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# Complex Metaprobe Production and Characterisation for Metabolic Studies in Relation to Bowel Health

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Research Conducted at
Stable Isotope Biochemistry Laboratory,
S.U.E.R.C.
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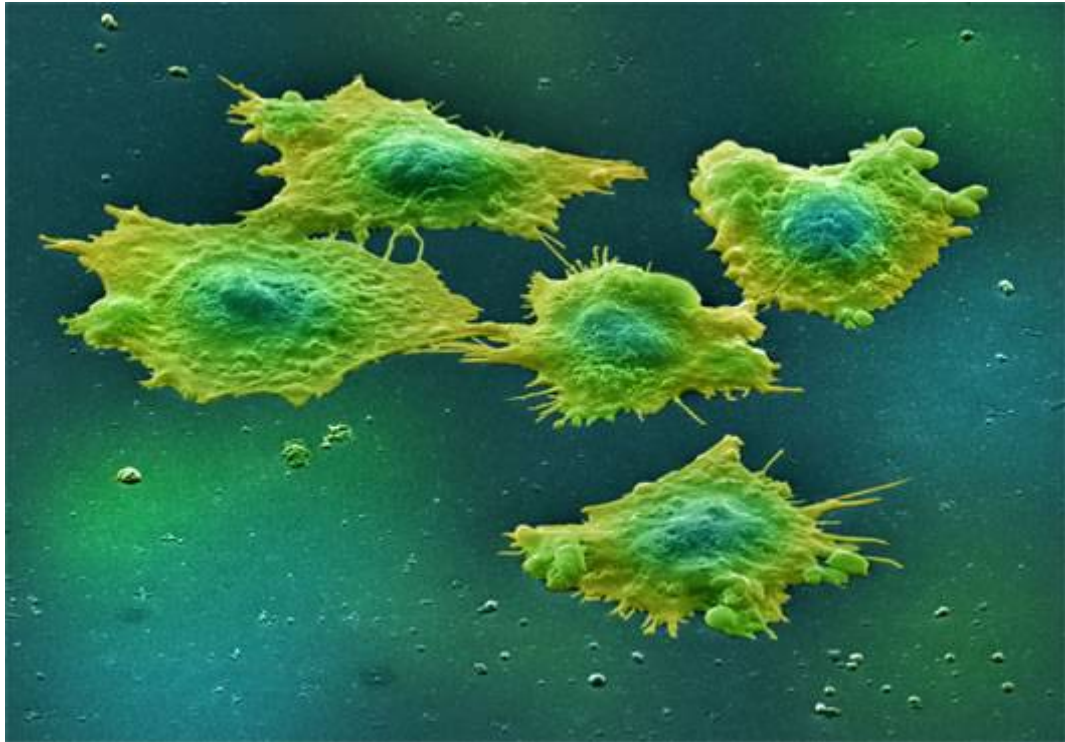
Colon Cancer Cells
Wellcome Trust Medical Photographic Library.

This Thesis is dedicated to my
Husband Keith and my children,
Laurence & Juliet

Barley grain sown, labelled, harvested & photographed by the author

"More than 40% of some cancers can be prevented by a mixture of healthy eating and exercise. Cutting down on red meat and alcohol whilst increasing intake of fruit and vegetables, could prevent thousands of cancer cases each year."

A quotation from a report entitled "Policy and Action for Cancer Prevention" funded by World Cancer Research Fund (2009)

**Abstract**

## Complex Metaprobe Production and Characterisation for Metabolic Studies in Relation to Bowel Health

Introduction Colorectal cancer is the third most common cancer in the UK and the second most common cause of cancer mortality.  There is increasing evidence that the presence of a systemic inflammatory response plays an important role in predicting survival in patients with colorectal cancer. The Glasgow Prognostic Score includes a measure of systemic inflammatory response and has been used successfully as a predictor of survival rate in cancer patients.

Butyric acid is one of the short chain fatty acids (SCFA) produced by colonic bacteria through fermentation of dietary non-digestible carbohydrate (NDC). This simple organic acid is known to have anti-inflammatory and possibly direct anti-neoplastic effects. The inaccessibility of the human colon means that little is known of the in vivo rate of butyrate production, its metabolic fate or the foodstuffs that may maximise its production.  Butyrate may be produced in greater quantities from other SCFA such as acetate and lactate rather than by direct production by saccharolytic bacteria.   Specialist organisms may be responsible for producing butyrate through conversion of other SCFA which are produced by fermentation in the colon.  The distal site of butyrate production may be a very important feature and the implication that slowly fermentable NDC, such as resistant starch, may be significant.

Previous Work The European Union project EUROSTARCH ([www.eurostarch.org](www.eurostarch.org)) investigated the way in which different starchy foods are digested and metabolised within the body.  The results of this project have informed the development of low GI foods.  Results have also increased our knowledge of NDC fermentation and promise to lead to improvements in human bowel health.  We pioneered the measurement of starch digestion and fermentation during this project.

What are Metaprobes? The term metaprobe is used to describe stable isotope labelled tracers. Complex metaprobes are produced by biological synthesis e.g. the introduction of $^{13}$C enriched $CO_2$ gas during the production of crops. Simple metaprobes are produced by chemical synthesis e.g. urea. We can produce complex metaprobes by labelling staple foods such as cereals using the stable isotope $^{13}$C incorporated from enriched $^{13}CO_2$ during anthesis of the crops. Complex metaprobes are unique tools with which to study human metabolism in health and disease and they can be used in several ways. The labelled cereals allow in vivo measurement of butyrate production from dietary NDC and to permit its production to be monitored.

Hypothesis 30% of cancer deaths in the UK are bowel related. Butyric acid produced naturally through fermentation of dietary carbohydrates, is a potent and natural anti-inflammatory agent which can protect against cancer. We urgently need to develop methods to quantify butyrate production in vivo.

Aims Three aims are recognised: optimising the preparation of complex metaprobes of barley and wheat grain; optimising yield and $^{13}$C-enrichment of whole grain and its starch and protein fractions; using complex metaprobes to compare SCFA production in vivo.

Plan of Work This project will include a review of complex metaprobe production for human nutrition and health studies in relation to bowel health. The practical work will include production and characterisation of complex metaprobes ($^{13}$C-labelled cereals) and their application to study butyrate production in a pilot study on volunteer human subjects.

I have successfully grown cereal crops with different varieties of barley and wheat in glasshouse conditions. During the anthesis phase, the plants were placed in growth cabinets at 18$^o$C with 18 hours of light in every 24 hours. The plants were labelled using $^{13}$C enriched $CO_2$ gas and are incubated for a further 24 hours and returned to the glasshouse. Examples of the harvest are Wheat var Paragon produced 1.7 Kg labelled grain at 0.278 ape $^{13}$C enrichment and Barley var Cellar produced 3.4 Kg labelled grain at 0.198 ape $^{13}$C enrichment. Some of the $^{13}$C labelled grain has already been used for human nutrition studies. The

$^{13}$C-enrichment of starch glucose has been measured by liquid chromatography isotope ratio mass spectrometry (LC-IRMS) analysis.

These cereal grains can be incorporated into common foodstuffs for in vivo studies of the production of butyrate and other SCFA. Volunteers were fed two meals made from $^{13}$C-enriched barley grain: porridge and whole grain barley. Whole grains being rich in resistant starch. Samples of urine will be used to measure butyrate production. Breath samples will be used to measure oxidation of the $^{13}$C-enriched barley, which is a global measure of digestion and fermentation. Laboratory procedures such as ultrafiltration and solid phase extraction will be applied to urine samples. These sample preparation procedures will be followed by SCFA analysis by GC-IRMS.

Future Direction Future clinical trials will be considered with Prof Donald C McMillan, University Dept of Surgery, Glasgow Royal Infirmary as a means of identifying the role of butyrate in colonic cancer.  The current screening process recognises three phases: early stage diagnosis; polyp development; diagnosis and treatment of advance stage colonic cancer.  We wish to study the role of butyrate and its production using our newly developed complex metaprobe tools.

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"I have finally come to the conclusion that a reliable set of bowels is worth more to a man than any quantity of brains"

Henry Wheeler-Shaw (1818-1885)

I wish to acknowledge

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# Chapter 1: Literature Review

# 1  Introduction

## 1.1 Carcinogenesis & Colorectal Cancer

Cancer is the leading cause of death worldwide among individuals aged 35-64 years of age and globally responsible for $> 0.5 \times 10^6$ deaths annually.  In the UK, approximately one in three people will contract this disease in their lifetime and one in four of the population will die from cancer or a cancer related disease. [39]  Colorectal cancer (CRC) is the third most common cancer in the UK and the second most common cause of cancer mortality.  In Scotland in 2009 with a population of 5,174,616, CRC is the second most common cause of death of both males and females after cardiovascular disease.  A personal history of inflammatory bowel disease, the main types of these inflammatory conditions being Crohn's disease and Ulcerative Colitis, has been associated with an increased risk for CRC. [26]

| Causes of Deaths | | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 |
|---|---|---|---|---|---|---|---|---|---|---|
| Neoplasm | | | | | | | | | | |
| | M | 7,609 | 7,891 | 7,923 | 7,763 | 7,800 | 7,782 | 7,847 | 7,926 | 7,841 |
| | F | 7,646 | 7,584 | 7,468 | 7,649 | 7,536 | 7,626 | 7,513 | 7,644 | 7,684 |
| Neoplasm of Colon | | | | | | | | | | |
| | M | 515 | 513 | 507 | 488 | 479 | 485 | 464 | 443 | 500 |
| | F | 537 | 549 | 468 | 478 | 438 | 481 | 458 | 456 | 440 |
| Neoplasm of Rectum | | | | | | | | | | |
| | M | 329 | 336 | 344 | 351 | 372 | 375 | 375 | 379 | 339 |
| | F | 235 | 246 | 259 | 295 | 279 | 234 | 271 | 285 | 306 |
| Diseases of the Circulatory system | | | | | | | | | | |
| | M | 11,058 | 10,455 | 10,405 | 10,379 | 9,761 | 9,434 | 8,798 | 8,999 | 8,497 |
| | F | 12,599 | 12,211 | 12.283 | 11,722 | 11,076 | 10.626 | 9,973 | 9,580 | 9,352 |
| Ischaemic Heart Diseases | | | | | | | | | | |
| | M | 6,578 | 6,258 | 6,190 | 6,200 | 5,814 | 5,629 | 5,099 | 5,260 | 4,852 |
| | F | 5,834 | 5,656 | 5,502 | 5,241 | 4,964 | 4,702 | 4,433 | 4,083 | 3,989 |

Table 1.1 Causes of death of males and females in Scotland 2000-2008

General Register for Scotland

Cancer (malignant neoplasm) is a class of disease associated with genetic abnormalities in the cell. Activation of a protein encoding oncogene, increases the chances of a normal cell developing into a tumour cell. Tumour suppressor genes will try to prevent DNA damage in these cells, but inactivation of tumour suppressors or up regulation of oncogenes, will result in DNA damage with apoptosis. (48) Carcinogenesis is a complex, multipath process often described as a somatic evolution, or an accumulation of mutations over the lifetime of a cell. (24)

## 1.1.2 Carcinogenic Stages

Carcinogenesis has three stages: the initiation stage where initiation agents, physical, biological or chemical, alter the molecular structure of cellular DNA with irreversible changes. This causes groups of cells displaying uncontrolled growth; the promotion stage, where promoting agents, which are a variety of substances including hormones and protein growth factors, influence genetic expression by binding to receptors on cell surfaces, the cytoplasm or nucleus; the progression stage, when morphological changes and tumour progression occur, leading to metastasis.

Overall survival after diagnosis of CRC is poor, even in those patients who undergo curative resection; more than one-third will die within 5 years. Dukes stage A, B, C, or D system or TNM staging (see Figs 1.1 & 1.2) are histopathological criteria for predicted survival rate in CRC. It is not only the intrinsic properties of tumour cells that determine invasion and metastasis, but the tumour environment. Tumour progression is a complex process that depends on interactions between tumour and host cells. Host cells often create a systemic inflammatory response, where pro-inflammatory cytokine expression and growth factors may promote further tumour growth. (44)

### 1.1.3 C-Reactive Protein (CRP)

C-reactive protein (CRP) concentration is used as a marker of systemic inflammation. It is a protein produced in the liver during episodes of acute inflammation. It is released into the bloodstream.   Its principle role is opsonisation or binding of cell debris and microbial invaders to facilitate their disposal (see glossary). CRP is expressed in response to interleukin-6 (IL6) (see glossary). (26) These facts help to explain why both anti-inflammatory and anti-neoplastic agents have received much research attention. As will be discussed in this thesis, dietary components may provide a natural source of such agents.

Raised CRP levels (>10 mg/L) and hypoalbuminaemia (<35 g/L) indicate a systemic inflammatory response. The Glasgow Prognostic Score (GPS) is a measure of systemic inflammatory response. It is a numerical score of 0, 1 or 2. A GPS of 0, indicates no biochemical abnormalities with a 94% overall survival rate, but elevated scores will lead to increased protein breakdown, nutritional decline and poor survival outcome in cancer patients. (45)  Raised CRP or hypoalbuminaemia score 1 on the GPS, whereas raised CRP together with hypoalbuminaemia score 2.

Dukes A

Dukes B
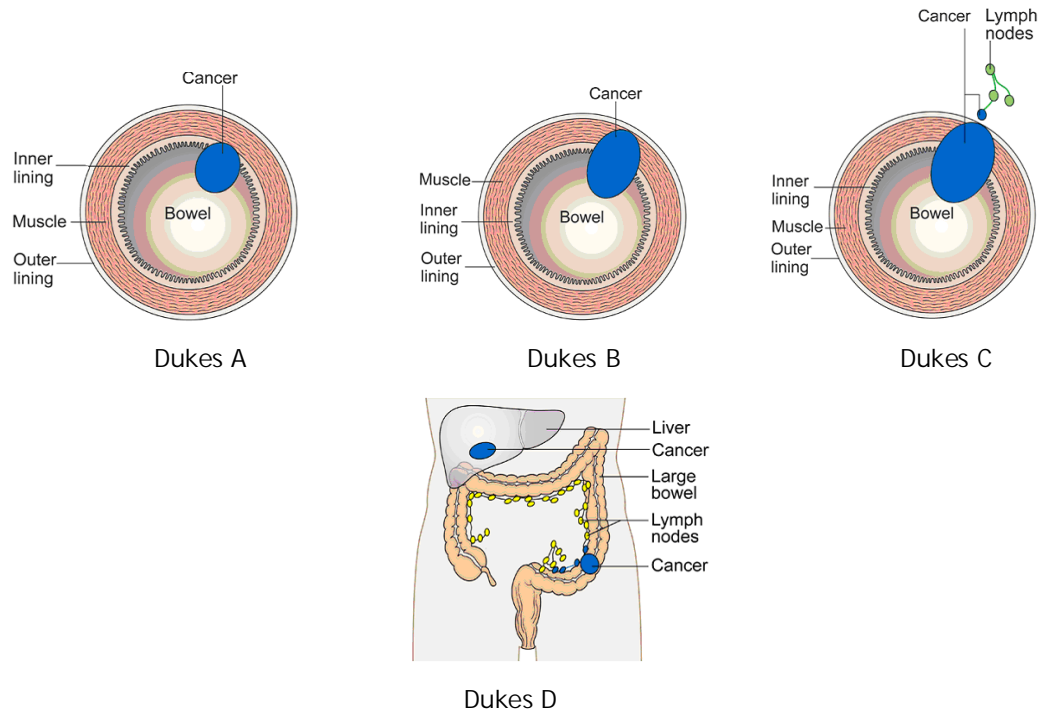
Dukes C

Dukes D

Dukes A – Tumour has invaded submucosa & muscle layer of bowel, but confined to the wall.

Dukes B – Tumour breached to muscle layer & bowel wall, no involvement in lymph nodes.

Dukes C – Tumour has spread to lymph nodes.

Dukes D – Distant visceral metastases, advanced cancer spread to liver & other organs.

Fig 1.1: Diagnosis of Colorectal Cancer using Dukes Staging System (Cancerhelp UK)
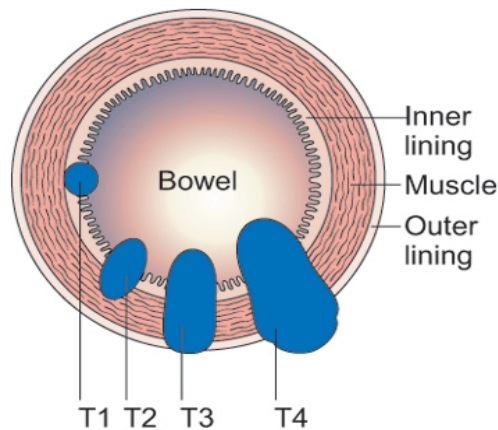


T1 – Tumour sited in inner layer of the bowel.

T2 – Tumour has grown into the muscle layer of the bowel wall.

T3 – Tumour has developed into outer lining or into adjacent organs or structures.

T4 – Tumour has spread into liver, small bowel or peritoneum.

Fig 1.2: T stages of Tumour Node Metastases (TNM) staging of Colorectal cancer. (Cancerhelp UK) See glossary for further explanation of both staging systems

Colorectal cancers (CRC), are cancerous growths in the colon, rectum or appendix. There are many reasons for developing colorectal cancer. Benign colorectal polyps can become malignant; inflammatory conditions such as chronic ulcerative colitis or a family history of the disease (genetic predilection) are all implicated. Diet and lifestyle factors (physical inactivity, smoking and high alcohol intake; >30 grams of alcohol per day) are significant factors in the development of CRC.

There are four main types of bowel cancer: Adenocarcinoma is tumours in the lining of the bowel; Sarcoma is tumours that grow in the muscle wall of the bowel; Carcinoid tumours occur in the appendix or ileum; Lymphoma, which are tumours in the lymph tissue of the bowel.

## 1.2 Diet and Lifestyle

Diet is important in the aetiology of CRC and other chronic diseases with red meats and diets containing high levels of saturated fatty acids considered primary negative factors. Dietary fibre consumption is associated with reduced CRC risk. Foods containing fibre, nutrients and phytochemicals, may offer cancer protection as increasing the amount of fibre lowered the risk of CRC.[7] Fruit, vegetable and cereals may also have a protective effect. Certain types of dietary carbohydrate may prevent this disease developing. Non-digestible carbohydrates (NDC) are an important component of dietary carbohydrate that passes undigested into the caecum, where they may be fermented. Short chain fatty acids (SCFA) are products of carbohydrate fermentation and may have important beneficial effects in the bowel, including anti-inflammatory and anti-neoplastic effects, which will be discussed further.

Growth, development and the maintenance of health require an adequate supply of nutrients available to all the cells in the body. The foods we eat are composed of a complex mixture of molecules. The most important energetically are macronutrients. Macronutrients largely consist of carbohydrates, fats and proteins and are insoluble. Macronutrients are digested in the small intestine and their simple digestion products are absorbed by diffusion or active

transport. (72)  Foods are grouped according to their macronutrient content. The five food groups according to British Dietary Association are: fruit and vegetables; bread, cereals and potatoes; meat, fish and alternatives; milk and dairy foods; foods containing fat, food and drinks containing sugar.  However, these groups include foods with different chemical composition.  The quality of a diet must consider the chemical structure, digestibility, metabolism and functionality of the foods as well as individual macronutrients. (31)

## A Healthy Diet Should Contain

- Fruit & Vegetables
- Bread, other cereals & potatoes
- Meat, fish & alternatives
- Milk & dairy foods
- Foods containing fat, foods & drinks containing sugar

7%
15%
33%
12%
33%
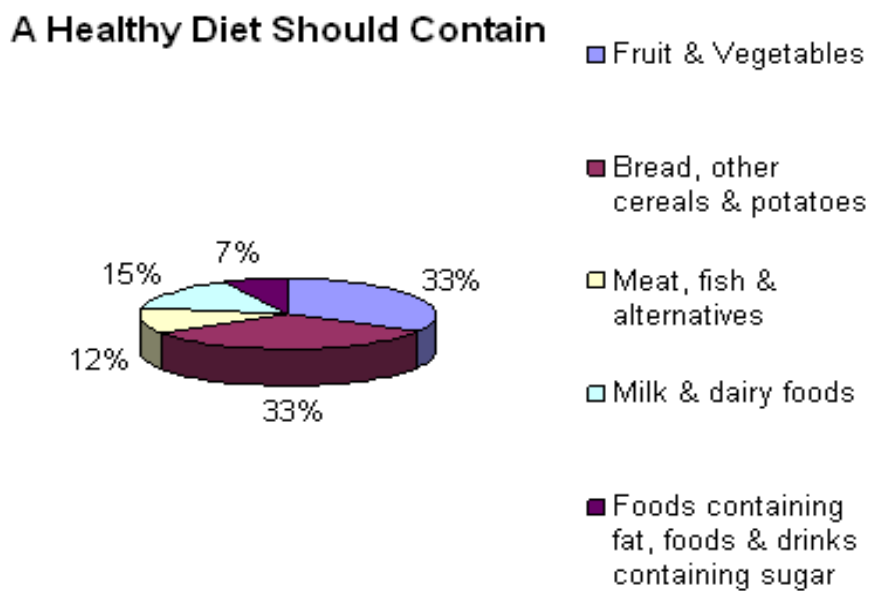
Fig 1.3: No single food contains all the nutrients required but consuming a wide variety of different foods each day is essential.  A healthy diet is selecting foods from each of the above group in the proportions as shown. (The British Dietary Association & Food Standards Agency, 2007)

## 1.2.1 A Healthy Diet

- Fruit and leafy vegetables (33%) are low in fat and calories and contain Vitamin C, carotenes, foliate, fibre, carbohydrates and potassium.

- Starchy foods such as bread, pasta, rice, cereals and potatoes should be the main part of most meals (33%).  Wholegrain foods digest more slowly and create a feeling of satiety.  Starchy foods are a good source of energy as they contain carbohydrate (starch), fibre, calcium, iron and vitamins B.

- Meat and fish (12%) are good sources of protein, iron, vitamins B12, D and E and the minerals: zinc; magnesium; selenium and copper.  Portion size should be approximately 50 – 100 g.

- Milk and dairy foods (15%) provide most of our calcium.  They also provide protein, zinc and vitamins B12, B2, A and D.  They should be included in the diet at least three times a day.

- Food containing fat, foods and drinks containing sugar (7%) provide energy as they contain fat and carbohydrates (sugar) but have few vitamins or minerals. This category is high in saturated fat and we should eat less than 30 g/per day of these foods.  Fatty foods, should be replaced with starchy foods and include food containing unsaturated fat to lower cholesterol levels in the blood.  (www.eatwell.gov.uk).

The World Health Organisation (WHO) has a Global Strategy on Diet, Physical Activity and Health to help fight heart disease, stroke, diabetes, cancer and obesity-related conditions.(56) This strategy encourages people to be more physically active and eat healthier diets and they recommend that a diet should include the following:

- achieve energy balance and a healthy weight
- limit energy intake from total fats and shift fat consumption from saturated fats (>5g saturates per 100g) to unsaturated fats (<1.5g saturates per 100g) while eliminating trans-fatty acids (trans fat is not a problem in UK diet)
- increase consumption of fruits and vegetables, and legumes, whole grains and nuts
- limit the intake of free sugars
- Limit salt (sodium) consumption from all sources and ensure that any salt purchased/used has been pre-iodized, that are mixed with a small amount of potassium iodide.  A high intake is considered 0.6 g/day sodium with a low intake as 0.3g/day.

WHO also recommends that saturated fats be replaced with unsaturated fats and carbohydrates. The optimal type of carbohydrate is the subject of intense research effort of which this thesis is a part. Carbohydrates can affect both physiological and metabolic processes by reducing risk factors for disease or the disease processes but excessive consumption of carbohydrates can be a risk factor for cardiovascular disease and is responsible for the rapidly increasing incidence of obesity and type II diabetes.

Affluent people of the developed world commonly use fat rather than carbohydrates as the main source of their diet. Populations of the developing world may have a staple diet composed almost entirely of cereals such as rice, with carbohydrates providing the overwhelming source of energy.  The more affluent people in the developing world are increasingly showing the diet-related problems of the West. Previous generations in the UK consumed a more wholesome diet high in complex carbohydrates and not pre-prepared meals.  To

understand how to achieve a balanced diet to compliment a healthy lifestyle, we must understand how the body digests and absorbs nutrients.
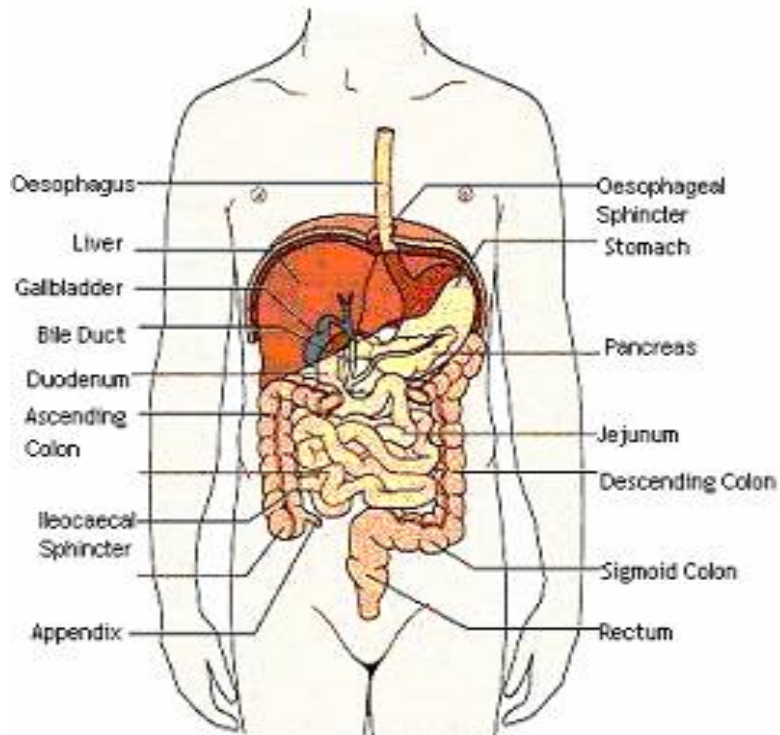


Fig 1.4: Anatomy of the Digestive System (ISD, Scotland)
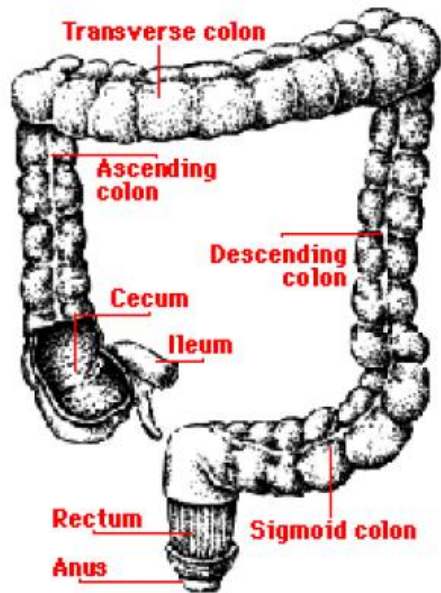


Figure 1.4a: The colon is divided into 4 sections, ascending, transverse, descending & sigmoid. (ISD Scotland)

# 1.3 Digestive Enzymes & Gastrointestinal Tract

The whole process of digestion consists of hydrolytic cleavage reactions. Dietary macronutrients are large polymeric structures, unable to be absorbed until hydrolyzed by enzymes in the gastrointestinal (GI) tract. The breakdown products include monosaccharides, amino acids and fatty acids, are able to be absorbed. Daily, there are approximately 30 g of digestive enzymes secreted. As the enzymes have narrow substrate specificity and only hydrolyze certain bonds, several enzymes have to co-operate in the digestion of complex nutrients.[47] (see Table 1.2). The pancreas secretes many of these digestive enzymes.

Here we will consider the full digestive fate of dietary macronutrients, particularly carbohydrates.

| Nutrient | Products Generates | Enzymes | Sites of Digestion |
|---|---|---|---|
| Starch, glycogen | Glucose | $\alpha$-Amylase, disaccharidases & oligosaccharidases | Saliva, intestinal lumen & brush border |
| Maltose | Glucose | Glucoamylase, sucrase | Brush Border |
| Sucrose | Glucose + fructose | Sucrase | Brush Border |
| Lactose | Glucose + galactose | Lactase | Brush Border |
| Proteins | Amino acids, tri & dipeptides | Pepsin, pancreatic & Brush border enzymes | Stomach, intestinal lumen & brush border. |
| Triglycerides | Fatty acids | Pancreatic lipase | Intestinal lumen |
| Nucleic acids | Nucleosides | DNAses, RNAses | Intestinal lumen. |
| Fibre | Acetate, propionate, lactate, $H_2$, $CH_4$, $CO_2$ | | Fermentation by colonic bacteria |

Table 1.2: Dietary Nutrients and their Fates in the GI Tract (Principles of Medical Biochemistry, Meisenberg et al, (47))

### 1.3.1 Mouth & Saliva

The first stage of the process of digestion begins in the mouth.  The digestive function of the mouth and the oesophagus are mastication, taste and swallowing with saliva secretion.  Three pairs of salivary glands, parotids, submandibular and sublingual, secrete saliva.  The main function of saliva is not digestion but the conversion of food into a homogenous mass during mastication.  The enzyme $\alpha-$amylase in saliva cleaves glycosidic bonds in starch. (See Table 1.2)

### 1.3.2 Stomach

Swallowing is a complex physiological act where food passes from the mouth to the stomach through the action of peristaltic waves in the oesophagus.  The stomach lies between the oesophagus and the duodenum (see fig 1.4). The primary function of the stomach is to hold the ingested food and to regulate its release into the duodenum.  Its secondary function is to churn and mix food along with the secretions of the stomach to produce chyme.  Paracrine secretions include histamine, which stimulates the exocrine secretion of gastric juice.  Gastric juice is highly acidic and contains the enzyme pepsin and other digestive factors.

The acidic gastric juice kills microorganisms and denatures dietary proteins.  Pepsin is a protease, which acts in acid and cleaves the bonds after the N terminal of the large hydrophobic amino acids (Phe,Try,Tyr) to produce peptone, oligopeptides and free amino acids.  Chyme passes from the stomach to the duodenum via the pyloric sphincter (see fig 1.4).

### 1.3.3 Small Intestine; duodenum, jejunum & ileum.

The small intestine is largely responsible for digestion and absorption. The small intestine comprises approximately six meters of tubing from the stomach to the colon.  It has three sections, the duodenum, the jejunum and the ileum. Many digestive enzymes enter the small intestine from the pancreas and the liver, whilst others are produced by cells lining the small intestine.  The surface of the small intestine has many small pits lying between epithelium covered intestinal

villi and secreting mucus goblet cells (Fig. 1.5). The large surface area of the villi, microvilli and the brush border, increases absorption. The peristalstic movements of the small intestine mix the chyme with digestive juices so that absorption is maximal. (74)
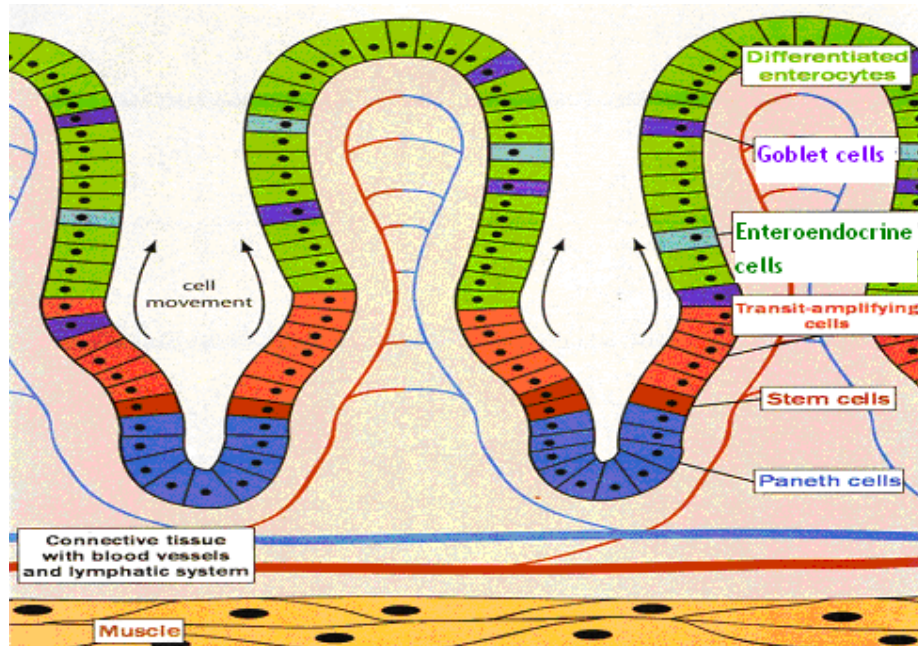


Fig 1.5: Intestinal Epithelium (Maintaining the Whole, Open University (72))

Digestion continues in the small intestine. As the chyme enters the duodenum it contains triglycerides, protein and carbohydrates including starch which has escaped hydrolysis by salivary amylase. Pancreatic secretions contribute a large range of digestive enzymes. The acid chyme, is buffered by bicarbonate ions in pancreatic juice, to achieve a neutral pH of 7. Proteins are broken down into tri and dipeptide fragments by trypsin and chymotrypsin and the further breakdown into amino acids occurs in the epithelial cells. Carbohydrates enter the duodenum as disaccharides (maltose, sucrose or lactose), dextrins and non-digestible carbohydrates (NDC). Disaccharides, are converted to monosaccharides by brush border enzymes. Fats are hydrolysed by lipase. Bile secretions include bile salts, which promote emulsification and dispersion of fats as micelles. Nucleic acids, are broken down by nucleases and nucleotidases into a mixture of purine and pyrimidine bases, phosphoric acid and pentose sugars, but these are minor macronutrients of little importance in terms of energy intake. (74)

Absorption of amino acids is rapid in the duodenum and jejunum but slower in the ileum. Monoglycerides, cholesterol and fatty acids form micelles, which are absorbed in the small intestine by passive diffusion. Long chain fatty acids (of more that 10-12 carbons units) are esterified into triglycerides and enter the lymphatic for transport to the liver. Undigested material, including NDC, passes into the large bowel. (76) NDC is comprised of non-starch polysaccharides (NSP), resistant starch (RS) and non-digestible oligosaccharides. (see section 1.4)

### 1.3.4 Large Intestine; caecum, colon & rectum.

The large intestine begins at the ileocaecal junction and consists of the caecum, to which the appendix attaches, the ascending, transverse and descending colon and the rectum (see fig 1.4a). The caecum and colon fill with undigested material carried by peristaltic waves from the ileum following relaxation of the ileocolic sphincter. This undigested material moves slowly taking between 12-24 hours depending on the diet, as it proceeds to the rectum. The volume of undigested material reduces in the colon by absorption of water in the caecum and the ascending colon. This absorption of water and ions is important in the maintenance of normal water and salt levels in the body. Sodium and amino acids are absorbed, but protein, fats, sugars and calcium are not absorbed. The colon secretes mucus from the goblet cells, which lubricates the faeces and neutralises any acid that has formed. Before we discuss the metabolic products produced from undigested macronutrients that reach the colon, we should consider carbohydrate nutrition in greater detail.

## 1.4 Dietary Carbohydrates; sugars, oligosaccharides & polysaccharides.

Carbohydrates are the major fuels of the body. The major dietary carbohydrates are in three principal (chemical) groups, sugars, oligosaccharides and polysaccharides. Each class of carbohydrate has a variety of physiological effects. The physiological effects of dietary fibre are that it reaches the colon without absorption in the gut. (28)

| Class | Sub-Group | Components |
|---|---|---|
| Stachyose | Monosaccharide | Glucose, galactic, fructose |
| | Disaccharides | Sucrose, lactose, trehalose |
| | Polyols | Sorbitol, mannitol |
| Oligosaccharides | Malto-oligosaccharides | Maltodextrins |
| | Other oligosaccharides | Raffinose, stacchyose |
| Polysaccharides | Starch | Amylose, amylopectin |
| | Non-starch polysaccharides | Cellulose, hemicellulose, pectins |

Table 1.3: Major Dietary Carbohydrates (FAO) (55)

"Complex carbohydrates" is a term, previously used to describe dietary fibre-rich polysaccharides in foods such as whole grain cereals. The term Non-Digestible Carbohydrates (NDC) is now preferred.

## 1.4.1 Polysaccharides: Starch & Resistant Starch

Starch is found in abundance in plant tissues especially in the endosperm of seeds, in root vegetables and certain starchy fruits. Natural starches comprise two main molecular structures: amylose, consisting of long linear chains of glucose, and amylopectin, consisting of short bound glucose chains of variable length with a high content of branching chains. The proportion of amylase to amylopectin is variable and is the major determinant of their digestibility in the small intestine.

Nutritionally, starch divides into glucogenic, which is available carbohydrate, and resistant starch (RS) which is undigested, but it is fermentable. Glucogenic foods can be further divided into providing rapidly and slowly available glucose (www.Eurostarch.org). There are four forms of resistant starch (see Table 1.5). $RS_1$ is physically enclosed starch in whole grains of cereals and in intact cell structures. $RS_2$, includes raw starch granules. $RS_3$ is retrograded amylose and amylopectin,

which can be strongly influenced by preparation and can be altered by genetic engineering in plants; $RS_4$ is chemically modified starch. Physically ($RS_3$) or chemically ($RS_4$) modified starches have important nutritional properties which can be exploited in the formulation of foods with low GI. RS is an important component of NDC.

| Types of Resistant Starch | Examples of Occurrence |
| --- | --- |
| $RS_1$; physically inaccessible | Partly milled grains and seeds. |
| $RS_2$; resistant granules | Raw potato, green banana, some legumes and high-amylose starches. |
| $RS_3$; retrograded | Cooked and cooled potato, bread and cornflakes. |
| $RS_4$; chemically modified | Etherised, esterified or cross-bonded starches (used in processed food) |

Table 1.4: Nutritional Classification of Resistant Starches (SCFA & Human Colonic Function, Topping et al 2001) (78)

## 1.4.2 Non-Starch Polysaccharides

Cellulose is a plant cell wall polysaccharide present in food, which is non-digestible and may be referred to as fibre, roughage or more commonly in recent years, non-starch polysaccharide (NSP). The enzymes of the human gut <u>cannot</u> digest most NSP. NSP is an important component of NDC. There are two types of NSP, insoluble and soluble. Insoluble NSP is present in wheat, rice, vegetables and pulses and soluble NSP, which is found in cereals, such as barley and oats, and legumes. Foods high in insoluble NSP increase the bulk of gut content, which has the benefit in reducing transit time. Low NSP diets can cause "pocketing" or diverticula in the gut, which can lead to constipation, inflammation and disease.

## 1.4.3 $\beta$–glucans

$\beta$-glucans are soluble NSP which are present in the bran and other components of dietary cereal grains, most abundant in barley and oats but to a much lesser degree in rye and wheat. $\beta$-glucans are also present in legumes. Wheat and rye

have a β-glucan content of 3-7%, but oats and barley have more, with some barley varieties having 14% β-glucan content. (1)

## 1.4.4. Oligosaccharides

Although not a major NDC component in a traditional diet, non-digestible oligosaccharides are widely present in nature, in roots (fructo-oligosaccharides (OS; such as inulin), cereals (e.g. arabinoxylan-OS in wheat), legumes (raffinose, stachyose and verbascose) and milk (galacto-OS). Their inclusion here is justified as they are becoming more common in the Western diet, as additives such as inulin (in breakfast cereals, yoghurts and sip feeds) which are favoured by the food industry. (79)

## 1.4.5. Fermentation of Macronutrients that reach the Colon Undigested.

The large intestine is the site of active bacterial protein synthesis, as carbohydrates stimulate intestinal bacteria. Bacteria synthesise protein from amino acids. Ammonium uptake and conversion to amino acids by colonic microflora is particularly efficient if fermentable carbohydrate is available. Colonic microflora, have an important role in substrate and energy provision in human tissues. The diet strongly influences bacterial metabolism. Specific components of diet such as NDC, may have selective effects on the colonic microbiota and on health.

| Carbohydrate | Protein | Other |
|---|---|---|
| Starch | Dietary | Intestinal glycoprotein |
| Non-starch polysaccharides | Endogenous enzymes | Mucopolysaccharides |
| Unabsorbed sugars | (e.g pancreatic enzymes) | |
| Raffinose, stacchyose | | |
| Polydextrose | | |
| Modified cellulose | | |

Table 1.5: Substrates for Fermentation (Fermentation in the Human Large Intestine, Cummings et al, 1987 (17))

Fermentation is an important large intestinal activity.  Fermentation is the process whereby anaerobic bacteria break down dietary and other endogenous substrates such as carbohydrates (see Table 1.3) to obtain substrates and energy for growth and cellular function. The colonic microflora has a largely anaerobic metabolism. It does not use oxygen as a terminal electron acceptor. Energy is released through anaerobic substrate-level phosphorylation reactions or fermentation.  Humans have a less efficient digestive system in comparison with many animals, as the digestion of most nutrients is incomplete.  Microflora breaks down carbohydrates that pass undigested from the upper gut.  Starch is only 70-90% digested and the residue is available for fermentation. Protein digestion is variable, but keratins and some plant proteins along with endogenous proteins which escape digestion, are available for fermentation. Plant polymers (cellulose, RS, $\beta$-gucan, inulin, pectin, lignin etc) are resistant to human digestive enzymes and are classed as NDC.  A large and diverse bacterial flora including bacteroides, bifidobacteria, lactobacilli and streptococci ferment this NDC. Fermentation produces gases (hydrogen, methane and carbon dioxide) and intermediate products such as lactate and ethanol. Fermentation also stimulates bacterial biomass and short-chain fatty acid (SCFA) production. SCFA can be absorbed through the colonic mucosa and metabolised. (47)

NDC consumption affects the rate of faecal movement and faecal bulk.  Bulky stools eliminate quickly from the colon and this is beneficial, as any bacteria in the colon may increase toxin production, such as particular products of protein fermentation.  Prolonged exposure to potential toxins may contribute to the incidence of colon cancer, but fibre can be protective as some types may bind to these toxins. In addition, SCFA production will reduce pH that may reduce protein fermentation and increase transit. Protein fermentation products can also be reduced through preference for carbohydrate.

## 1.4.6  Carbohydrate & Protein Fermentation

The major products of carbohydrate fermentation are the short chain fatty acids (SCFA), acetate, propionate and butyrate, which are formed in the colon and can be absorbed.  Colonic epithelial cells metabolise SCFA, especially butyrate.

SCFA absorption stimulates sodium and water absorption and is important for fluid homeostasis in the colon. Once absorbed, SCFA pass into the hepatic portal vein then to the liver where propionate and acetate are taken up. Butyrate is less significant in the liver as it is more likely to be metabolised in the colonocyte where it is a differentiating agent and is important in the maintaining the large bowel epithelium. (17) (see fig 1.6).

Products of protein fermentation include branched-chain fatty acids formed from branched-chain amino acids, ammonia, indoles, phenols and amines. Their production appears to be inversely dependent on the amount of carbohydrate available for fermentation. Fermentation lowers the pH of the colon and effects microbial growth, many enzymes and their activities. This is a symbiotic process through which the body can salvage energy from dietary components that would otherwise be lost by excretion. Fermentation is important in human metabolism. We have some control over fermentation as the colonic flora respond to the substrates made available through the diet. Our dietary choice and the processing of food prior to consumption can regulate these substrates. (19) The latter is particularly important in the case of RS, where food preparation for instance, can have large impact on RS content.
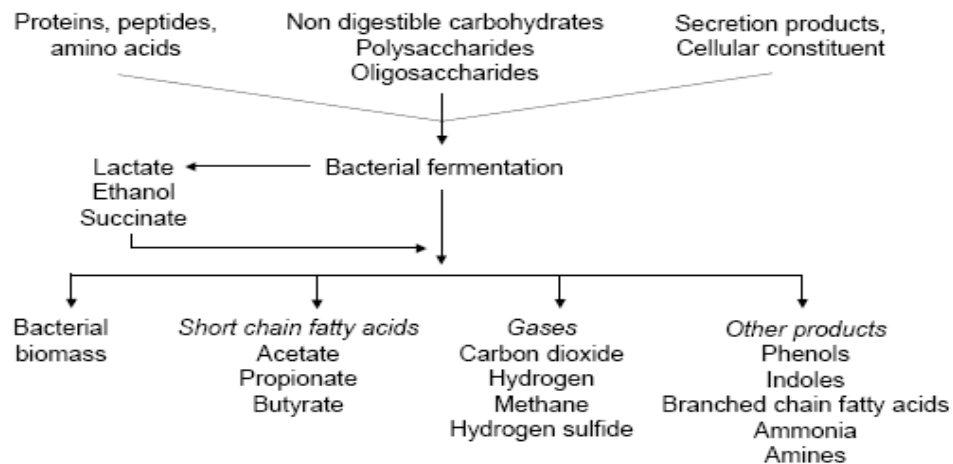
Fig 1.6: Fermentation in the Large Intestine (Resistant Starch, Topping et al, 2008 (77))

## 1.5 Short Chain Fatty Acids

Short chain fatty acids (SCFA) are the principal luminal anions of humans. They are weak acids with an acid dissociation constant (pKa) of ~4.8. Raising their concentrations through fermentation lowers digesta pH. The effects of SCFA are divided into those occurring in the lumen and those arising from their uptake and metabolism by the cells in the large bowel wall and elsewhere in the body. (78) SCFA are the major end products of fermentation. It is important to understand the nutritional and physiological factors that affect their production. SCFA have up to 6 carbons in length and the primary products of NDC formation are, acetic, propionic and butyrate acids with 2-4 carbon atoms. (49) (see table 1.6).

| Carbon Number | Formula | Common Name | Molecular Weight |
|---|---|---|---|
| 2 | $CH_3COOH$ | Acetic acid | 60.05 |
| 3 | $C_2H_5COOH$ | Propionic acid | 74.08 |
| 4 | $C_3H_7COOH$ | n-Butyric acid | 88.11 |

Table 1.6: Common name & physiochemical constants of three SCFA (Physiological & Clinical Aspects of SCFA, Cummings et al 1995 (16))

Short chain fatty acids, are sometimes referred to as volatile fatty acids. The term "fatty acids" is somewhat misleading. Long chain fatty acids, for instance stearic and palmitic acids are present in triglycerides. SCFA produced in the body are largely derived from carbohydrates. They are more polar than longer chain fatty acids. SCFA are not "fatty" and are completely miscible with water (16) although propionic acid and butyric acids form soaps like higher fatty acids.

Colonic microbiota, are essential contributors to the digestive process as the bacteria enable energy salvage from unabsorbed dietary carbohydrates through fermentation and absorption of SCFA. SCFA have the role of maintaining the gut epithelium and intestinal bacteria, are influenced by diet e.g. certain foods such as the carbohydrates oligosaccharides, may have prebiotic effects.

The prebiotic effect has been defined by Marcel Roberfroid, "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health." (71) **(see glossary)**

Investigation of food or food components as physiological stimuli, cannot be obtained from simple chemical analysis alone. It is important to understand dietary carbohydrate fermentation and to assess the production rate of these SCFA from foods. (18)
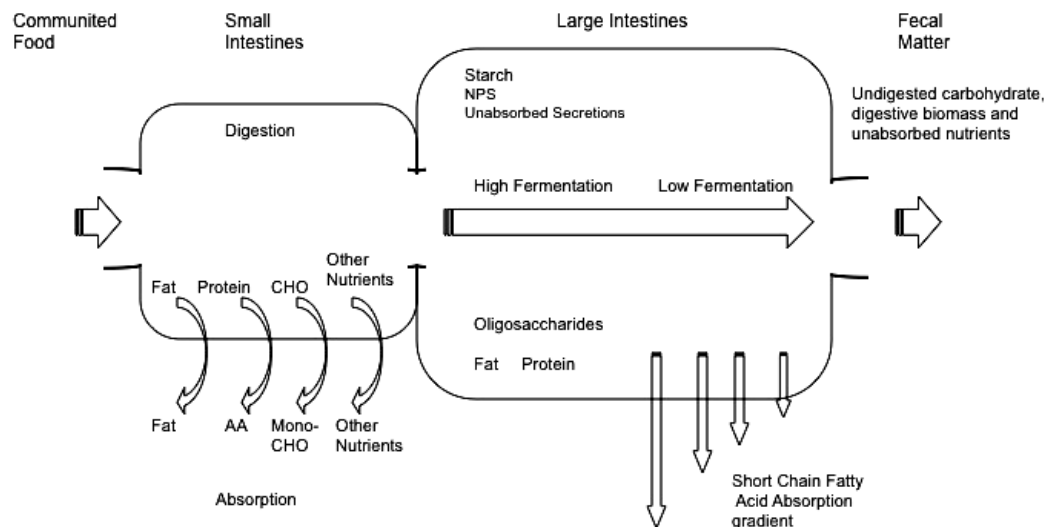


Fig 1.7 – This is the transition of food through the human gastrointestinal tract and digestion of nutrients in the small intestine with fermentation in the caecum and colon. Food becomes digestible by cooking, mastication in the mouth and macerating in the stomach. Digestion occurs in small intestine by intrinsic enzymes when nutrients are absorbed. Those food components and endogenous secretions not absorbed, pass through the ileocecal valve and are fermented. Fermentation, production and absorption of SCFA are high in the large bowel but progression into the faecal passage means fermentation and SCFA absorption decline. (Adapted from SFCA and Human Colonic Function, Topping et al 2001 (78))

There are two types of colonic contractions, tonic and peristaltic contractions. Transit rate depends on the number of these contractions and SCFA may induce tonic contractions and inhibit peristaltic activity, which increases fluid flow through the large intestine and reduces colonic transit time.  A large increase in SCFA concentration could stimulate the contractile activity of the colon. Alternatively, an increasing SCFA concentration may depress motility and increase fluid flow during fermentation.  Intestinal effects of SCFA depend on concentration and different effects may occur above or below a certain threshold. Excessive amounts of rapidly fermenting sugars might induce undesirable motor and sensitivity effects, more steadily fermented fibre might contribute to the regulation of gastrointestinal motility in a beneficial way. (14)

Whole grain diets are implicated in being protective against the development of type II diabetes, obesity, cardiovascular disease and cancer.  Motility effects may provide a mechanism for reduced glycaemic response following ingestion of meals containing fermentable carbohydrates. The so-called "second meal effect", where NDC taken in an evening meal can improve glucose tolerance after a standardised breakfast (53) could either be the result of motility changes or a direct result of the action of fermentation products, such as SCFA.  This 'late' second meal effect is more likely to be caused by fermentation products. Fermentation of dietary fibre by the gut microflora enhances SCFA, which are potentially chemoprotective against colon cancer.  Functional food containing wheat aleurone caused by butyrate, may prevent cancer by influencing cell cycle and cell death. (10)

### 1.5.1 Butyrate
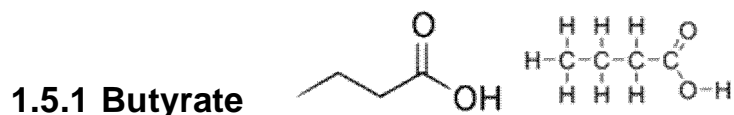
Butyric acid is one of the SCFA produced by intestinal bacteria through fermentation of dietary NDC.  This simple organic acid is known to have anti-inflammatory and possibly direct anti-neoplastic effects. (39) Anti-inflammatory action is particularly important as the presence of a systemic inflammatory response predicts survival in patients with colorectal cancer. (13) The inaccessibility of the human colon means little is known of the in vivo rate of butyrate production, its metabolic fate or the foodstuffs that may enhance its production. (56)

CRC may be preventable through a dietary regimen. There is an association between low risk of CRC with a high (35-50g/day) dietary fibre intake. There have been studies showing an increased total dietary fibre intake of 35g showed a 40% decline in colon cancer but not rectal cancer. (7) Other studies have shown no relationship between dietary fibre intake and colon cancer incidence. (25) (9) There is therefore uncertainty about the association of dietary fibre intake with colon cancer risk. The effects of dietary fibre on the incidence of colon cancer have been attributed to several factors, including the decreased exposure of the colonic epithelium to carcinogens and increased NDC fermentation by colonic anaerobic bacteria, producing SCFA. However, dietary fibre intake as assessed by diet diary may not accurately reflect NDC fermentation and the production of bioactive products, such as SCFA.

Butyrate is an inhibitor of cell growth and regulator of differentiation in coloncytes. Cell proliferation occurs in the base and middle portion of the crypt of the small intestine and not the mucosal surface, which is characteristic of a pre-neoplastic condition. (18) Butyrate is of particular interest as an inhibitor of histone deacetylase. Butyrate is present in the colonic lumen at high concentrations after fermentation of dietary fibre. It promotes cell cycle arrest (apoptosis) and cellular differentiation of CRC cells. Modulation of Wtn activity by butyrate may underlie the protective role of fibre against colon cancer. Mutation of the Wtn signalling pathway is one of the earliest events in colonic tumorigenesis. (9)

A high intake of fermentable dietary fibre can yield different concentrations of butyrate in the colonic lumen. Conflicting observations of butyrate action in vivo may be caused by: differences in the in vitro and in vivo experimental conditions; timing of butyrate administration with respect to tumour development; the amount and type of fibre utilised in the experiments; other effects of dietary fibre such as, transit time and the interaction of fibre with dietary fat. (9) The timing of exposure of colonic cells to butyrate appears to be a very relevant factor here (See fig 1.9). Butyrate may reduce colon cancer risk by suppressing the initiation phase of colonic tumorigenesis and early polyp formation, in the early stages of the neoplastic process, but butyrate seems to

be ineffective in suppressing the progression of adenomas to carcinomas. Therefore, some cancer cells may progress regardless of dietary fibre intake. (9)

Although high dietary fibre intake may not have a major effect on the risk of colorectal cancer, this maybe more related to specific fibre types, possibly those that are more likely to produce butyrate. A diet high in dietary fibre from whole plant foods is advisable, as this has been related to lowered risk of other chronic conditions such as cardiovascular disease and type II diabetes. (62)

## Fermentable Dietary Fiber

Intestinal Microflora

Fermentation → Anti-inflammatory cells

Butyrate Production by Intestinal Microflora

Butyrate Inhibits Adenoma Development of Butyrate Sensitive Early Lesions

Timing of Butyrate Administration

Little or No Inhibition by Butyrate of Progression of Established Adenomas

Initiation, Adenoma Formation

Early Colon Lesion, Butyrate Sensitive

Colon Adenoma

Progression

Colon Carcinomas Sensitive and Resistant to the Induction of Apoptosis by Butyrate

Fig 1.8: Primary factors which influence the preventive action of fermentable dietary fibre and inflammatory effects on colon cancer. Dietary fibre, are processed by intestinal microflora, yielding different levels of butyrate. In the initial phase of colonic tumourigensis, butyrate may suppress adenoma/polyp formation. This has great variability in human. ( Adapted from Butyrate & Wtn Signalling, Bordonaro et al, 2008 (9))

## 1.5.2 Biological Effects of SCFA

Observational studies showed that native East Africans, whose diet consisted of unrefined cereals, were at low risk of colorectal cancer compared with Europeans with a diet consisting of processed foods. (78) The human large bowel

is a complex bacterial ecosystem receiving a varied NDC load where the short chain fatty acids (SCFA) mainly acetate, propionate and butyrate, are produced. SCFA contribute to normal bowel function and may prevent pathologies through their actions in the lumen, the colon and through their metabolism by coloncytes and other tissues.

NSP are commonly the major components of dietary fibre. However, the result of NSP supplementation has been disappointing in the prevention and management of large bowel inflammatory disease (IBD) or colorectal cancer (CRC). RS could be as important as NSP in promoting large bowel health and preventing IBD and CRC. RS intake appears to be low in most affluent industrialised countries, so increasing its consumption by modifying consumer foods is one strategy to improve public health. One of the means by which RS may benefit bowel health is through increased butyrate production. (63) Novel vectors to deliver SCFA may also be useful clinically as it has been shown in animal models that acetylated, propionylated and butyrylated starches ($RS_4$) resist small intestinal amylolysis, but undergo microbial de-esterification, delivering SCFA to the colon. Such model compounds support the role of SCFA in lowering the risk of CRC and IBD. (5) To assess the benefit of a particular NDC in producing SCFA, we urgently need methods to measure SCFA production in vivo.

Metabolic and physiological responses have also been demonstrated following β-glucan consumption, mainly lowered cholesterol levels and moderated blood insulin and glucose level responses. One result of microbial fermentation of β-glucan in the large intestine and is increased SCFA production, in particular propionate, which has a hypocholesterolemic effect. (32) Some β–glucans are thought to be important by virtue of their capacity to alter small intestinal viscosity and therefore slowing digestion and absorption. Although this β–glucan effect is not prebiotic in the sense of increasing bifobacteria and lactobacilli, other classes of fermentable soluble β-glucan can significantly modulate microbial biomass and SCFA profile.(32) Consumption of products containing RS and β–glucans, increase faecal SCFA excretion. (8)

SCFA metabolism continues following transport via the portal circulation. Acetate acts as an energy substrate and induces an insulin response. Acetate can

escape hepatic metabolism, which includes fat synthesis, and will appear in the blood and urine after meals containing fermentable carbohydrates, but it quickly clears and oxidised by peripheral cells such as skeletal muscle. Propionate is gluconeogenic and may reduce hepatic cholesterol synthesis and lipogenesis. Both acetic and propionate acids are absorbed across the colonic epithelium and transported to the liver. Butyrate and to a lesser degree, propionate and acetate are oxidised by colonocytes. Butyric acid is a key mediator in the inflammatory process and its use as fuel maintains a normal colonocyte population. Butyrate is the most potent trophic factor, but all three SCFA contribute. The trophic effects of SCFA are important in maintaining gut integrity and preventing bacterial translocation in the very ill. Altered trophic effects of the large bowel mucosa may be a factor in tumour development. [78]

NSP are the principal components of dietary fibre-rich foods and established in the promotion of normal colonic function and prevention of functional disease. However, the interest and concern here is not to dwell on dietary $\beta$-glucans or NSP, but to focus on measuring SCFA production to facilitate the greater understanding of their dietary sources and their effect on the bowel and other tissues.

## 1.5.3 Measurement of SCFA production

A major focus of this work is to facilitate methods of measuring SCFA production in vivo. There are great difficulties in establishing the digestive function of the large intestine because of its inaccessibility. Occurrence of fermentation in the caecum and colon and measuring SCFA in the small and large intestine, have been previously measured in portal, hepatic and peripheral venous blood from persons suffering sudden death [19]. SCFA in the portal blood infers that substantial carbohydrate breakdown is occurring in the large intestine. There is very little advantage to medicine by measuring portal blood for SCFA after death. Only a very few reports on portal SCFA concentrations have been made in surgical patients and these will be strongly influenced by dietary restriction pre-operation. [20]

A current project in the Stable Isotope Biochemistry Laboratory, SUERC, which is funded by the UK Food Standards Agency (FSA), is entitled "Urinary versus faecal

biomarkers of colonic carbohydrate and protein fermentation." Studying the impact of non-digestible carbohydrates (NDC) on colonic health is challenging since there is no validated method at present to measure the extent and pattern of colonic fermentation in vivo. Urinary versus faecal biomarkers, is investigated by measurement of both urinary and faecal SCFA after the consumption of oligofructose. Bacterial degradation of oligofructose is associated with increased urinary excretion of SCFA. In this study, faecal concentrations of SCFA did not correspond to their intra-colonic production and so faecal samples are not suitable to assess colonic fermentation as they are not sampled near the production sites of caecum or acending colon or where SCFA have efficient absorption. Faecal sampling is also undesirable for subjects and experimenters alike. Colonic fermentation of carbohydrates and proteins are represented by urinary excretion. (Hanske et al., October 2010)

Circulating SCFA concentrations in blood or urine may provide a proxy for production, but their high clearance rate must result in insensitivity and reduced accuracy. Low concentrations require sensitive analytical methods. The naturally extended period of fermentation (up to 24 hours following a meal) impose timing issues when considering blood sampling. Analysis of SCFA concentration in plasma and urine have been performed, but very high extraction rates in body tissues will always make this an indirect measure of colonic SCFA production. (51) (65) (29))


The use of non-radioactive tracers provides a direct, safe and potentially non-invasive way to measure digestion and fermentation in human subjects in vivo. If known quantities of labelled SCFA are administered, SCFA production can be observed by dilution in vivo. Such an approach will measure the total (endogenous plus exogenous) production of each labelled SCFA. In the case of acetate, endogenous production (from acetyl CoA) may exceed that of exogenous production (from fermentation). The exogenous production term may be accurate but it will not indicate directly which class of macromolecule has been contributed the most (NDC from the test diet, from previous meals or endogenous compounds such as dead bacteria, mucus or digestive enzymes undergoing fermentation). Alternatively, foods can be labelled using stable isotopes (complex metaprobes, see section Metaprobes) and SCFA production can be observed in vivo following their administration. Such complex

metaprobes MUST provide the most definitive evidence of SCFA production from specific food types. By increasing the amount of $^{13}$C in plant material above that naturally present, e.g., enriching or labelling of cereals to produce complex metaprobes, digestion and metabolism of that food can be measured. Depending on the objective, this may by measuring $^{13}$C-glucose in blood plasma (to measure digestion), $^{13}$CO$_2$ in breath samples (to measure oxidation), $^{13}$C metabolites in faeces (to measure bioavailability) or measuring $^{13}$C-labelled SCFA in urine and plasma (to measure fermentation).

Information about the extent of carbohydrate digestion and fermentation is critical to our ability to explore the metabolic effects in vivo. Recently, consumption of $^{13}$C-labelled barley kernels rich in NDC, specifically grown as part of this MSc project, has been used to develop a method to distinguish between and assess carbohydrate digestion and fermentation. These studies will be discussed in detail in Chapter 5.

## 1.6 Stable Isotopes as Tracers

Human studies show that stable isotopes are extremely useful and safe for use in clinical practice and medical research. Stable isotopes are non-radioactive and present no risk to human subjects. [83] The increased availability and diversity of stable isotope labelled compounds and improved analytical methods for their quantitative analysis make them suitable tracers for in vivo studies of fermentation and other processes. [82] [23] [69] [49]

An isotope is a nuclide, which contains the same number of protons as other isotopes of that element, but a different number of neutrons. This does not affect its chemical properties. Isotopes are categorised by their atomic mass and are termed either stable or unstable. Radioactive isotopes are unstable and are less commonly used in medical research studies, due to concerns over hazards impacting health, safety and disposal. There are approximately 300 naturally occurring stable isotopes, but the stable isotopes of greatest interest in macronutrient metabolism are those of hydrogen, carbon, nitrogen and oxygen (See table 1.5). Isotopes have their atomic weights denoted in their prefix. Carbon atoms have 6 protons in the nucleus, the atomic mass (protons +

neutrons) shown in the prefix e.g.$^{12}$C has 6 protons and 6 neutrons, $^{13}$C has 6 protons and 7 neutrons. The radioactive $^{14}$C has 6 protons with 8 neutrons.

| Element | Isotope | Atomic Number | Neutrons | Natural Abundance (atom %) |
|---|---|---|---|---|
| Hydrogen | $^1$H | 1 | 0 | 99.985 |
| | $^2$H | 1 | 1 | 0.015 |
| Carbon | $^{12}$C | 6 | 6 | 98.892 |
| | $^{13}$C | 6 | 7 | 1.108 |
| Nitrogen | $^{14}$N | 7 | 7 | 99.6337 |
| | $^{15}$N | 7 | 8 | 0.3663 |
| Oxygen | $^{16}$O | 8 | 8 | 99.759 |
| | $^{17}$O | 8 | 8 | 0.037 |

Table 1.7: Natural Abundance of Stable Isotopes (Wolfe et al, 1992)   (82)

$^{12}$C and $^{13}$C are stable isotopes of carbon and are present in all organic materials. $^{13}$C forms approximately 1.1% of all carbon atoms in nature.  Enrichment is the term used to describe the abundance of a sample raised above a baseline level. The stable isotope of greater abundance is the major isotope, while the least abundant in the minor isotope.  Tracers in metabolic studies are compounds (molecules) in which one or more minor stable isotopes replace atoms of the major isotope.  The unlabelled endogenous compound is the tracee.

It is possible to synthesise "highly enriched" compounds in which 99% of the atoms in a particular position on the molecule have been replaced by the heavy isotope of that atom e.g. 99% of the carbons are $^{13}$C rather than $^{12}$C.  Highly enriched tracers can be "diluted" with unlabelled material to achieve enrichments of any desired percentage above natural abundance.

The use of a labelled tracer requires that the labelled molecule will trace the movement of the unlabelled molecule. Isotopic labelling is a technique for tracking the passage of a sample of substance through a system. The substance will be "labelled" using different isotopes and when these isotopes are detected during analysis, they have to have originated from the labelled substance. Since isotopes have different masses, they can be separated using mass spectrometry, which is the preferred technique for isotope analysis.

## 1.7 Mass Spectrometry: Gas Chromatography Mass Spectrometry (GCMS) and Isotope Ratio MS

The most versatile analytical instrument for stable isotopes, is a mass spectrometer (MS). A MS is an instrument that separates ions in a high vacuum according to their mass-to-charge ratio (m/z) by ionizing a sample and separating the ions of different masses. A MS records the relative abundance by measuring the intensities of ion currents.

The major components of a MS are the inlet system, ion source, mass analyser, detector and vacuum system. Gas Chromatography MS (GCMS) comprises a high-resolution chromatography system to separate volatile organic compounds interfaced to the ion source of (usually) a quadrupole mass spectrometer which posses unit resolution over 1000 mass units. Molecules that are not naturally volatile (amino acids; sugars) are modified prior to analysis to produce a volatile derivative. GCMS can be used very effectively to measure stable isotope enrichments of ~1% and greater. (67) More recently, LCMS have been applied to measure stable isotope enrichment of molecules separated by HPLC (46) with similar performance to GCMS. For enrichments below 1 atom %, Isotope Ratio MS is used. This low-resolution, magnetic sector MS, analyses simple gases ($CO_2$; $H_2$; $N_2$) by simultaneous measurement of all isotopic species using two or three high gain current detectors.

Most biomedical IRMS instruments in current use are continuous flow IRMS (CF-IRMS) where sample conversion to simple gases occurs on line. (67) (68) (66) CF-IRMS techniques are high throughput and sensitive. For the most accurate analysis, each sample gas is compared with a reference gas of known

43

composition.  They can be divided into two areas.  Bulk techniques, measure the abundance in the whole samples and include IRMS interfaced to elemental analysers.  The second group is compound-specific techniques which measure the abundance of isotopes in particular compounds e.g. fatty acids, which are separated on line by GC. More recently, LC-IRMS has been introduced which permits $^{13}$C analysis in individual compounds on-line following HPLC separation. (52)


### 1.7.1 Metaprobes: Complex and Simple

*"Metaprobes are dynamic markers"* (Vernon Young, 2000)

The use of stable isotopes as tracers in metabolism and nutrition should be expanded with experimental protocols and tracer compounds to probe the dynamic aspects of metabolism. (83) When isotopically labelled probes or tracers are administered to a subject, the rates of appearance, concentration and kinetic parameters of the product are measured in relation to the diminishing precursor.  The data and analysis will be concurrent with the metabolic or disease conditions under study. (84)

In the context of SCFA, measuring their production *in vivo* is difficult because of inaccessibility of sampling sites and low circulating concentrations.  Stable isotope tracer techniques are a way to measure SCFA production and to measure the concentration and enrichment of $^{2}$H-labelled SCFA by gas chromatography mass spectrometry (GCMS) and $^{13}$C-labelled SCFA by isotope ratio mass spectrometry (IRMS), the latter allowing measurement at much lower enrichment.  Improving both the preparation of plasma and urine samples and the mass spectrometry methods has allowed us to monitor isotopically labelled SCFA to be used as biological tracers to measure exogenous or colonic SCFA production. (49)

We exploit enriched stable isotope tracers in most areas of this research. In order to differentiate these techniques from uses of potentially hazardous radio-isotopes and also from exploiting natural variations in stable isotope abundance, the community is increasing using the term metaprobe (derived from *metabolic*

probe).  An increasing number of highly-enriched simple metaprobes can be purchased commercially, produced by chemical synthesis. When simple metaprobes are not available commercially, it may be feasible to synthesise them in the laboratory, (e.g., lactose ureides (50)) but complex metaprobes, which are plant or animal-derived products, are produced by biosynthesis, almost entirely by research teams.

Stable isotopes of carbon ($^{13}C$) are used to label compounds or nutrients, which are essentially identical to the usual substrate for a given physiological or metabolic pathway within the body.  These metaprobes, can be taken orally, or if soluble, given by i.v. infusion, and the metabolic processes can be followed. When the label is absorbed, its metabolism can be monitored in body fluids or tissues and breath, urine or faeces can monitor its excretion from the body.

# 1.8 Staple Cereals as Complex Metaprobes:

# Barley and Wheat

| Barley | Wheat |
|---|---|
| Kingdom: **Plantae- Plants** | Kingdom: **Plantae - Plants** |
| Subkingdom: **Tracheobionta - Vascular plants** | Subkingdom: **Tracheobionta - Vascular plants** |
| Superdivision: **Spermatophyta - Seed plants** | Superdivision: **Spermatophyta - Seed plants** |
| Division: **Magnoliophyta - Flowering plants** | Division: **Magnoliophyta - Flowering plants** |
| Class: **Liliopsida - Monocotyledons** | Class: **Liliopsida - Monocotyledons** |
| Subclass: **Commelinidae** | Subclass: **Commelinidae** |
| Order: **Cyperales** | Order: **Cyperales** |
| Family: **Poaceae - Grass family** | Family: **Poaceae - Grass family** |
| Genus: Hordeum – **barley** | Genus: **Triticum - wheat** |
| Species: Hordeum vulgare - **common barley** | Species: **Triticum aestivum - common wheat** |

Table: 1.8 Taxonomy of Barley & Wheat

"In the age of acorns, before the times of Ceres, a single barley-corn had been of more value to mankind than all the diamonds of the mines of India" (Henry Brooke 1703- 1783)

The word cereal derives from Ceres, the name of the pre-Roman goddess of harvest and agriculture. Cereals are members of the monocot grass family, Poaceae.

## 1.8.1 BARLEY

Barley (Hordeum vulgare L.) originated in the Eastern Mediterranean regions and varieties can be distinguished from other cereals by differences in head type and growth habit. There are Spring and Winter species of Hordeum vulgare, or Barley. There are two different genotypes of barley, the six-rowed barley where three kernels are formed at each node of the head and the two-rowed barley, where only a single kernel forms at each node.

Barley is an annual cereal and its cereal grains provide more edible energy than any other type of crop. Barley is a staple crop and a rich source of carbohydrates and is used for both malting, for animal foods and for nutritional foods often accompanied by a claim of health benefit (developing EU legislation

requires increasingly explicit evidence of health claims).  Malting is when the grains are allowed to germinate, soaked in water and then quickly dried with hot air to prevent any further germination.  This process of malting grain develops the enzymes required to hydrolyse starches into monosaccharides and disaccharides such as maltose which is commonly used in foods including breakfast cereals (i.e., Shreddies).   The most common use of malted barley is for brewing beer and distilling whisky.  Whole barley grain, scotch barley and pearl barley (see glossary for descriptions), can be added to soups and stews. Barley is rich in vitamin B6 and the minerals copper, phosphorus and zinc.  It is also rich in fibre, particularly the soluble NSP, $\beta$–glucan. (3)

 Barley varieties are defined in classes of Spring Barley and Winter Barley. Spring Barley is favoured by the malting market. Winter Barley is used for food, animal feed and bio fuel.  Barley-meal is wholemeal flour that is lighter than wholewheat and can be used to make porridge.  Most barley found in the supermarket is pearl barley (see Glossary).  In this project, we cultivated and used for consumption pearlised Spring Barley vars Cellar and Kirsty.


## 1.8.2 WHEAT

Wheat (triticum spp.) is a cultivated grass and after rice, is the second most produced cereal crop worldwide.  Wheat grain is a staple food for much of the world and its flour is used for bread, biscuits and pasta.  Other uses include semolina, bulgur wheat, breakfast cereals, cakes and pastry.  There are Winter and Spring wheats, Triticum aestivum. Durum wheat Triticum durum, has the highest density, protein content and gluten strength, of all wheats.  Durum kernels are larger than any other wheats and are amber coloured which gives pasta its yellow colour.  The vitamin and mineral content of these foods will vary depending on the proportion of germ, bran and endosperm present.  Wholegrain wheat contains several B vitamins, such as thiamin, riboflavin and niacin and vitamin E.  It contains nutritionally significant quantities of the minerals potassium, iron, magnesium, zinc and selenium (see glossary for terms).

In some developing nations, grain in the form of rice, wheat, or maize constitutes practically the entire diet. In developed nations, cereal consumption is more moderate and varied but is still substantial. It is often refined for food palatability but this reduces the NDC content.  If the grain is refined NDC and other components are removed as dietary fibre is lost with the outer layer of the grain. (57)

| Food | Specification | Water % | Ash % | Fat g | CHO g | Fibre g | Protein g | Food Energy kcal |
|---|---|---|---|---|---|---|---|---|
| Barley | Pearled | 11.1 | 0.9 | 1.0 | 78.8 | 0.5 | 8.2 | 349 |
| | Scotch | 10.8 | 1.3 | 1.1 | 77.2 | 0.9 | 9.6 | 348 |
| Wheat | Dry | 10.4 | 1.8 | 2.0 | 72.3 | 2.2 | 13.5 | 338 |
| | Cooked | 87.7 | 0.8 | 0.3 | 9.4 | 0.3 | 1.8 | 45 |
| | Germ | 11.5 | 4.3 | 10.9 | 46.7 | 2.5 | 26.6 | 363 |
| | Whole | 12 | 1.7 | 2.0 | 71.0 | 2.3 | 13.3 | 333 |

| Food | Specification | Calcium mg | Phosphorus mg | Iron mg | Sodium mg | Potassium mg |
|---|---|---|---|---|---|---|
| Barley | Pearled | 16 | 189 | 2.0 | 3 | 160 |
| | Scotch | 34 | 290 | 2.7 | Nil | 296 |
| Wheat | Dry | 45 | 398 | 3.7 | 2 | 370 |
| | Cooked | 7 | 52 | 0.5 | 212 | 48 |
| | Germ | 72 | 1118 | 9.4 | 3 | 827 |
| | Whole | 41 | 372 | 3.3 | 3 | 370 |

Table 1.9: The compositions of foods, raw & processed are subject to variation. Variety, climate, soil composition, stage of ripening and storage are important and may account for fluctuation. Nutrients can be lost through cooking as vitamins and minerals can be leached by water. The carbohydrate content is calculated by substracting the water, protein, fat and ash from the total mass. The values given are contents per 100 g edible portion of the food. (see Glossary for explanation of terms; Composition of Foods, Raw and Processed from Geigy Scientific Table 1, Ciba-Geigy Ltd, Basle, Switzerland).

## 1.8.3  Varieties of Wheat

Wheat varieties



soft red winter | hard red winter | hard red spring | durum | common white
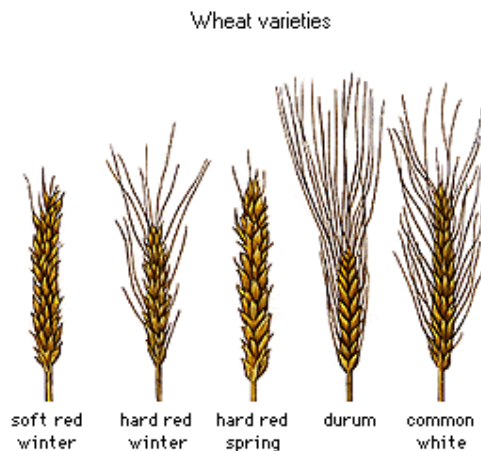
Wheat trading classes can be defined in (USA) classes of: Hard Red Spring; Hard Red Winter; Soft Red Winter; Hard White and Soft White wheat.  Hard Red Spring wheat, is brown coloured grain with high protein and is used for bread and bread flour.  Hard Red Winter is brown coloured grain with high is protein and is used for bread making but is also added to other flours to increase the protein levels for pastry flour.  Winter wheat is a key crop for Scotland as there is a large market for grain whisky production. Hard White wheat is an opaque, chalky, medium protein, wheat used for bread and brewing purposes.  Soft Red Winter wheat is a low protein wheat used for cakes, biscuits, self-raising flours and baking powder.  Soft White wheat is light coloured, very low protein and is grown in temperate moist areas.  Durum wheat, Triticum durum cv Lloyd,  which we cultivated and used in our nutritional studies, is very hard translucent grain form the Hard Spring classification and has high protein and gluten content.  It is used to make semolina flour for pasta. During this project, we undertook trials of winter wheat and Durum wheat varieties, but the major focus was on producing complex metaprobes of soft spring wheat varieties would be used for bread and biscuit making in Northern Europe. Thus, Spring Wheat var Paragon, with hard endosperm texture was cultivated and labelled. Winter Wheat Var Robigus, was used in growth trials along with a selection of hard wheat varieties (International Grain Council, www.igc.org.uk & HGA, www.hgca.com, www.sac.ac.uk; see Chpt 2)

## 1.8.4 Phytochemicals

Phytochemicals are non-nutritive plant chemicals that may have protective or disease preventive properties as the dietary fibres and antioxidants create anti-inflammatory effects.  Cancer prevention involves decreased cell proliferation and cell cycle arrest but there is limited understanding of the mechanisms involved. (59)

Most foods contain phytochemicals. Examples of phytochemicals in foods are lycopene in tomatoes and flavonoids in fruits.  Whole grains, vegetables, beans and fruits contain many phytochemicals and other compounds that protect against chronic disease such as phytate, phyto-oestrogens and vitamins. (73) Whole grains are both rich in NDC and antioxidants, which are independently and positively, linked to disease prevention. (see Fig 1.9 & glossary)

It should be emphasised that complex metaprobe production aimed at facilitating the study of SCFA production will also produce metaprobes of micronutrients such as vitamins and antioxidants, which can be exploited in studying these important phytochemicals.  Intrinsic labelling of plants using stable isotopes, can be used to assess the effects of food processing on the final nutrient bioavailability, but is not commercially available.  It is the only way to provide proteins, vitamins and phytochemicals in stable isotope labelled form, for human nutritional studies. (27)

Many further studies are necessary to clarify the relationship between colonic fermentation and glycaemia. Should NDC prove as important nutritionally as expected, every effort should be spent to encourage industry to minimise grain refining and to educate the public to increase their intake of whole grains. (71)
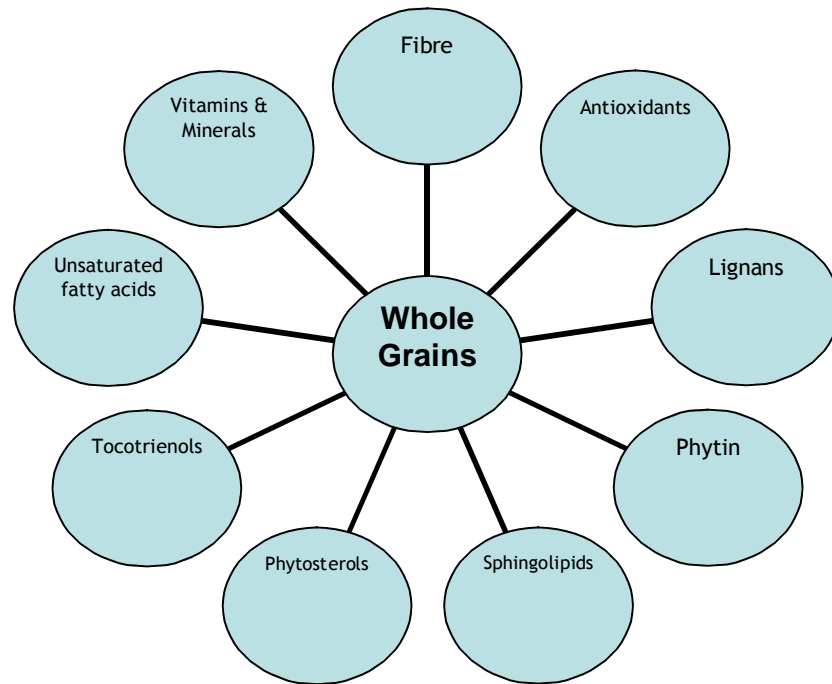


Fig 1.9: Phytonutrient content of whole grains in breads cereals and brown rice. Components in the whole grains associated with improved health status are lignans and tococtrienols.  The grain refining process results in the loss of dietary fibre, vitamins, lignans, phyto-oestrogens, phenolic compounds and phytic acid.  Refined grains have a higher concentration of starch as the bran and some germ have removed in the refining process. More research is required to report the protection of whole grain consumption in human subjects (see glossary for explanation of terms; Slavin et al, (73)).

## 1.9 Plant Morphology and Growth Cycle

The growth cycle of cereals have the following divisions: germination, seedling establishment and leaf production, tillering, stem elongation, pollination with kernel development and maturity.   Barley and wheat have three distinct growth phases, namely: vegetative phases; reproductive phases; grain filling phase. The vegetative phases include germination of seed, emergence of seedlings and the tillering phases. The reproductive phase begins with the initiation of floral primordia at the shoot apex. The spikelets differentiate on the spike and the

culm extends upwards during the jointing phase.  In barley the first node is not visible and must be found by touch.  The joints or internodes elongate and stems become erect.  Elongation of the secondary stems occurs later than the main stem.  The increase in the size of the spike and its growth inside the flag leaf sheaths are detectable as swelling in the sheaths.  The swelling of the flag leaf sheath is called booting.

Fig 1.10: The author assessing the growth of barley and wheat

## 1.9.1 Grain Filling

Grain filling involves different stages: flag leaf photosynthesis; translocation of carbon, which is stored in the internodes; respiration, allocation and partitioning and ear photosynthesis. (4)  During grain filling, mitosis in the zygote occurs between 18 – 30 hours after pollination.  This development has a rapid increase in length filling and the kernel is at the milk stage.  Around 15 days after anthesis, the grains start to turn yellow in colour.  After 30 days form anthesis cellular division in the endosperm is complete. Barley kernels more than any other cereal, produce more $\beta$-glucans at this stage.  Non-development of kernels or sterility of the heads frequently occurs and is associates with high

temperatures or drought. Maturation occurs when the grain dries up and shrinks. Harvesting can then commence.

The germination of barley and wheat occurs at a minimum temperature of $1^0$C but is usually around $10^0$C. (38) When the seed absorbs moisture, the radicle emerges and grows downward providing the anchorage for the plant and the means for absorbing both water and nutrients. The radicle develops lateral branches which remain active throughout the growing season. Barley and wheat have two types of root systems. The first is the seedling roots which develop from germination when the seed absorb moisture, to the tillering or shooting stage. The second system starts after tillering when the secondary crown roots appear. These crown roots anchor the plant and provide it with water and nutrients. (33)

The stems should be erect and made up of hollow, cylindrical internodes of joints separated by the nodes which bear the leaves. Approximately 3-4 weeks after plant emergence, the upper internodes of the stem begin to elongate. Leaves grow alternately along the stem. The leaves are linear lanceolate and formed of sheath, blade, auricles and ligule. The sheaths surround the stem completely. The ligule and the auricles distinguish barley from other cereal grains. Early in the plant's growth, the leaf blades are the major photosynthetic organs. The duration of leaf function is important for maximum grain yield as the functional leaf area declines during grain growth.

The last internode of the stem extends as a rachis which bears the spicules alternating on its nodes. Spikes of barley lack the terminal spikelet but wheat plants have which aids identification.


## 1.9.2 Pollination

Cereals, such as barley and wheat are self-pollinated. Pollination begins in the stigma and the pollen tube spreads towards both tip and the base. This event occurs around six weeks after crop emergence. Pollen formation is sensitive to stress, water deficiency and high temperature which will decrease the number

of kernels that form and may reduce yield.  Kernels begin to develop after pollination has occurred and the first or milk stage lasts for ten days.  This is an important stage as it determines the number of cells that will subsequently be used for storing starch.  The second stage lasts for ten days and is termed "soft dough" as the kernels are storing starch and growing rapidly.  The final stage or "hard dough" is when the kernel reaches maturity, loses moisture and becomes more solid. (3) (34-36) In terms of efficient labelling of the developing grain, the period after anthesis as the soft dough forms, appears the most effective for preparing complex metaprobes based on cereal grain.
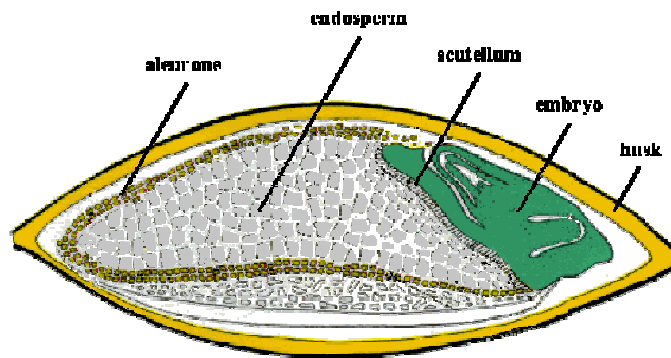


Fig 1.11: A Barley Grain Kernel. The embryo and endosperm are the carbohydrate store. They also include protein which supports the growth of the germinating embryo.  The layers of aleuorne cells contain a high protein content. The outer husk (hull) protects the grain and is strongly attached to the seed.  The hull is removed with varying efficiency during the pearling process depending on the procedures used.  A wheat kernel is not so strongly attached to the seed and has only one aleurone layer. The husk is lost at threshing, leaving only the  endosperm which now has lower starch content. (see glossary for explanation of terms; HGCA, Agriculture & Horticulture Development Board, www.hgca.com)
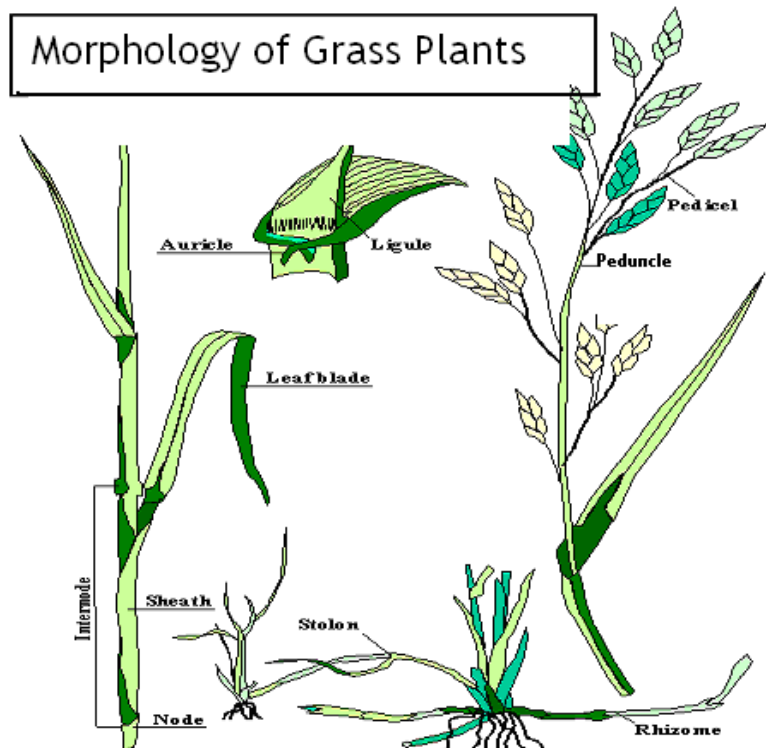
Fig 1.12: Morphology of Plant Grass (see glossary for description of terms)
Growth and Development of Spring Barley, Anderson PM et al. (3)

Recognizing the barley and wheat growth staging systems is important. By using the physiological maturity indicators, harvest decisions can be made that will maximize crop yield potential.  Crop development is measured on a decimal Zadoks scale, a two digit code referring to the principal stages of development from germination to kernel-ripening. (3) (see glossary for further explanation )

Fig 1.13: Anthesis (Photograph taken by the Author)

### 1.9.3 Photosynthesis   $6CO_2 + 6H_2O > C_6H_{12}O_6$ (glucose) $+ 6O_2$

Photoautotrophs use light as the source of energy to survive and grow. This process is termed photosynthesis.  During photosynthesis light energy is converted into chemical energy and stored as sugar. This occurs in the chloroplast.  Photosynthesis occurs in three stages; stages 1 & 2 are photolysis, which occurs in the granum of the chloroplast. Here, light is absorbed by chlorophyll and secondary pigments, which convert it to chemical energy. $H_2O$ splits into H and $O_2$ apart and $O_2$ is released from the chloroplast but the H+ acceptor is temporarily stored in ATP and NADPH.  Stage 3 occurs in the stroma of the chloroplasts and is the carbon fixation stage known as the Calvin Cycle. Plant Species that fix carbon only through the Calvin Cycle are known as C3

plants. During grain filling in cereals, flag leaf photosynthesis occurs but ear photosynthesis becomes more important. (www.biologycorner.com)
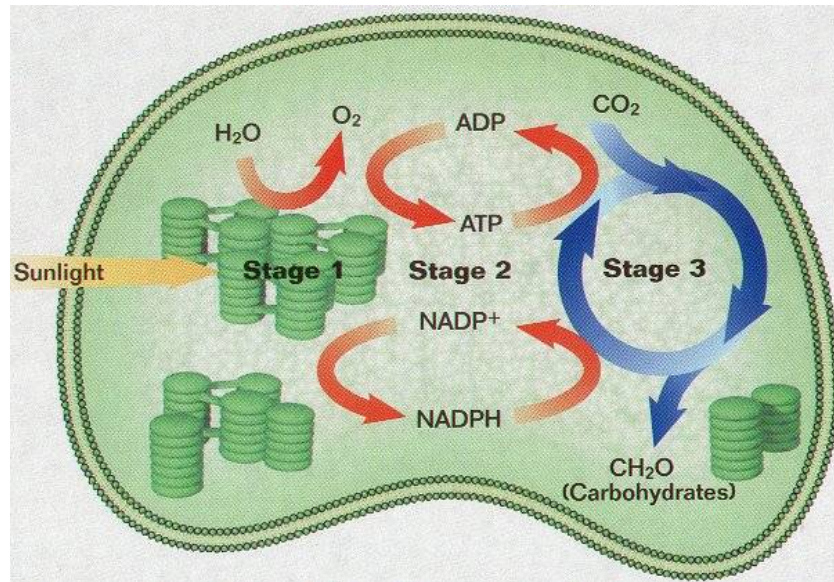


Fig 1.14: Photosynthesis in a Chloroplast
Stage 1 – Photolysis – Photochemical Reaction
Stage 2 – Photolysis – Thermochemical Reaction
Stage 3 – Calvin Cycle –Thermochemical Stage
(www.Biologycorner.com)

Many environmental factors affect the rate of photosynthesis, including light quality; light intensity; light period; carbon dioxide concentration and water availability.   In terms of producing labelled cereals, replacing or supplementing natural atmospheric $CO_2$ with $^{13}CO_2$ will cause plant organic matter to become $^{13}C$-enriched during photosynthesis. Doing this at the soft dough stage of grain development when starch synthesis is maximal, will boost the production of $^{13}C$ labelled starch and produce this complex metaprobe with greatest efficiency.

## 1.10 Production of Complex Metaprobes: $^{13}C$-labelling of Cereal Crops

Enrichment of starch with the stable isotope of carbon, $^{13}C$ offers a means of studying the digestion, absorption and metabolism of this complex carbohydrate

by in vivo. (30) Unlike many simple chemicals (simple metaprobes), $^{13}$C-labelled foods such as starch are not available commercially. Carbon isotopes are powerful non-invasive probes for characterising carbon metabolism in plants. Tracing carbon incorporation into the carbon-molecules of different plant tissues provides essential information about the photosynthetic activity in the plant, such as analysis of carbon in tissues such as grain, to which recently fixed carbon has been delivered. The use of isotopes enables quantification and partitioning of carbon and has no effect in the main processes: photosynthesis; allocation; partitioning and respiration, all involved in carbon metabolism. (61) Carbohydrate storage of fructose, glucose and fructan can be measured, but was not the aim of this project.

There are different approaches for labelling crops. Continuous labelling, also known as dynamic or steady-state labelling, where plants are held in a controlled $CO_2$ atmosphere for their whole growth cycle, leads to consistent results with predictable $^{13}$C-enrichment in the mature plant. This approach requires that plants are held within a sealed growth chamber for their entire life cycle, with failsafe facilities to control light, temperature, humidity and watering and atmosphere. This approach requires plants to be grown in a hydroponic (see glossary for explanation) or soil-free system in order to reduce the entry of unlabelled $CO_2$ from soil respiration. It is very expensive and has been used to label single small whole plants to high enrichment. Commercial systems are available, but these can only accommodate single plants of up to 1m tall (www.Isolife.nl), which is suitable for producing a few gram of labelled product.

Pulse labelling, when $^{13}CO_2$ is introduced for only a brief period in the growth cycle, was first used in metabolic studies of carbon translocation. It has been frequently adopted for labelling studies but may yield less predictable results in terms of the final enrichment of plant material. However, pulse labelling is economical as it can use existing facilities and is suited to larger scale production.

With the use of trials or pilot studies, it is possible to predict the enrichment of the final product. With the exception of a small number of labelling experiments that have exploited small natural abundance differences in natural sources of

$CO_2$ or in the plant's metabolism, the large majority of labelling experiments use commercial sources of highly enriched $CO_2$ which is very expensive (at the time of writing, £70 per Litre). Consideration of the quantity and enrichment of the product is paramount. In the series of labelling studies presented here, and through use of pilot experiments, it was realised that it was preferable to produce kg quantities of low enrichment staple foods than g quantities of high enrichment products. Typical human nutrition studies may recruit tens of subjects while typical meals under test may contain up to 100g starchy food under test. If these were to be only solely on finely ground flours, then homogenous mixing of a small quantity of highly enriched flour within the bulk of non-enriched flour may be possible. However, in the context of a study of the benefit of whole grains in the diet, and considering the expense of producing complex metaprobes with the sensitivity of our analytical techniques, the logic of producing kg quantities of low enrichment foodstuffs became clear. Therefore, a subject may only require one or two highly enriched whole grains in a slice of bread to study the fate of this metaprobe. By using traditional food preparation techniques, it would be impossible to control the exact quantity of metaprobe each subject received. It would be far better to label each whole grain in the bread used for the whole study, in a homogenous manner.

Approaches adopted to prepare ^{13}C-labelled plants for nutrition studies have been of two types. Firstly the apparatus to contain a [13]$CO_2$ enriched atmosphere for the pulse labelling period could be assembled in the field where the crops are growing. (60) Secondly and much more commonly, either the plants could be grown in containers, which can be individually sealed, temporarily moved to a labelling chamber or the glasshouse sealed temporarily to allow temporary control of its atmosphere (see Table 1.10). As the latter approach avoids moving heavy and delicate plants, it is more suitable from larger metaprobe production runs. However, it requires more sophisticated glasshouse designs sufficiently gas tight. Crude sealed plastic canopies and use of conventional environmental chambers has thus far been more common (see Table 1.10).

| Labelling Methods | Publication |
|---|---|
| At growth stage Z68, spring wheat plants var Wembley, were stripped of their leaves and enclosed in polypropylene coated bags, which have low permeability to $CO_2$. 100 mL $^{13}CO_2$ was introduced to each bag and left for 3 days. 150 mL $^{13}CO_2$ was then added to each bag and then left for 4 days. On a second attempt, the plants were placed in a gas-tight box for 6 days with a single dose of $^{13}CO_2$. | Labelling Wheat Flour with $^{13}C$. M Harding et al, 1994 (30) |
| Barley PW & BZ 594.35e * were maintained in a controlled environment chamber and illuminated continuously by both incandescent & fluorescent lamps. The plants were pulse-labelled with $^{13}CO_2$ on 3 occasions. | Carbohydrate Digestion in Humans from β-Glucan-Enriched Barley is Reduced. C Lifschitz et al, 2002 (40) |
| Pisum sativium var Baccara, had bags coated with polypropylene film, placed over the pots that had formed small pods of premature pea seeds. Plastic valves were inserted through the wall of the bag to allow injection of $^{13}CO_2$. The bags were left in a controlled environment, 14 hrs light @ $20^oC$ for 6 days. . | Production of $^{13}C$ labelled Pea Flour for use in Human Digestion and Fermentation Studies. C Edwards et al, 2002 (22) |

Table 1.10 Different Methods of Labelling Crops for Human Nutritional Studies (30)

* cultivar PW (prowashonupana) provided by ConAgra Oat Processing, Omaha, NE & cultivar BZ 594.35e. provided by Western Plant Breeders, Bozeman, MT

Labelling starchy foods with the stable isotope of carbon, $^{13}C$, offers a powerful means of studying the digestion, absorption and metabolism of complex carbohydrates, non-invasively. $^{13}C$-enriched starch can occur in nature depending upon the way atmospheric $CO_2$ is assimilated by some plants. There are two major photosynthetic pathways. Plants with the C3 pathway such as wheat and rice discriminate against $^{13}CO_2$ during fixation by ribulose-1,5 bisphosphate carboxylase. Plants using the C4 Hatch-Slack pathway (see glossary) also discriminate against $^{13}CO_2$ but less so, resulting in the organic carbon of

certain crops, notably tropical grasses (e.g., maize and sugar cane), being slightly enriched in $^{13}$C relative to C3 plants (temperate grasses, e.g., wheat, barley, rice). Naturally $^{13}$C enriched cornstarch (i.e., maize) has been used as natural complex metaprobes. (37) Although an elegant technique, its utility is largely limited to maize products and because the natural $^{13}$C enrichment is, too low to follow all products as their enrichment is reduced by dilution within complex body pools.

Table 1.11 lists studies in which labelled crops have been utilised for human consumption for the purposes of nutrition and fermentation studies.   The publications of which I am co-author are marked with an asterisk, have used the labelled grain produced for this project and will be discussed in full in Chapter 5.

| MAIN AUTHOR | TITLE OF PAPER/BOOK | YEAR |
|---|---|---|
| Boutton TW | $^{13}$C-labelled rice produced for Dietary studies (Abstract only) | 1987 |
| Boutton TW | Tracer studies with $^{13}$C-enriched substrates: humans and large animals. | 1988 |
| Lifschitz CH | Absorption of $^{13}$C-labelled rice in milk In milk by infants during acute Gastroenteritis. | 1991 |
| Svejcar TJ | Labelling of forages with $^{13}$C for nutrition and metabolism studies. | 1992 |
| Harding M | Labelling wheat flour with $^{13}$C. | 1994 |
| Livesey G | Influence of the physical form of barley grain On the digestion of its starch in the human small Intestine and implications for health. | 1995 |
| Normand S | Influence of dietary fat on postprandial Glucose metabolism (exogenous and Endogenous) using intrinsically $^{13}$C-enriched Durum wheat. | 2001 |
| Edwards CA | Production of $^{13}$C labelled pea flour for use in human digestion and fermentation. | 2002 |
| Christian MT | Starch digestion and fermentation in Infants | 2002 |
| *Priebe MG | An explorative study of in vivo digestive starch characteristics and postprandial glucose kinetics of wholemeal wheat bread | 2008 |
| *Wang H | A curve fitting approach to estimate the Extent of fermentation of indigestible carbohydrates. | 2008 |
| *Verbeke K | Influence of the type of Indigestible Carbohydrates on plasma and urine SCFA Profiles in healthy volunteers. | 2010 |

Table 1.11:  Published work using $^{13}$C labelled crops.

## The Objectives of this Project

- To discuss the significance of SCFA production to bowel health

- The principle aim of this work was to develop a means to prepare kilogram quantities of complex metaprobes of common staple carbohydrates

- To characterise these cereal metaprobes in quantitative terms by describing their production yield, their carbon and nitrogen content, their bulk $^{13}$C enrichment and the $^{13}$C enrichment of the starchy fraction of the grain

- To discuss the use of complex metaprobes prepared during this project and applied in human nutrition experiments to investigate SCFA production by colonic fermentation

- To consider the future use of complex metaprobes in human nutrition and in other areas

# Chapter 2: Materials and Methods

# 2  Wheat, Barley & Hay Crops

## 2.1 Pilot Studies

The principal objective of this project was to produce complex metaprobes of staple starchy carbohydrates.  To achieve this we planned pilot studies to grow and efficiently label the common cereals, barley and wheat.  Pilot studies were undertaken for a number of short term objectives: to develop a robust labelling procedure; to investigate varieties of Durum (hard) wheat suitable for labelling; to investigate if winter wheat can be labelled in the same way as spring wheat; and to investigate if hay can readily be labelled.

Locally-available plant growth facilities were surveyed and those at the Scottish Agricultural Centre (SAC), Kings Buildings, University of Edinburgh were chosen as SAC has the required facilities, namely an available glasshouse space, controlled growth cabinets and a single-ear thresher.  They also supplied pots, growth medium and different varieties of cereal and hay seeds.  Two staff from SUERC undertook all sowing, labelling and harvesting activities, while SAC staff watered the plants on a daily basis and applied pesticides, as necessary.

### 2.1.1  Initial Preparation for Labelling Plants with $^{13}CO_2$

Growth cabinets 600 L (Fi-totron 600, Fisons, UK) were prepared for use. (see Figure 2.2)  Each cabinet has programmable humidity, lighting and temperature control.  The humidity control was bypassed as the cabinet had to be sealed for gas addition.  Using a variety of rubber bungs and Duck tape, the drains and humidity pipe work were sealed.  The door seal was adjusted to optimise fit and the glass plate sealing the lid was checked.  A rubber bung fitted with three 4 mm OD PVC tubes was used to seal the port in the left hand side of the cabinet.  A similar port in right hand side was sealed with a plain rubber bung.  Each of the PVC tubes entered the growth cabinet and sealed using a Luer stopcock on the outside. One port was to be connected to the $^{13}CO_2$ gas cylinder, while the other two were to be connected to the pumped inflow and outflow of an Infra

Red Gas Analyser (IRGA; Guardian 3000, Edinburgh Sensors, UK; calibrated from 0-3000 parts-per-million $CO_2$) for monitoring the internal carbon dioxide concentration.

The successful sealing of the cabinet and subsequent cabinets, was checked by adding a pulse of unlabelled carbon dioxide from a standard laboratory compressed gas cylinder (BOC gases, UK). Lecture bottles, containing 30L $^{13}CO_2$ (99 atom % $^{13}C$, Cambridge Isotopes, from CK Gas, UK; or Spectra Gases, UK) were fitted with a 200psi regulator to which a 106 cm loop of 4 mm ID (6mm OD) copper pipe was attached, and isolated both ends by manual Nupro gas valves fitted with ¼'' Swagelok gas fittings (Valve and Fitting Company, Glasgow). The volume of the gas loop was estimated from its length to be 13.32 ml. The gas regulator allowed the pressure to be adjusted from zero to an operational limit of 90 psi (pounds-force per square inch). Using the valves to isolate and pressurise the loop, each loop of 13.32 mL when pressurised to 90 psi, would add 80 ml $CO_2$, raising the $CO_2$ partial pressure in the 600L growth cabinet by 133 ppm (parts-per-million). Eight loops of 80 psi would raise the partial pressure of the cabinet by 946 ppm, compared with a target of 1000 ppm. The result of this theoretical calculation could be monitored using the Guardian 3000 IRGA. Use of the valved loop allowed controlled addition of the expensive gas, while avoiding its catastrophic loss. This system (see Fig 2.1, 2.2, 2.3) proved robust in use.



Fig 2.1 : Fisons Fi-totron 600 L Growth Cabinets at SAC, University of Edinburgh

Fig 2.2: $^{13}CO_2$ labelling of cereal plants



Fig 2.3: 200 psi $CO_2$ regulator with compressed $^{13}CO_2$ cylinder

## 2.1.2 Labelling Trials: Sowing

A total of 87 pots (20cm; Stewarts Plastics, UK) were filled with growth medium (Shamrock Professional Growth Medium, Scotts, UK). 25 pots were planted at a density of 6 seeds per pot with Barley var Kirsty, being chosen as commonly grown in Northern Europe and being available from the temperature regulated seed store at SAC. Plants were grown on staging in a bay of the glasshouse at SAC (10m x 4m with 22 linear meters of 1 m depth decking).They were watered daily by SAC staff. A further 62 pots were planted at a density of 6 seeds per pot, with spring hard Durum Wheat var Lloyd, were sown manually. The plants were watered daily by SAC support staff. Anthesis was monitored visually and occurred approximately 50 days after sowing. (see Figure 2.4)

Fig 2.4: Wheat Plants in Anthesis

## 2.1.3 Labelling of the Pilot Study

Approximately one week after anthesis, plant pots were transferred to a growth cabinet, using a flat barrow to carry pots from the glasshouse to the growth cabinet room.  During the pilot trial when a single growth cabinet was in use, the infrared gas analyser (IRGA) output could be logged with a data logger (Data Hog, Skye Instruments, UK).  A figure of the $CO_2$ partial pressure in Growth

Cabinet 2 (SAC numbering) over five days of labelling, is demonstrated in Figure 2.5.  The addition of $CO_2$ each morning with a second smaller addition each afternoon can be clearly seen with $CO_2$ partial pressure declining from the combined effects of plant growth and leakage.  This was the system adopted to label the cereal plants over five days in the pilot studies and over a single day

during the metaprobe production phases. Following labelling, plants were returned to the glasshouse until maturity.

**Growth Chamber CO$_2$**
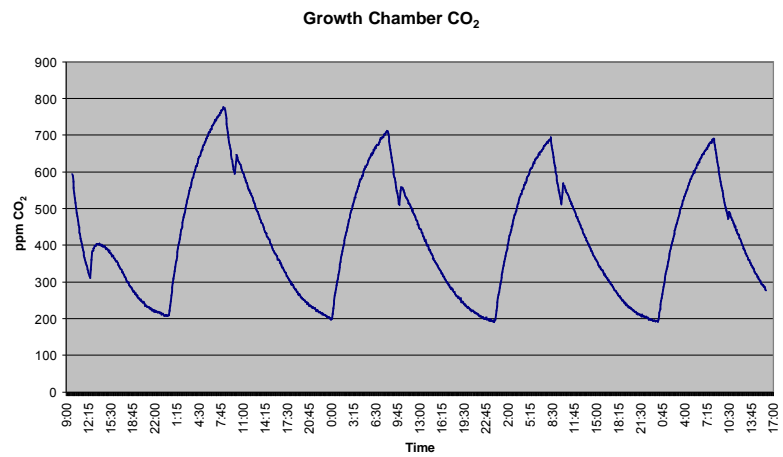


Fig 2.5: IRGA output using data logger over 5 days



Fig 2.6: Labelled plants are returned to glasshouse until maturation

## 2.1.4 Harvesting for the Pilot Study

Maturity was determined visually and the plants were harvested manually. Secateurs were used to cut the stem as close to the soil level as possible.  The grain heads were removed by hand and fed individually into a single ear thresher.  Growth cabinets were harvested individually. The grain, stems, root and chaff were individually weighted, bagged and transferred to SUERC for analysis. The grain was weighed and collected in brown paper bags marked with cabinet number and weight of grain minus the weight of the paper bag (9.57g). The straw and chaff from each growth cabinet was bagged in black plastic bags and labelled with weight and cabinet number.  On transfer to SUERC, plant samples were milled (Reustch Mixer Mill, UK) and analysed for their $^{13}$C enrichment and carbon content by continuous flow isotope ratio mass spectrometry CF-IRMS[66]; see section 2.8).

# 2.2 Durum Wheat Variety Study

Four varieties of Durum wheat suitable for bread and pasta making were chosen for growth trials after discussions with seed suppliers (Saaten Union, UK) and SAC staff.  Durum wheat vars Lloyd, Duramar, Orjaune and Combo, were used with 3 batches each containing 4 pots. Table 2.1 shows that seed density and soil type were also varied during the growth trial. Perlite Supercoarse 3.0-6.00 mm (Sinclair, UK) was added in a 1:2 ratio (1 part Perlite: 2 parts Shamrock potting medium) was added to improve soil aeration as these varieties are typically grown in drier Southern European soils.  The end point of this trial was the dry weight of grain harvest.  The appearance of the plants and grain was also noted.

| Variety of Durum or Spring Wheat | $N^o$ of Pots | $N^o$ of Seeds per Pot | Perlite Added |
| --- | --- | --- | --- |
| Lloyd | 4 | 10 | No |
| Lloyd | 4 | 20 | No |
| Lloyd | 4 | 30 | No |
| Lloyd | 4 | 10 | Yes |
| Lloyd | 4 | 20 | Yes |
| Lloyd | 4 | 30 | Yes |
| Duramar | 4 | 10 | Yes |
| Duramar | 4 | 20 | Yes |
| Duramar | 4 | 30 | Yes |
| Orjaune | 4 | 10 | Yes |
| Orjaune | 4 | 20 | Yes |
| Orjaune | 4 | 30 | Yes |
| Combo | 4 | 10 | Yes |
| Combo | 4 | 20 | Yes |
| Combo | 4 | 30 | Yes |

Table 2.1 Varieties of Wheat used in Pilot Study using Perlite

## 2.3 Hay Growth Trials

Growth trays measuring 38cm deep x 56 wide x 4 cm high (Stewarts plastics, UK) were chosen as three of these trays were a good fit to the base of the growth cabinet.  A common hay mix was chosen for sowing, comprising 50g perennial rye grass (Lolium perenne), 7.5g Timothy perennial grass (Phleum pratense) and 5g red clover (Trifolium pratense) seed.  The soil was 50/50 mix of John Innes No3 and Levington compost M3 75L was used to fill each tray and the seed was broadcast and watered.  Watering was continued daily and the seed grew vigorously to 15 cm tall after 48 days of growth.

The 1st trial used 3 growth trays and the 2nd trial used 18 growth trays.  Only 3 trays at a time would fit into each growth cabinet and labelled, for a period of 24 hours, to a target of 1000 ppm $^{13}CO_2$ in exactly the same manner as described

above. The labelled trays were then returned to the glasshouse until harvest 9 days later.  Harvesting was achieved by using coarse scissors to cut the crop 2 cm above soil level. Wet weight was recorded and the hay was transferred to SUERC for air drying and analysis. The trays from the second study were left in the glasshouse after harvest.  Watering was continued and a second hay crop was harvested after a further 4 weeks growth, without further labelling. It was again taken to SUERC for analysis.

## 2.4 Winter Wheat Trials var Robigus

To produce wheat suitable to make French style bread which would be high in starch, use of a soft winter wheat variety, var Robigus, was advised.  This is Nabim Group 3 soft wheat which produces yellow coloured flour and is low in protein (www.hgca.com).  In anticipation of producing kg quantities of grain, 225 pots with 9 seeds per pot were sown using Professional Growth Medium (Scotts, UK). The plants developed and become very bushy, producing a large number of leaves but no green stalks.  Few seed heads developed and therefore anthesis did not occur.  We were then advised that winter wheat seeds should have been vernalised for a longer period before germination. We disposed of these plants, prepared new pots with soil and sowed another 216 pots with 9 seeds per pot of wheat var Robigus that had been vernalised for a further 4 week period at 4$^{o}$C. For the second time, the plants became bushy with no seed heads.  This crop also failed to develop and was destroyed. Further trials of winter wheat in the glasshouse were abandoned.

## 2.5. Barley Production *var Kirsty*

### 2.1.2  Barley Sowing

A total of 216 20cm (rim diameter) plastic plant pots (Stewart Plastics, Scotland) were selected from stock at SAC and cleaned. They were filled manually with compost in the potting area of the SAC crop science facility. Shamrock Potting Professional Growing Medium (Scotts, UK) was used from 75kg bags. After filling with compost, all 216 pots were moved to the adjoining glasshouse, in groups of 9, using a flat barrow. Barley, var Kirsty, seeds were taken from the seed store

at SAC. The pots were planted at a density of 9 seeds per pot with Barley var Kirsty, being chosen as commonly grown in Northern Europe and being available from the temperature regulated seed store at SAC. Plants were growth on staging in a bay of the glasshouse at SAC. They were watered daily by SAC staff. Nine seeds per pot of spring Barley var Kirsty were sown manually with the aid of a 9-headed dibber. It was expected that each plant would produce 3 tillers on average. A 10m x 4m glasshouse bay fitted with 22 linear meters of 1 m depth decking was used for germination and maturation. Daily watering and pest control, as necessary, was performed by SAC support staff. Sowing was conducted in the month of May and germination occurred successfully some 10 days later. As the plants developed, long bamboo or fibreglass stakes were used to support the stalks using plant string. This counteracts the tendency of glasshouse grown cereals to be weaker than field grown plants and ensures maximum light for development.

## 2.5.2 Barley Labelling

Anthesis occurred 8 weeks after sowing when the barley stalks were about 80 cm tall. Anthesis of the maturing plants was determined visually by inspecting a representative group of plants. Approximately one week after anthesis, pots were loaded 9 at a time onto a flat barrow and wheeled to the plant growth chamber housed in a horticulture laboratory adjoining the glasshouse. Four Fisons Fi-totron 600 growth cabinets were used, which each took only 18 plants at a time. Labelling up to 216 pots of plants was thus achieved in 3 batches of 4 cabinets. In a similar way to the pilot study, each chamber was sealed and a 30L 'lecture bottle' cylinder of $^{13}CO_2$ gas was attached via a Luer stopcock connector to 4 mm PVC tubing leading inside the growth cabinet. By using individual pulses of known pressure and volume, the partial pressure of carbon dioxide inside the 600L cabinet was raised to approximately 1000 ppm.

The progress of gas addition was monitored using an Infra Red Gas Analyser (IRGA). Unlike the pilot studies, a second monitor, sensitive to $^{13}CO_2$ was used (Guardian Plus; Edinburgh Sensors, UK; calibrated to 0-30% $CO_2$ but actually measuring 0-3000 ppm $^{13}CO_2$. As the natural abundance of $^{13}C$ is close to 1% and the 0-30% scale relates to $CO_2$ at natural abundance, this meter reads 0-3000

ppm if the added $CO_2$ is fully enriched).  Each labelling period started in the morning, the exact time being noted. A smaller second addition of $^{13}CO_2$ was applied in the afternoon to return $CO_2$ partial pressure to 1000 ppm.

The growth cabinets have tungsten and fluorescent lights and were set for 16 hrs light per 24 hrs at a temperature of $18^0$C.

Labelling ceased after 24 hours when the cabinets opened and the plants removed and returned to the glasshouse. The growth chambers were pre-set to $16^0$C with 18 hours of simulated daylight (tungsten and fluorescent bulbs) in 24 hours.  One pot of barley was kept in the glasshouse as an unlabelled control, to allow us to compare the $^{13}$C content at natural abundance with those labelled $^{13}CO_2$.  The plants were returned to the glasshouse staging, 9 pots at a time. Each group of 18 pots were identified with paper labels and kept together in their cabinet numbers to retain their identity through the remaining production stages.

## 2.5.3 Barley Harvesting

Plant maturity was determined by visual inspection of the hardened seed head, browning of the plant and drying of the stem. Maturation occurred some 20 weeks after sowing. Using secateurs, each stem was cut as close as possible to the root.  The mature plants were placed in large plastic bags and carried (in groups representing the 18 pots labelled at one time) to the adjoining laboratory. Ears with seed heads were removed using secateurs and fed individually into a single ear thresher (SAC in-house design), with each batch of 18 pots being harvested individually.  The grain from all cabinets was not mixed until the yield and $^{13}$C enrichment had been determined after transfer to SUERC. The grain was weighed and collected in brown paper bags marked with cabinet number and weight of grain minus the weight of the paper bag (9.57g).  The straw from each growth cabinet was bagged in black plastic bags and labelled with weight and cabinet number.  All materials were transferred to SUERC for analysis.

## 2.6 Wheat Production var Lloyd

### 2.6.1 Wheat Sowing

A total of 200 pots of Spring Hard Durum wheat var Lloyd were sown in May using a density of 30 seeds per pot.   The similar procedure as described above for barley was used.

### 2.6.2 Wheat Labelling

In July, after 57 days of growth, wheat was in anthesis which was observed visually. Labelling commenced, with the same procedure used as was described above for barley. After 24 hours, the plants were returned to the glasshouse to mature.

### 2.6.3 Wheat Harvesting

The mature plants were harvested in August. The grain was threshed and the grain and straw were weighed and bagged for transfer to SUERC.

## 2.7. Wheat Production var Paragon

### 2.7.1 Wheat Sowing

A similar procedure was described above was used. The Spring Soft wheat, var Paragon, from Group 1 on the NABIM Wheat List (see Glossary) was chosen as being suitable for bread making. Paragon has low protein content (13%) and a 250 Hagberg Falling Number (HFN; see glossary).  216 pots with 9 seeds per pot were sown.

### 2.7.2 Wheat Labelling

By February, the soft wheat was in anthesis which was observed visually. Due to delays in growth cabinet availability, labelling commenced some 3 weeks after

anthesis. The same procedure was used as was described above for barley. After 24 hours, the plants were returned to the glasshouse to mature.

### 2.7.3 Wheat Harvesting

The mature plants were harvested in April. The grain and straw were separated, weighed and bagged for transfer to SUERC.

## 2.8. Barley Production, *var Cellar*

### 2.8.1 Barley Sowing

A further batch of spring barley was produced with 208 pots of spring barley var Cellar, being sown with 9 seed per pot. Exactly the same procedure was followed as was described above.

### 2.8.2 Barley Labelling

Approximately one week after anthesis, the barley pots were loaded, 9 at a time, onto a flat barrow and wheeled to the plant growth chambers. Three 600 L Fisons Fi-totron 600 growth cabinets were available for use. Each held a maximum of 18 plant pots, so labelling was undertaken in four sessions each using three cabinets. Labelling was achieved in batches so that all crops were eventually enriched with $^{13}$C-labelled $CO_2$ gas and monitored using the Guardian Plus infra red gas analyser, at a target of 1000 ppm $^{13}CO_2$. The growth chambers were set to $16^0$C with 18 hours of fluorescent light in 24 hours. After 24 hours labelling, the barley plants were returned to the glasshouse, staked and tied while remaining in groups to retain the identity of plants labelled together.

### 2.8.3 Barley Harvesting

After 155 days of growth, the barley and wheat was mature and it was manually harvested using the single ear thresher. All grain and straw was weighed, bagged and returned to SUERC for milling, analysis and storage.

## 2.9 Metaprobe Characterisation

### 2.9.1 Bulk $^{13}$C analysis

On transfer to SUERC all samples were air dried to constant weight and their weight recorded. Aliquots of samples grain and straw from each growth cabinet were ground to a fine powder using a mill (Reustch Mixer Mill, UK). This flour was then weighed into a tin combustion container (Elemental Microanalysis, Okehampton, UK) to 1.00 mg using a 5 figure electrobalance (Mettler Toledo, UK). The $^{13}$C abundance, carbon and nitrogen content were measured by continuous flow isotope ratio mass spectrometry (CF-IRMS), first introduced by Preston and Owens, 1983. [66]

Here an elemental analyser, using the principle of Dumas combustion, is used to covert organic samples by combustion into the simple gases, carbon dioxide and nitrogen (with water being removed on line using a magnesium perchlorate trap). Carbon dioxide and nitrogen gas are separated on-line by gas chromatography and a helium carrier is used to sweep these gases into the ion source of an IRMS, where they are ionised by EI ionisation. Total elemental content and isotope ratio are analysed from the total ion beam and ion beam ratio, respectively. The system gives total carbon content, $^{13}$C abundance, total nitrogen content, $^{15}$N abundance and carbon to nitrogen ratio. Total protein can be calculated from nitrogen data. This system is capable of rapid and precise total carbon and $^{13}$C analysis of samples of labelled plant tissue. [66] Results for $^{13}$C abundance were converted to units of atom percent, with reproducibility of bulk analysis better that 0.001 atom % $^{13}$C. Most results are expressed in units of enrichment (Atom % $^{13}$C excess) where $^{13}$C natural abundance has been subtracted from the measured $^{13}$C abundance of labelled material.

### 2.9.2 $^{13}$C Enrichment of starch

$^{13}$C enrichment of starch was analysed by converting grain starch to glucose using acid hydrolysis. The enrichment of glucose was then analysed by the new technique of liquid chromatography IRMS (LC-IRMS) under development in our laboratories at SUERC [52]. (see also section 1.7) 5 mL of deionised water and 10 mL

6M HCl were added to 35 mg of flour in a 20 mL crimp capped glass headspace analyser vial fitted with a PTFE-lined crimp cap (Chromacol UK). Samples were ultrasonicated for two minutes to ensure wetting and then hydrolysed at $60^{\circ}C$ for 6 hours in a shaking water bath (Techne, UK). Samples were cooled and 1 mL 1 mM fructose was pipetted into 30kDa cut-off centrifugal ultrafiltration devices (Ultra 4, Millipore, UK) as internal standard. 0.2 mL sample aliquot was pipetted into the device followed by 0.8 mL 1M NaOH to neutralise the hydrolysate. Samples of flour derived from each growth cabinet from Wheat var Paragon and Barley var Cellar were analysed. Reagent blanks and unlabelled barley flour were also analysed. The $^{13}C$ enrichment of the glucose produced was analysed by LC-IRMS using automated injection into strong anion exchange chromatography interfaced to an IsoPrime IRMS via a LIquiface interface (GVI, UK).[52] Results of starch $^{13}C$ enrichment were compared with bulk $^{13}C$ enrichment obtained by CF-IRMS of each cereal grain from each individual cabinet as described above. It is possible that some NSP were hydrolysed in this treatment also producing glucose. This would not alter the fact that this analysis differentiates glucose polymers from other macromolecules such as proteins. Nilsson et al reported that NSP comprised <15% of total carbohydrates in both wheat and barley grain. [54] The effect of differential labelling between NSP and starch, upon the measured tota carbohydrate $^{13}C$ signature, would be expected to be minor.

## 2.10 Grain for use in Human Nutrition Studies

Once characterised, each type of grain was fully mixed and the bulk was transported in cotton drawstring bags to our EU collaborators at the University Medical Centre Groningen, The Netherlands. This $^{13}C$ labelled whole grain was subsequently prepared for human nutrition studies which are discussed in Chapter 6.

## 2.11. Statistical Methods

The Student's t-test was used to test if the enrichment of grain starch was significantly different from its bulk $^{13}C$ signature.

## 2.12 Analysis of SCFA in human body fluids

The methods used to analyse SCFA concentration and enrichment in human studies is described in Chapter 6 and in our published method. (49) (80) (81)

# Chapter 3: Results

# 3  Metaprobe Production and Characterisation

## 3.1  Labelling Wheat, Barley & Hay for Pilot Study

### 3.1.1 Pilot labelling of Durum Wheat *var Lloyd*

The pilot study was to establish labelling methods and used a single growth cabinet.  The Durum wheat had 8 weeks growth from sowing to labelling and a total of 142.2 g of grain was produced. Batch 1 had 1 day of labelling in the growth cabinets but batches 2-4 had 5 days of labelling. The grain weighted mean enrichment of batches 2-4 was 1.31 ape $^{13}$C (Atom % excess $^{13}$C).  The total straw weighted mean enrichment was 0.450 ape $^{13}$C.

Table 3.1 displays the yield of entire plant structure from this wheat crop.

| Durum Wheat var Lloyd | Pots per Batch | Sowing density (per pot) | Days of Labelling | Date of Harvesting | Yield of Grain (g) | Yield of Stems & Leaves (g) | Yield of Roots (g) | Yield of Chaff (g) |
|---|---|---|---|---|---|---|---|---|
| Batch 1 | 12 | 6 | 1 | 11 Aug | 44.23 | 48.19 | 4.33 | 13.78 |
| Batch 2 | 15 | 6 | 5 | 13 Aug | 40.70 | 43.03 | 2.05 | 17.07 |
| Batch 3 | 15 | 6 | 5 | 18 Nov | 24.50 | 47.42 | 1.45 | 14.08 |
| Batch 4 | 20 | 6 | 5 | 2 Dec | 32.78 | 92.37 | 7.42 | 23.11 |

Table 3.1: Grain & straw Yield from Durum wheat var Lloyd

Table 3.2 displays analysis using CF-IRMS of $^{13}$C enrichment of entire plant structure from this wheat crop.  The column headed 'weight x ape' is the product of the weight of individual batches multiplied by their enrichment. It is an intermediate result of the calculation of the weighted mean enrichment of the whole crop.

| Durum Wheat var Lloyd | Section of Plant | Weight x ape | Carbon Content (g) | Nitrogen Content (g) | C:N Ratio | $^{13}C$ Enrichment (ape) |
|---|---|---|---|---|---|---|
| Batch 1 | Grain | 85.45 | 18.35 | 1.84 | 10.0 | 1.932 |
| | Stems & leaves | 69.36 | 19.15 | 0.92 | 20.8 | 1.439 |
| | Roots | 2.36 | 1.90 | 0.09 | 21.0 | 0.544 |
| | Chaff | 14.53 | 5.85 | 0.15 | 38.0 | 1.054 |
| Batch 2 | Grain | 304.54 | 16.93 | 1.53 | 11.1 | 7.483 |
| | Stems & leaves | 43.46 | 17.22 | 0.95 | 18.1 | 1.010 |
| | Roots | 1.29 | 0.90 | 0.04 | 23.7 | 0.627 |
| | Chaff | 66.85 | 6.96 | 0.45 | 15.4 | 3.916 |
| Batch 3 | Grain | 45.82 | 10.01 | 1.10 | 9.1 | 1.870 |
| | Stems & leaves | 16.65 | 15.79 | 1.19 | 13.3 | 0.351 |
| | Roots | 0.05 | 0.55 | 0.03 | 16.5 | 0.033 |
| | Chaff | 3.85 | 5.85 | 0.23 | 25.2 | 0.271 |
| Batch 4 | Grain | 55.74 | 13.90 | 1.49 | 9.3 | 1.700 |
| | Stems & leaves | 12.93 | 33.76 | 1.97 | 17.2 | 0.140 |
| | Roots | 2.03 | 3.05 | 0.18 | 16.6 | 0.273 |
| | Chaff | 1.10 | 0.41 | 0.02 | 17.7 | 1.048 |

Table 3.2: Analysis of $^{13}C$ enriched grain & straw from Durum wheat var Lloyd.

### 3.1.2. Pilot Study of Barley *var Kirsty*

The Barley var Kirsty was sown 2 batches of 10 & 15 pots each. Both batches were labelled for 5 days and produced a total of 336.6 g of grain and 484.4 total straw. The weighted mean enrichment of grain was 1.307 ape $^{13}C$ and the weighted mean enrichment of total straw was 0.1777 ape $^{13}C$.

Table 3.3 shows yield from entire plant structure.

| Barley var Kirsty | Pots per Batch Grain | Pots per Batch Straw | Sowing density (seeds per pot) | Days of Labelling | Date of Harvesting | Yield of Grain (g) | Yield of Stems & Leaves (g) | Yield of Roots (g) | Yield of Chaff (g) |
|---|---|---|---|---|---|---|---|---|---|
| Batch 1 | 10 | 10 | 6 | 5 | 18 Nov | **165.4** | **194.9** | **33.9** | **32.2** |
| Batch 2 | 15 | 15 | 6 | 5 | 2 Dec | **171.2** | **158.3** | **34.1** | **31.1** |

Table 3.3: Grain & straw yield from Barley var Kirsty

Table 3.4 show entire plant structure analysis of $^{13}C$ enrichment of this barley crop.

| Barley var Kirsty | Section of Plant | Weight x ape | Carbon Content (g) | Nitrogen Content (g) | C:N Ratio | $^{13}C$ Enrichment (ape) |
|---|---|---|---|---|---|---|
| Batch 1 | Grain | 128.29 | 67.28 | 5.19 | 13.0 | 0.776 |
|  | Stems & leaves | 46.51 | 85.42 | 4.08 | 21.0 | 0.239 |
|  | Roots | 1.20 | 15.28 | 0.64 | 24.0 | 0.035 |
|  | Chaff | 10.38 | 13.97 | 0.38 | 37.1 | 0.322 |
| Batch 2 | Grain | 311.57 | 70.18 | 4.74 | 14.8 | 1.820 |
|  | Stems & leaves | 19.87 | 68.35 | 2.25 | 30.4 | 0.126 |
|  | Roots | 1.98 | 15.12 | 0.61 | 24.7 | 0.058 |
|  | Chaff | 5.69 | 12.77 | 0.26 | 48.3 | 0.183 |

Table 3.4: Analysis $^{13}C$ enriched of grain & straw from Barley var Kirsty

### 3.1.3. Durum Wheat Trials

The results of this study, comparing the grain yield from 4 different varieties of Durum wheat, are shown in Table 3.5.  There was little difference in terms of the maximum grain yield between varieties, which each produced approximately 8 g grain per pot.  The variety Combo gave a more consistent yield.  Individual pots with a poorer yield corresponded to the visual quality of the plants, some

showing signs of fungal infection.  Generally, the use of perlite to aerate the soil showed little improvement.  There was an improvement in yield when seed density increased from 10 to 20 seeds per pot, but little further gain when a density of 30 seeds per pot were used.

| Variety of Durum Wheat | Sowing Density (per pot) | Perlite Added (1:1 blend) | Total Days of Growth | Average Height of Plant (cm) | Grain Yield (grams per 4 pots) |
|---|---|---|---|---|---|
| Lloyd | 10 | No | 155 | 55 | 10 |
| Lloyd | 20 | No | 155 | 55 | 40 |
| Lloyd | 30 | No | 155 | 55 | 40 |
| Lloyd | 10 | Yes | 155 | 55 | 10 |
| Lloyd | 20 | Yes | 155 | 55 | 14 |
| Lloyd | 30 | Yes | 155 | 55 | 45 |
| Duramar | 10 | Yes | 155 | 40 | 10 |
| Duramar | 20 | Yes | 155 | 40 | 40 |
| Duramar | 30 | Yes | 155 | 40 | 45 |
| Orjaune | 10 | Yes | 155 | 51 | 30 |
| Orjaune | 20 | Yes | 155 | 51 | 25 |
| Orjaune | 30 | Yes | 155 | 51 | 40 |
| Combo | 10 | Yes | 155 | 56 | 20 |
| Combo | 20 | Yes | 155 | 56 | 40 |
| Combo | 30 | Yes | 155 | 56 | 40 |

Table 3.5: Varieties of Durum Wheat sown with & without Perlite

## 3.1.4 Hay – Labelled and Naturally (Unlabelled) Hay Trials

### 3.1.4.1 Hay Trial 1

The 1st hay trials consisted of 3 trays of hay seed mix (see section 2.4) which were seeded by broadcast for labelling trials.  Two weeks later, 3 further trays were broadcast with the intention not to label but to be used as unlabelled controls to measure $^{13}C$ natural abundance and subtract this from all labelled batches of labelled hay.

The labelling 3 trays were broadcast on 12th May, labelled on 20th June and harvested on 26th June.  They had 48 days of growth in total.  The trays of

unlabelled crop, was broadcast on 26th May and harvested on 29th June with 34 days of total growth.  The harvested hay had been watered prior to harvest and wet weight was recorded.  The total weight of wet hay harvest was 1098 g.  The hay harvest was returned to SUERC to be aired dried at room temperature.  The dry weight was recorded after 12 days, 11th July, with an average loss of 155 g per tray.  At 50 days post harvest, 18th August, the dry hay was re-measured and weighed 932.40 g.  It was noted that moisture in the air of the laboratory air caused this variation in hay dry weight of approximately 0.7g per tray.   The labelled hay mix trays numbers 1, 2 & 3, had 48 total days of growth.   This product is for human consumption therefore air drying is appropriate.  Oven drying was not used during subsequent processing.

Table 3.6, shows the results of the yield of wet weight and dry weights.

| Date of Broadcasting of Hay Seed Mix | Date of Labelling | Tray N° | Growth Cabinet Code | Date of Harvest | Wet Weight (g per tray) | Dry Weight (g per tray) | Dry Weight (g per tray) |
|---|---|---|---|---|---|---|---|
| 12 May | 20 June | 1 | C7 | 26 June | 368.07 | 53.56 | 54.88 |
| 12 May | 20 June | 2 | C7 | 26 June | 340.67 | 52.34 | 55.05 |
| 12 May | 20 June | 3 | C7 | 26 June | 389.47 | 58.72 | 52.61 |

Table 3.6: Wet & dry hay yield from 1st Trials

Table 3.7 shows $^{13}$C analysis of the labelled and finely milled hay. The enrichment of this crop was 0.515 ape $^{13}$C.

| Tray 1,2,3 Sum | Growth Cabinet Code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | $^{13}$C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Sum | C7 | 84.73 | 72.87 | 4.54 | 16.04 | 0.515 |

Table 3.7: Analysis of $^{13}$C enriched hay from 1st Trial

### 3.1.4.2. Hay Trial 2

The 2<sup>nd</sup> hay trial, (see Table 3.8), a batch comprising 18 trays, was sown by broadcast on 26<sup>th</sup> July.  After 8 weeks, the hay had 15 cm of growth and obvious red clover shoots had appeared.  As there were only three growth cabinets available and trays sufficient for 6 cabinets, the labelling of 18 trays occurred over two days, 19<sup>th</sup> & 20th  September. This hay was harvested on 3<sup>rd</sup> October. The wet hay harvest was weighed at 4275 g and taken to SUERC to be air dried at room temperature.  Dry weight was taken after 16 days post harvest, with an average 200g weight loss and after 26 days from harvest with approximately 0.10g ± weight loss/gain. (see section 3.1.3.1)

These trays were returned to the glasshouse post labelling, and was allowed to produce a second hay crop without further labelling to study the translocation of labelled root carbon to the new shoots.  This second crop was harvested and weighed and its residual label was measured.



Fig 3.1: Trays of Hay from 2<sup>nd</sup> Hay Trial

Table 3.8 displays the wet yield harvest and the dry weight of hay 16 days and 26 days of air drying post harvest.

| Date of Broadcasting of Hay Seed Mix | Date of Labelling | Tray Nº | Growth Cabinet Code | Date of Harvest | Wet Weight (g per tray) | Dry Weight after 16 days (g) | Dry Weight after 26 days (g) |
|---|---|---|---|---|---|---|---|
| 26 July | 19 Sept | 1 | C5 | 3 Oct | 215 | 31.4 | 31.73 |
| 26 July | 19 Sept | 2 | C5 | 3 Oct | 180 | 27.94 | 28.19 |
| 26 July | 19 Sept | 3 | C5 | 3 Oct | 220 | 32.68 | 33.14 |
| 26 July | 19 Sept | 4 | C3 | 3 Oct | 210 | 28.91 | 28.93 |
| 26 July | 19 Sept | 5 | C3 | 3 Oct | 290 | 36.9 | 37.35 |
| 26 July | 19 Sept | 6 | C3 | 3 Oct | 270 | 36.27 | 36.58 |
| 26 July | 19 Sept | 7 | C7 | 3 Oct | 290 | 46.15 | 46.07 |
| 26 July | 19 Sept | 8 | C7 | 3 Oct | 270 | 38.08 | 37.97 |
| 26 July | 19 Sept | 9 | C7 | 3 Oct | 310 | 42.38 | 41.93 |
| 26 July | 20 Sept | 10 | C5 | 3 Oct | 210 | 37.51 | 37.56 |
| 26 July | 20 Sept | 11 | C5 | 3 Oct | 180 | 31.69 | 31.96 |
| 26 July | 20 Sept | 12 | C5 | 3 Oct | 220 | 37.06 | 37.26 |
| 26 July | 20 Sept | 13 | C3 | 3 Oct | 280 | 46.38 | 46.76 |
| 26 July | 20 Sept | 14 | C3 | 3 Oct | 250 | 39.3 | 39.47 |
| 26 July | 20 Sept | 15 | C3 | 3 Oct | 220 | 36.64 | 36.86 |
| 26 July | 20 Sept | 16 | C7 | 3 Oct | 250 | 38.3 | 38.15 |
| 26 July | 20 Sept | 17 | C7 | 3 Oct | 200 | 37.08 | 37.03 |
| 26 July | 20 Sept | 18 | C7 | 3 Oct | 210 | 37.32 | 37.00 |

Table 3.8: Wet & Dry Hay yield from 2nd Trial

Table 3.9 displays the $^{13}C$ enrichment of the labelled and milled hay from initial harvest and re-growth 2nd harvest.  The mean residual fraction of $^{13}C$ from the 1st crop was 11% of the 2nd crop.

| Tray N° | Weight x ape | Carbon Content (g per tray) | Nitrogen Content (g per tray) | C:N Ratio | 1st Crop $^{13}C$ Enrichment (ape) | $^{13}C$ enrichment 2nd crop (ape) | Residual Fraction (mean 11 % of 1st Trial) |
|---|---|---|---|---|---|---|---|
| 1 | 58.32 | 11.30 | 0.98 | 11.48 | 0.777 | 0.064 | 0.083 |
| 2 | 52.15 | 9.83 | 0.86 | 11.38 | 0.786 | 0.062 | 0.079 |
| 3 | 61.54 | 12.04 | 1.05 | 11.41 | 0.803 | 0.057 | 0.071 |
| 4 | 55.65 | 11.01 | 0.73 | 15.15 | 0.845 | 0.097 | 0.115 |
| 5 | 71.34 | 13.85 | 0.91 | 15.28 | 0.853 | 0.094 | 0.110 |
| 6 | 70.30 | 13.69 | 0.91 | 15.11 | 0.858 | 0.103 | 0.120 |
| 7 | 73.31 | 17.13 | 1.43 | 11.97 | 0.508 | 0.062 | 0.122 |
| 8 | 59.08 | 14.09 | 1.21 | 11.62 | 0.471 | 0.058 | 0.123 |
| 9 | 67.60 | 15.58 | 1.26 | 12.34 | 0.515 | 0.056 | 0.109 |
| 10 | 78.48 | 13.38 | 1.29 | 10.40 | 1.012 | 0.065 | 0.064 |
| 11 | 71.07 | 11.47 | 1.07 | 10.76 | 1.162 | 0.059 | 0.050 |
| 12 | 82.26 | 13.29 | 1.22 | 10.86 | 1.139 | 0.063 | 0.055 |
| 13 | 130.01 | 15.44 | 0.94 | 16.43 | 1.723 | 0.378 | 0.219 |
| 14 | 115.02 | 15.01 | 1.00 | 15.06 | 1.846 | 0.325 | 0.176 |
| 15 | 109.16 | 13.76 | 0.97 | 14.24 | 1.899 | 0.256 | 0.135 |
| 16 | 68.01 | 14.48 | 1.14 | 12.70 | 0.695 | 0.084 | 0.122 |
| 17 | 66.68 | 13.58 | 1.06 | 12.81 | 0.718 | 0.087 | 0.121 |
| 18 | 65.78 | 14.26 | 1.07 | 13.26 | 0.682 | 0.088 | 0.129 |

Table 3.9: Analysis of $^{13}C$ enriched hay from 2nd Trial.

The weighted mean enrichment for the hay was 0.968 ape $^{13}C$. The second crop contained on average, 11% of the enrichment of the 1st Trial hay crop.

## 3.2 Winter Wheat *var Robigus*

Neither of these two winter wheat trials succeeded to maturity.  Bushy, leafy plants resulted with very few seed heads developing.  Our experience shows that spring wheat varieties are much more successful candidates for labelling and taking maturity in a glasshouse.

## 3.3 Production of Barley *var Kirsty*

The results of the pilot study to produce Spring Barley var Kirsty are shown in the following tables. Batch 1 contained 6 individual sets of 5 x 17 pots and 1 x 18 pots.  Batch 2 contained 6 individual sets of 5 x 18 pots and 1 x 4 pots.  This explains the apparently low yield (90 g grain, 95 g straw) for one treatment in batch 2 in the following tables.  An unlabelled batch comprising 2 pots was analysed as control as a measure of $^{13}C$ natural abundance in the glasshouse.  All pots had 9 seeds per pot.

Table 3.10 displays the yield per cabinet of the labelled barley grain.   The total grain yield of Barley var Kirsty weighed 3830 g.

| Barley var Kirsty | Batches | Date of Sowing | Sowing Density (per pot) | Growth Cabinet Code | Date of Labelling | Date of Harvesting | Grain Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Grain | Batch 1 | 10 May | 9 | C2 | 29 June | 29 Sept | 250 |
| Grain | Batch 1 | 10 May | 9 | C3 | 29 June | 29 Sept | 280 |
| Grain | Batch 1 | 10 May | 9 | C4 | 29 June | 29 Sept | 330 |
| Grain | Batch 1 | 10 May | 9 | C5 | 29 June | 29 Sept | 400 |
| Grain | Batch 1 | 10 May | 9 | C6 | 29 June | 29 Sept | 390 |
| Grain | Batch 1 | 10 May | 9 | C7 | 29 June | 27 Sept | 330 |
| Grain | Batch 2 | 10 May | 9 | C2 | 30 June | 27 Sept | 390 |
| Grain | Batch 2 | 10 May | 9 | C3 | 30 June | 28 Sept | 390 |
| Grain | Batch 2 | 10 May | 9 | C4 | 30 June | 28 Sept | 90 |
| Grain | Batch 2 | 10 May | 9 | C5 | 30 June | 28 Sept | 330 |
| Grain | Batch 2 | 10 May | 9 | C6 | 30 June | 27 Sept | 310 |
| Grain | Batch 2 | 10 May | 9 | C7 | 30 June | 27 Sept | 330 |

Table 3.10: Grain yield from Barley var Kirsty

| Barley var Kirsty | Batches | Date of Sowing | Sowing Density (per pot) | Growth Cabinet Code | Date of Labelling | Date of Harvesting | Straw + Chaff Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Straw | Batch 1 | 10 May | 9 | C2 | 29 June | 29 Sept | 250 |
| Straw | Batch 1 | 10 May | 9 | C3 | 29 June | 29 Sept | 260 |
| Straw | Batch 1 | 10 May | 9 | C4 | 29 June | 29 Sept | 280 |
| Straw | Batch 1 | 10 May | 9 | C5 | 29 June | 29 Sept | 330 |
| Straw | Batch 1 | 10 May | 9 | C6 | 29 June | 29 Sept | 355 |
| Straw | Batch 1 | 10 May | 9 | C7 | 29 June | 27 Sept | 370 |
| Straw | Batch 2 | 10 May | 9 | C2 | 30 June | 27 Sept | 395 |
| Straw | Batch 2 | 10 May | 9 | C3 | 30 June | 28 Sept | 390 |
| Straw | Batch 2 | 10 May | 9 | C4 | 30 June | 28 Sept | 95 |
| Straw | Batch 2 | 10 May | 9 | C5 | 30 June | 28 Sept | 375 |
| Straw | Batch 2 | 10 May | 9 | C6 | 30 June | 27 Sept | 370 |
| Straw | Batch 2 | 10 May | 9 | C7 | 30 June | 27 Sept | 402 |

Table 3.11: Straw & chaff yield from Barley var Kirsty

Table 3.11 displays the yield of straw and chaff.  The roots were not harvested from this crop which had occurred previously in the pilot studies.  The total weight of straw and chaff was 3872 g.

Table 3.12, records the ^{13}C enrichment analysis of labelled and milled barley grain. The weighted mean enrichment of Barley var Kirsty grain was 0.138 ape [13]C.

| Barley var Kirsty | Growth Cabinet Code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | [13]C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Grain | C2 | 67.01 | 98.15 | 2.90 | 33.85 | 0.268 |
| Grain | C3 | 43.03 | 110.41 | 3.09 | 40.84 | 0.154 |
| Grain | C4 | 36.67 | 128.98 | 6.10 | 21.20 | 0.111 |
| Grain | C5 | 84.80 | 124.20 | 4.88 | 25.30 | 0.212 |
| Grain | C6 | 18.61 | 153.29 | 7.60 | 20.98 | 0.048 |
| Grain | C7 | 23.96 | 127.48 | 3.92 | 32.57 | 0.073 |
| Grain | C2 | 4.78 | 149.53 | 5.96 | 25.07 | 0.012 |
| Grain | C3 | 149.79 | 152.04 | 5.84 | 26.04 | 0.384 |
| Grain | C4 | 10.81 | 35.70 | 1.28 | 35.67 | 0.120 |
| Grain | C5 | 53.43 | 131.22 | 5.32 | 27.26 | 0.162 |
| Grain | C6 | 28.54 | 121.77 | 4.49 | 30.66 | 0.092 |
| Grain | C7 | 10.36 | 101.73 | 8.31 | 13.12 | 0.031 |

Table 3.12: Analysis of ^{13}C enriched grain from Barley var Kirsty

Table 3.13 records the ^{13}C enrichment analysis of labelled and milled barley grain, straw & chaff flour. The weighted mean enrichment of the barley straw was 0.082 ape [13]C.

| Barley var Kirsty | Growth Cabinet Code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | [13]C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Straw | C2 | 0.064 | 105.62 | 4.63 | 22.80 | 0.064 |
| Straw | C3 | 0.096 | 108.90 | 4.38 | 24.86 | 0.096 |
| Straw | C4 | 0.009 | 116.76 | 3.31 | 35.27 | 0.009 |
| Straw | C5 | 0.117 | 136.90 | 3.82 | 35.86 | 0.117 |
| Straw | C6 | 0.050 | 146.59 | 5.45 | 26.91 | 0.050 |
| Straw | C7 | 0.033 | 153.65 | 4.12 | 37.34 | 0.033 |
| Straw | C2 | 0.041 | 166.03 | 4.77 | 34.82 | 0.041 |
| Straw | C3 | 0.025 | 162.30 | 3.81 | 42.65 | 0.025 |
| Straw | C4 | 0.105 | 39.60 | 1.24 | 32.02 | 0.105 |
| Straw | C5 | 0.092 | 152.76 | 4.99 | 30.59 | 0.092 |
| Straw | C6 | 0.052 | 150.54 | 4.32 | 34.81 | 0.052 |
| Straw | C7 | 0.306 | 158.86 | 5.73 | 27.74 | 0.306 |

Table 3.13: Analysis of ^{13}C enriched straw & chaff from Barley var Kirsty

## 3.4 Production of Durum Wheat *var Lloyd*

The following tables demonstrate the results and analysis of the pilot labelling trials where 2 batches of hard Spring Durum Wheat var Lloyd were labelled. Batches 1 & 2 had a variety of pot numbers because of the capacity and number of growth cabinets available. The wheat crops were labelled and harvested. The harvest grain and straw were weighed, milled and analysed.

Table 3.14, displays the yield of grain in grams per cabinet from Durum wheat var Lloyd. The total weight of wheat grain is 507 g.

| Durum Wheat var Lloyd | Pots per Batch | Date of Sowing | Sowing density (per pot) | Growth Cabinet code | Date of Labelling | Date of Harvesting | Grain Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Batch 1 | 16 | 9 May | 30 | C2 | 5 Jul | 22 Aug | 78 |
| Grain | 17 | 9 May | 30 | C3 | 5 Jul | 22 Aug | 53 |
| Grain | 4 | 9 May | 30 | C4 | 5 Jul | 22 Aug | 8 |
| Grain | 17 | 9 May | 30 | C5 | 5 Jul | 22 Aug | 70 |
| Grain | 17 | 9 May | 30 | C6 | 5 Jul | 22 Aug | 73 |
| Grain | 17 | 9 May | 30 | C7 | 5 Jul | 22 Aug | 73 |
| Batch 2 | 18 | 10 May | 30 | C2 | 7 Jul | 23 Aug | 33 |
| Grain | 18 | 10 May | 30 | C3 | 7 Jul | 23 Aug | 45 |
| Grain | 4 | 10 May | 30 | C4 | 7 Jul | 23 Aug | 6 |
| Grain | 18 | 10 May | 30 | C5 | 7 Jul | 23 Aug | 29 |
| Grain | 18 | 10 May | 30 | C6 | 7 Jul | 23 Aug | 29 |
| Grain | 18 | 10 May | 30 | C7 | 7 Jul | 23 Aug | 9 |

Table 3.14 Grain yield from Durum wheat var Lloyd

Table 3.15, displays the yield of straw and chaff in grams per cabinet from Durum wheat var Lloyd. The total weight of wheat straw was 3084 g.

| Durum Wheat var Lloyd | Pots per Batch | Date of Sowing | Sowing density (per pot) | Growth Cabinet code | Date of Labelling | Date of Harvesting | Straw + Chaff Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Batch 1 | **16** | 9 May | 30 | C2 | 5 Jul | 22 Aug | 295 |
| Straw | **17** | 9 May | 30 | C3 | 5 Jul | 22 Aug | 315 |
| Straw | **4** | 9 May | 30 | C4 | 5 Jul | 22 Aug | 127 |
| Straw | **17** | 9 May | 30 | C5 | 5 Jul | 22 Aug | 270 |
| Straw | **17** | 9 May | 30 | C6 | 5 Jul | 22 Aug | 320 |
| Straw | **17** | 9 May | 30 | C7 | 5 Jul | 22 Aug | 320 |
| Batch 2 | **18** | 9 May | 30 | C2 | 7 Jul | 23 Aug | 270 |
| Straw | **18** | 9 May | 30 | C3 | 7 Jul | 23 Aug | 270 |
| Straw | **4** | 9 May | 30 | C4 | 7 Jul | 23 Aug | 110 |
| Straw | **18** | 9 May | 30 | C5 | 7 Jul | 23 Aug | 292 |
| Straw | **18** | 9 May | 30 | C6 | 7 Jul | 23 Aug | 305 |
| Straw | **18** | 9 May | 30 | C7 | 7 Jul | 23 Aug | 190 |

Table 3.15: Straw yield from Durum wheat var Lloyd

Table 3.16, shows the analysis and $^{13}C$ enrichment of the milled grain this wheat production crop.  The weighted mean enrichment of grain of 0.341 ape $^{13}C$.

| Durum Wheat var Lloyd | Growth Cabinet code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | $^{13}C$ Enrichment (ape) |
|---|---|---|---|---|---|---|
| Grain | C2 | 24.57 | 22.21 | 3.06 | 7.39 | 0.464 |
| Grain | C3 | 2.14 | 3.35 | 0.30 | 11.23 | 0.268 |
| Grain | C4 | 3.68 | 28.94 | 2.05 | 14.16 | 0.053 |
| Grain | C5 | 23.46 | 27.28 | 1.42 | 19.52 | 0.321 |
| Grain | C6 | 14.92 | 29.46 | 2.47 | 11.93 | 0.204 |
| Grain | C7 | 18.99 | 13.22 | 0.85 | 15.75 | 0.576 |
| Grain | C2 | 33.69 | 17.79 | 1.35 | 13.15 | 0.749 |
| Grain | C3 | 5.05 | 2.35 | 0.23 | 10.35 | 0.841 |
| Grain | C4 | 13.13 | 11.44 | 1.06 | 10.78 | 0.453 |
| Grain | C5 | 31.19 | 11.44 | 1.00 | 11.52 | 1.076 |
| Grain | C6 | 1.29 | 3.55 | 0.30 | 11.78 | 0.143 |
| Grain | C7 | 0.83 | 0.34 | 0.02 | 15.41 | 0.835 |

Table 3.16: Analysis of $^{13}C$ enriched grain from Durum wheat var Lloyd

Table3.17, shows the analysis and ^{13}C enrichment of the milled straw of this wheat production crop. The weighted mean enrichment of straw was 0.322 ape [13]C.

| Durum Wheat var Lloyd | Growth Cabinet code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | [13]C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Straw | C2 | 99.51 | 114.37 | 7.43 | 15.40 | 0.337 |
| Straw | C3 | 131.25 | 66.02 | 4.77 | 12.33 | 0.417 |
| Straw | C4 | 36.78 | 51.06 | 3.17 | 16.10 | 0.290 |
| Straw | C5 | 102.45 | 127.01 | 8.01 | 15.85 | 0.379 |
| Straw | C6 | 33.49 | 124.55 | 8.64 | 14.42 | 0.105 |
| Straw | C7 | 43.37 | 126.45 | 8.10 | 15.61 | 0.136 |
| Straw | C2 | 132.63 | 106.95 | 7.05 | 15.17 | 0.491 |
| Straw | C3 | 128.45 | 98.85 | 8.77 | 11.95 | 0.476 |
| Straw | C4 | 13.61 | 37.43 | 3.24 | 11.89 | 0.124 |
| Straw | C5 | 110.65 | 110.47 | 8.85 | 12.48 | 0.379 |
| Straw | C6 | 64.86 | 115.89 | 8.52 | 13.61 | 0.213 |
| Straw | C7 | 96.51 | 71.46 | 5.66 | 12.62 | 0.508 |

Table 3.17: Analysis of ^{13}C enriched straw from Durum wheat var Lloyd

## 3.5  Wheat *var Paragon*

There were 12 batches containing 18 pots each sown with Spring soft wheat var Paragon. Each pot contained 9 seeds. There were no controls within this crop. The ^{13}C natural abundance of previous barley and wheat crops had not varied and was used to calculate the ^{13}C enrichment of this crop. The average height of the plants was 89 cm and the crop had 161 days of growth. Both the

enrichment of $^{13}C$ and starch was measured from the grain from the wheat crop.
Enrichment of straw was obtained and recorded.
The following tables indicate the success of this crop.

Table 3.18, displays the gram yield per cabinet from the labelled grain harvest
from Wheat var Paragon.  The total grain yield was 1740 g. Cabinets 2, 3 & 7
contained a lower yield of grain and straw.  These plants were in poor condition
and showing signs of mildew.

| Soft Wheat var Paragon | Batch = 18 pots | Date of Sowing | Sowing density (per pot) | Growth Cabinet code | Date of Labelling | Date of Harvesting | Grain Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Grain | Batch 1 | 20 Nov | 9 | C2 | 5 Feb | 24 April | 195 |
| Grain | Batch 2 | 20 Nov | 9 | C2 | 13 Feb | 24 April | 165 |
| Grain | Batch 3 | 20 Nov | 9 | C2 | 14 Feb | 24 April | 120 |
| Grain | Batch 4 | 20 Nov | 9 | C3 | 5 Feb | 24 April | 175 |
| Grain | Batch 5 | 20 Nov | 9 | C3 | 13 Feb | 24 April | 125 |
| Grain | Batch 6 | 20 Nov | 9 | C3 | 14 Feb | 24 April | 140 |
| Grain | Batch 7 | 21 Nov | 9 | C5 | 5 Feb | 15 May | 175 |
| Grain | Batch 8 | 21 Nov | 9 | C5 | 13 Feb | 15 May | 165 |
| Grain | Batch 9 | 21 Nov | 9 | C5 | 14 Feb | 15 May | 140 |
| Grain | Batch 10 | 21 Nov | 9 | C7 | 5 Feb | 15 May | 135 |
| Grain | Batch 11 | 21 Nov | 9 | C7 | 13 Feb | 15 May | 95 |
| Grain | Batch 12 | 21 Nov | 9 | C7 | 14 Feb | 15 May | 110 |

Table 3.18: Grain yield from Wheat var Paragon

Table 3.19, display the gram yield per cabinet from the labelled straw harvest from Wheat var Paragon.  The total straw yield was 3140 g.  Cabinets 2,3 & 7 contained a lower yield of grain and straw.  These plants were in poor condition and showing signs of mildew.

| | Soft Wheat var Paragon | Date of Sowing | Sowing density (per pot) | Growth Cabinet code | Date of Labelling | Date of Harvesting | Straw Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Straw | Batch 1 | 21 Nov | 9 | C2 | 5 Feb | 24 April | **290** |
| Straw | Batch 2 | 21 Nov | 9 | C2 | 13 Feb | 24 April | **330** |
| Straw | Batch 3 | 21 Nov | 9 | C2 | 14 Feb | 24 April | **225** |
| Straw | Batch 4 | 21 Nov | 9 | C3 | 5 Feb | 24 April | **290** |
| Straw | Batch 5 | 21 Nov | 9 | C3 | 13 Feb | 24 April | **220** |
| Straw | Batch 6 | 21 Nov | 9 | C3 | 14 Feb | 24 April | **235** |
| Straw | Batch 7 | 21 Nov | 9 | C5 | 5 Feb | 15 May | **270** |
| Straw | Batch 8 | 21 Nov | 9 | C5 | 13 Feb | 15 May | **305** |
| Straw | Batch 9 | 21 Nov | 9 | C5 | 14 Feb | 15 May | **245** |
| Straw | Batch 10 | 21 Nov | 9 | C7 | 5 Feb | 15 May | **245** |
| Straw | Batch 11 | 21 Nov | 9 | C7 | 13 Feb | 15 May | **270** |
| Straw | Batch 12 | 21 Nov | 9 | C7 | 14 Feb | 15 May | **215** |

Table 3.19: Straw yield from Wheat var Paragon

Table 3.20, displays the $^{13}$C enrichment analysis of $^{13}$C enriched grain. The weighted mean enrichment for grain was 0.278 ape $^{13}$C.

| Soft Wheat var Paragon | Growth Cabinet code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet | C:N Ratio | $^{13}$C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Grain | C2 | 29.57 | 76.9 | 4.44 | 17.32 | 0.1516 |
| Grain | C2 | 24.34 | 65.1 | 4.02 | 16.18 | 0.1475 |
| Grain | C2 | 25.29 | 46.7 | 3.01 | 15.51 | 0.2108 |
| Grain | C3 | 43.85 | 68.8 | 4.84 | 14.23 | 0.2506 |
| Grain | C3 | 19.45 | 49.0 | 3.19 | 15.37 | 0.1556 |
| Grain | C3 | 14.98 | 54.6 | 3.34 | 16.37 | 0.1070 |
| Grain | C5 | 105.40 | 68.3 | 4.41 | 15.47 | 0.6023 |
| Grain | C5 | 59.03 | 65.3 | 3.91 | 16.73 | 0.3578 |
| Grain | C5 | 84.06 | 55.6 | 3.19 | 17.41 | 0.6004 |
| Grain | C7 | 35.32 | 53.3 | 3.51 | 15.18 | 0.2616 |
| Grain | C7 | 12.32 | 37.8 | 2.87 | 13.17 | 0.1297 |
| Grain | C7 | 31.11 | 43.5 | 2.61 | 16.64 | 0.2828 |

Table 3.20: Analysis of $^{13}$C enriched grain from Wheat var Paragon

Table 3.21, displays the $^{13}$C enrichment analysis of 13C enriched straw. The weighted mean enrichment for straw was 0.0566 ape $^{13}$C.

| Soft Wheat var Paragon | Growth Cabinet code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | $^{13}$C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Straw | C2 | 11.9 | 91.8 | 6.45 | 14.24 | 0.0410 |
| Straw | C2 | 20.2 | 113.2 | 6.22 | 1948 | 0.0611 |
| Straw | C2 | 5.0 | 80.6 | 3.02 | 26.82 | 0.0223 |
| Straw | C3 | 11.2 | 105.5 | 4.38 | 24.11 | 0.0387 |
| Straw | C3 | 15.6 | 82.0 | 4.05 | 20.55 | 0.0710 |
| Straw | C3 | 13.9 | 87.6 | 3.58 | 24.50 | 0.0591 |
| Straw | C5 | 15.4 | 99.7 | 4.55 | 2289 | 0.0570 |
| Straw | C5 | 37.6 | 112.1 | 6.68 | 16.77 | 0.1234 |
| Straw | C5 | 12.6 | 88.5 | 5.00 | 18.28 | 0.0516 |
| Straw | C7 | 17.7 | 88.6 | 4.15 | 21.60 | 0.0724 |
| Straw | C7 | 11.4 | 97.8 | 4.45 | 23.15 | 0.0424 |
| Straw | C7 | 4.9 | 77.4 | 2.50 | 30.89 | 0.0229 |

Table 3.21: Analysis of $^{13}$C enriched straw from Wheat var Paragon

## 3.6 Production of Barley *var Cellar*

This variety of Spring barley was selected from the SAC seed store. There were 12 batches of 18 pots with 9 seeds per pot. The unlabelled control was Batch 6 which comprised of 2 pots with 9 seeds per pot. This batch was not labelled to provide a measure of $^{13}$C natural abundance which would be used to calculate the $^{13}$C-enrichment of the labelled plants. The days of total growth varied according to anthesis and availability of growth cabinets. Batches 1-5 had 155 days of total growth and batches 7-12 had 176 total days of total growth.

Both the enrichment of $^{13}$C of the bulk and starch was measured in the grain. The $^{13}$C enrichment of the straw was also measured.

Table 3.22, displays the yield per cabinet from the barley grain. The total grain yield was 3406 g.

| Barley var Cellar | Batch = 18 pots | Date of Sowing | Sowing Density (per pot) | Growth Cabinet Code | Date of Labelling | Date of Harvesting | Grain Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Grain | Batch 1 | 3 May | 9 | C3 | 20 June | 5 Oct | 240 |
| Grain | Batch 2 | 3 May | 9 | C3 | 21 June | 5 Oct | 350 |
| Grain | Batch 3 | 3 May | 9 | C5 | 20 June | 5 Oct | 320 |
| Grain | Batch 4 | 3 May | 9 | C5 | 21 June | 5 Oct | 310 |
| Grain | Batch 5 | 3 May | 9 | C7 | 21 June | 5 Oct | 355 |
| Grain | Batch 7 | 15 May | 9 | C3 | 3 Jul | 5 Oct | 340 |
| Grain | Batch 8 | 15 May | 9 | C3 | 4 Jul | 5 Oct | 280 |
| Grain | Batch 9 | 15 May | 9 | C5 | 3 Jul | 5 Oct | 360 |
| Grain | Batch 10 | 15 May | 9 | C5 | 4 Jul | 5 Oct | 276 |
| Grain | Batch 11 | 15 May | 9 | C7 | 3 Jul | 5 Oct | 310 |
| Grain | Batch 12 | 15 May | 9 | C7 | 4 Jul | 5 Oct | 265 |

Table 3.22: Grain yield from Barley var Cellar

Table 3.23, displays the yield per cabinet from the barley straw.  The total straw yield was 3063 g.

| Barley var Cellar | Batch = 18 pots | Date of Sowing | Sowing Density (per pot) | Growth Cabinet Code | Date of Labelling | Date of Harvesting | Straw Yield (g per cabinet) |
|---|---|---|---|---|---|---|---|
| Straw | Batch 1 | 3 May | 9 | C3 | 20 June | 5 Oct | 281 |
| Straw | Batch 2 | 3 May | 9 | C3 | 21 June | 5 Oct | 358 |
| Straw | Batch 3 | 3 May | 9 | C5 | 20 June | 5 Oct | 297 |
| Straw | Batch 4 | 3 May | 9 | C5 | 21 June | 5 Oct | 268 |
| Straw | Batch 5 | 3 May | 9 | C7 | 21 June | 5 Oct | 329 |
| Straw | Batch 7 | 15 May | 9 | C3 | 3 Jul | 5 Oct | 302 |
| Straw | Batch 8 | 15 May | 9 | C3 | 4 Jul | 5 Oct | 281 |
| Straw | Batch 9 | 15 May | 9 | C5 | 3 Jul | 5 Oct | 278 |
| Straw | Batch 10 | 15 May | 9 | C5 | 4 Jul | 5 Oct | 338 |
| Straw | Batch 11 | 15 May | 9 | C7 | 3 Jul | 5 Oct | 293 |
| Straw | Batch 12 | 15 May | 9 | C7 | 4 Jul | 5 Oct | 337 |

Table 3.23: Straw yield from Barley var Cellar

Table 3.24, displays the $^{13}$C enrichment analysis from $^{13}$C enriched grain for Barley var Cellar. The weighted mean enrichment of grain was 0.198 ape $^{13}$C.

| Barley var Cellar | Growth Cabinet Code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | $^{13}$C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Grain | C3 | 30.12 | 104.99 | 5.99 | 1.21 | 0.126 |
| Grain | C3 | 41.26 | 145.10 | 9.33 | 1.20 | 0.118 |
| Grain | C5 | 88.03 | 132.01 | 7.73 | 1.36 | 0.275 |
| Grain | C5 | 50.76 | 127.22 | 7.95 | 1.24 | 0.164 |
| Grain | C7 | 112.75 | 148.23 | 8.70 | 1.40 | 0.318 |
| Grain | C3 | 36.63 | 138.98 | 8.39 | 1.19 | 0.108 |
| Grain | C3 | 25.94 | 109.72 | 7.02 | 1.17 | 0.093 |
| Grain | C5 | 80.71 | 148.28 | 8.04 | 1.30 | 0.224 |
| Grain | C5 | 59.13 | 113.12 | 6.52 | 1.29 | 0.214 |
| Grain | C7 | 61.77 | 86.57 | 5.11 | 1.23 | 0.199 |
| Grain | C7 | 89.69 | 109.54 | 6.22 | 1.42 | 0.338 |

Table 3.24: Analysis of $^{13}$C enriched grain from Barley var Cellar

Table 3.25, displays the $^{13}$C enrichment analysis from the $^{13}$C enriched straw from Barley var Cellar. The weighted mean enrichment of straw was 0.156 ape $^{13}$C.

| Barley var Cellar | Growth Cabinet code | Weight x ape | Carbon Content (g per cabinet) | Nitrogen Content (g per cabinet) | C:N Ratio | $^{13}$C Enrichment (ape) |
|---|---|---|---|---|---|---|
| Straw | C3 | 37.07 | 115.90 | 1.37 | 1.21 | 0.132 |
| Straw | C3 | 33.02 | 141.14 | 1.45 | 1.17 | 0.092 |
| Straw | C5 | 85.11 | 125.20 | 1.13 | 1.37 | 0.287 |
| Straw | C5 | 7.55 | 111.36 | 2.42 | 1.11 | 0.028 |
| Straw | C7 | 18.37 | 139.28 | 1.37 | 1.14 | 0.056 |
| Straw | C3 | 55.21 | 120.96 | 1.18 | 1.36 | 0.281 |
| Straw | C3 | 78.93 | 129.69 | 0.94 | 1.26 | 0.183 |
| Straw | C5 | 95.01 | 118 | 0.83 | 1.42 | 0.342 |
| Straw | C5 | 43.05 | 142.31 | 1.69 | 1.22 | 0.127 |
| Straw | C7 | 27.92 | 122.40 | 1.22 | 1.18 | 0.095 |
| Straw | C7 | 43.78 | 131.36 | 3.24 | 1.21 | 0.130 |

Table 3.25: Analysis of $^{13}$C enriched straw from Barley var Cellar

## 3.7 Metaprobe Characterisation

### 3.7.1 Bulk $^{13}$C Analysis & $^{13}$C Enrichment of Starch

Table 3.26 displays the bulk $^{13}$C enrichment and the $^{13}$C enrichment of starch (analysed by LC-IRMS as glucose) from the grain from both Wheat var Paragon and Barley var Cellar. Sections 2.9.1 and 2.9.2 describe this procedure. Note, Batch 6 control of barley was not labelled.

| Soft Wheat var Paragon | Grain Bulk $^{13}$C (ape) | Grain Starch Enrichment (ape) | Barley var Cellar | Grain Bulk $^{13}$C (ape) | Grain Starch Enrichment (ape) |
|---|---|---|---|---|---|
| C2 | | 0.1771 | C3 | 0.1077 | 0.0752 |
| C2 | 0.2108 | 0.2577 | C3 | 0.1255 | 0.0914 |
| C2 | 0.1516 | 0.1408 | C3 | 0.1179 | 0.1459 |
| C3 | 0.2506 | 0.3023 | C3 | 0.0926 | 0.0876 |
| C3 | 0.1070 | 0.1217 | C5 | 0.2242 | 0.2139 |
| C3 | 0.1556 | 0.1683 | C5 | 0.2751 | 0.2085 |
| C5 | 0.6023 | 0.7503 | C5 | 0.1638 | 0.1768 |
| C5 | 0.6004 | 0.6454 | C5 | 0.2143 | 0.1843 |
| C5 | 0.3578 | 0.4771 | C7 | 0.1993 | 0.1575 |
| C7 | 0.2616 | 0.2929 | C7 | 0.3385 | 0.3269 |
| C7 | 0.2616 | 0.2703 | C7 | 0.3176 | 0.3664 |
| C7 | 0.2828 | 0.3068 | | | |
| C7 | 0.1297 | 0.1156 | | | |

Table 3.26: Grain bulk $^{13}$C enrichment & starch $^{13}$C enrichment per cabinet from both barley & wheat crops

Analysis by paired Student's T test showed there was no significant difference between the $^{13}C$ enrichment of barley starch and the $^{13}C$ bulk enrichment. However, $^{13}C$ enrichment of wheat starch is significantly different by approx 14 % than the bulk $^{13}C$ signature of the grain. (see figures 3.2 & 3.3)

**Barley**

$y = 0.8559x + 0.0396$
$R^2 = 0.8733$
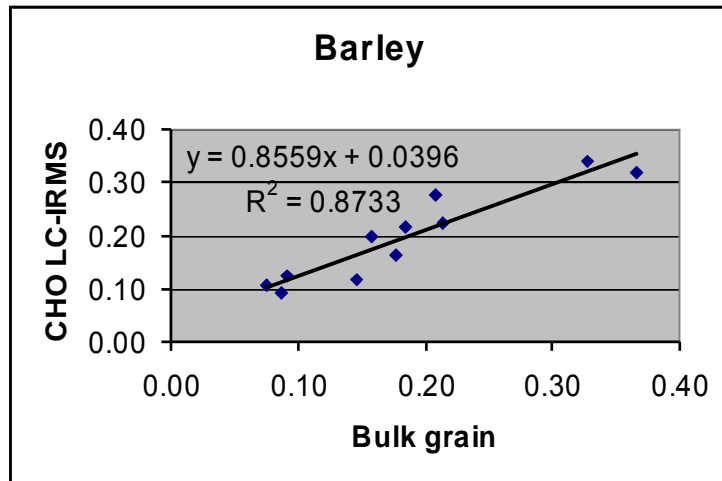
Fig 3.2: Scatter plot of bulk v starch in barley grains, paired T test showed P= 0.113

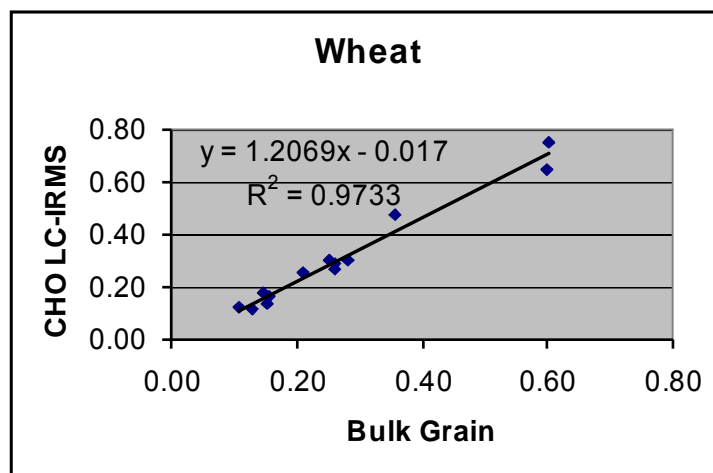**Wheat**

$y = 1.2069x - 0.017$
$R^2 = 0.9733$

Fig 3.3: Scatter plot of bulk v starch in wheat grains, paired T Test showed P= 0.006

# Chapter 4: Discussion

# 4 Complex Metaprobe Production

## 4.1 Pilot Studies

### 4.1.1 Labelling trials

(see Appendix A for photographs of all crops in their different growth stages)

The pilot trials were aimed to establish the conditions for optimal growth of cereal crops in the glasshouse. Tests included: sowing procedures where soil type and seed density were varied; the timings and methodology for labelling with $^{13}CO_2$; harvesting and elemental and isotopic analysis of grain and straw also had to proven and established.

For the first pilot study, it was decided to use spring barley and Durum wheat. Durum wheat has considerable interest amongst nutritionists), in hard wheat varieties used for bread and pasta making. SAC advised and provided Barley var Kirsty and Durum Whear var Lloyd seeds. Four batches of Durum wheat were prepared, each containing between 12 – 20 pots. All pots were planted at a seed density of 6 seeds per pot. (see table 3.1) Approximately 50 days after germination, the ear emerges from the flag leaf sheath as anthesis occurs. In the plant growth cycle, grain filling will occur approx 30 days after ear emergence? (The explanation of growth stages in section 1.9). Ear emergence is a crucial stage as non-development frequently occurs, which can be caused by high temperatures and/or drought or flooding/over watering.

Batch 1 was labelled in growth cabinet for 1 day. Batches 2, 3 & 4 were labelled for 5 days in the growth cabinet. It was expected, and was clearly observed (see Tables 3.2 & 3.4), that 5 day's labelling using a similar quantity of gas each day, resulted in a higher enrichment than a single day's labelling. It was also apparent that, even when the same growth cabinet was used, it could prove more or less prone to leakage on different occasions. Also, it became apparent that the infra-red gas analyser (IRGA: Guardian 3000) used to monitor $CO_2$ additions was inaccurate. Too often in the pilot study were large additions of $CO_2$ made with a low response on the IRGA. This was wasteful and discussions with the

manufacturer showed the reason was the model was insensitive to $^{13}CO_2$ and their alternative model the Guardian Plus, was sensitive only to $^{13}CO_2$, their use for $^{13}C$ labelling studies is unique. When this fact was established the manufacturer assured us that the Guardian Plus, calibrated to 0-30% $CO_2$ at natural abundance.  This would measure 0-3000 ppm $^{13}CO_2$, as it is designed to measure the minor isotope of carbon which has a natural abundance of close to 1%. A meter of this type was purchased and used for the subsequent metaprobe production phase. Despite the fact that each growth cabinet was manually prepared prior to labelling to minimise gas leakage, some leakage did occur.  It was also difficult to reserve these growth cabinets for long periods of time as they were required for other projects at SAC.  In the production phase (see below), 12 labelling sessions were frequently required.  This was accomplished by using 3 or 4 of such 600L growth cabinets in parallel.

Altogether, the labelling trials produced 142.2 g Durum wheat grain with a weighted mean enrichment of 1.671 ape $^{13}C$ and 366.6 g spring barley grain with a weighted mean enrichment 0.640 ape $^{13}C$. (see section 3.1.1 for explanation of weighted mean).As these trials were on a modest scale, we were able to sample and analyse all parts of the plant (see Tables 3.1 – 3.4). The straw, chaff was isolated from leaves and stem, and roots were sampled.  This would inform our calculations of growth efficiency following the grain production phase (see below).

The grain quality of the barley prepared in the labelling trials was acceptable and was to be used for a human nutrition pilot study (see Chapter 5) but the Durum wheat crop was poor. The wheat grains were small in size with little starch within the grain pods.  This may have been because of high temperatures in glasshouse or over watering by SAC staff (periods of under- and over-watering).

During this pilot study and the forthcoming production crops, we were restricted to equipment availability as dictated by SAC: glasshouse space, growth cabinets and the single ear thresher.  Labelling and harvesting was delayed on occasions because of this.  Despite efforts of preparing growth cabinets; leakage did occur in some. In cabinet 4, we discovered that the fluorescent lighting was not as

intense as the other cabinets. These were all disadvantages which had to be overcome in the future production crops.

The next pilot study was to try different Durum wheat varieties, while varying seed density and soil conditions.  We were advised by the crop scientists at SAC, to add Perlite (see glossary) to improve aeration in the potting medium used for the wheat seeds.

## 4.2 Durum Wheat Variety

As the previous crop of wheat had produced seeds of poor quality, it was important to produce Durum Wheat in pots in the glasshouse with a high yield. A plan was developed to test the success of other varieties of Durum wheat, while varying sowing density and soil conditions. (see table 2.1)

After 155 days of growth, the entire crop was harvested and the most successful wheat was the var Combo.  Although height of plant is not a priority and grain yield is, it was interesting to see the variations in the varieties.  The var Combo plants grew to 60 cm in height and produced the heaviest harvest at a total of 100 g grain from 12 pots (3 batches of 4 pots).  The var Orjaune grew to 56 cm height and produced a total of 95 g grain from 12 pots.  Both var Lloyds (no perlite/ added perlite) looked visually similar with a height of 55 cm.  No perlite wheat plants had a grain harvest total of 90 g from 12 pots.  Wheat var Lloyd with added perlite showed the smallest yield, with grain totalling 59 g from 12 pots.  The var Duramar had the shortest height at 40 cm and although the foliage looked striped which may be fungal infection, this variety produced a total of 95 g grain from 12 pots. (See table 3.5)

This wheat was unlabelled, but this pilot study did repeat others' observations that varieties other than Durum Wheat var Lloyd can grow successfully in a glasshouse in our northern climes. The addition of perlite made no visual difference to the condition of the plant, grains and straw.  Only the var Lloyd was divided into "no perlite" and "added perlite" to compare between the two. The added perlite produced substantially less grain, possibly resulting from the

crude watering conditions with manual daily watering causing periods of flood and drought, the latter especially on hotter days in the glasshouse.

## 4.3 Hay Trials

A pilot study to label hay was undertaken as there is considerable interest amongst nutritionists in having supplies of labelled milk and milk products for human nutrition trials.  Hay grows rapidly and can produce multiple crops each year. It is an efficient animal feed, supplying energy via digestible stem, leaf and seeds. If the objective was to label milk through supplementing the diet of milk cattle, labelled hay may prove a more efficient means to do this as compared with labelled grain.

The chosen hay mix of perennial rye grass, Timothy perennial grass and red clover (see section 2.3) looked green and prolific with much growth (15 cm). The red clover shoots were obvious among the green grass/hay.  Red clover is a legume and fixes atmospheric nitrogen.  It adds nitrogen to the soil which, in the future when $^{13}$C enriched hay may be fed to cattle to produce labelled milk, the red clover would also supply protein. The soil used was a mixture of growth medium and compost (see section 2.3).  The compost holds water to prevent the soil drying.

Access to the growth cabinets was limited and the labelling period was delayed. By the time of harvest the hay was overgrown, over-wet and had started to rot due to overwatering. The harvested hay was saturated but it was air dried, stored in paper bags and kept at room temperature.

Another advantage of growing hay is its re-growth potential. Table 3.9 shows that the second crop, harvested without further labelling, contained some 11% of the enrichment of the first.  This illustrates the potential benefit of hay labelling, where translocation of labelled organic carbon from the root stores is able to nourish further growth.

The efficiency of hay growth is compared with that of barley grain (see section 4.12 & Tables 3.6-3.9). The growth of hay and its high $^{13}$C transfer efficiency of 59.9% which was much higher than barley grain at 39.6%.

## 4.4 Winter Soft Wheat *var Robigus*

Winter varieties of wheat and barley are important in UK and Northern European agriculture. They produce high yields of disease-free grain. A study to establish if quantities of winter cereal grain could be prepared in the glasshouse for labelling was thus undertaken. During the soft Winter wheat var Robigus, nabim Group 3, in anticipation of producing kg quantities of grain, 225 pots with 9 seeds per pot were sown using. After 5 weeks of growth the foliage was prolific causing some browning of the leaves nearest the soil. We spaced out the plants for these leaves to receive more daylight. The plants continued appeared bushy with many leaves but poor stems and no ear development. Winter wheat needs to be vernalised, with seeds held for a period at low temperature to ensure ear development. To ensure developed stems and normal ear development, seeds should be kept at a low temperature of under 8°C but generally around 4°C, for 4-6 weeks. This was done and we were assured, should produce healthy stalks with seed heads.

We re-sowed 216 pots with 9 seeds per pot of vernalised wheat var Robigus but the same result was apparent. The glasshouse was probably too warm during the evening/night, with winter night time temperatures never being achieved. In practice, a much more sophisticated glasshouse may be necessary to prepare winter cereal varieties. This variety of crop should be grown in a glasshouse with an air temperature control unit installed to maintain temperature closer to field conditions.

Both of these crops were destroyed and growth trials of winter cereal varieties in this glasshouse were abandoned.

## 4.5 Scale of Production

Towards the end of the pilot study, we discussed the optimum scale of the production phase with our human nutrition partners within the EU programme EUROSTARCH ([www.eurostarch.org](www.eurostarch.org)). This was informed by early results from human nutrition studies.  In particular, with a focus on the nutritional benefit of the non-digestible carbohydrates within whole grains and kernels (polished whole grains), we sought to provide the optimal balance between amount of grain and its enrichment. Some foods products may be made using $^{13}$C-labelled whole grains incorporated into a matrix of unlabelled refined grain e.g. varieties of whole grain bread.  With the expense of the tracer, the large dilution in vivo caused by large body size and the greatly improved analytical techniques, it was readily apparent that it would be better to make such breads with every whole grain enriched to a low degree than a few grains labelled to a higher degree.  It readily imagined that a slice of whole grain bread (with just a few whole grains) could be produced at very variable enrichments.  The consensus amongst nutritionists was that the aim of the production phase should be to prepare kg quantities of suitable grain at low enrichment, rather than hundreds of grams at higher enrichment.

## 4.6 Quantitative Gas Addition

A new Infra Red Gas Analyser, sensitive to $^{13}CO_2$ (Guardian Plus 0-30%, Edinburgh Instruments, UK), was obtained and used to monitor gas additions. A linear increase in $CO_2$ partial pressure as each pulse of gas was added to the growth cabinet was observed which confirmed the method used for quantitative gas addition and the ability of the new meter to monitor this.
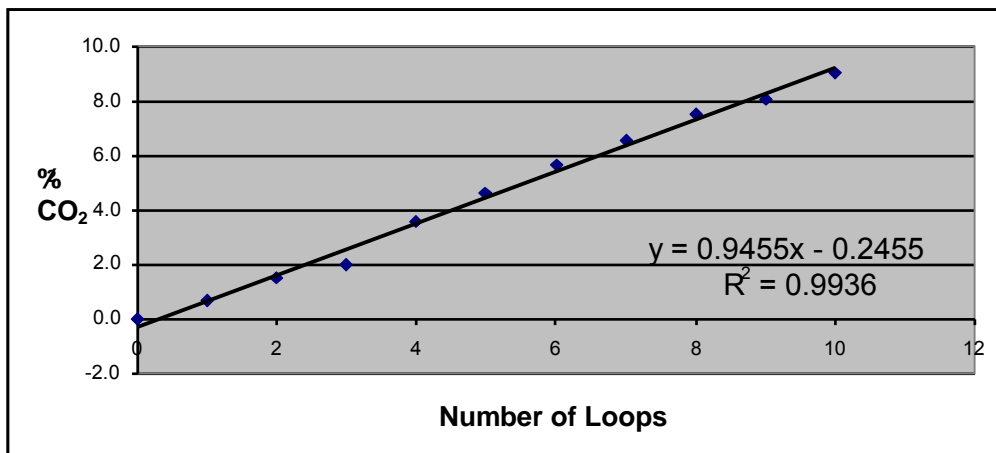
Fig 4.1: $^{13}CO_2$ response (partial pressure) of the IRGA following multiple additions from the gas loop (13.32 mL) attached to the growth cabinet

The y axis is given in units, % $CO_2$, from 0 to 10 %. This is equivalent to 0-1000 ppm $^{13}CO_2$. This gave an accurate means of monitoring gas addition for the production studies. The theoretical gradient of the line is 1.36% $CO_2$ per loop.

## 4.7 Production of Barley *var Kirsty*

The aim was to produce several kg of barley grain labelled at low enrichment. This was undertaken in 2 batches, each planted at a density of 9 seeds per pot but with a variable number of pots labelled. (see section 3.3). All had 1 day labelling after 50 days growth and were harvested after 142 days of growth.

The variation in the numbers of pots labelled per batch was determined by some being held without labelling to provide a value for natural $^{13}C$ abundance. These accounts for the variation in the grain and straw weight per cabinet (see Table 3.10). The variation in enrichment value is less likely to be connected to the number of pots per batch but more to the condition of that particular growth cabinet. The problem with some cabinets was different leakage rate and occasionally, inferior lighting.

The total barley grain produced from all cabinets was 3872 g with a weighted mean enrichment of 0.138 ape $^{13}C$. (see Table 3.10 – 3.13)   From a visual assessment of the grains, it appeared a successful crop. These enriched grains were taken to SUERC for analysis then transported to Centre of Medical Biomics,

University Medical Centre, Groningen, The Netherlands, to be used in future human nutrition studies.

## 4.8 Production of Durum Wheat *var Lloyd*

Learning from the pilot studies, 2 batches of various amounts of pots were (see section 3.4) sown with 30 seed density per pot.  After 57 days of growth the wheat appeared to be in anthesis and a single day's labelling occurred. Harvesting commenced after 105 days of growth.

This crop had a low yield with a total grain weight of 507 g.  This was more successful than the pilot study crop but represented no improvement in comparison to the Durum Wheat Variety study.  There could be many reasons for this: over watering; too large a temperature variation in the glasshouse conditions; too high a seed density; fungal infection. However, the conclusion was reached that hard wheat varieties do not develop reliably under glasshouses conditions, unless these were of a more sophisticated design.  The weighted mean enrichment was 0.341 ape $^{13}$C. (see Tables 3.14 – 3.17) This enrichment was higher than the barley crop and was likely to have been the result of lower absolute yield, with possible higher intracellular $^{13}$C enrichment during photosynthesis. Yield and efficiency are discussed further below.

The enriched wheat grain were taken to SUERC for analysis then transported to Centre of Medical Biomics, University Medical Centre, Groningen, The Netherlands to be used in future human nutrition studies.

## 4.9 Production of Soft Spring Wheat *var Paragon*

As we had no success producing Winter wheat in the glasshouse and problems producing quality hard spring wheat, we sought to produce a variety of soft Spring wheat that was used by industry for breadmaking and biscuit making.

216 pots of soft wheat var Paragon nabim Group 1 were sown with a density of 9 seeds per pot (totally 1944 seeds).  After 76 days, the wheat appeared to be in anthesis but the wheat was 89 cm in height (not including the pot) had little

turgor and was bending and drooping.  These wheat plants had not tillered well, unlike the barley. The advice to use a density of 9 seeds per pot was poor. Future work should increase seed density.  Labelling commenced but growth Cabinet 2 appeared to be less able to hold $CO_2$, with a resulting reduction in $^{13}C$ enrichment.

After 161 days of growth, this wheat crop was harvested.  The plants showed signs of mildew but produced 1740 g of apparently good quality grain.  See Tables 3.18 – 3.21) Visual assessment of the grains showed they were dense grains without being chipped or broken.

The weighted mean enrichment for the grain was 0.278 ape $^{13}C$,   As well as bulk $^{13}C$ analysis, the $^{13}C$ enrichment of grain starch was also analysed (see sections 2.9.1, 2.9.2, Table 3.26 & discussion, below)

These enriched grains were taken to SUERC for analysis and then transported to Centre of Medical Biomics, University Medical Centre, Groningen, The Netherlands for use in future human nutritional studies.

## 4.10 Production of Barley *var Cellar*

A second production batch of several kg spring barley grain was requested and var Cellar seeds were provided from the SAC store.  208 pots of Spring Barley var Cellar (this variety was previously used for the production of malt for brewing but no longer approved by Institute of Brewing & Distilling (IBD)) were sown with a density of 9 seeds per pot (totally 1872 seeds).  All pots were labelled after anthesis at 48 days of growth.  The plants were approx 80 cm in height but after removal from the growth cabinet, they had lost turgor and had to be staked and tied with plant string whilst placed in glasshouse to mature. Harvesting was split into two sessions at 155 days of total growth and 176 total days of growth because of availability of equipment as dictated by SAC.

The total grain yield was 3406 g with weighted mean enrichment of 0.198 ape $^{13}C$. (see Tables 3.22 – 3.25)  This time, Cabinet 3 appeared to be less able to

hold $CO_2$.  In addition to bulk $^{13}C$ analysis, the $^{13}C$ enrichment of grain starch was also analysed (see sections 2.9.1 & 2.9.2, table 3.26, and below)

The visual quality of grain was good and these enriched grains were taken to SUERC for analysis and then transported to Centre of Medical Biomics, University Medical Centre, Groningen, The Netherlands, for use in future human nutrition studies.

## 4.11 $^{13}C$ Analysis of Grain Starch in Comparison to Bulk Analysis

Table 3.26 compared the analysis of bulk grain with starch derived glucose from each of the batches from the last two production runs, Spring soft Wheat var Paragon and spring Barley var Cellar. This analysis is important in that, as grain is composed of a variety of organic molecules, we wish to be able to characterise this in as much detail as possible.  Following a request for further detail, we wished to comment on the likely starch enrichment as opposed to protein enrichment in the grain, to our nutrition colleagues. A food reference gives the following analysis of barley grain: Carbohydrate, 77.2%; protein 9.6%, fat 1.1%, with the balance being ash and water. The value for protein content is in line with our own grain nitrogen assay (see section 2.9.1, 2.9.2, 2.10, table 3.26). Although carbohydrate is the dominant species, differential labelling of protein could potentially cause bias in our subsequent studies. Grain fat content is so low that this is unlikely to contribute bias.

We compared the bulk enrichment (including protein) with starch-derived glucose enrichment (starch carbohydrate omitting some NDC and all protein).  A statistical analysis showed that, in barley var Cellar, there was no difference between starch $^{13}C$ and bulk $^{13}C$ enrichment, indicating even labelling of the macronutrient components of barley.  However, starch glucose of wheat var Paragon was slightly more enriched than its bulk signature and this was significant. As the availability of the growth cabinets was restricted, this wheat was labelled some 2-3 weeks later than planned. This resulted in a greater proportion of the label being incorporated into starch.

## 4.12 Efficiency of Labelling Grain and Straw with a Comparison of Grain *vs* Hay.

We wished to comment on the overall economy and efficiency of the labelling process, both in terms of its current scale and in order to predict likely economies of scale for future studies.  Furthermore, should labelling of milk and dairy products be desired in the future, we wished to compare the efficiency of grain production with that of hay.

Only the shoot (stem, leaf & chaff) is included in this comparison.  The root system is excluded.  If roots were to be included, as the results from the pilot study suggest, it would add 10% biomass to the straw analysed.  However, as this comparison on the basis of harvestable animal feed, the roots biomass has been excluded. Finally, the faster growth to harvest of hay has to be considered, as multiple crops can be produced each year.  Should the aim be to produce a digestible carbohydrate as animal feed for producing labelled milk and dairy products for human consumption, hay would appear to be a more efficient means of incorporating tracer into ruminant feed than cereal grain.

On the following page, Table 4.1 demonstrates the efficiency of transfer of $^{13}CO_2$.

| Plant Product | Variety of Crop | Total Yield (g) | Weighted Mean Enrichment (ape $^{13}$C) | Total Carbon (g) |
|---|---|---|---|---|
| 2nd Hay Trial | Dry Hay (x 3 cabinets) | 320 | 0.695 | 119 |
| Grain | Barley var Kirsty | 3830 | 0.138 | 1435 |
| Straw | Barley var Kirsty | 3872 | 0.082 | 1493 |
| Grain | Durum wheat var Lloyd | 507 | 0.341 | 171 |
| Straw | Durum wheat var Lloyd | 3084 | 0.322 | 1151 |
| Grain | Wheat var Paragon | 1740 | 0.278 | 685 |
| Straw | Wheat var Paragon | 3140 | 0.056 | 1125 |
| Grain | Barley var Cellar | 3406 | 0.198 | 1364 |
| Straw | Barley var Cellar | 3063 | 0.156 | 1398 |

| Plant Product with CropVariety | Excess $^{13}$C (g) | $^{13}$CO$_2$ total used (mL) | $^{13}$CO$_2$ total used (g excess $^{13}$C) | Efficiency (%) |
|---|---|---|---|---|
| 2$^{nd}$ Hay Trial | 0.82 | 3472.4 | 1.995 | 42.5 |
| Grain Kirsty | 1.98 | 14881.7 | 8.550 | 23.2 |
| Straw Kirsty | 1.22 | 14881.7 | 8.550 | 14.3 |
| Grain Lloyd | 0.58 | 13889.5 | 7.980 | 7.3 |
| Straw Lloyd | 3.70 | 13889.5 | 7.980 | 46.4 |
| Grain Paragon | 1.90 | 13889.5 | 7.980 | 23.9 |
| Straw Paragon | 0.63 | 13889.5 | 7.980 | 7.9 |
| Grain Cellar | 2.70 | 13641.5 | 7.838 | 34.5 |
| Straw Cellar | 2.18 | 13641.5 | 7.838 | 27.8 |

Table 4.1: Transfer efficiency of $^{13}$CO$_2$ to cereal crops

Table 4.1 shows the efficiency of transfer from the $^{13}$CO$_2$ gas cylinder to the grain, straw and hay of production barley and wheat crops.   The hay values

were calculated from the first three growth cabinets used for this crop, as here the gas addition was quantitative. As 1L of $^{13}CO_2$ contains 0.58 g $^{13}C$, then a 30 L cylinder of $^{13}CO_2$ was sufficient for approximately 2 x 3 kg production runs. Efficiency was calculated as g $^{13}C$ in crop per g $^{13}C$ in gas used, expressed as %.

The efficiency of transfer to grain was acceptable with Barley var Cellar having a higher transfer efficiency of the grains. The tracer was well incorporated into grain, hay and straw. Durum Wheat var Lloyd straw was more enriched than the grain, as it had low grain yield. If the objective was to supply digestible carbohydrates as feed for a ruminant mammals (see glossary) then the hay would provide more tracer than the grain.

The Spring Barley var Cellar crop produced 3406 g a total weight of grain in a total of 208 x 20cm pots. The yield was 16 g grain per pot or an area of 6.53 $m^2$. A total of ~14 L $^{13}CO_2$, was used with 34.5% $^{13}C$ transfer efficiency.

The Wheat var Paragon produced 1740 g total weight of grain in a total of 216 x 20 cm pots. The yield was 8 g grain per pot or an area of 6.78 $m^2$. A total of ~14L $^{13}CO_2$, was used with 23.9% $^{13}C$ transfer efficiency.

## 4.13 Comparing our Metaprobe Production with Previous Studies Producing Metaprobes

Table 1.10 illustrated the labelling methods applied in early studies using complex metaprobes. Table 1.11 listed published human nutrition studies using $^{13}C$ labelled crops. Sections 5.1 below, discuss earlier in vivo studies using complex metaprobes. Section 5.2 discusses the work undertaking using complex metaprobes used in this study. A brief comparison of the scale of metaprobe production in early studies is now given.

Boutton et al, (12) produced 6 x 0.5 $m^2$ plots of rice var Lemont. Each plot produced approximately 300 g rice with an enrichment of 1.41 – 2.40 ape $^{13}C$. This material was to be used by Lifschitz and co-workers. (42)

Although Svejcar et al, produced labelled forage principally for animal nutrition studies, they discussed its application for human nutrition.  There production was on 200 – 500 g scale and low $^{13}$C enrichment. (75)

Harding et al, (30) produced 8 x $^{13}$C Spring Wheat var Wembley plants enriched to 3.0 ape $^{13}$C.  Wheat flour from this source was to be used by this group and by Christian et al. (15).

Although Livesey et al,  did not apply $^{13}$C tracers, they are included here as their studies were some of the first to explore digestion and fermentation in human volunteers given various cereal grain based diets (43).

Normand et al, produced a small quantity (75 g) of $^{13}$C highly enriched Durum wheat for producing pasta for human nutrition studies (58).

Edwards et al, produced two small batches of $^{13}$C labelled peas at an enrichment of 2.36 and 8.64 ape $^{13}$C, respectively.  These were subsequently used to study starch fermentation in volunteer adults (22).

Each of these studies concerned small-scale production of complex metaprobes, on a scale of a few hundred grams and similar to our pilot studies.  We have since progressed to producing complex metaprobes on a kg scale.

# Chapter 5

# Discussion: Human Nutrition Experiments using Complex Metaprobes

# 5 Human Nutrition Experiments using Complex Metaprobes

## 5.1 Previous *in vivo* Studies using Complex Metaprobes

(see Tables 1.10, 1.11 & section 4.13 )

Boutton and co-workers (11) applied 13C-labelled rice starch to study digestion in infants.  Two subjects were used, with breath and stool outputs measured following oral dosage of infant formula amended with labelled rice starch.  Both subjects were found to oxidise a significant portion of the starch with some 64 – 50% of dose, respectively, appearing in breath.

Lifschitz and co-workers, applied $^{13}$C labelled rice cereal and was fed to 8 infants suffering acute gastroenteritis.  Analysis of faeces and breath tests showed good carbohydrate absorption by the infants and the meal provided a source of dietary energy from post admission to hospital and after rehydration. (41)

Livesey and co-workers (43) gave 4 adult volunteers with ileostomies, test meals consisting of different forms of barley rich in NDC. Faecal studies concluded that cereal structure is an important determinant of digestibility and energy value.

Normand and co-workers (58) of postprandial glucose metabolism of  pasta made from made from $^{13}$C-labelled Durum wheat.  Nine healthy adult female volunteers were recruited.  A comprehensive picture of glucose metabolism after ingestion of starchy food was provided for the first time.  Adding lipid to the starchy meal led to the moderation of the glycaemic response to the test meal. This was one of the very first examples of the kinetics of $^{13}$C glucose being followed in the circulation after a meal containing $^{13}$C labelled cereal.

Harding and co-workers [30] applied different test meals which included $^{13}$C enriched wheat flour in a single adult volunteer, to study the appearance of breath $CO_2$ following starch digestion and oxidation. Different preparations of the test meals resulted in altered digestion rates.

Christian and co-workers [15] used white wheat flour prepared by Harding et al to study starch digestion and fermentation in 13 infants. Mathematical modelling of breath $CO_2$ data revealed a significant proportion of the starch was fermented in the colon.

Edwards and co-workers [22] used the digestion and fermentation of uncooked pea starch in 2 adult volunteers. Breath $CO_2$ continued to appear after 15 hrs indicating fermentation was a significant process in the fate of the test meal. The authors concluded that the use of complex metaprobes proved an effective non invasive way to measure digestion and fermentation in vivo.

## 5.2 *In vivo* Studies using Complex Metaprobes Produced in this Study

The EU project EUROSTARCH (www.eurostarch.org) investigated the way in which different starchy foods are digested and metabolised within the body. The results have increased our knowledge of NDC fermentation and the techniques we pioneered hold promise to lead to improvements in human bowel health. The complex metaprobes in this study were applied within the EU funded Eurostarch project.

An example of the use of our labelled cereals is Priebe et al [70] where 4 healthy male subjects were given test meals of wholemeal wheat bread containing a portion of $^{13}$C –enriched Durum wheat var Lloyd or equivalent amount of glucose which is naturally labelled with $^{13}$C. The rate at which starch was digested was then studied. The authors found reduced appearance of exogenous glucose following the whole wheat meal and a reduced insulin response indicating the potential of whole grain products to moderate glycaemia.

This concurs with the second meal effect observed by Nilsson and co-workers (2006), where low GI (glycaemic index) and high GI foods where prepared with barley and wheat kernels, spaghetti and wheat bran. All meals had differing amounts of RS and DF but high amounts of NDC. The blood glucose response was measured after second meal. The second meal effect or overnight effect, contributes to long-term metabolic benefits but more studies are necessary to clarify the relationship between colonic fermentation and glucose metabolism. However, this study was one of the first to argue that carbohydrate products (SCFA) have a role to play in glucose homeostasis. (53)

[13]C-labelled Spring Barley var Kirsty grains were applied to estimate the extent of fermentation of NDC in a study of 17 adult volunteers in Wang and co-workers (81). After an overnight fast, volunteers ate 86g of cooked barley kernels (BK) and $^{13}CO_2$ and $H_2$ in breath samples measured every 30 mins for over 12 hours. Using the difference between area under curve (AUC) of the fitted digestion curve against AUC of the observed curve regarded as fermentation, an estimate was given of the total available ^{13}C in barley that was derived from colon fermentation. Curve fitting revealed that up to 25% of the barley meal was fermenting. The authors concluded that breath analysis provided a feasible and non-invasive method to assess digestion and fermentation of different starchy food. It should also be possible to examine individual differences in recovery of energy from the colon after starchy food consumption through estimation of SCFA production. This may beneficial in identifying any health problems within the gut. The next applications of our metaprobes concerned the measurement of SCFA production.

Verbeke and co-workers [80] studied five healthy adult volunteers who consumed two different types of barley meals made from $^{13}$C-labelled Spring Barley grain var Kirsty (from pilot study, section 3.1.2). These experiments met with approval from the Medical Ethics Committee of the University Medical Centre in Groningen, The Netherlands and the protocol complied with the Helsinki declaration and was approved by the Ethics Committee of the University of Leuven. One meal comprised cooked barley kernels (BK), which has a high content of RS and DF and the other meal comprised barley porridge (BP), with a high content of DF. The barley grains used for BP were pearled and milled before cooking. Urine and blood plasma samples from these healthy volunteers were returned to SIBL for SCFA analysis. We monitored $^{13}$C acetate, $^{13}$C propionate and $^{13}$C butyrate in the plasma and urine samples of those persons whom ingested these meals. Verbeke et al reported that the maximal $^{13}$C excretion was significantly shorter for the porridge meals (p=0.043) indicating faster digestion than that of the kernel meal.

An increase of $^{13}$C-acetate in serum was observed in the early stages (< 360 mins). A second peak in $^{13}$C-acetate enrichment is observed in the later stages and was higher after digestion of the BK meal. An increase in $^{13}$C-propionate enrichment was detected in the volunteers. An increase in $^{13}$C- butyrate enrichment was found in the fermentation phase, with even higher $^{13}$C- butyrate enrichment after the BK meal.

A conclusion from this study was that meals containing DF combined with RS resulted in altered systemic SCFA profiles as compared with meals containing DF alone. The metabolic consequences of this have yet to be established. Following the ingestion of a meal of the $^{13}$C-labelled barley, significant quantities of $^{13}$C-labelled SCFA were detected in the circulation providing unambiguous evidence of SCFA through colonic fermentation of NDC.


In future, crops could be chosen and studies could be designed, to achieve colonic delivery of known quantities of labelled SCFA. The most important message from this paper was that it represented the first report in humans of acetate, propionate and butyrate appearance in vivo from dietary carbohydrate.

SCFA products of carbohydrate fermentation are increasingly recognised as important contributors to the benefits of dietary fibre.  In vivo methods to measure the rate and extent of SCFA production in response to a particular meal are necessary to be developed and tested. They would be applied to measure the capacity of different foodstuffs to produce SCFA.

SCFA, especially butyrate, have been proposed to provide anti-inflammatory and anti-neoplastic effects that benefit bowel health.  SCFA have also been proposed as having systemic effects on improving glucose homeostasis, moderating inflammatory agents and reducing cholesterol synthesis.  Diets rich in fibre such as whole grains, which promote colonic fermentation of NDC, are the principal source of SCFA.

To investigate the interaction between colonic SCFA production and peripheral tissue, we need to obtain information about the extent of fermentation-derived SCFA on the systemic circulation and the affect of fermentable substrates on each of the SCFA and their circulation pattern.

Preston et al (in preparation) developed quantitative analysis of SCFA production from Verbeke et al and co-workers data. [80] To achieve this, they interpreted the in vivo dilution of known quantities of deuterated SCFA which had been infused simultaneously with the complex metaprobe test meals.

This analysis compared the BK treatment with the BP and showed the RS rich BK produced 34% more acetate and 22% more butyrate than the fermentable soluble NSP rich meal given in BP.  The BP treatment produced 27% more propionate than BK. The study was small and the power was low, so these trends where not significant.  However, in both treatments, acetate production was significantly greater than propionate and together, SCFA production accounted for some 30% of the complex metaprobe in the diet.  For the first time, this study applying complex metaprobes was able to quantify the in vivo production of acetic, propionic and butytric acids in human volunteers following fibre rich test meals.

# Chapter 6. Conclusions

# 6 The Future of Complex Metaprobe Production

This was a very interesting, exhausting and at times frustrating research field work. It was interesting to learn how to grow, label and harvest these varieties of barley and wheat. It was exhausting as it was manually achieved by two people. It was a frustrating time as we felt we were given some wrong advice. Winter wheat varieties failed to mature under glasshouse conditions and Durum wheat was regularly over-watered, leaving it prone to fungal disease.

However, the need to establish future trials to measure SCFA production from consumption of intrinsic labelled staple cereals and the effect on the human bowel and bowel disease is very necessary. At the moment, we have several hundred gram $^{13}$C-labelled grain remaining from our previous crops which could be used for nutrition trials. To upscale our production of crops, we would need to look at other sites suitable for larger crop growth and labelling.

## 6.1 Increasing the Scale of Metaprobe Production

We have located a modern glasshouse at the Scottish Crop Research Institute, Invergowrie, Dundee, which was designed from the outset to be hermetically sealed and suitable for isolating plants undergoing crop virus and GM plant research. It has forced ventilation which can be disabled and sealed to contain a discrete atmosphere. Sections are available that can accommodate four times the staging area that we used for producing ~3kg quantities of cereal grain. We previously used 200 x 20 cm pots but the glasshouse at SCRI can accommodate some 800 pots, or a planted area of some 25m$^2$. Simple scaling would suggest this would have the capacity to produce 12kg batches of $^{13}$C-labelled grain without having to move plants from glasshouse to growth chamber for labelling, and a sealed glasshouse would remove this labour-intensive stage of the procedure.

In chapter 4, we demonstrated the use of half a 30L bottle of $^{13}$CO$_2$ to produce a 3kg crop of labelled grain. A 12kg crop may the use two 30L bottles of $^{13}$CO$_2$,

which is cheaper and less time-consuming. Furthermore, as leakage from individual growth cabinets limited transfer efficiency, it is anticipated that a single large glasshouse would suffer reduced leakage, leading to increased efficiency. Our pilot study was small, the production study was bigger but future production batches could be considerably greater, producing metaprobes for larger human nutrition studies.

## 6.2 Potential Uses for Labelled Complex Metaprobes

There may be other uses for labelled complex metaprobes in other areas of human nutrition and in other than human nutrition studies. Animal nutrition, soil carbon cycle studies and bio fuel research, are some of the possibilities.

### 6.2.1 Animal Nutrition

As discussed in Chapter 4, feeding $^{13}$C-labelled hay and straw to cattle which are ruminant mammals, may prove successful in producing $^{13}$C-labelled milk. Hay and straw has low digestible energy and nutrient content but our analysis proved that hay and straw can be highly enriched. Studies would need to be devised and tested. $^{13}$C has no regulatory constraints and is considered a safe carbon tracer. Aside from milk production, metaprobes may prove a unique tool to study other aspects of ruminant nutrition such as meat production.

### 6.2.2 Soil Carbon Cycle Studies

Cereal straw is often ploughed in to the soil where it decomposes and improves soil condition. Its decomposition rate is of great interest to soil scientists. We have recently delivered a quantity of $^{13}$C-labelled wheat straw to BBSRC Rothamsted, North Wyke, to facilitate a pilot study of the degradation of lignin and cellulose from cereal straw in arable soils. They have recently published work showing plant cell wall polymers such as lignin and polysaccharides from dung incorporated into soil from cattle fed with $^{13}$C enriched forage. [21]

## 6.2.3 Biofuels

There is a potential application for industry to exploit metaprobes to facilitate biofuel production efficiency studies. Grain from a selection of crops with high yields of fermentable starch, may be used for bio-ethanol or bio-butanol production. Having a source of grain labelled with $^{13}$C would allow efficiency of different crops to be compared. However, diversion of grain for biofuel production has limited potential, with the possible exception of sugarcane production regions of the world such as Sao Paolo, Brazil. We do not wish to promote diversion of food crops for use as biofuels.

In contrast, there is more potential for use of agricultural by-products for bio fuel production. If straw can be efficiently converted into glucose by fungal celluloses (and thence to ethanol using yeast) this would greatly increase bio fuel production potential. The use of $^{13}$C-straw would facilitate selection of fungal enzyme based processes to hydrolyse cellulosic material to glucose. Metaprobes would be a unique tool to measure process efficiency. (www.rothamsted.bbsrc.ac.uk)

## 6.2.4 Human Nutrition

As discussed in Chapter 5, most of the metaprobe studies in human nutrition undertaken, concern the study of carbohydrate digestion and the resulting glycaemic response. Several other areas of human nutrition may benefit from metaprobe use. Some examples of micronutrient studies (vitamin and antioxidant nutrients) exist, e.g. labelled vitamin C in Bates et al [6]. Previous protein metabolism studies have used simple metaprobes, with only two examples of use of complex metaprobes (egg protein, Picou et al [64,64]; milk protein, Evenepoel et al [23]). Similarly, studies of fat metabolism have applied simple (chemical) metaprobes e.g $^{13}$C labelled mixed triglyceride breath tests, Armarri et al [2]) Recently complex metaprobes have become commercially available for such studies. Martek Biosciences Corporation USA, are now producing $^{13}$C labelled fatty acids for human nutritional use. (www.martek.com).

## 6.3 Metaprobes and Bowel Health

Unlike other examples where metaprobe derived from cereals have been applied to study starch digestion, most of the human studies using the complex metaprobes that we produced, have been aimed to study the systemic effects of fermentable NDC in the diet. In the context of bowel health, we believe butyrate to be the most important SCFA. There was no certainty that techniques were sufficiently sensitive to measure butyrate production in vivo. A key observation in our most recent study (Verbeke et al,[80]) was that our techniques are now sufficiently sensitive to observe butyrate production in vivo from labelled dietary NDC. This is the first such observation in humans. This opens the gates for future studies designed to assess butyrate production potential of different diets and to compare butyrate production from the same diet in different individuals, including those with bowel disorders associated with chronic inflammation. The latter would appear to offer a unique means to address the key question of whether there is a defect in butyrate production in subjects with inflammatory conditions of the bowel.

Chemoprevention of colorectal cancer with the reduced survival of neoplastic cells is of global concern in the fight against the progression of carcinogenesis. Knowledge of any defect in butyrate production and a means to determine which diets may maximise butyrate production are regarded as key areas where this new technology may be applied in the near future. Results from such studies may lead to novel intervention strategies and improved dietary recommendations in the fight against colorectal cancer.

# Glossary

Abundance – The abundance of an isotope refers to the number of heavy atoms compared to the total number of atoms of that element.  It is often expressed as "atom per cent" in tracer studies.

Acute-phase reactants – Plasma proteins which can elevated (positive) or reduced (negative) within one or two days after acute injury.

Adenosine Triphosphate (ATP) – A high energy molecule which transports chemical energy within cells, and is produced as an energy source in driving photosynthesis.

Aerobic/Anaerobic – Are with oxygen/without oxygen.

Albumin – A plasma protein made by the liver and transports essential fatty acids to adipose tissue.  Albumin accounts for approx 60% of total plasma protein.

Aleurone – Protein granules in a single cell layer of the endosperm in a grain kernel.

Alkyl – A univalent radical consisting of carbon and hydrogen atoms arranged in a chain.

Amylose – A highly branched linear polymer of glucose. Amylose and amylopectin are the two components of starch.  Amylose is found in wheat, rice and maize.

Amylose – A linear polymer of glucose and the other component of starch.

Anther – Pollen-bearing section of the stamen.

Anthesis – After the ear emerges from the flag leaf sheath anthesis occurs.  Each floret swells which forces apart the lemma and palea.  The stamens elongate (≥ 10 mm).  The anther degenerates and pollen is released.

Anti-neoplastics – Are cytotoxics which inhibit the development of tumor cells.

Antioxidants – Are substances or nutrients in our food which prevent oxidative damage to our body.  When cells use oxygen, they can produce free radicals which can lead to health problems.  Cardiovascular disease, diabetes and cancers are all contributed by oxidative damage.  Antioxidants enhance immune defence.

Apoptosis – Cell death.

Atom percent – The number of heavy atoms expressed as a proportion of the total number of atoms of that element.

Atom percent excess – The International System of Units (SI) of enrichment.

Atoms – Are made up of 3 particles, electrons, protons & neutrons.  Electrons are light particles with a negative charge, protons are heavy particles with a positive charge and neutrons are heavy particles but with no electrical charge.

Auricle - A small lobe or ear-shaped appendage frequently found at the base of leaves.

Auxins - Growth hormones found in plants

Blade - The lamina or flat expanded portion of the leaf which is usually green in colour.

Booting – The swelling of a flag leaf sheath during the reproductive phase.

Brush Border – Microvilli which line the villus surface of the small intestine.

C - Reactive Protein (CRP) – A protein produced in the liver during episodes of acute inflammation and is released into the blood stream.  CRP is made in response to interleukin-6.  CRP is a most sensitive acute-phase reactant.  This protein binds to bacterial polysaccharides and aids the immune response against bacteria.  It acts as an opsonin by preparing cellular debris for phagocytosis and elimation  by macrophages and leukocytes.  CRP is a sensitive but non-specific systemic marker of inflammation.

$C_3$ Carbon Fixation – Is a metabolic pathway for carbon fixation in photosynthesis.  This process converts carbon dioxide ($CO_2$) using the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) into 3-phosphoglycerate.  This reaction is the first step in the Calvin Cycle.   $C_3$ plants, such as wheat, have 3 carbon atoms per molecule and show a greater increase in photosynthesis and $CO_2$ concentration.

$C_4$ Carbon Fixation – Is a metabolic pathway for carbon fixation in photosynthesis.  $C_4$ plants, such as sugarcane and maize, also delivery $CO_2$ to the RuBisCo enzyme but it requires more energy in the form of ATP.  Since every $CO_2$ molecule has to be fixed twice, the $C_4$ pathway is more energy consuming than the $C_3$ pathway.  The $C_4$ pathway is an adaptive mechanism for minimising loss of photosynthetic carbon and is often referred to as the Hatch-Slack pathway.  (see Hatch-Slack pathway).

Calvin Cycle – The carbon fixation stage in photosynthesis, when a substrate is manipulated into various carbon compounds to produce energy.  It is named after Melvin Calvin (1911-1997).

Canonical Wtn Signalling – Is a series of events that occur when Wtn proteins bind to cell-surface receptors which ultimately change the amount of $\beta$–catenin that reaches the nucleus and so promotes specific gene expression.

Carbon Dioxide ($CO_2$) – A chemical compound composed of two oxygen atoms covalently bonded to a single carbon atom. At standard temperature and pressure, it is a gas and exists in the atmosphere in this state.  Carbon dioxide is used by plants during photosynthesis to make sugars, which may either be consumed in respiration or used as the raw material to produce other organic compounds needed for plant growth and development.  It is produced during respiration by plants, animals, fungi and some microorganisms.

Cellulose – The skeleton of the plant cell wall of insoluble fibres called microfibrils. In secondary cell walls it is entwined with lignin.

Chaff – Dry protective casings of cereal grain.

Chloroplasts - Small green structures in plants that contain chlorophyll, a photosynthetic pigment which converts light to energy.

Chromatography – The separation of mixtures into individual components by allowing them to pass through a columns of solid or liquid.

Chyme – A soup-like mixture of liquid and small particles of food within the stomach.

Cœliac disease – Is an autoimmune disorder of the small intestine.  This disease is caused by a reaction to gliadin, a gluten protein found in wheat but which can also occur in barley and rye.  Exposure to this protein causes the immune system

to reacts to the bowel tissue causing inflammatory reaction and villous atrophy of the lining small intestine.  This interferes with the absorption of nutrients because the intestinal villi are responsible for absorption.  The only effective treatment is lifelong gluten-free diet.  This disease is caused by a reaction to wheat proteins and not the same as a wheat allergy.

Colonocytes – Mature non-dividing colonic epithelial cells.

Colorectal Polyp - A fleshy growth (polyp) occurring on the lining of the colon or rectum.

Crohn's Disease  - Also called colitis,  is an autoimmune disease which causes inflammation of the intestines and gastrointestinal tract. It causes abdominal pain, diarrhea, vomiting, or weight loss. This disease was named after American gastroenterologist Burrill Bernard Crohn.

Culm – Woody, hollow stems.

Cytokine - A small protein released by cells that has a specific effect on the interactions between cells, on communications between cells or on the behavior of cells. The cytokines includes the interleukins, lymphokines and cell signal molecules, such as tumor necrosis factor and the interferons, which trigger inflammation and respond to infections.

Cytotoxic T Cells – Destroy infected cells and tumour cells.

Differentiation – Modification in gene expression which alters the size, shape, activity and potential of a cell.

Dukes System – Colorectal cancer diagnosis using Dukes A, B, C or D staging system. Originally published by C E Dukes in 1932 and was intended for rectal cancer only as it did not include distant metastasis.

Embryo - A rudimentary plant enclosed within a seed which becomes a sporophyte.  Sporophytes are formed from the union of two gametes, male and female sex cells.

Endosperm – Nutritive tissue within a grain kernel.

Enrichment – The abundance of a sample above the baseline level, e.g. $^{13}CO_2$– enrichment of cereal plants.

Enteroendocrine Cells – Specialised endocrine cells of the gastrointestinal tract. They produce serotonin, gastric inhibitory peptide and vasoactive intestinal peptide.

Epithelium Cells – Specialised cells which perform digestive or absorptive roles.

Fatty Acids – Are carboxylic acid and are an important source of energy.  They are either saturated or unsaturated, depending on their lengths and number of double-bonds.  Saturated fats have no double-bonds and unsaturated have one or more double-bonds.

Functional Foods – The presence of bioactive substances that affect physiology or cellular and molecular biology.

Glasgow Prognostic Score (GPS) – Combination of an elevated C-reactive protein concentration which adds to increased protein breakdown and hypoalbuminaemia or progressive nutritional decline equals systemic inflammatory which is used as a predictor of survival rate in colon cancer.

Glumes – Lowermost scale of the spikelet.

Glycaemic Index (GI) – Measurement of the incremental glucose response per gram of carbohydrate. Carbohydrates that break down quickly during digestion relealse glucose rapidly into the bloodstream are classes as High GI. Carbohydrates the breakdown slowly, release glucose more gradually and are Low GI.

Glycoproteins - Are proteins that contain oligosaccharide chains (glycols) covalently attached to their polypeptide side-chains.

Goblet Cells – These are glandular columnar epithelial cells which secrete mucus.

Grain Kernel: Consists of three parts: the innermost germ, the endosperm which surrounds the germ and the bran that enveloped both.  Most of the kernel's nutrients are locked into the germ and bran.  Whole grain products provide us with the full nutrient content of the grain kernel.

Gramineae – Grasses, mainly herbaceous but including some woody plants and cereals, bamboo, reeds and sugar cane.

Grana - A stack of thylakoid disks within the chloroplast.

Growth Factors - A naturally occurring protein which stimulates cell growth.

Hagberg Falling Number (HFN) – Is the measure of the gelling properties of flour made from wholegrain. The minimum value is 62 and ranges to 325.  Values greater the 250 are required for bread making. Poor gel formation results from the enzyme alpha-amylase which may form during or after ripening.

Hatch-Slack Pathway – The $C_4$ pathway was discovered by M.D. Hatch and C.R. Slack in Australia in 1966.  (see $C_4$ carbon fixation)

Histone deacetylases (HDAC) - A class of enzymes that remove acetyl groups from an ε-N-acetyl lysine amino acid on a histone.

Husk or Hull – The outer shell or coating of a seed.

Hydroponics – A system of growing plants using mineral nutrients solvents such as gravel or perlite.

Hypoalbuminaemia – Low albumin levels on the blood serum.

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate immune response to trauma, especially burns or other tissue damage leading to inflammation.

Inflammatory Bowel Disease (IBD) – Covers a group of inflammatory conditions of the colon and small intestine. The major types are Crohn's disease and Ulcerated Colitis.

Interleukin-6 (IL-6) is a protein that in humans is encoded by the IL6 gene.

Internode – The area between any two adjacent nodes.

Irritable Bowel Syndrome – Is a bowel disorder which causes chronic abdominal pain.

Isotopes - In early 1900's Frederick Soddy, a chemist in the University of Glasgow, showed that different types of atoms of the same chemical element have different atom masses.  Whilst explaining to Dr Margaret Todd, that several elements occupied each position in the periodic table numbers, she suggested naming this "isotopes", the Greek name for "at the same place".  Isotopes have

the same number of protons and atomic number but different numbers of neutrons.  Soddy applied this term and won the Nobel Prize for Chemistry in 1921.

Isotopic Labelling – Is to analyse the passage of a substance through a known system. The substance is 'labelled' by including unusual isotopes in its chemical composition. If these unusual isotopes are later detected in a certain part of the system, they will be identified as coming from the labelled substance.

Isotopomers - A molecule with an isotopic tracer incorporated in it.

Junk Food – Food which is high in energy density but low in nutrient density.

Lanceolate - Resembling a lance, much longer than broad, widest in the middle and tapering to a pointed apex.

Leaf - An outgrowth of a plant that grows from a node in the stem. Most leaves are flat and contain chloroplasts; their main function is to convert energy from sunlight into chemical energy (food) through photosynthesis.

Lemma – A scale of the spikelet which surrounds and protects the flower.

Lignan -  A group of chemical compounds found in plants.  It is one of the major phytooestrogens and acts as an antioxidant.

Lignin – A phenolic polymer and is an integral part of the secondary plant cell walls.

Ligule - A membrane appendage at the tip of the leaf of most grasses.

Lumen – The inside lining of the human gut.

Lymphatic System – A system of thin tube that run throughout the body carrying a clear fluid called lymph e.g tonsils, liver and spleen.

Macromolecule – A large molecule and applies to the biopolymers; nucleic acids, proteins, carbohydrates and lipids.

Mastication – Chewing, biting and grinding of food within your mouth until it becomes soft enough to swallow. This is essential for good digestion.

Meiosis – Is the reductional division of chromosomes in cells, which produces either gametes or spores.

Metastasis – Transmission of cancerous cells spreading to other parts of the body using lymph glands or blood vessels.  This results in a secondary tumour.

Micelles – During digestion, ingested fats are hydrolysed by lipase and bile salt and dispersed as small particles.

Mitotic – Cell division by which the nucleus divides resulting in two new nuclei. This division has four stages: prophase; metaphase; anaphase; telophase.

Mucopolysaccharides - Glycosaminoglycans or mucopolysaccharides are long unbranched polysaccharides consisting of a repeating disaccharide unit.

Nabim – The nabim Wheat Guide is representative organisation of the UK Flour Milling industry and lists varieties of and guidance on wheat.  Varieties are divided into Groups 1, 2 & 3 depending on yield, Group 1 being the highest yield.

Nicotinamide adenine dinucleotide phosphate (NADPH) - In chloroplasts, NADP is reduced in the electron chain of the light reactions of photosynthesis. The NADPH produced, is used as reducing power for the biosynthetic reactions in the Calvin cycle.

Node - the part of the stem of a plant from which a leaf, branch, or aerial root grows; each plant has many nodes.

Oligosaccharides - A complex carbohydrate containing three to eight units of simple sugars.

Oncogene - A gene when mutated converts a normal cell into a cancerous cell.

Palea - A scale of the spikelet which surrounds and protects the flower.

Paneth Cells - Are found in the intestinal tract, contain zinc and lysozyme.

Pascal Units (Pa) - The SI unit of pressure and is defined a one newton per square metre.  kPa = 1000 Pa.

Passive diffusion - Is the absorption of biochemicals and molecular substances moving from higher to lower concentration across a cell membrane.

Pearl Barley - Grain which has the hull and bran removed and then polished, called "pearling."

Peduncle - A stalk supporting an inflorescence (flower bud).

Peristalsis - Muscle contractions that move food through the digestive system, bile to the gallbladder and urine from the kidneys into the bladder.

Perlite - A generic term for naturally occurring siliceous rock.  When it is heated to a suitable temperature, it expands to twenty times its original volume.  When added to potting compost, it improves aeration, draining and insulation.  Perlite also encourages quicker germination and improved seedling growth.

Photosynthesis - The process in which plants convert sunlight, water, and carbon dioxide into food (sugars and starches), oxygen and water. Chlorophyll is essential to the photosynthetic process.

Phototropism - Bending a plant in response to sunlight. This reaction is caused by the growth hormone auxin which is contained within the stem.

Phytate - Phytic acid is found in the hull of nuts, seeds and grains. It is a phytonutrient antioxidant effect and may prevent or inhibit some cancers by depriving cells of minerals (mainly iron) which they need to produce.

Phytochemicals - Non-nutrients plant chemicals that may have protective or disease preventive properties.  Most foods contain phytochemicals except sugar or alcohol, which are refined.  Whole grains, vegetables, beans and fruits contain many phytochemicals. Examples of phytochemical are; alkaloids; caroteniods; flavoniods.

Phyto-oestrogens - Phenolic compounds known as flavonoids.  Found in whole grain cereals, seeds and legumes.  May regulate cholesterol and have protective action against cancer and cardiovascular disease.

Phytosterols or Plant Sterols - Occur naturally in whole grains and is an important dietary supplement. A daily diet of 0.8 g of plant sterols will reduce cholesterol and risk of cardiovascular disease.

Pistil - The seed-bearing organ of a flower normally consisting of an ovary, style and stigma.

Polydextrose - Is a food ingredient classified as soluble fibre and is frequently used to increase the non-dietary fiber content of food.

Prebiotics - are non-digestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system which are beneficial to the health of the body. They were first identified and named by Marcel Roberfroid in 1995.  Prebiotics are carbohydrates, such as oligosaccharides, and they are nutritionally classed as soluble fibre.

Probiotics – Live microorganisms added to fermented foods such as yoghurts, for health benefits.  Common microbes used are lactic acid bacteria, bifidobacteria, yeast and bacilli. The term "Probiotics" was first introduced in 1953 by W Kollath.

Protein – Essential ingredients of all living matter.  The proteins of foods have to be broken down so that amino acids can be rearranged.

Pyrimidine Base – A nitrogenous base and includes cytosine, thymine and uracil. It has one ring of atoms in its structure.

Rachis - The main flower stem to which the outer parts are attached.

Radicle – Primary root.

Raffinose – An important oligosaccharide carbohydrate found in some vegetables (peas, beans).

Rhizome - Horizontal stem of a plant that is usually found underground, often sending out roots and shoots from its nodes.

Ruminant – Mammals with a highly specialised digestive system.  Ruminant's stomach contains four compartments with fermentation chamber for fermenting proteins and carbohydrates and converting cellulose to SCFA. The semi-digested plant-based foods from the first two chambers are separated into solids and liquids.  The solid or "cud" is regurgitated and chewed slowly to breakdown particle size.  Examples of ruminating mammals are cattle, sheep and goats.

Scotch Barley – Barley grain which has had fibrous outer hull removed.

Scutellum – The large, shield-shaped cotyledon of the embryo of a grass plant, specialised for the absorption of food from the endosperm.

Sheath - A gelatinous envelope surrounding a plant.

Sigmoid Colon – Part of the large intestine, is S-shaped (Sigmoid) and susceptible to perforation and has the highest pressure of all the intestinal organs.

Somatic Evolution – An accumulation of mutations in human body non-reproductive cells during a lifetime and the effects these mutations have on the fitness of those cells. Somatic evolution is important in the aging process as well as in the development of diseases including carcinogenesis.

Sphincter – Constriction in a tubular organ, such as the gut and surrounded by a ring of skeletal muscle during digestion.

Sphingolipids – Are extremely versatile molecules which comprises of a complex range of lipids in which fatty acids are linked.  They have important biological functions as cell messengers of the immune system.

Spicules - A small pointed appendage, a diminutive spike or prickle.

Spikelets - The primary inflorescence (cluster of flowers) of grasses is the spikelet, a small structure consisting of a short axis with attached overlapping scales of lemma, glumes and palea.

Sporophyte - The sporophyte produces spores by meiosis.

Stacchyose - An important oligosaccharide carbohydrate found in some vegetables (peas, beans).

Starch - Starch is a polysaccharide carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. Starch is produced by all green plants as an energy store and is a major food source for humans.

Stem Cells - Cells found in multi-cellular organisms are characterised by their ability to renew themselves through mitotic cell division.

Stigma - The tip of a pistil which is receptive to the pollen grains and upon which they germinate.

Stomata – Are pores in a plant's leaves mostly on the underside of the leaf. Guard cells open and close the stoma using turgor pressure, controlling the loss of water vapour and other gases from the plant.

Straw – The dry stalks of cereal plants after the grain and chaff have been removed.

Stroma - Part of the chloroplasts in plant cells, located within the inner membrane of chloroplasts, between the grana.

Substrate-level phosphorylation - The formation of high-energy phosphate bonds by phosphorylation of ADP to ATP coupled to cleavage of a high-energy metabolic intermediate.

Sugar – A complex carbohydrate and a crystalline substance of sucrose, lactose and fructose.

Thylakoid disks - Disk-shaped membrane structures in chloroplasts that contain chlorophyll. Chloroplasts are made up of stacks of thylakoid disks; a stack of thylakoid disks is called a granum.  Photosynthesis takes place on thylakoid disks.

Tiller – A shoot that sprouts from the base of a grass.  This is the Phenological stage, in which tillers appear in gramineae.

TNM (Tumour, Node, Metastases) – The TNM stages are replacing Dukes' Staging system when identifying the different stages of bowel cancer.  There are 4 tumour sizes, T1,T2,T3,T4 which indicate the position of the tumour. There are 3 node stages, N0, N1, N2, which describe the number of lymph nodes containing cancer cells and two stages of metastasis spread, M0, M1.

Tocotrienols – An essential nutrient.  They are natural compounds found in barley, wheat germ, nuts and other grains.

Transit Amplifying Cells – Cells in the lining of the small intestine which undergo continuous cellular turnover.

Tumor Suppressor Gene – An anti-oncogene protecting cells from becoming cancerous.  It can inactivate cancerous cells.

Turgor - Pressure exerted by fluid in a cell that presses the cell membrane against the cell wall and makes living plant tissue rigid. Loss of turgor, resulting from the loss of water from plant cells, causes flowers and leaves to wilt.

Unsaturated Fatty Acids - See fatty acids.

Villi – Finger-like projections in the small intestine, which contain a network of capillaries and a central lymphatic vessel.  The epithelium of villi, are further

dived into microvilli which form the brush border which contains the enzymes of digestion.

Vernalised - For plants to flower at the right time they must experience a period of cold to trigger a process of vernalisation. Vernalised comes from the Latin word 'vernus' meaning 'of the spring' therefore if the seed does not get cold enough, flowering is much delayed or may not occur.

Wheat allergy - Or wheat hypersensitivity is a food allergy but also can be a respiratory or contact allergy. It is limited to the seed storage proteins of wheat and there are many allergenic components wheat e.g. serine proteinase inhibitors, glutelins and prolamins.

Wnt Signalling – A pathway which describes a complex network of proteins. Wnt signaling pathway refers to a sequence of proteins that change in behaviour to affect other proteins. This pathway commenced when a Wnt protein binds to a cell surface protein called Frizzled. Various other proteins in the pathway then interact, and eventually result in the build-up of a protein called ß-catenin, which enters the cell nucleus, where it combines with various transcription factors to affect gene expression.
The name "Wnt" originates from the "wingless" gene in fruit flies (which, when mutated, yields flies without wings), and the Int genes found in mouse tumors.

Xylem - Transport tissue found in vascular plants. Its function is to conduct water and dissolved mineral nutrients from roots to other parts of the plant.

Zadok's Scale – Is a cereal development and growth scale. This decimal scale is based on ten cereal growth stages, 0-9. 0 = germination and 9 = ripening. Each primary growth stage in then sub-divided into ten secondary stages, 00-99. This scale is named after Jan C Zadoks, the Dutch phytopathologist.

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# Appendix A

The many stages of cereal growth
and metaprobe characterisation.