibility of energy over the total digestive tract. However, apparent faecal digestibility values alone do not provide information about the uptake of nutrients from different parts of the gastrointestinal tract.

Upper-tract digestion (mouth to terminal ileum) provides the majority of the energy that humans gain from food. The contribution to maintenance energy requirements to the human host resulting from fermentation of dietary material by colonic bacteria is quantitatively less important, estimated to contribute between 3 and 11% for Western diets low in dietary fibre (DF) (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984). This contribution is not small enough to be neglected altogether, however, and would be expected to be more important for diets rich in cereals, fruits and/or vegetables. Knowledge of the quantities of specific energy yielding nutrients (primarily carbohydrate, protein and fat) absorbed in the upper-tract and the large intestine respectively would provide useful information that could be used to more accurately calculate dietary energy yields.

It is difficult to determine ileal nutrient digestibility directly in humans (Darragh and Hodgkinson, 2000) and the use of human ileostomates is impractical for routine application. Ileostomates, moreover, may not be suitable models due to histological and microbial changes that occur, post-operation (Dowsett *et al.*, 1990). An animal model, however, may be used to provide predictions of the loss of nutrients in the upper digestive tract of humans. The growing rat, being an omnivore and having a similar small intestinal transit time as adult humans (3 - 4 h) (DeSesso and Jacobson,

2001) was accepted here as a suitable model. The laboratory rat has several logistical advantages over larger animals.

The aim of the present study was to predict the uptake of energy and nutrients in the human upper digestive tract and the loss of energy from the large bowel separately for four diverse diets varying in the amount and type of dietary fibre to ascertain to what extent these variables are affected by the type of diet, with comparisons made within diets rather than across diets. Human faecal gross energy excretion data, combined with rat ileal gross energy excretion data (used to predict human ileal energy excretion) were used to determine respective predicted gross energy losses in the small and large intestines and the ratio of colonic loss to small intestinal energy uptake.

Materials and methods

Human balance study

Participants

Twenty five healthy women were recruited for the balance study. Of these, twenty one women, aged 20 – 59 y completed the study (**Table 1**). Participants were carefully selected, based on the criteria that none were taking medication during the study nor had a history of medical problems that may have interfered with the aims of the study. Especially in the 3 mo prior to the trial, participants had not experienced gastrointestinal illness or taken any antibiotics or laxatives.

Informed written consent was obtained from all volunteers, and the study was approved by the Massey University Human Ethics Committee (PN 04/128). All participants were highly motivated and had a genuine interest in the objectives of the study and the experimental diets were well received. In the days immediately preceding the study, participants as a group met the research team to establish a good rapport and discuss individually the details of the trial. To ensure compliance throughout the study, participants met with the lead researcher at least once every other day.

Table 1

Participants' characteristics¹

Diet	Wheat Bran	Pectin	Low Fibre	High Fibre
Number of women, <i>n</i>	5	6	4	6
Age, <i>y</i>	35.4 ± 6.4	44.7 ± 5.9	46.0 ± 0.4	39.7 ± 4.7
Height, <i>cm</i>	164.0 ± 1.5	163.2 ± 1.1	155.3 ± 3.6	160.3 ± 2.2
Body weight, <i>kg</i>	78.6 ± 4.7 ^a	63.7 ± 1.6 ^{ab}	69.5 ± 8.7 ^{ab}	55.8 ± 4.3 ^b

¹ All data are expressed as means ± SE.

^{ab} Means in a row without common superscript letters are significantly different, *P*<0.05.

Study plan

The study consisted of a 10 d adaptation period followed by a 7 d balance period involving total collection of faeces and urine. Four experimental diets were randomly allocated to participants such that each participant received one diet, with a constant daily intake of food ingredients throughout the 17 d period. Participants consumed the diets as six daily meals: breakfast (between 0600 and 1000 h), morning snack (between 1000 and 1200 h), lunch (between 1200 and 1500 h), afternoon snack (between 1500 and 1700 h), dinner and evening snack (1700 h onwards). Daily food intake was set for each woman according to individual dietary metabolisable energy needs determined prior to the trial based on a 7 d food record compiled by the participants when consuming their normal diets and analysed using the Foodworks® database (Xyris Software, QLD, Australia). Participants were free-living in their own homes during the adaptation period. During the balance week, participants were housed together in an apartment complex where they were free to come and go as necessary, with a member of the research team present 12 h per day to maintain regular contact with participants.

Diets

Two types of diet were studied: mixed fibre diets, as commonly consumed as part of a 'Western Diet', and simplified diets each containing a single source of DF. The four experimental diets (**Table A1**, see Appendix) were designated: Wheat Bran (WB), Pectin (PE), Low Fibre (LF) and High Fibre (HF). WB and PE diets contained wheat bran (WB) (All-Bran®, Kellogg, Australia) and high methoxyl citrus pectin (PE) (7050 acid/milk pectin, Cerestar, Krefeld, Germany), respectively, as sole sources of dietary fibre. An isotonic nutritional supplement (Osmolite®, Abbott Labs, IL) was included in both these diets to ensure nutritional adequacy. The wheat bran was included as a ready-to-eat breakfast cereal, a snack bar and a pudding base. The pectin was included as a thickener in drinks (shakes and blackcurrant drink). The other two diets were mixed-fibre, containing cereals, legumes, fruits and vegetables in a low fibre (LF) or high fibre (HF) form. The HF diet contained DF from a wider range of sources than the LF diet, and included resistant starch (bread), oligosaccharides (yoghurt) and DF

from a greater variety of cereal sources (wheat bran, rye and oats). Diets were formulated using foods commonly consumed as part of a 'Western Diet' and designed to be highly palatable to prevent participant drop-out during the study. For all diets, DF was distributed over the six daily meals. Two weeks prior to the commencement of the 17 d study, participants on the WB or HF diets were provided with ready-to-eat wheat bran breakfast cereal and instructed to gradually increase the amount consumed daily. The purpose was to minimise any effects from suddenly consuming large amounts of wheat bran. Thus for the WB and HF diets, participants were habituated to the fibre sources for a total of 31 d.

All food was provided and cooked under standardised conditions at the Institute of Food, Nutrition and Human Health (Massey University) and was weighed accurately and packed for participants to collect. The weight of each food item for each participant was related to individual dietary energy requirements, such that all participants on a given diet consumed proportionally the same amount of each food item.

Balance technique

Faeces and urine were collected separately over a 7 d period, from 0800 h on study d 11 until 0800 h on study d 18 following the methodologies of Zou *et al.* (2007). For the purposes of chemical analysis of the diets, a sample of each ingredient was taken daily during the 7 d balance period. Ingredients were accurately weighed according to their proportion in the diet and composite diet samples prepared, homogenised, freeze-dried and finely ground (1 mm mesh). As such, a representative sample of each diet as consumed was obtained and nutrient and DF content determined.

Rat Study

Animals and housing

A total of 112 Sprague-Dawley male rats were obtained (Small Animal Production Unit (SAPU), Massey University) at approximately 7 wk of age. The trial was approved by

the Massey University Animal Ethics Committee (PN 04/84). Rats were randomly allocated to individual cages and to the four diets (WB, PE, LF, HF), resulting in 28 rats consuming each of the four diets. Rat weights (mean \pm standard error of the mean (SEM)) on study d 0 were 314 (\pm 7)g, 323 (\pm 7)g, 326 (\pm 8)g and 310 (\pm 8)g for animals consuming the WB, PE, LF and HF diets respectively. Animals were individually housed at SAPU in wire-bottomed stainless steel cages in a temperature- ($22\pm 1^\circ\text{C}$) and humidity- ($55\pm 10\%$) controlled environment with a 12 h light/dark cycle (0600 – 1800 h) for the duration of the trial.

Diets

The base for each of the four rat diets (**Table A2**, see Appendix) comprised the same mixtures of ingredients as used in the human balance study. Ingredients were weighed accurately and after homogenising in a food processor, diets were freeze-dried and finely ground (1 mm mesh). A standard vitamin and mineral premix (Plant and Food Research Ltd., Palmerston North, New Zealand), to meet the requirements of the growing rat (National Research Council, 1995) and an indigestible marker (titanium dioxide), were added to each base diet before mixing.

The determined chemical composition of the dietary material given to the women and also used as a base material for the rat diets is given in **Table A3** (see Appendix). The experimental diets met the nutrient requirements of the growing rat (National Research Council, 1995).

Food was offered *ad libitum* for 10 min each hour between 0800 h and 1600 h daily to train rats to a frequent feeding regimen. Hourly rather than single-meal feeding was undertaken to ensure that digesta would be available for collection from the terminal ileum when required. Rats had free access to clean drinking water at all times.

Collection of ileal digesta

On study d 14, from 4 h after the first meal, rats were randomly selected to be

euthanised by an overdose of CO₂ gas, followed by decapitation. The contents of the terminal 20 cm of the ileum were then removed as described by Rutherford and Moughan (2003), rapidly frozen (-20°C) and freeze-dried. After discarding digesta samples from coprophagous rats, samples of ileal digesta from individual rats were pooled within a diet to give sufficient quantities of material for chemical analysis and finely ground (1 mm mesh). This resulted in two samples of digesta for the PE diet (1 x *n*=14, 1 x *n*=13) and three samples for each of the HF diet (2 x *n*=8, 1 x *n*=9), the WB (2 x *n*=9, 1 x *n*=10) diet and the LF diet (2 x *n*=9, 1 x *n*=10).

Chemical analyses

On each day during the balance period of the human study, samples of all meals (as eaten) were weighed in proportion to their contribution to the diet, homogenised, ground to a fine powder (1 mm mesh) and frozen. At the end of the balance period, all daily diet samples were combined in equal proportion, homogenised and frozen to provide a composite weekly sample for analysis. The diet samples were analysed for dry matter (DM), ash, gross energy (GE), total N, total sugars, total starch, total fat, fatty acids (FA), non-starch polysaccharides (NSP) and DF (total, insoluble and soluble). Rat ileal digesta samples were analysed for DM, ash, titanium dioxide, GE, total N, total sugars, total starch, FA and NSP. Human faeces were analysed for ash, N, fat, starch, sugars, total DF and NSP. Pooled weekly urine samples were analysed for GE.

GE of the faeces and diets was determined in duplicate using an automatic adiabatic bomb calorimeter (AC-350, Leco Corporation, St. Joseph MI, USA) (Miller and Payne, 1959). For GE content of urine, samples were freeze-dried in plastic bags prior to analysis. GE determination of rat ileal digesta samples was performed using 50 mg samples wrapped in plastic of known GE content.

DM and ash determination of diets, digesta and human faeces were performed in duplicate by drying samples in a Watvic convection oven (Watson Victor Ltd., Wellington, New Zealand) at 105°C for 16 h (AOAC, 2000), followed by ashing in a Muffle furnace (Electrofurn, New Zealand) at 550°C for 16 h (AOAC, 2000). The

titanium dioxide content of the rat diets and digesta was measured on a UV spectrophotometer following the method of Short *et al.* (1996).

Human faecal samples were analysed for N using the Leco total combustion method (AOAC, 2000) on a Leco TruSpec CN (Carbon/Nitrogen) Determinator (Leco Corporation, St. Joseph, MI, USA). Given limited quantities of rat ileal digesta, rat digesta samples were analysed using microanalysis with an elemental analyser (EA 1108, Carlo Elba, Italy). N analysis of diets was performed with duplicate samples using both the total combustion method and the microanalysis method and the mean protein content given (Table A3, see Appendix). Crude protein was calculated using an N to protein conversion factor of 6.25 (Kraisid *et al.*, 2003).

Determination of NSP in diet samples, human faeces and rat digesta was undertaken using a modified Englyst method (Englyst and Cummings, 1984, 1988). Constituent sugars of NSP were measured using colorimetry. Total DF of the diet samples and human faecal samples, along with insoluble and soluble fibre fractions, was determined using an enzymatic-gravimetric method (AOAC, 2000) with a commercial kit (Total Dietary Fibre Kit Assay, Megazyme, Ireland) and alpha-amylase/amyloglucosidase/protease enzymes.

Total fat in human faecal samples was determined using Soxhlet extraction (AOAC, 2000) (Soxtec HT6 fat analyzer, Tecator, Hoganas, Sweden). Total fat in diet samples was determined using the Mojonnier method (AOAC, 2000). Given the limited quantity of ileal digesta, total fat content of digesta samples was determined as the total lipid FA expressed as triacylglycerol as suggested by the FDA (Ali *et al.*, 1997; FDA, 2004). Individual FA were determined in duplicate using GC (Sukhija and Palmquist, 1988; Wu *et al.*, 1994) on a 6890 GC FID (Agilent Technologies, Santa Clara, CA). Data for diet samples (Table A3, see Appendix) are given as the mean of the values determined using the two methods (Mojonnier and GC).

Total sugars and starch determination were performed in duplicate on human faecal

and rat ileal digesta samples. Total sugars were defined as simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl esters with free or potentially reducing groups. Sugars were determined using the phenol sulphuric colorimetric method (Dubois *et al.*, 1956) and total starch determination was performed in duplicate using a commercial kit (Total Starch Kit, Megazyme, Ireland) (AOAC, 2000). Total carbohydrate (CHO) (%) was calculated as the difference between 100 and the sum of the percentage of water, protein, total fat and ash.

Calculations

Human balance study

Metabolisable Energy

Dietary metabolisable energy (ME) values (MJ/kg) were calculated as the difference between GE intake of the food (MJ/d) and the GE excreted in the faeces (MJ/d) and the urine (MJ/d), as a function of the daily food intake (kg/d).

$$ME \text{ (MJ/kg)} = \frac{GE_{\text{food}} - GE_{\text{faeces}} - GE_{\text{urine}}}{\text{Food intake}} \quad (\text{Eq. 1})$$

Total tract digestibility (apparent faecal digestibility of nutrients)

The apparent faecal digestibility (%) of all nutrients (protein, fat, total CHO, starch and sugars) and analogously, other dietary components (TDF and NSP), was calculated as the difference between the daily intake (g/d) and that excreted in the faeces (g/d), relative to the daily intake (g/d).

$$\text{Apparent faecal digestibility of nutrient (\%)} = \frac{\text{Nutrient}_{\text{food}} - \text{Nutrient}_{\text{faeces}}}{\text{Nutrient}_{\text{food}}} \times \frac{100}{1}$$

(Eq. 2)

Predicted ileal output

The predicted daily human ileal output of each nutrient (g/d) (protein, fat, total CHO, starch, sugars) was calculated by multiplying the daily dietary intake of the nutrient (g/d) for a given human participant by the rat ileal output (%) of the nutrient (see Eq. 6).

Predicted human ileal nutrient output (g/d)

$$= \frac{\text{diet nutrient intake (g/d)} \times (100 - \text{rat ileal digestibility of nutrient (\%)})}{100} \quad (\text{Eq. 3})$$

The predicted human ileal output of GE, NSP and DM were calculated in an analogous manner.

Predicted uptake (upper-tract)

The predicted uptake (upper-tract) of each nutrient (g/d) for humans (protein, fat, total CHO, starch, sugars) was calculated by subtracting the predicted human ileal output (g/d) of the nutrient for a given human participant (see Eq. 3) from the actual daily dietary intake of the nutrient (g/d) for that participant.

Predicted human nutrient uptake (upper-tract) rate (g/d)

$$= \text{actual daily intake of nutrient (g/d)} - \text{predicted ileal output of nutrient (g/d)}$$

(Eq. 4)

The predicted human uptake (upper-tract) of GE and NSP were calculated in an analogous manner.

Predicted colonic loss

The predicted daily colonic loss of each nutrient (g/d) (protein, fat, total CHO, starch,

sugars) in the human participants was calculated by subtracting the actual human faecal excretion (g/d) of the nutrient for a given human participant from the predicted ileal output of the nutrient (g/d) for that participant (see Eq. 3).

$$\begin{aligned} & \text{Predicted human nutrient colonic loss (g/d)} \\ & = \text{predicted ileal output of nutrient (g/d)} - \text{actual human faecal nutrient excretion (g/d)} \\ & \text{(Eq. 5)} \end{aligned}$$

The predicted human colonic losses of GE and NSP were calculated in an analogous manner.

Rat ileal digestibility

The apparent ileal digestibility of each nutrient (protein, fat, total CHO, starch, sugars) was calculated using the ratio of the nutrient (mg/g DM_{diet}) to titanium dioxide (mg/g DM_{diet}) in the diet ((Nutrient/Ti)_{diet}) and the ratio of the nutrient (mg/g DM_{ileal}) to titanium dioxide (mg/g DM_{ileal}) in the ileal digesta ((Nutrient/Ti)_{ileal}):

$$\begin{aligned} & \text{Apparent ileal digestibility of nutrient (\%)} \\ & = \frac{(\text{Nutrient/Ti})_{\text{diet}} - (\text{Nutrient/Ti})_{\text{ileal}}}{(\text{Nutrient/Ti})_{\text{diet}}} \times \frac{100}{1} \text{ (Eq. 6)} \end{aligned}$$

The apparent ileal digestibility of organic matter (OM), DM, GE and NSP were calculated in an analogous manner.

Statistical analysis

Twenty five human participants were allocated to four diets in a randomised parallel study, such that each participant consumed a single diet for the duration of the trial. The same experimental design was used for the rat study with 118 rats consuming the same four base human diets. For digestibility comparisons between diets, the data were subjected to a one way analysis of variance (ANOVA) and tested for normal

distribution and homogeneity of variance. Results were considered statistically significant at $P < 0.05$. Where a statistically significant main effect was found, means were subjected to multiple comparison using Tukey's test (Muller and Fetterman, 2002). An analogous approach was used for determining inter-diet differences in energy intake, excretion (ileal, faecal and urinary) and ME. Standard error (SE), rather than standard deviation (SD) was used as the measure of statistical variability through the thesis. Not only is this approach in line with common practice, but the work was concerned with making inferences about population means based on sample means and in such cases, SE is an appropriate measure. Minitab (version 14.2, Minitab Inc., PA, USA) was used for all statistical analyses.

Results

Human balance study

The diets were well received and there were no compliance issues. Participants reported no adverse effects from the diets and remained weight stable (± 2 kg) during the trial. Four women chose not to complete the study due to reasons unrelated to the trial. No significant differences ($P > 0.05$) were found between diets with regard to daily energy intakes and excretions, both faecal and urinary, nor the determined dietary metabolisable energy values, which ranged from 19.3 to 21.8 MJ/kg DM (**Table 2**).

Table 2

Daily intakes and faecal and urinary excretions of gross energy, and determined apparent faecal energy digestibility and metabolisable energy¹

	Diet				SEM
	Wheat Bran	Pectin	Low Fibre	High Fibre	
Number of women, <i>n</i>	5	6	4	6	
Daily intake, MJ/d	9.4	8.1	10.0	8.7	0.5
Faecal excretion, MJ/d	0.62	0.29	0.39	0.68	0.06
Urinary excretion, MJ/d	0.55 ^a	0.47 ^{ab}	0.27 ^{bc}	0.25 ^c	0.04
Faecal dE, %	93.6 ^{ab}	96.2 ^a	96.3 ^a	92.1 ^b	0.6
Determined ME, MJ / kg DM	20.3	21.8	20.1	19.3	0.4

¹ Data are expressed as means \pm pooled SEM.

^{abc} Means in a row without common superscript letters are significantly different, $P < 0.05$.
dE, digestibility of energy

The apparent digestibility of dietary energy ranged from 92 to 96%, differing significantly ($P < 0.05$) only for the HF diet compared to the PE and LF diets (Table 2). Apparent protein digestibility varied among the diets with that for the HF diet (88%) being significantly lower ($P < 0.05$) than for the WB and PE diets (both 95%) (**Table 3**).

No significant differences ($P>0.05$) were found between diets with regard to fat digestibility and both starch and sugars were virtually completely digested and absorbed (Table 3).

Table 3

Apparent faecal digestibility of nutrients in the experimental diets¹

	Diet				SEM
	Wheat Bran	Pectin	Low Fibre	High Fibre	
Number of women, <i>n</i>	5	6	4	6	
Apparent faecal digestibility, %					
Crude protein	94.7 ^a	95.4 ^a	93.6 ^{ab}	87.5 ^b	1.0
Fat	97.8	97.7	98.2	96.9	0.3
Total CHO	91.5 ^b	97.6 ^a	97.1 ^a	93.3 ^b	0.7
Starch	99.78	99.86	99.90	99.79	0.03
Sugars	99.84 ^{ab}	99.93 ^a	99.74 ^{bc}	99.54 ^c	0.05
TDF	40.4 ^b	74.2 ^a	77.8 ^a	59.5 ^{ab}	4.4
NSP	47.7 ^c	89.0 ^a	78.0 ^{ab}	62.8 ^{bc}	4.2

¹ Data are expressed as means \pm pooled SEM.

^{abc} Means in a row without common superscript letters are significantly different, $P<0.05$.

CHO, carbohydrate; NSP, non-starch polysaccharides; TDF, total dietary fibre

Rat ileal digestibility study

Daily energy and nutrient intakes varied significantly ($P < 0.05$) for one or more diets for all analysed dietary components (**Table 4**).

Table 4

Daily food and nutrient intakes for the growing rats¹

	Diet				SEM
	Wheat Bran	Pectin	Low Fibre	High Fibre	
Groups of rats (pooled), <i>n</i>	3	2	3	3	
Diet component					
Dry matter, <i>g/d</i>	14.8 ^{ab}	13.3 ^b	15.1 ^a	15.3 ^a	0.2
Gross energy, <i>kJ/d</i>	291.1 ^{ab}	264.8 ^b	303.0 ^a	300.7 ^a	4.9
Protein, <i>g/d</i>	4.0 ^a	3.4 ^b	2.5 ^c	2.5 ^c	0.1
Fat, <i>g/d</i>	2.00 ^{bc}	1.75 ^c	2.37 ^a	2.13 ^{ab}	0.04
Total CHO, <i>g/d</i>	7.6 ^b	7.3 ^b	9.3 ^a	9.5 ^a	0.2
Starch, <i>g/d</i>	1.1 ^b	0.5 ^c	3.4 ^a	3.5 ^a	0.1
Sugars, <i>g/d</i>	5.5 ^b	6.5 ^a	4.4 ^c	3.9 ^c	0.1
TDF, <i>g/d</i>	0.95 ^b	0.51 ^c	0.99 ^b	1.37 ^a	0.03
NSP, <i>g/d</i>	0.65 ^b	0.64 ^b	1.17 ^a	1.06 ^a	0.03

¹ Data are expressed as means \pm pooled SEM.

^{abc} Means in a row without common superscript letters are significantly different, $P < 0.05$.

CHO, carbohydrate; NSP, non-starch polysaccharides; TDF, total dietary fibre

Rat ileal (mouth to terminal ileum) apparent nutrient digestibility (%) and the ileal apparent digestibility of energy (%) was significantly different ($P < 0.05$) between one or more diets for all analysed dietary components, including individual FA (**Table 5**). The upper-tract digestibility of the NSP fraction was highly variable. Starch was virtually completely absorbed anterior to the terminal ileum for all of the diets.

Table 5Apparent ileal nutrient digestibility for the four human diets fed to growing rats¹

	Diet				SEM
	Wheat Bran	Pectin	Low Fibre	High Fibre	
Groups of rats (pooled), <i>n</i>	3	2	3	3	
Ileal digestibility, %					
Gross Energy	83.9 ^{ab}	83.1 ^{ab}	85.8 ^a	79.4 ^b	0.9
Crude Protein	84.7 ^a	77.6 ^b	82.8 ^{ab}	78.4 ^b	1.0
Total Fat	96.8 ^a	93.5 ^{ab}	90.4 ^b	90.2 ^b	0.9
Total CHO	79.3 ^b	87.1 ^a	86.3 ^a	79.0 ^b	1.3
Starch	99.2 ^{ab}	99.7 ^a	98.8 ^b	98.9 ^b	0.1
Sugars	85.4 ^b	92.6 ^a	85.4 ^b	78.3 ^c	1.7
NSP	1.0 ^b	56.2 ^a	7.9 ^b	-0.8 ^b	6.9
Dry matter	77.8 ^{ab}	79.9 ^{ab}	82.9 ^a	75.9 ^b	1.0
Organic matter	82.8 ^{ab}	85.2 ^{ab}	86.3 ^a	80.3 ^b	0.9
Fatty acids					
Saturated					
12:0	98.8 ^a	97.6 ^{ab}	97.1 ^{ab}	96.5 ^b	1.1
14:0	98.5 ^a	95.7 ^b	93.3 ^b	93.0 ^b	2.5
16:0	95.3 ^a	87.2 ^b	82.0 ^b	84.1 ^b	5.9
18:0	92.1 ^a	80.3 ^b	75.8 ^b	80.3 ^b	7.2
Monounsaturated					
16:1	98.9 ^a	93.6 ^b	97.3 ^a	96.9 ^a	2.0
18:1(n-9)	98.6 ^a	96.8 ^{bc}	97.0 ^b	95.3 ^c	1.4
18:1(n-7)	62.9 ^b	92.3 ^a	90.8 ^a	95.0 ^a	14.4
Polyunsaturated					
18:2	96.0 ^a	97.5 ^a	97.0 ^a	92.2 ^b	2.2
18:3	99.0 ^a	98.8 ^a	98.4 ^a	95.7 ^b	1.5
Other ²	93.4 ^a	78.5 ^b	49.7 ^c	77.2 ^b	17.6

¹ Data are expressed as means ± pooled SEM.² Unidentified fatty acids.^{abc} Means in a row without common superscript letters are significantly different, *P*<0.05.

Discussion

In the present work, both the site (small intestine, colon) and form (protein, fat, CHO) of energy disappearance for four mixed human diets of varying DF composition (wheat bran, pectin and mixed low/high fibre) were investigated to determine more completely the apparent uptake of energy from the gut. A human balance study was undertaken to determine the apparent faecal digestibility of energy-providing nutrients and an animal model (growing rat) was used to predict apparent ileal nutrient digestibility in humans.

A critical assumption in the present study is that the growing rat is a valid model for the adult human for determining the digestibility of nutrients in the upper digestive tract (mouth to terminal ileum). Being simple-stomached omnivores, the anatomy and histology of the digestive tracts and the physiology of digestion is similar in the two species (Kararli, 1995). Transit time of food through the small intestine is 3 – 4 h in both species (DeSesso and Jacobson, 2001). Comparative studies have shown the rat to be a valid model for total tract nutrient digestibility in adult humans for a wide range of diets (Bach Knudsen *et al.*, 1994; Forsum *et al.*, 1982; Nyman *et al.*, 1986; Rich *et al.*, 1980), including total tract digestibility of energy, fat, protein and NSP in mixed diets for humans (Wisker *et al.*, 1997; Wisker *et al.*, 1996). Comparable comparative studies have not been undertaken for upper-tract digestibility, but it is well established that the growing pig is a valid model for the adult human for ileal protein digestibility (Deglaire *et al.*, 2009; Moughan and Rowan, 1989; Rowan *et al.*, 1994) and good agreement has also been shown between the growing rat and the growing pig for ileal protein digestibility over a very wide range of diets and sources of protein (Donkoh *et al.*, 1994; Moughan *et al.*, 1984; Moughan *et al.*, 1987; Picard *et al.*, 1984; Rutherford and Moughan, 2003; Smith *et al.*, 1987). The growing rat also offers logistical advantages. The terminal anaesthesia method used with the rat digestibility assay is straightforward and more ethically acceptable than using cannulated animals because the need for surgery is avoided and there is no ongoing discomfort for the animal.

Rats are readily available, easily housed and cared for and are a more economical option than the pig as an animal model.

In the presently reported work, significant differences ($P<0.05$) in the faecal digestibility of energy in humans were observed between the HF diet (92%) compared to the PE and LF diets (both 96%). Significant ($P<0.05$) differences in faecal digestibility were also observed between one or more diets for all of the analysed dietary components, except for fat, and starch (Table 3).

There was a significant difference ($P<0.05$) in apparent ileal (rat model) protein digestibility (Table 5) between the WB (85%) and PE (78%) diets, even though the protein sources for both diets were very similar (refer Table A1). Ileal effluent contains not only undigested dietary material, but also endogenous protein and fat, and the endogenous contribution to ileal output varies between diets (Boisen and Moughan, 1996; Duran-Montge *et al.*, 2007). This endogenous material is not differentiated from that of exogenous origin during normal chemical analyses, consequently reducing apparent digestibility values for dietary protein and fat. The higher ileal digestibility of fat for the WB diet (97%) compared to the LF and HF diets (both 90%) appeared to be a result of the inter-diet differences in the ileal uptake of most, but not all, of the individual fatty acids (Table 5). The differences in individual fatty acid digestibility did not seem to be related to either total fat intake or the fatty acid profile of the diets (refer Table A3, Appendix). There may be interactive effects between the fatty acids and other dietary components in the upper-tract.

Pectin has been documented to be well fermented in the hindgut, but poorly digested in the upper-tract (Saito *et al.*, 2005; Sandberg *et al.*, 1983). While our findings point to extensive hindgut fermentability, the PE diet had a not inconsiderable ileal NSP digestibility of 56%, compared to values of 10 - 15% reported elsewhere (Saito *et al.*, 2005; Sandberg *et al.*, 1983). It is possible that the quantity of NSP in the diet may have been overestimated for this diet, during extraction of DF at high temperatures, as can occur with pectin-rich foods in particular (Bach Knudsen, 2001). This may be the

cause of the seemingly high ileal NSP digestibility for the PE diet, because relative to the TDF content of the diet, the NSP content appeared to be considerably higher than expected. However, it is also possible that extensive fermentation may have occurred prior to the terminal ileum due to bacterial activity (Drasar and Hill, 1974; Neut *et al.*, 2002), which is more likely to occur with highly fermentable DF such as pectin. It is also possible that the microflora in the rat upper-tract may be more abundant and more active than in humans, although this was not seen with the LF and HF diets which also contained a number of highly fermentable ingredients. In general, further investigation is warranted to determine if both humans and rats rank diets the same in terms of ileal digestibility.

Total CHO was quantitatively the largest component of ileal dry matter output for most of the diets, being approximately double that of combined protein and fat ileal output for the WB and HF diets and 1.5 times that for the LF diet. Undigested CHO available for hindgut fermentation was predicted to be between 26 g/d (PE) and 54 g/d (HF) in the present study. These findings agree with literature estimates for humans of between 20 and 40 g/d of undigested CHO for Western diets, and up to 50 - 60 g/d for diets rich in cereals / fruits / vegetables (Cummings, 1996; Elia and Cummings, 2007).

The known food intakes of the participants and the ileal nutrient digestibility values (laboratory rat) allowed calculation of predicted human ileal nutrient outputs, and predicted nutrient uptakes in the upper digestive tract. Also, data for actual faecal nutrient output in the human participants and the predicted ileal nutrient outputs allowed calculation of the predicted colonic losses of nutrients and gross energy (**Table 6**).

Table 6

Actual daily intakes and faecal excretions and derived values (predicted) for human ileal output¹, upper-tract uptake and colonic loss for several dietary components²

	Diet			
	Wheat Bran	Pectin	Low Fibre	High Fibre
Number of women, <i>n</i>	5	6	4	6
Diet component				
Energy, <i>MJ/d</i>				
Actual daily intake	9.4	8.1	10.0	8.7
Predicted uptake (upper-tract)	7.9	6.7	8.6	6.9
Predicted ileal output	1.5	1.4	1.4	1.8
Actual faecal excretion	0.6	0.3	0.4	0.7
Predicted colonic loss	0.9	1.1	1.0	1.1
Protein, <i>g/d</i>				
Actual daily intake	134.8	113.7	87.0	79.8
Predicted uptake (upper-tract)	114.2	88.2	72.0	62.6
Predicted ileal output	20.6	25.5	15.0	17.2
Actual faecal excretion	7.6	5.0	5.8	9.8
Predicted colonic loss	13.0	20.5	9.2	7.4
Fat, <i>g/d</i>				
Actual daily intake	65.6	51.3	85.4	66.9
Predicted uptake (upper-tract)	63.5	48.0	77.2	60.3
Predicted ileal output	2.1	3.4	8.2	6.6
Actual faecal excretion	1.6	1.1	1.7	2.0
Predicted colonic loss	0.6	2.3	6.6	4.6
Total CHO, <i>g/d</i>				
Actual daily intake	214.2	198.5	283.3	258.2
Predicted uptake (upper-tract)	169.8	172.8	244.3	204.1
Predicted ileal output	44.4	25.7	38.9	54.1
Actual faecal excretion	18.8	4.5	8.5	17.3

Predicted colonic loss	25.5	21.2	30.4	36.9
<i>Starch, g/d</i>				
Actual daily intake	39.7	16.2	135.8	121.2
Predicted uptake (upper-tract)	39.4	16.1	134.2	119.9
Predicted ileal output	0.3	0.05	1.6	1.3
Actual faecal excretion	0.09	0.02	0.2	0.3
Predicted colonic loss	0.2	0.03	1.5	1.0
<i>Sugars, g/d</i>				
Actual daily intake	162.1	176.4	127.2	95.8
Predicted uptake (upper-tract)	138.5	163.3	108.6	75.0
Predicted ileal output	23.6	13.1	18.6	20.8
Actual faecal excretion	0.3	0.1	0.4	0.4
Predicted colonic loss	23.3	13.0	18.2	20.4
<i>NSP, g/d</i>				
Actual daily intake	28.3	17.9	20.2	23.1
Predicted uptake (upper-tract)	0.3	10.0	1.6	-0.2
Predicted ileal output	28.0	7.8	18.6	23.3
Actual faecal excretion	11.4	0.6	3.4	8.3
Predicted colonic loss	12.8	5.9	13.0	10.2
<i>Dry matter, g/d</i>				
Actual daily intake	432.3	376.0	471.5	420.4
Predicted uptake (upper-tract)	336.4	300.6	390.9	319.1
Predicted ileal output	95.9	75.5	80.5	101.3
Actual faecal excretion	34.1	15.0	19.0	34.1
Predicted colonic loss	61.9	60.5	61.5	67.2

¹ Ileal output is predicted based on human daily intake and mean rat apparent ileal digestibility (refer Table 5).

² Data are expressed as means.

A comparison of predicted human ileal and actual human faecal outputs (Table 6) showed that for some nutrients (starch) there was little difference between ileal and faecal excretion rates due to a high degree of absorption in the upper-tract. However,

the daily quantity of protein excreted at the ileal level was between two (HF) and five (PE) times higher than that at the faecal level. Estimates of protein entering the colon are given as 5 – 20 g/d in the literature (Cummings, 1996), which is slightly lower than the 15 g/d (LF) to 26 g/d (PE) predicted here. Large differences were also observed between the ileal and faecal outputs of total fat, with the ileal excretion being as much as three times that at the faecal level (LF diet). In the case of the PE diet, the combined total ileal output of protein and fat (29 g/d) was higher than total CHO (26 g/d) output.

The ratio of predicted nutrient loss (colon : upper-tract) for all determined dietary nutrients (except NSP) was no larger than 0.3 (HF, sugars), indicating that apparent upper-tract nutrient digestibility exceeded that in the colon by at least a threefold difference. The largest difference between the sites of uptake was for starch (data not shown) with ratios ranging from 0.002 (PE) to 0.01 (all other diets). In the case of NSP, the predicted uptake in the colon was calculated relative to total tract uptake (**Figure 1A**).

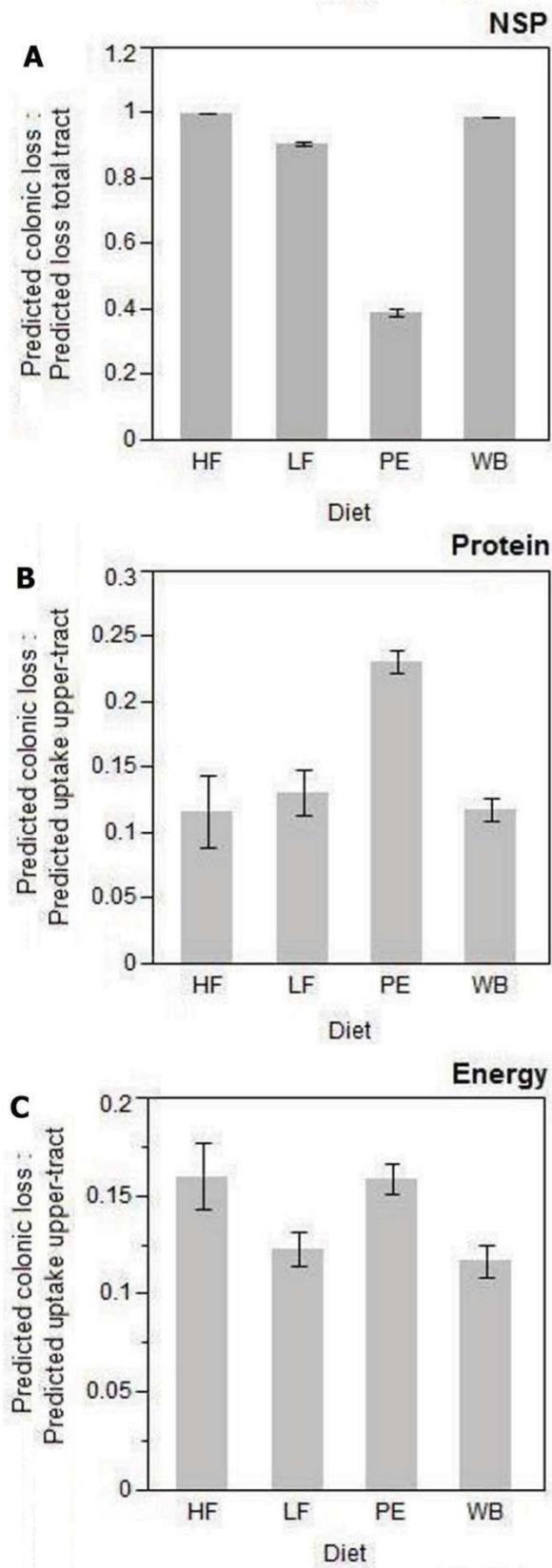


Figure 1. Ratio of predicted colonic loss : predicted total tract loss of NSP (A) and the ratio of predicted colonic loss : predicted upper-tract uptake of Protein (B) and Gross

Energy (C). Values are means \pm pooled SEM, $n=6$ participants / diet except for WB diet ($n=5$) and LF diet ($n=4$).

When calculating the ratio of predicted loss (colon) : predicted loss (total tract) for the HF diet it was assumed that there was no uptake of NSP in the upper-tract. This was necessary to account for a small apparent net gain of NSP (assumed as analytical error) in the upper-tract for the HF diet. Only the PE diet exhibited more extensive NSP uptake in the upper-tract than in the colon with a ratio of predicted colonic loss : predicted loss (total tract) of 0.4. The three remaining diets were observed to have colonic NSP loss far exceeding the NSP uptake in the upper-tract with ratios of 0.9 - 1. There was a twofold difference in the ratio of protein loss in the colon to the upper-tract uptake (Figure 1B) for the PE diet (0.2) compared to the other three diets (all approximately 0.1). The ratio of gross energy loss in the colon to the upper-tract uptake ranged from 0.12 (LF and WB) to 0.16 (HF and PE) (Figure 1C). The considerably larger uptake of energy in the upper-tract reaffirms the much larger contribution upper-tract digestion makes to total tract digestion. Despite some general similarities in the relative portion of energy uptake/loss in the upper-tract and colon across the diverse diets, the ratio of the predicted uptake of specific nutrients was unique to each diet and no two diets exhibited the same predicted uptake ratios across all analysed dietary components. Moreover, the present data highlight the quantitative importance of hindgut digestion to the overall digestive process.

Conclusion

Apparent faecal digestibility data are unable to differentiate between upper-tract and colonic uptake for different diets despite the fact that it is important to be able to predict both the site (upper-tract or colon) and degree of absorption of each nutrient because (i) nutrients vary in the efficiency by which they yield energy that is ultimately useful to the body (net ATP gains), and (ii) the energy made available to the body via short chain fatty acids (SCFA) from nutrients fermented in the hindgut is less than that obtained from direct nutrient uptake in the upper-tract. There is a need to be able to determine separately the contribution of nutrient absorption in the upper-tract and colon. The current reliance on generic digestibility values or total tract digestibility data does not allow a completely accurate quantification of the energy available from the many diverse diets consumed by different populations globally. This has implications for the nutritional status of communities in both developing and developed nations. Calculating the quantitative uptake of each nutrient in the upper and lower digestive tracts separately provides more useful information than does total tract digestibility data alone.

Literature cited

- Ali, L. H., Angyal, G., Weaver, C. M. & Rader, J. I. (1997). Comparison of capillary column gas chromatographic and AOAC gravimetric procedures for total fat and distribution of fatty acids in foods. *Food Chemistry*, 58(1-2), 149-160.
- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Bach Knudsen, K. E. (2001). The nutritional significance of "dietary fibre" analysis. *Animal Feed Science and Technology*, 90, 3-20.
- Bach Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, W. & Eggum, B. O. (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat. *British Journal of Nutrition*, 71, 471-487.
- Boisen, S. & Moughan, P. J. (1996). Dietary influences on endogenous ileal protein and amino acid loss in the pig - A review. *Acta Agriculturae Scandinavica, Section A - Animal Science*, 46, 154-164.
- Cummings, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet*, 321(8335), 1206-1209.
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H. & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, 70, 443-459.
- Darragh, A. J. & Hodgkinson, S. M. (2000). Quantifying the digestibility of dietary protein. *Journal of Nutrition*, 130, 1850S-1856S.
- Deglaire, A., Bos, C., Tomé, D. & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39, 209-228.

-
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994). The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, *49*, 57-71.
- Dowsett, J., Gibney, M. J. & Kennedy, N. P. (1990). Bacterial fermentation occurs in the terminal ileum of ileostomates. *Proceedings of the Nutrition Society*, *49*, 110.A. (abs.).
- Drasar, B. S. & Hill, M. J. (1974). *Human Intestinal Flora*. New York: Academic Press.
- Dubois, M., Gillies, K. A., Hamilton, J. A., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350-356.
- Duran-Montge, P., Lizardo, R., Torrallardona, D. & Esteve-Garcia, E. (2007). Fat and fatty acid digestibility of different fat sources in growing pigs. *Livestock Science*, *109*(1-3), 66-69.
- Elia, M. & Cummings, J. H. (2007). Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *European Journal of Clinical Nutrition*, *61* S40-S74.
- Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst (Cambridge UK)*, *109*, 937-942.
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, *71*, 808-814.
- FDA. (2004). *Code of Federal Regulations, Title 21, Volume 2, Chapter I, Part 101.9*. Washington DC: Department of Health and Human Services, Food and Drug Administration.
- Forsum, E., Goranzon, H. & Thilen, M. (1982). Protein evaluation of mixed diets in young adults, growing pigs, and growing rats. *American Journal of Clinical Nutrition*, *36*, 505-513.
- Kararli, T. T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharmaceutics & Drug Disposition*, *16*, 351 - 380.

-
- Kraisid, T., MacLean Jr., W. C. & Warwick, P. M. (2003). *Food energy: methods of analysis and conversion factors: report of a technical workshop, Rome, 3-6 December 2002*. Rome: Food and Agriculture Organization of the United Nations.
- McBurney, M. I. & Thompson, L. U. (1989). Dietary fiber and energy balance: integration of the human ileostomy and *in vitro* fermentation models. *Animal Feed Science and Technology*, 23, 261-275.
- McBurney, M. I., Thompson, L. U. & Jenkins, D. J. A. (1987). Colonic fermentation of some breads and its implications for energy availability in man. *Nutrition Research*, 7, 1229-1241.
- McNeil, N. I. (1984). The contribution of the large intestine to energy supplies in man. *American Journal of Clinical Nutrition*, 39, 338-342.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, 13, 501-508.
- Moughan, P. J. & Rowan, A. M. (1989). The pig as a model animal for human nutrition research. *Proceedings of the Nutrition Society of New Zealand*, 14, 116-123.
- Moughan, P. J., Smith, W. C. & James, K. A. C. (1984). Preliminary observations on the use of the rat as a model for the pig in determination of apparent digestibility of dietary proteins. *New Zealand Journal of Agricultural Research*, 27, 509-512.
- Moughan, P. J., Smith, W. C. & Kies, A. K. (1987). Comparison of the ileal digestibility of amino acids in ground barley for the growing rat and pig. *New Zealand Journal of Agricultural Research*, 30, 59-66.
- Muller, K. E. & Fetterman, B. A. (2002). *Regression and ANOVA: An Integrated Approach Using SAS Software*. Cary, NC: SAS Publishing.
- National Research Council. (1995). Nutrient requirements of the laboratory rat. In *Nutrient Requirements of Laboratory Animals* (4th rev. ed., pp. 11-79). Washington, DC: National Academy Press.

-
- Neut, C., Bulois, P., Desreumaux, P., Membre, J. M., Lederman, E., Gambiez, L., Cortot, A., Quandalle, P., van Kruiningen, H. & Colombel, J. F. (2002). Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *American Journal of Gastroenterology*, 97(4), 939-946.
- Nyman, M., Asp, N. G., Cummings, J. H. & Wiggins, H. (1986). Fermentation of dietary fibre in the intestinal tract: comparison between man and rat. *British Journal of Nutrition*, 55, 487-496.
- Picard, M., Bertrand, S., Duron, M. & Maillard, R. (1984). *Comparative digestibility of amino acids using 5 animal models: intact cockerel, caeectomised cockerels, rat deprived of large intestine, piglet with an ileo-caecal cannulation, piglet with an ileo-rectal shunt*. Paper presented at the Proceedings of the IVth European Symposium of Poultry Nutrition, Tours, France.
- Rich, N., Satterlee, L. D. & Smith, J. L. (1980). A comparison of *in vivo* apparent digestibility as determined using human and rat pancreatins and commercially available proteases. *Nutrition Reports International*, 21, 285-299.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K. & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71, 29-42.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, 87, 292-300.
- Saito, D., Nakaji, S., Fukuda, S., Shimoyama, T., Sakamoto, J. & Sugawara, K. (2005). Comparison of the amount of pectin in the human terminal ileum with the amount of orally administered pectin. *Nutrition* 21, 914-919.
- Sandberg, A.-S., Ahderinne, R., Andersson, H., Hallgren, B. & Hulten, L. (1983). The effect of citrus pectin on the absorption of nutrients in the small intestine. *Human Nutrition Clinical Nutrition*, 37(3), 171-183.
- Short, F. J., Gorton, P., Wiseman, J. & Boorman, K. N. (1996). Determination of titanium oxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, 59, 215-221.

-
- Smith, W. C., Moughan, P. J., Pearson, G. & James, K. A. C. (1987). Comparative bioavailable energy values of five ground cereal grains measured with growing rats and pigs. *Animal Feed Science and Technology*, 18, 143-150.
- Sukhija, P. S. & Palmquist, D. L. (1988). Rapid method for determination of total fatty-acid content and composition of feedstuffs and feces. *Journal of Agricultural and Food Chemistry*, 36(6), 1202-1206.
- Wisker, E., Bach Knudsen, K. E., Daniel, M., Eggum, B. O. & Feldheim, W. (1997). Energy values of non-starch polysaccharides: comparative studies in humans and rats. *Journal of Nutrition*, 127, 108-116.
- Wisker, E., Bach Knudsen, K. E., Daniel, M., Feldheim, W. & Eggum, B. O. (1996). Digestibilities of energy, protein, fat and nonstarch polysaccharides in a low fiber diet and diets containing coarse or fine whole meal rye are comparable in rats and humans. *Journal of Nutrition*, 126(2), 481-488.
- Wu, J. R., James Jr., D. W., Dooner, H. K. & Browse, J. (1994). A mutant of arabidopsis deficient in the elongation of palmitic acid. *Plant Physiology*, 106(1), 143-150.
- Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, 86, 1649-1656.

Appendix to Chapter III

Table A1

Food ingredients and foods included in the human diets

Ingredient	Wheat	Pectin	Low Fibre	High Fibre
	Bran Diet	Diet	Diet	Diet
Amount (g/MJ) ¹				
Milk (high calcium, 0.1% fat) ²	78.73	88.67	26.19	31.78
Chicken	11.66	13.51	3.27	3.31
Mayonnaise ³	2.19	2.53	1.31	0.93
Sugar	2.92	3.55	1.57	1.32
Cheese spread ⁴	1.46	1.69	3.27	-
Butter (61% fat) ⁵	1.75	-	2.62	1.46
Cheddar cheese ⁶	2.92	3.38	-	1.99
Wheat bran cereal ⁷	11.96	-	-	3.31
Citrus pectin ⁸	-	3.38	-	-
Custard	8.75	-	-	-
Cheese omelette	10.21	11.82	-	-
Jelly	35.43	32.93	-	-
Osmolite ^{®9} (isotonic supplement)	43.74	59.11	-	-
Fish (Hoki)	14.58	16.89	-	-
Cream (12% fat) ¹⁰	0.73	0.84	-	-
Marshmallow chocolate bar ¹¹	4.37	7.77	-	-
Blackcurrant drink ¹²	-	16.89	5.24	-
Whole-wheat cereal ¹³	-	-	3.93	-
Water crackers ¹⁴	-	-	3.14	-
Wheat bread (white) ¹⁵	-	-	13.62	-
Honey	-	-	0.65	-
Dairy dessert ¹⁶	-	-	19.64	-
Popcorn ¹⁷	-	-	3.27	2.65

Peaches (canned)	-	-	15.72	6.62
Apples (canned)	-	-	15.72	13.24
Tomato ketchup	-	-	2.62	1.99
Cucumber	-	-	5.24	5.30
Alfalfa sprouts	-	-	1.96	1.99
Carrots (frozen, boiled)	-	-	10.48	21.19
Green beans (frozen, boiled)	-	-	9.17	18.54
Chicken nuggets ¹⁸	-	-	16.50	16.68
Wheat bran cereal ¹⁹	-	-	-	6.62
Toasted muesli ²⁰	-	-	-	6.62
Rye crackers ²¹	-	-	-	1.99
Wheat bread (white, high fibre) ²²	-	-	-	10.33
Yoghurt (contains oligosaccharides) ²³	-	-	-	19.86

¹ MJ refers to the allocated daily dietary energy intake of an individual subject.

² Calci-trim®, Meadow Fresh, New Zealand; ³ Real Mayonnaise, Best Foods, Canada; ⁴ Kraft Foods, Australia; ⁵ Lite Dairy Spread, Anchor, New Zealand; ⁶ Edam, Valumetric®, New Zealand; ⁷ All-Bran®, Kellogg, Australia; ⁸ 7050 acid/milk pectin, Cerestar, Krefeld, Germany; ⁹ Abbott Laboratories, IL; ¹⁰ Lite Cream, Anchor, New Zealand; ¹¹ Pinky®, Cadbury, New Zealand; ¹² Ribena® syrup, GlaxoSmithKline, New Zealand; ¹³ Weet-Bix®, Sanitarium, New Zealand; ¹⁴ Original Water Crackers, Arnotts, Australia; ¹⁵ Nature's Fresh®, Quality Bakers, New Zealand; ¹⁶ CalciYum®, Swiss Maid, New Zealand; ¹⁷ Lite 'n' Buttery, Pop'n Good, New Zealand; ¹⁸ Glovers, New Zealand; ¹⁹ Sultana Bran®, Kellogg, Australia; ²⁰ Vitacrunch, Flemings, New Zealand; ²¹ Kavli® crisp bread, Kavli, Bergen, Norway; ²² Nature's Fresh Fibre White®, Quality Bakers, New Zealand; ²³ Apricot flavour, Metchinkoff, Fonterra, New Zealand.

Table A2

Formulation of the rat diets

Ingredient	Wheat Bran	Pectin	Low Fibre	High Fibre
	Diet	Diet	Diet	Diet
Composition, g/kg ¹				
Human diet mix (wet) ²	956.1	973.2	970.7	964.6
Vitamin Mix ³	21.3	13.0	14.2	17.2
Mineral Mix ⁴	21.3	13.0	14.2	17.2
Titanium dioxide	1.3	0.8	0.9	1.0

¹ per kg wet diet.² The human diet mix (refer Table A1) was used as the base for the rat diet.³ Containing the following (g/kg vitamin mix): retinol palmitate, 0.1; cholecalciferol, 0.0005; α -tocopherol acetate, 4; menadione, 0.06; thiamin-HCl, 0.1; riboflavin, 0.14; nicotinic acid, 0.4; calcium pantothenate, 0.4; pyridoxine-HCl, 0.16; biotin, 0.02; folic acid, 0.04; cyanocobalamin, 0.001; myo-inositol, 4; choline chloride, 30; sucrose, 960.6.⁴ Containing the following (g/kg mineral mix): calcium, 126; chloride, 156; magnesium, 21; phosphorus, 97; potassium, 105; sodium, 39; chromium, 0.039; copper, 0.214; iron, 9; manganese, 2; zinc, 0.964; cobalt, 0.00058; iodine, 0.003; molybdenum 0.003; selenium, 0.003; sucrose, 443.8.

Table A3

Determined chemical composition¹ of the four experimental diets given to the women and used as a base material for diets given to growing rats

Component	Wheat Bran Diet	Pectin Diet	Low Fibre Diet	High Fibre Diet
Dry matter ¹ , <i>g per kg diet</i>	936.3	906.0	926.7	926.3
Organic matter, <i>g</i>	961.9	966.6	966.5	961.7
Gross energy, <i>MJ</i>	21.7	21.4	21.2	20.7
Crude protein, <i>g</i>	299.5	305.5	189.5	192.1
Fat, <i>g</i>	139.2	116.6	151.2	146.1
Total CHO, <i>g</i>	523.2	544.5	625.7	623.5
Starch, <i>g</i>	91.8	43.0	288.1	288.3
Sugars, <i>g</i>	374.9	469.1	269.8	227.8
Dietary fibre				
Total, <i>g</i>	68.3	45.1	69.0	100.4
Insoluble, <i>g</i>	60.8	NR ²	54.0	76.8
Insoluble, <i>% of total DF</i>	89.1	NR ²	78.2	76.5
Soluble, <i>g</i>	7.5	NR ²	15.0	23.6
Soluble, <i>% of total DF</i>	10.9	NR ²	21.8	23.5
NSP, <i>g</i>	65.4	47.5	42.9	55.0
Ash, <i>g</i>	38.1	33.4	33.5	38.3
Fatty acids, <i>g</i>				
Saturated				
12:0	1.1	0.6	1.5	1.3
14:0	5.1	3.1	3.4	5.3
16:0	21.6	15.3	30.1	35.6
18:0	8.7	6.7	6.5	8.9
Monounsaturated				
16:1	1.5	1.4	1.7	2.0
18:1(n-9)	45.4	38.3	41.0	44.5

18:1(n-7)	3.7	2.5	3.4	3.4
Polyunsaturated				
18:2	15.4	10.6	18.6	14.8
18:3	3.7	3.0	2.2	2.7
Other ³	15.4	11.5	7.8	8.8

¹ Reported as per kg dry matter for all diet components, except for dry matter (per kg freeze-dried diet)

² NR: Not reported. Insoluble and soluble fibre fractions are not given for the Pectin Diet as the results obtained were untenable.

³ Other unidentified fatty acids that were measured but not included in the table.

CHO, carbohydrate; DF, dietary fibre; NSP, non-starch polysaccharides

CHAPTER IV

The 'Combined Model'

This chapter introduces the framework of a model, which combines predictive nutrient uptake and colonic loss data obtained using in vivo and in vitro digestibility assays respectively, with a simple mathematical model that uses stoichiometric relationships to predict ATP yields based on nutrient uptake. The digestibility assays predict human upper-tract nutrient digestibility (as modelled by the rat upper digestive tract) and colonic digestibility, predicted by fermenting rat ileal digesta in an in vitro digestion system containing human faecal bacteria. The studies described in subsequent chapters develop and validate the in vitro hindgut digestibility assay and the dual (in vivo – in vitro) digestibility assay.

Introduction

The ability to predict absolute quantities of nutrients available for cellular metabolism offers a more accurate means of determining the energy available to the body. It has been shown previously that considerable variation exists among different types of diets and nutrients for the colonic : small intestinal energy uptake (Coles *et al.*, 2010). There is, therefore, a need to be able to calculate the quantitative uptake of each nutrient in the upper and lower digestive tract separately. This provides more useful information than does total tract digestibility data alone. To address this need, we propose the development of a model (referred to as the 'Combined Model') to predict the upper-tract, hindgut and total tract uptakes of energy in humans. The Combined Model allows for the prediction of ATP yields from the catabolism of nutrients predicted (based on digestibility assays) to be absorbed from the upper- and lower-digestive tract, respectively. The model has particular relevance to the design and evaluation of specialised weight-loss foods.

Description of model

The Combined Model brings together predictive nutrient uptake and colonic loss data obtained using *in vivo* and *in vitro* digestibility assays, with a simple mathematical model to predict ATP yields based on absorbed nutrients. When combined, the digestibility assays (referred to as the 'dual digestibility assay') predict human upper-tract digestibility, as modelled by the rat upper digestive tract, and colonic digestibility, predicted by fermenting rat ileal digesta in an *in vitro* digestion system containing human faecal bacteria (Coles *et al.*, 2005). The dual digestibility assay provides information on the predicted uptake of nutrients and SCFA (short chain fatty acids) from the upper- and lower-digestive tracts, respectively. Such data can then be used with stoichiometric relationships to predict (the potential) ATP available to the body from the absorbed nutrients. The stoichiometry relates to the hypothetical state whereby energy intake \leq energy expenditure and all absorbed nutrients are catabolised. This assumption is an oversimplification since after consumption of a main meal excess energy will be temporarily stored for later use.

In addition to predicting upper-tract nutrient uptake, the yield of SCFA from hindgut fermentation also needs to be quantified to allow prediction of colonic energy uptake. The growing rat is not a suitable model for predicting hindgut fermentation in humans (Bach Knudsen *et al.*, 1994). Also, while carbohydrate present in the faeces is mainly of dietary origin, virtually all faecal fat and protein is of endogenous or bacterial origin (Elia and Cummings, 2007; Walker, 1975). The inability of apparent faecal digestibility data to isolate the material of dietary origin necessitates an alternative means of predicting energy uptake in the colon. The *in vitro* fermentation assay used in the Combined Model to predict large intestinal digestibility aims to account for the dynamic and complex nature of colonic fermentation through incubating the substrate with live bacteria sourced from fresh human faecal samples.

The proposed Combined Model (**Figure 1**) characterises the flow of dietary energy through the digestive tract (diet, terminal ileum, faeces) in terms of either gross energy

(kJ/d) or absolute quantity (g/d) of individual dietary components (protein, starch, sugars, fat, DF). Chemical analysis of one or more of these parameters is performed on the diet and the ileal digesta to determine uptake in the upper-tract. The Combined Model also allows human ileal output (kJ energy/d or g nutrient/d) to be predicted based on dietary intakes (kJ/d or g/d), as demonstrated previously (Coles *et al.*, 2010). Residual ileal material in the *in vitro* fermentation vessel post-incubation is measured in terms of OM fermented or SCFA production. The amount of OM fermented during hindgut *in vitro* fermentation can be calculated based on the *in vitro* hindgut OMD (organic matter digestibility) and then converted to SCFA yields, which can be further equated with energy uptakes from estimated equivalents in the literature (Cummings and Macfarlane, 1997).

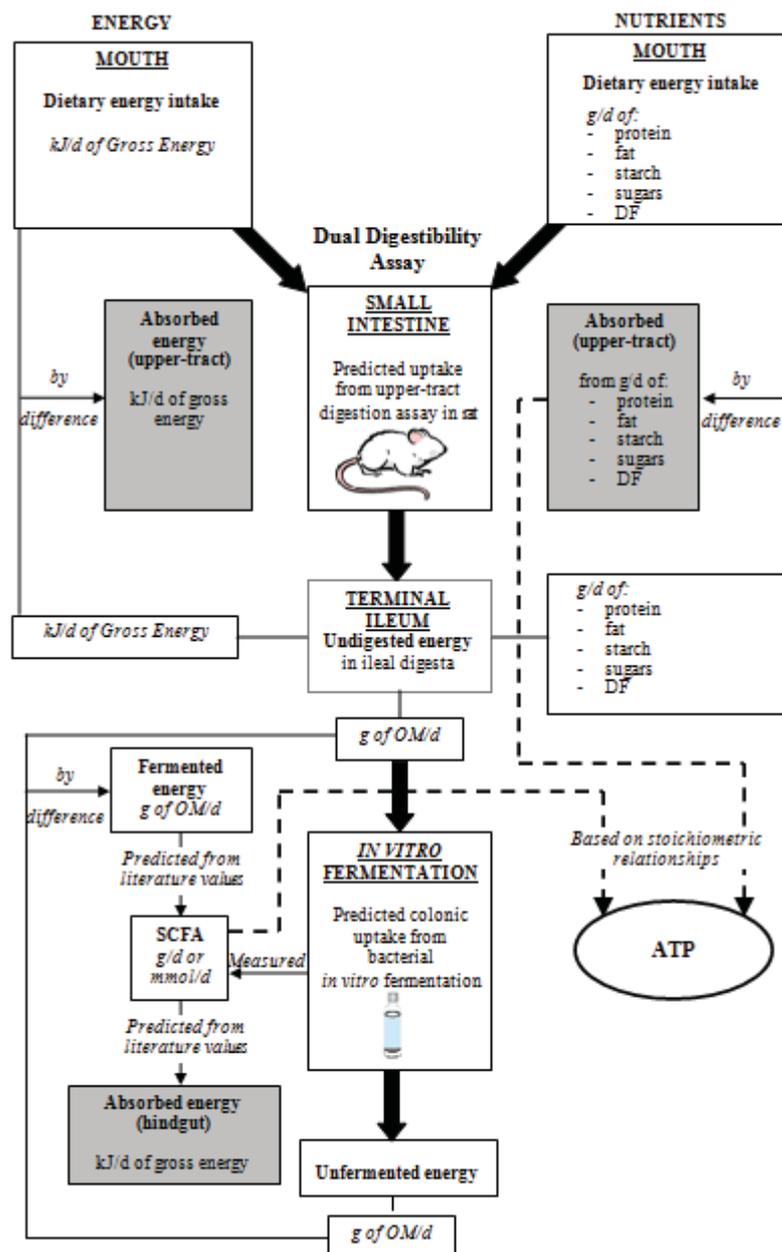


Figure 1. Schematic diagram of a Combined Model of digestion (combination of *in vivo* and *in vitro* digestion assays and stoichiometric chemical relationships) over the total tract, showing inputs and outputs at each stage. Left-hand side: model can be used to predict absorbed energy over the upper tract, hindgut and total tract in kJ/d. Right-hand side: model can be used to predict absorbed energy (g/d) in the upper tract in terms of protein / fat / starch / sugars / DF. The stoichiometry relates to the hypothetical state whereby energy intake \leq energy expenditure and all absorbed nutrients are catabolised.

Discussion

The upper-tract digestibility of not only gross energy, but also of most macronutrients, can be predicted using the Combined Model. The model's flexibility in this regard allows for customisation depending on the depth and type of required information or laboratory equipment / expertise available. For example, analysis of amino acids or fatty acids in both the diet and ileal digesta can be included to provide a detailed profile of the upper-tract uptake of these components.

The Combined Model also allows for the separate characterisation of undigested dietary and endogenous nutrient flows. Upper-tract endogenous losses may be predicted based on dietary dry matter intake and included in the model. Differentiation between hindgut fermentation of dietary and endogenous materials could potentially be predicted by the model through *in vitro* fermentation of endogenous material in addition to total digesta. Quantification of endogenous losses in the upper-tract and hindgut would allow 'real' or 'true' as opposed to 'apparent' ileal / faecal digestibility to be determined, which is considered to be more accurate than apparent digestibility (Darragh and Hodgkinson, 2000).

The rat, the pig and man are all simple-stomached omnivores with similar gastrointestinal anatomy and physiology (Kararli, 1995; Moughan and Rowan, 1989), including a small intestinal transit time of 3 - 4 h (DeSesso and Jacobson, 2001; Wilfart *et al.*, 2007). However, the growing rat was chosen here over the growing pig as an animal model for human upper-tract digestion due to a number of economic and logistical advantages. Digestibility studies with pigs demand a greater degree of animal husbandry, veterinary intervention and post-surgery recovery time, as well as increased food, housing and labour costs compared to rats. Furthermore, the terminal anaesthesia method used with the rat digestibility assay is straightforward and more ethically acceptable than using cannulated pigs because the need for surgery is avoided and there is no ongoing discomfort for the animal. Although there is a lack of comparative studies between upper-tract digestibility in the rat and human, it is well

established that the growing pig is a valid model for the adult human for ileal protein digestibility (Deglaire *et al.*, 2009; Moughan and Rowan, 1989; Rowan *et al.*, 1994) and good agreement has also been shown between the growing rat and the growing pig for ileal protein digestibility over a very wide range of diets and sources of protein (Donkoh *et al.*, 1994; Moughan *et al.*, 1984; Moughan *et al.*, 1987; Picard *et al.*, 1984; Rutherford and Moughan, 2003; Smith *et al.*, 1987).

The use of rat ileal digesta as a substrate for *in vitro* fermentation has its limitations, however, with the main drawback being the limited quantities of digesta obtained, particularly with highly viscous diets (such as pectin) or diets that are highly digestible in the upper-tract. The use of a larger animal, such as the pig, could potentially overcome this issue. The Combined Model requires fresh faecal samples to be obtained from human subjects on demand for the *in vitro* fermentation assay and while this is physiologically preferable, it is not ideal from a pragmatic point of view and there is a need for validated alternatives for the model.

Conclusion

The proposed model differentiates between the location (upper-tract or hindgut) of nutrient uptake and determines the contribution of each nutrient to upper-tract energy uptake. This allows diets to be defined in terms of actual energy available for cellular metabolism (ATP), which is a true measure of the net or available energy provided by a diet at the cellular level. Application of the Combined Model in this way, by quantitatively linking nutrient upper-tract / hindgut uptake of the various energy-yielding nutrients to cellular ATP yields, following the optimisation and validation of the model, is the subject of the following chapters.

Literature cited

- Bach Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, W. & Eggum, B. O. (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat. *British Journal of Nutrition*, 71, 471-487.
- Coles, L. T., Moughan, P. J., Awati, A., Darragh, A. J. & Zou, M. L. (2010). Predicted apparent digestion of energy-yielding nutrients differs between the upper and lower digestive tract in rats and humans. *Journal of Nutrition*, 140(3), 469-476.
- Coles, L. T., Moughan, P. J. & Darragh, A. J. (2005). *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, 123, 421 – 444.
- Cummings, J. H. & Macfarlane, G. T. (1997). Role of intestinal bacteria in nutrient metabolism. *Journal of Parenteral and Enteral Nutrition*, 21(6), 357-365.
- Darragh, A. J. & Hodgkinson, S. M. (2000). Quantifying the digestibility of dietary protein. *Journal of Nutrition*, 130, 1850S-1856S.
- Deglaire, A., Bos, C., Tomé, D. & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, 102(12), 1752-1759.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39, 209-228.
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994). The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, 49, 57-71.
- Elia, M. & Cummings, J. H. (2007). Physiological aspects of energy metabolism and gastrointestinal effects of carbohydrates. *European Journal of Clinical Nutrition*, 61 S40-S74.

-
- Kararli, T. T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharmaceutics & Drug Disposition*, 16, 351 - 380.
- Moughan, P. J. & Rowan, A. M. (1989). The pig as a model animal for human nutrition research. *Proceedings of the Nutrition Society of New Zealand*, 14, 116-123.
- Moughan, P. J., Smith, W. C. & James, K. A. C. (1984). Preliminary observations on the use of the rat as a model for the pig in determination of apparent digestibility of dietary proteins. *New Zealand Journal of Agricultural Research*, 27, 509-512.
- Moughan, P. J., Smith, W. C. & Kies, A. K. (1987). Comparison of the ileal digestibility of amino acids in ground barley for the growing rat and pig. *New Zealand Journal of Agricultural Research*, 30, 59-66.
- Picard, M., Bertrand, S., Duron, M. & Maillard, R. (1984). *Comparative digestibility of amino acids using 5 animal models: intact cockerel, caeectomised cockerels, rat deprived of large intestine, piglet with an ileo-caecal cannulation, piglet with an ileo-rectal shunt*. Paper presented at the Proceedings of the IVth European Symposium of Poultry Nutrition, Tours, France.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K. & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71, 29-42.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, 87, 292-300.
- Smith, W. C., Moughan, P. J., Pearson, G. & James, K. A. C. (1987). Comparative bioavailable energy values of five ground cereal grains measured with growing rats and pigs. *Animal Feed Science and Technology*, 18, 143-150.
- Walker, A. R. P. (1975). Effect of crude fiber intake on transit time and the absorption of nutrients in South African Negro schoolchildren. *American Journal of Clinical Nutrition*, 28(10), 1161-1169.

Wilfart, A., Montagne, L., Simmins, H., Noblet, J. & van Milgen, J. (2007). Effect of fibre content in the diet on the mean retention time in different segments of the digestive tract in growing pigs. *Livestock Science*, 109(1-3), 27-29.

CHAPTER V

Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter

The first step in developing a valid in vitro hindgut digestibility assay involved identifying assay variables that significantly influence predicted dry matter digestibility (DMD). This was investigated by measuring DMD in response to gross variation in pH, duration of incubation, presence of shaking during incubation and the concentration of faecal inoculum or digestive enzymes for three published in vitro hindgut digestibility assays.

*Data published in: Coles, L. T., Moughan, P. J., Awati, A., & Darragh, A. J. (2011). Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter. *Food Chemistry*, 125(4), 1351 - 1358.*

Abstract

In vitro assays have been developed to predict dry matter digestibility (DMD) in the human colon, but there is little information on the effect of assay variables. The effect of altering pH, duration of incubation, presence of shaking during incubation and the concentration of faecal inoculum or digestive enzymes on DMD was investigated for three *in vitro* hindgut digestibility assays. Three mixed human diets varying in the type and ratio of soluble and insoluble dietary fibre were used as substrates. The pH, duration of incubation and the concentration of inoculum relative to substrate significantly ($P < 0.05$) affected predicted DMD for the two *in vitro* methods employing faecal inocula. The method using synthetic enzymes showed little sensitivity to alteration of assay variables and gave highly variable results and for this reason was not pursued further. Shaking did not affect ($P > 0.05$) digestibility for any method or diet. The different methods led to large differences in predicted hindgut DMD within each of the three diets. *In vitro* hindgut digestibility assay variables need to be optimised and the predicted DMD data validated against *in vivo* data.

Introduction

Most energy containing compounds released from food during digestion in humans and simple-stomached animals are absorbed in the upper digestive tract. Although the energetic contribution from volatile fatty acids that are mainly absorbed in the large intestine should not be ignored, this component is difficult to quantify using human studies and no suitable animal model exists. The digestion of foods in humans is a complex process with digestibility of the energy-yielding nutrients being affected by numerous factors. The nutrient composition of each food and also the mix of foods in the diet influence digestibility.

In vitro digestibility assays offer a routine means of assessment to study hindgut digestibility whilst taking into account complex dietary factors. A sound *in vitro* digestibility method should be robust enough to not be greatly affected by small fluctuations and variations in the system conditions, yet it should also be sensitive enough that often small differences between substrates can be detected. It should also be repeatable from one time and place to another to provide consistency and to allow global standardisation. Historically, few *in vitro* methodologies developed for studying fermentation in the human colon meet all these criteria and an explanation as to how a particular assay has been optimised to simulate *in vivo* colonic digestibility is seldom provided (Coles *et al.*, 2005). It is largely unknown how altering fundamental assay variables such as enzyme / inoculum concentrations, pH and mixing, quantitatively affects *in vitro* digestibility of the substrate.

The aim of the study here was to ascertain the sensitivity of *in vitro* hindgut dry matter digestibility assays to gross variation in underlying system conditions. Specifically, the objectives were to firstly identify key assay variables for consideration for optimisation as part of future work, and secondly, to compare the selected *in vitro* hindgut dry matter digestibility assays, which are the preferred methods based on a review of the literature (Coles *et al.*, 2005), with respect to estimated hindgut dry matter digestibility. The effects of pH, duration of incubation, concentration of the inoculum

or digestive enzymes and mixing during incubation were investigated. Three previously published *in vitro* assays developed to simulate the digestibility of organic matter in the hindgut of simple-stomached mammals were selected. The assays were applied to mixed human diets varying in the ratio and origin of soluble and insoluble dietary fibre.

Materials and methods

In vitro hindgut digestion methods

Three *in vitro* hindgut digestibility methods demonstrating potential for simulating digestion in the human colon were identified (Coles *et al.*, 2005). The methods were the “Boisen method” (Boisen and Fernández, 1997), the “Barry method” (Barry *et al.*, 1995) and the “Edwards method” (Edwards *et al.*, 1996).

The Boisen method is primarily based on measuring the DMD / organic matter digestibility (OMD) of the substrate by incubating it with a formulation of commercially available synthetic enzymes known to be produced by colonic bacteria. The Barry and Edwards methods are based on the incubation of the substrate with live faecal microbiota and measuring the dry matter (DM) / organic matter (OM) loss of the substrate during the *in vitro* fermentation. The Barry and Edwards methods were originally developed to measure non-starch polysaccharide (NSP) digestibility and resistant starch digestibility respectively. The methods used here reproduced the originally published protocols as closely as possible, although slight modifications were introduced to suit the objectives of the study.

Modified Boisen method

The test substrate (100 mg) was added to a mixture of 1.8 ml of EDTA (0.2 M), 10 ml of sodium citrate buffer (0.1 M, pH 4.8), 0.1 ml chloramphenicol solution (0.1% in 96% ethanol) and 0.1 ml Viscozyme® (Sigma-Aldrich Co.) in a 28 ml glass McCartney bottle (Biolab Ltd., Auckland, New Zealand). The bottle was capped and placed upright in a horizontally shaking water bath (50 stokes/min, 39°C) for 18 h. The filtering, drying and ashing of the bottle contents were then performed as described by Boisen and Fernández (1997).

Modified Barry method

A carbonate buffer (pH 7.3) containing 10 ml/L of nutritive solution (Barry *et al.*, 1995), was boiled and then maintained at 37°C under a flow of CO₂ until required. A 200 g/L batch of inoculum was prepared by processing 250 ml of buffer and 50 g of pooled human faeces contributed to equally from four donors (w/w) (see below) in a Stomacher® for 2 min. The inoculum was twice filtered through six layers of muslin cloth. All procedures were performed under strict anaerobic conditions.

Inoculum (10 ml) was added to a dried and pre-weighed 28 ml glass McCartney bottle (Biolab Ltd., Auckland, New Zealand) containing 100 mg of test substrate. The bottles were flushed with oxygen-free nitrogen (OFN) and placed upright in a horizontally shaking water bath (37°C, 50 strokes/min) for 24 h. Bottles were then autoclaved (120°C, 8 min) to ensure no further microbial activity. The bottles were then dried in an oven (80°C) and placed in a Muffle furnace (500°C, 4 h) for ashing to determine DM (Methods 930.15 and 925.10 (AOAC, 2000)) and OM (Method 942.05 (AOAC, 2000)) respectively.

Modified Edwards method

A sodium phosphate buffer (0.1 M, pH 7) containing no nutritive solution, was boiled and then cooled under a stream of OFN and then maintained at 37°C until required. A 320 g/L batch of inoculum was prepared by homogenising 250 ml of buffer with 80 g of pooled human faeces for 7 min in a Stomacher®. The protocol then followed that described above for the modified Barry method except that 5 ml of inoculum and 5 ml of buffer were added to each incubation bottle to give a final concentration of 160 g/L.

Inoculum

Inoculum necessary for both the modified Barry and Edwards methods was prepared from fresh faeces collected from four healthy human subjects (3 female, 1 male) consuming an unspecified 'Western' diet. Subjects consumed their habitual diets and the content of the diets was not recorded as part of the study. Faeces were collected

into pre-warmed, insulated containers flushed with CO₂ and were processed in the laboratory within 30 min. Equal quantities of faeces from each subject were homogenised in a Stomacher® machine with buffer solution according to the quantities specified by each *in vitro* fermentation method to produce a pooled faecal inoculum. Subjects were not taking medication nor had a history of medical problems that may have interfered with digestion. In the 3 months prior, subjects had not experienced gastrointestinal illness or taken antibiotics or laxatives. Informed written consent was obtained from all volunteers, and the study was approved by the Massey University Human Ethics Committee (PN 04 /127). The same batch of inoculum was used for all runs testing a given variable within a given method.

Substrates

Three complete diets formulated for adult humans were used as the substrates (**Table 1**). All foods in a given diet were accurately weighed, homogenised, freeze-dried and ground to pass through a 1 mm mesh. Two of the experimental diets contained a single source of dietary fibre which was rich in either insoluble or soluble dietary fibre. In the first case, wheat bran (WB) was included at 6.8% of diet DM. In the second case, citrus pectin (PE) was included at 4.5% of diet DM. The third diet, referred to as the Mixed Fibre diet (MF), contained dietary fibre at 6.9% of diet DM from a range of foods including cereals, legumes, fruits and vegetables.

Table 1

Determined chemical composition of the three substrates (per kg dry matter)

Component	Wheat Bran Diet	Pectin Diet	Mixed Fibre Diet
Gross energy, <i>MJ</i>	21.7	21.4	21.2
Crude protein, <i>g</i>	311.7	302.3	184.5
Fat, <i>g</i>	151.8	133.4	181.1
Total CHO ¹ , <i>g</i>	498.4	530.9	600.9
Starch, <i>g</i>	91.8	43.0	288.1
Sugars, <i>g</i>	374.9	469.1	269.8
Dietary fibre			
Total, <i>g</i>	68.3	45.1	69.0
Insoluble, <i>g</i>	60.9	25.1	54.0
Insoluble, <i>as % of TDF</i>	89.1	55.7	78.2
Soluble, <i>g</i>	7.5	20.0	15.0
Soluble, <i>as % of TDF</i>	10.9	44.3	21.8
NSP, <i>g</i>	94.0	67.3	63.7
Ash, <i>g</i>	38.1	33.4	33.5

¹ Total CHO (by difference) = 100 - Moisture% - Fat% - Crude Protein% - Ash%

CHO, carbohydrate; NSP, non-starch polysaccharides; TDF, total dietary fibre

Experimental design

The effects of variation in pH, duration of incubation, concentration of the inoculum/enzyme solution and the importance of mixing during incubation on substrate DMD were tested for each of the three *in vitro* digestion methods. Mixing was undertaken through bottles being shaken horizontally in an upright position during incubation to prevent material being trapped in the cap and interfering with total DM recovery.

The four factors were tested at their standard (as originally published) value, a higher value and a lower value, whilst keeping all other factors constant at the standard value. Duration of incubation was set at the standard level ± 6 h for the modified Boisen method as previous results indicated digestion may be incomplete at less than 12 h and should be complete by 24 h (Boisen and Fernández, 1997). For the modified Barry and Edwards methods a lower incubation time of 18 h and a higher incubation time of 48 h were chosen as these are commonly the shortest and longest times used with other *in vitro* digestibility assays that use a faecal inoculum. The high and low pH levels for the modified Barry and Edwards methods were set at the standard value ± 1 pH unit, in keeping with the normal range in physiological pH within the proximal colon (Cummings, 1996). For the modified Boisen method a low level of pH 3.6 and a high level of pH 5.4 were adopted because the optimum pH for Viscozyme[®] is between pH 3.3 and pH 5.5 (S. Boisen, pers. comm.). For all three methods the high and low levels for enzyme/inoculum concentration were set at the standard value $\pm 50\%$ to give a range of extreme values considered high enough to detect any effect of concentration on DMD. Mixing was either absent or performed at 50 strokes / min. Possible interactions between experimental variables were not considered. Details of the experimental factors and the levels at which they were tested are summarised in **Table 2**. In addition to these four experimental factors, repeatability and a comparison between the two methods employing faecal inocula was also investigated as a complementary study, separate from the main goals of the study (see Discussion for details).

Table 2

In vitro hindgut digestion methodologies and the levels at which experimental variables were tested¹

Variable	<i>In vitro</i> method		
	Boisen ²	Edwards ³	Barry ⁴
Duration, <i>h</i>	12, <u>18</u> , 24	18, <u>24</u> , 48	18, <u>24</u> , 48
pH	3.6, <u>4.8</u> , 5.4	6.0, <u>7.0</u> , 8.0	6.3, <u>7.3</u> , 8.3
Inoculum or enzyme conc., <i>g/L</i>	0.004, <u>0.008</u> , 0.012	80, <u>160</u> , 240	100, <u>200</u> , 300
Mixing	Not shaken, <u>Shaken</u> ⁵	Not shaken, <u>Shaken</u>	Not shaken, <u>Shaken</u>

¹ When a given variable was varied, all other variables were kept constant according to the standard protocol

² Based on the methodology of Boisen and Fernández (1997)

³ Based on the methodology of Edwards *et al.* (1996)

⁴ Based on the methodology of Barry *et al.* (1995)

⁵ Magnetic stirring on a hotplate, rather than shaking in a water bath is used in standard protocol

Underlining indicates the level for this variable in the standard (as originally published) protocol

Variation in inoculum was not investigated as an assay variable because it is well established (Barry *et al.*, 1995) that source of inoculum has an effect on predicted digestibility. Although different batches of inoculum were sometimes used for different runs (different days) within the same method, the same batch of inoculum was always used within a run testing the effect of a given variable. Since digestibility was being compared between levels of a given variable (e.g. pH 6, pH 7 and pH 8) and not between variables (e.g. pH and duration), there was no need to use the same batch of inoculum for all assay runs.

Each factor was investigated in triplicate for each of the three substrates and for each of the three methods. Three bottles containing inoculum but no substrate (blanks) were also included in each run to determine the DM/OM present in the system at Time 0 not contributed by the substrate.

Chemical analyses

Diet samples were analysed for DM, ash, gross energy (GE), total N, total fat, total sugars, total starch, dietary fibre (total, insoluble and soluble) and NSP.

DM and ash were determined in duplicate by drying samples in a Watvic convection oven (Watson Victor Ltd., Wellington, New Zealand) at 105°C for 16 h (Methods 930.15 and 925.10 (AOAC, 2000)), followed by ashing in a Muffle furnace (Electrofurn, New Zealand) at 550°C for 16 h (Method 942.05 (AOAC, 2000)).

Gross energy (the heats of combustion of protein, fat and carbohydrate) was determined in duplicate using an automatic adiabatic bomb calorimeter (AC-350, Leco Corporation, St. Joseph MI, USA) (Miller and Payne, 1959).

Total N was determined in duplicate samples by the total combustion method (Method 986.06 (AOAC, 2000)) on a Leco TruSpec CN Carbon/Nitrogen Determinator (Leco Corporation, St. Joseph, MI, USA). Crude protein was calculated by multiplying N by a factor of 6.25.

Total fat was determined in duplicate using acid hydrolysis/Mojonnier extraction (Method 954.02 (AOAC, 2000)).

Total sugars were determined using the phenol sulphuric colorimetric method on duplicate samples (Southgate, 1991). Total starch determination was performed in duplicate using a commercial kit (Total Starch Kit, Megazyme, Sydney, Australia) (Method 996.11 (AOAC, 2000)). Starch was hydrolysed with thermostable alpha-amylase and amyloglucosidase (AMG). The D-glucose quantitatively released during hydrolysis was quantitatively measured in a colorimetric reaction employing peroxidase.

Total dietary fibre and insoluble and soluble fibre fractions were determined using an enzymatic-gravimetric method (Method 991.43 (AOAC, 2000)) with a commercial kit (Total Dietary Fibre Kit Assay, Megazyme, Ireland) and alpha-amylase/AMG/protease enzymes.

NSP was also separately determined to obtain a complete dietary fibre profile for each test diet using a modified Englyst method (Englyst and Cummings, 1984, 1988) with constituent sugars measured using colorimetry.

Calculations

DMD was calculated as follows:

$$\text{In vitro DMD (\%)} = \frac{S_0 + B_0 - T_i}{S_0} \times \frac{100}{1} \quad (\text{Eq. 1})$$

Where S_0 is the DM in the substrate (mg) at time 0, B_0 is the DM measured in the blank (control) at time 0 (mg) and T_i is the total DM in the bottle after incubation (time i) (mg). For the modified Barry and Edwards methods, and in keeping with standard practice (Bourquin *et al.*, 1996; Goni and Martin-Carron, 1998; Sunvold *et al.*, 1995),

the DM digestibility of the inoculum itself was ignored and it was assumed that any loss in total DM during incubation was a loss from the substrate.

Statistical analyses

The data for each variable within each method were subjected to a one way analysis of variance (ANOVA). If a statistically significant effect was found, the levels of that variable were compared for each diet using Tukey's test. An analogous approach was used for comparisons between methods (modified Edwards and Barry methods) using the same batch of faeces and performed under standard operating conditions for each method. Results were considered statistically significant at $P < 0.05$. Minitab (version 14.2, Minitab Inc., PA, USA) was used for all statistical analyses.

Results

The modified Boisen assay showed little sensitivity to changes in assay variables, with no clear trend in DMD (data not shown). Large variations also existed between some replicates for this method and for reasons unknown to the authors, the method gave a number of aberrant mean observations. Consequently, it was decided not to pursue application of the modified Boisen method.

pH

The effect of altering pH on the mean DMD of the three dietary substrates is shown in **Table 3**. For the modified Edwards method, there was a significant difference ($P < 0.05$) in DMD between the lowest pH level tested (pH 6) and the higher pH levels (pH 7 and pH 8) for the WB and MF diets. This method consistently gave the highest DMD at pH 7 and the lowest DMD at pH 6, irrespective of diet, although there was no significant difference ($P > 0.05$) between DMD at pH 7 and pH 8. The modified Barry method gave a significant difference in DMD between the lowest pH level tested (pH 6.3) and the two higher pH levels (pH 7.3 and pH 8.3). The highest DMD for a given diet was always found at pH 6.3, irrespective of diet. There was no significant difference between the DMD at pH 7.3 and pH 8.3 for any of the diets.

Table 3

Effect of pH¹ on mean² dry matter digestibility for two *in vitro* methods and three experimental diets

Method	pH	Dry Matter Digestibility, %					
		Wheat Bran Diet		Pectin Diet		Mixed Fibre Diet	
		Mean	SE	Mean	SE	Mean	SE
Edwards ³	6.0	61.7 ^a	1.5	63.3 ^a	2.2	60.7 ^a	1.2
	7.0	75.5 ^b	1.5	80.0 ^b	3.0	73.0 ^b	0.6
	8.0	73.0 ^b	1.0	72.0 ^{ab}	1.5	71.3 ^b	3.3
Barry ⁴	6.3	95.7 ^a	4.1	92.2 ^a	5.6	100.4 ^a	4.1
	7.3	49.6 ^b	1.3	54.9 ^b	0.4	46.8 ^b	6.7
	8.3	42.2 ^b	2.8	46.5 ^b	1.7	49.1 ^b	1.3

¹ Only pH was varied, all other variables remained at their standard level for the method. Edwards: 24 h incubation, bottles shaken, 160 g/L inoculum concentration; Barry: 24 h incubation, bottles shaken, 200 g/L inoculum concentration

² mean, $n=3$ replicates except for the following: $n=2$: Edwards, Wheat Bran Diet pH 7; Barry, Pectin Diet pH 8.3.

³ Based on the methodology of Edwards *et al.* (1996)

⁴ Based on the methodology of Barry *et al.* (1995)

SE, standard error

^{ab} Means with different letters within the same column and method were significantly ($P<0.05$) different

Enzyme / inoculum concentration

The mean DMD results for the three experimental diets at the different levels of inoculum/enzyme concentration are presented in **Table 4**. A significant ($P<0.05$) effect for two or more diets tested was found for the modified Edwards and Barry assays. DMD tended to increase with an increasing concentration of the inoculum.

Table 4

Effect of concentration of inoculum¹ on mean² dry matter digestibility for each of the two *in vitro* methods and three experimental diets

Method	Concentration, g/L ³	Dry Matter Digestibility, %					
		Wheat Bran Diet		Pectin Diet		Mixed Fibre Diet	
		Mean	SE	Mean	SE	Mean	SE
Edwards ⁴	80	58.0 ^a	6.0	71.7 ^a	2.0	66.7 ^a	1.5
	160	75.0 ^b	2.3	79.7 ^{ab}	1.2	73.0 ^a	0.6
	240	71.3 ^{ab}	1.3	83.3 ^b	2.7	75.7 ^a	4.1
Barry ⁵	100	53.5 ^a	2.9	53.3 ^a	1.3	54.0 ^a	1.5
	200	57.4 ^a	4.0	61.1 ^b	1.8	56.3 ^{ab}	1.9
	300	61.7 ^a	4.3	63.8 ^b	1.9	61.8 ^b	0.3

¹ Only concentration of inoculum was varied, all other variables remained at their standard level. Edwards: 24 h incubation, bottles shaken, pH 7; Barry: 24 h incubation, bottles shaken, pH 7.3

² mean, $n=3$ replicates

³ 'Concentration' refers to concentration of inoculum in the system. The concentration of the substrate in the system (relative to the inoculum) was as follows: Edwards 5, 10 and 15 g/L (for inoculum concentrations of 80, 160 and 240 g/L respectively; Barry 5, 10 and 15 g/L (for inoculum concentrations of 100, 200 and 300 g/L respectively)

⁴ Based on the methodology of Edwards *et al.* (1996)

⁵ Based on the methodology of Barry *et al.* (1995)

SE, standard error

^{ab} Means with different letters within the same column and method were significantly ($P<0.05$) different

Duration of incubation

Results illustrating the effect of duration of incubation on mean DMD are given in **Table 5**. The effect of duration of incubation on DMD when comparing 18 h and 48 h incubation times was statistically significant with the modified Barry method for all three diets and with the modified Edwards method for both the WB and MF diets. Specifically, in these cases, the DMD at 48 h was always higher than that at 18 h. While for both the modified Edwards and modified Barry methods there was a numerical increase in DMD from 18 h to 24 h across all diets, the difference was only significant for the PE diet using the modified Barry method. Likewise, both methods showed a numerical increase in DMD from 24 h to 48 h for all diets, but the difference was only statistically significant for the WB diet using the modified Edwards method and the MF diet using the modified Barry method.

Table 5

Effect of duration of incubation¹ on mean² dry matter digestibility for two *in vitro* methods and three experimental diets

Method	Duration, <i>h</i>	Dry Matter Digestibility, %					
		Wheat Bran Diet		Pectin Diet		Mixed Fibre Diet	
		Mean	SE	Mean	SE	Mean	SE
Edwards ³	18	68.7 ^a	1.8	74.3 ^a	0.3	69.0 ^a	1.2
	24	69.0 ^a	0.6	77.7 ^a	2.8	71.7 ^{ab}	1.3
	48	79.3 ^b	3.7	82.0 ^a	2.5	77.3 ^b	2.3
Barry ⁴	18	41.7 ^a	0.7	42.7 ^a	1.2	42.3 ^a	1.9
	24	45.3 ^{ab}	2.4	49.3 ^b	0.3	44.7 ^a	0.3
	48	52.0 ^b	1.5	52.3 ^b	1.2	52.7 ^b	0.9

¹ Only duration of incubation was varied, all other variables remained at their standard level. Edwards: pH 7, bottles shaken, 160 g/L inoculum concentration; Barry: pH 7.3, bottles shaken, 200 g/L inoculum concentration

² mean, *n*=3 replicates

³ Based on the methodology of Edwards *et al.* (1996)

⁴ Based on the methodology of Barry *et al.* (1995)

SE, standard error

^{ab} Means with different letters within the same column and method were significantly ($P < 0.05$) different

Mixing of materials during incubation

Mean DMD of the experimental diets when bottles were mechanically shaken or not shaken in the water bath are shown in **Table 6**. There was no effect of mixing ($P>0.05$) for both the modified Edwards and Barry methods, regardless of the diet being tested.

Table 6

Effect of mixing¹ during incubation on mean² dry matter digestibility for two *in vitro* methods and three experimental diets

Method	Mixing	Dry Matter Digestibility, %					
		Wheat Bran Diet		Pectin Diet		Mixed Fibre Diet	
		Mean	SE	Mean	SE	Mean	SE
Edwards ³	Yes	68.7	4.8	76.0	3.2	72.0	1.0
	No	70.3	1.5	65.3	6.1	69.0	3.3
Barry ⁴	Yes	60.9	2.9	59.9	2.7	58.3	3.5
	No	57.9	3.3	60.0	3.6	59.2	1.6

ANOVA was applied and for each method in each diet there was no significant ($P>0.05$) effect of mixing

¹ Only shaking of bottles (either shaken or not shaken) during incubation was varied, all other variables remained at their standard level. Edwards: pH 7, 24 h incubation, 160 g/L inoculum concentration; Barry: pH 7.3, 24 h incubation, 200 g/L inoculum concentration

² mean, $n=3$ replicates

³ Based on the methodology of Edwards *et al.* (1996)

⁴ Based on the methodology of Barry *et al.* (1995)

SE, standard error

Discussion

There is currently no standard *in vitro* method for predicting the digestibility of dietary organic matter in the human colon or in the colon of simple-stomached animals. Although several *in vitro* hindgut digestibility assays have been proposed, there has been little work reported on the relative importance of the various assay variables, and usually little justification given for the values of the variables chosen (Coles *et al.*, 2005). Generally, there has been no attempt made to optimise assay variables, or if so, such optimisation has not been reported. Any variable that has a large effect on digestibility should be optimised to ensure that the predictions made by the *in vitro* system are accurate and valid when compared to *in vivo* digestibility data. Failure to do so may result in *in vitro* assays that have the ability to correctly rank substrates, but are unable to give accurate absolute data.

The effect of manipulating system conditions for three published *in vitro* digestibility assays designed to simulate hindgut digestion in simple-stomached animals and considered (Coles *et al.*, 2005) to show promise for practical application was investigated. Each method was tested with three different substrates, each being a mixed human diet with varying proportions of soluble and insoluble dietary fibre. The effect of altering pH, concentration of inoculum/digestive enzymes, incubation time and mixing during incubation were determined.

Despite the artificial nature of *in vitro* systems, it is logical that closely mimicking *in vivo* conditions is more likely to produce results in line with *in vivo* data. Human body temperature, for example, is well known and for this reason, incubation temperature was not investigated as a variable. Although it is known that peristaltic movement in the colon aids in mixing the colonic contents, previous findings on the necessity of shaking/stirring during *in vitro* incubation (Stevenson *et al.*, 1997) call into question its widespread inclusion in *in vitro* digestibility assays.

Adaptation of donors

In the present study, donors consumed an unspecified 'Western' diet such that each subject consumed his/her regular diet. The contents of the donor's diet were not regulated as part of the trial or reported by the donors. Donors were not adapted to the test substrates in accordance with the findings of Barry *et al.* (1995) who found that a 2 week adaptation to pectin or sugar beet fibre did not influence *in vitro* hindgut fermentation, irrespective of the type of substrate tested (pectin, sugar beet or soya bean fibre) or length of fermentation.

Boisen assay

Generally, the results for the modified Boisen method showed no consistent pattern with changes in assay variables. In many cases the standard errors were large and the method gave rise to several unexplained untenable mean observations. It was concluded that the modified Boisen method in its present form is not suitable for determining *in vitro* hindgut dry matter digestibility for our purposes and was not pursued further.

pH

With the modified Barry method the difference in DMD between pH 6.3 and the higher pH values was considerable. It is suspected that during incubation the pH may have dropped much lower than the starting pH when testing at pH 6.3. This may account for the large difference in DMD between the lowest pH level (pH 6.3) and the two higher pH levels (pH 7.3 and pH 8.3). Some drop in pH is to be expected during incubation with faecal bacteria due to the production of SCFA (short chain fatty acids). However, it is possible that the pH in this case may have dropped much lower than pH 5 when the inoculum was prepared for pH 6.3, pre-incubation. A pH drop of up to 1.6 units has been observed previously with the Barry method (24 h incubation, pH 7.3 buffer) (Barry *et al.*, 1995) and a pH drop of between 1 to 2 pH units has also been observed during a 24 h incubation with the Edwards assay (Edwards *et al.*, 1996),

which uses a pH 7 buffer system that is more easily regulated, pre-incubation. It was more difficult to regulate the pH of the carbonate buffer used for the modified Barry method during inoculum preparation than the phosphate buffer used in the modified Edwards method. Too much CO₂ causes a drop in pH, whilst the pH rises too high if not enough CO₂ is added. As a result, the acidic nature of the environment, rather than increased bacterial activity, may have been responsible for the higher DMD at the low pH level tested with the modified Barry method. A buffer that is too acidic may also affect the viability of the bacteria. With the modified Edwards method, digestibility was considerably lower at the lower pH but did not differ at the higher pH level. It is concluded that pH is an important assay variable, influencing DMD considerably. The standard pH appeared to give the best differentiation in DMD between the diets (based on expected *in vivo* digestibility) for the modified Edwards and Barry methods.

Enzyme / inoculum concentration

Inoculum concentration appears to be a key determinant of DMD for the modified Edwards and Barry assays. At the standard faecal inoculum concentration the ratio of substrate to inoculum for the modified Barry and Edwards methods was 100 mg substrate : 10 ml inoculum (w/v), equivalent to 10 g substrate / L of inoculum. The more fermenting or digesting potential there is in the *in vitro* system, the higher the DMD is expected to be. This is only true up to a certain point, however, as the system may become short of substrate (Awati *et al.*, 2006). At this point, the system cannot support further fermentation of the substrate. The bacteria's access to the substrate may also be hindered by the sheer quantity of the inoculum compared to substrate in the system. Conversely, insufficient supplies of bacteria may limit digestibility in the *in vitro* system. Barry *et al.* (1995) found inoculum activity to be the limiting factor when the substrate was added at levels higher than 10 g / L inoculum. Lower concentrations of inoculum led to lower estimates of digestibility for some of the diets. Our results support the need to determine the appropriate inoculum concentration through further experimental work.

Duration of incubation

The degree of *in vitro* digestion at a given point in time is not only determined by the length of time that the faecal bacteria have had access to the substrate for, but the substrate's physical form and chemical make-up are also critical. It has been observed previously that different substrates have different fermentation profiles over time (Barry *et al.*, 1995). *In vivo* colonic transit times are highly variable, commonly in the range of 54 h for subjects consuming a Western diet (Cummings, 1996), although the degree of fermentation in various parts of the colon varies. However, a 24 h incubation time is fairly standard for most *in vitro* digestibility assays developed for simulating the human colon. Edwards *et al.* (1996) argue that this duration corresponds with the transit time in the proximal colon, the main fermenting chamber in humans (Metcalf *et al.*, 1987). The lack of scientific justification by other workers indicates that a duration of 24 h is likely chosen largely due to its convenience in the laboratory. Our results show that the modified Edwards and Barry assays are sensitive to variation in the duration of incubation. The higher digestibility at 48 h compared to 18 h for both methods and all three diets (except for Pectin Diet, modified Edwards method) was notable.

Mixing of materials during incubation

From a practical perspective, shaking water baths are considerably more expensive than standard water baths and are not available in all laboratories. They are also generally smaller than other water baths and this greatly limits the number of bottles that can be processed as a single batch. It is generally wise to limit any unnecessary complexity when developing a method to avoid adding another variable and possible source of error. Since it appears to make no difference whether or not the bottles are shaken, there is clearly no need to do so.

Repeatability

By default, each main experiment for a given *in vitro* method contained bottles run at the standard levels (see underlined values in Table 1). In most cases, more than one main experiment for a given *in vitro* method was trialled on a single day (using the same batch of faecal inoculum), resulting in multiple bottles (three, six or nine) at the standard level per day. Given that the main experiments were trialled over two or three days, there was the opportunity to compare the repeatability of each *in vitro* method on different days using those bottles run at the standard level. Mean DMD on two or three different days when each *in vitro* method was trialled using the standard protocol are given in **Table 7**. The modified Edwards method showed no significant difference ($P>0.05$) in DMD between the different days that observations were made, but this was not the case for the modified Barry method where often large differences across days were apparent. As such, the modified Edwards method appears to be more repeatable than the modified Barry method. This may be a result of the difficulty in maintaining a steady pH for the inoculum pre-incubation, as discussed earlier. The results in Table 7 also show that when considering the methods as a group across all diets, the methods gave a large range in DMD. Overall, the modified Edwards method gave the highest DMD values (range 71 – 79%) and the modified Barry method the lowest values (range 45 – 60%). The method adopted clearly has a significant impact on the prediction of DMD. This highlights the difficulty in comparing results across different *in vitro* methods and reinforces the need to develop and validate a standardised *in vitro* method.

Table 7

Comparison of mean dry matter digestibility for three diets on different days for two *in vitro* methods using standard protocols¹

Method	Occasion	Dry Matter Digestibility, %								
		Wheat Bran Diet			Pectin Diet			Mixed Fibre Diet		
		<i>n</i>	Mean	SE	<i>n</i>	Mean	SE	<i>n</i>	Mean	SE
Edwards ²	Day 1	8	73.0 ^a	1.4	9	79.2 ^a	1.3	9	72.5 ^a	0.5
	Day 2	6	70.5 ^a	2.4	6	75.6 ^a	1.7	6	73.5 ^a	0.9
Barry ³	Day 1	6	53.5 ^{ab}	2.6	6	58.0 ^a	1.6	5	51.5 ^{ab}	3.8
	Day 2	6	58.2 ^b	1.8	6	59.7 ^a	1.4	6	57.8 ^a	1.7
	Day 3	3	45.2 ^a	2.4	3	49.4 ^b	0.5	3	44.8 ^b	0.2

¹ Standard protocol refers to the normal levels of the variables as published. Edwards: pH 7, 24 h incubation, bottles shaken, 160 g/L inoculum concentration; Barry: pH 7.3, 24 h incubation, bottles shaken, 200 g/L inoculum concentration

² Based on the methodology of Edwards *et al.* (1996)

³ Based on the methodology of Barry *et al.* (1995)

SE, standard error

^{ab} Means with different letters within the same column and method were significantly ($P < 0.05$) different

It may be expected that consistency of the inoculum on different experimental days would be the largest variable, provided sound experimental technique was otherwise practiced. Although the same donors were used for providing faeces on all occasions, it has been found previously that variation in SCFA production and ratios from a given donor from one day to another consuming their regular diet was similar to variation between donors (Barry *et al.*, 1995). It would therefore seem difficult to maintain consistency of faecal samples for *in vitro* hindgut digestibility methods, even when the donors used are kept constant. However, a wide range of fermentation profiles of colonic substrates would also be expected *in vivo*. This may in part explain variation across days (Table 7).

Comparison of methods

To observe the effect of method *per se*, independent of any effects due to the batch of faeces used, the mean DMD of the diets was compared for the modified Barry and modified Edwards methods under standard operating conditions using inoculum prepared as each method required, but using the same batch of faeces. Data were taken from the main experiments on a single day when both the modified Edwards and Barry methods were performed (**Table 8**).

Table 8

Comparison of mean¹ dry matter digestibility for two *in vitro* methods using standard protocols² with the same batch of faeces used for the inocula and three experimental diets

Method	Dry Matter Digestibility, %					
	Wheat Bran Diet		Pectin Diet		Mixed Fibre Diet	
	Mean	SE	Mean	SE	Mean	SE
Edwards ³	72.3 ^a	1.5	76.0 ^a	2.1	74.7 ^a	1.5
Barry ⁴	55.6 ^b	0.6	59.5 ^b	1.5	57.3 ^b	1.3

¹ mean, $n=3$ replicates

² Standard protocol refers to the normal levels of the variables as published. Edwards: pH 7, 24 h incubation, bottles shaken, 160 g/L inoculum concentration; Barry: pH 7.3, 24 h incubation, bottles shaken, 200 g/L inoculum concentration

³ Based on the methodology of Edwards *et al.* (1996)

⁴ Based on the methodology of Barry *et al.* (1995)

SE, standard error

^{ab} Means with different letters within the same column were significantly ($P<0.05$) different

Both methods ranked the three diets the same (PE>MF>WB), although for the modified Edwards method the differences in DMD between diets were not statistically significant ($P>0.05$), while for the modified Barry method they were significant ($P<0.05$) only when comparing the WB diet to the PE diet. The absolute differences in DMD between the two methods were large and statistically significant ($P<0.05$), with the modified Edwards method always giving considerably higher DMD values than the modified Barry method. The methodology for preparation of the inoculum for the two methods was very similar, yet the differences in DMD were large. The possible effect of a large pH drop during incubation decreasing DMD as a result of the buffer solution with the modified Barry method, but not the modified Edwards method, has been discussed earlier. One other possible explanation for the differences in DMD between the methods lies in differences in the duration of homogenisation of the inoculum for the two methods. The modified Edwards method calls for the inoculum to be processed for 7 min, whereas for the modified Barry method, it is only for 2 min. One of the purposes of homogenising the inoculum is to physically separate the bacteria from the faecal matrix. The short processing time in the modified Barry method may not allow sufficient time to fully achieve this, thus less bacteria may be present in the filtrate resulting in lower substrate DMD values. The short homogenising time may also increase the variability between batches of faeces depending on the level of fibrous material in a given faecal sample.

Preparing faecal inocula and sourcing donors to provide a stool sample on demand is a practical drawback for the modified Barry and Edwards assays. However, the results for these two assays are promising, both showing the ability to differentiate between the DMD of different substrates, and general agreement in ranking substrates. The modified Edwards method is the more straightforward of the two assays in terms of buffer and inoculum preparation and does not require the addition of a nutritive solution.

There was often a lack of statistical significance between sometimes quite large absolute differences in DMD at different levels of a given assay variable, and

sometimes the observed standard errors were relatively high. By way of example, DMD for the Pectin Diet for the modified Edwards method at pH 7 was 80% and at pH 8 was 72% (Table 3), yet this difference (8% units) was not statistically significant. In practice, triplicate runs may not be sufficient to detect actual differences in DMD and a greater number of bottles at each variable / method / diet combination may be desirable.

Conclusion

There is a lack of detailed experimental work reported for the development of most *in vitro* assays used to study digestibility in the human colon. It has been shown here, however, that variation in assay conditions have important effects on predicted digestibility values. In the present study, several assay variables gave rise to statistically significant effects on the hindgut DMD of three mixed human diets containing various types and amounts of dietary fibre. Particularly, altering the pH, the duration of incubation and the concentration of inoculum in the *in vitro* system played an important role. Despite its almost universal use in *in vitro* fermentation assays, shaking of bottles during incubation seems unnecessary and had no affect on predicted DMD. The present study has demonstrated that variation in key assay variables influences *in vitro* hindgut DMD to different degrees across the three assays investigated. The next important step in developing a robust *in vitro* hindgut digestibility assay is the determination of optimal assay conditions and this will be the subject of a further study in our laboratory.

Literature cited

- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Awati, A., Williams, B. A., Bosch, M. W., Li, Y. C. & Verstegen, M. W. A. (2006). Use of the *in vitro* cumulative gas production technique for pigs: An examination of alterations in fermentation products and substrate losses at various time points. *Journal of Animal Science*, *84*, 1110-1118.
- Barry, J.-L., Hoebler, C., Macfarlane, G. T., Mathers, J. C., Reed, K. A., Mortensen, P. B., Nordgaard, I., Rowland, I. R. & Rumney, C. J. (1995). Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *British Journal of Nutrition*, *74*, 303-322.
- Boisen, S. & Fernández, J. A. (1997). Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. *Animal Feed Science and Technology*, *68*, 277-286.
- Bourquin, L. D., Titgemeyer, E. C. & Fahey Jr., G. C. (1996). Fermentation of various dietary fiber sources by human fecal bacteria. *Nutrition Research*, *16*(7), 1119-1131.
- Coles, L. T., Moughan, P. J. & Darragh, A. J. (2005). *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, *123*, 421 – 444.
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Edwards, C. A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J. C., Nagengast, F., Rumney, C. & Quehl, A. (1996). *In vitro* method for quantification of the fermentation of starch by human faecal bacteria. *Journal of the Science of Food and Agriculture*, *71*, 209-217.
- Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst (Cambridge UK)*, *109*, 937-942.

-
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, *71*, 808-814.
- Goni, I. & Martin-Carron, N. (1998). *In vitro* fermentation and hydration properties of commercial dietary fibre-rich supplements. *Nutrition Research*, *18*(6), 1077-1089.
- Metcalfe, A., Phillips, S. F., Zinsmeister, A. R., MacCarty, R. L., Beart, R. W. & Wolff, B. G. (1987). Simplified assessment of segmental colonic transit. *Gastroenterology*, *92*, 40-47.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, *13*, 501-508.
- Southgate, D. A. T. (1991). *Determination of Food Carbohydrates* (2nd ed.). Barking, UK: Elsevier Science Publishers.
- Stevenson, A., Buchanan, C. J., Abia, R. & Eastwood, M. A. (1997). A simple *in vitro* fermentation system for polysaccharides - the effects of fermenter fluid surface area/fluid volume ratio and amount of substrate. *Journal of the Science of Food and Agriculture*, *73*, 101-105.
- Sunvold, G. D., Hussein, H. S., Fahey Jr., G. C., Merchen, N. R. & Reinhart, G. A. (1995). *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *Journal of Animal Science*, *73*, 3639-3648.

CHAPTER VI

Optimisation of inoculum concentration and incubation duration for an *in vitro* hindgut digestibility assay

In the work described in Chapter V, the effects of gross changes in assay variables were investigated and it was found that inoculum concentration and incubation duration are key variables. The aim here was to optimise these two key variables for a published in vitro hindgut digestibility assay using ileal digesta pertaining to a mixed human diet as the substrate. Firstly, hindgut dry matter digestibility at six different inoculum concentrations for each of the two modified in vitro hindgut digestibility assays was investigated. Secondly, and based on these findings, the most promising in vitro hindgut digestibility method was chosen and then tested at various incubation durations (18, 24, 48 h) but with a fixed inoculum concentration (160 g / L). The study also sought to investigate the digestibility of the faecal inoculum itself and the importance of correcting for this in the in vitro hindgut digestion assay.

Abstract

The aim was to optimise inoculum concentration and incubation duration for a published *in vitro* hindgut digestibility assay using ileal digesta pertaining to a mixed human diet as the substrate. The study also sought to investigate the digestibility of the inoculum itself and the importance of correcting for this in the *in vitro* hindgut digestion assay. For two assays (Barry and Edwards), hindgut dry matter digestibility (DMD) generally increased with inoculum concentration. A sharp increase in DMD observed at high inoculum concentrations may have been related to problems with filtering the inoculum. An inoculum concentration of 160 g/L was considered optimal based on close agreement of observed values with previously published *in vivo* hindgut dry matter digestibility for similar diets. The Edwards method was chosen for optimisation of the duration of incubation. Ileal substrate organic matter digestibility (OMD) with the Edwards method increased with increasing time of incubation for all diets. An incubation duration of 18 h using a mean inoculum digestibility value for calculation purposes was considered optimal based on observed *in vivo* hindgut DMD values in humans, but there was little difference in estimated *in vitro* hindgut DMD between 18 and 24 h incubation durations. Although considerably lower than the OMD of the substrate (no less than 51% after 48 h), the OMD of the inoculum (13% after 48 h) itself was of significance in calculating estimated digestibility. The optimised Edwards assay gave realistic hindgut OMD values ranging from 55 to 79% (Wheat Bran diet and Pectin diet respectively) using an 18 h incubation duration.

Introduction

It is important to be able to determine the hindgut digestibility of dry matter and energy for diverse dietary sources because hindgut fermentation is estimated to contribute up to 3 - 11% of maintenance energy needs in humans consuming a typical Western diet low in dietary fibre (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984). *In vitro* digestibility assays offer a practical and routine means of assessment to study hindgut digestibility.

In an earlier study (Coles *et al.*, 2011) of two published *in vitro* hindgut digestibility assays, (Barry *et al.*, 1995; Edwards *et al.*, 1996) whereby the effects of gross changes in assay variables were investigated, it was found that inoculum concentration and incubation duration are key variables. It was also found that shaking of the water bath during incubation did not affect hindgut DMD. Although pH was found to be an important variable, the standard pH employed in the two methods allowed the best differentiation of DMD across diverse types of substrate. The standard pH values (pH 7.0 Edwards, pH 7.3 Barry), moreover, led to a slightly acidic environment during incubation, as pH drops during the incubation period, and the resultant slight acidity accords with *in vivo* conditions in the proximal colon (Cummings, 1996).

In general, assay variables have not been reported to be optimised in previously published *in vitro* hindgut digestibility assays. These *in vitro* hindgut digestibility assays may be adequate for ranking of substrates in order of digestibility, but have not been shown to provide valid absolute quantitative data. The work described here details the systematic optimisation of the two important *in vitro* system variables, the optimisation of which remains unresolved: the concentration of faecal inocula and duration of incubation.

The work involved three parts. Firstly, hindgut DMD at six different inoculum concentrations for each of the two modified *in vitro* hindgut digestibility assays (Barry

et al., 1995; Edwards *et al.*, 1996) was investigated. Secondly, and based on these findings, the most promising *in vitro* hindgut digestibility method was chosen and then tested at various incubation durations but with a fixed inoculum concentration. Thirdly, the *in vitro* hindgut digestibility of the faecal inoculum itself was investigated to determine the need to account for dry matter lost from the inoculum during incubation.

Ileal digesta was used as the substrate for the *in vitro* studies, rather than the diet as eaten. For the purposes of investigating inoculum concentration on hindgut DMD, ileal digesta from broiler chickens were used due to this being easier and less expensive to obtain in large quantities than ileal digesta from the rat. Although from an anatomical and physiological perspective the rat and human upper-digestive tracts are more similar than that of the chicken and the human, for the purposes of the trial only relative comparisons were required between inoculum concentration levels and chicken ileal digesta were considered more similar in form to human ileal digesta than the diet as eaten.

Materials and methods

The study involved two experiments: 'Experiment A' to optimise the concentration of the inoculum and 'Experiment B' to optimise the duration of incubation and to investigate the need to correct for digestion of the inoculum itself. Ileal digesta rather than dietary material were used as the substrate for digestion in both experiments.

The methodologies for the two *in vitro* assays were slightly modified versions of the protocols of Barry *et al.* (1995) and Edwards *et al.* (1996), and have been described fully previously (Coles *et al.*, 2011). Briefly, chicken or rat ileal digesta were incubated in glass bottles with live human faecal inocula in a standard (non-shaking) water bath at 37°C. At the conclusion of the incubation, bottles were autoclaved to cease bacterial activity and dried in an oven and then ashed to determine dry matter (DM) and organic matter (OM) remaining post-incubation. For the preparation of human faecal inocula, faecal samples were provided by the same four donors as used on previous occasions in our laboratory (Coles *et al.*, 2011), under approval from the Massey University Human Ethics Committee (PN 04 /127). The use of broiler chickens and rats to provide digesta samples was approved by the Massey University Animal Ethics Committee for Experiment A (MUAEC 02/21) and Experiment B (PN 04/84) respectively.

Experiment A - concentration of the inoculum

Six different concentrations of inoculum were tested for each of the two *in vitro* methods (**Table 1**). Three bottles containing substrate and two bottles containing no substrate (blanks) were included for each method-concentration combination. The triplicate bottles within a diet used the same pooled group of substrate (chicken digesta). All bottles within a method, including blanks, contained the same quantity and source of inoculum. Buffer pH, incubation duration and temperature were held at their standard values for each *in vitro* method (refer Table 1). Blanks were sampled at time 0. At the end of the prescribed incubation period, all bottles were immediately autoclaved to cease fermentation and the material analysed for DM and ash.

Table 1

Inoculum concentrations tested¹ for two *in vitro* hindgut digestion methodologies

	<i>In vitro</i> method	
	Edwards ²	Barry ³
	60	75
	80	100
	160 ⁴	200 ⁴
Inoculum concentration, g/L	240	300
	320	400
	400	500

¹ Only concentration of inoculum was varied, all other parameters remained at their standard level for the method. Edwards: 24 h incubation, pH 7; Barry: 24 h incubation, pH 7.3

² Based on the methodology of Edwards *et al.* (1996)

³ Based on the methodology of Barry *et al.* (1995)

⁴ level in standard protocol

Substrate

For Experiment A, ileal digesta from chickens were used as the substrate. Eight broiler chickens were fed *ad libitum* one of two freeze-dried, powdered experimental diets (Low Fibre, LF or High Fibre, HF diets, **Table 2**) for three days. At the end of the feeding period, birds were sacrificed and ileal digesta were collected and immediately frozen (-20°C) prior to being freeze-dried, pooled across birds within a diet and finely ground (1 mm mesh).

Table 2

Ingredient compositions of four experimental diets (g/kg diet) (as weighed prior to freeze drying)

Ingredient	Wheat Bran Diet	Pectin Diet	Low Fibre Diet¹	High Fibre Diet
Milk (high calcium, 0.1% fat)	376.8	370.1	154.0	172.5
Chicken	55.8	56.4	19.2	18.0
Mayonnaise	10.5	10.6	7.7	5.0
Sugar	3.5	-	9.2	7.2
Cheese spread	7.0	7.0	19.2	-
Butter (61% fat)	8.4	-	15.4	7.9
Cheddar cheese (Edam)	14.0	14.1	-	10.8
Wheat bran cereal ²	57.2	-	-	18.0
Citrus pectin ³	-	14.1	-	-
Custard	41.9	-	-	-
Cheese omelette	48.8	49.3	-	-
Jelly crystals	28.8	27.9	-	-
Osmolite ⁴	209.3	246.7	-	-
Fish (Hoki)	69.8	70.5	-	-
Cream (12% fat)	3.5	3.5	-	-
Marshmallow chocolate bar	20.9	32.4	-	-
Blackcurrant drink (Ribena®)	-	70.5	30.8	-
Whole-wheat cereal (Weet-Bix®)	-	-	23.1	-
Water crackers	-	-	18.5	-
Wheat bread (white)	-	-	80.1	-
Honey	-	-	3.8	-
Dairy dessert	-	-	115.5	-
Popcorn	-	-	19.2	14.4
Peaches (canned, in juice)	-	-	92.4	35.9
Apples (canned, in juice)	-	-	92.4	71.9

Tomato ketchup	-	-	15.4	10.8
Cucumber	-	-	30.8	28.8
Alfalfa sprouts	-	-	11.5	10.8
Carrots (boiled)	-	-	61.6	115.0
Green beans (boiled)	-	-	53.9	100.6
Chicken nuggets	-	-	97.0	90.6
Wheat bran cereal ⁵	-	-	-	35.9
Toasted muesli	-	-	-	35.9
Rye crackers (Kavli®)	-	-	-	10.8
Wheat bread (white, high fibre)	-	-	-	56.1
Yoghurt (contains oligosaccharides)	-	-	-	107.8
Vitamin mix ⁶	21.3	13.0	14.2	17.2
Mineral mix ⁷	21.3	13.0	14.2	17.2
Titanium dioxide	1.3	0.8	0.9	1.0

¹ referred to as the Mixed Fibre diet in the previous study (Coles *et al.*, 2011)

² All-Bran®, Kellogg, Australia

³ 7050 acid/milk pectin, Cerestar, Krefeld, Germany

⁴ Abbott Laboratories, IL

⁵ Sultana Bran, Kellogg, Australia

⁶ Vitamin mix (supplied by Plant and Food Research Ltd., Palmerston North, New Zealand) containing (per kg of vitamin mix): 100 mg vitamin A (retinol), 500 µg vitamin D₂, 4 g vitamin E (α-tocopherol acetate), 60 mg vitamin K₃, 100 mg vitamin B₁ (thiamin HCl), 140 mg vitamin B₂ (riboflavin), 400 mg vitamin B₃ (nicotinic acid), 400 mg B₅ (pantothenic acid), 160 mg vitamin B₆ (pyridoxine HCl), 20 mg vitamin B₇ (biotin), 40 mg B₉ (folic acid), 1 mg vitamin B₁₂ (cyanocobalamin), 4 g myo-inositol, 30 g choline (as choline chloride), 960.6 g sucrose

⁷ Mineral mix (supplied by Plant and Food Research Ltd., Palmerston North, New Zealand) containing (per kg mineral mix): 126 g Ca, 156 g Cl, 21 g Mg, 97 g P, 105 g K, 39 g Na, 39 mg Cr, 214 mg Cu, 9 g Fe, 2 g Mn, 964 mg Zn, 580 µg Co, 3 mg I, 3 mg Mo, 3 mg Se, 443.8 g sucrose.

Inoculum

Freshly collected, uncontaminated human faecal samples were delivered to the laboratory in a pre-warmed container filled with CO₂. Subjects contributing faeces consumed their habitual diets and the content of the diets was not recorded as part of

the study. The faecal inoculum was prepared under strict anaerobic conditions as described previously (Coles *et al.*, 2011), accounting for the required inoculum concentration (Table 1).

Experiment B - duration of incubation

Based on results from Experiment A (inoculum concentration trial), the modified Edwards method was the only *in vitro* method selected for Experiment B. An inoculum concentration of 160 g/L, a buffer pH of 7 and an incubation temperature of 37°C were used at three durations (18, 24, 48 h), with no shaking of the bottles during incubation. Inoculum preparation was performed under strict anaerobic conditions following the same protocol as for Experiment A. Triplicate bottles (WB, LF and HF diets) or duplicate bottles (PE diet) for each substrate were included at each incubation duration, with each triplicate / duplicate bottle for a given diet using a different pooled group of substrate (rat digesta). Additionally, blank bottles containing 5 ml of buffer and 5 ml of inoculum were included to determine the digestibility of the inoculum when no substrate was present in the system. A total of 15 blank bottles were sampled, with four blank bottles for each of 0, 18 and 24 h, and three bottles at 48 h. At the end of the prescribed incubation period, bottles were immediately autoclaved to cease fermentation and the material analysed for DM and ash.

Substrate

Rat ileal digesta were used as the substrate. While obtaining chicken digesta is easier and less expensive when large quantities are required (Experiment A), from an anatomical and physiological perspective the rat and human upper-digestive tracts are more similar. A total of 118 Sprague-Dawley male rats (7 wk of age, mean BW 318 g) were obtained from the Small Animal Production Unit (SAPU), Massey University. Animals were housed individually in wire-bottomed stainless steel cages in a temperature- (22 ± 1°C) and humidity- (55 ± 10%) controlled environment with a 12 h light/dark cycle (06:00 – 18:00 h).

Rats were randomly allocated to cages and one of four diets varying in the ratio of soluble : insoluble dietary fibre and source of dietary fibre (Table 2). The diets are referred to herein as Wheat Bran (WB) diet, Pectin diet (PE), Low Fibre (LF) diet (referred to as Mixed Fibre (MF) Diet in a previous study (Coles *et al.*, 2011)) and High Fibre (HF) diet. Each diet contained added vitamins and minerals as required for the growing rat (National Research Council, 1995) and an indigestible marker (titanium dioxide). All diet ingredients were accurately weighed, mixed, homogenised in a food processor, freeze-dried and finely ground (1 mm mesh). Twenty nine rats consumed each of the PE and HF diets, and 30 rats consumed each of the WB and LF diets.

Food was offered *ad libitum* hourly (10 min meal) between 0800 and 1600 h daily. The frequent feeding regimen was to ensure that digesta would be available for collection from the terminal ileum when required. Rats had free access to water at all times.

On study d 14, from 4 h after the first meal, animals were randomly selected to be sacrificed by an overdose of CO₂ gas. After confirmation of death, neural stimulation was immediately halted by decapitation. The stomach was inspected for evidence of coprophagy, observable by the presence of dark flecks in the stomach contents. The final 20 cm of the ileum was dissected from the body and ileal contents flushed with reverse osmosis water into a plastic bag. The digesta were immediately frozen (-20°C) prior to being freeze-dried and finely ground (1 mm mesh). After discarding digesta from animals that may have practised coprophagy, samples of ileal digesta from individual rats were pooled across rats within a diet to give sufficient quantities of digesta for chemical analysis. This resulted in two samples for the PE diet (1 x $n=14$, 1 x $n=13$) and three samples for each of the HF diet (2 x $n=8$, 1 x $n=9$), the WB (2 x $n=9$, 1 x $n=10$) diet and the LF diet (2 x $n=9$, 1 x $n=10$).

Chemical analyses

Chicken ileal digesta samples were analysed for DM and ash. Rat ileal digesta samples were analysed for DM, ash, total fat, nitrogen and total non-starch polysaccharides

(NSP). Diet samples were analysed for DM, ash, gross energy (GE), nitrogen, total fat, starch, total sugars, and total, soluble and insoluble NSP. All analyses were performed using duplicate samples, except for NSP which was performed using single samples. The chemical composition of the diets are given in **Table 3**.

Table 3

Determined chemical composition of four experimental diets (per kg dry matter¹)

Component	Wheat Bran Diet	Pectin Diet	Low Fibre Diet	High Fibre Diet
Dry matter, <i>g</i>	904.3	867.3	879.3	904.9
Gross energy, <i>MJ</i>	19.7	19.9	20.1	19.7
Crude protein, <i>g</i>	268.6	256.6	162.3	163.1
Fat, <i>g</i>	135.3	132.0	156.7	139.5
Total CHO ² , <i>g</i>	511.8	548.5	613.2	622.8
Starch, <i>g</i>	75.2	34.6	224.0	225.4
Sugars, <i>g</i>	376.0	487.7	291.1	253.1
NSP				
Total, <i>g</i>	83.4	61.2	62.5	99.0
Insoluble, <i>g</i>	49.2	19.1	28.0	62.3
Insoluble, % of total NSP	59.0	31.2	44.8	62.9
Soluble, <i>g</i>	34.2	42.1	34.5	36.7
Soluble, % of total NSP	41.0	68.8	55.2	37.1
Ash, <i>g</i>	84.2	63.0	67.8	74.7

¹ Reported as per kg dry matter for all diet components, except for dry matter (per kg freeze-dried diet)

² Total CHO (by difference) = 100 - Moisture% - Fat% - Crude Protein% - Ash%

CHO, carbohydrate; NSP, non-starch polysaccharides

DM and ash determination were performed using the AOAC standard procedures (Methods 930.15 and 925.10 (AOAC, 2000)) by drying samples in a Watvic convection

oven (Watson Victor Limited, Wellington, New Zealand), followed by ashing in a Muffle furnace (Electrofurn, New Zealand) (Method 942.05 (AOAC, 2000)).

GE (the heats of combustion of protein, fat and carbohydrate) content was determined using an automatic adiabatic bomb calorimeter (AC-350, Leco Corporation, St. Joseph MI, USA) (Miller and Payne, 1959).

Total N analysis was performed with the Leco total combustion method (Method 986.06 (AOAC, 2000)) on a Leco TruSpec CN Carbon/Nitrogen Determinator (Leco Corporation, St. Joseph, MI, USA). Crude protein was calculated using an N to protein conversion factor of 6.25 (Kraisid *et al.*, 2003).

Total fat was determined using acid hydrolysis/Mojonnier extraction (Method 954.02 (AOAC, 2000)).

Total sugars were defined as simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl esters with free or potentially reducing groups. Sugars were determined using the phenol sulphuric colorimetric method (Dubois *et al.*, 1956). Total starch determination was performed using a commercial kit (Total Starch Kit, Megazyme, Ireland) according to AOAC Method 996.11 (AOAC, 2000). Starch was hydrolysed with thermostable alpha-amylase and amyloglucosidase (AMG). The D-glucose quantitatively released during hydrolysis was quantitatively measured in a colorimetric reaction employing peroxidase.

Total carbohydrate (CHO) (%) was determined as the difference between 100 and the sum of the percentage of water, protein, total fat and ash. NSP content was determined using a modified Englyst method (Englyst and Cummings, 1984, 1988) with constituent sugars measured using colorimetry.

Calculations

No correction was made for OM in the blanks, as is common practice, when calculating the hindgut organic matter digestibility (OMD) of the ileal substrate for Experiment A (i.e. calculation method I_{\min} , as described below, was used). However, for Experiment B, hindgut OMD was determined by correcting for the OM in the blanks in one of three ways (I_{\min} , I_{mean} and I_{\max}) as described below:

I_{\max}

Here the inoculum OM present in the total system was assumed to be fermented to the same degree as in the blank regardless of whether or not substrate was present in the system. In this case, the OM remaining in the bottle (substrate plus inoculum) at the end of the incubation ($OM_{\text{bottle residual}}$) was adjusted by subtracting the OM remaining in the blank ($OM_{\text{blank residual}}$) for the corresponding incubation time.

$$OMD_{I_{\max}} (\%) = \frac{OM_{\text{substrate}} - (OM_{\text{bottle residual}} - OM_{\text{blank residual}})}{OM_{\text{substrate}}} \times \frac{100}{1} \quad (\text{Eq. 1})$$

where $OM_{\text{substrate}}$ is the OM (mg) contributed by the ileal digesta substrate prior to incubation, $OM_{\text{bottle residual}}$ is the OM (mg) remaining in the bottle at the end of incubation from both the ileal substrate and the inoculum and $OM_{\text{blank residual}}$ is the OM (mg) remaining at the end of incubation (18, 24 or 48 h) for the blank.

I_{\min}

Here the OMD (%) of the inoculum when incubated with substrate was assumed to be negligible and the OM contributed by the inoculum at time i (end of incubation) was assumed to be the same as that at time 0 ($OM_{\text{blank initial}}$). Therefore, no correction was made for the OMD of the inoculum when determining the OMD of the substrate.

$$OMD_{I_{min}} (\%) = \frac{OM_{substrate} - (OM_{bottle\ residual} - OM_{blank\ initial})}{OM_{substrate}} \times \frac{100}{1} \quad (Eq. 2)$$

$OM_{blank\ initial}$ is the OM (mg) measured in the blank pre-incubation (0 h).

It is known that when fermentable carbohydrate is available in the incubation system microbes will have a preference for this over inoculum OM as an energy source (Awati *et al.*, 2006). Equally, when little fermentable substrate is present in the system, inoculum OM can be fermented to some degree. Thus, in reality, the OMD of the substrate is likely to lie somewhere between the values of I_{max} and I_{min} . Pragmatically, a mean value (I_{mean}) can be adopted:

I_{mean}

$$OMD_{I_{mean}} (\%) = \frac{OMD_{I_{min}} + OMD_{I_{max}}}{2} \quad (Eq. 3)$$

Ileal substrate DMD (%) was calculated in an analogous manner to ileal substrate OMD.

Inoculum DMD (%) was calculated in an analogous manner to inoculum OMD:

$$Inoculum\ OMD (\%) = \frac{OM_{blank\ initial} - OM_{blank\ residual}}{OM_{blank\ initial}} \times \frac{100}{1} \quad (Eq. 4)$$

Statistical analysis

The data for each variable within each method were subjected to a one way analysis of variance (ANOVA). In the case of the duration of incubation trial (Experiment B), if a significant overall effect was found, Tukey's test was used to compare means within each diet (Muller and Fetterman, 2002). Results were considered statistically significant at $P < 0.05$. For Experiment A, where a significant overall effect was found,

regression analyses were performed on mean DMD inoculum concentration data for each assay using linear, quadratic and cubic models to obtain best fits (R^2) to the data (Muller and Fetterman, 2002). The program Minitab (version 14.2, Minitab Inc., PA, USA) was used for all statistical analyses.

Results

The chemical composition of the chicken and rat ileal digesta are given in **Table 4**. The NSP content of the rat ileal digesta ranged from 134 g/kg (PE diet) to 414 g / kg (HF diet). A large difference was seen between the NSP content of the rat ileal digesta of the PE diet (134 g/kg) and the LF diet (338 g/kg) despite the chemical composition of the two diets (as ingested) having a similar NSP content (61 and 63 g/kg respectively).

Table 4
Determined chemical composition of the chicken and rat ileal digesta (per kg dry matter¹)

Component	Wheat Bran Diet		Pectin Diet		Low Fibre Diet		High Fibre Diet	
	Rat	Chicken	Rat	Chicken	Rat	Chicken	Rat	Chicken
Dry matter, <i>g</i>	910.9	887.1	864.6	915.3	885.7	911.6		
Organic matter, <i>g</i>	707.7	689.9	853.8	747.0	845.9	757.3		
Crude protein, <i>g</i>	176.2	280.5	·	152.4	·	142.3		
Fat, <i>g</i>	15.4	33.3	·	78.3	·	46.1		
Total CHO ² , <i>g</i>	516.1	376.1	·	516.3	·	568.9		
NSP, <i>g</i>	373.4	133.9	·	337.3	·	413.9		

¹ Reported as per kg dry matter for all diet components, except for dry matter (per kg freeze-dried digesta)

² Total CHO (by difference) = 100 - Moisture% - Fat% - Crude Protein% - Ash%

CHO, carbohydrate; NSP, non-starch polysaccharides

· indicates that digesta were not analysed for this component

Experiment A - concentration of inoculum

The effect of inoculum concentration on the mean DMD of the ileal digesta substrate for each of the two *in vitro* hindgut digestibility methods is shown in **Table 5**.

Table 5

Effect of concentration of inoculum on the mean¹ dry matter digestibility of ileal digesta for two diets for each of the two *in vitro* hindgut digestibility methods

Method	Inoculum Concentration, g/L	Hindgut Dry Matter Digestibility, %			
		Low Fibre Diet digesta		High Fibre Diet digesta	
		Mean	SE	Mean	SE
Edwards ²	60	48.7	0.3	49.0	0.6
	80	55.0	2.5	52.0	1.9
	160	59.7	2.7	56.3	2.0
	240	60.3	0.3	61.7	0.9
	320	64.0	1.0	68.0	0.9
Barry ³	400	73.7	2.0	75.3	2.6
	75	30.3	2.9	28.7	0.3
	100	36.0	4.5	30.0	0.6
	200	39.3	2.0	34.3	0.7
	300	38.0	1.5	36.0	1.5
	400	52.0	1.5	47.0	0.7
	500	71.0	2.6	73.7	4.4

ANOVA was applied and for each method in each diet there was a significant ($P<0.05$) effect of inoculum concentration

¹ $n=3$ replicates except for the following where $n=2$: Edwards 80 g/L (Low Fibre Diet). Each replicate within a diet used the same pooled group of chicken digesta.

²Based on the methodology of Edwards *et al.* (1996)

³Based on the methodology of Barry *et al.* (1995)

SE, standard error

The modified Edwards method gave digestibility values in the range of 49 – 74% for the LF diet and 49 – 75% for the HF diet. For the modified Barry method, the digestibility values were generally much lower than those for the modified Edwards method at similar inoculum concentrations as noted earlier (Coles *et al.*, 2011), ranging from 30 – 71% for the LF diet and 29 – 74% for the HF diet. There was a trend observed for both methods, of increasing DMD with increasing inoculum concentration.

The absolute differences in DMD between consecutive inoculum concentrations tended to be higher at the higher concentration levels. There was a significant ($P < 0.05$) effect of inoculum concentration for each method in each diet. Fitted line plots of DMD versus mean inoculum concentration are presented for the LF and HF diets for both the modified Barry (**Figure 1a** and **1c**, respectively) and Edwards methods (**Figure 1b** and **1d**, respectively). Best fits were found using a cubic regression, with R^2 values of 98.0% (Barry method, LF), 98.5% (Edwards method, LF), 99.9% (Barry HF) and 99.7% (Edwards method, HF). When the concentration of inoculum increased beyond 240 g/L or 300 g/L for the modified Edwards and Barry methods respectively, there was a sharp increase in DMD.

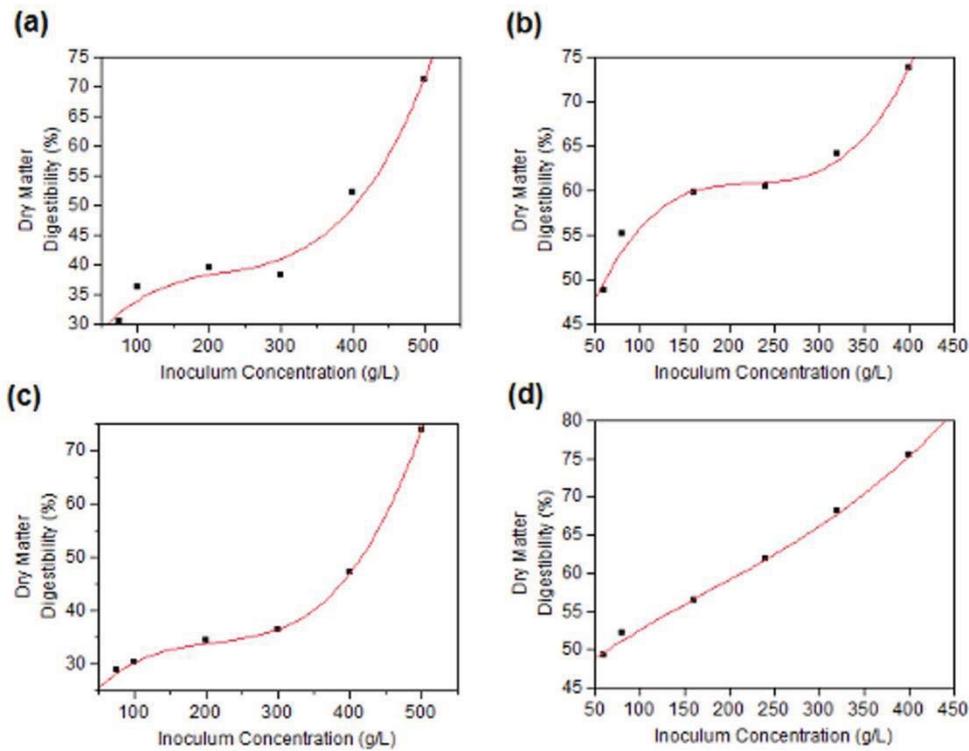


Figure 1. Mean ($n=3$) hindgut dry matter digestibility versus inoculum concentration for the: (a) Barry method, Low Fibre Diet; (b) Edwards method, Low Fibre Diet; (c) Barry method, High Fibre Diet and (d) Edwards method, High Fibre Diet.

Inoculum digestibility

DMD and OMD values for the inoculum (160 g/L), when incubated with no added substrate at various incubation times for the modified Edwards method, are given in **Table 6**. Mean OMD ranged from 9.1% (18 h) to 12.8% (48 h) and comparably, DMD from 5.0 to 7.1%.

Table 6

Mean¹ dry matter digestibility and organic matter digestibility for blanks containing inoculum alone (160 g/L) at three incubation durations using the modified Edwards method²

Duration, h	Dry Matter Digestibility, %		Organic Matter Digestibility, %	
	Mean	SE	Mean	SE
18	5.0	0.1	9.1	0.2
24	5.5	0.1	10.0	0.3
48	7.1	0.5	12.8	0.5

¹ $n=4$ replicates except for 48 h duration where $n=3$

² Based on the methodology of Edwards *et al.* (1996)

SE, standard error

Experiment B - duration of incubation

Data resulting from the three different calculation methods (I_{\min} , I_{mean} and I_{\max}) for correction for the blank for three different durations of incubation are given in **Table 7**. Data are given as rat ileal digesta OMD after fermentation in the *in vitro* digestibility system (modified Edwards assay). Overall there was a significant ($P<0.05$) effect of incubation duration for each calculation method and for each diet, with the exception of the PE diet for which the difference in OMD between incubation times was not significant ($P>0.05$) for any calculation method. There was a general trend of an increasing OMD with increasing incubation duration. For I_{mean} and with the exception of the PE diet, differences between the 24 h and 48 h durations were statistically significant ($P<0.05$). Statistical significance ($P<0.05$) across all three calculation methods within a diet was consistent except for HF using calculation method I_{\max} where there was no statistical significance seen between the OMD at the 18 h and 24 h durations, which was not the case for I_{\min} and I_{mean} .

Table 7

Effect of three incubation durations on substrate organic matter digestibility after *in vitro* digestion (Edwards method¹) for four experimental diets

	Calculation method ²	Duration, h	Organic Matter Digestibility ³ , %											
			Wheat Bran Diet			Pectin Diet			Low Fibre Diet			High Fibre Diet		
			Mean	SE		Mean	SE		Mean	SE		Mean	SE	
I_{min}		18	67.1 ^a	1.3		91.6 ^a	1.6		72.8 ^a	1.2		67.9 ^a	0.3	
		24	67.8 ^a	1.0		95.2 ^a	0.7		77.1 ^a	0.8		72.8 ^b	1.2	
		48	84.1 ^b	0.1		104.2 ^a	0.1		87.2 ^b	1.0		83.7 ^c	0.2	
I_{mean}		18	55.1 ^a	1.3		78.9 ^a	1.6		61.5 ^a	1.2		56.7 ^a	0.3	
		24	54.6 ^a	0.9		81.3 ^a	0.7		64.7 ^a	0.7		60.1 ^b	1.3	
		48	67.3 ^b	0.2		86.4 ^a	0.1		71.3 ^b	0.9		68.0 ^c	0.3	
I_{max}		18	43.1 ^a	1.2		66.3 ^a	1.6		50.3 ^a	1.2		45.5 ^a	0.3	
		24	41.5 ^a	0.9		67.4 ^a	0.6		52.3 ^a	0.6		48.3 ^a	1.3	
		48	50.5 ^b	0.2		68.7 ^a	0.1		55.4 ^b	0.8		52.3 ^b	0.3	

¹ Based on the methodology of Edwards *et al.* (1996)

² I_{min}, I_{mean} and I_{max} are calculation methods using minimal, mean or maximal inoculum organic matter respectively. Refer text.

³ n=3 replicates except for the following where n=2: Pectin Diet (all durations, all calculation methods), High Fibre Diet (24 h duration, all calculation methods). Each replicate within a diet contained a different group of pooled digesta.

SE, standard error

abc Means with different letters within the same column and calculation method were significantly ($P < 0.05$) different

The highly digestible nature of the PE diet was observed at all durations, with the OMD considerably higher than those found for the other three diets. Differences among the other three diets were less distinct, although the LF diet appeared to be more fermentable than the WB and HF diets.

Across all diets, most of the fermentation occurred in the first 18 h and in most cases no difference ($P>0.05$) in fermentation of the substrate occurred between 18 and 24 h. The fermentative loss occurring between 24 h and 48 h, although in most cases being statistically significant ($P<0.05$), was small in comparison to that which occurred in the first 18 h of incubation.

Discussion

The study sought to optimise two key assay variables as part of the development of a validated *in vitro* hindgut digestibility assay using ileal digesta pertaining to human diets as the substrate. It has been established previously (Coles *et al.*, 2011) that shaking of the bottles during incubation is not necessary in such an assay and that the pH levels suggested by the developers of the original *in vitro* assays (Edwards: pH 7.0, Barry: pH 7.3) are suitable. Inoculum concentration and duration of incubation have been previously identified, however, as important variables requiring optimisation (Coles *et al.*, 2011).

Substrate for *in vitro* hindgut digestibility

Ideally, the substrate for an *in vitro* hindgut digestibility assay should resemble, physically and chemically, the material entering the colon. Relying on simple chemical pre-treatment of foods to mimic upper tract digestion is not ideal as the food may be under- or over-digested and endogenous materials (materials of body origin) are not present. Given the close similarities between the rat and human upper digestive tracts and thus diet OM digestibility (DeSesso and Jacobson, 2001), our aim was to use rat ileal digesta as the substrate for the *in vitro* hindgut digestibility assay. However, for determining the optimum inoculum concentration, chicken ileal digesta were used as a substrate as they were easier and less expensive to obtain. Rat digesta were used (as per the intended finalised *in vitro* hindgut digestibility assay) for optimising the effect of incubation duration.

Experiment A - concentration of inoculum

The observed trend of increasing DMD with increasing inoculum concentration for the modified Edwards and Barry methods was expected due to the greater potential fermenting ability in the system at higher inoculum concentration. The marked increase in DMD at high inoculum concentrations (>240 g/L or >300 g/L for the modified Edwards and Barry methods respectively) may have been due to an increase

in bacteria present in the system, possibly adhered to faecal material not removed during filtering, as removal of faecal solids when filtering the inoculum during preparation became difficult at these higher concentrations. It is also possible that increased faecal solids in the system may have resulted in the inoculum itself being fermented to a greater degree.

In vivo hindgut digestibility data determined with human subjects in separate studies (Coles *et al.*, 2010) indicated that the LF diet has a higher hindgut DMD than the HF diet and here this directional response was only replicated for the Edwards method at inoculum concentrations of 80 g/L and 160 g/L. Of these two concentrations, the 160 g/L concentration produced results numerically closest to the expected hindgut DMD of the diets based on the *in vivo* data and was therefore chosen for future work. It was also noted, that as inoculum concentration exceeded 240 g/L for the modified Edwards method, it became increasingly difficult to achieve adequate filtration.

The modified Barry method generally resulted in lower DMD values than the modified Edwards method. Results in our laboratory (Coles *et al.*, 2010; ML Zou, unpublished results) for seven complete human diets indicate actual hindgut DMD values ranging from approximately 50% for poorly fermented cereal diets up to approximately 80% for highly fermentable diets. In the context of the modified Barry method, DMD values of around 30 – 40% (for inoculum concentrations < 400 g/L) appear low, whereas DMD values for the modified Edwards method of between 50 – 70% are realistic. Furthermore, the preparation of the inoculum for the modified Barry method is more involved than that of the modified Edwards method and there is a need for the addition of CO₂ to produce and maintain the correct pH. The modified Edwards method was thus selected for further optimisation.

Inoculum digestibility

In previous studies (Bourquin *et al.*, 1996; Goni and Martin-Carron, 1998; Sunvold *et al.*, 1995) it has been assumed that the DMD of the inoculum itself is zero (calculation method I_{min}). Although such an assumption may be acceptable for making relative

comparisons between diets, when making absolute comparisons accounting for the inoculum OMD becomes more important.

In the present study, inoculum on its own was degraded but the DMD and OMD values for the inoculum itself were much lower than what is typically observed for the OMD of dietary and other substrates when incubated in an *in vitro* hindgut digestibility system. However, to appreciate the relative importance of the observed inoculum DMD/OMD the ratio of the inoculum to the ileal substrate must be considered. By way of example, when the inoculum concentration is 160 g/L the total combined weight of OM (substrate plus inoculum) at the end of the incubation is typically around 180 to 190 mg. Of this, the minimum weight of OM contributed by the inoculum (calculation method I_{\max}) is around 150 mg, but could be as much as 170 mg (calculation method I_{\min}). Even a highly indigestible substrate such as WB or HF, contributes only around 20 mg of OM (calculation method I_{\min}) post-incubation, given that the pre-incubation ratio of substrate to inoculum is 100 mg : 10 ml. Therefore, the inoculum is expected to contribute between 80 - 90% (w/w) of the OM remaining at the end of the incubation.

The lowest possible digestibility coefficient for the inoculum in the system is zero (I_{\min}). An upper limit to the digestibility of the inoculum (I_{\max}) is likely set by the digestibility of the inoculum when incubated alone, as added material is considered to be a preferable substrate for the bacteria (Awati *et al.*, 2006). Faeces are composed of material that has already been fermented for some time in the colon. It is to be expected, therefore, that the digestibility of the inoculum would be lower than that of the ileal substrate. Furthermore, in a system with no substrate the bacteria are forced to survive on the faecal material present in the system. In this case the OMD of the inoculum would likely be higher than, or similar to, that in a system where more fermentable ileal substrate is present. SCFA (short chain fatty acid) production, like OM loss, is an indicator of the degree of fermentation occurring *in vitro*. Other similar studies by several other workers have found that fermenting the inoculum on its own, with no substrate present, produces low levels of SCFAs, typically around one third to

half the amount produced when the inoculum is fermented with a range of substrates (Adiotomre *et al.*, 1990; Birkett *et al.*, 2000; Silvester *et al.*, 1995).

The three different calculation methods (I_{\min} , I_{mean} and I_{\max}) led to very different results (Table 7). This illustrates the importance of using the appropriate value for the inoculum OMD. The untenable OMD value for the PE diet (104%) using I_{\min} suggests that I_{\min} likely overestimates the actual OMD. In reality, and as argued above, the OMD of the inoculum likely lies between I_{\min} and I_{\max} . Pragmatically, the use of I_{mean} is recommended in practice, but this awaits experimental validation.

Experiment B - duration of incubation

The pattern of fermentative loss of OM over time (Table 7) is in agreement with that found in other studies that illustrate that SCFA production begins to plateau rapidly within the first 12 h of fermentation (Barry *et al.*, 1995; Birkett *et al.*, 2000; Silvester *et al.*, 1995). Also, at longer incubation times there may be less viable bacteria, which further reduces the rate of fermentation (Vince *et al.*, 1976).

The diets, for the most part, were ranked the same for hindgut digestibility at all three incubation durations. Thus, for an *in vitro* hindgut digestibility method required to provide only relative comparisons of diets it would be possible theoretically to use any of these durations. While such a rationale is not ideal even for relative comparisons, it is unacceptable when the objective is to predict absolute quantities of OM digested *in vivo*. Human faecal and rat ileal digestibility data from our laboratory, using the same four diets as studied here (Coles *et al.*, 2010), indicated that approximately 10 – 12% of OM lost over the total tract disappears during hindgut fermentation. This corresponds to an OMD of 60 - 80% for the OM entering the colon for the two extreme diets (diets WB and PE respectively). When using the I_{mean} calculation method, an 18 h *in vitro* incubation (OMD of 55.1% and 78.9% for WB and PE respectively) gave data in good agreement with these observed values, and thus an 18 h incubation period was accepted as optimal. It was noted that there was little difference in the OMD (%) for

the I_{mean} calculation method between 18 and 24 h, and that in practice an incubation period between 18 and 24 h would appear to be suitable.

Conclusion

The modified Edwards hindgut digestibility method, as systematically developed and optimised here, offers a means of predicting hindgut OMD in humans. An inoculum concentration of 160 g/L and an incubation duration of 18 h are considered optimal for this method, based on using a mean inoculum digestibility value (I_{mean}) for calculation purposes. It is important to correct the hindgut digestibility values for digestion of the inoculum itself.

Literature cited

- Adiotomre, J., Eastwood, M. A., Edwards, C. A. & Brydon, W. (1990). Dietary fibre: *in vitro* methods that anticipate nutrition and metabolic activity in humans. *American Journal of Clinical Nutrition*, *52*, 128-134.
- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Awati, A., Williams, B. A., Bosch, M. W., Li, Y. C. & Verstegen, M. W. A. (2006). Use of the *in vitro* cumulative gas production technique for pigs: An examination of alterations in fermentation products and substrate losses at various time points. *Journal of Animal Science*, *84*, 1110-1118.
- Barry, J.-L., Hoebler, C., Macfarlane, G. T., Mathers, J. C., Reed, K. A., Mortensen, P. B., Nordgaard, I., Rowland, I. R. & Rumney, C. J. (1995). Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *British Journal of Nutrition*, *74*, 303-322.
- Birkett, A. M., Mathers, J. C., Jones, G. P., Walker, K. Z., Roth, M. J. & Muir, J. G. (2000). Changes to the quantity and processing of starchy foods in a Western diet can increase polysaccharides escaping digestion and improve *in vitro* fermentation variables. *British Journal of Nutrition*, *84*, 63-72.
- Bourquin, L. D., Titgemeyer, E. C. & Fahey Jr., G. C. (1996). Fermentation of various dietary fiber sources by human fecal bacteria. *Nutrition Research*, *16*(7), 1119-1131.
- Coles, L. T., Moughan, P. J., Awati, A. & Darragh, A. J. (2011). Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter. *Food Chemistry*, In Press.
- Coles, L. T., Moughan, P. J., Awati, A., Darragh, A. J. & Zou, M. L. (2010). Predicted apparent digestion of energy-yielding nutrients differs between the upper and lower digestive tract in rats and humans. *Journal of Nutrition*, *140*(3), 469-476.
- Cummings, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet*, *321*(8335), 1206-1209.

-
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H. & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, *70*, 443-459.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, *39*, 209-228.
- Dubois, M., Gillies, K. A., Hamilton, J. A., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350-356.
- Edwards, C. A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J. C., Nagengast, F., Rumney, C. & Quehl, A. (1996). *In vitro* method for quantification of the fermentation of starch by human faecal bacteria. *Journal of the Science of Food and Agriculture*, *71*, 209-217.
- Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst (Cambridge UK)*, *109*, 937-942.
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, *71*, 808-814.
- Goni, I. & Martin-Carron, N. (1998). *In vitro* fermentation and hydration properties of commercial dietary fibre-rich supplements. *Nutrition Research*, *18*(6), 1077-1089.
- Kraisid, T., MacLean Jr., W. C. & Warwick, P. M. (2003). *Food energy: methods of analysis and conversion factors: report of a technical workshop, Rome, 3-6 December 2002*. Rome: Food and Agriculture Organization of the United Nations.
- McBurney, M. I. & Thompson, L. U. (1989). Dietary fiber and energy balance: integration of the human ileostomy and *in vitro* fermentation models. *Animal Feed Science and Technology*, *23*, 261-275.

-
- McBurney, M. I., Thompson, L. U. & Jenkins, D. J. A. (1987). Colonic fermentation of some breads and its implications for energy availability in man. *Nutrition Research*, 7, 1229-1241.
- McNeil, N. I. (1984). The contribution of the large intestine to energy supplies in man. *American Journal of Clinical Nutrition*, 39, 338-342.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, 13, 501-508.
- Muller, K. E. & Fetterman, B. A. (2002). *Regression and ANOVA: An Integrated Approach Using SAS Software*. Cary, NC: SAS Publishing.
- National Research Council. (1995). Nutrient requirements of the laboratory rat. In *Nutrient Requirements of Laboratory Animals* (4th rev. ed., pp. 11-79). Washington, DC: National Academy Press.
- Silvester, K. R., Englyst, H. N. & Cummings, J. H. (1995). Ileal recovery of starch from whole diets containing resistant starch measured *in vitro* and fermentation of ileal effluent. *American Journal of Clinical Nutrition*, 62, 403-411.
- Sunvold, G. D., Hussein, H. S., Fahey Jr., G. C., Merchen, N. R. & Reinhart, G. A. (1995). *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. *Journal of Animal Science*, 73, 3639-3648.
- Vince, A. J., Down, P. F., Murison, J., Twigg, F. J. & Wrong, O. M. (1976). Generation of ammonia from non-urea sources in a faecal incubation system. *Gastroenterology*, 74, 313-322.

CHAPTER VII

Validation of a dual *in vivo* – *in vitro* assay for predicting the digestibility of dietary energy in humans

The validation of a dual in vivo - in vitro digestibility assay for separately predicting the upper-tract, hindgut and total tract digestibility of energy in humans is described. Upper-tract and large-intestinal organic matter digestibility (OMD) were predicted using an animal model (rat) and an in vitro hindgut fermentation assay, respectively. Rat ileal digesta were obtained from rats fed one of four complete human diets (wheat bran, pectin, mixed low fibre, mixed high fibre) and then fermented with human faecal inocula using the in vitro hindgut fermentation system. To assess the validity of the dual digestibility assay, apparent total tract OMD of the four diets as determined with a human balance study was compared with that predicted using the dual digestibility assay.

Abstract

This work describes the validation of a dual *in vivo* - *in vitro* digestibility assay ('dual digestibility assay') for separately predicting the upper-tract, hindgut and total tract digestibility of energy in humans, as estimated using organic matter digestibility (OMD). Upper-tract OMD was predicted using an animal model (rat) with digesta from the terminal ileum collected from rats fed one of four complete human diets (wheat bran, pectin, mixed low fibre, mixed high fibre). Large intestinal OMD was predicted using an *in vitro* hindgut fermentation assay employing human faecal inoculum with the rat ileal digesta as the substrate. A comparison of total tract OMD of the four diets from a human balance study (OMD_{human}) with that predicted using the dual digestibility assay OMD_{dual} showed no significant differences ($P > 0.05$). OMD_{human} and OMD_{dual} were significantly correlated ($r = 0.953$, $P = 0.047$). The dual digestibility assay is a valid and accurate means of predicting the uptake of dietary energy (as grams of organic matter) in humans over the total tract. The assay is able to separately quantify the digestibility of dietary energy in the upper and lower tracts.

Introduction

Total tract digestibility studies with humans are invasive, demand considerable time, effort and financial resources and do not differentiate between upper-tract and colonic nutrient uptakes. Alternative methods, involving animal models, that allow such differentiation, are needed. The laboratory rat is a suitable choice for an animal model due to its low cost and ease of use (Pearson *et al.*, 1999; Wisker and Bach Knudsen, 2003). The gastrointestinal tract of rats and humans are similar anatomically and both species are omnivorous. There are, however, some important physiological differences, mostly concerning hindgut fermentation. Fermentation is largely dependent on body size, and larger animals such as the pig and man, digest dietary fibre (DF) to a greater extent than the rat (Van Soest *et al.*, 1983). Large intestinal transit time in rats is around 15 h, compared to 3 – 4 d in humans and the rat has a caecum accounting for 26% of the total anatomical length of the large intestine (DeSesso and Jacobson, 2001). Bach Knudsen *et al.* (1994) and Wisker *et al.* (1997) support the use of the rat model for determining the digestibility of energy, protein and fat in mixed diets for humans, but question its applicability for determining non-starch polysaccharide (NSP) digestibility as advocated by others (Nyman *et al.*, 1986). Measurement of upper-tract digestibility only, however, avoids most of the physiological differences between the species (Van Soest *et al.*, 1983). Although relative to body size, humans have an upper-tract absorptive capacity more than four times that of the rat, small intestinal transit time in both species is around 3 – 4 h (DeSesso and Jacobson, 2001). Studies have confirmed the validity of rats as a model for upper-tract digestibility in pigs (Donkoh *et al.*, 1994; Moughan *et al.*, 1984) and also the use of pigs as a model for upper-tract digestibility in humans (Darragh and Moughan, 1995; Monsma *et al.*, 2000). The laboratory rat is accepted here as a suitable animal model for upper-tract (mouth to terminal ileum) nutrient digestibility in humans.

The complex and dynamic process of bacterial fermentation occurring in the human colon is more difficult to simulate *in vivo* with an animal model, although *in vitro* digestibility assays have shown some success in this regard. In an attempt to closely

replicate *in vivo* colonic conditions these assays most commonly use human faecal bacteria to ferment the substrate. Given the shortcomings of published *in vitro* hindgut fermentation assays available for use with humans (Coles *et al.*, 2005), a new hindgut *in vitro* assay has been developed based on the systematic identification and optimisation of *in vitro* assay parameters (Coles *et al.*, 2011) (see chapters V and VI). An advantage of the latter *in vitro* hindgut assay is that ileal digesta are used as the substrate fermented in the system, allowing for a seamless digestibility model of the total tract. Estimates of upper-tract (ileal) nutrient digestibility obtained using the laboratory rat can be combined with estimates of hindgut fermentation determined *in vitro*, to produce a “dual digestibility assay”. The aim of the present work was to validate the dual *in vivo* – *in vitro* digestibility assay (“dual digestibility assay”). Validation of the assay involved comparison of total tract organic matter digestibility (OMD) from a human balance study with total tract OMD predicted using the assay.

Materials and methods

Human balance study

Subjects

Twenty four healthy women were recruited for the balance study. Of these, twenty one subjects, aged 20 – 59 y, chose to complete the study. Subjects were carefully selected, based on the criteria that none were taking medication during the study nor had a history of medical problems that may have interfered with the aims of the study. Especially in the 3 months prior to the trial, subjects had not experienced gastrointestinal illness or taken any antibiotics or laxatives. Subject characteristics are given in **Table 1**, Chapter III.

Informed written consent was obtained from all volunteers, and the study was approved by the Massey University Human Ethics Committee (PN 04/128). All subjects were highly motivated and had a genuine interest in the objectives of the study and the experimental diets were well received. In the days immediately preceding the study, subjects as a group met the research team to establish a good rapport and discuss individually the details of the trial. To ensure compliance throughout the study, subjects met with one of the authors at least once every other day.

Study plan

The study consisted of a 10-d adaptation period followed by a 7-d balance period involving total collection of faeces. Four experimental diets were randomly allocated to subjects such that each subject received one diet, with a constant daily intake of food ingredients throughout the 17-d period. Subjects consumed the diets as six daily meals: breakfast (0600 – 1000 h), morning snack (1000 – 1200 h), lunch (1200 – 1500 h), afternoon snack (1500 – 1700 h), dinner and evening snack (1700 h onwards). Food intake was controlled according to individual dietary energy needs determined prior to the trial based on a 7-d food record compiled by the subjects when consuming their normal diets and analysed using the Foodworks® database (Xyris Software, QLD,

Australia). Subjects were free-living in their own homes during the adaptation period. During the balance week, subjects were housed together in an apartment complex where they were free to come and go as necessary, with a member of the research team present 12 h per day to maintain regular contact with participants.

Diets

The four experimental diets (**Table A1**, see Appendix, Chapter III) were designated: Wheat Bran (WB), Pectin (PE), Low Fibre (LF) and High Fibre (HF). WB and PE diets contained wheat bran (WB) (All-Bran[®], Kellogg, Australia) and high methoxyl citrus pectin (PE) (7050 acid/milk pectin, Cerestar, Krefeld, Germany), respectively, as sole sources of dietary fibre. An isotonic nutritional supplement (Osmolite[®], Abbott Labs, IL) was included in both these diets to ensure nutritional adequacy. The wheat bran was included as a ready-to-eat breakfast cereal, a snack bar and a pudding base. The pectin was included as a thickener in drinks (shakes and blackcurrant drink). The other two diets were mixed-fibre, containing cereals, legumes, fruits and vegetables in a low fibre (LF) or high fibre (HF) form. The HF diet contained DF from a wider range of sources than the LF diet, and included resistant starch (bread), oligosaccharides (yoghurt) and DF from a greater variety of cereal sources (wheat bran, rye and oats). For all diets, DF was spread throughout the six daily meals. Two weeks prior to the study, participants on the WB or HF diets were provided with ready-to-eat wheat bran breakfast cereal and instructed to gradually increase the amount consumed daily. The purpose was to avoid any adverse effects from suddenly consuming large amounts of wheat bran. Thus for the WB and HF diets, participants were habituated to the fibre sources for a total of 31 d.

All food was provided, and was cooked under standardised conditions at the Institute of Food, Nutrition and Human Health (Massey University) and was weighed accurately and packed for participants to collect. The weight of each food item for each subject was related to individual dietary energy requirements, such that all subjects on a given diet consumed proportionally the same amount of each food item.

Balance technique

Faeces were collected over a 7 d period, from 0800 h on study d 11 until 0800 h on study d 18 following the methodologies of Zou *et al.* (2007). For the purposes of chemical analysis of the diets, a sample of each ingredient was taken daily during the 7 d balance period. Ingredients were accurately weighed according to their proportion in the diet and composite diet samples prepared, homogenised, freeze-dried and finely ground (1 mm mesh). As such, a representative sample of each diet as consumed was obtained and nutrient and DF content determined.

Rat study

Animals and housing

A total of 112 Sprague-Dawley male rats were obtained (Small Animal Production Unit (SAPU), Massey University) at approximately 7 wk of age, with a mean body weight (\pm SEM) of 318 (\pm 4)g. The trial was approved by the Massey University Animal Ethics Committee (PN 04/84). Rats were randomly allocated to individual cages and to the four diets (WB, PE, LF, HF), resulting in 28 rats consuming each of the four diets. Rat weights (mean \pm SEM) on Day 0 were 314 (\pm 7)g, 323 (\pm 7)g, 326 (\pm 8)g and 310 (\pm 8)g for animals consuming the WB, PE, LF and HF diets respectively. Animals were individually housed at SAPU in wire-bottomed stainless steel cages in a temperature- (22 \pm 1°C) and humidity- (55 \pm 10%) controlled environment with a 12h light/dark cycle (06:00 – 18:00) for the duration of the trial.

Diets

The base for each of the four rat diets (**Table A2**, see Appendix, Chapter III) comprised the same mixture of ingredients as used in the human balance study. Ingredients were weighed accurately and after homogenising in a food processor, diets were freeze-dried and finely ground (1mm mesh). A standard vitamin and mineral premix as required for the growing rat (National Research Council, 1995) and an indigestible marker (titanium dioxide), were added to each diet before mixing.

Food was offered *ad libitum* for 10 min each hour between 0800 h and 1600 h daily to train rats to feed continuously during this time period. Hourly feeding was undertaken to ensure a semi-continuous flow of digesta at the terminal ileum. Rats had free access to clean drinking water at all times.

Collection of ileal digesta

On study d 14, from 4 h after the first meal, animals were randomly selected to be euthanised by an overdose of CO₂ gas. After confirmation of death, neural stimulation was immediately halted by decapitation. An incision was made along the mid-ventral line and the skin and musculature were folded back to expose the viscera. The stomach was inspected for evidence of coprophagy, observable by the presence of dark flecks in the stomach contents. The contents of the terminal 20 cm of the ileum were then removed as described by Rutherford and Moughan (2003), rapidly frozen (-20°C) and freeze-dried. After discarding digesta samples from coprophagous rats, all ileal digesta from an individual rat were pooled with that from other rats within a diet to give sufficient quantities of material for chemical analysis and finely ground (1 mm mesh). This resulted in two samples of digesta for the PE diet (1 x *n*=14, 1 x *n*=13) and three samples for each of the HF diet (2 x *n*=8, 1 x *n*=9), the WB (2 x *n*=9, 1 x *n*=10) diet and the LF diet (2 x *n*=9, 1 x *n*=10).

***In vitro* fermentation method**

Fresh faeces were obtained from four healthy human subjects (3f, 1m) consuming their normal western diets. Faeces were collected into pre-warmed, insulated containers flushed with CO₂ and processed in the laboratory within 30min after collection. Subjects were not taking medication nor had a history of medical problems that may have interfered with the aims of the study. In the 3 months prior, subjects had not experienced gastrointestinal illness or taken antibiotics or laxatives. Informed written consent was obtained from all volunteers, and the study was approved by the Massey University Human Ethics Committee (PN 04 /127).

A phosphate buffer (0.1 M, pH 7) was boiled, cooled under a stream of oxygen-free nitrogen (OFN) and maintained at 37°C until required. A 320 g/L batch of inoculum was prepared by processing 160 ml of buffer and 80 g of pooled faeces in a Stomacher® for 7 min. The inoculum was twice filtered through six layers of muslin cloth. All procedures were performed under a constant flow of CO₂ to ensure anaerobic conditions.

A total of 100 mg prepared rat ileal digesta (see above) were weighed into a dried and pre-weighed 28 ml glass McCartney bottle (Biolab Ltd., Auckland, New Zealand). To each bottle was added 5 ml of inoculum and 5 ml of buffer to give a final concentration of 160 g/L. The bottles were flushed with OFN and placed upright in a standard water bath (37°C) for 18 h. Bottles were then autoclaved (120°C, 8 min) and the caps removed prior to drying in an oven (80°C) and ashing in a Muffle furnace (500°C, 4 h) to determine dry matter (DM) and organic matter (OM) respectively.

Chemical analyses

On each day during the balance period of the human study, samples of all meals (as eaten) were weighed in proportion to their contribution to the diet, homogenised, ground to a fine powder (1 mm mesh) and frozen. At the end of the balance period, all daily diet samples were combined in equal proportion, homogenised and frozen to provide a composite weekly sample for analysis. The diet samples were analysed for DM, ash, gross energy (GE), total N, total sugars, total starch, total fat, fatty acids (FA), NSP and DF (total, insoluble and soluble). Rat ileal digesta samples were analysed for DM and ash. Human faeces were analysed for DM, ash and GE. Rat diet and digesta samples were also analysed for titanium dioxide.

DM and ash determination of diets, digesta and human faeces were performed in duplicate by drying samples in a Watvic convection oven (Watson Victor Ltd., Wellington, New Zealand) at 105°C for 16 h (AOAC, 2000), followed by ashing in a

Muffle furnace (Electrofurn, New Zealand) at 550°C for 16 h (AOAC, 2000). The titanium dioxide content of the rat diets and digesta was measured on a UV spectrophotometer following the method of Short *et al.* (1996).

GE (the heats of combustion of protein, fat and carbohydrate) of the diets and human faeces was determined in duplicate using an automatic adiabatic bomb calorimeter (AC-350, Leco Corporation, St. Joseph MI, USA) (Miller and Payne, 1959). GE determination of digesta samples was performed using 50mg samples in sections of plastic wrap of known GE content.

Crude protein was calculated using an N to protein conversion factor of 6.25 (Kraisid *et al.*, 2003). N analysis was performed with duplicate samples using two methods: (a) Leco total combustion method (AOAC, 2000) on a Leco TruSpec CN (Carbon/Nitrogen) Determinator (Leco Corporation, St. Joseph, MI, USA) and (b) microanalysis using an elemental analyser (EA 1108, Carlo Elba, Italy).

Individual FA were determined in duplicate by GC (Sukhija and Palmquist, 1988; Wu *et al.*, 1994) on a 6890 GC FID (Agilent Technologies, Santa Clara, CA). Total fat was determined in duplicate by two methods: (a) acid hydrolysis/Mojonnier extraction (AOAC 954.02 (AOAC, 2000)) and (b) total lipid FA as determined by GC, expressed as triacylglycerol as suggested by the FDA (Ali *et al.*, 1997; FDA, 2004).

Total sugars were defined as simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl esters with free or potentially reducing groups. Sugars were determined using the phenol sulphuric colourimetric method on duplicate samples (Dubois *et al.*, 1956). Total starch determination was performed in duplicate using a commercial kit (Total Starch Kit, Megazyme, Ireland) (AOAC, 2000). Total carbohydrate (CHO) (%) was calculated as the difference between 100 and the sum of the percentage of water, protein, total fat and ash. Total CHO was calculated using fat and N as determined by the Mojonnier and total combustion methods, respectively, as described above.

Total DF, along with insoluble and soluble fibre fractions, was determined using an enzymatic-gravimetric method (AOAC, 2000) with a commercial kit (Total Dietary Fibre Kit Assay, Megazyme, Ireland) and alpha-amylase/amyloglucosidase/protease enzymes. NSP content was determined using a modified Englyst method (Englyst and Cummings, 1984, 1988). Constituent sugars of NSP were measured using colorimetry.

Calculations

Human balance study

Total tract digestibility (apparent faecal digestibility) of DM and OM

The total tract (apparent faecal) digestibility of DM/OM (%) in the human balance study was calculated as the difference between the DM/OM in the food (g/d) and that excreted in the faeces (g/d), relative to DM/OM in the food (g/d), as shown in Equation 1 for DM digestibility (and analogously for OM digestibility).

$$\text{Apparent faecal digestibility of DM (\%)} = \frac{DM_{\text{food}} - DM_{\text{faeces}}}{DM_{\text{food}}} \times \frac{100}{1} \quad (\text{Eq. 1})$$

Dual *in vivo* – *in vitro* digestibility assay

Rat ileal digestibility

The apparent ileal digestibility of OM (%) was calculated using the ratio of OM (mg/g DM_{diet}) to titanium dioxide (mg/g DM_{diet}) in the diet ((OM/Ti)_{diet}) and the ratio of OM (mg/g DM_{digesta}) to titanium dioxide (mg/g DM_{digesta}) in the ileal digesta ((OM/Ti)_{ileal}).

$$\text{Apparent ileal digestibility of OM (\%)} = \frac{(OM/Ti)_{\text{diet}} - (OM/Ti)_{\text{digesta}}}{(OM/Ti)_{\text{diet}}} \times \frac{100}{1} \quad (\text{Eq. 2})$$

Hindgut OMD with *in vitro* fermentation assay

OMD of the rat ileal substrate was determined by correcting for the OM present in the blanks, as described elsewhere (see Chapter VI) and calculated as follows:

$$\text{Hindgut OMD (\%)} = \frac{OM_{\text{fermented}}}{OM_{\text{ileal substrate}}} \times \frac{100}{1} \quad (\text{Eq. 3})$$

$$= \frac{OM_{\text{ileal substrate}} - \left(OM_{\text{bottle residual}} - \left(\frac{OM_{\text{blank residual}} + OM_{\text{blank initial}}}{2} \right) \right)}{OM_{\text{ileal substrate}}} \times \frac{100}{1} \quad (\text{Eq. 4})$$

where $OM_{\text{ileal substrate}}$ is the OM (mg) in the ileal substrate prior to incubation, $OM_{\text{bottle residual}}$ is the total OM (mg) remaining in the bottle at the end of incubation (from both the ileal substrate and the inoculum), $OM_{\text{blank initial}}$ is the OM (mg) of the blank at the start of incubation and $OM_{\text{blank residual}}$ is the OM remaining of the blank OM (mg) at the end of incubation.

$OM_{\text{ingested equivalent}}$

The OM content of the diet (OM_{diet}) per g of DM was known from chemical analysis. However, the mean absolute quantity of OM ingested by the group of rats for each pooled ileal digesta sample to give x amount (mg) of OM present in the ileal substrate ($OM_{\text{ileal substrate}}$, mg), referred to as $OM_{\text{ingested equivalent}}$ (mg), needed to be calculated. This calculation was based on the apparent ileal digestibility of the diet (%) as calculated using Equation 2 and the absolute quantity of OM (mg) in the ileal digesta sample used for the *in vitro* fermentation ($OM_{\text{ileal substrate}}$).

$$OM_{\text{ingested equivalent}} (\text{mg}) = \frac{OM_{\text{ileal substrate}} \times 100}{100 - \text{ileal OM digestibility (\%)}} \quad (\text{Eq. 5})$$

Total tract OMD from dual in vivo – in vitro digestibility assay

OMD over the total tract for the dual *in vivo – in vitro* digestibility assay (%) was defined as the mean OM ingested for the pooled group of rats ($OM_{\text{ingested equivalent}}$, mg) corrected for the OM contributed by the substrate at the end of the *in vitro* incubation ($OM_{\text{bottle residual}}$, mg), corrected for the OM in the blank (mg), relative to the OM ingested (OM_{ingested}).

Total tract in vivo - in vitro OMD (%)

$$= \frac{(OM_{\text{ingested equivalent}} - OM_{\text{ileal substrate}}) + OM_{\text{fermented}}}{OM_{\text{ingested equivalent}}} \times \frac{100}{1} \quad (\text{Eq. 6})$$

$$= \frac{OM_{\text{ingested equivalent}} - (OM_{\text{bottle residual}} - \left(\frac{OM_{\text{blank residual}} + OM_{\text{blank initial}}}{2} \right))}{OM_{\text{ingested equivalent}}} \times \frac{100}{1} \quad (\text{Eq. 7})$$

Statistical analysis

Data were subjected to a one way analysis of variance (ANOVA). Results were considered statistically significant at $P < 0.05$. If a statistically significant effect was found, the means were compared for each diet using Tukey's test (Muller and Fetterman, 2002). The program Minitab (version 14.2, Minitab Inc., PA, USA) was used for all statistical analyses.

Results

The determined chemical composition of the dietary material given to the human subjects and also used as a base for the rat diets is given in Table A3 (see Appendix, Chapter III).

Human balance study

The diets were well received and there were no compliance issues. Subjects reported no adverse effects from the diets and remained weight stable (± 2 kg) during the trial. **Table 1** shows the faecal bulking (g wet faeces/g DM), DM and OM intakes and excretion rates (g/d) and the DM and OM digestibility (%) for each diet. The apparent faecal digestibility of DM and OM for the HF diet were significantly ($P < 0.05$) lower than for the PE and LF diets, while no significant difference ($P > 0.05$) was observed between the WB, PE and LF diets. The total tract (apparent faecal) OM digestibility of the diets were ranked as HF < WB < LF < PE, with OMD values ranging from 93% (HF) to 97% (PE).

Table 1

Mean¹ daily intake, faecal excretion and faecal digestibility of dry matter and organic matter and faecal bulking for four diets consumed by the subjects

Component	Parameter	Diet				SEM
		Wheat Bran Diet	Pectin Diet	Low Fibre Diet	High Fibre Diet	
Dry matter	Daily intake, g	432.3 ^a	376.0 ^a	471.5 ^a	420.4 ^a	24.0
	Faecal excretion, g/d	34.1 ^a	15.0 ^a	19.0 ^a	34.1 ^a	3.2
	Faecal bulking, g wet faeces / kg diet DM	272.4 ^{ab}	144.5 ^b	144.7 ^b	364.0 ^a	30.9
	Faecal digestibility, %	92.4 ^{ab}	95.8 ^b	96.1 ^b	91.8 ^a	0.6
Organic matter	Daily intake, g	414.6 ^a	363.5 ^a	455.7 ^a	405.0 ^a	23.0
	Faecal excretion, g/d	28.0 ^{ab}	10.6 ^a	16.0 ^{ab}	29.0 ^b	2.9
	Faecal digestibility, %	93.5 ^{ab}	96.9 ^b	96.7 ^b	92.8 ^a	0.6

¹ Mean, n=6 subjects, except for Wheat Bran Diet (n=5) and Low Fibre Diet (n=4)

^{ab} Means with different superscripts within the same row were significantly ($P < 0.05$) different DM, dry matter; SEM, standard error of the mean

Dual *in vivo* - *in vitro* digestibility assay

In vivo upper-tract OMD: rat study

The diets were readily consumed by the animals. Despite the large size of the animals at the time of digesta sampling, limited quantities of ileal digesta were obtained, particularly for the PE diet. There was also evidence that some rats had practised coprophagy, requiring that four animals be excluded from the trial. Consequently, there was insufficient digesta for the necessary chemical analyses if each rat was treated as an experimental unit. Therefore, pooling of digesta for the rats consuming the same diet was necessary. The rat ileal digestibility of OM (%) is shown in **Table 2**. Ileal OMD ranked the diets as HF<WB<PE<LF and ranged from 80% (HF) to 86% (LF), with only the difference between the HF and LF diets being significant ($P<0.05$).

Table 2

Mean¹ ileal (rat) and large intestinal (*in vitro*) organic matter digestibility for the four experimental diets

Model	Organic Matter Digestibility, %				SEM
	Wheat Bran Diet	Pectin Diet	Low Fibre Diet	High Fibre Diet	
Rat ileal	82.8 ^{ab}	85.2 ^{ab}	86.3 ^a	80.3 ^b	0.9
<i>In vitro</i> hindgut	55.1 ^a	78.9 ^b	61.5 ^a	56.7 ^a	2.7

¹ Mean, $n=3$ groups of rats, except for Pectin Diet ($n=2$)

^{ab} Means with different superscripts within rows were significantly ($P<0.05$) different

SEM, standard error of the mean

Hindgut *in vitro* OMD: *in vitro* fermentation assay

Considerable variation was seen for the hindgut (*in vitro*) OMD (Table 2) due to the range in potential fermentability of the diets. Of the undigested OM reaching the terminal ileum, between 55% (WB) and 79% (PE) appeared to be fermented. The hindgut OMD ranking of the diets was WB<HF<LF<PE.

Total tract OMD: dual *in vivo* - *in vitro* digestibility assay

Table 3 shows predicted values for total tract OMD determined using the dual digestibility assay (OMD_{dual}), which combines the results of rat ileal OMD and hindgut *in vitro* OMD. The range of total tract OMD_{dual} values, like ileal OMD, was not large, ranging from 92% (HF) to 97% (PE). Total tract OMD was predicted exactly by the dual digestibility assay for the PE diet (96.9%), while good agreement (OMD_{human} versus OMD_{dual}) was found for the other three diets: 93.5% versus 92.3% (WB), 96.7% versus 94.8% (LF) and 92.8% versus 91.5% (HF). OMD_{dual} ranked the total tract digestibility of the four diets as HF<WB<LF<PE, in agreement with actual total tract OMD (OMD_{human}).

Table 3

Comparison of mean¹ predicted (determined using dual *in vivo-in vitro* digestibility assay) and actual (human *in vivo* balance study) total tract organic matter digestibility for the four experimental diets

Diet	Total Tract Organic Matter Digestibility, %		SEM
	Human <i>in vivo</i>	Dual digestibility assay	
Wheat Bran Diet	93.5	92.3	0.5
Pectin Diet	96.9	96.9	0.4
Low Fibre Diet	96.7	94.8	0.5
High Fibre Diet	92.8	91.5	0.9

¹ Mean, *n*=6 subjects for human *in vivo*, except for Wheat Bran Diet (*n*=5) and Low Fibre Diet (*n*=4); mean, *n*=3 replicates for dual digestibility assay, except for Pectin Diet (*n*=2)

Differences between 'Human *in vivo*' and 'dual digestibility assay' were not significant (*P*>0.05) for any diet

SEM, standard error of the mean

Discussion

Studies to quantify the overall digestibility of food organic matter and energy in humans over the total digestive tract have relied historically on human balance studies, due to a lack of validated alternatives. Here, an alternative approach is presented in the form of a dual *in vivo* – *in vitro* digestibility assay (“dual digestibility assay”), allowing the prediction of the total tract digestibility of dietary energy in humans. The dual digestibility assay brings together upper-tract (ileal) digestibility, as determined using an animal model (rat upper-tract), and colonic digestibility, as predicted by fermenting rat ileal digesta in an *in vitro* system with human faecal bacteria.

The intent of developing the dual digestibility assay was to enable prediction of the uptake of absolute quantities of energy-yielding nutrients (OM) from different parts of the digestive tract. This is shown diagrammatically in **Figure 1**, where the inputs and outputs of OM in both the human digestive system and the dual digestibility assay are illustrated for comparative purposes. The assay needs to be validated. Thus, in the present work total tract OMD was predicted using the dual digestibility assay and compared with values obtained from a human balance study, for four mixed human diets. Diets were formulated to represent the extremes in DF likely to be encountered in a Western human diet, recognising that the type and level of DF inclusion is known to affect the apparent digestibility of the energy-yielding nutrients (Baer *et al.*, 1997; Calloway and Kretsch, 1978; Farrell *et al.*, 1978; Kelsay *et al.*, 1978). These extremes in DF were in terms of both degree of fermentability (WB and PE diets) and quantity (LF and HF diets) of DF.

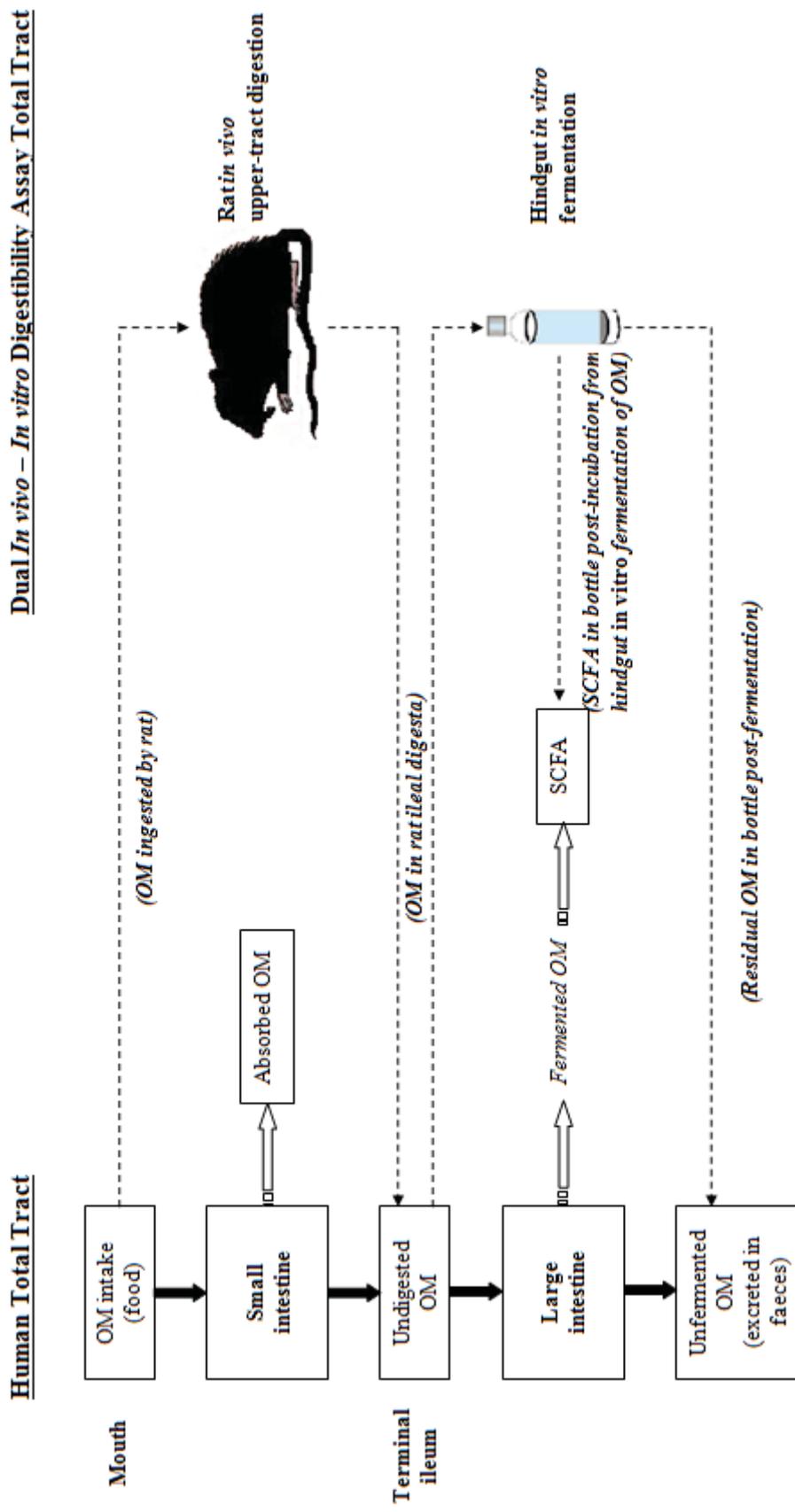


Figure 1. Flow of OM in human digestive system and dual *in vivo* - *in vitro* digestibility assay over the total tract, showing inputs and outputs at each stage. OM, organic matter; SCFA, short chain fatty acids

Current methodologies for determining both GE and OM are known to be accurate. However, compared to GE, determination of OM is affordable and straightforward, requiring limited specialised laboratory equipment and technical expertise. OM determination can also be undertaken with small sample sizes (100 mg or less), which is important given the limited quantities of digesta available from the rat. Furthermore, using OMD as a measure of the digestibility of energy means that the entire *in vitro* portion of the dual digestibility assay methodology can be performed in a single vessel, limiting loss of material during analysis. Once the rat digesta have been obtained and prepared, the *in vitro* fermentation is simple and OMD results are obtained 75 h from the beginning of the 18 h fermentation period. Multiple runs are easily performed simultaneously, in most cases limited only by the capacity of the water bath. Given the advantages of quantifying OM rather than GE, OMD was used as a measure of the digestibility of energy in the dual digestibility assay.

Comparison of total tract OMD: human *in vivo* OMD and predicted OMD from the dual digestibility assay

Total tract OMD obtained from the human study and that predicted using the dual digestibility assay ranked the four diets the same: HF<WB<LF<PE. Predicted and actual OMD were not significantly ($P>0.05$) different for any of the diets.

A Pearson's Correlation was performed using the mean OMD data for the human study and the predicted values from the dual digestibility assay. A correlation coefficient (r) of 0.953 ($P=0.047$) was obtained. A plot of OMD_{dual} versus OMD_{human} (**Figure 2**) indicates that the PE diet falls on the line $x = y$, while good agreement was also seen for the other diets.

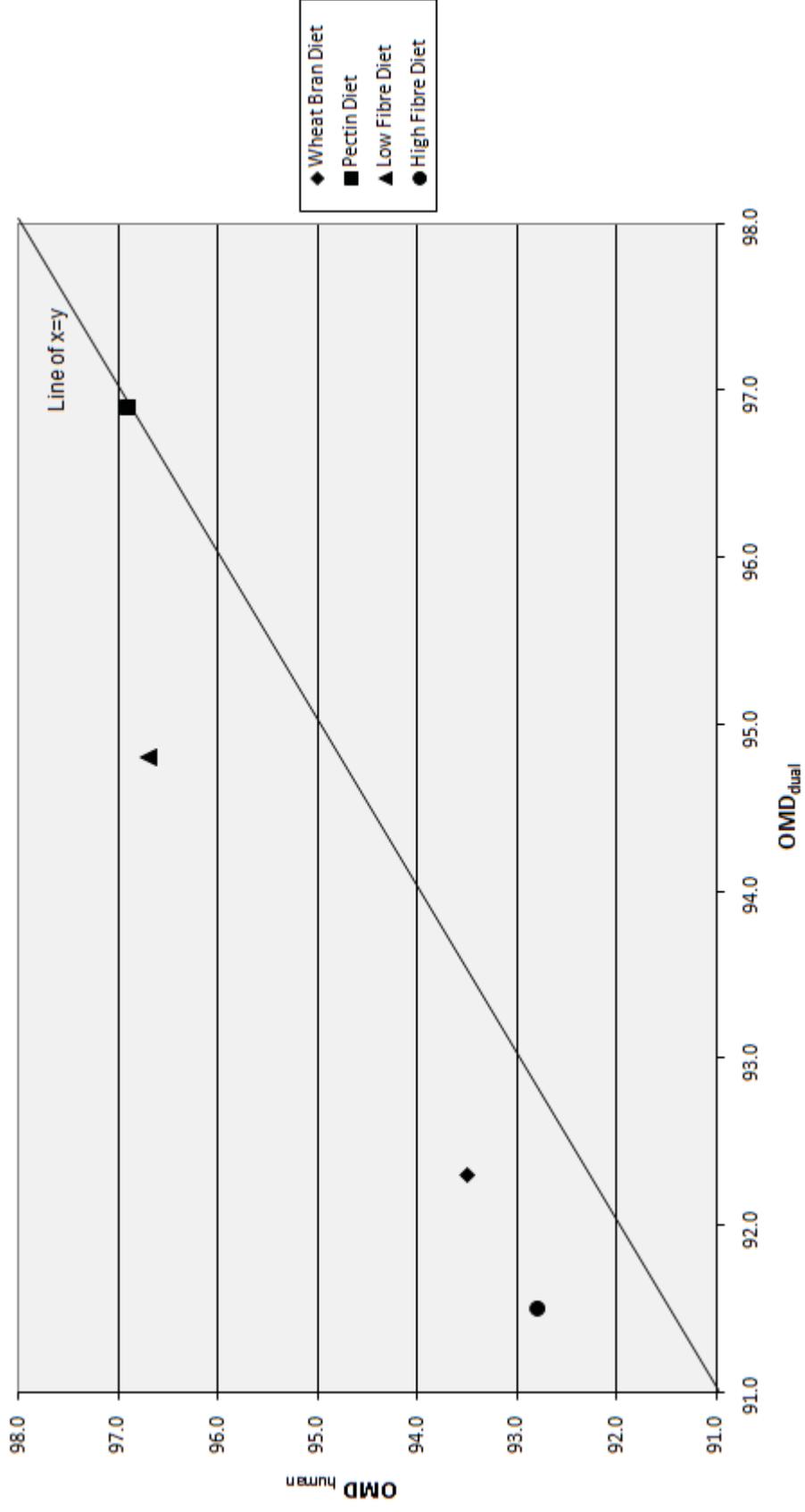


Figure 2. Total tract OMD (%) as determined using the dual digestibility assay (OMD_{dual}) versus OMD from a human balance study (OMD_{human}) for four mixed human diets. OMD, organic matter digestibility.

To extend the number of diets included in the correlation analysis and the OMD range, the dual digestibility assay was used to predict the digestibility of three additional mixed diets using OMD data obtained in a previous human balance study (Zou *et al.*, 2007). **Figure 3** shows the relationship between actual (human *in vivo*) and predicted (dual digestibility assay) OMD for the additional diets (Fruit and Vegetable Diet, Refined Diet and Cereal Diet), as well as those from the present study. The dual digestibility assay predicted the OMD of the three additional diets within 1% of actual OMD (OMD_{human}) and a Pearson's Correlation including all seven diets gave a correlation co-efficient (*r*) of 0.961 (*P*=0.001).

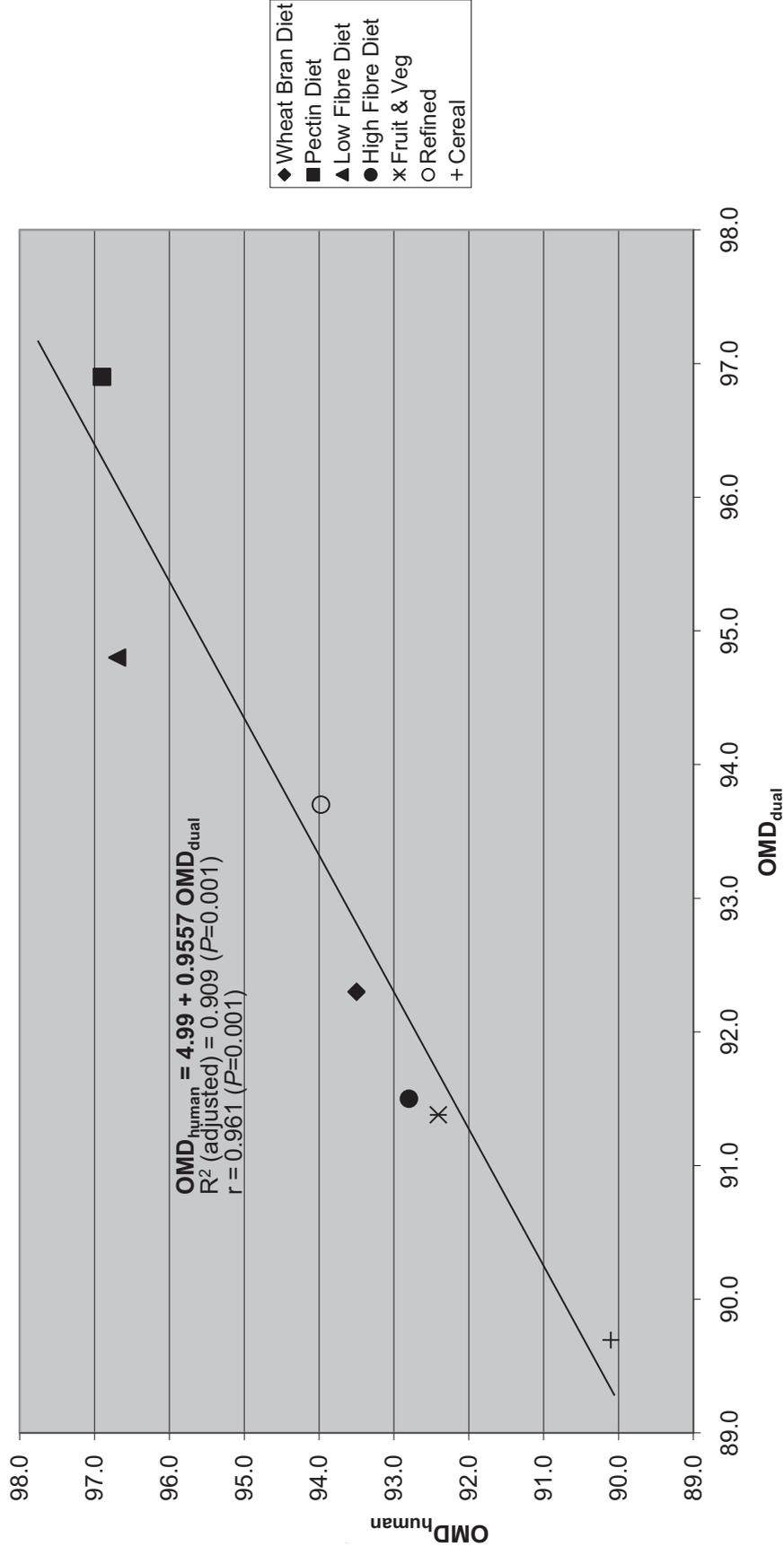


Figure 3. Total tract OMD (%) as determined using the dual digestibility assay (OMD_{dual}) versus OMD from a human balance study (OMD_{human}) for four mixed human diets from the present study and three additional mixed human diets (Zou *et al.* 2007). OMD, organic matter digestibility.

Conclusion

The dual digestibility assay performs well using diets ranging in OMD from approximately 90% to 97%. To the authors' knowledge, the dual digestibility assay is the only validated methodology available that is able to predict the total tract digestibility of energy in humans as well as separately quantifying the contribution from the upper-tract and lower digestive tract. Uniquely, it is able to predict the uptake of absolute quantities of dietary energy (expressed as g of OM) from mixed human diets, thus having the potential to quantify the energy available for metabolic processes. The dual digestibility assay has particular practical application in nutritional research, such as in the assessment and development of novel weight-loss foods and ingredients. The metabolisable energy content of these novel foods is of considerable importance but often difficult to accurately predict due to their unknown digestibility *in vivo*.

The dual digestibility assay is sensitive enough to detect small differences between diets and gives a variation no larger than that found with human balance studies. The dual digestibility assay offers a valid and simpler means of determining the digestibility of energy in man than that possible with balance studies, without sacrificing accuracy or precision.

Literature cited

- Ali, L. H., Angyal, G., Weaver, C. M. & Rader, J. I. (1997). Comparison of capillary column gas chromatographic and AOAC gravimetric procedures for total fat and distribution of fatty acids in foods. *Food Chemistry*, 58(1-2), 149-160.
- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Bach Knudsen, K. E., Wisker, E., Daniel, M., Feldheim, W. & Eggum, B. O. (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and the rat. *British Journal of Nutrition*, 71, 471-487.
- Baer, D. J., Rumpler, W. V., Miles, C. W. & Fahey Jr., G. C. (1997). Dietary fiber decreases the metabolizable energy content and nutrient digestibility of mixed diets fed to humans. *Journal of Nutrition*, 127, 579-586.
- Calloway, D. H. & Kretsch, M. J. (1978). Protein and energy utilization in men given a rural Guatemalan diet and egg formulas with and without added oat bran. *American Journal of Clinical Nutrition*, 31, 1118-1126.
- Coles, L. T., Moughan, P. J., Awati, A. & Darragh, A. J. (2011). Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter. *Food Chemistry*, In Press.
- Coles, L. T., Moughan, P. J. & Darragh, A. J. (2005). *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, 123, 421 – 444.
- Darragh, A. J. & Moughan, P. J. (1995). The three-week-old piglet as a model for studying protein digestion in human infants. *Journal of Pediatric Gastroenterology and Nutrition*, 21, 387-393.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39, 209-228.

-
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994). The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, *49*, 57-71.
- Dubois, M., Gillies, K. A., Hamilton, J. A., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350-356.
- Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst (Cambridge UK)*, *109*, 937-942.
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, *71*, 808-814.
- Farrell, D. J., Girлие, L. & Arthur, J. (1978). Effects of dietary fibre on the apparent digestibility of major food components and on blood lipids in men. *Australian Journal of Experimental Biology and Medical Science*, *56*, 469-479.
- FDA. (2004). *Code of Federal Regulations, Title 21, Volume 2, Chapter I, Part 101.9*. Washington DC: Department of Health and Human Services, Food and Drug Administration.
- Kelsay, J. L., Behall, K. M. & Prather, E. S. (1978). Effect of fiber from fruits and vegetables on metabolic responses of human subjects I. Bowel transit time, number of defecations, fecal weight, urinary excretions of energy and nitrogen and apparent digestibilities of energy, nitrogen, and fat. *American Journal of Clinical Nutrition*, *31*, 1149-1153.
- Kraisid, T., MacLean Jr., W. C. & Warwick, P. M. (2003). *Food energy: methods of analysis and conversion factors: report of a technical workshop, Rome, 3-6 December 2002*. Rome: Food and Agriculture Organization of the United Nations.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, *13*, 501-508.

-
- Monsma, D. J., Thorsen, P. T., Vollendorf, N. W., Crenshaw, T. D. & Marlett, J. A. (2000). *In vitro* fermentation of swine ileal digesta containing oat bran dietary fiber by rat cecal inocula adapted to the test fiber increases propionate production but fermentation of wheat bran ileal digesta does not produce more butyrate. *Journal of Nutrition*, *130*, 585-593.
- Moughan, P. J., Smith, W. C. & James, K. A. C. (1984). Preliminary observations on the use of the rat as a model for the pig in determination of apparent digestibility of dietary proteins. *New Zealand Journal of Agricultural Research*, *27*, 509-512.
- Muller, K. E. & Fetterman, B. A. (2002). *Regression and ANOVA: An Integrated Approach Using SAS Software*. Cary, NC: SAS Publishing.
- National Research Council. (1995). Nutrient requirements of the laboratory rat. In *Nutrient Requirements of Laboratory Animals* (4th rev. ed., pp. 11-79). Washington, DC: National Academy Press.
- Nyman, M., Asp, N. G., Cummings, J. H. & Wiggins, H. (1986). Fermentation of dietary fibre in the intestinal tract: comparison between man and rat. *British Journal of Nutrition*, *55*, 487-496.
- Pearson, G., Moughan, P. J., Dong, G. Z. & Morel, P. C. H. (1999). Protein quality in blood meal. I. The rat as a model animal for determining apparent ileal amino acid digestibility in the growing pig. *Animal Feed Science and Technology*, *79*, 301-307.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, *87*, 292-300.
- Short, F. J., Gorton, P., Wiseman, J. & Boorman, K. N. (1996). Determination of titanium oxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, *59*, 215-221.
- Sukhija, P. S. & Palmquist, D. L. (1988). Rapid method for determination of total fatty-acid content and composition of feedstuffs and feces. *Journal of Agricultural and Food Chemistry*, *36*(6), 1202-1206.

-
- Van Soest, P. J., Jeraci, J., Foose, T., Wrick, K. & Ehle, F. (1983). Comparative fermentation of fibre in man and other animals. In G. Wallace & L. Bell (Eds.), *Fibre in Human and Animal Nutrition: Proceedings of Dietary Fibre in Human and Animal Nutrition Symposium* (pp. 75-80). Palmerston North, New Zealand: The Royal Society of New Zealand.
- Wisker, E. & Bach Knudsen, K. E. (2003). The rat as a model for pigs: comparative values for the digestibility of NSP and other macronutrients. *British Journal of Nutrition*, *90*, 373-383.
- Wisker, E., Bach Knudsen, K. E., Daniel, M., Eggum, B. O. & Feldheim, W. (1997). Energy values of non-starch polysaccharides: comparative studies in humans and rats. *Journal of Nutrition*, *127*, 108-116.
- Wu, J. R., James Jr., D. W., Dooner, H. K. & Browse, J. (1994). A mutant of arabidopsis deficient in the elongation of palmitic acid. *Plant Physiology*, *106*(1), 143-150.
- Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, *86*, 1649-1656.

CHAPTER VIII

A model to predict the ATP equivalents of macronutrients absorbed from food

A series of predictive equations for determining ATP costs / yields were developed and applied to the uptake of each energy-yielding nutrient (as predicted separately in the upper-digestive tract and the hindgut using a validated dual in vivo - in vitro digestibility assay), for an adult human in a state of weight-loss (sub-maintenance energy intakes).

Abstract

Calculating the physiologically available energy of food at the cellular level (ATP), based on known stoichiometric relationships and predicted nutrient uptake from the human digestive tract may be more accurate than using currently available factorial or empirical models for estimating dietary energy. The objective was to develop a model that can be used for describing the ATP costs / yields associated with the total tract uptake of the energy-yielding nutrients for an adult human in a state of weight-loss (sub-maintenance energy intakes). A series of predictive equations for determining ATP yields / costs were developed and applied to the uptake of each energy-yielding nutrient, as predicted separately in the upper-digestive tract and the hindgut using a dual *in vivo* – *in vitro* digestibility assay. The costs associated with nutrient ingestion, absorption and transport and with the synthesis and excretion of urea produced from amino acid catabolism were also calculated. ATP yields (not including costs for digestion, absorption and transport) were predicted as 28.9 mol ATP / mol glucose; 4.7 – 32.4 mol ATP / mol amino acid and 10.1 mol ATP / mol ethanol, while yields for fatty acids ranged from 70.8 mol ATP / mol lauric acid (C12) to 104 mol ATP / mol linolenic acid (C18:3). The energetic contribution of hindgut fermentation was predicted to be 101.7 mmol ATP / g organic matter fermented. The model allows prediction of the physiologically available energy (ATP) of foods and has particular application for the development of foods such as specialised weight-loss products.

Introduction

There has been considerable discussion in recent years on the most appropriate means of predicting the energy available to humans from foods, mostly relating to the use of factorial models versus empirical models (Livesey, 1995; Zou *et al.*, 2007). While the application of these models has shown their usefulness to varying degrees, there may be more accurate means for determining the “available” energy content of foods (Elia and Livesey, 1988; Livesey, 1984; Zou *et al.*, 2007). An alternative approach would be to calculate available energy in terms of ATP yields, based on known stoichiometric relationships and predicted uptakes of nutrients from the digestive tract. Ideally, the nutrient uptake would be separately quantified in absolute terms for each energy-yielding substrate in the upper-tract (mouth to terminal ileum) and for short-chain fatty acid (SCFA) uptake as a result of colonic fermentation. The ATP yield may be corrected for energetic costs (ATP equivalents) directly related to dietary intake. Such costs include the energetic cost of digesting, absorbing and transporting the nutrients to the main site of catabolism (liver) and excretion of urea synthesised during dietary amino acid catabolism.

Two important components to allow the development of such an alternative model for predicting food energy values, namely the prediction of the degree of absorption of dietary energy-containing compounds and the prediction of subsequent ATP yields, are now available. Recently, a means of predicting the quantitative uptake of each substrate available for oxidation across the total tract has been proposed in the form of a dual *in vivo* – *in vitro* digestibility assay (Coles *et al.*, 2011; Moughan *et al.*, 2009). The biochemical pathways involved in nutrient oxidation and therefore the ATP yields from the catabolism of absorbed nutrients have been known for some time with considerable work being undertaken in the 1970’s and 1980’s. However, knowledge of the underlying cell energetics in the human body has grown considerably in the past two decades, largely due to advancements in an understanding of cell biochemistry. There is presently more accurate information available on the mitochondrial efficiency of ATP production, which provides a more accurate prediction of ATP yields.

The current contribution provides an overview of a model that can be used for describing the energetic costs and yields associated with the ingestion and digestion of food in terms of ATP. The specific ATP costs and yields as a result of the uptake of each energy-yielding substrate are predicted quantitatively.

Experimental methods

Model overview

The proposed model relates to an adult human consuming food at or below maintenance energy levels. The model predicts the energy yielded from nutrient catabolism but does not describe the utilisation of that energy. The model does not include basal energy costs as these were considered to be largely independent of dietary intake. Basal energy costs relate to those functions that are essential for life, such as cell function and replacement, brain function, the maintenance of body temperature and the uninterrupted work of cardiac and respiratory muscles. The energy costs relating to the processes of urinary and faecal excretion are not well characterised and thus were not included in the model, but can be included in the future. The cost of urea synthesis and excretion was included in the model as an inherent cost of amino acid catabolism. ATP was considered to be the end product of the catabolism of all absorbed macronutrients which were divided into crude protein, crude fat and carbohydrate. Ethanol, while not considered a major energy source for the majority of the population was included for completeness. Carbohydrate was further classified according to its ability / inability to be digested in the upper-tract, as either available carbohydrate or unavailable carbohydrate (UC) respectively. The available carbohydrates consist primarily of starch and sugars. The unavailable carbohydrates collectively constitute a range of substrates including hemicellulose, cellulose, lignin, pectins, and gums. While not included here, the model can also incorporate other dietary components such as polyols, synthetic fats and organic acids (e.g. citric acid).

In the case of a subject whose dietary energy intake is less than the total energy requirement, the amino acids, fatty acids and glucose produced as a result of digestion and absorbed in the upper digestive tract ultimately undergo oxidation in cells. Even in the case where body weight is constant, all consumed nutrients will be oxidised over the course of a few days (Flatt and Tremblay, 1998). It is therefore assumed that all the absorbed nutrients provide the body with ATP directly, rather than material being

stored for later use. Part of the UC undergoes fermentation by colonic bacteria in the hindgut, resulting in the formation of SCFA, which are absorbed by the body and oxidised.

ATP yield (ATP_a)

The model assumes that all absorbed nutrients are ultimately catabolised and supply energy. ATP is an energy equivalent and may be regarded as the universal currency of energy in the body even though other energy intermediaries exist (e.g. creatinine). ATP does not accumulate in cells, but rather, is constantly being used for metabolic or external work. ATP yield (ATP_a) refers to the energy released in the cell (available ATP) and subsequently made available to the body for this work upon the oxidation of exogenous energy-yielding nutrients. ATP_a was defined as the summation of ATP gained as a result of catabolism of nutrients arising from digestion (upper-tract) and fermentation (hindgut), less the direct ATP cost of catabolism, which is the metabolic energy cost of processing each nutrient into ATP once it arrives at the site of catabolism (liver). The ATP cost of catabolism includes the ATP used for activation (conversion to a compound that can enter the tricarboxylic acid (TCA) cycle) in the case of glucose (GL), fatty acids (FA), ethanol (EtOH) and short chain fatty acids (SCFA). Amino acids (AA) enter the glycolysis / TCA cycle at various points (α -ketoglutarate, acetyl co-enzyme A, pyruvate and serine) depending on their structure. The quantity of ATP, NADH, FADH₂ and NADPH involved in the conversion of each AA to these entry point compounds is detailed in Table 3 of van Milgen (2002). The ATP necessary for the synthesis and excretion of urea in the case of AA is also included in the ATP cost of catabolism. Other catabolic costs include the cost of transferring NADH from the cytoplasm to the mitochondrion, the cost of supplying NADPH required during the oxidation of some nutrients (e.g. unsaturated FA, lysine, tryptophan, phenylalanine and histidine) and the ATP cost of converting one intermediate substrate into another. All of these metabolic costs are accounted for by ATP_a.

ATP_a is calculated by summing the predicted molar ATP yields from the catabolism of absorbed GL, AA, FA, glycerol and EtOH from upper-tract digestion and SCFA from hindgut fermentation, while allowing for the energetic cost of any directly related metabolic processes. ATP yields arising from the catabolism of each nutrient (**Table 1**) were calculated using the equations given in the metabolic framework proposed by van Milgen (2002), with an additional equation added for the metabolism of ethanol (see sub-section on ethanol). The van Milgen framework does not place constraints on the cellular P/O ratios of NADH and FADH₂. For the purposes of the model described here, the following general assumptions were made and applied to all nutrients:

- All absorbed nutrients are catabolised directly in the liver, rather than being temporarily stored or converted to intermediary compounds
- Recycling of absorbed nutrients or their products was not considered, except in the case of urea produced as a result of AA catabolism
- P/O ratios of 2.31 for NADH and NADPH, and 1.38 for FADH₂ were applied
- The transfer of NADH from the cytoplasm to the mitochondria was at an equivalent cost of 0.25 ATP (van Milgen, 2002). This is relevant for glucose, ethanol, glycerol, propionate and all AA.
- Conversion of mitochondrial GTP to ATP yields 0.75 ATP, rather than 1 ATP and as such, the reaction of α -ketoglutarate to oxaloacetate yields 0.75 ATP (van Milgen, 2002)

Table 1

ATP yields (ATP_a) of glucose, amino acids, fatty acids, SCFA and ethanol and the costs associated with their digestion (ATP_d) and absorption / transport (ATP_t)

Model component	ATP yield or cost	
ATP yields		
ATP_a		
Glucose	28.9	mol ATP / mol GL
Amino acids	see Table 2	
Fatty acids	see Table 3	
SCFA (produced in hindgut)	see Table 3	
Glycerol	see Table 3	
Ethanol	10.1	mol ATP / mol EtOH
ATP costs		
ATP_d	28	mol ATP / kg OM_{D+F} ¹
ATP_t		
Glucose	0.5	mol ATP / mol GL
Amino acids	0.5	mol ATP / mol AA
Fatty acids	0.75	mol ATP / mol FA
SCFA (produced in hindgut)	0	mol ATP / mol SCFA
Glycerol	0	mol ATP / mol GY
Ethanol	0	mol ATP / mol EtOH

¹ OM_{D+F} refers to ingested OM that is digested and fermented by the body

AA, amino acid; EtOH, ethanol; FA, fatty acid; GL, glucose; GY, glycerol, OM, organic matter; SCFA, short chain fatty acid

Carbohydrate catabolism

Monosaccharide carbohydrates in foods consist mainly of glucose, fructose and galactose (Livesey, 1984). The heat of combustion (routinely measured by bomb calorimetry) and the ATP and NADH produced in the cytoplasm and mitochondria and

the mitochondrial FADH_2 produced during oxidation are the same for glucose, fructose and galactose. The ATP yield and heat of combustion of glucose was therefore taken as being representative of all dietary monosaccharides and all sugars absorbed in the upper-digestive tract were considered equivalent to glucose.

Catabolism of available glucose proceeds via aerobic glycolysis with each GL producing 2 pyruvate, using 2 ATP but generating 4 ATP and 2 NADH. Each pyruvate enters the TCA cycle, yielding 4 NADH, 1 FADH_2 and 1 GTP. It was assumed that glycolysis uses the glutamate-aspartate shuttle, rather than the more costly glycerol phosphate (α GP) shuttle as the glutamate-aspartate shuttle is the dominant shuttle system in the liver (Houston, 2006). The corresponding ATP yield from glucose catabolism was determined as 28.9 mol ATP/mol GL based on Equations 1 – 4 from van Milgen (2002). A molar mass of 180 g/mol was used to equate this ATP yield to 160.3 mmol ATP/ g GL.

Protein and amino acid catabolism

Equations 21- 40 from van Milgen (2002) were used to determine ATP equivalents produced from the catabolism of AA (**Table 2**), calculated using free molecular weights and considering only primary metabolic pathways. Free molecular weights, rather than 'bound' molecular weights were used because the ATP values derived here may be applied to an ileal digestibility assay (see below) which measures AA rather than N and chemical analysis results from laboratories for AA (mg) are routinely reported using free molecular weights.

Table 2ATP yields from the catabolism¹ of amino acids

Amino Acid	Free AA molar mass	ATP yield	
	<i>g/mol</i>	<i>mol ATP/mol AA</i>	<i>mmol ATP/g AA</i>
Lys	146.2	26.2	179.3
Met	149.2	19.7	132.2
Cys	121.2	11.5	95.0
Thr	119.2	16.0	134.6
Trp	204.2	32.4	158.7
Ile	131.2	30.0	228.7
Leu	131.2	29.0	221.1
Val	117.2	23.3	198.5
Phe	165.2	28.4	171.8
Tyr	181.2	30.7	169.3
His	155.2	15.9	102.5
Arg	174.2	20.2	116.0
Ser	105.1	9.2	87.6
Gly	75.1	4.7	62.2
Ala	89.1	11.5	129.2
Glu	147.1	18.0	122.4
Gln	146.2	15.8	108.4
Pro	115.1	21.5	186.4
Asp	133.1	11.5	86.5
Asn	132.2	9.3	70.7

¹ Amino acids are assumed to be deaminated and catabolised via the tricarboxylic cycle (gluconeogenesis was not considered)

AA, amino acids

Amino acids are absorbed from the lumen of the gastrointestinal tract as free amino acids or small peptides. The latter are hydrolysed within the enterocyte to amino acids (Sanford, 1992). Upon reaching the liver, AA are catabolised by deamination followed

by either gluconeogenesis or catabolism via the TCA cycle, although it was assumed here that all AA follow the latter route. The ammonia produced from deamination is disposed of via ureagenesis. The cost of urea synthesis was equated to 4 mol ATP/mol urea produced (Armstrong, 1969; Birkett and de Lange, 2001b; Gerrits *et al.*, 1997). Transportation across the cytoplasmic membrane of the NADH produced during ureagenesis was calculated at a cost of 0.25 ATP. There is a lack of literature on the energetic cost of excreting synthesised urea. Birkett and de Lange use a value of 6 mol ATP / mol of urinary N to account for both the synthesis and excretion of urea and all non-metabolisable materials in the urine (Birkett and de Lange, 2001b). In the present work, the value quoted by Martin and Blaxter (1965) as adopted by Gerrits (1997) of 0.1 mol ATP/mol urea excreted was applied on the basis that all AA produce 0.5 mol urea per AA oxidised, with the exception of lysine, arginine, glutamine and asparagine (each producing 1 mol urea) and histidine (1.5 mol urea). It was further assumed that at least some of the urea produced as a waste product of protein metabolism is recycled via ureolysis. Urea passes into the large intestine where it is metabolised by colonic bacteria (Moran and Jackson, 1990). In line with previous findings that have found approximately 20 – 30% of the synthesised urea is not excreted in the urine but is recycled and remains within the body's metabolic N pool in normal subjects, it was assumed here that 25% of synthesised urea is recycled (Groff and Gropper, 2000; Long *et al.*, 1978; Walser and Bodenlos, 1959). The cost associated with metabolising the recycled urea was not considered. It has been documented that the degree of urea recycling varies under different dietary conditions and physiological states, although no account of this was considered here (Langran *et al.*, 1992). The total net ATP requirement for the synthesis and excretion of urea from AA catabolism was therefore estimated at 4.33 mol ATP / mol urea. The potential energy value contained within each mole of excreted urea was considered a loss to the body. However, based on a gross energy of 10.5 MJ/kg urea (Boisen and Verstegen, 2000), a molecular mass of 60.07 g/mol urea and a ΔG (free energy) of -59.8 kJ/mol ATP (Flatt and Tremblay, 1998; Stryer *et al.*, 2002), the equivalent ATP of 0.00293 mol ATP/mol excreted urea was considered negligible and therefore ignored in the calculation of the ATP yielded from AA.

Triacylglycerol and fatty acid catabolism

The digestion of triacylglycerol (TAG) was assumed to liberate fatty acids and glycerol, which are subsequently available for absorption. **Table 3** gives ATP yields for individual FA and glycerol. 'Bound' molecular weights, rather than 'free' molecular weights were used for the calculation of ATP_a as mmol ATP / g FA since it is assumed that all dietary FA originate from TAG.

Table 3

ATP yields from catabolism of glycerol, short chain fatty acids and fatty acids

	Molar Mass ¹	ATP yield	
	<i>g/mol</i>	<i>mol ATP/mol</i>	<i>mmol ATP/g</i>
Glycerol	92	16.5	179.1
Acetic acid	60	7.1	117.6
Propionic acid	74	12.8	173.1
Butyric acid	88	19.8	225.1
10:0 ²	172	58.1	337.5
Lauric (12:0)	183	70.8	386.9
Myristic (14:0)	211	83.6	396.0
Palmitic (16:0)	239	96.3	403.0
Palmitoleic (16:1)	237	94.9	400.5
Stearic (18:0)	267	109.1	408.5
Oleic (18:1)	265	107.7	406.3
Linoleic (18:2)	263	105.4	400.6
Linolenic (18:3)	261	104.0	398.4
20:0 ³	295	121.8	412.9

¹ Bound molecular weight, assumed to be equal to the free molecular weight - 17 g/mol

² Used for all fatty acids with less than twelve C

³ Used for all fatty acids with more than eighteen C

ATP yields from fatty acid (FA) catabolism are dependent on the number of C atoms and double bonds in the fatty acid, as well as whether there are an even or odd number of C atoms. Fatty acids are iteratively reduced through consecutive cycles of β -oxidation, with each cycle producing 1 mole each of NADH, FADH₂ and acetyl-CoA and shortening the C chain by two C atoms. Unsaturated FA are first converted by an isomerase (for odd numbered double bonds) or a reductase and an isomerase (for even numbered double bonds) into suitable intermediate forms before undergoing β -oxidation. While the details of these pathways will not be discussed here, the result is 1 less FADH₂ being generated for each double bond present and in addition, 1 less NADPH being generated for each even numbered double bond.

Each acetyl-CoA generated through β -oxidation enters the TCA cycle and yields a further 3 NADH and 1 FADH₂, as well as 1 GTP. Fatty acids containing an even number of C atoms produce one extra acetyl-CoA during β -oxidation than those FA of an odd C chain length.

ATP yields for saturated FA and glycerol were calculated using Equations 14 and 19 respectively as given by van Milgen (2002). ATP yields of unsaturated FA containing an even numbered double bond (e.g. linoleic and linolenic acids) were calculated using van Milgen's Equation 15 (van Milgen, 2002), while those containing only one double bond (i.e. palmitoleic and oleic acids) were calculated as for their saturated counterparts but with one less FADH₂ to account for the enoyl-CoA isomerise replacing acyl-CoA dehydrogenase in one cycle. The model only accounts for FA of C chain length of 18 or less.

Catabolism of glycerol can proceed via one of two pathways which vary in ATP yields (Equations 19 and 20 in van Milgen (2002)). The primary pathway produces acetyl-CoA, which then enters the TCA cycle. The secondary pathway proceeds via gluconeogenesis and becomes more important during times of starvation. Quantitatively, 6.7 moles less of ATP are produced when glycerol catabolism proceeds via gluconeogenesis. Under normal physiological conditions the contribution of

glycerol to gluconeogenesis is relatively minor compared to the other major precursors of gluconeogenesis (alanine, glutamine and lactate). In both obese and lean humans glucose from glycerol contributed only around 10% of the total glucose production as a result of gluconeogenesis after a 17 h fast (Chevalier *et al.*, 2006). In fasted (16 h - 18 h) dogs, glycerol was estimated to be responsible for only 5% of total carbon used for gluconeogenesis (Rolfe and Brown, 1997). It is difficult to estimate the proportion of glycerol catabolised via each of the two pathways as it would be expected to vary depending on the body's physiological energy status and the time lapsed since the last meal. Therefore, ATP yields from glycerol were calculated on the basis of catabolism proceeding via the primary pathway.

Ethanol catabolism

Approximately 5 – 10% of ingested ethanol is excreted unaltered, primarily in the expired breath and urine (Rubin and Strayer, 2008). Total ethanol absorption in the stomach is estimated to be in the range of 10 – 30% (Levitt *et al.*, 1997), with the remaining ethanol absorbed in the small intestine. Ethanol is absorbed intact into the portal vein and transported unaltered, primarily to the liver. Upon arrival at the liver, ethanol is degraded to acetaldehyde and then to acetate, producing 1 NADH at each step (Bamworth, 2005; Groff and Gropper, 2000). The acetate enters normal metabolic pools, largely outside the liver, and is activated to acetyl-CoA (at a cost of 2 ATP) and enters the TCA cycle producing 3 NADH, 1 FADH₂ and 1 GTP (Lands and Zakhari, 1991). This pathway (alcohol dehydrogenase (ADH)) is considered the most important for individuals with moderate ethanol intake, although a second pathway, known as the microsomal ethanol oxidising system (MEOS) may be induced in chronic alcoholics (Bamworth, 2005; Lieber, 1988). The equations provided by van Milgen (2002) did not include the two steps from ethanol to acetaldehyde and acetaldehyde to acetate. Two additional equations were thus added to account for these steps based on the NADH, FADH₂ and ATP yields above. Total ATP yields for ethanol were calculated using these two additional equations in conjunction with Equation 16 (acetate to acetyl-CoA) and Equations 3 and 4 (TCA cycle) (van Milgen, 2002). The cost of activation of acetate to acetyl-CoA is included in the ATP yields. Accounting for the

fact that only approximately 90% of ingested ethanol is metabolised (Rubin and Strayer, 2008), the ATP yield was calculated as 10.1 mol ATP/mol EtOH or 218.2 mmol ATP/g EtOH using a molar mass of 46.1 g/mol.

SCFA catabolism

The main substrate for hindgut fermentation is undigested dietary carbohydrate (Cummings, 1996) and in the model it was assumed that all organic matter (OM) fermented in the hindgut produces SCFA as the only product of energetic value to the host. SCFA are rapidly absorbed and subsequently metabolised by the body, with the main SCFA being acetic, propionic and butyric acids. Branched chain fatty acids produced from the fermentation of protein were not considered. Equations 16 – 18 in van Milgen (2002) were used to determine the respective ATP yields from the three major SCFA (Table 3).

Proportionally, acetic acid is always the predominant SCFA produced during hindgut fermentation in humans and is rapidly converted to acetyl-CoA. The minor pathway of propionic acid conversion to acetyl-CoA was not considered and it was assumed that propionic acid is converted to glucose in the liver (McDonald *et al.*, 1995). Butyric acid, like acetic acid, is converted to acetyl-CoA.

Lactic acid is also produced as an end product of microbial fermentation, primarily in the stomach and upper-tract, while SCFA remain the primary product of fermentation in the hindgut (Serena *et al.*, 2007). Any fermentation occurring before the terminal ileum was ignored. However, the lactic acid produced as a result of the fermentation of starch / sugars in the upper-tract is implicitly accounted for as part of the digested starch / sugars (assumed to be absorbed as glucose).

Prediction of uptake of substrates using a dual *in vivo* / *in vitro* digestibility assay

The model described here details the ATP yields (ATP_a) from nutrients once they have been absorbed and are available for cellular metabolism. However, it is not correct to assume that nutrients are absorbed in the same proportions as present in the diet or that a generic digestibility value applies to all nutrients or diets. The digestibility and hindgut fermentability of dietary components are difficult to determine accurately in humans *in vivo*. We have developed and validated a dual *in vivo* – *in vitro* digestibility assay to predict energy absorbed in the upper and lower digestive tract of humans (Coles *et al.*, 2011) (see chapters V - VII). The model is based on an *in vivo* measurement of upper-tract (mouth to terminal ileum) nutrient uptake using the growing rat or pig as an animal model for digestion in humans coupled with *in vitro* measurement of hindgut fermentation based on an incubation assay using a human faecal inoculum. The dual digestion model has been fully described elsewhere (Coles *et al.*, 2011) (see chapters V - VII) and has been shown to accurately predict organic matter digestibility in humans over a wide range of diets (see Chapter VII). The dual digestion model predicts the quantities of AA, FA, starch (ST) and sugars (SU) absorbed in the upper-tract and the SCFA produced during *in vitro* hindgut fermentation of the ileal substrate. Equations describing ATP generation from absorbed nutrients, such as those described here, can be linked with the outputs from the dual digestibility assay to provide a detailed estimate of ATP yield, whilst accounting for the specific digestibility of a food or diet (**Figure 1**). The overall predictive model described here, combining the *in vivo* and *in vitro* digestion assays and the equations to predict ATP is referred to as the ‘Combined Model’.

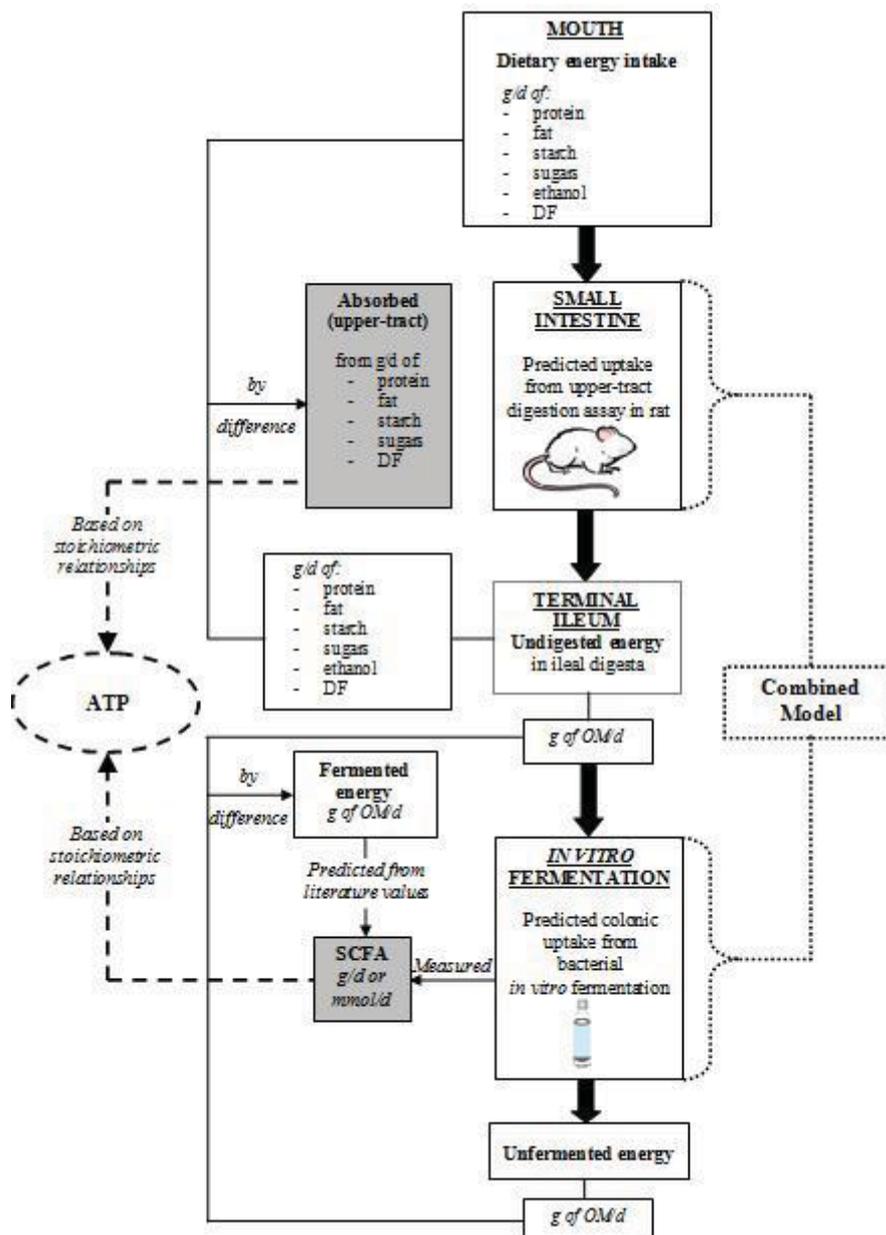


Figure 1. Schematic diagram of a Combined Model of digestion (combination of *in vivo* and *in vitro* digestion assays and stoichiometric chemical relationships) over the total tract, showing inputs and outputs at each stage. Model can be used to predict absorbed energy (g/d) in the upper-tract in terms of protein / fat / starch / sugars / ethanol / dietary fibre. The stoichiometry relates to the hypothetical state whereby energy intake \leq energy expenditure and all absorbed nutrients are catabolised. DF, dietary fibre; OM, organic matter; SCFA, short chain fatty acids.

Chemical analysis of the ingested diet allows quantification of ST, SU, FA, AA (ST_d , SU_d , FA_d and AA_d respectively). Chemical analysis of ileal digesta sampled from growing rats or pigs (*in vivo* assay) allows quantification of the same nutrients at the ileal level (ST_i , SU_i , FA_i and AA_i) and calculation of the absorbed quantities of each of these nutrients. The predicted absorbed quantities of nutrients and the predicted SCFA produced during *in vitro* fermentation can be used to derive the total ATP yield (ATP_a) resulting from catabolism of all nutrients absorbed across the digestive tract:

Calculation of ATP yields from nutrient catabolism

ATP yield from starch and sugars (ATP_{ST+SU})

The ATP yield from monosaccharide (ATP_{ST+SU} , mmol) was based on the quantities of ST and SU digested as given by Equation 1.

$$ATP_{ST+SU} = ((ST_d + SU_d) - (SU_i + ST_i)) \times \text{ATP yield for glucose (mmol ATP) / g GL} \quad (\text{Eq. 1})$$

where ST and SU are given in glucose equivalents. ST_d and SU_d are the ST and SU present in the diet (g / dry matter diet) respectively, ST_i and SU_i are the ST and SU present in ileal digesta (g / dry matter diet) respectively, and 'mmol ATP / g GL' is the ATP (mmol) yielded per gram of glucose.

ATP yield from fatty acids (ATP_{FA})

The ATP yield from FA (ATP_{FA} , mmol) was calculated according to Equation 2:

$$ATP_{FA} = \frac{179.1 \times n \text{ mol FA}}{3} + \sum ((FA_d - FA_i) \times \text{ATP yield for FA (mmol ATP) / g FA}) \quad (\text{Eq. 2})$$

where 179.1 is the ATP yield (mmol ATP / g glycerol) from catabolising glycerol (see Table 3), ' n mol FA' is the sum of the moles of all fatty acids, FA_d is the FA present in the diet (kg / dry matter diet) and FA_i is the FA present in the ileal digesta (kg / dry matter diet).

It was assumed that the majority of dietary FA (12 C and above) available for absorption originate from triacylglycerol (TAG) in the diet, rather than as non-esterified fatty acids (NEFA). Therefore, when calculating the ATP yield (mmol /g FA), the 'bound' molecular weight for each FA was used rather than the 'free' molecular weight. The bound molecular weight was taken as the free molecular weight for the FA less 17 g / mol (18 Da / mol).

ATP yield from amino acids (ATP_{AA})

ATP from AA was calculated using the ATP yield from the sum of all AA in dietary (AA_d , g) and ileal (AA_i , g) samples:

$$ATP_{AA} = \sum((AA_d - AA_i) \times ATP \text{ yield for AA (mmol ATP)/g AA}) \quad (\text{Eq. 3})$$

ATP yield from SCFA (ATP_{SCFA})

The sum of ATP generated from the catabolism of the individual SCFA (ATP_{SCFA} , mmol) produced as a result of hindgut fermentation of OM was used to predict the energy contribution from hindgut fermentation. Individual SCFA were limited to acetic acid, propionic acid and butyric acid, with ATP yields for each SCFA defined in terms of mmol ATP / g SCFA produced, as quantified in Table 3.

$$ATP_{SCFA} = \sum(g \text{ SCFA produced} \times ATP \text{ yield for SCFA (mmol ATP)/g SCFA}) \quad (\text{Eq. 4})$$

Quantification of the three primary SCFA produced during *in vitro* fermentation may also be predicted based on the OM fermented using the difference between OM in the ileal digesta (OM_i) and unfermented OM at the end of *in vitro* incubation (OM_{uf}).

$$ATP_{SCFA} = ((OM_i - OM_{uf}) \times mmol \text{ ATP/g OM fermented}) \quad (\text{Eq. 5})$$

A molar ratio of 60:20:18 for acetic : propionic : butyric acid was assumed for colonic fermentation in humans (Cummings and Macfarlane, 1997). This ratio is supported by

theoretical and experimental findings and equates to a yield of 63 g SCFA per 100 g of carbohydrate fermented (Cummings and Macfarlane, 1997), similar to the value of 61 g SCFA per 100 g reported by Livesey and Elia (1995). An ATP yield of 10.4 mol ATP/mol SCFA produced is equated to 101.7 mmol ATP/g carbohydrate fermented and applied generically to all OM fermented in the hindgut. These figures are based on the molar ratios of the respective SCFA given above (60:20:18) and a value of 63 g SCFA per 100g of carbohydrate fermented. Molar masses used for acetic, propionic and butyric acid are given in Table 3.

$$ATP_{SCFA} = ((OM_i - OM_{uf}) \times 101.7 \text{ mmol ATP/g OM fermented}) \quad (\text{Eq. 6})$$

where OM_i is OM in the ileal digesta (g) and OM_{uf} is the unfermented OM at the end of incubation (g).

The indirect route of energy supply to the body (via SCFA produced from bacterial fermentation) in the hindgut results in some inefficiency and loss of potential ATP compared to upper-tract digestion, through a loss of energy from the heat of fermentation. Consequently, ATP yields from nutrients absorbed in the upper-tract (160 mmol ATP / g GL; 62 – 229 mmol ATP /g AA, 338 – 413 mmol ATP / g FA and 218 mmol ATP / g EtOH), are generally greater than the ATP yield from hindgut fermentation of OM (110 mmol /g fermented OM).

Discussion

A model is described for predicting ATP yields for foods consumed by humans, based on the predicted absorbed nutrient content of foods (dual *in vivo* / *in vitro* digestibility assay) and derived values for ATP yields for each nutrient. The model is designed specifically for an adult human under negative energy balance so it is applicable to the design and evaluation of weight-loss food products. In such a scenario it is assumed that all absorbed nutrients will ultimately be catabolised to meet the body's energy requirements. The model does not account for the temporary storage of nutrients after a main meal, although it is recognised that ATP values derived here are affected by the physiological state (fed, post-absorptive, fasting, starvation) determining the preferred metabolic substrate to meet energy requirements of the body. The assumptions made here are general and do not include extreme scenarios (trauma, sepsis, starvation).

Energetic efficiency

ATP yields derived here for each nutrient may be translated into kJ equivalents. The ΔG for the hydrolysis of ATP is most commonly stated as 7.3 kcal / mol ATP under standard conditions. However, *in vivo* conditions may not be representative of standard conditions and so there is some possible degree of error in this figure. The free hydrolysis of ATP under typical cellular conditions, and in muscle, is approximately -59.8 kJ/mol (-14.3 kcal/mol) (Flatt and Tremblay, 1998; Stryer *et al.*, 2002). This value was used here for calculating the calorific equivalent of ATP yields. Energetic efficiency values (%) as MJ / MJ of GE were calculated for selected nutrients as determined using the present model ATP_a values and compared with values reported in the literature (Boisen and Verstegen, 2000) (**Table 4**). The present values were not dissimilar to those reported by Boisen and Verstegen (2000), but it would appear that in most cases currently reported values (Boisen and Verstegen, 2000) of ATP_a may be overestimated.

Table 4

Gross Energy¹, Physiological Energy² and Energetic Efficiency³ values of selected nutrients

Nutrient	Gross Energy ¹ , MJ / kg	Physiological Energy ² , MJ / kg	Energetic Efficiency ³ , %	
			Model value	Literature value ¹
Glucose	15.6	10.7	68	67
Lys	23.5	11.7	50	49
Met	18.6	8.6	46	34
Cys	18.4	6.4	35	37
Thr	17.2	9.0	53	53
Trp	27.5	9.9	36	44
Ile	27.6	15.1	55	60
Leu	27.6	14.6	53	58
Val	25.0	13.4	53	59
Phe	28.2	11.0	39	44
Tyr	24.9	10.7	43	49
His	21.7	6.6	30	32
Arg	21.4	7.4	34	40
Ser	13.8	6.0	44	47
Gly	12.9	4.7	36	38
Ala	18.2	9.2	51	51
Glu	17.6	8.0	45	49
Gln	12.9	7.0	55	38
Pro	23.7	12.6	53	55
Asp	12.1	5.7	47	55
Asn	14.6	4.7	32	40
Glycerol	18.0	11.1	62	63
Acetic Acid	14.6	7.0	48	59
Propionic Acid	20.8	10.4	50	61
Butyric Acid	24.9	13.5	54	64
Lauric (12:0)	36.4	23.1	64	67
Palmitic (16:0)	39.1	23.7	61	67
Stearic (18:0)	39.9	24.1	60	67
Oleic (18:1)	39.7	24.0	60	67
Ethanol	29.7	13.0	44	61

¹ As summarised by Boisen and Verstegen (2000)

² Calculated based on ATP_a for given nutrient using 'in chain' molecular weights for glucose, amino acids and long chain fatty acids. See Table 1 for values of ATP_a.

³ Energetic Efficiency (%) = Physiological Energy (MJ / kg) / Gross Energy (MJ / kg) x 100 / 1

Urea recycling

The fate of endogenous urea entering the large intestine is that it is converted to ammonia through bacterial degradation, which is then partly absorbed (most likely through passive non-ionic diffusion) into the portal vein and transported in the form of NH_4^+ to the liver where the majority is metabolised to urea via the urea cycle (Wrong and Vince, 1984). It was assumed that there is no cost for the absorption and transport of the ammonia from the large intestine. The conversion of ammonia back to urea via the urea cycle is at a cost of 3 ATP, although this was not factored into the model. The extent of urea recycling in humans is difficult to predict and is known to be affected by dietary protein intake (Danielsen and Jackson, 1992). However, most estimates lie within the range of 20 to 30% (Groff and Gropper, 2000; Walser and Bodenlos, 1959) and here, a figure of 25% was adopted and incorporated into the calculation of ATP_a . The accuracy of the actual figure used would seem of limited importance given that the ATP cost of urea excretion is small compared to the ATP produced via AA catabolism. This conclusion is supported by Livesey (1984) who examined the numerical effect on ATP yields of different assumptions on the extent of urea recycling and concluded that this source of error was small.

Extent of gluconeogenesis from amino acids

It is unknown to what extent free AA undergo gluconeogenesis in the liver. While it has been assumed here that all free AA undergo direct oxidation, some certainly will proceed via the gluconeogenesis route. Flatt and Tremblay (1998) base their calculations on 35% of the free AA being used for gluconeogenesis and the remainder being deaminated and catabolised via the TCA cycle. However, Lindsay (1976) states that approximately 90% of the AA catabolised by monogastric animals proceeds via oxidation. The actual scenario will vary depending on the fed/fasted state of the individual. The liver does not instantaneously switch from the gluconeogenic state to the glycolytic state in the early fed state (Salway, 2004). Rather, the gluconeogenic state persists for some time after a meal. The ability of an AA to act as a substrate for

gluconeogenesis is limited to those glucogenic AA, with alanine, glutamate and aspartate being quantitatively the most important AA for gluconeogenesis (Brosnan, 2003; Garlick and Reeds, 2000; Rolfe and Brown, 1997). The GL produced from AA catabolism via gluconeogenesis (in most cases 0.5 mol GL) enters the TCA cycle to yield 28.9 mol ATP / mol GL. However, the number of moles of ATP used to produce this GL varies significantly among the AA. For methionine, there is an overall ATP gain of 19.8 mol ATP, whereas for histidine there is an ATP cost of 7.9 mol ATP. The net ATP_a gains (including costs for urea synthesis and excretion) for producing 0.5 mol GL from the main glucogenic AA are: alanine, -5.2 mol ATP; glutamate, 2.6 mol ATP; aspartate, -4 mol ATP. If these are added to the ATP gain from 0.5 mol GL (14.4 mol ATP), the overall ATP_a for each mole of these AA undergoing gluconeogenesis can be estimated at 2.3, 1.0 and 1.1 mol ATP less for each mole of alanine, glutamate and aspartate respectively, than if they were directly oxidised via the TCA cycle to produce ATP_a (Table 2). Although Livesey (1984) suggests that the choice of catabolic pathway may be an important consideration for dietary protein, the results presented here are more in agreement with Birkett and de Lange (2001b) who calculated little difference in overall ATP yields for the two catabolic pathways for AA (providing GL is non-limiting). Birkett and de Lange also undertook model simulations using exclusively either the gluconeogenic pathway or the direct oxidation pathway for AA catabolism and found very small differences in retained energy at the whole animal level, thereby confirming that the choice of pathway appears to be unimportant (Birkett and de Lange, 2001b). The presently described model can be altered to include different assumptions about alternative pathways and the different scenarios simulated and evaluated.

Net ATP yield (ATP_{net})

A model to quantify the net daily cytoplasmic ATP yields from ingested food is illustrated (**Figure 2**). ATP_a is an estimate of the ATP produced from the catabolism of absorbed nutrients less the energetic cost of catabolism as described earlier (see section on 'ATP yield (ATP_a)'), which includes the cost of urea synthesis and excretion and the cost of activation. As defined here, ATP_a does not take into account the energy expended to ingest and digest these nutrients (ATP_d), nor the costs of

absorption / transportation to the liver (ATP_t), considered here to be the primary site of catabolism. These two energetic costs (Table 1), which in part vary with dietary chemical composition, are deducted from the available ATP produced (ATP_a) to give the net ATP yield (ATP_{net}) (Equation 7).

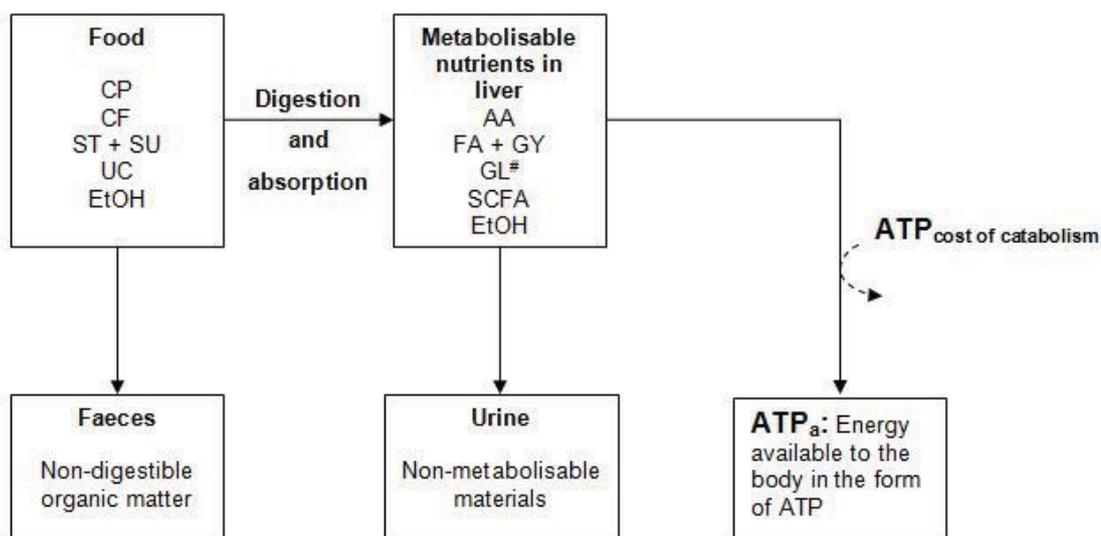


Figure 2. Overview of model (adapted from Birkett and de Lange (2001a)). AA, amino acids; ATP_a , energy yielded in terms of ATP from the metabolism of dietary nutrients; CF, crude fat; CP, crude protein; EtOH, ethanol; FA, fatty acids; GL, glucose; GY, glycerol; SCFA, short chain fatty acids; ST, starch; SU, sugar; UC unavailable carbohydrate (carbohydrate not digested in upper digestive tract). #GL includes all monosaccharides (galactose, fructose etc. See text).

$$ATP_{net} = ATP_a - ATP_d - ATP_t \quad (Eq. 7)$$

The model considers only “first pass” metabolism and allows calculation of the net yield of ATP (ATP_{net}) available to the body from dietary intake, after accounting for ATP_d and ATP_t .

In the case of the Combined Model, ATP_a can be calculated as the total of the ATP yields from equations 1 – 6 and the overall equation for determining the net ATP yield (ATP_{net}) is given as:

$$ATP_{net} = ATP_{ST+SU} + ATP_{FA} + ATP_{AA} + ATP_{EtOH} + ATP_{SCFA} - ATP_d - ATP_t \quad (Eq. 8)$$

ATP costs (ATP_d and ATP_t)

Digestion (ATP_d)

ATP_d is a function of the digestible OM intake and is the energetic cost associated with the intake and digestion of CP, CF, ST, SU, UC and EtOH. The ingested OM that is digested and fermented by the body (OM_{D+F}) was used as a basis to calculate ATP_d (mol ATP / kg OM_{D+F}). OM_{D+F} was calculated as the difference between OM intake (OM_i) and unfermented OM (OM_{uf}). A value of 28 mol ATP/kg faecal digestible dry matter (DM) was used by Birkett and de Lange (2001a) and was applied here to give an ATP_d value of 28 mol ATP / kg OM_{D+F}.

Transportation (ATP_t)

ATP_t refers to the energetic cost of absorbing, transporting and handling each energy-yielding nutrient. This cost varies depending on the substrate (Table 1), but in all cases was defined as the cost to transfer the nutrient from the intestinal lumen to the major site of catabolism, which was assumed to be the liver. Active transport requires energetic input to absorb the nutrient from the intestinal lumen through the enterocyte, while passive diffusion is a non-energy requiring process (Mercadante, 2006).

Glucose is absorbed by both passive diffusion and active transport systems and transported to the liver at an estimated cost of 0.5 mol ATP / mol GL (Blaxter, 1989; Flatt and Tremblay, 1998). While the different energy costs of absorption of the different monosaccharides may affect cytoplasmic ATP yield, this was not accounted for here and the absorption and transport costs for all monosaccharides were taken as being equivalent to glucose.

It was assumed that digested protein is absorbed and transported to the liver as free amino acids at an estimated cost of 0.5 mol ATP / mol AA (Flatt and Tremblay, 1998).

The model assumes that all absorbed AA are transported to the liver and catabolised, and protein turnover and its energetic costs were not considered. The amount of protein returned to the body to replace losses will vary considerably depending on a number of factors including protein intake, body mass (lean / obese), nutritional status (weight losing / weight stable) and fed / fasted state (Fearon *et al.*, 1988; Waterlow, 2006). Whole-body protein breakdown has been estimated to be in the range of 2.2 – 4.6 g/kg/d (Fearon *et al.*, 1988). The use of absorbed AA to meet AA maintenance needs was ignored in the present version of the model but can be readily incorporated.

Ethanol is absorbed by passive diffusion with no ATP costs involved in its transport to the liver.

In the case of lipids, the absorption process depends on the carbon-chain length. Short and medium chain FA are absorbed intact by passive diffusion at no energetic cost and like GL, AA and EtOH, these are transported to the liver via the portal vein (Nelson and Ackman, 1988). FA of carbon-chain length less than 8 or 10 may be absorbed directly from the stomach intact into the venous circulation (Nelson and Ackman, 1988). The uptake and transport of SCFA from bacterial fermentation in the colon is assumed to be similar to those SCFA in the upper-tract in that there is no energetic cost involved in their uptake and transport (Mercadante, 2006). FA of carbon-chain length of C10 to C14 may be absorbed intact from the small intestine (Nelson and Ackman, 1988). FA of carbon-chain length longer than C14 are absorbed from the intestinal lumen into the mucosal cell as two NEFA and a 2-monoglyceride by passive diffusion across the microvillus membrane at no energetic cost (Johnston and Borgstrom, 1964). These components are then reconstituted to TAG in the endoplasmic reticulum (ER) in the enterocytes and formed into chylomicrons which then enter the blood via the lymphatic system. The process of reesterification in the ER is an energy requiring process. Flatt and Tremblay (1998) quote a value of 2.3 mol ATP per TAG reesterified in the adipose tissue and the liver and the same value was assumed here for TAG reesterified in the ER. Although some FA may be absorbed intact and will not require reconstituting, it was assumed that TAG are reesterified in the ER at a cost of 0.75 mol

ATP / FA for all absorbed FA, but not including SCFA. FA reach the liver as chylomicron remnants via the general circulation (Nelson and Ackman, 1988). The model assumes that all absorbed dietary FA are ultimately catabolised by the liver, for a person in a state of weight-loss. The model does not consider any energetic costs once the absorbed FA reach the liver, such as the cost of reesterifying the NEFA by the liver and reexporting the resulting TAG as lipoproteins.

Accuracy of ATP_{net}

The costs ATP_d and ATP_t are not known with a high degree of accuracy. In contrast, ATP yields are based on known stoichiometry and as such, can be determined accurately. Consequently, ATP_a can be predicted with accuracy and is likely to be estimated more accurately than ATP_{net} , which includes the energetic costs of ATP_d and ATP_t . In spite of this, it needs to be borne in mind that ATP_d and ATP_t are relatively smaller energetic transactions (compared to the yield of ATP from catabolism, ATP_a), and that errors in estimating these energy costs are of less significance overall. In the case of ATP_d , while the exact cost of those processes relating to digestion is not known precisely, the enthalpy of the enzymic hydrolyses of the three main energy yielding nutrients (protein, lipid and polysaccharides) in the lumen of the gut is estimated to be only 0.1 - 0.2% of the energy of the substrates hydrolysed (Blaxter, 1989). This heat, along with heat produced during the other metabolic processes related to the digestion, absorption and assimilation of nutrients as a result of meal feeding is that broadly referred to as dietary induced thermogenesis (DIT). The exact causes of DIT are still not entirely understood (McCue, 2006) and it is therefore difficult to quantify DIT. The model proposed here, however, accounts for the energetic costs of digestion, absorption, transport, the production and excretion of urea from AA catabolism and those costs inherently necessary as part of nutrient catabolism, such as activation. Heat produced from these processes as a result of energy expended to digest and metabolise the potential energy from nutrient intake is therefore a component of the model. Processes such as protein turnover and glycogen production are not incorporated in the model, so any ATP used and consequently heat produced as a result of such processes is not considered.

P/O ratios

The most significant source of error in the model is likely limited to the selection of the cellular P/O ratio used to calculate ATP yields (ATP_a). The P/O ratio describes the ATP produced per oxygen atom reduced by the electron transport chain during oxidative phosphorylation. Historically, it was assumed that these ratios were integers (3 for NADH and 2 for $FADH_2$) (Hinkle, 2005). More recently, with the advance of chemiosmotic theory, non-integer values (2.31 for NADH and 1.38 for $FADH_2$) have been derived. Brand (2005) discusses these ratios in detail, but briefly, the P/O ratio is based on the proton/O ratio and the proton/ATP ratio. The proton/O ratio describes the number of protons (currently believed to be 10 for NADH and 6 for $FADH_2$) that are translocated across the mitochondrial membrane by the electron transport complexes as a consequence of the oxidation of 1 NADH (or $FADH_2$) and the concomitant consumption of 1 oxygen atom. The relocated protons provide the proton gradient that powers the ATP synthase to produce 1 ATP (currently believed to be 3.3 protons for every 1 ATP synthesised) and the adenine nucleotide translocase to transport that ATP from the mitochondrion (currently believed to be 1 for every ATP transported). Overall, for every NADH oxidised 10 protons are translocated, while 4.33 protons are required for every ATP produced. Consequently, 2.31 ($10/4.33$) ATP molecules are synthesised for every NADH. Similarly, for $FADH_2$ 6 protons are translocated, giving a ratio of 1.38 ($6/4.33$).

However, these theoretical P/O ratios may still be higher than what occurs *in vivo*. Slip reactions, proton leakage and the activity of uncoupling proteins may not be fully accounted for by the accepted P/O ratios. Slip may occur when the proton pumps are not perfectly coupled, resulting in fewer protons pumped across the mitochondrial membrane and therefore less ATP synthesised, effectively lowering the P/O ratio (Brand, 2005). Although it would seem that slip does not occur under normal physiological cell conditions, there is still some uncertainty around this (Brand, 2005; Brown, 1992; Kadenbach, 2003; Murphy, 1989). The phenomenon of proton leakage, where protons leak back across the coupling membrane and are therefore unavailable for ATP synthesis, is more widely accepted. Several *in vitro* studies have shown that

proton leakage occurs not only in isolated mitochondria, but also in intact cells, accounting for around 20% of the total cellular respiration rate, dependent largely on the species and type of cell (Brand, 2005). It has been calculated that only 60% of an hepatocyte's total oxygen consumption may be used for ATP synthesis, effectively lowering the P/O ratio considerably (Brand, 2005). However, it is still uncertain as to whether *in vitro* observations are completely transferable to the *in vivo* situation and to what extent proton leakage should be accounted for when predicting the P/O ratio (Marcinek *et al.*, 2004; Rolfe *et al.*, 1999). The specialised uncoupling proteins (UCP) present in some mitochondria are most probably used for transporting anionic fatty acids and may create proton permeability by allowing H⁺ pumped out of the mitochondria by electron transport to flow back in (Groff and Gropper, 2000; Hinkle, 2005). While UCP may be important with regard to physiological energy metabolism, they are not seen as affecting mechanistic P/O ratios, at least in liver and heart (Hinkle, 2005). Overall, corrections to the P/O ratios on account of uncoupling, including slip, proton leakage and uncoupling proteins appear to be small or of limited relevance (Hinkle, 2005), although there is still a need to confirm the extent of protein leakage *in vivo*.

Conclusion

Previous models proposed for predicting the energetic value of foods in terms of ATP are most often based on P/O ratios that are now viewed as outdated by cell bioenergeticists. In combination with the *in vivo* – *in vitro* digestibility assays, the model presented here allows a diet to be defined in terms of potential ATP yields based on currently accepted P/O ratios and the experimentally determined digestibility of the specific diet across the total human digestive tract. The accuracy of the model could potentially be improved if the extent of proton leakage *in vivo* and the cost of digestion (ATP_d) could be determined with greater certainty. The model is a valuable tool for the research and development of foods such as specialised weight-loss products that need to provide accurate known quantities of dietary energy for subjects undergoing weight loss.

Literature cited

- Armstrong, D. G. (1969). Cell bioenergetics and metabolism. In W. Lenkheit, K. Breirem & E. Crasemann (Eds.), *Handbuch der Tierernaehrung (Handbook of Animal Nutrition)* (pp. 385-414). Hamburg: Paul Parey.
- Bamworth, C. W. (2005). Beer, carbohydrates and diet. *Journal of the Institute of Brewing*, 111(3), 259-264.
- Birkett, S. & de Lange, K. (2001a). Calibration of a nutrient flow model of energy utilization in growing pigs. *British Journal of Nutrition*, 86, 675-689.
- Birkett, S. & de Lange, K. (2001b). A computational framework for a nutrient flow representation of energy utilization by growing monogastric animals. *British Journal of Nutrition*, 86, 661-674.
- Blaxter, K. L. (1989). *Energy Metabolism in Animals and Man*. Cambridge, United Kingdom: Cambridge University Press.
- Boisen, S. & Verstegen, M. W. A. (2000). Developments in the measurement of the energy content of feeds and energy utilisation in animals. In P. J. Moughan, M. W. A. Verstegen & M. I. Visser-Reyneveld (Eds.), *Feed Evaluation, Principles and Practice* (pp. 57-76). Wageningen: Wageningen Pers.
- Brand, M. D. (2005). *The efficiency and plasticity of mitochondrial energy transduction*. Paper presented at the Keilin Memorial Lecture, Glasgow.
- Brosnan, J. T. (2003). Interorgan amino acid transport and its regulation. *Journal of Nutrition*, 133(6), 2068S-2072S.
- Brown, G. C. (1992). The leaks and slips of bioenergetic membranes. *FASEB Journal*, 6, 2961-2965.
- Chevalier, S., Burgess, S. C., Malloy, C. R., Gougeon, R. & Marliss, E. B. (2006). The greater contribution of gluconeogenesis to glucose production in obesity is related to increased whole-body protein catabolism. *Diabetes*, 55(3), 675-681.
- Coles, L. T., Moughan, P. J., Awati, A. & Darragh, A. J. (2011). Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter. *Food Chemistry*, In Press.

-
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H. & Macfarlane, G. T. (1997). Role of intestinal bacteria in nutrient metabolism. *Journal of Parenteral and Enteral Nutrition*, 21(6), 357-365.
- Danielsen, M. & Jackson, A. A. (1992). Limits of adaptation to a low protein diet in normal man: urea kinetics. *Clinical Science (London)*, 83(1), 103-108.
- Elia, M. & Livesey, G. (1988). Theory and validity of indirect calorimetry during net lipid synthesis. *American Journal of Clinical Nutrition*, 47, 591-607.
- Fearon, K. C. H., Hansell, D. T., Preston, T., Plumb, J. A., Davies, J., Shapiro, D., Shenkin, A., Caiman, K. C. & Burns, H. J. G. (1988). Influence of whole body protein turnover rate on resting energy expenditure in patients with cancer. *Cancer Research*, 48, 2590-2595.
- Flatt, J.-P. & Tremblay, A. (1998). Energy expenditure and substrate oxidation. In G. A. Bray, C. Bouchard & W. P. T. James (Eds.), *Handbook of Obesity* (1st ed., pp. 513-537). New York: Marcel Dekker.
- Garlick, P. J. & Reeds, P. J. (2000). Proteins. In J. S. Garrow, W. P. T. James & A. Raplh (Eds.), *Human Nutrition and Dietetics* (10th ed.). London: Churchill Livingstone.
- Gerrits, W. J. J., Dijkstra, J. & France, J. (1997). Description of a model integrating protein and energy metabolism in preruminant calves. *Journal of Nutrition*, 127, 1229-1242.
- Groff, J. L. & Gropper, S. S. (2000). *Advanced Nutrition and Human Metabolism* (3rd ed.). Belmont, CA, USA: Wadsworth.
- Hinkle, P. C. (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta*, 1706, 1-11.
- Houston, M. E. (2006). *Biochemistry primer for exercise science* (3rd ed.). Champaign, IL: Human Kinetics.
- Johnston, J. M. & Borgstrom, B. (1964). The intestinal absorption and metabolism of micellar solutions of lipids. *Biochimica et Biophysica Acta*, 84, 412-423.
- Kadenbach, B. (2003). Intrinsic and extrinsic uncoupling of oxidative phosphorylation. *Biochimica et Biophysica Acta*, 1604(2), 77-94.

-
- Lands, W. E. M. & Zakhari, S. (1991). The case of the missing calories. *American Journal of Clinical Nutrition*, 54, 47-48.
- Langran, M., Moran, B. J., Murphy, J. L., & Jackson, A. A. (1992). Adaptation to a diet low in protein: effect of complex carbohydrate upon urea kinetics in normal man. *Clinical Science (London)*, 82, 191-198.
- Levitt, M. D., Li, R., Demaster, E. G., Elson, M., Furne, J. & Levitt, D. G. (1997). Use of measurements of ethanol absorption from stomach and intestine to assess ethanol metabolism. *American Journal of Physiology*, 273, G951-G957.
- Lieber, C. S. (1988). The influence of alcohol on nutritional status. *Nutrition Reviews*, 46, 241-254.
- Lindsay, D. B. (1976). Amino acids as sources of energy. In D. J. Cole, K. N. Boorman, P. J. Buttery, D. Lewis, R. J. Neale & H. Swan (Eds.), *Protein Metabolism and Nutrition*, EAAP Publication no 16 (pp. 183-195). London: Butterworths.
- Livesey, G. (1984). The energy equivalents of ATP and the energy values of food proteins and fats. *British Journal of Nutrition*, 51, 15-28.
- Livesey, G. (1995). Metabolizable energy of macronutrients. *American Journal of Clinical Nutrition*, 62, 1135S-1142S.
- Livesey, G. & Elia, M. (1995). Short chain fatty acids as an energy source in the colon: metabolism and clinical implications. In J. H. Cummings, J. L. Rombeau & T. Sakata (Eds.), *Physiological and Clinical Aspects of Short Chain Fatty Acids* (pp. 427-481). Cambridge, United Kingdom: Cambridge University Press.
- Long, C. L., Jeevanadam, M., & Kinney, J. M. (1978). Metabolism and recycling of urea in man. *American Journal of Clinical Nutrition*, 31(8), 1367-1382.
- Marcinek, D. J., Schenkman, K. A., Ciesielski, W. A. & Conley, K. E. (2004). Mitochondrial uncoupling *in vivo* in mouse skeletal muscle. *American Journal of Physiology*, 286, C457-C463.
- Martin, A. K. & Blaxter, K. L. (1965). The energy cost of urea synthesis in sheep. In K. L. Blaxter (Ed.), *Energy Metabolism* (pp. 83-91). London, UK: Academic Press.
- McCue, M. D. (2006). Specific dynamic action: A century of investigation. *Comparative Biochemistry and Physiology*, 144, 381-394.

-
- McDonald, P., Greenhalgh, J. F. D., Edwards, R. A. & Morgan, C. A. (1995). *Animal Nutrition* (5th ed.). New York: Longmans.
- Mercadante, S. (2006). Diarrhea, Malabsorption, and Constipation. In A. M. Berger, J. L. Shuster & J. H. V. Roenn (Eds.), *Principles and practice of palliative care and supportive oncology* (3rd ed., pp. 923). Philadelphia, PA: Lippincott Williams & Wilkins.
- Moran, B. J. & Jackson, A. A. (1990). ¹⁵N-urea metabolism in the functioning human colon: luminal hydrolysis and mucosal permeability. *Gut*, *31*, 454-457.
- Moughan, P. J., Coles, L. T. & Rutherford, S. M. (2009). *Describing dietary energy – do we need a new approach?* Paper presented at the Joint Annual Scientific Meeting of the Nutrition Society of Australia & Nutrition Society of New Zealand
- Murphy, M. P. (1989). Slip and leak in mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta*, *977*, 123-141.
- Nelson, G. J. & Ackman, R. G. (1988). Absorption and transport of fat in mammals with emphasis on n-3 polyunsaturated fatty acids. *Lipids*, *23*, 1005-1014.
- Rolfe, D. F. S. & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological Reviews*, *77*(3), 731-759.
- Rolfe, D. F. S., Newman, J. M. B., Buckingham, J. A., Clark, M. G. & Brand, M. D. (1999). Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver to SMR. *American Journal of Physiology*, *276*, C692-C699.
- Rubin, R. & Strayer, D. S. (2008). *Rubin's Pathology: Clinicopathologic Foundations of Medicine* (5th ed.). Philadelphia: Lippincott Williams and Wilkins.
- Salway, J. G. (2004). *Metabolism at a Glance* (3rd ed.). Oxford, England: Blackwell Publishing.
- Sanford, P. A. (1992). *Digestive System Physiology* (2nd ed.). Suffolk, Great Britain: Edward Arnold.

-
- Serena, A., Jørgensen, H. & Bach Knudsen, K. E. (2007). The absorption of lactic acid is more synchronized with the absorption of glucose than with the absorption of short-chain fatty acids - A study with sows fed diets varying in dietary fibre. *Livestock Science*, 109(1-3), 118-121.
- Stryer, L., Berg, J. M. & Tymoczko, J. L. (2002). *Biochemistry* (5th ed.). New York: W.H. Freeman and Co. Ltd.
- van Milgen, J. (2002). Modeling biochemical aspects of energy metabolism in mammals. *Journal of Nutrition*, 132, 3195-3202.
- Walser, M. & Bodenlos, L. J. (1959). Urea metabolism in man. *Journal of Clinical Investigation*, 38, 1617-1626.
- Waterlow, J. C. (2006). *Protein turnover*. Wallingford, United Kingdom: CABI.
- Wrong, O. M. & Vince, A. (1984). Urea and ammonia metabolism in the human large intestine. *Proceedings of the Nutrition Society*, 43, 77-86.
- Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, 86, 1649-1656.

CHAPTER IX

Available energy content of three meal replacement formulations determined using a model to predict the cellular ATP yield of absorbed macronutrients in the adult human

The objective of the final experimental study was to demonstrate the practical application of the 'Combined Model' to predict the available (ATP) energy content of weight-loss foods. The uptake of each energy-yielding nutrient in the human was predicted separately in the upper-digestive tract (rat ileal digestibility) and the hindgut (in vitro digestibility assay based on human faecal inoculum) for three commercial meal replacement formulations. A series of predictive equations for estimating net ATP yields (based on stoichiometric relationships) were applied to the predicted nutrient uptakes for each diet.

Abstract

A model has been developed to predict the uptake of energy-yielding nutrients and subsequent physiologically available energy of food at a cellular level (ATP) for an adult human in a state of weight-loss. The objective was to demonstrate the practical application of the model to predict the available energy (ATP) content of weight-loss foods. The uptake of each energy-yielding nutrient in the human was predicted separately in the upper-digestive tract (rat ileal digestibility, $n=11$ rats per diet) and the hindgut (*in vitro* digestibility assay based on human faecal inoculum) for three commercial meal replacement formulations (Diet A a weight-gain supplement and diets B and C weight-loss formulations). A series of predictive equations for estimating net ATP yields (based on stoichiometric relationships) were applied to the predicted nutrient uptakes for each diet. Predicted total tract digestibility of energy varied from 86% (Diet B) to 99% (Diet A). Available energy (ATP energy) was estimated to be 9.6, 8.3 and 7.3 kJ/g for Diets A, B and C, respectively and metabolisable energy (ME) ranged from 17.5 – 18.1, 13.6 – 18.4 and 12.5 – 15.4 kJ/g for Diets A, B and C, respectively depending upon the ME model. Most, but not all of the ME systems ranked the diets in accordance with the predicted available energy (Diet A>B>C). When the energy content of each diet was compared to that of a baseline food (dextrin), some ME models gave considerably different ratios compared to that predicted by the Combined Model (*in vivo* / *in vitro* / stoichiometric). By way of example, for Diet C a ratio of 0.96 (Atwater and FDA models) was found versus 0.75 (Combined Model). The Combined Model may provide a more accurate means of predicting the dietary energy content of foods than some current ME models and has application in the development of specialised weight-loss foods.

Introduction

The current prevalence of obesity in humans with its accompanying health disorders has led to the development of novel foods, many of which are aimed at reducing the available calorie content to assist in maintaining or reducing body weight. Estimation of the energy content of foods (metabolisable energy, ME) currently relies on various factorial or empirical models. There is evidence, however, that such models may not accurately predict the energy content of foods with more extreme nutrient compositions, especially low-fat, high fibre foods (Brown *et al.*, 1998; Zou *et al.*, 2007). The energetic values assigned to protein and dietary fibre in particular vary between individual models and are difficult to define generically due to the diversity in chemical composition and digestibility of these two components (Ferrer-Lorente *et al.*, 2007; Livesey, 1990). It is these same two dietary components that are often included in novel weight-loss products in significant quantities based on their satiating effect (Howarth *et al.*, 2001; Paddon-Jones *et al.*, 2008; Slavin, 2005; Veldhorst *et al.*, 2008) and/or lower metabolic energy yields compared to fat, simple sugars and starch. The generic nature of factorial or empirical models means that they may significantly over- or under-estimate the energy content of novel foods (Livesey, 1995).

It has been suggested that the available energy content of foods could be more accurately quantified in terms of potential ATP generation (physiological energy) (Livesey, 1984; Zou *et al.*, 2007). ATP is the universal currency of energy in the body, and the metabolic pathways that yield ATP from the catabolism of nutrients are well known. However, until recently there has not been consensus on the quantities of ATP yielded from these reactions as a result of varying estimates of the theoretical P/O ratios during cellular metabolism (Brand, 2005). Furthermore, prediction of ATP yields relies on being able to quantify the nutrients available for metabolism based on uptake from the digestive tract. Ideally, nutrient uptake should be determined separately in the upper-tract and the hindgut (Coles *et al.*, 2010), but it is impractical to obtain such measures *in vivo* in humans (Darragh and Hodgkinson, 2000).

Recently, our research group has proposed a model (referred to as the 'Combined Model') to allow quantification of cellular ATP yields in humans based on predictions of the uptake of energy-yielding nutrients from both the upper digestive tract (mouth to ileum) and the hindgut, and mathematical stoichiometric relationships linking quantities of absorbed nutrients to ATP generation (see chapters V - VIII). Nutrient uptake in the human is predicted using a dual *in vivo* / *in vitro* digestibility assay that predicts upper-tract nutrient uptake based on an *in vivo* animal model (rat or pig) and colonic energy yield based on an *in vitro* hindgut digestibility assay utilising a human faecal inoculum.

In the present study, the aim was to apply the Combined Model to (i) predict ileal, hindgut and total tract nutrient uptakes for three meal replacement formulations and (ii) predict the associated ATP yields and costs for each nutrient to determine available energy as ATP. The overall objective was to demonstrate the practical application of the Combined Model to predict net ATP yields (available energy) and to compare the predicted available energy content (kJ) with that determined using a number of published traditional factorial and empirical ME based models. It was particularly pertinent to investigate whether the rankings of the diets, in terms of their abilities to deliver utilisable energy to the body, differed according to method of dietary energy estimation.

Materials and methods

The study sought to obtain estimates for humans of nutrient uptake from the upper-tract (mouth to ileum) and colon, separately, for three different diets. Colonic uptakes were predicted using an *in vitro* hindgut digestibility assay and upper-tract nutrient uptakes were predicted using the growing rat as a model for humans.

Stoichiometric relationships were applied to the predicted nutrient uptakes across the total tract to predict net cellular ATP generated for each of the diets in an adult human consuming food at sub-maintenance energy intakes whereby it can be assumed that all absorbed nutrients are catabolised.

Diets

The experimental diets (referred to herein as diets A, B and C) were based on three commercial meal replacement shake mixtures in powder form. Diet A was based on a weight-gain supplement, whereas diets B and C were based on weight-loss preparations. Vitamins and minerals (supplied by Plant and Food Research Ltd., Palmerston North, New Zealand) were added to each mixture as required for the growing rat (National Research Council, 1995) along with an indigestible marker (titanium dioxide) (**Table 1**). All diet ingredients were accurately weighed and then thoroughly homogenised and the chemical composition of the three diets determined (**Table 2**). Both diets B and C contained high levels of NSP (142 and 132 g/kg respectively) compared to Diet A (22 g/kg). Diet C had a particularly high protein content (382 g/kg) and low fat (14 g/kg) and starch (5 g/kg) contents.

Table 1

Ingredient composition (g/kg air-dry weight) of the three experimental diets

Component	g/kg
Meal replacement formulation (A, B or C)	897.5
Vitamin Mix ¹	50.0
Mineral Mix ²	50.0
Titanium dioxide	2.5

¹Containing the following (g/kg vitamin mix): retinol palmitate, 0.1; cholecalciferol, 0.0005; α -tocopherol acetate, 4; menadione, 0.06; thiamin-HCl, 0.1; riboflavin, 0.14; nicotinic acid, 0.4; calcium pantothenate, 0.4; pyridoxine-HCl, 0.16; biotin, 0.02; folic acid, 0.04; cyanocobalamin, 0.001; myo-inositol, 4; choline chloride, 30; sucrose, 960.6.

²Containing the following (g/kg mineral mix): calcium, 126; chloride, 156; magnesium, 21; phosphorus, 97; potassium, 105; sodium, 39; chromium, 0.039; copper, 0.214; iron, 9; manganese, 2; zinc, 0.964; cobalt, 0.00058; iodine, 0.003; molybdenum 0.003; selenium, 0.003; sucrose, 443.8.

Table 2

Determined chemical composition of the three experimental diets (per kg dry matter¹)

Component	Diet A	Diet B	Diet C
Dry matter, <i>g per kg diet</i>	930.9	936.6	953.8
Organic matter, <i>g</i>	930.2	901.7	904.1
Ash, <i>g</i>	65.0	92.1	91.4
Gross energy, <i>MJ</i>	19.2	18.7	16.8
Crude protein, <i>g</i>	144.4	93.1	382.4
Fat, <i>g</i>	121.2	155.7	14.3
Total CHO ² , <i>g</i>	664.7	652.9	507.5
Starch, <i>g</i>	173.6	77.5	4.7
Sugars, <i>g</i>	399.6	309.6	380.6
NSP			
Total, <i>g</i>	22.1	142.0	132.3
Insoluble, <i>g</i>	7.6	81.0	11.5
Insoluble, % of total NSP	34.5	57.1	8.7
Soluble, <i>g</i>	14.4	60.9	120.8
Soluble, % of total NSP	65.3	42.9	91.3
Titanium dioxide, <i>g</i>	1.7	1.7	1.6
Fatty acids			
SFA			
C12, <i>g</i>	11.6	0.2	0.2
C14, <i>g</i>	5.2	0.4	0.0
C16, <i>g</i>	8.7	10.5	2.2
C18, <i>g</i>	4.0	9.7	0.6
MUFA			
C16:1, <i>g</i>	0.1	0.1	0.1
C18:1 (n-9), <i>g</i>	57.1	112.8	2.2
C18:1 (n-7), <i>g</i>	0.9	2.0	0.2
PUFA			
C18:2, <i>g</i>	22.1	14.8	6.0
C18:3, <i>g</i>	2.3	0.3	0.7
Other FA ³ , <i>g</i>	4.0	4.1	2.6

¹ Reported as per kg dry matter for all diet components, except for dry matter (per kg diet)

² Total CHO (by difference) = 100 - Moisture% - Fat% - Crude Protein% - Ash%

³ Other FA refers to the sum of other unidentified fatty acids not included in the table

CHO, carbohydrates; FA, fatty acids; MUFA, monounsaturated fatty acids; NSP, non-starch polysaccharides; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids

***In vivo* digestibility assay**

Upper-tract nutrient digestibility was determined based on collection of samples of ileal digesta from the growing rat. The ileal digesta were also used as the substrate for the *in vitro* fermentation part of the assay. The study was approved by the Massey University Animal Ethics Committee (PN 06/18).

Animals and housing

A total of 99 Sprague-Dawley female rats (7 wk of age, mean BW 204 g) were obtained from the Small Animal Production Unit (SAPU), Massey University. Animals were individually housed in wire-bottomed stainless steel cages in a temperature- ($22\pm 1^\circ\text{C}$) and humidity- ($55\pm 10\%$) controlled environment with a 12 h light/dark cycle (0600 h – 1800 h) for the duration of the trial. Rats were randomly allocated to cages and the three diets (Diet A, B or C), resulting in 33 rats consuming each diet.

Food was offered *ad libitum* for 10 min per hour between 0800 and 1600 h daily to train rats to feed continuously during every meal and provide a semi-continuous flow of digesta at the terminal ileum on the collection day. Rats had free access to water at all times.

On Day 14, from 4 h after the first hourly meal, animals were randomly selected to be sacrificed by overdose of CO_2 gas. After confirmation of death, neural stimulation was immediately halted by decapitation. The stomach was inspected for evidence of coprophagy, observable by the presence of dark flecks in the stomach contents. The contents of the terminal 20 cm of the ileum were then removed as described by Rutherford and Moughan (2003), rapidly frozen (-20°C) and freeze-dried. After discarding digesta samples from coprophagous rats, samples of ileal digesta from individual rats were pooled within a diet to give sufficient quantities of material for chemical analysis and finely ground (1 mm mesh). This resulted in two composite samples of digesta for Diet A (1 x $n=14$, 1 x $n=13$ animals), three samples for Diet B (3 x $n=11$ animals) and four samples for Diet C (4 x $n=8$ animals).

***In vitro* hindgut digestibility assay**

Ileal digesta obtained from rats consuming the three meal replacement formulations were used as the substrate for the *in vitro* hindgut digestibility assay. Faecal samples for the *in vitro* hindgut fermentation were provided by the same four human donors as used on previous occasions in our laboratory (Coles *et al.*, 2011), under approval from the Massey University Human Ethics Committee (PN 04 /127).

The methodology for the *in vitro* hindgut digestibility assay was based on a modified version of the Edwards *et al.* (1996) method and is described in detail elsewhere (Coles *et al.*, 2011). In brief, an inoculum concentration of 160 g/L, a buffer of pH 7 and an incubation temperature of 37°C were used. Bottles were incubated for 18 h with no shaking during incubation. The faecal inoculum was prepared under strict anaerobic conditions using freshly collected, uncontaminated human faecal samples which were delivered to the laboratory in a pre-warmed container filled with CO₂. Rat ileal digesta were used as the substrate for the hindgut assay with triplicate *in vitro* bottles for each group of pooled rat digesta within a diet. Triplicate blank bottles containing 5 ml of buffer and 5 ml of inoculum were also sampled at 0 and 18 h to determine the digestibility of the inoculum when no substrate was present in the system. Short chain fatty acids (SCFA) were sampled from all sample bottles at 18 h and blanks at 0 h, by carefully removing 2 ml of bottle contents. At the end of the prescribed incubation period, all bottles were immediately autoclaved to cease fermentation and analysed for dry matter (DM) and ash. The DM and organic matter (OM) present in the SCFA sample were determined and during calculations the determined quantities were used to correct for the DM/OM loss from the system.

Chemical analyses

Diet samples were analysed for DM, ash, titanium dioxide, gross energy (GE), crude protein, fatty acids (FA), starch, total sugars, and total, soluble and insoluble non-starch polysaccharides (NSP). Rat digesta samples were analysed for DM, ash, titanium

dioxide, GE, crude protein, FA, starch, total sugars. *In vitro* fermentation residues were analysed for DM, ash and SCFA.

DM and ash determination were performed in duplicate using the AOAC standard procedures (AOAC, 2000) by drying samples in a Watvic convection oven (Watson Victor Limited, Wellington, New Zealand), followed by ashing in a Muffle furnace (Electrofurn, New Zealand). The titanium dioxide content of the diets and digesta was measured on a UV spectrophotometer following the method of Short *et al.* (1996).

GE was determined in duplicate using an automatic adiabatic bomb calorimeter (AC-350, Leco Corporation, St. Joseph MI, USA) (Miller and Payne, 1959). GE determination of digesta samples was performed using 50mg samples in sections of plastic wrap of known GE content.

Microanalysis of total N was performed on duplicate samples, using an elemental analyser (EA 1108, Carlo Elba, Italy). Crude protein was calculated using an N to protein conversion factor of 6.25 (Kraisid *et al.*, 2003).

Given the limited quantity of ileal digesta, total fat content of diet and digesta samples was determined as the total lipid FA expressed as triacylglycerol as suggested by the FDA (Ali *et al.*, 1997; FDA, 2004). Individual FA were determined in duplicate by GC on a 6890 GC FID (Agilent Technologies, Santa Clara, CA) (Sukhija and Palmquist, 1988; Wu *et al.*, 1994).

Total sugars were defined as simple sugars, oligosaccharides, polysaccharides, and their derivatives, including the methyl esters with free or potentially reducing groups. Sugars were determined using the phenol sulphuric colorimetric method on duplicate samples (Dubois *et al.*, 1956). Total starch determination was performed in duplicate using a commercial kit (Total Starch Kit, Megazyme, Ireland) according to the AOAC method (AOAC, 2000). Total carbohydrate (CHO) (%) was determined as the difference between 100 and the sum of the percentage of water, protein, total fat and ash. NSP

was determined using a modified Englyst method (Englyst and Cummings, 1984, 1988) and constituent sugars measured using colorimetry.

SCFA in *in vitro* fermentation residues were determined using GC (Wronlowska *et al.*, 2006). The sample was deproteinised using metaphosphoric acid. The supernatant was injected directly into the gas chromatograph (Carlo Erba 5380, capillary column Alltech ATTM-1000, 15m x 0.53mm ID, 1.00um film) with hydrogen as the carrier gas, FID (flame ionisation detector), and iso-caproic acid as an internal standard.

Calculations

Upper-tract nutrient digestibility

The apparent ileal digestibility of each nutrient was calculated using the ratio of the nutrient (mg) to titanium dioxide (mg) in the diet ($(\text{Nutrient}/\text{Ti})_{\text{diet}}$) and the ratio of the nutrient (mg) to titanium dioxide (mg) in the ileal digesta ($(\text{Nutrient}/\text{Ti})_{\text{ileal}}$).

$$\text{Apparent ileal digestibility of nutrient (\%)} = \frac{(\text{Nutrient}/\text{Ti})_{\text{diet}} - (\text{Nutrient}/\text{Ti})_{\text{ileal}}}{(\text{Nutrient}/\text{Ti})_{\text{diet}}} \times \frac{100}{1}$$

(Eq. 1)

The ileal digestibility of GE and other dietary components was calculated in an analogous manner.

In vitro hindgut OM digestibility

Organic matter digestibility (OMD) of the ileal substrate was determined by correcting for the OM present in the blanks, as described elsewhere (see Chapter VI) and calculated as follows:

$$OMD (\%) = \frac{OM_{substrate} - (OM_{bottle\ residual} - \left(\frac{OM_{blank\ residual} + OM_{blank\ initial}}{2}\right))}{OM_{substrate}} \times \frac{100}{1} \quad (Eq. 2)$$

where $OM_{substrate}$ is the OM (mg) contributed by the ileal substrate prior to incubation, $OM_{bottle\ residual}$ is the OM (mg) remaining in the bottle at the end of incubation from both the ileal substrate and the inoculum, $OM_{blank\ initial}$ is the OM (mg) of the blank at the start of incubation and $OM_{blank\ residual}$ is the OM remaining of the blank OM (mg) at the end of incubation. Ileal substrate DMD was calculated in an analogous manner to ileal substrate OMD.

SCFA

The quantity of each SCFA produced during *in vitro* hindgut fermentation was calculated as the difference between the concentration of SCFA (μmol per bottle) at the beginning of *in vitro* incubation, as observed in the blank ($SCFA_{blank\ initial}$) (μmol), and that at the end of *in vitro* incubation ($SCFA_{bottle\ residual}$) (μmol).

$$SCFA\ produced\ (\mu\text{mol}\ per\ bottle) = SCFA_{bottle\ residual} - SCFA_{blank\ initial} \quad (Eq. 3)$$

OM_{ingested equivalent}

The OM content of the diet (OM_{diet}) per g of DM was known from chemical analysis. However, the mean absolute quantity of OM ingested ($OM_{ingested\ equivalent}$, mg) by the group of rats for each pooled ileal digesta sample to give x amount (mg) of OM present in the ileal substrate ($OM_{ileal\ substrate}$, mg) needed to be calculated. This calculation was based on the apparent ileal digestibility of the diet (%) as calculated using Equation 1 and the absolute quantity of OM (mg) in the ileal digesta sample used for the *in vitro* fermentation ($OM_{ileal\ substrate}$).

$$OM_{\text{ingested equivalent}} \text{ (mg)} = \frac{OM_{\text{ileal substrate}} \times 100}{100 - \text{ileal OM digestibility (\%)}} \quad (\text{Eq. 4})$$

Total tract OMD from dual *in vivo* – *in vitro* digestibility assay

OMD over the total tract for the dual *in vivo-in vitro* digestibility assay (%) was defined as the mean OM ingested for the pooled group of rats ($OM_{\text{ingested equivalent}}$, mg) corrected for the OM contributed by the substrate at the end of the *in vitro* incubation ($OM_{\text{bottle residual}}$, mg), corrected for the OM in the blank (mg), relative to the OM ingested (OM_{ingested} , mg).

Total tract *in vivo - in vitro* OMD (%)

$$= \frac{(OM_{\text{ingested equivalent}} - OM_{\text{ileal substrate}}) + OM_{\text{fermented}}}{OM_{\text{ingested equivalent}}} \times \frac{100}{1} \quad (\text{Eq. 5})$$

$$= \frac{OM_{\text{ingested equivalent}} - (OM_{\text{bottle residual}} - \left(\frac{OM_{\text{blank residual}} + OM_{\text{blank initial}}}{2} \right))}{OM_{\text{ingested equivalent}}} \times \frac{100}{1} \quad (\text{Eq. 6})$$

Total predicted ATP yield (ATP_a)

The ATP yield (ATP_a) for each diet (per kg DM diet) (Equation 7) was calculated as the sum of ATP gains from starch and sugars ($ATP_{\text{ST+SU}}$), fatty acids (ATP_{FA}), amino acids (ATP_{AA}) and SCFA (ATP_{SCFA}).

$$ATP_a = ATP_{\text{ST + SU}} + ATP_{\text{FA}} + ATP_{\text{AA}} + ATP_{\text{SCFA}} \quad (\text{Eq. 7})$$

The upper-tract *in vivo* digestibility of crude protein, starch, sugars and FA (per kg DM diet) and the primary hindgut SCFA produced (per kg DM diet) during *in vitro*

fermentation (acetic acid, propionic acid and butyric acid) were determined using the dual *in vivo* – *in vitro* digestibility assay. These were translated into predicted ATP yields (ATP_a , mol ATP/ kg DM diet) using the values given in Chapter VIII. The ATP yield for starch was taken as 178.11 mmol/g starch, which was calculated as 1.11 times the ATP yield of glucose based on a molecular weight of 162 mol/g glucose, rather than 180g/mol as in free glucose.

The digestibility of individual amino acids (AA) was not determined in the present study and the uptake of AA was determined based on crude protein digestibility. A generic ATP yield for all proteins was calculated as 130.9 mmol / g protein. This value was calculated by determining the AA content of 32 common food proteins from FAO tables (FAO, 1981), and summing the associated ATP yields for each AA in the food on a proportional basis using the free AA molar mass to obtain the total ATP yielded for each AA for each of the 32 foods (mmol ATP / g protein) (**Table 3**). The ATP yield values for each AA were taken from Chapter VIII, but with 0.5 mol / AA subtracted to account for the cost of absorption and transport (ATP_t), that is included in these published ATP_a yields. The ATP_a from the catabolism of AA (ATP_{AA} , mmol) was therefore determined as:

$$ATP_{AA} = (N_d - N_i) \times 6.25 \times 130.9 \quad (Eq. 8)$$

where N_d and N_i are the nitrogen in the diet (g) and ileal digesta (g) respectively and 6.25 is the conversion factor from N (g) to protein (g) (Kraisid *et al.*, 2003). It is acknowledged that there is a wide range of products and subsequent ATP yields for the different AA and a single value for all proteins may not be fully representative of the ATP yield.

Table 3ATP yields¹ from the catabolism of amino acids for selected food proteins²

Food	mmol ATP / g protein²
Cow's milk cheese	168.2
Spinach leaf	146.7
Whole cow's milk	161.8
Whole hen's egg	149.8
Potato	109.6
Beef meat	139.5
Pilchard-like fish	150.9
Lamb flesh	138.9
Chicken meat	131.2
Tuna-like fish	129.7
Salmon-like fish	131.3
Mollusk	142.6
Cabbage	98.6
Haddock-like fish	149.9
Chick-pea	135.4
Polished rice	142.3
Crustacean	144.7
Pea	131.0
Soybean	152.0
Lentil	136.9
Sesame	152.3
Bean	128.1
Whole wheat	146.9
Hazelnut	165.1
Cassava	99.1
Carrot	105.5
Apple	111.0
Almond	153.5
Tomato	103.4
Banana	110.9
Orange	109.7
Oats	140.3

¹ ATP yield based on amino acid ATP yields given in Chapter VIII. Protein conversion factors are as given in FAO (1981)

² Amino acid content data for each protein obtained from FAO (1981)

In the present study, it was also investigated whether the ATP yield from SCFA in the hindgut could be accurately predicted using the digestibility of OM. The digestibility of OM was translated into predicted ATP_{SCFA} using the value of 101.7 mmol ATP/g fermented OM, as given elsewhere (see Chapter VIII). The ATP yielded per gram of triacylglycerol (TAG) was calculated based on a generic TAG consisting of palmitic acid, stearic acid and oleic acid. This combination of FA is the typical pattern of adipose fat in humans (Flatt and Tremblay, 1998) and adipose tissue is known to largely be a reflection of dietary intake (Summers *et al.*, 2000). The sum of ATP yielded from the catabolism of these three FA, as well as glycerol, was used to calculate the total ATP yield of 329.5 mmol ATP / mol TAG. When calculating the ATP yield on a mmol ATP / g TAG basis, the 'bound' molecular weights of the three FA contributing to the TAG were used and it was assumed that the bound molecular weight of each FA was equal to the 'free' molecular weight less 17 g / mol (18 Da / mol). A molar mass of 810.5 g / mol was calculated for the generic TAG and converted to 406.6 mmol ATP / g TAG.

$$ATP_{FA} = (TAG_d - TAG_i) \times 406.6 \text{ mmolATP/gTAG} \quad (\text{Eq. 9})$$

where TAG_d is the TAG present in the diet (kg / DM diet), TAG_i is the TAG present in the ileal digesta (kg / DM diet), and 'mmol ATP / g TAG' is the ATP (mmol) yielded per gram of TAG.

Statistical analyses

Data for each diet for a given parameter were compared using a one way analysis of variance (ANOVA). If a statistically significant effect was found, the means were subjected to multiple comparison using Tukey's test (Muller and Fetterman, 2002). Results were considered statistically significant at $P < 0.05$. The program Minitab (version 15, Minitab Inc., PA, USA) was used for all statistical analyses.

Results

Animal Study

The rats readily consumed all the diets. One rat (Diet C) died during the study from unrelated causes.

Upper-tract nutrient digestibility

There were significant differences ($P<0.05$) for the apparent ileal digestibility of GE for the three diets (**Table 4**). Diet A had the highest GE digestibility (94%), while Diet B had a relatively low GE digestibility (68%) compared to both Diets A and C. Diet B also exhibited a very low apparent ileal protein digestibility (29%) compared to the other diets (Diet A: 89%, Diet C: 93%) (Table 4). Total CHO digestibility (Diet A: 93%, Diet B: 60%, Diet C: 72%) and starch digestibility (Diet A: 100%, Diet B: 97%, Diet C: 93%) were significantly ($P<0.05$) different for all diets, but there was no significant difference ($P>0.05$) in the digestibility of sugars (Table 4). The difference between Diet A and the other two diets for ileal fat digestibility (Diet A: 98% Diet B: 93% Diet C: 94%) was statistically significant ($P<0.05$) (Table 4). FA digestibility for Diet B was lower than that for one or both of the other diets for all fatty acids and this difference was statistically significant ($P<0.05$) for all fatty acids except lauric acid (C12) (**Table 5**).

Table 4

Mean¹ ileal excretion² (g or MJ per kg digesta DM) and apparent ileal digestibility (%) of gross energy, crude protein, fat, total carbohydrate³, starch, sugar, DM and OM in the growing rat fed three diets

Component	Parameter	Diet			SEM
		Diet A	Diet B	Diet C	
Gross energy	Ileal excretion, MJ/kg	13.7 ^{ab}	14.6 ^a	13.1 ^b	0.3
	Ileal digestibility, %	93.5 ^a	68.4 ^c	83.6 ^b	3.5
Crude protein	Ileal excretion, g/kg	170.6 ^a	164.5 ^a	132.0 ^b	6.5
	Ileal digestibility, %	89.3 ^a	28.8 ^b	92.7 ^a	10.6
Total fat	Ileal excretion, g/kg	25.8 ^a	26.7 ^a	3.8 ^b	4.1
	Ileal digestibility, %	98.1 ^a	93.0 ^b	94.4 ^b	0.8
Total CHO ³	Ileal excretion, g/kg	495.4 ^b	606.5 ^a	618.6 ^a	17.6
	Ileal digestibility, %	92.8 ^a	60.1 ^c	71.8 ^b	4.4
Starch	Ileal excretion, g/kg	2.6 ^b	5.9 ^a	1.6 ^c	0.7
	Ileal digestibility, %	99.9 ^a	96.9 ^b	93.0 ^c	1.0
Sugars	Ileal excretion, g/kg	396.6 ^a	82.8 ^c	175.5 ^b	41.0
	Ileal digestibility, %	91.0	89.2	90.3	0.4
DM	Ileal excretion, g/kg	938.1 ^a	958.0 ^b	964.2 ^b	3.8
	Ileal digestibility, %	90.9 ^a	59.4 ^c	78.9 ^b	4.4
OM	Ileal excretion, g/kg	691.7 ^c	797.6 ^a	754.4 ^b	13.7
	Ileal digestibility, %	93.0 ^a	62.7 ^c	81.5 ^b	4.3

¹ Mean of pooled groups of rat ileal digesta. Diet A, $n=2$ samples (1 x $n=14$ animals, 1 x $n=13$ animals); Diet B, $n=3$ samples (3 x $n=11$ animals); Diet C, $n=4$ samples (4 x $n=8$ animals)

² Expressed as g per kg of ileal digesta dry matter, except for DM (g per kg digesta)

³ Total CHO (by difference) = 100 - Moisture% - Fat% - Crude Protein% - Ash%

^{abc} Means with different superscripts within the same row were significantly ($P<0.05$) different CHO, carbohydrate; DM, dry matter; OM, organic matter; SEM, standard error of the mean

Table 5

Mean¹ ileal excretion² (g/kg digesta dry matter) and apparent ileal digestibility (%) of fatty acids in the growing rat fed three diets

Component	Parameter	Diet			SEM	
		Diet A	Diet B	Diet C		
SFA	C12	Ileal excretion, g/kg	0.67 ^a	0.04 ^b	0.02 ^b	0.10
		Digestibility, %	99.4	90.2	97.7	2.7
	C14	Ileal excretion, g/kg	0.81 ^a	0.10 ^b	0.04 ^c	0.11
		Digestibility, %	98.6 ^a	90.2 ^{ab}	73.7 ^b	4.2
	C16	Ileal excretion, g/kg	3.80 ^a	3.33 ^a	0.59 ^b	0.53
		Digestibility, %	96.0 ^a	86.6 ^b	94.1 ^a	1.5
C18	Ileal excretion, g/kg	3.28 ^b	4.96 ^a	0.49 ^c	0.71	
	Digestibility, %	92.5 ^a	78.2 ^b	82.4 ^{ab}	2.2	
MUFA	C16:1	Ileal excretion, g/kg	0.12 ^a	0.07 ^b	0.04 ^b	0.01
		Digestibility, %	88.9 ^a	68.7 ^b	86.1 ^a	3.6
	C18:1 n9	Ileal excretion, g/kg	7.11 ^b	11.78 ^a	0.43 ^c	1.82
		Digestibility, %	98.9 ^a	95.5 ^b	94.9 ^b	0.6
	C18:1 n7	Ileal excretion, g/kg	0.49 ^a	0.29 ^{ab}	0.13 ^b	0.06
		Digestibility, %	95.3 ^a	93.8 ^a	86.2 ^b	1.7
PUFA	C18:2	Ileal excretion, g/kg	1.83 ^a	2.05 ^a	0.44 ^b	0.27
		Digestibility, %	99.3 ^a	94.0 ^b	98.4 ^a	0.8
	C18:3	Ileal excretion, g/kg	0.16 ^a	0.05 ^{ab}	0.01 ^b	0.02
		Digestibility, %	99.4 ^a	93.0 ^b	99.7 ^a	1.2
	Other FA	Ileal excretion, g/kg	6.48 ^a	2.98 ^b	1.52 ^b	0.74
		Digestibility, %	85.4 ^a	68.9 ^b	87.0 ^a	3.3
Sum of FA	Ileal excretion, g/kg	24.8 ^a	25.6 ^a	3.7 ^b	3.9	

¹ Mean of pooled groups of rat ileal digesta. Diet A, $n=2$ samples (1 x $n=14$ animals, 1 x $n=13$ animals); Diet B, $n=3$ samples (3 x $n=11$ animals); Diet C, $n=4$ samples (4 x $n=8$ animals)

² Expressed as g per kg of ileal digesta dry matter

^{abc} Means with different superscripts within the same row were significantly ($P<0.05$) different

FA, fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; SFA, saturated fatty acids

***In vitro* hindgut OM digestibility and SCFA production**

There were no significant ($P>0.05$) differences between the hindgut OMD of Diet A (89%) and Diet C (84%) (Table 6). However, the hindgut OMD of Diet B (57%) was significantly ($P<0.05$) lower when compared to the other two diets. Total SCFA production of 719, 567 and 785 μmol per *in vitro* fermentation bottle was observed for Diets A, B and C respectively (Table 7).

Table 6

Mean¹ hindgut organic matter digestibility (OMD) (%) for three experimental diets as determined using the hindgut *in vitro* digestibility assay and predicted total tract OMD²

Model	OMD ¹ , %			SEM
	Diet A	Diet B	Diet C	
<i>In vitro</i> hindgut	88.7 ^a	57.2 ^b	84.2 ^a	5.6
Total tract ²	99.2 ^a	86.2 ^b	97.2 ^a	2.2

¹ Mean of pooled groups of rat ileal digesta. Diet A, $n=2$ samples (1 x $n=14$ animals, 1 x $n=13$ animals); Diet B, $n=3$ samples (3 x $n=11$ animals); Diet C, $n=4$ samples (4 x $n=8$ animals)

² Total tract digestibility in rat predicted based on ileal flows and hindgut *in vitro* digestibility

^{abc} Means with different superscripts within the same row were significantly ($P<0.05$) different

OMD, organic matter digestibility; SEM, standard error of the mean

Table 7

Mean¹ short chain fatty acid production ($\mu\text{mol per bottle}$)² of the three ileal substrates during *in vitro* hindgut fermentation

SCFA	Concentration at end of <i>in vitro</i> incubation ¹ ,			SEM
	$\mu\text{mol per bottle}$ ²			
	Diet A	Diet B	Diet C	
Acetic	387.4 ^b	327.7 ^c	472.3 ^a	25.3
Propionic	129.8 ^a	101.3 ^b	135.8 ^a	6.2
iso-Butyric	16.0 ^a	12.7 ^b	11.5 ^b	0.8
n-Butyric	112.0 ^a	76.1 ^b	108.5 ^a	6.1
iso-Valeric	21.0 ^a	18.8 ^a	14.7 ^b	1.1
n-Valeric	36.6 ^a	20.9 ^c	27.7 ^b	2.3
n-Caproic	16.6 ^a	9.4 ^c	14.7 ^b	1.1
Total	719.3 ^a	566.9 ^b	785.1 ^a	34.7

¹ Mean of pooled groups of rat ileal digesta. Diet A, $n=2$ samples (1 x $n=14$ animals, 1 x $n=13$ animals); Diet B, $n=3$ samples (3 x $n=11$ animals); Diet C, $n=4$ samples (4 x $n=8$ animals)

² $\mu\text{mol per in vitro}$ fermentation bottle

^{abc} Means with different superscripts within the same row were significantly ($P<0.05$) different SCFA, short chain fatty acid; SEM, standard error of the mean

Predicted ATP gains and costs

Nutrient uptakes (per kg dry matter) predicted using the chemical composition of the diets and the digestibility data obtained from the dual *in vivo* – *in vitro* digestibility assay were used to calculate ATP gains and costs. The predicted net ATP yields from absorbed nutrients (ATP_a) (**Table 8**) were calculated using two different ways of calculating hindgut SCFA uptake; using the OM fermented or the sum of individual SCFA produced. The two calculation methods gave similar ATP_a yields. Diet C had the lowest overall ATP_a value, whereas Diet A, as expected had the highest available energy content.

Table 8

Predicted ATP yields (ATP_a) (mol/kg DM diet) for three meal replacement formulations

Metabolic substrate	Predicted ATP yield, <i>mol/kg diet DM</i>		
	Diet A	Diet B	Diet C
<i>Upper-tract absorption</i>			
Protein ¹	16.9	3.5	46.4
Starch	30.9	13.4	0.8
Sugars	58.3	44.3	55.1
TAG	48.3	58.9	5.5
<i>Hindgut absorption</i>			
Organic matter	5.7	18.8	13.6
SCFA			
Acetic acid	2.5	9.6	7.1
Propionic acid	1.5	5.4	3.7
Butyric acid	2.0	6.2	4.6
Total SCFA	6.1	21.2	15.4
<i>Total predicted ATP yield</i>			
using hindgut OM	160.1	138.9	121.4
using SCFA	160.4	141.2	123.1

¹ The ATP yield for protein includes the cost of amino acid transport

DM, dry matter; TAG, triacylglycerol; OM, organic matter; SCFA, short chain fatty acids

Discussion

The model applied in the present study allowed prediction of the net ATP yield (i.e. ATP yield net of direct ATP costs of catabolism) subsequent upon metabolism of the absorbed energy-yielding substrates for three meal replacement formulations. The quantities of nutrients predicted to be absorbed from two regions of the digestive tract (mouth to terminal ileum and the hindgut) and subsequently available for cellular metabolism were determined using a dual *in vivo* - *in vitro* digestibility assay (see chapters V - VII). The *in vivo* portion of the dual digestibility assay uses the laboratory rat as an animal model for predicting upper-tract nutrient digestibility in humans. The *in vitro* portion of the dual digestibility assay uses rat ileal digesta as the substrate in a hindgut *in vitro* fermentation system using human faecal bacteria. The potential ATP yield from the metabolism of the absorbed nutrients was predicted using a series of mathematical equations derived using known stoichiometric relationships (see Chapter VIII).

The Combined Model (*in vivo* / *in vitro* digestion / stoichiometric predictions) allows for the prediction of ATP yields from the catabolism of nutrients predicted to be absorbed from the upper- and lower-digestive tract separately, thereby providing more useful information than total tract digestibility data alone. The use of experimentally determined digestibility data for the actual food/diet in question is preferable to relying on generic factors for each of the energy yielding nutrients. The upper-tract digestibility of not only gross energy, but also of most macronutrients, can be predicted using the Combined Model.

It is difficult to determine nutrient uptakes directly in humans (Darragh and Hodgkinson, 2000). The rat is an omnivore and has a similar small intestinal transit time as adult humans (3 - 4 h) (DeSesso and Jacobson, 2001). The laboratory rat was accepted here as a suitable animal to predict the loss of nutrients in the upper digestive tract of humans.

The rat model, although offering some logistical advantages, does have the limitation of providing low quantities of ileal digesta. The use of a larger animal, such as the pig, would increase the limited quantities of ileal digesta obtained from the rat for the *in vitro* fermentation step in the assay. The *in vitro* digestibility assay could also be improved from a pragmatic point of view by finding a valid alternative to the requirement for fresh faecal samples to be obtained from human subjects on demand.

Total tract organic matter digestibility

The predicted total tract OMD of Diet B (86%) (Table 6) was lower than that previously observed for high dietary fibre (DF) diets studied using the dual *in vivo* - *in vitro* digestibility assay, which have been in the range of approximately 90 – 92% (see Chapter VII). The high predicted total tract OMD observed with Diet A (99%) was expected given the low NSP content and highly digestible ingredients of this diet. Despite the upper-tract digestibility of Diet C being lower compared to Diet A, the highly fermentable nature of Diet C meant that over the total tract it was almost completely digested with an OMD of 97%. The OMD ranking of the three diets in the upper-tract was the same as in the hindgut (Diet A>Diet C>Diet B). However, the relative proportion of each diet and specific nutrients digested at each site varied. These differences in digestibility in the two areas of the digestive tract were clearly demonstrated using the digestibility assay. Overall, the dual digestibility assay was able to differentiate between the diets in several aspects of digestibility and provides considerably more information than just a simple ranking of diets.

Predicted ATP yields

There were differences between the three meal replacement formulations, in terms of the predicted ATP yields (ATP_a). The basis on which ATP_a values were derived for hindgut fermentation (SCFA versus OM fermented) made little difference to the calculated ATP gains. For the remainder of the discussion here, the ATP_a figures calculated from OM fermented will be used, which were 160, 139 and 121 mol ATP/kg DM for Diets A, B and C, respectively.

SCFA are the primary end product of hindgut fermentation and can be used as a measure of the degree of OM fermentation. Diets A and C produced similar hindgut OM digestibility values and Diet B had a much lower hindgut OMD than the other two diets (Table 6), which corresponded closely to the observed SCFA production rates (Table 7). All three test diets produced acetic, propionic and butyric acids in the approximate ratio of 3:1:1, respectively. A similar pattern of molar SCFA ratios has been found both in human colonic contents and with other *in vitro* fermentation studies, as summarised elsewhere (Cummings, 1996) and estimated to be approximately 60:20:18 (acetic, propionic and butyric acids, respectively). This molar ratio from the literature was used here to predict SCFA yields from fermented OM. A significant deviation from these generic molar ratios may cause the calculation of ATP from directly measured SCFA production rates to be more accurate than predicting SCFA production from the OM fermented in the *in vitro* hindgut digestibility system. However, given the relatively small contribution of hindgut fermentation to total ATP yields, the overall effect is likely not to be of any great practical significance. Hindgut fermentation of OM contributed around only 4% of total ATP_a yield for Diet A, while higher contributions were seen for diets B (14%) and C (11%). These results were expected given that diets B and C were formulated to have a high DF content for the satiating effect purported to be imparted by DF (Howarth *et al.*, 2001; Slavin, 2005), while Diet A was designed primarily as a nutritional supplement (for weight-gain rather than for weight-loss) and exhibited high upper-tract digestibility for all nutrients. The values observed for the contribution of hindgut fermentation to total energy gains in humans are largely in agreement with values of 3 – 11% quoted in the literature (Cummings, 1983; Cummings and Macfarlane, 1991; McBurney and Thompson, 1989; McBurney *et al.*, 1987; McNeil, 1984).

Diet C contributed the majority of its total energy from the upper-tract digestion of protein (43%) and starch and sugars (52%), with a minimal contribution from TAG (5%). A similar disproportionate split between protein and fat was seen for Diet B, except that upper-tract energy gains from fat contributed 49% of total ATP_a, and protein 3%.

The relatively low protein content of Diet B (93 g/kg DM diet), may have partly accounted for the particularly low apparent ileal protein digestibility value observed (29%). This low degree of protein digestibility was unexpected given that the protein component of Diet B was dairy-based (buttermilk powder and milk protein concentrate). It is possible that the highly viscous nature of the ileal digesta observed with Diet B may have increased the presence of endogenous protein in the ileal digesta sample, thereby reducing the apparent digestibility of the diet.

Tabulated food energy values are currently calculated by the use of various factorial and empirical models, which vary from country to country. These models calculate 'metabolisable' energy and may be suitable for food tabulation and food labelling purposes. However, for the purposes of foods specially formulated for weight-loss, the 'available' (ATP) energy, as predicted here, gives a more accurate estimate of the energy that is actually physiologically available to the cell and takes into account the efficiency of utilisation of each energy-yielding dietary nutrient. In the present study, the ranking of diets obtained using nine different factorial and empirical models (Atwater, 1910; Brown *et al.*, 1998; FDA, 1993; Holland *et al.*, 1991; Levy *et al.*, 1958; Livesey, 1991, 2002; Miller and Judd, 1984; Miller and Payne, 1959; Southgate, 1975) (**Table 9**) for determining ME were compared with the diet rankings made according to predicted 'available' energy (ATP_a) values (Table 8). The ME values were calculated for each diet as kJ equivalents using the chemical composition of the diets (**Table 10**), whilst predicted ATP_a values were translated into kJ equivalents using a value of -59.8 kJ/mol ATP (-14.3 kcal/mol ATP) (see Chapter VIII). The ME models use various definitions for the indigestible carbohydrate fraction and are most often determined using the Southgate (1969) or total dietary fibre (TDF) (AOAC, 2000) methods. In the present study, NSP was used instead, as TDF was not measured. This may have resulted in a slight underestimation of the total ME content for those models that are based on TDF.

Table 9Models used to predict ME¹ of the three experimental diets (kJ/g dry diet)²

Model and reference	Model
Factorial models	
Atwater (1910)	$ME_{\text{Atwater}} = 16.7P + 37.6F + 16.7C$
Holland <i>et al.</i> (1991) ³	$ME_{\text{British}} = 16.7P + 37.6F + 15.7Cm$
FDA (1993)	$ME_{\text{FDA}} = 16.7P + 37.6F + 16.7(C - \text{isDF})$
Brown <i>et al.</i> (1998), Livesey (2002)	$ME_{\text{Atwater modified}} = 16.7P + 37.6F + 16.7AC + 8.4UC$
Empirical models	
Levy <i>et al.</i> (1958)	$ME_{\text{Levy}} = 0.976E - 33.3N - 250$
Miller and Payne (1959)	$ME_{\text{M\&P}} = 0.95E - 31.4N$
Southgate (1975)	$ME_{\text{Southgate}} = 0.977E - 16.7UC - 27.6N$
Miller and Judd (1984)	$ME_{\text{M\&J}} = (0.95E - \text{DF}\%) - 31.4N$
Livesey (1991)	$ME_{\text{Livesey}} = 0.96E - 8.4U - 30N$

¹ ME (kJ/g dry diet), metabolisable energy; P (g), dietary protein; F (g), dietary fat; C (g), total carbohydrate by difference; Cm (g), determined available carbohydrate expressed as equivalent weight of monosaccharide (1.1 x starch (g) + sugars (g)); isDF (g), insoluble dietary fibre; AC, available carbohydrate (determined by difference); UC (g), unavailable carbohydrate determined as Southgate dietary fibre or total dietary fibre; E(kJ/g), gross energy of diet; N(g), dietary nitrogen; DF%, total dietary fibre as a percentage of the dry weight of the diet; U (g), unavailable complex carbohydrate; FDA, Food and Drug Administration.

² Table taken from Zou *et al.* (2007)

³ For legislative purposes, the Atwater approach is used in the European Union; fibre has zero energy by default (C = total carbohydrate minus total dietary fibre).

Table 10

Available energy¹ and metabolisable energy (ME)² based on factorial and empirical models expressed as kJ/g dry diet³ and kJ/kj gross energy (GE)⁴

Model	Available ¹ or metabolisable ² energy		
	Diet A	Diet B	Diet C
ATP _a (Combined Model)	9.574	8.304	7.260
ME _{Atwater}	18.067	18.313	15.397
ME _{British}	16.238	13.609	12.977
ME _{FDA}	18.058	18.378	15.392
ME _{Atwater modified}	17.883	17.135	14.300
ME _{Levy}	17.740	17.492	14.097
ME _{M&P}	17.535	17.285	14.027
ME _{Southgate}	17.772	15.474	12.504
ME _{M&J}	17.534	17.283	14.026
ME _{Livesey}	17.573	16.300	13.170

¹ Available Energy based on predicted ATP yields (Combined Model)

² Metabolisable energy (ME) as predicted using published factorial and empirical ME models

³ kJ/g dry diet

⁴ kJ/kj gross energy (GE)

With the exception of the British Food Tables model (16.2 kJ/g), the estimated ME content for Diet A was similar for all models from the literature, ranging from 17.5 – 18.1 kJ/g. The low NSP content of Diet A meant that differences in the models were not large. However, for the two diets with higher NSP content the calculated ME content varied widely from 13.6 kJ/g to 18.4 kJ/g for Diet B and from 12.5 – 15.4 kJ/g for Diet C using the models from the literature.

The available energy predicted using the Combined Model (present study) ranked the diets as diet A>B>C. All ME models investigated also ranked the diets as diet A>B>C with the exception of the Atwater model and the FDA model, which both ranked the diets as diet B>A>C. Some ME models, therefore, may not be suitable for ranking foods formulated for weight-loss in terms of physiologically available energy.

The predicted 'available energy' values for the Combined Model and the four factorial ME systems were compared with that of dextrin as a baseline, assuming that dextrin consists entirely of starch, with a GE equivalent to the heat of combustion of starch (17.5 kJ/g) (Livesey, 1992), which was completely digested, absorbed and metabolised as glucose (**Table 11**). The calculated available energy for dextrin was predicted as 0.58 kJ/kJ GE (10.15 kJ/g) using the Combined Model, whereas for the ME models ME was predicted as 0.99 kJ/kJ GE (17.3 kJ/g) (British Food Tables model) or 0.95 kJ/kJ GE (16.7 kJ/g) (other ME models). The British Food Tables model gave relatively close agreement for the ratio of metabolisable energy (ME) (meal replacement formulation: dextrin); (Diet A: 0.86, Diet B: 0.74, Diet C: 0.78) when compared to predicted available energy (ATP_a) (Diet A: 0.86, Diet B: 0.77, Diet C: 0.75). The three other ME systems (Atwater, FDA and Atwater Modified), generally gave similar comparable ratios (meal replacer: dextrin) to each other but considerably higher than was predicted using the Combined Model. In particular, both the Atwater and FDA models gave a ratio of 0.96 for Diet C: dextrin, compared to 0.75 for the predicted available energy. This again demonstrates that current ME systems may not be the most appropriate means of determining the dietary energy content of some foods, particularly weight-loss diets, and that the Combined Model may be a more accurate measure of the ATP made

available to the body, having particular application for the development of such diets. The Combined Model predicted the generation of quite different amounts of 'available energy' relative to that for the baseline dextrin for the three diets, compared to the other ME based models.

Table 11

Available energy¹ and Metabolisable Energy (ME)² of dextrin and the ratio of energy meal replacement formulation : energy dextrin³

Model	Available ¹ or Metabolisable ² Energy of dextrin, kJ/kJ GE ⁴	Ratio of energy meal replacement formulation : energy dextrin ³		
		Diet A	Diet B	Diet C
Combined Model	0.580	0.859	0.766	0.746
ME _{Atwater}	0.954	0.985	1.027	0.961
ME _{British}	0.987	0.856	0.738	0.783
ME _{FDA}	0.954	0.984	1.031	0.961
ME _{Atwater modified}	0.954	0.975	0.961	0.893

¹ Available energy based on predicted ATP yields (Combined Model)

² Metabolisable energy as predicted using published factorial ME models

³ Calculated using predicted available or metabolisable energy values of meal replacement and dextrin as kJ/kJ gross energy

⁴ kJ/kJ gross energy, where the GE of dextrin was taken as the heat of combustion of starch at 17.5 kJ/g, taken from Livesey (1992)

GE, gross energy; ME, metabolisable energy

Conclusion

To the authors' knowledge, the Combined Model is the first published model to separately predict the upper-tract and hindgut uptake of all major energy yielding nutrients of a food in humans and convert this into predicted available energy (ATP) at the cellular level using recent estimates of cellular P/O ratios. The model is able to differentiate between diets in terms of both digestibility and predicted ATP yields. The model follows the complete process of food assimilation from mouth to the primary site of metabolism (liver) by simulating the digestive and absorptive processes across the total tract and converting the uptake of energy into energy ultimately available to the body at the cellular level (ATP). The model can be applied in practice to refine the development of weight-loss foods.

Literature cited

- Ali, L. H., Angyal, G., Weaver, C. M. & Rader, J. I. (1997). Comparison of capillary column gas chromatographic and AOAC gravimetric procedures for total fat and distribution of fatty acids in foods. *Food Chemistry*, 58(1-2), 149-160.
- AOAC. (2000). *Official Methods of Analysis* (Vol. 17). Washington DC: Association of Official Analytical Chemists.
- Atwater, W. O. (1910). Principles of nutrition and nutritive values of food. *United States Farmers' Bulletin*, 142.
- Brand, M. D. (2005). *The efficiency and plasticity of mitochondrial energy transduction*. Paper presented at the Keilin Memorial Lecture, Glasgow.
- Brown, J., Livesey, G., Roe, M., Faulks, R., Poppitt, S., Wilkinson, J. & Elia, M. (1998). Metabolizable energy of high non-starch polysaccharide-maintenance and weight-reducing diets in men: experimental appraisal of assessment systems. *Journal of Nutrition*, 128, 986-995.
- Coles, L. T., Moughan, P. J., Awati, A. & Darragh, A. J. (2011). Influence of assay conditions on the *in vitro* hindgut digestibility of dry matter. *Food Chemistry*, In Press.
- Coles, L. T., Moughan, P. J., Awati, A., Darragh, A. J. & Zou, M. L. (2010). Predicted apparent digestion of energy-yielding nutrients differs between the upper and lower digestive tract in rats and humans. *Journal of Nutrition*, 140(3), 469-476.
- Cummings, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet*, 321(8335), 1206-1209.
- Cummings, J. H. (1996). *The Large Intestine in Nutrition and Disease*. Brussels, Belgium: Institute Danone.
- Cummings, J. H. & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, 70, 443-459.
- Darragh, A. J. & Hodgkinson, S. M. (2000). Quantifying the digestibility of dietary protein. *Journal of Nutrition*, 130, 1850S-1856S.

-
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, 39, 209-228.
- Dubois, M., Gillies, K. A., Hamilton, J. A., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.
- Edwards, C. A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J. C., Nagengast, F., Rumney, C. & Quehl, A. (1996). *In vitro* method for quantification of the fermentation of starch by human faecal bacteria. *Journal of the Science of Food and Agriculture*, 71, 209-217.
- Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst (Cambridge UK)*, 109, 937-942.
- Englyst, H. N. & Cummings, J. H. (1988). Improved method for the measurement of dietary fibre as non-starch polysaccharides in plant foods. *Journal of AOAC International*, 71, 808-814.
- FAO. (1981). *Amino-acid content of foods and biological data on proteins*. Rome, Italy: Food Policy and Food Science Service Nutrition Division, FAO.
- FDA. (1993). Nutritional labeling of food regulations. Title 21 of the Code of Federal Regulations (21CF). *Federal Register*, 53, 2175.
- FDA. (2004). *Code of Federal Regulations, Title 21, Volume 2, Chapter I, Part 101.9*. Washington DC: Department of Health and Human Services, Food and Drug Administration.
- Ferrer-Lorente, R., Fernandez-Lopez, J. A. & Alemany, M. (2007). Estimation of the metabolizable energy equivalence of dietary proteins. *European Journal of Nutrition*, 46, 1-11.
- Flatt, J.-P. & Tremblay, A. (1998). Energy expenditure and substrate oxidation. In G. A. Bray, C. Bouchard & W. P. T. James (Eds.), *Handbook of Obesity* (1st ed., pp. 513-537). New York: Marcel Dekker.

-
- Holland, B., Welch, A. A., Unwin, I. D., Buss, D. H., Paul, A. A. & Southgate, D. A. T. (1991). *McCance and Widdowson's The Composition of Foods* (5th ed.). Cambridge, United Kingdom: Royal Society of Chemistry, Ministry of Agriculture, Fisheries and Food.
- Howarth, N. C., Saltzman, E. & Roberts, S. B. (2001). Dietary fiber and weight regulation. *Nutrition Reviews*, 59(5), 129-139.
- Kraisid, T., MacLean Jr., W. C. & Warwick, P. M. (2003). *Food energy: methods of analysis and conversion factors: report of a technical workshop, Rome, 3-6 December 2002*. Rome: Food and Agriculture Organization of the United Nations.
- Levy, L. M., Bernstein, L. M. & Grossman, M. I. (1958). *The calorie content of urine of human beings and the estimation of the metabolizable energy of human foodstuffs* (No. 26). Denver, CO: US Army Medical Research and Nutrition Laboratory.
- Livesey, G. (1984). The energy equivalents of ATP and the energy values of food proteins and fats. *British Journal of Nutrition*, 51, 15-28.
- Livesey, G. (1990). Energy values of unavailable carbohydrate and diets: an inquiry and analysis. *American Journal of Clinical Nutrition*, 51, 617-637.
- Livesey, G. (1991). Calculating the energy value of foods - towards new empirical formulas based on diets with varied intakes of unavailable complex carbohydrates. *European Journal of Clinical Nutrition*, 45, 1-12.
- Livesey, G. (1992). The energy values of dietary fibre and sugar alcohols for man. *Nutrition Research Reviews*, 5, 61-84.
- Livesey, G. (1995). Metabolizable energy of macronutrients. *American Journal of Clinical Nutrition*, 62, 1135S-1142S.
- Livesey, G. (2002). Functional attributes of foods not diets will enable consumer choice. In A. Palou, M. Bonnet & F. Serra (Eds.), *Study on Obesity and Functional Foods in Europe, Cost Action 918* (pp. 366-375). Brussels, Belgium: European Commission.

-
- McBurney, M. I. & Thompson, L. U. (1989). Dietary fiber and energy balance: integration of the human ileostomy and *in vitro* fermentation models. *Animal Feed Science and Technology*, *23*, 261-275.
- McBurney, M. I., Thompson, L. U. & Jenkins, D. J. A. (1987). Colonic fermentation of some breads and its implications for energy availability in man. *Nutrition Research*, *7*, 1229-1241.
- McNeil, N. I. (1984). The contribution of the large intestine to energy supplies in man. *American Journal of Clinical Nutrition*, *39*, 338-342.
- Miller, D. S. & Judd, P. A. (1984). The metabolizable energy value of foods. *Journal of the Science of Food and Agriculture*, *35*, 111-116.
- Miller, D. S. & Payne, P. R. (1959). A ballistic bomb calorimeter. *British Journal of Nutrition*, *13*, 501-508.
- Muller, K. E. & Fetterman, B. A. (2002). *Regression and ANOVA: An Integrated Approach Using SAS Software*. Cary, NC: SAS Publishing.
- National Research Council. (1995). Nutrient requirements of the laboratory rat. In *Nutrient Requirements of Laboratory Animals* (4th rev. ed., pp. 11-79). Washington, DC: National Academy Press.
- Paddon-Jones, D., Westman, E., Mattes, R. D., Wolfe, R. R., Astrup, A. & Westerterp-Plantenga, M. (2008). Protein, weight management, and satiety. *American Journal of Clinical Nutrition*, *87* (suppl), 1558S-1561S.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, *87*, 292-300.
- Short, F. J., Gorton, P., Wiseman, J. & Boorman, K. N. (1996). Determination of titanium oxide added as an inert marker in chicken digestibility studies. *Animal Feed Science and Technology*, *59*, 215-221.
- Slavin, J. L. (2005). Dietary fiber and body weight. *Nutrition*, *21*, 411-418.
- Southgate, D. A. T. (1969). Determination of carbohydrates in foods. II. Unavailable carbohydrates. *Journal of the Science of Food and Agriculture*, *20*, 331-335.

-
- Southgate, D. A. T. (1975). Fiber and other unavailable carbohydrates and energy effects in the diet. In P. L. White & N. Selvey (Eds.), *Proceedings of the Western Hemisphere Nutrition Congress IV* (pp. 51-55). Acton, MA: Publishing Science Group Inc.
- Sukhija, P. S. & Palmquist, D. L. (1988). Rapid method for determination of total fatty-acid content and composition of feedstuffs and feces. *Journal of Agricultural and Food Chemistry*, *36*(6), 1202-1206.
- Summers, L. K. M., Barnes, S. C., Fielding, B. A., Beysen, C., Ilic, V., Humphreys, S. M. & Frayn, K. N. (2000). Uptake of individual fatty acids into adipose tissue in relation to their presence in the diet. *American Journal of Clinical Nutrition*, *71*, 1470-1477.
- Veldhorst, M., Smeets, A., Soenen, S., Hochstenbach-Waelen, A., Hursel, R., Diepvens, K., Lejeune, M., Luscombe-Marsh, N. & Westerterp-Plantenga, M. (2008). Protein-induced satiety: Effects and mechanisms of different proteins. *Physiology & Behavior*, *94*, 300-307.
- Wronlowska, M., Soral-Smietana, M., Krupa, U. & Biedrzycka, E. (2006). *In vitro* fermentation of new modified starch preparations - changes of microstructure and bacterial end-products. *Enzyme and Microbial Technology*, *40*(1), 93-99 Sp. Iss. SI.
- Wu, J. R., James Jr., D. W., Dooner, H. K. & Browse, J. (1994). A mutant of arabidopsis deficient in the elongation of palmitic acid. *Plant Physiology*, *106*(1), 143-150.
- Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, *86*, 1649-1656.

CHAPTER X

General discussion and conclusions

A global obesity epidemic has been declared (WHO, 2000). Although a multifaceted approach using a variety of techniques to combat obesity is recommended, one means of counteracting the problem may be through the provision of foods specifically designed for weight-loss using nutritional science based on valid and accurate theory and experimentation. The general objective when developing weight-loss foods is normally to minimise the energy delivered to the body (i.e. available energy, ATP) per gram of food, whilst (ideally) imparting maximum satiety. To accurately predict the available energy (ATP) provided by a food, the uptake of dietary energy needs to be separately quantified in the upper- and lower-digestive tract and translated into the ATP delivered to the body upon post-absorptive catabolism at the cellular level. Current methods of comparing the energy content of foods generally rely on metabolisable energy (ME) systems. However, ME systems do not accurately describe the uptake of dietary energy in the gut because the gross energy contents of dietary proteins, fats, and carbohydrates are not constants and the digestibilities of these nutrients are also variable, may be affected by the presence of other dietary components and depend on the site of uptake (upper-tract or hindgut) (Zou *et al.*, 2007). Further, the amount of energy per unit nitrogen excreted in urine varies across diverse diets and foods. Every diet is essentially unique and this cannot be consistently and accurately accounted for when using generic factors. The accurate determination of digestibility is particularly important for novel foods as these are often formulated to have reduced digestibility that may be significantly different from what is expected based on determined chemical composition alone.

A model was therefore proposed (Chapter IV), developed (Chapters V, VI, VII and VIII) and practically demonstrated (Chapter IX) to more accurately predict the actual energy a given food delivers in terms of ATP, as specific to adults in the catabolic state (i.e. sub-maintenance energy requirements) and with application to weight-loss foods. The development process involved devising a routine means of (i) predicting the uptake from the gut of all energy-yielding components of food (sugars, starch, fats, protein, alcohol) as a result of upper-tract digestion; (ii) predicting the production of short chain fatty acids (SCFA) in the hindgut; and (iii) predicting the available energy (mol of

ATP) as a result of the post-absorptive catabolism of known quantities of absorbed nutrients and SCFA (as predicted by (i) and (ii), respectively).

It is important to be able to predict both the site (upper-tract or colon) and degree of absorption of each nutrient because (i) nutrients vary in the efficiency by which they yield energy that is ultimately useful to the body (net ATP gains), and (ii) the energy made available to the body via SCFA from nutrients fermented in the hindgut is less than that obtained from direct nutrient uptake in the upper-tract. Ideally, the upper-tract digestion of foods would be determined in humans directly. However, there are shortcomings of determining ileal digestibility in humans (ileostomy or intubation studies), such as the heavy demand on resources, the need for a number of willing subjects and the ethical acceptability of using such methods for the routine evaluation of foods. There are also questions regarding the validity of data from ileostomised subjects due to a prolific ileal microbiota, and the potential alteration in digestive function (Christl and Scheppach, 1997; Dowsett *et al.*, 1990). Although comparative studies have not been undertaken for upper-tract nutrient digestibility between the rat and humans, it is well established that the growing pig is a valid model for the adult human for ileal protein digestibility (Deglaire *et al.*, 2009; Moughan and Rowan, 1989; Rowan *et al.*, 1994) and good agreement has also been shown between the growing rat and the growing pig for ileal protein digestibility over a very wide range of diets and sources of protein (Donkoh *et al.*, 1994; Moughan *et al.*, 1984; Moughan *et al.*, 1987; Picard *et al.*, 1984; Rutherford and Moughan, 2003; Smith *et al.*, 1987). Furthermore, the rat and man are both simple-stomached omnivores with similar gastrointestinal anatomy and physiology (Kararli, 1995; Moughan and Rowan, 1989), including a small intestinal transit time of 3 - 4 h (DeSesso and Jacobson, 2001). Throughout the experimental work described herein, the growing rat was chosen over the piglet as an animal model due to economic and logistical advantages in that pigs demand a greater degree of animal husbandry, veterinary intervention and post-surgery recovery time, as well as increased food, housing and labour costs compared to digestibility studies with rats. Furthermore, the terminal anaesthesia method used with the rat digestibility assay is straightforward and more ethically acceptable than using cannulated pigs

because the need for surgery is avoided and there is no ongoing discomfort for the animal.

In Chapter III of this dissertation, the extent to which the type of diet (quantity and source of DF) affects upper-tract and hindgut nutrient digestibility was investigated. This was undertaken using four diverse diets containing either a single DF source (Wheat Bran and Pectin diets) or mixed fibre sources (Low Fibre and High Fibre diets) for two separate studies; a human balance study to predict apparent faecal digestibility and a rat digestibility trial to predict human apparent ileal energy and nutrient excretion. Although there were general similarities in the relative amounts of energy uptake/loss in the upper-tract and colon across the diverse diets, the ratio of the predicted uptake of specific nutrients (colon : upper-tract) was unique to each diet and no two diets exhibited the same predicted uptake ratios across all analysed dietary components. It was reaffirmed that total energy uptake at each site of digestion (upper-tract and hindgut) cannot be predicted using apparent faecal digestibility data alone and hence, there is a need to be able to calculate the quantitative uptake of each nutrient in the upper and lower digestive tracts separately.

In response to this need, a 'Combined Model' was proposed (Chapter IV) to allow prediction of the upper-tract, hindgut and total tract uptakes of energy and nutrients in humans. The Combined Model allows for the prediction of ATP yields from the catabolism of nutrients predicted (based on rat ileal and *in vitro* hindgut digestibility assays) to be absorbed from the upper- and lower-digestive tract, respectively. Whilst the use of the growing rat (or pig) as an animal model is considered a valid means of predicting digestibility in the human upper-tract, no such alternative exists for predicting the uptake of energy from the human hindgut. Although several *in vitro* hindgut digestibility assays have been proposed for this purpose, none meet the criteria necessary for the assay to be used with confidence to quantitatively predict the colonic uptake of energy in man (i.e. values of assay variables have been experimentally optimised, the assay has been validated with human *in vivo* data from controlled studies and the method is applicable for a range of fermentative

substrates). It was deemed necessary, therefore, to develop a robust and valid *in vitro* hindgut digestibility assay through a systematic process of experimentation. This process firstly involving the identification (Chapter V) and optimisation (Chapter VI) of key assay variables affecting *in vitro* dry matter digestibility (DMD) and secondly, the validation (Chapter VII) of the *in vitro* hindgut digestibility assay (when combined with predicted upper-tract digestibility using the growing rat as an animal model) against human *in vivo* digestibility data.

In Chapter V, the effect of altering pH, duration of incubation, presence of shaking during incubation and the concentration of faecal inoculum or digestive enzymes on DMD was investigated for three promising published *in vitro* hindgut digestibility assays (Barry *et al.*, 1995; Boisen and Fernández, 1997; Edwards *et al.*, 1996). Three mixed human diets varying in the type and ratio of soluble and insoluble DF were used as substrates. It was found that the method using synthetic enzymes (Boisen and Fernández, 1997) showed little sensitivity to alteration of assay variables and produced highly variable results and for this reason was not pursued further. Shaking of bottles during incubation did not affect ($P>0.05$) digestibility for any method or diet. This was a somewhat unexpected result given that shaking is commonly, although seemingly unnecessarily, used with *in vitro* hindgut digestibility assays. For the other three assay variables tested (pH, duration of incubation and the concentration of inoculum relative to substrate), a significant ($P<0.05$) effect on predicted DMD was observed for the two *in vitro* methods employing faecal inocula (Barry *et al.*, 1995; Edwards *et al.*, 1996).

Any assay variable that has a large effect on digestibility should be optimised to ensure that the predictions made by the *in vitro* system are accurate and valid when compared to *in vivo* digestibility data. Failure to do so may result in *in vitro* assays that have the ability to correctly rank substrates, but are unable to give accurate absolute data. Although pH was also found to be a key assay variable it was argued (Chapter VI) that the pH levels suggested by the developers of the original *in vitro* assays (Edwards: pH 7.0, Barry: pH 7.3) are suitable, waiving the need for pH to be investigated further.

It remained therefore, for the optimal values for two assay variables (inoculum concentration and duration of incubation) to be determined and this was the subject of the study described in Chapter VI. Throughout this study to optimise key assay variables, ileal digesta pertaining to mixed human diets were used as the substrate for fermentation based on the premise that the substrate for an *in vitro* hindgut digestibility assay should resemble, physically and chemically, the material entering the colon.

The first part of the study predicted hindgut DMD at six different inoculum concentrations (whilst keeping all other assay variables constant) for each of the two modified *in vitro* hindgut digestibility assays (the Barry and Edwards methods). Results indicated that an inoculum concentration of 160 g/L was optimal based on close agreement of observed values with previously published *in vivo* hindgut DMD for similar diets.

Based on these findings, the second part of the study involved selecting the most promising of the two assays (Edwards method) and predicting hindgut DMD at various incubation durations (18, 24, 48 h) using a fixed inoculum concentration (160 g faeces (wet weight) / L buffer). An incubation duration of 18 h, using a mean inoculum digestibility value for calculation purposes, was considered optimal based on observed *in vivo* hindgut DMD values in humans, although there was little difference in estimated *in vitro* hindgut DMD between 18 and 24 h incubation durations. Overall, the optimised Edwards assay gave realistic hindgut organic matter digestibility (OMD) values ranging from 55 to 79% (Wheat Bran diet and Pectin diet respectively) using an 18 h incubation duration.

As an addition to the study, the digestibility of the faecal inoculum itself and the importance of correcting for this in the *in vitro* hindgut digestion assay was investigated. Although considerably lower than the OMD of the substrate (no less than 51% after 48 h), the OMD of the inoculum itself (13% after 48 h) was of significance in calculating estimated digestibility. It is therefore important to correct

the hindgut digestibility values for digestion of the inoculum and it was argued that for calculation purposes, a mean inoculum digestibility (relative to minimum and maximum inoculum digestibility) was suitably valid (pragmatically), although this awaits experimental validation.

The ultimate purpose of developing the *in vitro* hindgut digestibility assay was to combine the assay with predictions of *in vivo* upper-tract digestibility using the rat as an animal model to create a valid dual *in vivo* – *in vitro* digestibility assay that could be used to predict absolute quantities of energy and nutrient uptake across the entire digestive tract. However, in order to ensure that the dual assay could fulfil its purpose it was necessary to undertake validation of the method using reliable human *in vivo* data. As such, the study described in Chapter VII sought to compare apparent total tract OMD as predicted using human *in vivo* data from a balance study (OMD_{human}) with that predicted using the dual assay (OMD_{dual}) for each of four complete human diets (wheat bran, pectin, mixed low fibre, mixed high fibre).

No significant differences ($P>0.05$) were found between OMD_{human} and OMD_{dual} for any of the diets and the two sets of data gave a correlation coefficient of 0.953 ($P=0.047$). Furthermore, removing diet PE from the correlation improved the correlation to 0.998 ($P=0.039$). To extend the number of diets included in the correlation analysis and the OMD range, the dual digestibility assay was used to predict the digestibility of three additional mixed diets using OMD data obtained in a previous human balance study (Zou et al., 2007). The dual digestibility assay predicted the OMD of the three additional diets within 1% of actual OMD (OMD_{human}) and a Pearson's Correlation including all seven diets gave a correlation co-efficient (r) of 0.961 ($P=0.001$). It was concluded that the dual assay is a valid and accurate means of predicting the uptake of dietary energy in humans in absolute terms (as grams of organic matter) for a wide range of mixed diets over the total tract (OMD range: 90 – 97%), as well as separately in the upper-tract and hindgut.

The overall goal of the experimental work described in this dissertation was to calculate the predicted available energy content of a diet (mol ATP / g diet). Having established the validity of the dual digestibility assay, there only remained a need to translate absorbed quantities (g) of the energy-yielding nutrients and SCFA into quantities of ATP (mol). Estimates of ATP yields from the catabolism of the various nutrients (e.g. glucose) have been available for some time. However, it is only recently that accurate information on the mitochondrial efficiency of ATP production has emerged, providing a more accurate prediction of ATP yields. It was therefore deemed necessary to develop new estimates of catabolic ATP yields and costs for glucose (taken as representative of starch and sugars), each fatty acid and amino acid (as well as a 'generic' triacylglycerol and protein), ethanol and each SCFA (and organic matter fermented in the hindgut), based on recent estimates of cellular P/O ratios (2.31 for NADH and 1.38 for FADH₂) (Brand, 2005) and ATP costs of catabolism.

Thus, Chapter VIII describes the development of a series of predictive equations for determining ATP yields / costs (in moles) and their application to the uptake of each energy-yielding nutrient (in grams), as predicted separately in the upper-digestive tract and the hindgut using the dual *in vivo* - *in vitro* digestibility assay and as applied to the adult human in a state of weight-loss. The costs associated with nutrient ingestion, absorption and transport and with the synthesis and excretion of urea produced from amino acid catabolism were also calculated. ATP yields (not including costs for digestion, absorption and transport) were predicted as 28.9 mol ATP / mol glucose; 4.7 – 32.4 mol ATP / mol amino acid and 10.1 mol ATP / mol ethanol, while yields for fatty acids ranged from 70.8 mol ATP / mol lauric acid (C12) to 104 mol ATP / mol linolenic acid (C18:3). The energetic contribution of hindgut fermentation was predicted to be 101.7 mmol ATP / g organic matter fermented. It is to be noted that these estimates of ATP yields differ considerably from 'textbook values' (e.g. 36 – 38 mol ATP / mol glucose) which are generally based on the more traditional integral P/O ratios of 3 for NADH and 2 for FADH₂ (Hinkle, 2005).

Together with the dual *in vivo* – *in vitro* digestibility assay, the mathematical equations presented in Chapter VIII constitute the ‘Combined Model’. The Combined Model allows a diet to be defined in terms of potential ATP yields based on recently proposed P/O ratios and the diet’s experimentally determined nutrient digestibility across the total human digestive tract. The model is therefore a valuable tool for the research and development of foods that need to provide accurate known quantities of dietary energy for subjects undergoing weight-loss. The practical application of the Combined Model for this purpose (to predict the available energy (ATP) content of weight-loss foods) was demonstrated in Chapter IX. Firstly, the uptake of each energy-yielding nutrient in the human was predicted separately in the upper-digestive tract (rat ileal digestibility, $n=11$ rats per diet) and the hindgut using the dual digestibility assay for three commercial meal replacement formulations (Diet A, a weight-gain supplement and diets B and C, weight-loss formulations). The new predictive equations for estimating net ATP yields (as given in Chapter VIII) were then applied to the predicted nutrient uptakes for each diet to determine the predicted available energy content (mol ATP / g diet). The metabolisable energy (ME) content of the diets was also determined using a number of published traditional factorial and empirical ME based models and compared with the predicted available energy content (kJ) to investigate whether the rankings of the diets, in terms of their abilities to deliver utilisable energy to the body, differed according to method of dietary energy estimation.

The Combined Model was successfully able to differentiate between the diets in terms of both total tract gross energy digestibility, which ranged from 86% (Diet B) to 99% (Diet A), and predicted available energy (ATP energy), which was estimated to be 9.6, 8.3 and 7.3 kJ/g for Diets A, B and C, respectively. Determined ME ranged from 17.5 – 18.1, 13.6 – 18.4 and 12.5 – 15.4 kJ/g for diets A, B and C, respectively depending upon the ME model and most, but not all of the ME systems ranked the diets in accordance with the predicted available energy (Diet A>B>C). When the energy content of each diet was compared to that of a baseline food (dextrin), some ME models gave considerably different ratios compared to that predicted by the Combined Model e.g. for Diet C a ratio of 0.96 (Atwater and FDA models) was found versus 0.75 (Combined

Model). As such, the Combined Model may provide a more accurate means of predicting the dietary energy content of foods than some current ME models.

The Combined Model has the potential to be used as a tool for the product development of specialist weight-loss foods. The model can be used to screen ingredients during the early stages of the product development process, particularly novel ingredients designed to have reduced digestibility (and/or impart a high degree of satiety), to identify those ingredients that deliver the lowest predicted available (ATP) energy to the body. Alternatively (or additionally), the model can be used in combination with sensory evaluation to test ingredients once incorporated into potential weight-loss foods. The advantage of using the model to test whole foods or diets is that possible interactions between dietary components affecting nutrient uptake from the gut can be taken into account, which is not possible using generic digestibility factors.

Whilst the use of the growing rat has a number of logistical advantages over the pig as an animal model for human upper-tract digestion, quantities of digesta obtained are much smaller and this increases the number of animals required, particularly for diets that are highly digestible or highly viscous. The use of fresh human faecal inoculum for use in the *in vitro* hindgut digestibility assay requires a number of willing subjects to produce samples on demand and this is impractical if the assay is to be used for routine evaluation.

In summary, the main contributions of the experimental work described in this dissertation are as follows:

- A dual *in vivo* – *in vitro* digestibility assay was developed through systematic experimentation to separately predict the uptake of energy from the upper-tract and hindgut in humans. The methodology and analytical methods for the *in vitro* hindgut digestibility portion of the assay in particular are straightforward and hindgut and total tract OMD results are rapidly available (after 75 h from the beginning of *in vitro* incubation of the ileal digesta).

-
- Through comparison with human *in vivo* data, the dual *in vivo* – *in vitro* digestibility assay was shown to be a valid means of predicting the uptake of energy across the human total tract for a diverse range of mixed diets. There was excellent agreement between total tract OMD as predicted with the dual assay (OMD_{dual}) and human *in vivo* data (OMD_{human}). No significant difference ($P>0.05$) was found between OMD_{human} and OMD_{dual} for any diet and the two sets of data gave a correlation coefficient of 0.953 ($P=0.047$) when all diets were included in the correlation, or 0.998 ($P=0.039$) after removal of the Pectin Diet from the correlation.
 - A series of mathematical equations were developed to predict the available energy (ATP) yields from nutrient uptake (as a result of upper-tract digestion) and SCFA uptake (as a result of hindgut fermentation), and associated ATP costs of nutrient ingestion, digestion, absorption and transport. The equations are based on recent estimates of cellular P/O ratios and are applicable to the adult human in a state of weight-loss (sub-maintenance energy requirements).
 - When the ‘Combined Model’ (dual *in vivo* – *in vitro* digestibility assay + stoichiometric predictive equations) was applied to three meal replacement formulations, the model was successfully able to differentiate between the diets in terms of both energy digestibility and predicted ATP yields. This demonstrates that the model has practical application, particularly in the research and development of weight-loss foods, where it may be a more accurate means of predicting the dietary energy content than some current ME models.

Although other workers have explored the concept of a ‘dual digestibility assay’ to span nutrient uptake over the total tract (see Table 2, Chapter II), there is no such published method (to the authors’ knowledge) that has been validated against human total tract nutrient digestibility data and can therefore provide absolute digestibility data to relate predicted nutrient uptake to the predicted available (ATP) energy delivered by the food at the cellular level. Whilst estimates of ATP yields from the uptake of the various energy-yielding nutrients in humans have been made, these estimates either use outdated cellular P/O ratios (Livesey, 1984), have not equated

ATP yields to dietary intake (mol ATP/ g food)(van Milgen, 2002), are only applicable to a limited number of nutrients (Ferrer-Lorente *et al.*, 2007) or use generic digestibility factors (Birkett and de Lange, 2001). To the authors' knowledge, the Combined Model is the first published model, validated against human *in vivo* data to separately predict the upper-tract and hindgut uptake of all major energy yielding nutrients of a food in humans and convert this into predicted available energy (ATP) at the cellular level using recent estimates of P/O ratios. The model follows the complete process of food assimilation from mouth to the primary site of nutrient metabolism (liver) by simulating the digestive and absorptive processes across the total tract and converting the uptake of nutrients and energy into energy ultimately available to the body at the cellular level (ATP). The ability to quantitatively define a food in terms of ATP content (mol ATP / g food) is considered to be the 'Holy Grail' in terms of describing the energy content of a food because it directly relates dietary energy intake to the quantity and form (ATP) of useful energy ultimately delivered at the cellular level (i.e. from mouth to mitochondrion). The ideal index of available energy would be in terms of 'mol ATP / unit satiety', however this was beyond the scope of the work presented here.

Recommendations for future research

Based on the findings of this research a number of recommendations are made for future research to improve the potential usefulness of the Combined Model.

1. The investigation of another animal model (pig) is warranted in order to increase quantities of ileal digesta obtained.
2. An alternative to the requirement for fresh human faecal inoculum needs to be found from a pragmatic perspective.
3. It remains for the most appropriate means of accounting for the digestibility of the inoculum itself to be experimentally validated for the *in vitro* hindgut digestibility assay (currently a mean value of the minimum and maximum inoculum digestibility is used).
4. The accuracy of the model could potentially be improved if the extent of proton leakage *in vivo* and the cost of digestion (ATP_d) could be determined with

greater certainty.

5. Linking the predicted available (ATP) energy content of a food with predictions of satiety would provide a useful measure for product developers of weight-loss foods to identify ingredients and foods which may deliver minimal available energy to the body whilst also depressing post-meal hunger.

Literature cited

- Barry, J.-L., Hoebler, C., Macfarlane, G. T., Mathers, J. C., Reed, K. A., Mortensen, P. B., Nordgaard, I., Rowland, I. R. & Rumney, C. J. (1995). Estimation of the fermentability of dietary fibre *in vitro*: a European interlaboratory study. *British Journal of Nutrition*, *74*, 303-322.
- Birkett, S. & de Lange, K. (2001). Calibration of a nutrient flow model of energy utilization in growing pigs. *British Journal of Nutrition*, *86*, 675-689.
- Boisen, S. & Fernández, J. A. (1997). Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. *Animal Feed Science and Technology*, *68*, 277-286.
- Brand, M. D. (2005). *The efficiency and plasticity of mitochondrial energy transduction*. Paper presented at the Keilin Memorial Lecture, Glasgow.
- Christl, S. U. & Scheppach, W. (1997). Metabolic consequences of total colectomy. *Scandinavian Journal of Gastroenterology - Supplement*, *222*, 20-24.
- Deglaire, A., Bos, C., Tomé, D. & Moughan, P. J. (2009). Ileal digestibility of dietary protein in the growing pig and adult human. *British Journal of Nutrition*, *102*(12), 1752-1759.
- DeSesso, J. M. & Jacobson, C. F. (2001). Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology*, *39*, 209-228.
- Donkoh, A., Moughan, P. J. & Smith, W. C. (1994). The laboratory rat as a model animal for determining ileal amino acid digestibility in meat and bone meal for the growing pig. *Animal Feed Science and Technology*, *49*, 57-71.
- Dowsett, J., Gibney, M. J. & Kennedy, N. P. (1990). Bacterial fermentation occurs in the terminal ileum of ileostomates. *Proceedings of the Nutrition Society*, *49*, 110.A. (abs.).
- Edwards, C. A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J. C., Nagengast, F., Rumney, C. & Quehl, A. (1996). *In vitro* method for quantification of the fermentation of starch by human faecal bacteria. *Journal of the Science of Food and Agriculture*, *71*, 209-217.

-
- Ferrer-Lorente, R., Fernandez-Lopez, J. A. & Alemany, M. (2007). Estimation of the metabolizable energy equivalence of dietary proteins. *European Journal of Nutrition*, 46, 1-11.
- Hinkle, P. C. (2005). P/O ratios of mitochondrial oxidative phosphorylation. *Biochimica et Biophysica Acta*, 1706, 1-11.
- Kararli, T. T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharmaceutics & Drug Disposition*, 16, 351 - 380.
- Livesey, G. (1984). The energy equivalents of ATP and the energy values of food proteins and fats. *British Journal of Nutrition*, 51, 15-28.
- Moughan, P. J. & Rowan, A. M. (1989). The pig as a model animal for human nutrition research. *Proceedings of the Nutrition Society of New Zealand*, 14, 116-123.
- Moughan, P. J., Smith, W. C. & James, K. A. C. (1984). Preliminary observations on the use of the rat as a model for the pig in determination of apparent digestibility of dietary proteins. *New Zealand Journal of Agricultural Research*, 27, 509-512.
- Moughan, P. J., Smith, W. C. & Kies, A. K. (1987). Comparison of the ileal digestibility of amino acids in ground barley for the growing rat and pig. *New Zealand Journal of Agricultural Research*, 30, 59-66.
- Picard, M., Bertrand, S., Duron, M. & Maillard, R. (1984). *Comparative digestibility of amino acids using 5 animal models: intact cockerel, caeectomised cockerels, rat deprived of large intestine, piglet with an ileo-caecal cannulation, piglet with an ileo-rectal shunt*. Paper presented at the Proceedings of the IVth European Symposium of Poultry Nutrition, Tours, France.
- Rowan, A. M., Moughan, P. J., Wilson, M. N., Maher, K. & Tasman-Jones, C. (1994). Comparison of the ileal and faecal digestibility of dietary amino acids in adult humans and evaluation of the pig as a model animal for digestion studies in man. *British Journal of Nutrition*, 71, 29-42.
- Rutherford, S. M. & Moughan, P. J. (2003). The rat as a model animal for the growing pig in determining ileal amino acid digestibility in soya and milk proteins. *Journal of Animal Physiology and Animal Nutrition*, 87, 292-300.

Smith, W. C., Moughan, P. J., Pearson, G. & James, K. A. C. (1987). Comparative bioavailable energy values of five ground cereal grains measured with growing rats and pigs. *Animal Feed Science and Technology*, 18, 143-150.

van Milgen, J. (2002). Modeling biochemical aspects of energy metabolism in mammals. *Journal of Nutrition*, 132, 3195-3202.

WHO. (2000). *Obesity: Preventing and managing the global epidemic*. Geneva: World Health Organization.

Zou, M. L., Moughan, P. J., Awati, A. & Livesey, G. (2007). Accuracy of the Atwater factors and related food energy conversion factors with low-fat, high-fiber diets when energy intake is reduced spontaneously. *American Journal of Clinical Nutrition*, 86, 1649-1656.