DEVELOPMENT OF NUTRIENT-RICH TEFF BREAD AND ITS EFFECTS ON IRON STATUS AND EXERCISE PERFORMANCE IN FEMALE RUNNERS

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Abstract

Public awareness of the health benefits associated with less-utilised yet nutritious grains has led to a growing demand for healthier cereal products. This has resulted in an interest in improving the nutritional properties of refined white wheat bread, which is one of the main staple foods for most Western nations. Teff is a small-grained cereal that is rich in nutrients and particularly abundant in iron. Therefore, Teff offers the potential to provide a healthier alternative bread product. Female athletes, especially runners are at risk of iron deficiency due to increased iron loss, inadequate dietary iron and limited iron bioavailability in the diet. Good nutrition has been suggested as the first line of action to prevent iron deficiency in this population. As cereals and cereal products are a main contributor to iron in the diet, the modification of dietary iron intakes through a staple food offers a good opportunity to improve the iron status of physically active females.

The aims of this research project were 1) to develop a novel, iron-rich bread product by incorporating Teff grain; 2) to explore dietary iron intervention by the means of a staple food product and to measure the effects of this intervention on iron status and exercise performance in female runners.

The results indicated that the addition up to 20% of Teff (flour weight) into the bread formulation significantly ($P<0.05$) increased dietary iron levels without detrimental effects on bread quality. By the use of enzyme combinations, this level was increased to 30%, giving a product that provides over 75% RNI for dietary iron if daily amount of 200g of bread is consumed. A cohort of 11 female runners reported inadequate daily dietary iron intake of 11 mg/day, which was associated with overall compromised iron status. A 6-week dietary intervention resulted in significantly ($P<0.05$) higher total iron intakes and improved iron tissue supply but not enlarged iron stores. In terms of exercise performance, there were significant ($P<0.05$) improvements in submaximal VO_2 at anaerobic threshold and time-to-exhaustion but not maximal $VO_{2max\ peak}$. Moreover, improvements in submaximal gas exchange parameters and endurance were significantly ($P<0.05$) correlated to improved iron status.

It was concluded that Teff bread is a promising iron-rich staple food alternative. It offers the opportunity to improve habitual dietary iron intakes. Favourable trends were observed between improved iron intakes, iron status and exercise performance in this study. Further research is advised to determine the bioavailability of iron from Teff bread and to confirm these findings using larger groups of participants.

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List of abbreviations

A	Amylase
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AT	Arrival time
ATP	Adenosine triphosphate
ATr	Anaerobic threshold
AX	Arabinoxylans
BU	Brabender units
CE	Catechin equivalents
CV	Coefficient of variance
DHA	Dehydro-L-ascorbic acid
DDS	Degree of dough softening
DDT	Dough development time
DRV	Dietary reference value
DST	Dough stability time
EAR	Estimated Average Requirement
EPS	Exopolysaccahrides
FFQ	Food frequency questionnaire
GO	Glucose oxidase
Hb	Haemoglobin
ID	Iron deficiency
IDA	Iron deficiency anaemia
L	Lipase
LAB	Lactic acid bacteria
LRNI	Lower Reference Nutrient Intake
NMES	Non-milk extrinsic sugars
NSP	Non-starch polysaccharides
RBC	Red blood cells
RNI	Reference Nutrient Intake
RVA	Rapid Visco analyser
SH	Sulphydryl
sFe	Serum iron

sFer	Serum ferritin
SS	Disulphite
sTRF	Soluble serum transferrin
sTsfR	Soluble serum transferrin receptor
sTsfR-F index	Serum soluble transferrin receptor /log ferritin ratio
TIBC	Total iron binding capacity
TKW	Thousand kernel weight
VO_{2max}	Maximum oxygen uptake
WA	Water absorption
WAI	Water absorption index
WE-AX	Water-extractable arabinoxylans
WU-AX	Water-unextractable arabinoxylans
X	Xylanase

Introduction

Bread is a popular staple food of global importance and is one of the oldest prepared foods, dating back to the Neolithic era (Mondal and Datta 2008). Bread consumption is high in most countries. In the UK the average individual consumes 177 g of bread a day (Bates *et al.,* 2009). Bread is an important source of protein, dietary fibre, B group vitamins, minerals and antioxidants.

Recently, growing interest in ethnic and specialty breads, such as pitta bread and Italian ciabatta, has resulted in the development of new bread products that incorporate less-utilised grains (Department for Environment Food and Rural Affairs 2008a). Cereal grains, such as rye, oats, buckwheat, sorghum, millet, quinoa and Teff were all used for the manufacture of specialty breads. These breads not only offer good alternative to traditional wheat breads, but also provide some additional health related functionalities. Research suggests that a diet rich in wholegrains is associated with reduced risk of coronary heart disease, certain cancers and diabetes (Knill and Kennedy 2003). This is suggested to be due to the nutritional properties of wholegrain products, such as the high level of fibre, low glycaemic index, high levels of antioxidants and minerals. Processing of these ancient grains meets challenges with respect to the texture and sensory quality of the final products. Optimisation of ingredients and their proportions, dough improvers, and baking processes have all been employed in order to produce high standard healthier bread products with less-utilised grains (Flander *et al.,* 2007).

Research indicates that cereals and cereal products are the main contributors to total dietary iron intake in the general Western population (Pynaert *et al.,* 2007). In addition to this, the modification of staple foods, such bread or pasta, to more healthy alternatives is perceived to be more beneficial by the consumers than improvements of hedonistic foods, such as biscuits (Dean *et al.,* 2007).

Teff (*Eragrostis tef*) is a cereal grain originating in Ethiopia. It is favoured for its nutritional profile and is gaining popularity in Western countries (Kulp and Ponte 2000). Teff is a good source of carbohydrate, fibre (National Research Council 1996; USDA 2007), and contains more iron, calcium and zinc than other cereal grains, including wheat,

barley and sorghum (Mengesha 1966; Abebe *et al.,* 2007). Teff is an excellent source of essential amino acids, and it contains higher lysine content, an amino acid that is most often deficient in cereals, than other grains (Jansen *et al.,* 1962). Hence, the nutritional profile of Teff indicates that it could be used in producing a healthy cereal product.

The physico-chemical properties of Teff indicate that there is a great potential to be used in a broad range of food applications. Teff flour has high water absorption capacity, which relates to the higher degree of swelling of the Teff starches, which have a small and uniform granule size, hence, providing larger surface area and thus higher water absorption (Bultosa *et al.,* 2002; Bultosa 2007). Teff starch has slow retrogradation tendency (Bultosa *et al.,* 2002), hence, it could have a potentially positive impact on shelf life of baked products. Teff is preferred for making Ethiopian flatbread *injera* in terms of flavour quality, texture and softness (Zegeye 1997; Taylor *et al.,* 2003).

Interestingly, of the underdeveloped countries, only Ethiopia has low prevalence of iron deficiency anaemia, which is mainly attributed to the regular consumption of Teff *injera* among the population (Hofvander, 1968). This is because Teff grain is particularly high in iron (Mengesha 1966; Abebe *et al.,* 2007). Iron plays a central role in oxygen transport and aerobic energy transfer (McArdle *et al.,* 2010), thus it could be suggested Teff could potentially be incorporated into a habitual diet, where increased total dietary iron is desired.

Female athletes, especially runners, are at greater risk of iron deficiency due to increased iron loss in the gastrointestinal tract, sweat, urine and menstruation, also due to increased haemolysis during endurance training (Suedekum and Dimeff 2005). Furthermore, inadequate dietary iron intake, coupled with limited bioavailability of iron in the diet, may present even a greater risk of iron-deficiency for female athletes.

Good nutrition to achieve adequate iron balance has been suggested as the first line of action in the prevention of iron deficiency in female athletes (Beard and Tobin 2000). It has been reported that increasing iron intake through dietary means rather than supplementation is more beneficial to the individual's iron status (Lyle *et al.,* 1992).

Recent studies concluded that bread is one of the most preferred foods and the major contributor to energy, carbohydrate and fibre intake for high-level athletes (Iglesias-

Gutierrez *et al.,* 2008). Thus, the modification of dietary intake of iron through a staple food, for example a Teff enriched bread product, offers a good opportunity to improve iron status of physically active females which in turn may aid sport and exercise performance.

This research aimed to develop a new bread product incorporating highly nutritious Teff grain that has organoleptic properties comparable to conventional bread products. Further to this, any associations between regular Teff bread consumption, iron status and sport and exercise performance were to be identified.

Aims and Objectives

The aims of this research were:

1) To evaluate the effects of Teff grain incorporation on nutritional properties, texture and shelf life of breads; and consumer acceptability of the product using sensory taste panels.

2) To develop acceptable iron-rich Teff bread using straight dough and sourdough bakery operations that can be used in a subsequent intervention study.

3) To identify associations between regular Teff consumption, iron status and sport and exercise performance in female runners using an intervention study.

4) To contribute to the knowledge of the effects of Teff grain on the quality of bread products and its role as a staple food to potentially improve iron status and subsequently to aid sport and exercise performance in female runners population.

Chapter 1 Teff bread development - Literature Review

1.1 Bread consumption and consumer acceptability

1.1.1 Bread consumption in the UK

Bread is an important commodity worldwide and one of the staple foods in the UK. Bread accounts for over 10% of average daily total energy intake and contributes to 18% of carbohydrate, 10% protein and 19% non-starch polysaccharides (NSP) for the general adult population in the UK (Bates *et al.,* 2009).

Overall consumption of bread has steadily declined in the past decade, which can be mainly attributed to the increased consumption of other starchy foods, such as pasta, breakfast cereals and snacks (Department for Environment Food and Rural Affairs 2008b). A recent National Diet and Nutrition Survey (Bates *et al.,* 2009) showed that an individual adult in the UK consumes 177g of bread a day. However, due to the rising prices of imported wheat, the value of flour increased resulting in a higher retail price of bread. UK retail value sales for bread were at nearly £2.6 billion in 2008, which showed value growth over 30% since 2003 (Mintel 2009). Although white wheat bread still accounts for the highest proportion of the bread market (45%), wholegrain, specialty and ethnic breads showed the highest sales increase in 2008 (Mintel 2009). This reflects the general trend and the shift towards purchasing more health-orientated and premium breads.

1.1.2 Consumer preferences

Food liking and preferences play an important role in food choice and purchase decisions (Mela 2001). The consumers' quality perception relies on intrinsic (appearance, colour, shape and aroma) and extrinsic (packaging, labeling, price and brand) features of the product (Bech *et al.,* 2001). Many researchers have investigated consumers' beliefs, attitudes and preferences of bread products (Mialon *et al.,* 2002; Kihlberg *et al.,* 2005; Arvola *et al.,* 2007; Dean *et al.,* 2007; Dewettinck *et al.,* 2008; Gellynck *et al.,* 2009).

Research conducted by Gellynck *et al*. (2009) revealed interesting changes in consumers' bread quality perception over the last decade. Although white wheat bread is still perceived as a 'basic' and 'traditional food' by most of responders, it is also considered to be 'expensive', 'boring', 'not tasty', 'untrendy' and having 'short-life'. However, the same study found that specialty breads and breads used as a part of a meal (sandwich) scored higher preferences in different consumer segments. This suggests that consumers' attitudes towards the types of bread might be changing. Another study reported that consumers were

aware of the health benefits of wholegrain breads and believed them to be more nutritionally balanced and healthier than white bread (Arvola *et al.,* 2007). Furthermore, wholegrain breads, rather than other starchy products such as biscuits or pasta, seem to portray a positive health image (Dean *et al.,* 2007). Although wholegrain breads are perceived to be nutritious and beneficial to health, consumers are more reluctant to try them. White bread was scored to be tastier, more favourable to consume but less nutritionally valued than wholemeal bread (Mialon *et al.,* 2002), while addition of wholegrain flour showed decreased scores in sensory characteristics, such as flavour and aroma (Kihlberg *et al.,* 2005).

Bread liking and preference can be also strongly affected by nutritional or health related information provided to the consumer. Mialon and colleagues (2002) observed that liking and likelihood of consumption were increased for white bread labeled high in fibre, whilst the preference did not change for wholegrain bread. Average scores for liking of bread also were higher for breads with wholegrain amaranth flour with a health statement and lower for white bread (Kihlberg *et al.,* 2005).

The research on preferences and liking of bread indicate that consumers are aware of wholegrain health benefits, however, sensory attributes, especially flavour and aroma, seem to be the main obstacles for the consumption of wholemeal bread. The likelihood of consumption and appeal of wholegrain breads may be increased by increasing the availability of nutritional information and health information of the products. Furthermore, gradual incorporation of wholegrains into white bread may be an option to increase their habitual intake, thus imparting their long term beneficial health effects (Marquart *et al.,* 2006).

1.2 Breadmaking Technology

Bread is a leavened product obtained by the fermentation of cereal flour sugars by the action of yeast and natural flour enzymes (Mondal and Datta 2008). The baking process transforms dough, made of flour, water and leavening agents, into a high quality product with unique sensory features.

1.2.1 Ingredients used in breadmaking

A number of different ingredients are used in breadmaking. Flour and water are essential and form the basis of all bread formulation mixes. Additional ingredients are used to produce bread products with specific desired characteristics.

Recently, there has been a growing interest in using less utilised whole grain flours. However, in order to produce an acceptable product with favourable textural and sensory properties, it is essential to understand how the basic ingredients in conventional bread technology work and how different proportions of these with the addition of functional agents can help in achieving a desired bread product.

1.2.1.1 Cereal flour

Flour is the major ingredient in bread recipes. It is able to absorb water and form cohesive and visco-elastic dough, which is a key function in breadmaking.

A number of different cereal flours, including wheat, rye, corn, oats, sorghum and millets have been used in different types of bread for many years (Kulp and Ponte 2000). Environmental and cultural differences influence the type of cereals used for breadmaking (Cauvain and Young 1998). Wheat bread is a staple bread of the UK, North America and other Western countries. Rye bread is an example of a staple bread in Germany, Eastern Europe, Russia and Scandinavia, whilst flat breads made from corn, sorghum and millets are popular in South and Latin America, the Middle East and African countries.

All cereal grains consist of germ, endosperm and bran (Kulp and Ponte 2000). High grade flours mainly consist of endosperm, whereas low grade flour and whole-meal flours contain of all parts of the cereal grain. Although any cereal flour can be used for breadmaking, wheat flour is the preferred grain for leavened bread manufacture (Kulp and Ponte 2000). This is due to wheat flour's ability to form a gluten network, which is essential for producing leavened bread with a fine open structure (Coultate 2002).

1.2.1.2 Water

Water has an impact on the properties of the dough and the final bread. Water plays a key role in dispersion and solubilisation of the dry ingredients, as well as in the formation of the gluten network (Cauvain and Young 2000). The appropriate level of water is crucial in order to obtain optimum consistency and dough performance (Cauvain and Young 1998). Tests for water absorption (refer to section 1.2.3.1) have become essential in order to ensure product reproducibility within the baking industry.

1.2.1.3 Yeast

There are around 1,000 known yeast species, of which *Saccharomyces cerevisiae* is the most widely used and studied (Walker and Moselio 2009). Yeasts have been used for thousands of years and have been used in fermentation processes, including bread leavening and alcoholic beverage production.

Baker's yeast (*Saccharomyces cerevisiae*) metabolises various sugars, including sucrose, glucose and maltose (Bekatorou *et al.,* 2006) and produces CO_2, which leavens the dough, and ethyl alcohol, which evaporates during the baking process. Baker's yeast has an optimum temperature between 28°C and 32°C and an optimum pH of 4-5 for growth (Spicher and Brummer 1995). Hence, proving conditions reflect these optimum conditions in order to obtain good dough expansion. Conventional bread usually requires 1-6% yeast for leavening; whilst levels of 8-9% yeast have an undesirable effect on bread taste and aroma (Spicher and Brummer 1995).

1.2.1.4 Salt

Salt (sodium chloride) is used for a variety of purposes in bread manufacture. Firstly, salt is a major contributor to bread flavour. It also modifies perception of other flavours that might be in bread: enhancing sweetness from added sugar and masking metallic bitterness from the bran fraction in wholegrain flours (Cauvain and Young 2006; Miller and Hoseney 2008).

Salt also has a stabilising effect on dough fermentation. In doughs with no or low levels of salt, excessive yeast fermentation leads to formation of a gassy and runny dough resulting in an open crumb grain and poorer texture baked product (Spicher and Brummer 1995; Miller and Hoseney 2008).

Research indicates that addition of 2% of salt (based on flour weight) increases dough development time (1.9min *v* 2.3min), stability (3.0min *v* 15.5min) and reduces the degree of softening (115BU *v* 40BU) (Wehrle *et al.,* 1997). This shows that an optimum level of

salt would lead to stronger doughs with more stability and better tolerance to the effects of overmixing.

1.2.1.5 Other ingredients

Other optional ingredients are used to improve rheological properties of dough in order to produce better final products.

Sugar is added to the standard recipe in order to promote yeast fermentation and improve gas production during fermentation. Furthermore, the level of salt can be reduced by the addition of sugar (Cauvain and Young 1998), which is beneficial where salt reduction is desirable from a nutritional aspect. However, excessive levels of sugar in the bread recipe can cause excessive fermentation. This has a negative impact on dough handling properties as the resultant dough can be 'gassy' and runny (Cauvain and Young 1998).

Fat is another optional ingredient, which is used to improve loaf volume and shelf-life as well as to produce finer crumb structure. Fats act on dough by formation of a film between starch and gluten layers (Spicher and Brummer 1995). This has a positive effect on gas cells stabilisation in the dough, as the lipid layer surrounds gas bubbles and prevents coalescence (Sroan and MacRitchie 2008). Indeed, addition of fat (at the level of 0.2% on flour basis) was shown to restrict rapid gas loss at the end of proving (Mousia *et al.,* 2007). As a result, fat content of 5-6% of flour weight can increase loaf volume by 15-25% (Stauffer 1998).

Ascorbic acid is used as a dough conditioner at levels of 100-200ppm of the flour weight. The mechanism of dough improvement is not well understood, however, it is generally accepted that it acts as an antioxidant and helps the gluten development process (Elkassabany and Hoseney 1978). Ascorbic acid is oxidised to dehydro-L-ascorbic acid (DHA) by oxygen or other oxidants. DHA then oxidises sulphydryl (SH) to disulphite (SS) groups in the gluten network forming inter- and intramolecular bonds, hence, strengthening the gluten matrix and hardening the dough system (Nakamura and Kurata 1997). The addition of ascorbic acid was shown to increase loaf volume and give finer crumb structure (Collins 1966).

1.2.2 Processes during breadmaking

The qualities of bread rely on both optimum formula and processing conditions. Production of bread consists of mixing, proving and baking. Each of these processes is responsible for different quality properties of bread.

1.2.2.1 Mixing

Mixing is an integral part of breadmaking which is characterised by homogenisation of the ingredients and the development of the dough structure (Cauvain and Young 1998). Several important processes occur during the mixing phase: dispersion and hydration of ingredients, gluten development and dough aeration.

Dough, in breadmaking, is defined as a semi-solid mass that resists mixing (Cauvain and Young 1998). Dough development involves a number of changes, including mixing, kneading, air incorporation, and creation of gluten structure. During the early stage of mixing all of the ingredients are blended to give a homogenous dough mass. The protein network is softened by hydration, the starch granules are less firmly attached to protein, flour lipids are uniformly distributed and soluble materials are fully dissolved in the aqueous medium. This phase is essential for forming the matrix in which reactions take place during the dough development (Cauvain and Young 1998). During the latter stages of mixing, the hydration of flour proteins and the input of energy result in the formation of the gluten network. The low-molecular weight *gliadins* are responsible for the viscosity while the high-molecular weight *glutenins* are required for the elasticity of the dough (Goesaert *et al.,* 2005). Gluten proteins are linked together by disulphide bonds and provide structure for the dough. The gluten matrix is responsible for the retention of air bubbles incorporated during mixing and uniform expansion of the dough under the influence of carbon dioxide (CO_2) gas produced by the yeast during fermentation. Therefore, the gluten protein network plays a major role in determining final product quality parameters, such as loaf volume and crumb structure (Goesaert *et al.,* 2005). As starch is the largest fraction of flour, during dough stages of bread making it acts as a filler and contributes to dough volume together with gluten network (Hug-Iten *et al.,* 1999).

All air incorporation is achieved during mixing (Scanlon and Zghal 2001). Little air is incorporated during hydration and blending stages. The majority of air bubbles are entrapped during latter stages of mixing when the dough develops resistance to mixing. During mixing CO_2 starts to be produced by yeast. Two other gases are also present in dough, oxygen and nitrogen. Oxygen is used up very quickly and very little of it is left by the end of mixing. Nitrogen acts as a nucleating agent for the development of CO_2 bubbles formed during fermentation (Scanlon and Zghal 2001).

During mixing, the resistance of dough increases until it reaches an optimum consistency and finally decreases if overmixed. This can be monitored by dough recording devices, such as the Farinograph, which is discussed in detail in section 1.2.3.1.

1.2.2.1.1 Role of gluten in breadmaking

Gluten refers to the protein fraction of wheat flour and is formed as a network in dough during mixing. Gluten is a structure-building protein in breadmaking and its viscosity and extensibility properties are essential for good gas holding properties and good crumb structure in bakery products (Schofied 1994). Hence, breadmaking properties and the final bread product's quality properties are heavily affected by the quality of gluten in flour. The absence of gluten would result in a dough that has batter-like consistency and the final bread product that has quality defects (Gallagher 2009).

A rise in the incidence of coeliac disease and the awareness of the health benefits of wholegrains has seen a gradual move towards inclusion of gluten-free whole-grain flour into grain-based products (Marquart *et al.,* 2006). As dilution or replacement of gluten in a breadmaking formula has such a detrimental effect on final products characteristics, historically alternative cereal grain based products were reported to have poor quality properties and low consumer acceptability (Gallagher 2009). This has put more pressure on cereal technologists to produce bread products with improved structure, mouthfeel, acceptability and shelf-life (Gallagher *et al.,* 2004).

1.2.2.2 Fermentation

Fermentation starts during the mixing phase and continues during proving in a controlled atmosphere. Dough entering the prover has a lower temperature (25-32^{o}C) than the equipment (Cauvain and Young 2006). The warm and moist conditions during proving are held to encourage the production and retention of CO_2. During proving, the dough undergoes a series of interlinked reactions. Starch is converted into sugars by enzymes. Alpha-amylases naturally present in flour are a range of enzymes which catalyse the hydrolysis of both amylase and amylopectin into short-chain dextrins, while beta-amylases hydrolyse part of the starch to maltose (Martin and Hoseney 1991; Cauvain and Young 1998). The simple sugars produced are then metabolised by the yeast, which then produces CO_2 and alcohol.

The dough expands during fermentation due to the action of CO_2, which diffuses into the air bubbles, incorporated during mixing process. This causes these air cells to grow and the dough to expand and the density of the dough is reduced (Scanlon and Zghal 2001). The number of cells is not increased during proving, only the volume and size of the cells. The gas bubble diameter increases from around 100μm before baking to 2mm in the baked bread (Cauvain and Young 2006). Other compounds produced by the yeast activity are mainly acids, which contribute to the flavour and aroma development.

1.2.2.3 Baking

The baking process is the key step in achieving the final product's texture, colour and flavour qualities. The main changes influencing bread quality during baking are volume expansion, starch gelatinisation, protein denaturation, crust formation, inactivation of yeast, non-enzymatic (Maillard) browning and moisture loss (Pyler 1998).

Initial stages of baking are significant to bread crust formation. As temperature rises, water evaporation starts and the surface reaches 100°C, which results in a moisture loss (Cauvain and Young 1998). During later stages of baking, the higher temperature of the surface of the dough in the oven and movement of the heat toward the centre of the dough results in water content redistribution to the centre of loaf, mainly due to evaporation and condensation (Mondal and Datta 2008). By the end of baking, there is a significant moisture difference between the crust and the crumb (Cauvain and Young 2006).

There are two major structural changes in a baking process. The first one is expansion of the dough, which is then followed by solidification, during which the transformation of the dough to a solid baked product occurs. This transformation is mainly induced by starch gelatinisation and protein denaturation (Therdthai and Zhou 2003). Both processes result in a light and porous structure formation of the final baked product (Primo-Martin *et al.,* 2006).

1.2.2.3.1 Expansion

The first structural change during baking is the expansion of the dough in the oven until its cellular structure is fixed. The production of carbon dioxide (CO_2) by the yeast increases during initial stages of baking until yeast are inactivated and destroyed at around 55°C (Therdthai and Zhou 2003). Air bubbles extend and expand the dough. This transforms the dough into a sponge-like structure in which air cells are interconnected and the final product is composed of crumb and crust. This expansion is the main factor determining loaf volume and, ultimately, bread quality. The expansion ceases by several factors including yeast inactivation and resistance of dough (Mondal and Datta 2008).

1.2.2.3.2 Starch gelatinisation

In most cereal starches, gelatinisation starts at around 55-60°C and finishes at 85-90°C (Cauvain and Young 1998; Therdthai and Zhou 2003). In the presence of water, when the temperature reaches the gelatinisation stage, swelling and disruption of starch granules occurs. Starch gelatinisation leads to formation of a continuous starch network, consisting of swollen, interconnected and partially or fully disrupted starch granules, where amylose polymers are found in the centre of granules and amylopectin in the outer zones (Hug-Iten

et al., 1999). The viscosity of the system changes from viscous semi-liquid dough to near-rigid bread. In the crust a continuous protein network and discontinuous starch can be observed, moving from the crust to crumb, the increase of starch gelatinisation, due to higher moisture content, can be noticed (Primo-Martin *et al.,* 2007).

1.2.2.3.3 Protein denaturation

The protein in the dough will start to coagulate at 70-80°C (Cauvain and Young 2006). Heat transfer induces further polymerisation of the glutenins by thiol-disulphide interchange reactions and changes in the solubility of the gliadins (Therdthai and Zhou 2003). These thermal effects on protein lead to the strengthening effect on gluten network and hence, better crumb structure and texture (Therdthai and Zhou 2003). Protein denaturation and starch gelatination during the baking process result in the transformation from viscous dough to an elastic and solid bread material (Cauvain and Young 1998).

1.2.2.3.4 Colour and flavor formation

Colour and flavour development during the baking process are important characteristics of bread quality contributing to consumer preference. Crust colour formation is mainly attributed to non-enzymatic browning reactions, including Maillard reactions and caramelisation (Therdthai and Zhou 2003). Maillard reactions take place in the presence of amino compounds and reducing sugars at low water concentration at temperatures above 50°C and in neutral or slightly alkaline conditions (Coultate 2002). Maillard reactions produce colour (melanoidins), flavour and aroma (aldehydes and ketones) components associated with bread crust characteristics. Caramelisation is a degradation of sugars, which occurs at a temperature above 120°C, in the presence of low water concentration and pH levels between 3 to 9. This reaction plays an important role in developing sensory characteristics of bread crust (Ramirez-Jimenez *et al.,* 2000).

1.2.3 Analysis of flour, dough and bread characteristics

1.2.3.1 Farinograph parameters

The farinograph is defined as a dynamic torque measuring dough testing instrument (Shuey 1972). The resistance of dough during continuous mixing at a constant temperature is transmitted to a dynamometer, which is connected to a lever, scales and recording system, which produces a curve on a chart (Shuey 1975).

The farinograph curve shows important physical properties of flour. Several farinogram parameters are used to interpret flour and dough characteristics (Figure 1.1).

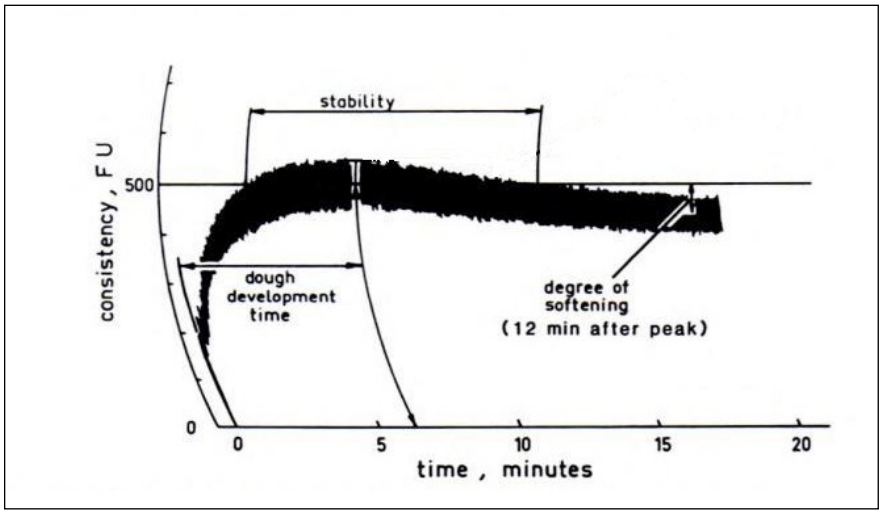

Figure 1.1 Representative farinograph curve and parameters used in interpreting farinogram (Dendy and Dobraszczyk 2001)

1.2.3.1.1 Water absorption (WA)

It is critical for the dough to have optimum water levels and to be mixed to the point of optimum development. Therefore, WA is one of the most important flour parameters, which can be determined by the Brabender Farinograph. Water absorption is the amount of water absorbed by the flour to achieve dough of optimum consistency for breadmaking (Cauvain and Young 1998). Sufficient water is added to the flour to produce dough with a maximum viscosity of 500 Brabender units (BU), the level that has been found to provide the optimum baking performance dough (D'Appolonia and Kunerth 1984). Farinograph's burette reading gives this water absorption value. The water absorption of flour is influenced by the amount of damaged starch, presence of non-starch polysaccharides, protein content and particle size of the flour. It is desired that flours for breadmaking technology have a high water absorption capacity in order to produce a higher yield of dough.

1.2.3.1.2 Arrival time (AT)

AT is the time required for the curve to reach the 500 BU line after water was introduced during mixing (Dendy and Dobraszczyk 2001). This value indicates the rate at which water is taken up by the flour. Generally, arrival time increases with higher protein content (D'Appolonia and Kunerth 1984) due to the fact that more water is absorbed by the flour. Also, arrival time increases as particle size of flour is reduced (D'Appolonia and Kunerth 1984). This can be explained by the fact that smaller particle size results in a larger surface area, which contributes to higher water absorption rate, hence, longer time is required for dough development.

1.2.3.1.3 Dough development time (DDT)

DDT is the time from water addition to the flour until the dough reaches the point of the maximum consistency, expressed by the highest point in the farinograph curve just before the first indication of weakening (Shuey 1975). During mixing, water hydrates the flour component and the dough is developed. Therefore, DDT is related to the optimum mixing time in order for the flour fully to absorb water and for the dough to reach its optimum (500 BU) consistency.

1.2.3.1.4 Dough stability time (DST)

DST is defined as the difference in time between the point where curve first intersects the 500 BU line (arrival time) and the point where the curve leaves 500 BU line (departure time) (D'Appolonia and Kunerth 1984). This value gives an indication of flour's tolerance to mixing. Usually, flour with good breadmaking properties has high stability time and is more tolerant to mixing.

1.2.3.1.5 Degree of dough softening (DDS)

DDS is the difference in height, measured in Brabender/farinograph units (expressed as BU or FU), between the centre of the curve at the highest peak, and the centre of the curve at a point of 12 min later (Cauvain and Young 1998). It generally gives the rate of dough breakdown and the strength of flour. Weaker flours tend to have higher values of DDS.

1.2.3.1.6 Departure time (DT)

DT is the time from the introduction of water until the top of curve leaves the 500 BU line (D'Appolonia and Kunerth 1984). Stronger flours usually have longer departure times compared to weak flours.

Farinograph results are also useful for predicting finished product texture characteristics (Atkins and Larsen 1990; Sebecic and Sebecic 1996; Bason *et al.,* 2004). Table 1.1 presents correlation coefficients between farinograph parameters, loaf volume, levels of protein and damaged starch content.

Table 1.1 Correlation coefficients for farinograph parameters, loaf volume, levels of protein and damaged starch in flour

	Loaf volume	WA	DDT	DST
WA	0.01			
DDT	0.45*	0.618***		
DST	0.32	-0.60**	0.810***	
DDS	-	-0.321**	-0.572***	-0.852***
Protein	0.58**	0.47*	0.72***	0.26
Damaged starch	-	0.820*	-	-

Key: - data not given or analysed. *P<0.05. **P<0.01. ***P<0.001. Correlations values adapted from Bason *et al*. (2004), Atkins and Larsen (1990) and Sebecic and Sebecic (1996)

High WA values in tested wheat flour resulted in stronger doughs with longer mixing times, higher stability and a lower degree of softening (Bason *et al.,* 2004). Stronger flours, which have a higher protein content, showed medium strength significant correlations with increased WA capacity, higher loaf volume and longer DDT (Atkins and Larsen 1990). The content of damaged starch during milling is an important factor affecting the WA. There is a strong positive correlation between WA and damaged starch, which is statistically significant (Sebecic and Sebecic 1996).

1.2.3.2 Bread volume

Bread volume is one of the principal components of bread quality. Volume is particularly important in wholemeal and gluten-free grain breads in which loaf volumes tend to be lower than the breads made from white wheat flour. This was suggested to be due to dilution of functional gluten proteins (Pomeranz *et al.,* 1977), poorer gluten hydration during mixing due to the fibre components' ability to bind water (Lai *et al.,* 1989) and physical disruption of the starch-gluten matrix by the bran components (Gan *et al.,* 1992).

The seed displacement method, usually rape, canola or pearled barley, is the most common method for determining bread specific volume (Cauvain and Young 2006). Although new instruments, including image analysis techniques and laser sensors, have become commercially available, the seed displacement method is still used by most researchers. In fact, a recent study comparing the rapeseed displacement method and a laser sensor technique showed a significant correlations between the two (r^2=0.993, P<0.05) when measuring bread loaf volume (Caley *et al.,* 2005).

1.2.3.3 Texture analysis

Texture refers to the physical characteristics that can be sensed by touch and related to product deformation and disintegration during time (Bourne 1982). Texture is an important property in bread, which strongly influences consumers' acceptability of the product.

The most common bread texture attribute that is chosen for bread quality determination is crumb firmness, which is defined as a peak force of the first compression of the baked product (Kilcast 2004). Crumb firmness is usually determined by a number of compression tests, these can be either subjective or objective.

The subjective compression test would involve 'squeeze test', where a panelist compresses the crumb of the sliced bread to evaluate the spring-back of the crumb (Cauvain and Young 2006).

The objective compression tests are carried out by the compression of bread crumb through a standard distance, where the force required to deform the crumb is measured (Kilcast 2004). Changes in starch polymers (amylopectin and amylose) in the crumb cell walls are the main contributing factor to firming of the bread crumb over time (Scanlon and Zghal 2001). Hence, the same crumb compression technique is used to assess the firming of the crumb during shelf-life.

1.2.3.4 Cellular structure/Image analysis

The term crumb grain is defined as the exposed cell structure of the crumb, when a loaf of bread is sliced (Kamman 1970). The crumb structure of bread is a contributing factor in determining mechanical strength and texture, visual appearance and the overall quality. Indeed, crumb cellular structure accounts for approximately 20% of the weighting in judging bread quality (Pyler 1998).

Parameters, such as the number and size of cells, spatial distribution of cells (uniformity) and cell wall thickness, are all used to evaluate cellular structure of the crumb. Good quality bread should possess structural qualities, such as thin but strong cell walls, so they can recover from deformation and a fine, uniform crumb grain with smaller, evenly sized cells (Liu and Scanlon 2003). This is usually obtained from strong white wheat flours. Low grade flours usually produce breads with a coarser structure, consisting of higher volume irregular cells with thicker walls and higher crumb density.

Subjective assessment of crumb structure involves scoring a combination of parameters visually. However, this technique relies on the expertise of the panelists to assess the products accurately and consistently (Cauvain and Young 2006). The more accurate and reliable evaluation of bread crumb structure is obtained by objective techniques, such as

digital imaging (Wang and Coles 1994), this captures an image of a bread slice by scanner or video camera and analyses the crumb parameters by means of a software system.

1.2.3.5 Bread staling

The staling of bread involves several processes, such as starch retrogradation, interactions between starch molecules and gluten proteins, and a redistribution of moisture (Willhoft 1973; Ribotta and Le Bail 2007). All these changes contribute to the chemical and physical changes of the crust and crumb, hence, decreasing product acceptance by the consumer (Cauvain and Young 1998).

1.2.3.5.1 Starch retrogradation

Starch is a major component of flour and plays a very important role in structure formation, textural properties and keeping quality of bread (Cauvain and Young 1998). During bread storage, rearrangements in the starch fractions, amylose and amylopectin, lead to the structural transformation of bread crumb, including rapid gelation of amylose and slow recrystallisation of amylopectin (Hug-Iten *et al.,* 1999). This process is called retrogradation, during which molecules reassociate to form a double helix crystalline structure (Cauvain and Young 1998). The first theory of bread staling was presented by Schoch and French in 1947 (Figure 1.2). In this retrogradation model, changes in branched amylopectin chains from widely spread in the swollen starch granule of fresh bread into aligned side by side in the stale bread, was proposed to be the main contributor to bread staling.

The polymers of starch, amylopectin and amylose, have different roles in retrogradation. Evidence suggests that amylopectin is the main cause of bread staling because it contributes to long-term rheological and structural changes, whereas amylose is responsible for short-term changes (Biliaderis 1990). Initially, amylose and amylopectin are in an amorphous state after gelatinisation due to the baking process. In fresh bread amylose and amylopectin are partially separated in the swollen and gelatinised granules. Small amounts of amylose become more soluble, due to increased swelling, and leach out of the granules (Keetels *et al.,* 1996). On cooling this solution of amylose retrogrades to an insoluble gel, contributing to the loaf structure and initial firmness of the bread crumb. However, it is considered to be stable during storage (Cauvain and Young 1998). A recent study looked at gelatinised wheat and maize starch pastes with different levels of amylose (Tang and Copeland 2007). Researchers demonstrated that the amylose molecules' ability to form networks is a key for initial retrogradation on cooling and setting of crumb structure. In fresh bread, branched amylopectin chains are unfolded and distributed within

swollen granules. During bread ageing amylopectin chains gradually align with each other by intermolecular associations (Cauvain and Young 1998). This results in crystallisation of amylopectin and increased rigidity of the internal granule structure (Ring *et al.,* 1987). Amylopectin retrogradation leads to formation of a crystalline pattern, which contributes to increased crumb firmness during staling of bread (Miles *et al.,* 1985). The relative crystallinity of amylopectin in the crumb was found to increase from 0% in fresh crumb to 26-34% in bread rolls after 20 days storage, although 1-day old bread showed no amylopectin retrogradation (Primo-Martin *et al.,* 2007). This was also confirmed by another study, which showed amylopectin retrogradation to have a strong relationship with bread firmness during bread storage (r^2=0.86, P<0.05) (Leon *et al.,* 2002).

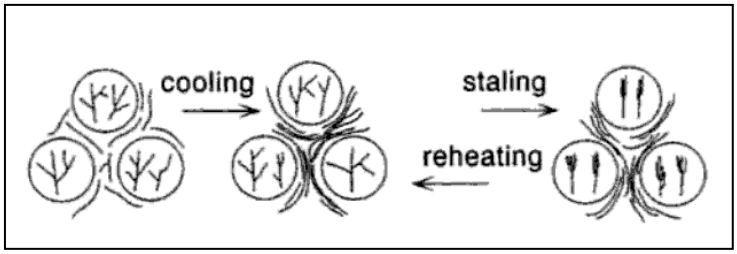

Figure 1.2 Mechanism of starch retrogradation (Schoch and French 1947)

1.2.3.5.2 Gluten

The effect of gluten on bread staling has been investigated by several researchers. The anti-firming effect on bread crumb was observed when protein content of flour was increased (Kim and Dappolonia 1977). The researchers concluded that breads made from stronger flours had a slower rate of staling when compared to breads made from weaker flours. The indirect positive effect of gluten was explained by the dilution of the starch fraction and its effect on loaf volume (Willhoft 1973). Indeed, the evidence shows that decreasing specific loaf volume significantly increases crumb firmness (Axford *et al.,* 1968). Another study also confirmed these findings, showing gluten from weaker flours had stronger interactions with starch granules than that from stronger flours (He and Hoseney 1990). However, more recent evidence showed that there is little or no effect of gluten on crumb firming. Martin *et al*. (1991) found that bread crumb firming was caused by entanglements and weak hydrogen cross-links between protein fibrins and starch granules, which suggests that starch-protein interactions are more important in bread staling than starch retrogradation. These findings, however, were contradicted by other researchers (Every *et al.,* 1998; Ottenhof and Farhat 2004). A study carried out by Every *et al.* (1998) looked at 0-15% gluten breads with similar specific loaf volumes. The authors suggested that although starch-protein interactions have an effect in bread staling, quantitatively starch-starch

interactions are more important because flour contains much higher proportions of starch than gluten. Similar observations were reported by Ottenhof and Farhat (2004). In their study, the presence of gluten had no effect on kinetics or on starch fragment retrogradation. Goesaert *et al*. (2009) proposed a mechanism by which the gluten network may play a role in bread staling. During water migration, water is lost from different networks in crumb, which results in an increase in firmness of the starch network as well decrease in the flexibility of the gluten network. All these processes will result in a drier, firmer and less elastic texture.

1.2.3.5.3 Moisture redistribution

During storage, the water content of bread changes due to water migration from crumb to crust and water incorporation into the starch crystalline structure (Primo-Martin *et al.,* 2007; Ribotta and Le Bail 2007). Moisture redistribution in bread has been shown to contribute to the processes involved in bread staling. Primo-Martin *et al*. (2007) demonstrated that water activity increased from 0.53 in fresh bread to 0.92 in staled 20 day old bread crust. Water migration from crumb starts rapidly after baking, the crumb of freshly baked bread loses 6% its moisture within first 2 hours of cooling, and over 15% after 30 days of storage (He and Hoseney 1990). Furthermore, the moisture content of crumb has been shown to be inversely proportional to rate of firming. This is because water acts as a plasticiser and when water content decreases, it accelerates the formation of cross-links between starch and proteins, which are known to be contributors to bread staling. He and Honesey (1990) showed that bread with higher moisture content firmed more slowly and had a lower final firmness when compared with lower moisture bread.

1.3 Incorporation of other grains into leavened breads

In recent years, consumer demand for healthier bread alternatives has resulted in industry's interest in improving the nutritional properties of refined white wheat bread. However, lack of gluten-forming proteins in most of the nutrient-rich cereals limits the application in conventional breadmaking.

Many researchers have looked at incorporating less-utilised and nutritious grains into breads and their effects on textural, nutritional and sensory properties (Dhingra and Jood 2002; Flander *et al.,* 2007; Holtekjolen *et al.,* 2008; Mlakar *et al.,* 2008b; Mlakar *et al.,* 2008a; Lipilina and Ganji 2009; Skrbic *et al.,* 2009; Zannini *et al.,* 2009).

Barley was incorporated into leavened breads by several researchers. Skrbic *et al.* (2009) supplemented white and wholegrain bread with hull-less barley flour and flakes. Authors reported that addition of barley to white wheat bread resulted in a significant improvement

in flavour profile, fibre, zinc and selenium content. However, a significant decrease (P<0.05) in bread quality parameters, such as volume, crumb elasticity and crumb uniformity were also observed. This is because barley contains hordeins, gluten forming proteins which do not possess the same dough development properties as gliadins and glutenins, found in wheat flour. A significant reduction in the leavening performance and CO_2 retention of the dough was seen as the barley flour content was increased (Zannini *et al.,* 2009). In wholegrain bread supplementation with barley flour did not significantly affect texture and flavour, whilst still improving zinc content (P<0.05) (Skrbic *et al.,* 2009). Another study investigated incorporation of barley at different levels and the effects on organoleptic and nutritional properties (Dhingra and Jood 2002). Levels up to 15% of barley (based on flour weight) improved mineral and amino acid lysine content without significantly affecting sensory properties of the breads. However, when barley replaced 40% of wheat flour, sensory analysis showed high scores for bitterness, off-odour and off-flavour, which were mainly attributed to high levels of phenolic compounds (Holtekjolen *et al.,* 2008).

Oats were also used in breadmaking to improve the nutritional profile of refined breads (Oomah 1983; Krishnan *et al.,* 1987; Flander *et al.,* 2007; Salehifar and Shahedi 2007). Oomah (1983) incorporated 25% of oat flour into white wheat breads. Dough development time, dough strength and loaf volume decreased, while mixing tolerance increased. Furthermore, there was no significant difference in the rate of gas production in the doughs. This suggests that functional gluten dilution was responsible for lower loaf volume in oat bread. Similar findings were reported by another study (Krishnan *et al.,* 1987), where wheat breads were supplemented with oat bran. Water absorption increased with higher levels of oat, whereas bread volume, crumb grain and texture parameters decreased. However, breads containing up to 10% oat bran had quality parameters and sensory scores that were comparable to control bread. In the study carried out by Salehifar and Shahedi (2007), breads produced with 30% and 40% oat flour had poor quality parameters and were scored as unacceptable by the sensory panelists, due to bitterness. Another recent study (Flander *et al.,* 2007) obtained acceptable oat bread by optimising ingredient levels and process conditions. Whole grain oats were used for white wheat flour replacement (51/100g) in straight dough breadmaking process. The highest loaf volume (3.7 ml/g), lowest bread firmness (1.37 N after 2 hours) and acceptable sensory properties (crust colour, thickness, flavour and crumb richness) were attained by adding gluten (15.2%, flour weight) and using longer proving times (75 min) with higher proving (40°C) and baking (210°C) temperatures.

The grain amaranth has also been used in straight dough breadmaking (Mlakar *et al.,* 2008a; Mlakar *et al.,* 2008b). The addition of up to 20% increased water absorption, resistance to extension but decreased the extensibility and viscosity of the dough. This effect was attributed to gluten dilution, as amaranth lacks gluten forming proteins. The positive effect on crumb colour was observed in 10% amaranth substitution as well as acceptable sensory scores and comparable loaf volume to control bread.

Teff grain has also been incorporated into leavened bread (Ben-Fayed *et al.,* 2008; Mohammed *et al.,* 2009). Teff breads, containing 10, 20 and 30% Teff had significantly lower specific volumes and firmer crumb compared to white wheat bread (Ben-Fayed *et al.,* 2008). Furthermore, only 10% Teff bread had an acceptable flavour and was comparable to wheat bread, whereas breads containing higher levels of Teff were judged significantly less acceptable. Similar results were found by Mohammed *et al*. (2009). In their study, all Teff breads (incorporation at 5, 10, 15 and 20% flour blends) had significantly lower sensory acceptability when compared to white wheat bread.

Overall, research indicates that acceptable breads could be produced with different cereal flour supplementation; however, difficulties in dough handling properties, texture, sensory and keeping qualities are encountered with higher levels of gluten-lacking grains.

1.4 Ingredients and processes used in the improvement of leavened breads

1.4.1 Enzymes

Enzymes are biological catalysts and directly or indirectly affect the structure and physicochemical properties of flour components and therefore, play a role in the functionality of flour and quality of bread (Cauvain and Young 1998; Goesaert *et al.,* 2006). Enzymes are generally recognised as safe (GRAS) (Olempska-Beer *et al.,* 2006) because they are inactivated during baking, hence, are not required to be labelled. Enzymes used in breadmaking can be divided into several groups according to their substrate: gluten cross-linking enzymes, oxidative enzymes and polysaccharide degrading enzymes.

1.4.1.1 Xylanase

Endo-1,4-ß-xylanase is a polysaccharide degrading enzyme, which hydrolyses arabinoxylan (AX), an important component in plant cell walls, leading to a decrease in

polymerisation (Goesaert *et al.,* 2006). This leads to release of free sugars such as pentoses, which are used during fermentation by the yeasts, resulting in a higher rate of CO_2 evolution. Indeed, researchers investigating the effects of xylanase on dough expansion during fermentation concluded that the volume of dough supplemented with the xylanase was remarkably higher compared to a control (185% *v* 144%) (Shah *et al.,* 2006). Arabinoxylans consist of water-unextractable (WU-AX) and water-extractable fractions (WE-AX) in cereals. WU-AX have a strong water holding capacity, which has detrimental effects on dough properties by disrupting of the formation of the protein films during dough mixing (Wang *et al.,* 2003). Xylanases solubilise WU-AX, which decreases water-holding capacity, and degrade WE-AX, leading to a decrease in viscosity (Gruppen *et al.,* 1993). This redistribution of water from arabinoxylan fraction in the flour to the starch and gluten phases of dough by the action of xylanase is favourable in the formation of the gluten network and promoting starch gelatinisation (Ingelbrecht *et al.,* 2000). Shah *et al.* (2006) showed that the addition of xylanase to wholemeal dough resulted in a decrease in water absorption from 72% to 64% as a result of solubilisation of WU-AX by the enzyme. Favourable effects on loaf volume have been reported as well. Findings show that specific loaf volume increased by 56% and density decreased by 36% in xylanase supplemented breads (Shah *et al.,* 2006). Another study reported positive effects on bread specific loaf volume (+35%) and crumb firmness (-40%) (Jiang *et al.,* 2008). Therefore, xylanase can have a positive effect on dough stability, loaf volume, crumb cellular structure and shelf-life.

Although an optimum dosage of xylanase can improve dough and the final product's properties, higher dosages can have an adverse effect. Overdose can cause excessive degradation of arabinoxylans to pentosans, weakening in gluten and poorer machinability (due to excessive 'softening' effect), leading to a stickier dough, and a gummy and chewy bread texture (Collar *et al.,* 2000).

1.4.1.2 α-Amylase

α-Amylase is an amylolytic enzyme which hydrolyses α-(1,4)- and α-(1,6)- linkages in the starch polymers creating short-chain unbranched dextrins (Cauvain and Young 1998; Goesaert *et al.,* 2006). Fermentable sugars can be used by the yeast for CO_2 production during proving. Several studies looked at the effects of fungal α-amylase on wholegrain wheat breads. The authors suggested that gas generation and retention during fermentation significantly increased with the addition of this enzyme (Kim *et al.,* 2006; Sanz Penella *et al.,* 2008).

Another important effect of α-amylase is the ability to delay starch gelatinisation during baking due to increased levels of dextrins. This reduces viscosity of the gelling starch and allows a greater time for the dough to rise in the oven before crumb setting, hence, increasing final loaf volume (Cauvain and Young 1998). A study investigating the effects of α-amylase confirmed that doughs containing higher α-amylase levels continued to expand further during baking, producing higher loaf volumes (Cauvain and Chamberlain 1988).

An antistaling effect of α-amylase was suggested to be due to its ability to hydrolyse branched amylopectin into smaller components, therefore, weakening starch networks and limiting amylopectin crystallisation (Cauvain and Young 1998; Leon *et al.,* 2002; Goesaert *et al.,* 2009). Higher dextrin content due to α-amylase action has a positive effect on decreasing crumb firmness during bread storage (Leon *et al.,* 2002) by interference in amylopectin crystallisation.

Fungal α-amylase is preferred to cereal or bacterial α-amylase because it has a lower thermal inactivation temperature. This means that the activity of the enzyme is destroyed at an earlier stage of baking. This reduces starch hydrolysis, hence, excessive formation of dextrins, which can cause stickiness and gumminess of the crumb (Cauvain and Young 1998).

1.4.1.3 Glucose oxidase

Glucose oxidase (GO) catalyses oxidation of glucose to gluconic acid and hydrogen peroxide (Bankar *et al.,* 2009). The strengthening effect of GO was explained by the formation of disulphide and non-disulphide bonds between gluten storage proteins (gliadins and glutenins) (Haarasilta and Pullinen 1992; Rasiah *et al.,* 2005; Bonet *et al.,* 2006) and gelation of water-soluble pentosans by the action of hydrogen peroxide (Vemulapalli and Hoseney 1998; Vemulapalli *et al.,* 1998). GO addition significantly increased the tenacity, elasticity and viscosity and reduced extensibility of dough (Vemulapalli *et al.,* 1998; Rosell *et al.,* 2003). Doughs supplemented with GO were strong and dry, which was attributed to limited water mobility due to gelation of pentosans (Vemulapalli *et al.,* 1998). As a result of such changes in dough characteristics, GO was shown to have positive effects on bread quality, in terms of improved specific volume (3.67 *v* 3.80ml/g) (Caballero *et al.,* 2007b), more elastic and cohesive crumb texture (Caballero *et al.,* 2007b), reduced crumb firmness (726N *v* 311N) and produced better shaped loaves (Bonet *et al.,* 2006). Furthermore, another study demonstrated that GO acted on glutenin subunits of damaged wheat by forming crosslinks between proteins, rebuilding the gluten network (Bonet *et al.,* 2007). However, high doses of GO (>0.005%, *w/w*)

resulted in over-oxidation and excessive crosslinking of the gluten network, causing decreased dough extensibility (Primo-Martin *et al.,* 2005) and negative effects on crumb hardness and specific volume (Bonet *et al.,* 2006).

1.4.1.4 Lipase

Lipase hydrolyses ester bonds in triglycerides yielding mono- and diglycerides and free fatty acids (Reetz 2002). Several mechanisms of action were proposed to explain the beneficial effect of lipase on dough. Non-polar flour lipids become bound when water is added to flour (Goesaert *et al.,* 2006). This has a negative effect on breadmaking, particularly on loaf volume; hence, hydrolysis of these compounds has a softening effect on bread crumb. Furthermore, hydrolysis increases the amount of emulsifying compounds, such as mono- and diglycerides, in the dough, which have a positive effect in stabilising gas cells (Goesaert *et al.,* 2006). An indirect effect of lipase action was postulated to be the release of polyunsaturated fatty acids, which are oxidised by the flour lipoxygenase enzyme, and as a result produces a whiter bread crumb colour (Olesen *et al.,* 1994; Castello *et al.,* 1999).

An antistaling effect of lipase was suggested to be due to increased levels of monoglycerides by hydrolysis and the formation of amylose-lipid complexes (Johnson and Welch 1968).

Experimental research data indicates that the addition of lipase resulted in advantageous effects on dough machinability, volume and crumb firmness (Olesen *et al.,* 1994). Lipase supplemented doughs had a better tolerance to longer fermentation times by improved stability of the gluten network and produced breads with finer and more homogeneous crumb pore structure (Poulsen *et al.,* 1998).

1.4.1.5 Enzyme combinations

Enzymes with different functional activities can induce synergistic effects on dough properties and quality parameters of bread. Enzyme combination effects on breadmaking performance have been of interest to many researchers (Martinez-Anaya and Jimenez 1997; Collar *et al.,* 2000; Leon *et al.,* 2002; Primo-Martin and Martinez-Anaya 2003; Primo-Martin *et al.,* 2003; Primo-Martin *et al.,* 2005; Caballero *et al.,* 2007a; Caballero *et al.,* 2007b; Stojceska and Ainsworth 2008).

The synergistic effect of α-amylase and endo-xylanase (A+X) was found to be due to the reduced viscosity of the gelling starch (as a result of the xylanase action) and an increased production of dextrins (as a result of the amylase action), which results in a better gas generation by the yeast (Martinez-Anaya and Jimenez 1997). The doughs treated with

A+X combination have decreased resilience, but increased extensibility, which lowers the dough consistency (Collar *et al.,* 2000). As a result, the produced breads were reported to have increased loaf volumes by 25-41% and reduced crumb firmness by 33-60% after 3 days storage (Martinez-Anaya and Jimenez 1997).

Xylanase and glucose oxidase (X+GO) is another combination that is frequently used in order to improve breadmaking performance of flours. The possible synergistic effect of X+GO has been proposed to be by diminishing negative effects of individual enzymes; GO promotes gelation of water soluble arabinoxylans which in excessive amounts limits water holding capacity, and high-molecular weight arabinoxylan crosslinks, which makes the dough dry and stiff and has a negative effect on bread quality (Primo-Martin and Martinez-Anaya 2003). Xylanase interferes with this by generating smaller ferulic acid containing arabinoxylan fragments, hence, diminishing negative aspect of GO supplementation (Primo-Martin *et al.,* 2005). The addition of GO also corrects the decrease in dough consistency and dough machinability, which occurs as a result of the action of xylanase (Primo-Martin and Martinez-Anaya 2003). Primo-Martin *et al.* (2003) demonstrated that supplementation of X with GO resulted in dough with greater extensibility ($P<0.01$) and lower resistance to extension ($P<0.05$) compared to the control dough. Furthermore, enzymatic addition significantly improved gluten quality index (GI) (91% *v* 99%, $P<0.001$) when compared to a control dough. The favourable effect of X+GO combination was also observed in terms of specific volume (4.48ml/g *v* 5.38ml/g) and bread crumb firmness (20N *v* 14N) (Primo-Martin and Martinez-Anaya 2003; Caballero *et al.,* 2007a).

Amylolytic enzymes were shown to have a synergistic effect in combination with gluten network strengthening enzymes, such as transglutaminase and glucose oxidase (Caballero *et al.,* 2007b; Caballero *et al.,* 2007a). Amylase provides increased gas production, whilst glucose oxidase has gluten strengthening effects, hence, combination of amylase and glucose oxidase (A+GO) can improve functional properties of dough, which lacks these qualities.

Combination of α-amylase and lipase (A+L) was shown to have a synergistic effect through the formation of a more thermostable amylose-lipid complex (Leon *et al.,* 2002). This results in a reduction of the starch available to retrograde and allows amylose-surfactant complexation, which contributes to an antistaling effect. A+L supplementation resulted in slower crumb firming, inhibition of starch retrogradation, higher specific volume (5.41 ml/g *v* 6.12 ml/g) and better sensory acceptability in fresh and 48 h aged bread (Leon *et al.,* 2002).

1.4.2 Sourdough fermentation

Sourdough is a dough leavened by a starter, which usually contains bacteria and yeast (Gisslen 2005). The sourdough starter can be a firm dough or a liquid dough and is acidic as a result of fermentation by microorganisms (Decock and Cappelle 2005). As a result the sourdough baking process improves the texture and flavour of bread as well as mineral bioavailability and starch digestibility (Figure 1.3).

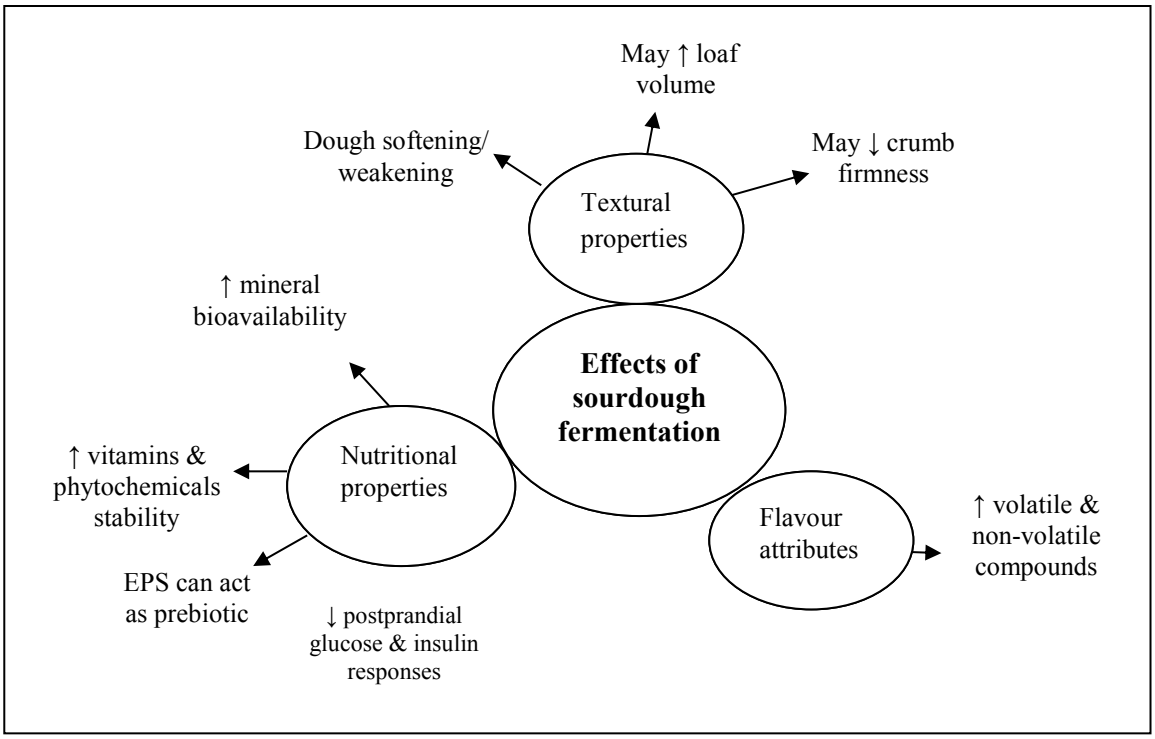

Figure 1.3 Effects of sourdough fermentation on bread properties

Sourdoughs are classified into several types (De Vuyst and Neysens 2005; Decock and Cappelle 2005; Corsetti and Settanni 2007). Type I sourdough is a traditional sourdough, where part of a previous fermentation is used as a starter or a starter is prepared by continuous refreshment in order to maintain the microorganisms activity. Type II sourdough is characterised by a liquid starter with long fermentation periods. Type III sourdough is a dried preparation, which is preferred by industrial bakeries as it provides less variation in the end product.

Sourdough starters vary in their consistency. It can be either prepared as firm dough (sponge) or liquid. This will influence the flavour profile (Decock and Cappelle 2005). The sponge sourdough will produce more acetic acid and less lactic acid, while liquid sourdough results in higher levels of lactic acid. Acetic acid is responsible for shorter and harder gluten structure (Lorenz 1981), sharp acidic taste and has an antimicrobial and antimould effect (Rosenquist and Hansen 1998), whereas lactic acid accounts for more

elastic gluten structure (Lorenz 1981) and slow acting and mild acidic taste in bread (Decock and Cappelle 2005).

1.4.2.1 Cultures

The main microflora in sourdough is represented by lactic acid bacteria (LAB) and yeasts, where yeast/LAB ratio is usually 1:100 (Corsetti and Settanni 2007). LAB has two main families of bacteria: heterofermentive and homofermentive and is often called 'aromatic' microflora (Arendt *et al.,* 2007). LAB utilises soluble carbohydrates (glucose, fructose and maltose) and produces lactic acid, CO_2, acetic acid and ethanol (Robert *et al.,* 2006). Both heterofermentive and homofermentive LAB have an influence on bread texture and crumb structure, however, only acidification by heterofermentive LAB leads to formation of characteristic sensory attributes of sourdough (Spicher and Brummer 1995). So far around 50 different species of *Lactobacilli* were found in sourdough (Hammes *et al.,* 2005), the most frequently occurring were *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum*; and more than 25 species of yeasts, especially in *Saccharomyces* and *Candida* species (Arendt *et al.,* 2007). Interactions between LAB and yeast have been shown to be important in better leavening and CO_2 production, flavour quality and inhibition of spoilage bacterial flora (Gobbetti 1998).

1.4.2.2 Textural properties

The pH of the fermented sourdough ranges from 3.5 to 4.3 and mainly depends on the type of flour and ash content (Arendt *et al.,* 2007). Acidification of dough will affect structure-forming components, including gluten, starch and arabinoxylans. At the pH of 4 and below, acidity causes increased swelling and softness of the gluten together with increased elasticity of gluten network (Schober *et al.,* 2003; Moore *et al.,* 2007). Solubility of gluten increases to 85% as a result of disentanglement of gluten protein network which has a softening and emulsifying effect on dough (Takeda *et al.,* 2001). As the fermentation time increases, a decrease in dough elasticity and viscosity is observed during sourdough acidification (Clarke *et al.,* 2004). It was suggested by Clarke *et al.* (2004) that large protein aggregates, which are responsible for dough structure, are broken down into smaller aggregates during sourdough fermentation, thus resulting in a softer and less elastic dough structure. Acidification has an influence on other rheological properties of dough as well. It results in slightly shorter dough development times (2.3min *v* 1.5-2.0), significantly firmer doughs at the beginning of mixing phase (0.5min *v* 3.0min) with less stability (15.5min *v* 2.0min) and greater sensitivity to overmixing after longer mixing times (9 min

and over) (Wehrle *et al.,* 1997). However, addition of salt (2% flour weight) can strengthen the dough structure and reduce the effects of overmixing (Wehrle *et al.,* 1997).

The sourdough process can have a positive, negative or no effect on loaf volume and texture. The positive influence was suggested to be due to the ability of LAB to increase metabolic activity of yeast, hence, increasing production CO_2 and enhanced gluten ability to retain CO_2 due to acidification (Gobbetti *et al.,* 1995a; Gobbetti 1998). The loaf volume was increased significantly when 5-20% of sourdough was added to the wheat flour dough (Hansen and Hansen 1996). A similar effect was obtained in 10-25% sourdough wheat breads started with *Lactobacillus plantarum* and *Lactobacillus brevis* (Esteve *et al.,* 1994). Sourdough fermentation has also significantly improved specific volume (3.7 ml/g *v* 4.2ml/g; $P<0.05$) and crumb firmness (320g *v* 205g; $P<0.05$) of white wheat with added bran bread (Katina *et al.,* 2006b). Improved loaf volume was also reported in another study, where the sourdough process improved specific volume by 0.2-0.5 ml/g and reduced crumb firmness in wholemeal rye and wheat breads using *Lactobacillus brevis* culture (Katina *et al.,* 2006a). However, this trend was not observed in gluten-free breads, as sourdough did not improve loaf volume and only slightly improved crumb firmness, which was suggested to be due to the lack of a gluten network (Moore *et al.,* 2007). The increase in specific volume of sourdough bread also depends on the level of sourdough starter used in the final dough. Specific volume was significantly higher in 20% sourdough wheat bread (3.15 ml/g *v* 3.4 ml/g), however, 40% sourdough bread did not show any improvement (3.15 ml/g) (Crowley *et al.,* 2002). In the same study, crumb structure and crumb firmness of bread containing 20% sourdough were similar to textural properties of standard bread, while 40% sourdough bread had a denser crumb structure. Corsetti *et al*. (2000) reported a significant increase in loaf volume of sourdough bread when compared to straight dough bread (533 ml *v* 764 ml). However, the same study failed to show improvements in initial and storage crumb firmness (11.0 N sourdough bread *v* 9.7 N control bread). Sourdough addition did not change the loaf volume or density in wheat bread when *Lactobacillus plantarum* was used (Robert *et al.,* 2006).

In summary, the improvements in texture properties of sourdough breads mainly depend on the particular LAB strain characteristics and cereal flour properties.

1.4.2.3 Flavour

The main compounds responsible for sourdough bread flavour are organic acids, alcohols, esters and carbonyls (Corsetti and Settanni 2007). The total amount of flavour compounds can increase up to 100 fold during sourdough fermentation (Katina *et al.,* 2004). Sourdough bread has higher levels of these flavour compounds compared to chemically

acidified bread and has been shown to have a higher sensory acceptability in taste panels as well as more favourable textural properties (Hansen and Hansen 1994b; Katina *et al.,* 2006b). The pleasant aromatic flavour of wheat sourdough was associated with higher levels of flavour compounds produced by LAB and yeast, including acetic, methylbutanol, methylpropanoic acids and phenylethanol (Hansen and Hansen 1996).

Organic acids such as acetic and lactic acids are non-volatile compounds, which acidify the dough, and contribute to the flavour (Salim ur *et al.,* 2006). Production of acetic and lactic acids can be influenced by the starter culture and the type of flour. Research shows that sourdoughs from low-grade, wholemeal and higher ash content (0.55-1%) flours had higher levels of these organic acids compared to high extraction rate flours (Hansen and Hansen 1994a; Gobbetti *et al.,* 1995b; Katina *et al.,* 2004).

Volatile compounds, including methylpropanol, methylbutanol, other iso-alcohols, aldehydes, diacetyls and carbonyls are produced by the action of LAB. However, higher levels of these compounds are produced when a combination of LAB, yeast strains, enzymes and substrates are used during sourdough fermentation (Salim ur *et al.,* 2006). In fact, addition of yeast to sourdough fermentation results in the development of a larger amount of volatile compounds, including alcohols, esters and carbonyls (Damiani *et al.,* 1996). Furthermore, combination of yeast with *Lactobacillus plantarum* showed a significant improvement in flavour when compared to sourdough made only with *Lactobacillus plantarum* (Hansen and Hansen 1996). Addition of the yeast strain *Saccharomyces cerevisiae* to LAB starter also improved formation of volatile compounds without causing excessive acidity in wheat sourdoughs (Katina *et al.,* 2004).

Overall, starter combinations of LAB and yeast with optimum processing conditions results in a final product that has a more complete flavour compound profile (Gobbetti *et al.,* 1995b).

1.4.2.4 Mineral bioavailability and stability of vitamins

Wholemeal grain products are a good source of minerals, including iron, calcium, potassium, magnesium, zinc and phosphorus. However, the bioavailability and solubility of these minerals can vary due to high levels of phytate, which is naturally present in grain bran (Harland and Morris 1995).

Bioavailability of minerals can be influenced by sourdough bread technology (Poutanen *et al.,* 2009). It has been showed that phytases naturally occurring in grains have an optimum pH of 5 (Turk *et al.,* 1996). Even a moderate drop in pH up to 5.5 using sourdough fermentation resulted in 70% reduction of initial phytate content in flour compared to 40% reduction in control dough (Leenhardt *et al.,* 2005). Turk *et al*. (1996) showed 96% of the

initial phytate hydrolysis with addition of lactic acid to the dough compared to only 64% degradation in a control dough. Furthermore, baker's yeast was shown to express phytase activity and degrade phytate (Turk *et al.,* 2000). Lactic acid fermentation of cereal flours resulted in a significant reduction of phytate, which was independent of the LAB strain used (Reale *et al.,* 2007). However, it should be noted, that the positive effect of sourdough fermentation is provided by favourable conditions for the endogenous phytase activity as LAB do not act on phytate breakdown directly. Sourdough fermentation was also found to be effective for solubilising minerals, hence, improving bioavailability of minerals, including iron, calcium, magnesium and zinc in whole wheat flours, but less effective with bran (Turk *et al.,* 1996; Lioger *et al.,* 2007).

Cereal foods are also an important source of vitamins, especially thiamine, vitamin E and folate. Research indicates that fermentation can affect overall retention of vitamins and phytochemicals in the baking process (Poutanen *et al.,* 2009). Sourdough fermentation of rye flour doubled the levels of folate and phenolic compounds, but decreased vitamin E content, which was suggested to be due to oxidation (Liukkonen *et al.,* 2003). After baking, sourdough rye bread contained the highest antioxidant capacity and higher levels of folate and phenolic compounds compared to control bread (Liukkonen *et al.,* 2003). In another study, wheat sourdough bread with added yeast had the highest level of folate (Kariluoto *et al.,* 2004). The content of thiamine decreased when using conventional breadmaking technology, however, it was maintained by increasing fermentation time (Batifoulier *et al.,* 2005). Furthermore, the same study showed that riboflavin content was enriched by 30% when yeast and sourdough fermentation was used.

1.4.2.5 Starch digestibility

Dietary carbohydrates are the major source of plasma glucose. In the Western diets, the majority of carbohydrate comes from rapidly digestible starch in foods like baked products, breakfast cereals and snacks (Poutanen *et al.,* 2009). Hence, these products have high glycaemic responses. Starchy foods, especially white bread, have high glycaemic index (GI) as a result of their usually porous structure and high level of starch gelatinisation (Fardet *et al.,* 2006). As a result these processed starches are more rapidly digested when compared to native starches.

Several mechanisms were proposed for the favourable effect of sourdough technology to reduce the glycaemic response. The formation of acids during sourdough processes was shown to reduce starch hydrolysis, hence, to lower the rate of starch digestion in bread (Liljeberg *et al.,* 1995). Postprandial glycaemic response was also significantly lower (P<0.001) after ingestion of sourdough wheat bread *in vivo* compared to straight dough

wheat bread (Scazzina *et al.,* 2009). Also, the consumption of sourdough bread or bread with lactic acid reduced blood glucose increments by 26% (Liljeberg and Bjorck 1996) and insulin response by 45% in healthy subjects (Liljeberg *et al.,* 1995). It has been also suggested that organic acids, especially, acetic and propionic acid may prolong gastric emptying (Liljeberg and Bjorck 1996) and hence, result in prolonged satiety. In addition, crumb structural differences (more rigid and less porous structure) in rye sourdough compared to straight dough wheat bread was reported to produce significantly lower insulin responses (22151pmol min/L *v* 16389pmol min/L; $P<0.05$) in healthy female subjects, when compared to a standard bread portion that provided 50g available carbohydrate as a part of breakfast (Juntunen *et al.,* 2003).

1.4.2.6 Prebiotic compounds

Prebiotics can significantly contribute in maintaining the integrity of gut microflora (Gibson and Roberfroid 1995).

Some sourdough starter cultures are able to synthesise exopolysaccharides (EPS), compounds such as glucan, fructans and gluco- and fructo-oligosaccharides (Poutanen *et al.,* 2009), which have potential in promoting gut health as a prebiotic food component (Ganzle *et al.,* 2009). Research indicates positive sourdough effects on gut microflora (Tieking and Ganzle 2005). Levain type EPS produced by *Lactobacillus sanfranciscensis* showed an enrichment of *Bifidobacterium* species in the gut (Dal Bello *et al.,* 2001; Korakli *et al.,* 2002). Other starter cultures, such as *Lactobacillus reuteri*, *Lactobacillus pontis*, and *Lactobacillus frumenti*, were shown to produce EPS at levels ranging from 0.5 to 2 g/kg of flour during sourdough fermentation (Tieking *et al.,* 2003). The optimum fermentation conditions for synthesis of EPS by *Lactobacilli reuteri* and *L.sanfranciscensis* were pH value of 4.7, liquid sourdough medium (mass dough/mass flour = 5.5) and 10% sucrose solution (Kaditzky and Vogel 2008). Research in this area is still at its very early stages and more investigation of the potential effects on gut function is required.

1.4.2.7 Synergistic activity of sourdough and enzymes

The application of combination of sourdough and various enzymes is commonly used in the breadmaking industry to improve both textural and sensory attributes of baked products. Many interactions can be expected as enzymes influence the growth of the microflora, whilst the lactic acid bacteria affect the environmental conditions of different enzymes by altering pH and providing additional substrate.

The combined effects of sourdough and enzymes have been studied by several researchers (Martinez-Anaya *et al.,* 1998; Andreu *et al.,* 1999; Di Cagno *et al.,* 2003; Katina *et al.,*

2006b). A significant increase in loaf volume was observed in sourdough bread with addition of fungal α-amylase (910ml *v* 833ml standard sourdough) (Corsetti *et al.,* 2000). The shelf-life of this bread was also improved as both initial firmness (6.3 N *v* 11.0 N) and crumb firmness after 120 hours of storage (21.1 N *v* 24.9 N) were significantly lower than the control sourdough. Sourdough fermented by *Lactobacillus hildegardii* and xylanase treatments also improved loaf volume, however, only when water-insoluble pensosans were added (Corsetti *et al.,* 2000). This suggests that a positive effect can be achieved if flour contains higher levels of water-insoluble pentosans. However, water-insoluble pentosans normally have a detrimental effect on loaf volume when using straight breadmaking processes.

Positive effects of a combination of *Lactobacillus brevis* and enzyme combination (α-amylase, xylanase and lipase) were also observed in bran supplemented bread (Katina *et al.,* 2006b). In the study, sourdough and enzyme mixture supplemented breads yielded significantly higher loaf volumes compared to a reference bran bread (4.5ml/g *v* 3.7ml/g) and did not differ from white wheat bread (4.5ml/g). Authors also reported positive effects on the shelf-life and microstructure of sourdough and enzyme treated bread. The crumb softness was significantly improved during a 6-day storage period and was comparable to that of white wheat bread. Microstructure investigations revealed changes in the crumb cellular structure of sourdough and enzyme mixture bran bread. Compared to the control, the cell walls in the bran particles were much more swollen. Furthermore, starch granules leached more amylose and formed a more evenly distributed protein network around the granules. The more uniform structure of sourdough and enzyme mixture bread may explain improvements in crumb firmness. Authors reported a lower amount of starch birefringence in this bread, which shows a positive effect of sourdough fermentation on the gelatinisation of starch granules. It is also reported that enzymes have antistaling effect due to hydrolysis of starch side chains.

Interactions between specific LAB strains and enzymes (GO, lipase, xylanase and α-amylase) were studied in sourdoughs (Di Cagno *et al.,* 2003). The authors emphasised the importance of LAB selection based on the type of enzyme used in order to improve rheological and texture properties. Positive synergistic effects of sourdough and enzyme were observed in terms of increased production of lactic (29.7-36.8mmol/l *v* 23.3mmol/l) and acetic acids (13.8-28.2mmol/l *v* 8.4mmol/l) and promoted greater stability and softening during fermentation. This suggests that appropriate selection of LAB strain and enzyme may have a positive effect on baking performance.

Similar positive findings were reported by Martinez-Anaya *et al.* (1998). Combinations of enzyme and starter cultures significantly (P<0.01) improved loaf volume and shape, decreased crumb firmness, gumminess, chewiness. Although all starter culture combinations (*Lactobacillus sanfranciscensis*, *L. brevis*, *L.fructivorans*, *L.plantarium* and *Candida milleri*) with α-amylase, xylanase and lipase combinations improved bread quality, the greatest loaf volume and best shape was yielded by *L.sanfranciscensis* and *C.milleri* in combination with α-amylase, xylanase and lipase using strong wheat flour. Bread made from lower grade flour yielded slightly lower volume. This may be explained by the lower level of functional gluten proteins and higher levels of bran particles in lower grade flour. However, the overall improvement was greater in lower grade wheat flour than strong wheat flour. This suggests that breads made from flours with lower gluten content and higher fibre and mineral content would benefit more from the addition of enzyme and sourdough combinations than those made from strong white flours.

In summary, research indicates that combination of enzymes and sourdough starters can be a way of improving bread nutritional and quality properties, especially if lower grade flour is used.

1.5 Teff grain

1.5.1 Historical view and origin of *Eragrostis tef*

The cereal Teff (*Eragrostis tef*) is an ancient grain. It is a C_4, self-pollinated, annual cereal. Teff originated in Ethiopia (Vavilov 1951), where it is a significant crop. Teff has a close morphological resemblance to wild species of *Eragrostis*, mainly *E.pilosa*, which supports the theory that Teff originated from these wild species (Bai *et al.,* 2000).

The name 'Teff' is derived from the local language Amharic and means 'lost' (Vinning and McMahon 2006), which refer to exceptionally small size of this grain.

Teff is a highly adaptive crop. It can be grown up to 3,000 metres above sea levels, it also tolerates drought and water logging better than most other cereal crops (Tesfaye 2000). Teff grows well in various soil types- from sandy to heavy clay soils, and under mildly acidic to slightly alkaline conditions (Tesfaye 2000). Although Teff can be grown under diverse agro-ecological conditions, it is highly labour intensive and expensive, due to the small size of Teff seeds and relatively low yielding, whereas, other world staple foods are rarely expensive.

Recently, 13 Teff grain varieties were released by the Ethiopian Teff improvement program by the Debre Zeit Agricultural Research Centre for production (Tefera *et al.,* 2001). Tef grain DZ-01-196 is the most preferred by the farmers for market purposes (Belay *et al.,* 2006) due to its very white colour, whereas brown varieties of Teff are mainly selected for home use. This is due to the fact that white varieties of Teff are more expensive, however, considered to be superior for *injera* bread making.

1.5.2 Present usage Teff around the world

Teff is an important staple cereal in Ethiopia and Eritrea. It is cultivated as a major cereal in Ethiopia and represents 19% of the total cereal production, with the largest share area (23.42%, about 2.6 million hectares) under cereal cultivation (Central Statistical Agency 2008). Furthermore, Teff production and yield has increased over 22% and 15%, respectively, in the last 2 years, the greatest change compared to all other cereal cultivars (Table 1.2).

Table 1.2 Comparison of crop agriculture in Ethiopia for 2006/07 and 2007/08 (Central Statistical Agency 2008)

Crop	Area (hectare)			Production (quintal)		
	2007/08	2006/07	% Change	2007/08	2006/07	% Change
Cereals	8,730,001	8,471,920	3.42	137,169,907	128,797,926	6.50
Teff	2,565,155	2,404,674	3.05	29,929,235	24,377,495	22.77
Barley	984,943	1,019,314	6.67	13,548,071	13,521,480	0.20
Wheat	1,424,719	1,473,917	-3.37	24,630,639	24,630,639	-6.03
Maize	1,767,389	1,694,522	-3.34	37,764,397	37,764,397	-0.71
Sorghum	1,533,537	1,464,318	4.30	26,591,292	23,160,409	14.81
Finger millet	399,268	374,072	4.73	5,379,915	4,844,089	11.06
Oats	30,556	32,798	6.74	365,858	362,432	0.95

Foods from grain Teff are the main staples for the majority of Ethiopians. Teff flour is widely used for making a fermented flat bread *injera*, traditional alcoholic beer *tella*, local spirit *katikalla*, sweet dry unleavened bread *kitta*, gruel *mu* and porridge (National Research Council 1996).

Although the main Teff cultivation and production remains in Ethiopia and Eritrea, recently some Western countries began to use Teff as well. Countries like USA, Canada, Australia, The Netherlands and South Africa and Kenya are using Teff as forage crop, thickening agent for soups, gravies and stews (Bultosa and Taylor 2004b). Food manufacturers are also exploring the possibility of using Teff as a novel food and ingredient. Products, including Teff flour, gluten-free beer and energy cereal bars, have been introduced to the Netherlands market (Soil & Crop Improvement BV 2003). Teff also found its place as a health food product in the USA. Teff products such as Teff breakfast cereals, Teff waffles and Teff bread are appearing in the USA (Tesfaye 2000).

1.5.3 Teff *injera*

Injera is a pancake-like fermented flat bread usually prepared from Teff flour. It is the traditional staple food of Ethiopia and accompanies most of the meals (Urga *et al.,* 1997). The typical injera is described as thin, round, soft, flexible, porous with honeycomb-like 'eyes' and sour-tasting flat bread (Stewart and Getachew 1962). Although it can be prepared from Teff, barley, sorghum, maize or combination of these, Teff flour is much more preferred than any other cereals (Stewart and Getachew 1962; Gifawesen and Bisrat 1982; Zegeye 1997).

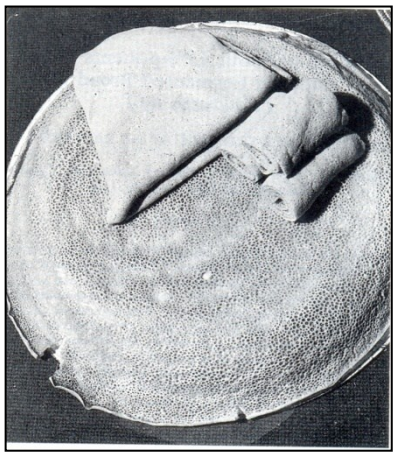

Figure 1.4 Ethiopian Teff injera (Parker et al., 1989)

Injera is made from a batter-like dough which is pre-fermented for 2-3 days (Figure 1.5). Fermentation is usually initiated spontaneously by addition of water to Teff flour, allowing the naturally occurring microorganisms to grow (Gashe 1985). Primary fermentation can also be initiated by addition of the starter, *irsho*, which is a small amount of batter saved from previous dough (Parker *et al.,* 1989). After the initial stage of fermentation, yellow liquid settles over the dough, which is discarded and used as *irsho* for the next batch.

During the second stage of fermentation, some of the dough is diluted, boiled ('*absit*') and returned to fermented paste. At this stage, during boiling some starch granules become gelatinised, swollen and mishapen and attached to ungelatinised starch from the primary fermentation dough (Parker *et al.,* 1989). During fermentation stages, the most predominant fermenting organisms are yeast (*Saccharomyces* species) (Gifawesen and Bisrat 1982) and lactic acid bacteria (*Lactobacillus* species) (Gashe 1985). These microorganims result in pH fall to 4.0, gas production and dough rising and are responsible for desired final product acidity and flavour (Umeta and Faulks 1988).

The fermented Teff dough has a consistency of batter. It is poured onto the hot oiled surface of the clay griddle, called *metad*, and baked covered with a lid to retain the steam.

Microscopic evaluation of a baked *injera* structure show that Teff flour starch is fully gelatinized into a matrix, with embedded fragments of bran layers, protein bodies, outer layers of endosperm, microorganisms and gas bubbles (Umeta and Faulks 1988; Parker *et al.,* 1989). The soft and spongy structure of Teff *injera* is important for its keeping qualities and acceptance. Teff injera is preferred because of the flavor, freshness and better keeping qualities than other injeras made from sorghum or maize (Zegeye 1997).

Physical and nutritional properties of Teff grains are comparable to those of the other major world cereals. Furthermore, the well balanced nutrient composition of Teff grain and its physicochemical characteristics indicate that it has the potential to be used in food applications where an improved nutritional profile is desired.

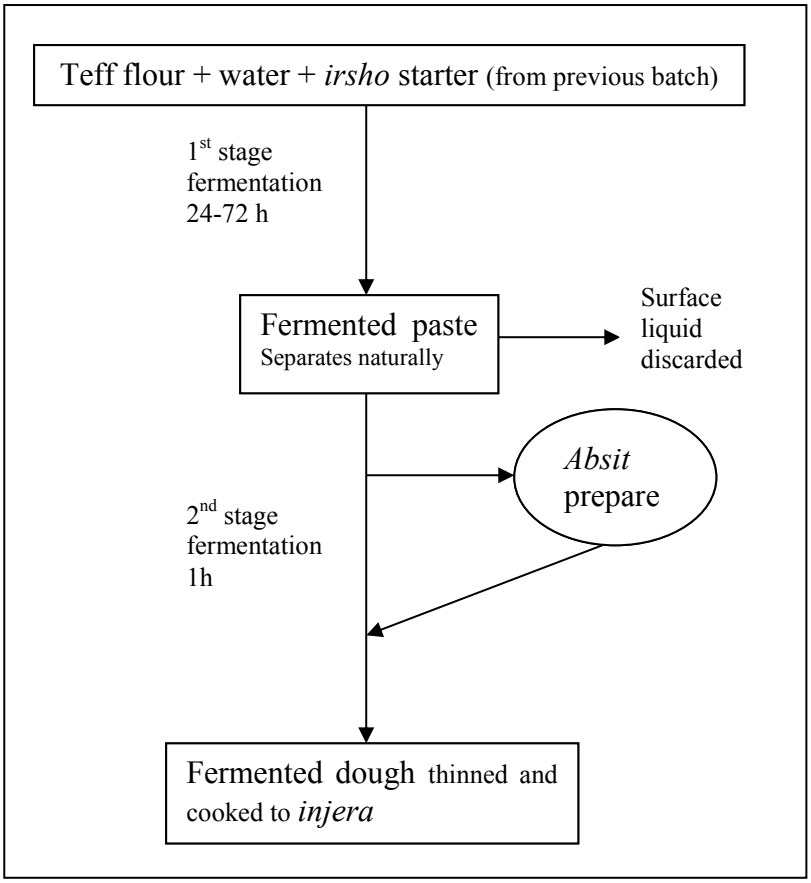

Figure 1.5 Traditional method of making Teff injera (adapted from Parker et al., 1989)

1.5.4 Teff properties

1.5.4.1 Structure of Teff grain

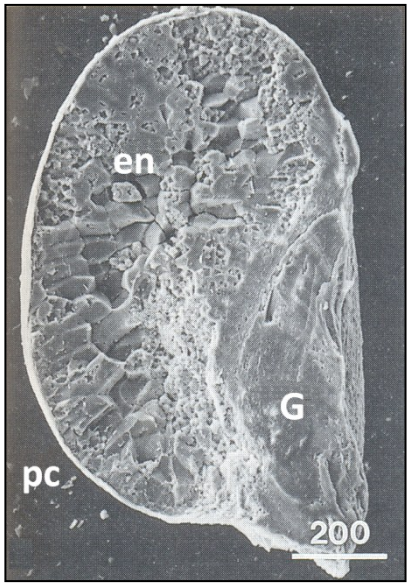

Figure 1.6 Cross-sectional view of Teff grain showing endosperm (en), germ (G) and pericarp (pc) X200 (Parker *et al.*, 1989)

Teff seeds are oval in shape. The cross-sectional view of the grains shows that the germ is large in proportion to the rest of the kernel (Parker *et al.,* 1989; Umeta and Parker 1996; McDonough and Rooney 2000) (Figure 1.6) compared to other cereal grains. The thin outer layer, pericarp, forms a bran, which protects the seed. In the red and brown varieties of Teff, the inner surface of bran has pigmented material, rich in tannins and polyphenols similar to those in sorghum and finger millet (Umeta and Parker 1996; McDonough and Rooney 2000). The endosperm represents the largest proportion of the Teff seed, with non-uniformly distributed protein bodies mainly in the outer layer; and starch granules found in the central part of endosperm (McDonough and Rooney 1985). The starch granules found in Teff are smooth and polygonal in shape, 2-6 μm in diameter and are the largest proportion of the carbohydrate fraction in Teff (Bultosa *et al.,* 2002). The granule size is comparable to rice (2-10 μm) (Juliano 1992), oat (2-14 μm) (Zheng and Sosulski 1997), small millet (0.8-10 μm) (Kumari and Thayumanavan 1998) and buckwheat (2-14 μm) (Zheng and Sosulski 1997) starch granules. The Teff starch granule is smaller compared to wheat (<10 and 10-35 μm) (Evers 1973), rye (2-3 and 22-36 μm) (Jane *et al.,* 1994) and barley (2-3 and 12-32 μm) (Tang *et al.,* 2001) starch granules, but is slightly larger than amaranth (1-2 μm) (Jane *et al.,* 1994) and quinoa (0.5-3 μm) (Zheng and Sosulski 1997) starch granules.

1.5.4.2 Physicochemical properties of Teff grain

The colour of Teff grain ranges from milky white to almost dark brown, but most common are white, red and dark brown colours (National Research Council 1996). Darker colouration of red and brown varieties of Teff grain indicate higher levels of pigmented material, which has been identified as tannins and polyphenols (Parker *et al.,* 1989). Lighter coloured grains have a milder taste, which may be explained by the higher iron and polyphenolics content in darker grains (Mengesha 1966; Besrat *et al.,* 1980).

Teff is an exceptionally small grain. The grain length ranges between 0.50-1.30 mm, and thousand kernel weight (TKW) averages at 0.3g (Umeta and Parker 1996; Bultosa 2007). On average, wheat TKW is 30g (Shroyer *et al.,* 2001), which means that 100 grains of Teff weigh as much as one grain of wheat.

Teff is a tropical cereal and thus has similar physicochemical properties to other tropical grains, including maize and rice.

Gelatinisation onset, peak and conclusion temperatures of Teff starches were reported to be 68.0, 74.0 and 80.0°C (Bultosa *et al.,* 2002) and can be compared to rice starch (65.0, 74.5, 78.0°C) and maize starch (65.0, 74.5, 78.0°C) temperatures. During baking of the *injera*, Teff starch is completely gelatinised. This forms a steam-leavened, spongy matrix in which bubbles of gas, microorganisms, fragments of bran and the outer layer of endosperm are embedded (Parker *et al.,* 1989). Teff starch is the major contributor to the texture and keeping properties of Ethiopian bread *injera* (Parker *et al.,* 1989). Gelatinised starches have a tendency to undergo retrogradation, a process that is mostly dependent on amylose and amylopectin ratios, during cool storage (Coultate 2002). Retrogradation can negatively affect the texture and shelf-life of foods. Bultosa and Taylor (2004) studied retrogradation of Teff and other cereals starches during a 21-day storage period at different temperatures. Although the mean amylose content of the Teff starches is typical to other native cereal starches like maize, sorghum and wheat (Bultosa *et al.,* 2002), Teff starches were shown to have lower retrogradation tendency (due to slower gelation) than maize starches (Bultosa and Taylor 2004a), which suggests that Teff could be beneficial for food applications where starch staling is preferred to be reduced or shelf-life of the product to be extended.

The water absorption index (WAI) of Teff starches has been reported to be considerably higher when compared to maize starch (108% *v* 86%) (Bultosa *et al.,* 2002). This can be related to the small Teff starch granule size and narrower granule diameter ranges (Teff starch granule- 2-6µm; maize- 5-30µm; wheat- 2-55µm; barley- 0.9-44.9µm (Bultosa *et al.,* 2002). The smaller the granule size, the larger surface area, and, therefore, the higher the water absorption. This suggests that Teff can contribute to higher volume of baked

products, such as *injera*, compared with foods made from other millet grain flours (Zegeye 1997).

1.5.5 Teff nutritional value

There is a growing interest in Teff grain utilisation because of its superior nutritional profile (Table 1.3). Teff seeds and flour have a similar nutritional composition as Teff grain gives a 99% return in flour (Roosjen 2005). This is in contrast to wheat which gives 60-80% return in flour (Tesfaye 2000).

Table 1.3 Teff nutritional value

Nutrient/ Component	Value per 100g				
	Units	(USDA 2007)	(National Research Council 1996)	(Griffith and Castell-Perez 1998)	(Bultosa 2007)
Moisture	g	8.82	11.00	8.9	10.53
Energy	kcal	367.00	336.00	*	*
Protein	g	13.30	9.60	14.6	10.40
Carbohydrate	g	73.13	73.00	80.1	*
Fat	g	2.38	2.00	2.9	2.30
Fibre	g	8.00	3.00	6.2	3.30
Ash	g	2.37	2.90	2.4	2.45
Vitamin C	mg	*	88.00	*	*
Calcium	mg	180.00	159.00	*	*
Iron	mg	7.63	5.80	*	*
Zinc	mg	3.63	2.00	*	*

1.5.5.1 Carbohydrate and fibre

Carbohydrate is the largest fraction of nutrients in cereals. The carbohydrate content in Teff has been reported to be 73g/100g by USDA (2007) and National Research Council (1996); and 80g/100g by Griffith and Castell-Perez (1998). The carbohydrate content is usually obtained as 'a percent difference'; therefore, it is highly influenced by the content of other nutrients. Griffith and Castel-Perez (1998) found carbohydrate content by subtracting protein, fat, ash and non-starch polysaccharides (NSP) content of whole Teff grains. Determination of NSP does not take into account all components of total dietary fibre, which may affect calculations for carbohydrate content. USDA (2007) and the National Research Council (1996) reported a carbohydrate content of 73g/100g for Teff

flour, which is similar to other cereal grains, such as white wheat flour-75g/100g, rye flour- 76g/100g, but lower than maize flour (92g/100g), and higher than soya flour-(28g/100g) and brown wheat flour (69g/100g) (McCance and Widdowson 2002). The differences in carbohydrate content in Teff and other cereal grains flour could be explained by different levels of protein and fibre. Soya flour has high level of protein (37g/100g) and fibre (11g/100g) (McCance and Widdowson 2002) compared to Teff flour. White wheat flour has lower fibre content than brown wheat flour; therefore, white wheat flour has similar carbohydrate levels as Teff flour. Teff has similar carbohydrate content to other tropical grains, such as rice and millets, except maize, which contains higher amount of carbohydrates. This could be explained by the fact that maize flour contains significantly less protein (0.6g/100g in maize flour (McCance and Widdowson 2002), 13g/100g in Teff flour (USDA 2007)).

The dietary fibre content of Teff reported by researchers varies (Table 1.3). This could be because of different analytical techniques used to determine total dietary fibre. Bultosa (2007) reported crude fibre content to be 3.3g/100g for Teff grain. This value is notably lower than that reported by Castel-Perez (1998) for NSP (6.2g/100g) and USDA (2007) for total dietary fibre (8.0g/100g). Secondly, different varieties of Teff grain have different levels of fibre. Only Bultosa (2007) stated the varieties of Teff grains that were analysed. The findings showed that brown varieties of Teff grain had higher content of crude dietary fibre (3.7-3.8g/100g) compared to white Teff seeds (2.6-3.5g/100g). Taking into account Bultosa (2007) and National Research Council (1996) dietary fibre values, Teff flour has similar levels to white flour (3.1g/100g), but lower fibre content than rye flour (12g/100g) or brown wheat flour (6g/100g) (McCance and Widdowson 2002).

1.5.5.2 Protein content and amino acid profile

Teff is considered to be good source of protein. In a typical Ethiopian's diet 41g of 65g total daily protein content comes from Teff products (Jansen *et al.,* 1962). The protein content of Teff reported by researchers is presented in Table 1.3. Although protein content values in Teff vary from 9.6g/100g (National Research Council 1996) to 14.6g/100g (Griffith and Castell-Perez 1998), overall Teff contains similar or higher protein levels to wheat (11.9g/100g), maize (10.8g/100g), rice (8.6g/100g), sorghum (12.1g/100g), and finger millet (7.9g/100g) (Baptist and Perera 1956). The high protein content of Teff grain is believed to be due to a relatively large germ. Also protein content was shown to be higher in smaller seeds, because larger grains had increased levels of starch due to larger proportion of endosperm (Lester and Bekele 1981). The main fractions of Teff seed proteins are albumin, glutelins and globulins. However, Teff is quite unique from other

cereals because it contains low prolamin and high albumin fractions, which is contrary to most other cereals (Bekele 1995). The albumin fraction is rich in lysine, whereas prolamins contain only a small amount of lysine. As prolamins are the main protein constituents in cereals like, barley and sorghum, it explains why these grains are deficient in the essential amino acid lysine.

Jansen *et al*. (1962) studied individual amino acid levels in 6 varieties of Teff seeds (Table 1.4). The experimental results showed that, except for lysine, the balance among essential amino acids in Teff is similar to the Food and Agriculture Organisation (FAO) pattern, which states the amount of amino acid content considered adequate by FAO standards. The FAO pattern is used as a template for amino acid profile scoring in foods, based on studies of amino acid requirements. The closest matches for FAO pattern are whole egg, cow's milk and human milk. Cereals, compared to whole egg, are usually deficient in the essential amino acid lysine (Harper and Yoshimura 1993). Although lysine is considerably lower in Teff than in whole egg protein, the ratio of other essential amino acids in Teff is high for a cereal grain. Furthermore, lysine content in Teff is one of the highest compared to other cereals.

Table 1.4 Essential amino acids in some cereal grains

Amino acid (g/100g protein)	**Teff***	**Wheat †**	**Maize †**	**Sorghum †**	**Finger millet†**	**FAO pattern ‡**	**Whole egg‡**
Lysine	3.1	3.0	2.8	1.8	3.1	4.2	6.6
Methionine	2.8	0.6	0.8	0.8	1.9	2.2	3.8
Tryptophan	1.3	1.2	0.4	0.8	1.1	1.4	1.4
Threonine	3.3	2.2	2.9	2.4	2.9	2.8	4.2
Isoleucine	4.1	2.4	4.3	2.9	3.1	4.2	7.5
Leucine	7.7	4.5	10.0	8.7	5.9	4.8	9.4
Valine	5.3	3.1	4.0	5.2	6.3	4.2	7.2
Phenylalanine	4.9	2.0	2.6	2.6	2.7	2.8	5.8

Adapted from
*(Jansen *et al.,* 1962)
†(Baptist and Perera 1956)
‡ (Food and Agriculture Organisation 1991)

Coeliac disease is a genetically based autoimmune condition caused by permanent sensitivity to protein gluten (Anderson 2008). Coeliac patients require life-long exclusion of gluten as the only cure. Recent advances in the development of gluten-free cereal based

products made it possible to incorporate some less-utilised grains and, therefore, provide more variety to the coeliac population (Gallagher *et al.,* 2004). Teff is a gluten-free cereal, and, therefore, can be used as a substitute for wheat in food applications where exclusion of gluten is required.

Cereals like wheat, barley, rye and even oats, a cereal that previously was considered to be a safe option for a coeliac individual, contain gluten or gluten homologues. Whereas, recent findings have indicated that gluten homologues were not detected in Teff grain (Spaenij-Dekking *et al.,* 2005). This suggests that Teff may be used as a good alternative cereal for coeliac sufferers.

1.5.5.3 Antioxidants

The research indicates that of the antioxidants in grains, phenolic compounds seem to be the main contributors to the antioxidant activity (Adom and Liu 2002). Although antioxidants in fruits and vegetables have received more attention from researchers and contain high levels of antioxidants, grains and grain products contribute to the largest food intake according to the nutritional guidelines (Food Standards Agency 2001), hence providing a considerable contribution to the antioxidant content in the diet.

The research on antioxidants and antioxidant activity in Teff is very sparse. The only published data on vitamin C content of Teff is by National Research Council (1996). Investigators reported 88mg/100g of vitamin C in Teff grains. This value is about average for a cereal grain. Dykes and Rooney (2006) reviewed phenolic compound levels in millets, including sorghum, finger millet, pearl millet and Teff. It was reported that Teff grain contained 0.09-0.15 mg/100mg catechin equivalents (CE) of phenolic compounds. The main phenolic compounds in Teff were ferulic acid (285.9µg/mg), vanillic acid (54.8µg/mg), cinnamic acid (46µg/mg), coumaric acid (36.9µg/mg) and protocatechuic acid (25.5µm/mg) (Dykes and Rooney 2006).

1.5.5.4 Iron

There has been much discussion and conflicting evidence about the iron content of Teff. Table 1.5 presents the findings from several studies. The interest in the iron content of Teff was primarily due to the fact that of all the African countries only Ethiopia has a low prevalence of iron deficiency anaemia. There was evidence suggesting that absence of iron deficiency anaemia was correlated with Teff consumption (Wick 2002). One of the first findings to be published was from the Ethiopian Nutrition Survey in 1959. The investigators reported 105mg/100g of iron in Teff seeds. However, this was disputed by the results obtained by the following study (Almgard 1963), which reported 5.2 and

5.9mg/100g iron in clean white and red Teff seeds, respectively. The report also suggested that high iron content in Teff was due to soil contamination rather than intrinsic iron.

Mengesha (1966) and Hofvander (1968) analysed washed samples. They reported 11.5-21.5mg/100g and 19.3-24.4mg/100g iron content in white and red Teff varieties, respectively. On the contrary, Assefa (1978) studied Teff seeds that were grown on a non-fertilised medium and found only 3.26mg/100g of iron in them.

Areda *et al*. (1993) determined iron content of 17 cultivars after acid-washing and water washing. Their results showed that both treatments significantly (P<0.05) reduced iron content of Teff seeds, which indicated extrinsic iron contamination. The most recent study (Abebe *et al.,* 2007) investigated iron content in selected Teff grains. Their findings showed that Teff contained almost 38mg/100g and more than 150mg/100g of iron in locally purchased white and red Teff grains, respectively.

Table 1.5 Iron content of Teff from previous studies

STUDY	SAMPLES	WHITE TEFF SEEDS		RED TEFF SEEDS	
		Iron content (mg/100g)			
		mean	range	mean	range
Nutrition Survey (1959)	6 purchased	105	-	-	-
Almgard (1963)	cleaned seeds	5.9	-	5.2	-
Mengesha (1966)	6 uncontaminated seeds	11.5	9.5-13.2	19.3	11.6-24.1
Hofvander (1968)	3 acid-washed	21.5	13.0-26.9	24.4	20.0-24.8
Food Crop Table (1968)	45 purchased	18.9	4.0-72.0	58.9	70.0-144.0
Assefa (1978)	uncontaminated seeds	3.3	-	-	-
Besrat (1980)	19 acid-washed & cleaned	5.3	3.6-6.8	6.0	4.5-7.8
Mamo (1987)	cleaned seeds	5.1	-	4.9	-
Areda *et al.* (1993)	17 acid and deionised water washed	6.7	3.7-11.2	6.9	3.0-12.3
Abebe (2007)	17 purchased	37.7	-	>150	-

The high variance in iron content of white and red Teff varieties shows that contamination from soil rather than intrinsic iron may be responsible for the high levels of iron in Teff. This was suggested to be due to the traditional process of threshing the grain under the hooves of cattle, contaminating the grain with iron containing soil (Bothwell *et al.,* 1979). Also, a small size of Teff seeds reflects a greater possibility of soil contamination, compared to other larger cereal grains. Therefore, the methods of sample preparation prior to iron determination have to be taken into a consideration when comparing levels of iron in Teff and other grains. Besrat *et al.* (1980), Mamo (1987) and Areda *et al.* (1993) found differences in iron content of uncleaned, acid-washed and water cleaned Teff seeds. The samples of white teff seeds contained 5.3mg/100g of iron, compared to 9.31mg/100g iron of uncleaned seeds; acid treated red Teff seeds contained 6.00mg/100g of iron, while unwashed red seeds 12.14mg/100g (Besrat *et al.,* 1980). Also, Besrat (1980) and Areda et al (1993) results showed that the acid-washing method was less effective in removing all exogenous material, especially in heavily contaminated samples. Teff samples, which were both acid-washed and hand-cleaned, contained the least iron (white Teff- 5.15mg/100g iron in acid-washed and hand-cleaned seeds, 5.23mg/100g iron in acid-washed; red Teff- 6.36mg/100g iron in acid-washed and hand-cleaned seeds, 7.96mg/100g in acid-washed samples) (Besrat *et al.,* 1980).

All studies reported similar differences between iron levels in white and red varieties of Teff. As in other darker coloured cereal grains, red varieties of Teff contained higher levels of iron. This has been proposed to be due to higher levels of pigmented material, such as tannins and polyphenols (Umeta and Parker 1996).

Although research indicates that Teff does not contain as high levels of iron as it was previously thought, it still contains higher amounts of this mineral than other grains, such as millet, wheat, barley, maize and sorghum. Furthermore, nutritional iron deficiency is not a serious problem in Ethiopia in general (Hofvander 1968), including susceptible population segments, such as children and women of reproductive age. This suggests that Teff grain products provide adequate levels of dietary iron in the Ethiopian population. It is also plausible that given a relatively low incidence of anaemia in Ethiopia, Teff may provide a high level of bioavailable iron.

Iron deficiency anaemia (IDA) arises from low dietary intake of iron, low bioavailability of non-haem dietary iron and high levels of absorption inhibitors in the diet, such as phytate, tannins and other minerals in the diet.

A study by Gebre-Medhin *et al.* (1976) compared IDA and anaemia of pregnancy in different areas of Ethiopia. The study findings concluded that irrespective of socio-

economic class women who were regularly consuming Teff products had normal levels haematological parameters and did not suffer from either of the deficiencies. A more recent study found significant association between parasitic infection and anaemia in pregnancy (P<0.01), confirming non-dietary causation of the condition (Desalegn 1993). Another study (Wolde-Gebriel *et al.,* 1993) looked at nutritional status of over 14, 000 school children in Ethiopia. They concluded that clinical anaemia cases (18.6%) were not nutritional in origin but due to the intestinal parasites. A study of young adult Ethiopian males also confirmed adequate haemoglobin and haemotocrit levels in these subjects (Abdulkadir *et al.,* 1979), confirming the findings of Hofvander (1968). The most recent study indicated that IDA may become a public concern in Ethiopia as findings indicated the existence of mild to moderate IDA among women of reproductive age (Umeta *et al.,* 2008). The authors explained the higher prevalence rate of anaemia in certain rural areas due to type of staple foods consumed, such as corn, milk and sorghum. These foods are much lower in iron and rich in iron absorption inhibitors, which may contribute to higher prevalence of anaemia.

In summary, although the high iron content of Teff grain was questioned by several researchers, haematological investigation of iron deficiency anaemia provides strong support to the hypothesis that in areas where Teff is consumed as a staple food IDA is not a major public health problem.

1.5.5.5 Other minerals

Other minerals that have been studied in Teff were zinc, calcium, copper, magnesium, manganese, potassium and sodium. Table 1.6 presents key minerals and trace elements composition in Teff reported by several researchers.

Table 1.6 Minerals and trace elements in mixed red and white Teff grains

Study	Zinc (mg/100g)	Calcium (mg/100g)	Copper (mg/100g)	Magnesium (mg/100g)	Potassium (mg/100g)
Mengesha, 1966	6.77	176.0	0.68	182	280
National Research Council, 1996	2.00	159	0.70	170	401
Abebe *et al*., 2007	3.86	147.0	-	-	-
USDA, 2007	3.63	180	0.80	184	427

The zinc content of Teff grains reported by the researchers varies from 2.00 mg/100g to 6.77mg/100g. The zinc content of Teff is similar to other cereal grains, such as barley, wheat or maize (Abebe *et al.,* 2007; USDA 2007).

Teff is notably higher in calcium than other cereals. Teff grains contain between 147-180mg/100g of calcium, whereas wheat grains contain 3.33mg/100g (USDA 2007), barley- 46mg/100g and maize- 16mg/100g of calcium (Mengesha 1966). The reported calcium content of Teff was relatively consistent between studies. This suggests that soil contamination did not affect this value. Indeed, soils in Ethiopia are clay-rich and not calcareous (WHO 1996).

The copper and magnesium content of Teff (Cu 0.68-0.80mg/100g; Mg 170-184mg/100g) is similar to that of sorghum grain (Cu 0.67mg/100g; Mg 180mg/100g) (Mengesha 1966), but is slightly higher than that in hard wheat grain (Cu 0.63mg/100g; Mg 93mg/100g) (Mengesha 1966; USDA 2007) and barley (Cu 0.35mg/100g; Mg 130mg/100g) (Mengesha 1966). The potassium levels in Teff range between 280- 427mg/100g, which is similar when compared to wheat, barley and sorghum (Mengesha 1966; USDA 2007).

1.5.6 Bioavailability of nutrients

Cereal grains are rich in phytic acid and tannins, which are potential inhibitors of mineral and trace element absorption. Phytate has been reported to inhibit absorption of iron, zinc, calcium, magnesium and manganese in humans (Hurrell 2003). Phytic acid inhibits the absorption of minerals by forming insoluble complexes in the gastrointestinal tract that cannot be absorbed or digested by humans due to the absence of intestinal phytase enzymes (Hurrell 2003). Because of the high density and negatively charged groups, phytate chelates mineral cations and forms insoluble complexes.

1.5.6.1 Phytate levels in Teff

Phytate levels of 528mg/100g have been reported in mixed Teff grains (Abebe *et al.,* 2007), 389mg/100g in unfermented Teff *injera*, and 126mg/100g in fermented Teff *injera* (Umeta *et al.,* 2005). Teff grains have a higher phytate level than barley (452mg/100g), but lower than maize (1443mg/100g) (Abebe *et al.,* 2007). Both unfermented and fermented wheat and sorghum *injeras* have lower amount of phytate than Teff *injeras*.

Phytate begins to lose its inhibitory effect on minerals when phytate:mineral molar ratios are less than 1.0 or 15.0 for iron and zinc, respectively (Hurrell 2003). Therefore, molar

ratios, rather than actual phytate content, are a more accurate measurement for predicting mineral bioavailability in foods.

Table 1.7 shows the phytate content and phytate molar ratios for iron, zinc and calcium in Teff grains and different cereal *injeras*. Although Teff grains and Teff injera have higher phytate content compared to sorghum and wheat *injeras*, the phytate:iron ratio is notably lower in Teff products and hence the bioavailability is in fact higher. Ratios above 1.0, which considerably compromise iron absorption, were found in unfermented sorghum and Teff *injeras* and fermented wheat *injera*. Only Teff grains and fermented Teff *injera* had significantly lower ratios of 0.3, which shows better iron bioavailability.

Table 1.7 Phytate, tannin content and ratios in Teff grains and *injeras*

PRODUCT		Phytate content (mg/100g)	Phy:Fe (molar ratio)	Phy:Zinc (molar ratio)	Ca:Phy (molar ratio)	Tannin content
Mixed Teff grains*		528	<0.3	13.6	-	-
Teff	Unfermented	389	1.1	28.2	2.7	60.1
***injera*†**	Fermented	126	0.3	10.8	8.2	49.8
Sorghum	Unfermented	325	3.0	38.6	0.7	53.2
***injera*†**	Fermented	75	0.8	11.1	2.4	49.8
Wheat	Fermented	137	3.3	9.4	2.8	21.2
***injera*†**						

Adapter from *(Abebe *et al.,* 2007), †(Umeta *et al.,* 2005)

A study with human subjects showed a significant reduction in iron bioavailability and absorption ($P<0.05$) when high phytate content grain sorghum meal was given to female subjects compared to low phytate content sorghum cultivar meal (Gillooly *et al.,* 1984). Furthermore, the increase in soluble iron content in sorghum and finger millet grains was strongly related to enzymatic degradation of phytate ($P<0.001$) (Svanberg *et al.,* 1993). Fermentation, such as lactic acid induced fermentation, is a natural way of increasing phytate breakdown in cereals (Svanberg *et al.,* 1993), hence, potentially improving bioavailability of iron. Indeed, fermentation of *injera* resulted in a 3-4 fold reduction in the phytate molar ratios for iron and zinc (Umeta *et al.,* 2005). Other food processes such as cooking, soaking, and germination were reported to have positive effects on phytate degradation in sorghum and finger millet (Matuschek *et al.,* 2001).

Phyate:zinc molar ratios above 15.0 indicate poor zinc availability (Hurrell 2003) and may result in zinc deficiency. Indeed, a study investigating the effects of phytate on zinc absorption in young men, found that zinc absorption fell by about 50% when phytate:zinc ratio was 15.0 (Turnlund *et al.,* 1984). All fermented *injeras* had lower molar ratios than 15.0, whereas, unfermented injeras had considerably higher phytate:zinc ratios. Teff grains had a lower ratio of 13.6, which indicates adequate zinc availability. Although barley contained lower phytate content of 452mg/100g compared to Teff grains, phytate:zinc molar ratios of both cereals were the same (13.6) (Abebe *et al.,* 2007).

1.5.6.2 Tannin levels in Teff

Tannins are complex polyphenol compounds that have been shown to reduce bioavailability of iron and zinc (Hemmingway and Laks 1992). Tannins form insoluble complexes with iron and zinc, reducing absorption in the gastrointestinal tract (Brune *et al.,* 1989). Cereals and cereal-based foods are abundant in tannins, hence, compromising bioavailability of these minerals.

The tannin content of wheat, Teff and sorghum *injeras* is presented in Table 1.7. Both Teff and sorghum *injeras* contained similar amount of tannins (60.1mg/100g, 53.2mg/100g, respectively), which was reduced during fermentation (49.8mg/100g for both *injeras*). In contrast wheat *injera* contained a notably lower amount of tannin (21.2mg/100g). The most plausible explanation for this is that during the milling of small-grained cereals, such as Teff, sorghum or finger millet, the whole grain is used to produce flour, whereas larger wheat grains offer the possibility to remove the bran. As most tannins are concentrated in the outer layer of grain pericarp, the use of white wheat flour explains lower content of tannin in wheat *injera*.

Effects of tannin content and *in vitro* iron accessibility in grain products were investigated in several studies (Matuschek *et al.,* 2001; Matuschek and Svanberg 2002). When tannic acid and green tea extract were added to wheat bread, a significant inhibitory effect on mineral bioavailability was reported by Matuschek and Svanberg (2002).

Several studies looked at how different levels of tannins in cereals affect the bioavailability of iron and zinc in human subjects. High and low tannin sorghum cultivars meals were given to normal and anaemic human subjects and the iron availability compared between the groups (Radhakrishnan and Sivaprasad 1980). The investigators concluded that iron absorption in normal subjects was similar for low and high tannin sorghum meals, while in the anaemic subjects, iron absorption from low tannin sorghum was significantly higher. However, when iron absorption was determined after accounting for the phytate content, the strong association between low and high tannin sorghum meals disappeared. This

suggests that tannins have a lesser role in determining iron bioavailability, especially when high phytate levels are present in a cereal cultivar.

1.5.6.3 Other factors affecting bioavailability

The bioavailability of minerals can be affected by the content of inhibitors, preparation of the food, other nutrients present in the food and the interrelationships between minerals.

1.5.6.3.1 Food processing conditions

Food processing is important, as it can greatly affect mineral bioavailability (Hurrell 2003). Food processing techniques that increase the activity of native enzymes (e.g. phytase), hence degrading absorption inhibitors, are soaking, germination, cooking and fermentation. Soaking of whole maize, millet, rice and sorghum seeds for 24 hours significantly reduced phytate content ($P<0.05$), however, only slightly decreased phytate:zinc molar ratio (Lestienne *et al.,* 2005). This suggests that soaking alone would not improve bioavailability of minerals; however, in combination with other treatments, it may be useful in improving bioavailability of nutrients.

Fermentation, a process used in breadmaking, influences phytate levels in cereals. The phytate content of bread containing bran was reduced by 60% after 2 hours of fermentation and by 85% after 2 days (Navert *et al.,* 1985). The study also investigated zinc absorption from single meals, containing milk, butter and test bread, in 42 healthy male and female volunteers using radioisotope technique. The results showed that the amount of zinc absorbed after breakfast was significantly increased as fermentation of bread was prolonged ($P<0.05$). Fermentation during Teff *injera* preparation showed a decrease in phytic acid and tannin content by 75% and 55%, respectively, after 96 hours of fermentation (Urga *et al.,* 1997). Furthermore, fermentation of Teff has resulted in an increase of the dialysable portion of iron, phosphorus and zinc content from 9%, 16% and 7%, respectively, to 24%, 60% and 43% (Ramachandran and Bolodia 1984), which may account for the enhancement of these minerals bioavailability in Teff *injera*.

1.5.6.3.2 Calcium

Calcium has been identified in reducing bioavailability of iron and zinc. The calcium:phytate molar ratios in foods above 6.0 are critical and indicate reduced bioavailability of iron and zinc. Calcium:phytate molar ratio was below the critical ratio of 6.0 in all cereal *injeras* except fermented Teff injera (Table 1.7). The high calcium level of fermented Teff injera may reduce both zinc and iron bioavailability.

Dietary calcium has also been identified in reducing bioavailability of both haem and non-haem iron. Several studies looked at direct bioavailability of haem and non-haem iron from a single meal in healthy subjects (Hallberg *et al.,* 1991; Hallberg *et al.,* 1993b). Halberg *et al.* (1991) initiated a study to compare iron absorption in 126 subjects after a meal containing wheat bread rolls with added calcium at levels of 40 and 600 mg. The study findings show dose-related inhibitory effects of calcium on iron absorption. Iron absorption was reduced by 50-60% at doses of 600 mg calcium. The following study looked at the calcium inhibitory effect on haem iron from meat sources (Hallberg *et al.,* 1993b). Results confirmed a direct inhibitory effect on haem iron absorption. In the following study, researchers investigated iron absorption from the whole diet in 21 female volunteers during a 10-day period (Gleerup *et al.,* 1995). Results showed that about 30-50% more iron was absorbed when no milk or cheese was served with meals. The importance of calcium and iron ingestion patterns was demonstrated by the previous study (Gleerup *et al.,* 1993). Study subjects were given either water or calcium from milk and cheese as a part of breakfast meal. After 2 hours, study participants were given a meal containing 2.1mg of iron. Using the single-meal absorption test, this study showed that consuming dietary calcium 2 hours before a meal did not affect the absorption of dietary iron.

Although short-term studies have shown that ingestion of calcium and iron from the same meal inhibits iron absorption, long-term studies failed to demonstrate the same effect. Snedeker *et al*. (1982) examined iron utilisation in 9 adult males over a 39-day period. Subjects' diets were supplemented with different levels of calcium. Subjects whose diets contained the highest intakes of calcium (2382 mg/day) excreted significantly more iron in their urine when compared to other groups (consuming 780 mg calcium) (Snedeker *et al.,* 1982). However, in all study diets this did not significantly (P<0.05) affect plasma iron, serum transferrin and serum ferritin. Another long-term study conducted on 57 premenopausal women indicated similar findings (Sokoll and Dawson-Hughes 1992). The researchers compared plasma ferritin levels in the calcium treated (1,000 mg/day) and control groups after 12 weeks intervention. The study findings showed no significant difference between iron status in healthy premenopausal women. Minihane and Fairweather-Tait (1998) investigated both short-term and long-term calcium supplementation effects on non-haem iron absorption. In the short-term study, non-haem iron absorption from low-calcium (<320 mg/day) and moderately high iron (15 mg/day) diet was compared to calcium supplemented diet (1200 mg/day). In the presence of calcium iron absorption fell significantly (P<0.001). The long-term effect of consuming calcium with meals was investigated in 11 iron-replete adults during a 6-month period.

There were no significant changes in haematologic indices between calcium treated and control groups.

Evidence from short-term and long-term studies proposed an adaptive mechanism of iron absorption that counters the effect of calcium (Minihane and Fairweather-Tait 1998). It was hypothesised that mucosal cells are stimulated to produce high affinity proteins that result in more efficient iron absorption in the presence of continuous low concentrations of iron. However, this was challenged by Hallberg (1998). It was argued that iron stores are highly regulated and difficult to change in iron-replete person. Therefore, studies using serum ferritin as indirect measurement of iron absorption may be inappropriate unless the investigation is considerably longer (e.g. years) and have a large sample size (Hallberg 1998).

Overall, studies on the direct effect of calcium on iron absorption show that calcium inhibits iron absorption. Although there is some conflicting evidence over long-term effects of calcium on haem and non-haem iron absorption, a general conclusion for those population groups with high iron requirements (adolescents, child bearing age and pregnant women) is to restrict or avoid high calcium intake with main meals, which contain most of the dietary iron.

Chapter 2 Teff bread development - Methodology

2.1 Bread product development

An overview of experimental work for Teff bread development is shown in Figure 2.1. Due to its nutritional profile, cereal Teff was chosen for the improvement of nutritional properties, in particular dietary iron, of wheat breads. The incorporation level (0-30% flour replacement) of Teff was chosen according to the literature search on Teff processing qualities (Ben-Fayed *et al.,* 2008; Mohammed *et al.,* 2009). The experimental design was chosen to screen the most important factors for the quality of bread: specific volume, crumb firmness during an 8-day shelf-life period, nutrients composition and sensory attributes.

The experimental data revealed a significant decrease in all quality parameters of 30% Teff bread. Therefore, this bread was chosen for the improvement stage. Single enzymes and enzyme-combinations were chosen according to supplier's recommendation and literature search. As addition of enzymes affected dough properties, processing conditions were optimised.

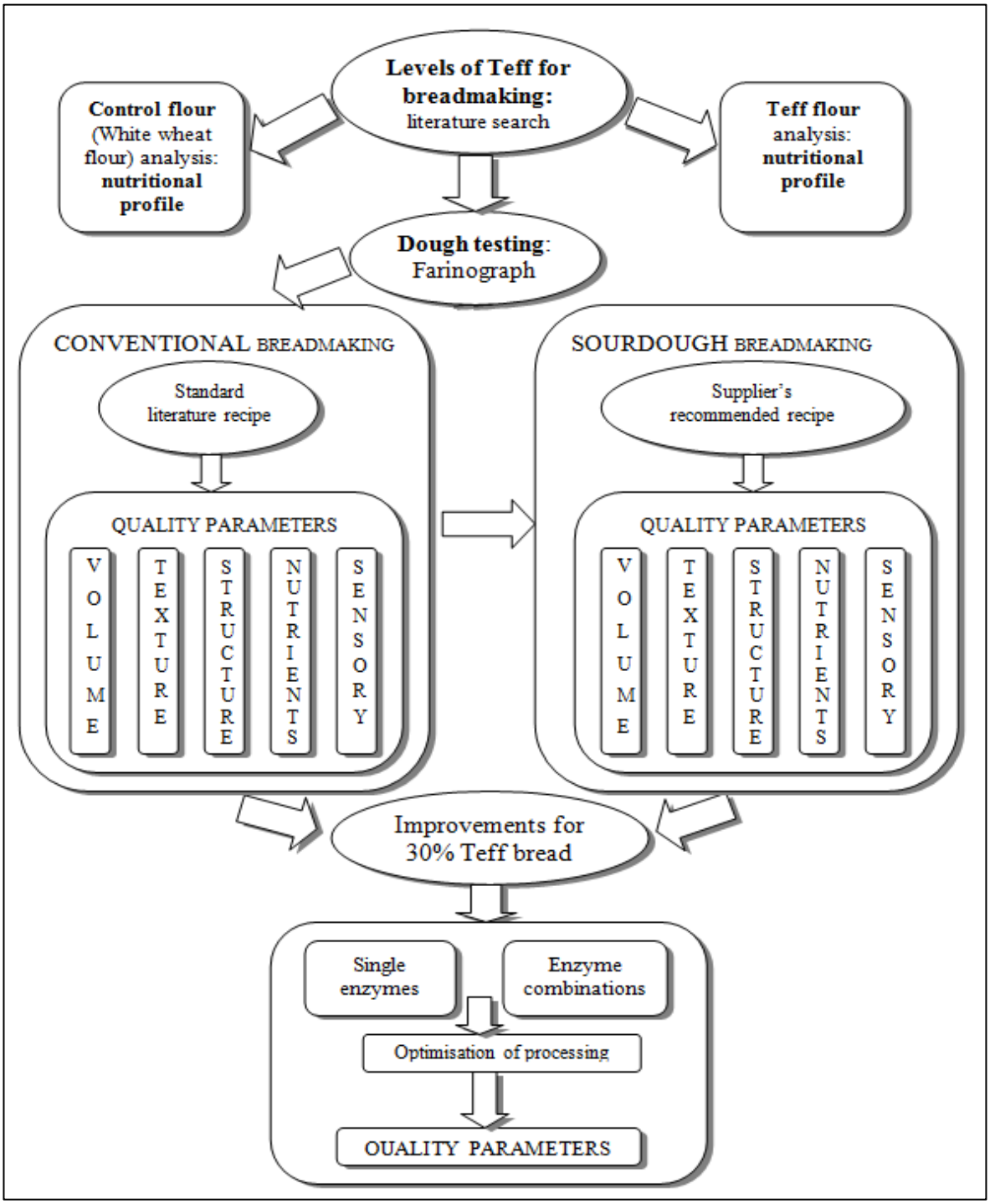

Figure 2.1 Research design for Teff bread development

2.1.1 Flour characteristics measurement

Farinograph measurements on the flours (Table 2.2) were carried out using a Brabender Farinograph (mixer bowl 300g, Brabender OHG, Duisburg, Germany) using an official method (ICC 1992).

Measurements obtained from the Farinograph were: flour water absorption (WA) by the burette's reading, dough development time (DDT), dough stability time (DST) and degree of dough softening (DDS) determined on 600 Brabender units (BU) line.

Pasting properties of flour blends (with corrected values for moisture content) were measured using a Rapid Visco Analyzer RVA-4 (Newport Scientific Pty. Ltd, Warriewood, NSW, Australia). Experiments were performed according to an official AACC method (AACC 1999).

The pasting temperature (the temperature where viscosity first increases by at least 25 cp over a 20 s period), peak time (the time at which peak viscosity occurred), peak viscosity (the maximum hot paste viscosity), holding strength or trough viscosity (the trough at the minimum hot paste viscosity), final viscosity (the viscosity at the end of test after cooling to 50°C and holding at this temperature), breakdown (peak viscosity − holding strength or trough viscosity) and setback (final viscosity − holding strength) were calculated from the pasting curve, using Thermocline version 2.2 software Newport Scientific Pty. Ltd. (Warriewood, Australia).

2.1.2 Materials

The ingredients used for bread making were: strong white wheat flour (10.2-11.7% protein content, Smiths Flours Mills, Worksop, UK), white Teff flour (Soil & Crop Improvement, Romhof, Holland), compressed yeast (Fermipan, Gist-Bracades, Holland), vegetable fat shortening (Cardowan Creamers Ltd, Glasgow, UK), sugar and salt (purchased from a local supermarket in Manchester) and improver (Diamond, British Arkady, Manchester, UK).

Commercial sourdough cultures LV1 and LV2 were obtained from Fermed International Ltd (Worcester, UK). LV1 consisted of 98% yeast (*Saccharomyces chevalieri*) and 2% bacteria (*Lactobacillus casei, Lactobacillus brevis*). LV2 consisted of 2-5% bacteria (*Lactobacillus brevis*), 35-45% yeast and 50-60% dried wheat flour.

Commercial enzymes, xylanase (EC 232-800-2), amylase (EC 232-588-1), glucose oxidase (EC 232-601-0) and lipase (EC 232-619-9) were supplied by Novoenzymes Ltd (Denmark).

2.1.2.1 Straight dough bread making

The ingredients were added to produce dough for 300±12g loaves (variation due to various levels of dough weight loss during baking).

The ingredients added were based on a g/100g flour weight (Table 2.1). All runs were processed in 1000g flour weight batches. One loaf per batch was used for analysis.

Table 2.1 Bread Recipe

Ingredient	Amount (g/100g)
Flour	100
Water (30° C)	Brabender Farinograph value (61.3-64.1)
Salt	2
Sugar	6
Shortening	4
Yeast (compressed)	3
Improver	1

Four different blends of wheat/Teff flour (Table 2.2) were used to bake three replicate batches of each one loaf of each blend was used for analysis.

Table 2.2 Formulations of four types of flours used in bread making

Bread	Wheat flour (%)	Teff flour (%)
White wheat bread	100	0
10% Teff bread	90	10
20% Teff bread	80	20
30% Teff bread	70	30

Mixing of the ingredients was carried out in Hobart mixer (Process Plant and Machinery Ltd, UK) at speed 2 for 10 min. The dough was divided into 340g pieces to account for moisture loss, moulded by hand and placed into pre-greased 454 g tins. The dough was proved for 55 min at 40°C and 85% humidity in Foster RBC MK3 prover (Norfolk, UK) and baked in the oven (Teknitronic reel oven, Teknigas Ltd, UK) for 20 mins at 200°C.

All bread loaves were stored in the plastic bags and kept in a dry place for further analysis. The bread samples were analysed for the quality parameters and prepared for the nutritional composition analysis within 24 hours of manufacture.

2.1.2.2 Sourdough bread making

Teff flour was incorporated into sourdough bread by replacing white wheat flour by 10, 20 and 30% (flour weight) in the starter flour blend and final dough flour.

Two sourdough cultures (LV1 and LV2) were used to produce white wheat (control), 10, 20 and 30% Teff sourdough breads, giving a total of 8 varieties for analysis (one loaf for each variety of blend taken from 3 repeated batches). Both cultures were added to flour at the level of 0.5% of flour weight. Sourdough bread formulations for wheat, 10, 20 and 30% Teff breads were used following the supplier recommended method (Figure 2.2). The processing conditions for sourdough breads were 55 min for proving at 40°C and 85% humidity.

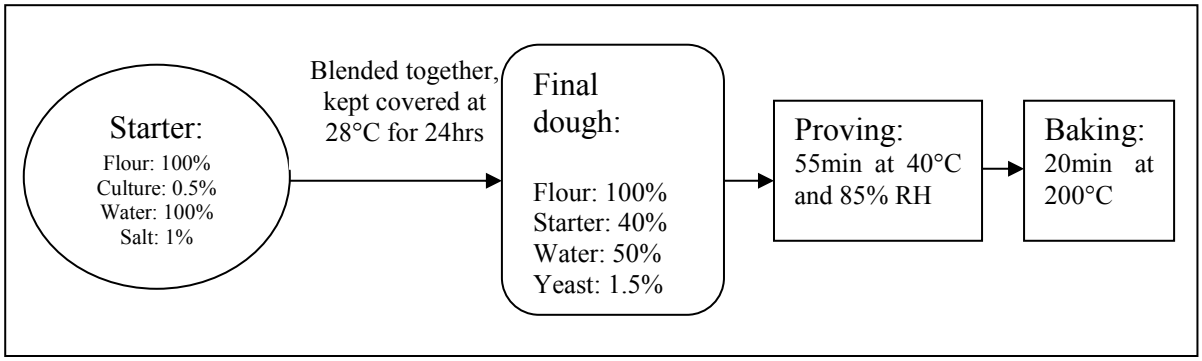

Figure 2.2 Sourdough bread making

2.2 Nutritional composition of flour blends and breads

All nutritional composition analyses were performed on freshly baked (within 24 hours) breads. All samples were prepared according to the recognised methods for each nutrient. Results were expressed as g/100g of sample for the total dietary fibre, protein and fat content, mg/100g for iron content and as Trolox equivalent antioxidant capacity (TEAC, mM Trolox equivalent per 100g of sample) for the total antioxidant capacity.

The total dietary fibre content was determined using Total Dietary Fibre Assay Kits TDF-100A and TDF-C10 (Sigma-Aldrich Inc, St Louis, MO, USA) based on an enzymatic and gravimetric method (AOAC 1997a). The samples of fresh bread were dried and gelatinised with heat stable α-amylase and then enzymatically digested with protease and amyloglucosidase to remove the protein and starch present in the sample. Ethanol was added to precipitate the soluble dietary fibre. The residue was then filtered and washed with ethanol and acetone. After drying, the residue was weighed. Half of the samples were analysed for crude protein and the others were ashed. Total dietary fibre was calculated as the weight of the residue less the weight of the protein and ash.

Protein content was estimated from the crude nitrogen content of the sample determined by the Kjeldahl method (AOAC 1997b). The samples were heated with aqueous sulphuric acid with potassium salt catalyst in the digestion apparatus. The samples were then neutralised with sodium hydroxide in the distillation unit and titrated with hydrochloric acid to determine nitrogen content of the samples. The crude protein content was estimated using Nitrogen factors (N x 6.25 for Teff flour and Teff breads; N x 5.7 for wheat flour) (Sawyer *et al.,* 1991).

Total fat content was determined based on a gas chromatographic technique using a peer-reviewed AOAC Caviezel method (Pendl *et al.,* 1998). The samples and internal standard (tridecanoic acid) were added to the butanol solvent. Fat was extracted and simultaneously saponified by potassium hydroxide. The fatty acid potassium salts were converted to fatty acids by adding an acidic aqueous salt solution, which produced a 2-phase system. The upper phase, containing the fatty acids and IS, were then injected into the fat determination Büchi system. After gas chromatographic separation, the fat content was calculated from IS and fatty acid peak areas.

Iron content was determined using 2,2 Dipyridyl method (AOAC 1980). The samples were ashed, then placed over boiling water for 30 minutes for digestion by hydrochloric acid. The digests were cooled down, diluted to 100 ml with distilled water and filtered. The iron content was measured using a Spectrophotometer (Shimadzu UV-160A) at 520nm.

The total antioxidant capacity was carried out according to the $ABTS^{+}$ method (Re *et al.,* 1999). The samples were extracted with methanol at 35°C for up to 24 hours in shaking water bath. After cooling, the extracts were centrifuged at 5000 rpm for 10 minutes and filtered. The pre-formed radical cation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) was generated by oxidation of ABTS with potassium persulphate and then it was reduced in the presence of hydrogen-donating antioxidants from the food samples. The absorption was read using a Spectrophotometer (Shimadzu UV-160A) at 734 nm.

2.3 Evaluation of bread quality characteristics

Bread loaves were weighed and the loaf volume was measured by the rapeseed displacement method within 24 hours of bread baking (AACC 2000). Analyses were

carried out in triplicate for each batch and specific loaf volume of breads was calculated as volume/weight (ml/g).

Crumb firmness was measured in triplicate for each batch using TA-XT2i texture analyser (Stable Micro Systems, Surrey, UK) fitted with 25 mm cylinder probe using bread 40% compression test (AACC 1998) to assess crumb firmness during an 8-day shelf-life period. A force-time curve was recorded and analysed by Texture Exponent 32 software (Stable Micro Systems, Surrey, UK).

Image analysis of bread slices was carried out in triplicates for each batch using a C-Cell analyser (Calibre Control International Ltd, Appleton, UK). Slice area (mm^2), brightness (0-dark to 255-white), number of cells, wall thickness (mm), cell diameter (mm), cell volume (mm^3) and number of cells/mm^2 were studied (AACC 2012).

Sensory analysis was conducted using taste panels, consisting of 50 untrained assessors. Panellists were asked to mark a 10 cm line in accordance to their preference (Figure 2.3). The attributes for sensory evaluation were adapted from the study of descriptive sensory quality of bread (Heenan *et al.,* 2008) and are presented in Table 2.3.

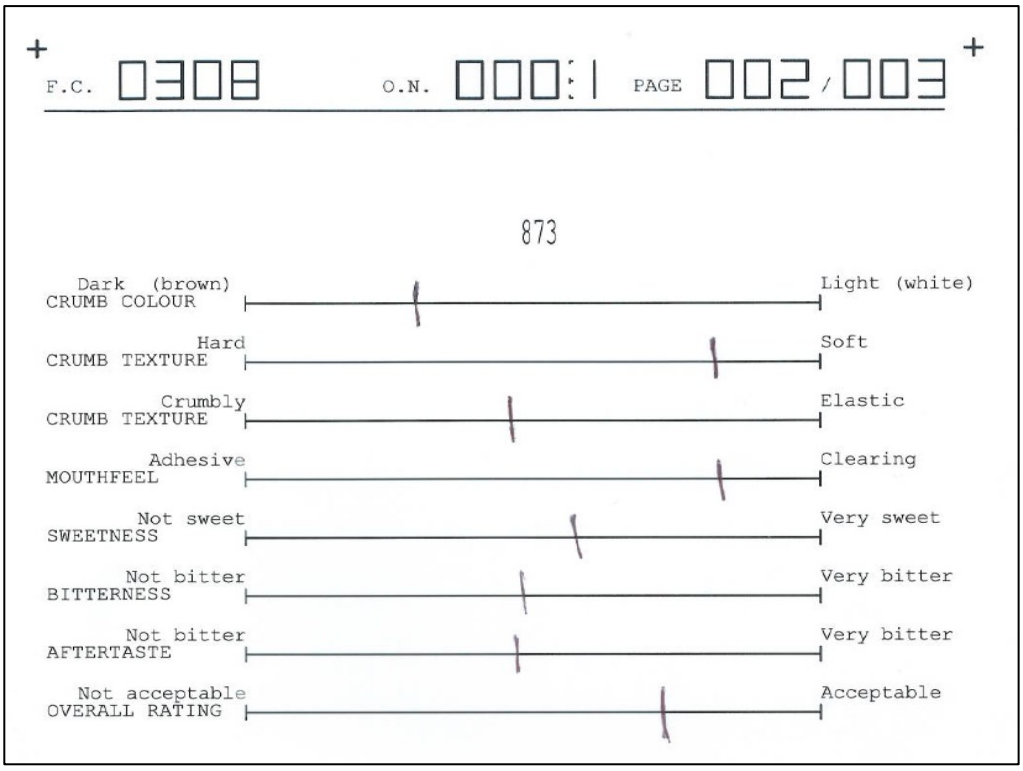

Figure 2.3 Example of sensory evaluation scoring form

Table 2.3 Attributes selected for bread taste panels (adapted from Heenan *et al*. (2008)**).**

Attribute	Description
Appearance:	
Crust lightness	Dark ↔ Light
Crust texture	Rough ↔ Smooth
Crumb colour	Dark brown ↔ White
Crumb Texture:	
Softness	Hard ↔ Soft
Elasticity	Crumbly ↔ Elastic
Mouthfeel	Adhesive ↔ Clearing
Flavour:	
Sweetness	Not sweet ↔ Very sweet
Bitterness	Not bitter ↔ Very bitter
Aftertaste:	
Bitterness	Not bitter ↔ Very bitter
Overall Acceptability	Not acceptable ↔ Very acceptable

2.4 Enzymes used for Teff bread development

Four commercial enzymes were used for straight dough bread making and sourdough bread making during the product development stage of the study (Table 2.4). All enzymes were in powder form and added to the flour blend simultaneously before the mixing to ensure homogeneity.

Table 2.4 Commercial enzymes used for breadmaking

Enzyme	Abbreviation	Product name	EC No
Xylanase (endo-1,4-)	X	Pentopan® Mono BG	232-800-2
Alpha-amylase (fungal)	A	Fungamyl® 2500 SG	232-588-1
Glucose oxidase	GO	Gluzyme® 10000 BG	232-601-0
Lipase	L	Lipopan® F BG	232-619-9

The following enzyme combinations were selected according to supplier recommendations and evidence from literature search: X+A, A+GO, GO+X and L+A.

2.4.1 Optimisation of the breadmaking process for the enzyme-supplemented breads

Dough parameters (dough handling, loss of dough volume during proving and/or from prover to oven) and final product characteristics (shape of a loaf, specific volume, and crumb firmness) were used as quality measures during the optimisation stage. The optimum process conditions were chosen according the best bread produced. The products were compared to a control white wheat bread. Therefore, the process conditions that produced Teff breads with the largest specific loaf volume, the most uniform crumb structure and the lowest crumb firmness were considered as optimum. Table 2.5 shows variables tested during the optimisation trials.

Table 2.5 Testing trial design for the optimisation of the breadmaking process for 30% Teff straight-dough and sourdough breads

Process variables		Xylanase (X)	Amylase (A)	Glucose oxidase (GO)	Lipase (L)
Levels of enzyme treatment (ppm)	Single treatment	60	9	20	60
		30	3	10	30
	Combination treatment	30	3	10	30
		15	1.5	5	15
Proving temperature (^{o}C)	Single and combination treatments	30	30	30	30
		35	35	35	35
		40	40	40	40
		45	45	45	45
Proving time (min)	Single and combination treatments	40	40	40	40
		30	30	30	30

The final enzyme treatment levels were: 30 ppm for X, 3 ppm for A, 10 ppm for GO, and 30 ppm for L in single enzyme supplementation. The enzyme treatment levels of 15 ppm for X, 1.5 ppm for A, 5 ppm for GO and 15 ppm for L were used for the combination supplementation. The final processing conditions as obtained from the optimisation baking trials for enzyme-supplemented straight-dough and sourdough doughs were 30 min proving time at 30°C, 85% relative humidity; and 20 min baking time at 200°C.

2.5 Statistical Analysis

Statistical tests were carried out using SPSS 16.0 (SPSS Inc., Chicago, Illinois, US). A significance level of $P<0.05$ was used. Data were presented as mean ± 1 standard deviation.

Data distribution and normality were assessed using skewness and kurtosis values, and Kolmogorov-Smirnov test. Log transformation was applied to the variables that were non-normality distributed. Descriptive statistics and differences in mean values of investigated factors were compared using one-way independent measures analysis of variance (ANOVA). These tests were applied for the nutritional composition, bread quality characteristics and sensory data to determine significant differences between the flour blends and breads. Pearson's correlations and linear multiple regression were used to study relationship between bread sensory attributes and overall acceptability.

Chapter 3 Teff bread development - Results

3.1 Nutritional properties

3.1.1 Flours used for breadmaking

A number of nutrients, including protein, fat, total dietary fibre, iron and antioxidant levels, were determined in wheat and Teff flour used in the breadmaking.

Wheat and Teff flours did not differ significantly in protein and fat content (Table 3.1). The obtained value of 13.7g/100g of protein content in Teff flour is similar to the literature values, which vary from 9.6g/100g (National Research Council 1996) to 14.6g/100g (Griffith and Castell-Perez 1998). The dietary fibre content of Teff flour was significantly higher than that in corresponding wheat flour. Levels of fibre content in Teff flour reported by other researchers vary from 3.00g/100g (Bultosa 2007) to 8.0g/100g (USDA 2007), which are similar to the present study's findings.

Teff flour contained significantly higher levels of dietary iron than control wheat flour. This is in agreement with the majority of other researchers who compared Teff grain with other cereal grains (Mengesha 1966; Areda *et al.,* 1993; Abebe *et al.,* 2007). Nevertheless, the level of iron in Teff flour in the present study was 14±1 mg/100g, which is notably lower than the amounts of 38 mg/100g and 105 mg/100g reported by Abebe (2007) and the National Survey (1959).

Total antioxidant capacity was also significantly higher in Teff flour than wheat flour.

Table 3.1 Nutritional properties of wheat and Teff flours used for breadmaking

Nutrient	White wheat flour	Teff flour
Protein content (g/100g)	14.1±0.60 a	13.7±0.50 a
Fat content (g/100g)	3.1±0.05 a	2.9±0.03 a
Dietary fibre content (g/100g)	4.3±0.33 a	5.7±0.36 b
Iron content (mg/100g)	2.85±0.40 a	14.41±1.19 b
Total antioxidant capacity (TEAC/100g)	2.00±0.47 a	3.91±1.89 b

Means±std dev in the same row followed by a different letter are significantly different ($P<0.05$; Independent samples t-test)

3.1.2 Wheat and Teff breads

In general, wheat and Teff breads did not differ significantly in their content of protein, fat and dietary fibre. There were significant differences in iron content and total antioxidant capacity (Table 3.2).

Table 3.2 Nutritional properties of breads

Sample	Protein content (g/100g)	Fat content (g/100g)	Dietary fibre content (g/100g)	Iron content (mg/100g)	Total antioxidant capacity (mM TEAC/100g)
Wheat bread	10.5±0.25 a	4.1±0.33 a	4.1±0.35 a	2.39±0.27 a	1.39±0.07 a
10% Teff bread	10.6±0.35 a	4.0±0.03 a	4.2±0.41 a	3.13±0.41 b	2.03±0.08 b
20% Teff bread	10.9±0.07 a	3.8±0.09 a	4.3±0.44 a	4.08±0.65 c	2.14±0.07 b
30% Teff bread	11.0±0.02 a	3.7±0.16 a	4.5±0.32 a	5.62±1.22 d	2.41±0.04 c

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

Table 3.3 shows the contribution to Dietary Reference Values (DRVs), set by the Food and Nutrition Board of the National Research Council (Department of Health 1991), of selected nutrients for wheat and Teff enriched breads. Teff breads would contribute to similar intakes of protein and fibre but notably higher intake of dietary iron when compared to white wheat bread if 200g of bread would be consumed daily (Table 3.3).

Table 3.3 Contribution of nutrients to the relevant DRVs consuming an average daily portion (4-5 slices, approx. 200g) of wheat and Teff breads

Nutrient	Gender	DRVs*	Contributions to DRVs (%)			
			Wheat bread	10% Teff bread	20% Teff bread	30% Teff bread
Protein	Male	56 g/day	38%	38%	39%	39%
	Female	46 g/day	46%	46%	47%	48%
Dietary fibre	Adults	18 g/d	46%	47%	48%	50%
Iron	Male	8.7 mg/d	55%	72%	94%	129%
	Female	14.8 mg/d	32%	42%	55%	76%

*Dietary Reference Values (DRVs) for adults aged 19-70 years of age set by Department of Health (1991)

The nutritional data suggests that Teff bread is promising as healthy cereal product alternative, in terms of protein, fat and dietary fibre profile and particularly high level of iron and antioxidant capacity.

3.2 Rheological properties of wheat and Teff flour blends used for breadmaking

Addition of Teff flour had pronounced effects on dough properties. Rheological properties obtained by Farinograph and RVA of Teff blends are presented in Table 3.4.
Representative farinograms for each flour blend are shown in Figure 3.1, whilst pasting profile curves are presented in Figure 3.2.

Table 3.4 Selected rheological properties of Teff supplemented doughs

	Teff in dough (%)	0%	10%	20%	30%
FARINOGRAPH PARAMETERS	Water absorption (%)	61.3±0.2 a	62.2±0.2 b	62.8±0.2 c	64.1±0.1 d
	Dough stability (min)	8.0±0.5 a	7.5±0.7 a,b	6.6±0.1 b	5.2±0.1 c
	Dough development (min)	2.5±0.2 a	5.4±0.8 b	4.5±0.5 b	5.2±0.2 b
	Degree of softening (BU)	61.7±6.2 a	108.3±6.2 b	143.3±4.7 c	175.0±4.1 d
RAPID VISCO ANALYSER PARAMETERS	Peak Viscosity (RVU)	48.97±4.5 a	49.58±1.3 a	50.97±3.8 b	59.04±3.4 c
	Hot Paste Viscosity (RVU)	29.21±2.1 a	29.82±0.4 a	30.89±1.1 a	35.38±1.8 b
	Setback Viscosity (RVU)	31.17±2.6 a	35.34±1.0 a	41.32±2.6 b	51.06±2.0 c

Means±std dev in the same row followed by a different letter are significantly different (P<0.05; One-way independent ANOVA)

Water absorption (WA) of wheat flours blended with Teff flour significantly increased (P<0.05). This indicates a higher water absorption capacity of Teff flour. Ben-Fayed *et al*. (2008) and Mohammed *et al.* (2009) found a similar trend for Teff supplemented doughs. Teff flour has high water absorption capacity, which relates to the higher swelling degree of the gel phase of Teff starches and possibly small and uniform size of Teff starches granules, hence, providing larger surface area and the higher water absorption (Bultosa *et al.,* 2002), which confirms the findings from this study. WA was also shown to be

increased in wheat doughs supplemented with oat bran and amaranth flour (Krishnan *et al.,* 1987; Mlakar *et al.,* 2008a). Higher WA is one of the contributing factors for increased dough development time (DDT), which significantly (P<0.05) increased in Teff supplemented doughs. Similar findings were also obtained with amaranth flour supplemented wheat dough (Tosi *et al.,* 2002), which suggests that increased WA due to small size of amaranth and Teff grains might prolong DDT.

Dough stability time (DST) significantly decreased when Teff addition was at the highest levels (20 and 30%) (P<0.05). Degree of dough softening (DDS) has also significantly increased (P<0.05). As DST and DDS values give indication of dough tolerance and the rate of breakdown, this suggests that dough containing higher levels of Teff flour have less stability during mixing phase and are more prone to early and rapid breakdown. Similar results were obtained by Salehifar and Shahedi (2007) who studied the addition of oat flour to white wheat bread. This was found to increase water absorption, the degree of softening and decreased dough stability.

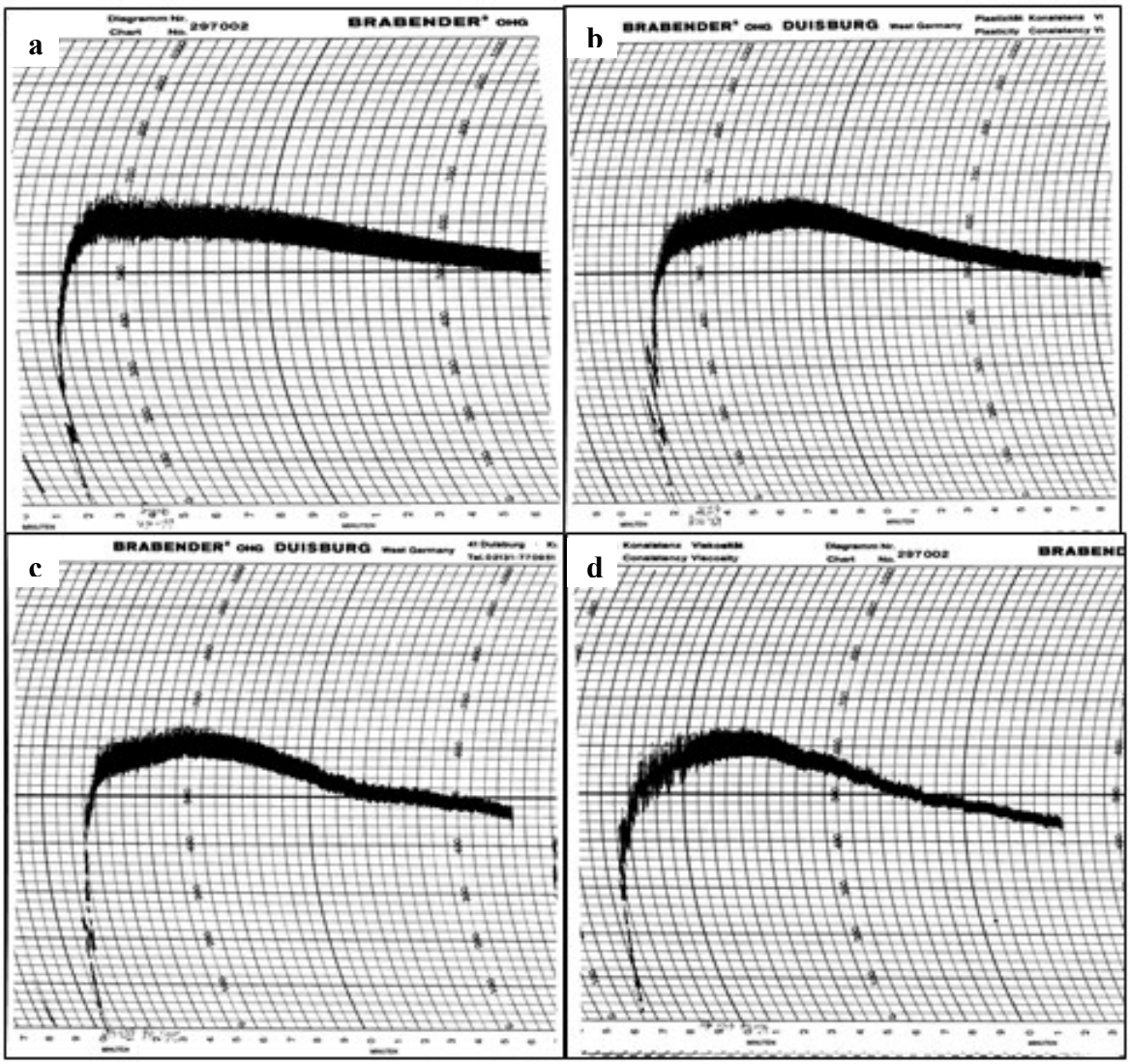

Figure 3.1 Farinograms of flour blends

a-strong white wheat flour, b- 10% Teff flour lend, c- 20% Teff flour blend, d- 30% Teff flour blend

Pasting profiles (Table 3.4, Figure 3.2) showed that there is a gradual increase in peak, hot paste and setback viscosities as the levels of Teff flour is increased.

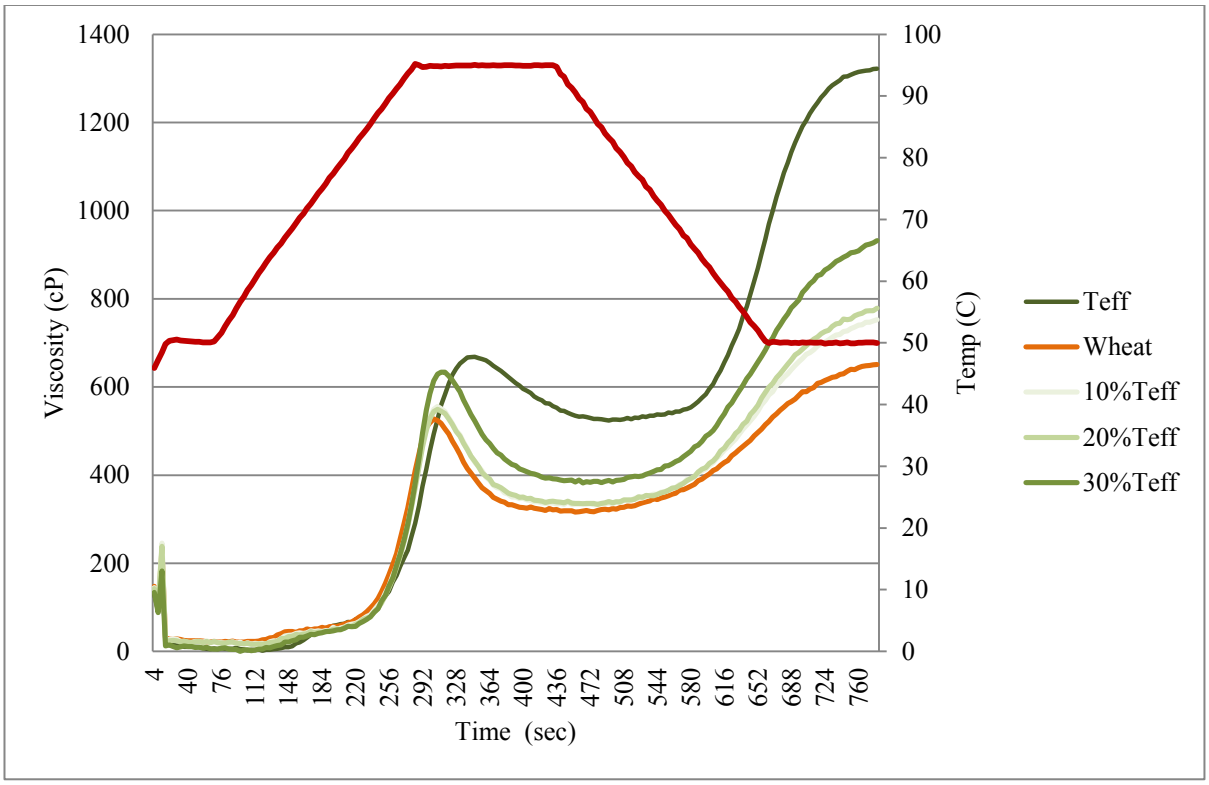

Figure 3.2 Pasting curves for Teff and wheat flours and their blends tested by RVA

3.3 The addition of Teff into straight dough breadmaking

Teff flour was incorporated into straight dough breadmaking at the levels of 0, 10, 20 and 30% (flour basis). The cross sections of the breads are shown in Figure 3.3.

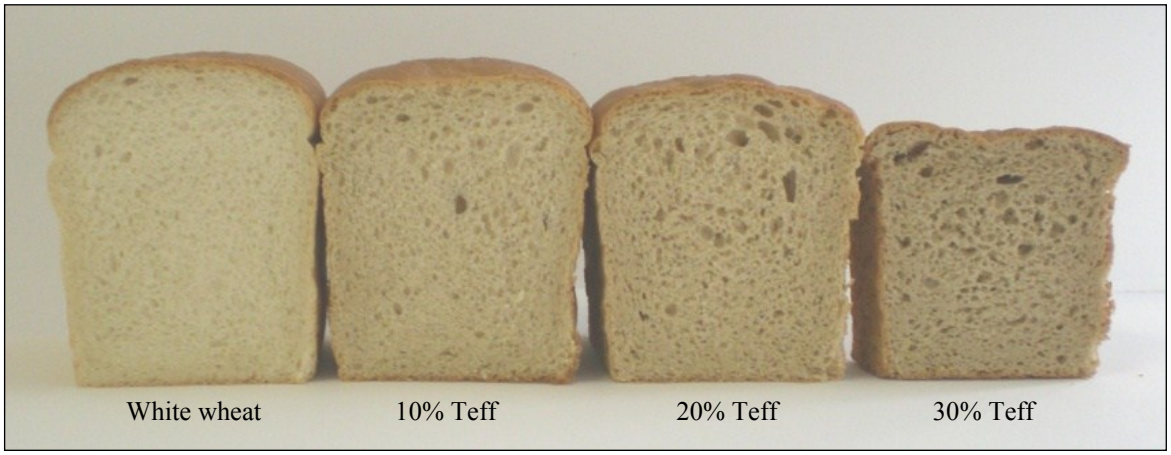

Figure 3.3 Straight dough breads

Specific loaf volume and loaf density of straight dough breads are detailed in Table 3.5. The results show that incorporation of Teff flour reduced loaf volume when 10 and 20% of Teff flour was incorporated. However, only addition of 30% Teff resulted in a significant decrease in loaf volume. Nevertheless, the increase in the level of Teff flour in

breadmaking formulation was negatively associated with specific volume (r=-0.83, *P*<0.001). A similar trend was observed in other studies (Ben-Fayed *et al.,* 2008; Mohammed *et al.,* 2009). Both studies found a gradual decrease in loaf volume as the level of Teff flour was increased. Because of the reduced loaf volume, the density of bread loaf was increased significantly for the highest level of Teff addition.

Table 3.5 Specific loaf volume and density of straight dough breads

BREAD	Specific loaf volume (ml/g)	Density (g/ml)
White Wheat bread	3.54±0.10 a	0.28±0.03 a
10% Teff bread	3.46±0.03 a	0.29±0.09 a, b
20% Teff bread	3.22±0.15 a, b	0.31±0.02 b
30% Teff bread	2.73±0.08 b	0.37±0.06 c

Means±std dev in the same column followed by a different letter are significantly different (*P*<0.05; One-way independent ANOVA)

Crumb firmness for wheat and Teff flour breads is presented in Figure 3.4.

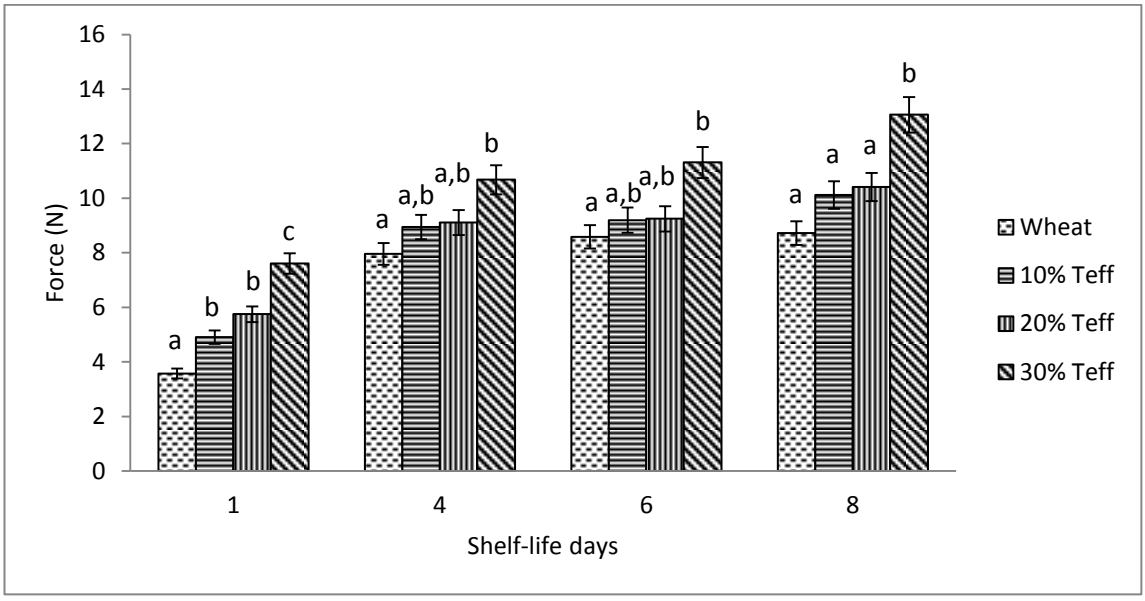

Figure 3.4 Changes in crumb firmness of straight dough breads during an 8-day shelf-life period

[a,b,c] Different superscripts indicate that means are significantly different (*P*<0.05; One-way independent ANOVA)

Initial crumb firmness (Figure 3.4) was significantly higher in Teff breads and the increase was gradual with higher Teff supplementation. Loaf volume is a major determining factor of crumb firmness (Axford *et al.,* 1968). In the present study all Teff breads had lower volumes than the control bread; therefore, it could be suggested that the increased crumb

firmness is a direct effect of this. During 8-day shelf-life period, all Teff breads had higher crumb firmness compared to white wheat bread, as the level of Teff positively correlates with increased crumb firmness (r=0.96; $P<0.001$). However, it should be noted that after four days of shelf-life, crumb firmness of 10 and 20% Teff breads was not significantly higher than that of wheat bread. However, at the highest level of Teff addition, crumb firmness during shelf-life was negatively affected.

Tested parameters of crumb grain of straight dough breads are presented in Table 3.6.

Table 3.6 Crumb cellular properties of wheat and Teff breads

SLICE	Slice Area (mm^2)	Slice Brightness (score)	Number of Cells	Wall Thickness (mm)	Cell Diameter (mm)	Cell Volume (mm^3)	Number of cells (mm^2)
Wheat bread	9123± 111 a	148± 2.8 a	5292± 376 a	0.454± 0.01 a	1.864± 0.12 a	5.70± 0.4 a	0.580± 0.04 a
10% Teff bread	8997± 275 a	126± 0.4 b	4881± 205 a, b	0.473± 0.01 b	1.979± 0.03 a, b	6.64± 0.2 b	0.542± 0.01 a
20% Teff bread	8468± 194 a	115± 1.1 c	4466± 182 b	0.482± 0.01 b	2.107± 0.05 a, b	7.75± 0.2 c	0.527± 0.01 a
30% Teff bread	6753± 102 b	103± 2.8 d	3739± 212 c	0.502± 0.01 c	2.251± 0.03 b	8.38± 0.3 c	0.554± 0.07 a

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

Analysis of crumb structure revealed changes in cellular parameters. Slice area has decreased in breads supplemented with Teff (r=-0.84, $P<0.001$). However, the significant decrease was only present in the highest level of Teff bread. Other cellular parameters, including cell wall thickness, cell diameter and volume had significantly increased in 20 and 30% Teff breads, whilst number of cells had notably decreased.

Slice brightness has significantly decreased with increased levels of Teff flour in bread (r=-0.98, $P<0.001$). As Teff flour is wholegrain flour, this could be expected, as bran particles, containing phenolic components, would cause a darker colour. Ben-Fayed *et al.* (2008) found breads containing 10-30% Teff flour to have significantly lower values for brightness, but higher values for yellowness and redness factors compared to corresponding wheat control bread.

Sensory evaluation of straight dough breads, as shown in Figure 3.5, revealed that increasing the level of Teff significantly (P<0.05) decreased crust and crumb lightness, whilst bitter flavour and aftertaste increased (P<0.05) in breads with 20 and 30% Teff flour. The overall acceptability was also significantly lower for 20 and 30% Teff breads, which was significantly correlated to bitter aftertaste (r= -0.62, P<0.01). This was also further supported by the multiple regression model (Table 3.7). Bitter flavour and bitter aftertaste contributed to 21 and 40%, respectively, of overall acceptability in this model. When non-significant overall acceptability contributors such as crust and crumb lightness, crust smoothness and sweet flavour were removed from this model, even stronger associations between bitter flavour and aftertaste were observed. Bitter flavour contributed to 22% of overall acceptability prediction score (beta coefficient value 0.224, P<0.01), whilst bitter aftertaste explained 46% of reduced acceptability (beta coefficient value 0.457, P<0.001). Similar findings were reported by Ben-Fayed *et al.* (2008) and Mohammed *et al*. (2009), as only bread with levels of Teff at 5 to 10% were judged as acceptable in the sensory tests.

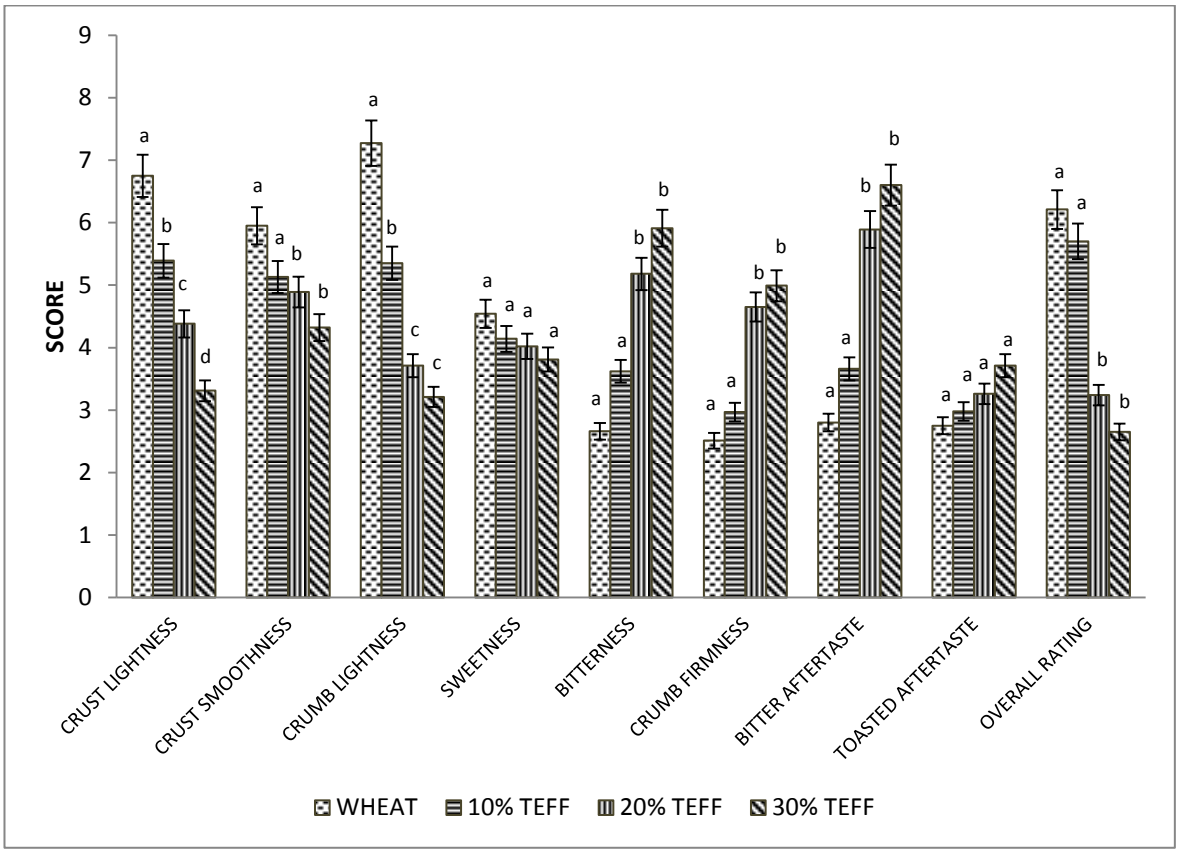

Figure 3.5 Sensory scores for straight dough breads judged by 50 panellists

[a,b,c,d] Different superscripts indicate that means are significantly different (P<0.05; One-way independent ANOVA)

Table 3.7 Multiple Regression Model for overall sensory evaluation

Variable	Beta coefficient value	% contribution to overall acceptability	*P* value
Crust lightness	-0.06	6%	0.4
Crust smoothness	0.045	5%	0.9
Crumb lightness	0.117	11%	0.5
Sweet flavour	-0.073	7%	0.1
Bitter flavour	0.207	21%	0.02*
Crumb firmness	0.014	1%	0.8
Bitter aftertaste	0.391	40%	0.001*
Toasted aftertaste	0.016	2%	0.8

Model summary: r=0.7, $P<0.001$
*represents a significant ($P<0.005$) individual contribution

3.4 Teff bread development using enzyme technology

3.4.1 Optimisation of baking process for enzyme-supplemented breads

Initial results indicated that straight dough bread recipe and processing conditions with supplier's recommended enzyme addition levels had a negative impact on the quality of the final product.

Initial enzyme supplementation levels, as recommended by the supplier, were 45ppm for xylanase, 4.5ppm for amylase, 15ppm for glucose oxidase and 45ppm for lipase. However, these quantities resulted in an overdose effect. The doughs, which had risen in the prover, lost the volume in the oven, hence, affecting the final volume and loaf shape. Figure 3.6 illustrates the bread product characteristics.

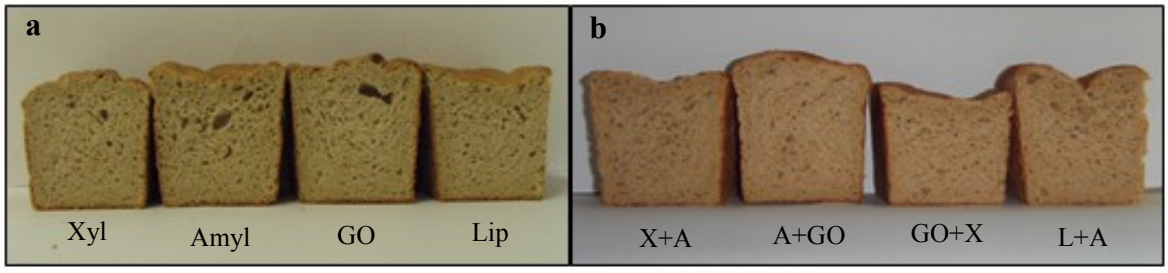

Figure 3.6 The effects of addition of high levels of single enzyme (a) and combination (b) on straight dough 30% Teff breads

Reduction of enzyme addition levels to the lowest recommended level for single enzyme addition and half of that for the combinations resulted in an improvement in bread quality,

in terms of higher loaf volume and better loaf shape (not collapsed at the top, similar to the shape of a control white wheat bread). This suggests that initial levels may have resulted in an overdose effect, which was observed by other researchers as well (Collar *et al.,* 2000). Therefore, lowest recommended levels for single enzyme supplementation and half of those dosages for combination supplementation were used for straight dough and sourdough breads (Table 2.5).

Table 3.8 Enzyme levels adjustments for 30% Teff conventional and sourdough breads

Enzyme combination (abbreviation)	Supplier recommended level	Level adjusted for single supplementation	Level adjusted for combinations
Xylanase (X)	30-60ppm	30ppm	15ppm
Amylase (A)	3-9ppm	3ppm	1.5ppm
Glucose oxidase (GO)	10-20ppm	10ppm	5ppm
Lipase (L)	30-60ppm	30ppm	15ppm

Initially, the same processing conditions as used for unsupplemented breads (55 min proving at 40°C and 85% humidity) were followed for enzyme-supplemented breads. However, decrease in dough quality parameters (handling, loss of dough volume from prover to the oven) resulted a decrease in bread quality (shape of loaves, specific volume, crumb firmness).

The effect on proving temperature and time were studied on the optimum single enzyme addition (Figure 3.7).

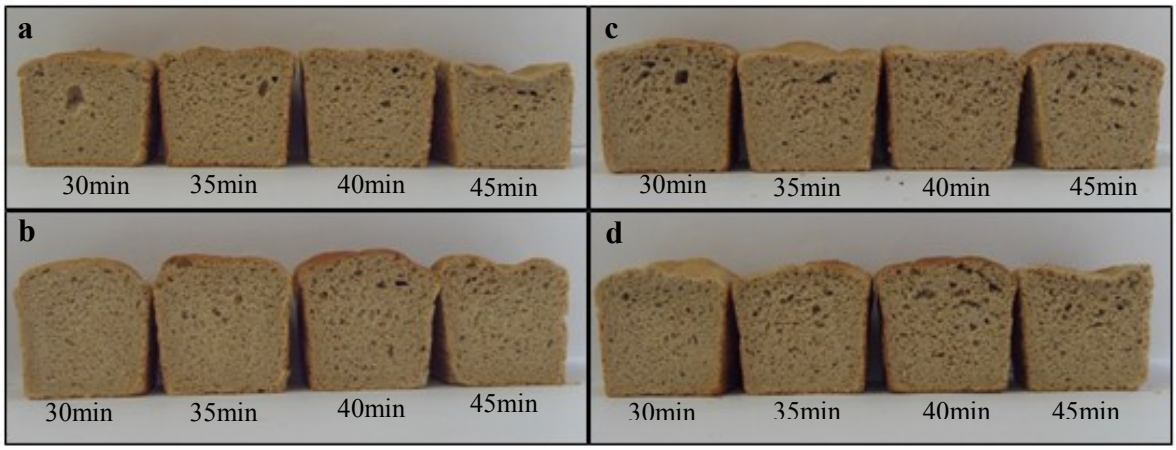

Figure 3.7 The effects of proving time on breads with added single enzymes
a- xylanase, b- amylase, c- glucose oxidase, d- lipase

The proving temperature was reduced from 40°C to 30°C, which corresponds to the optimum temperature for most enzymes (Cauvain and Young 1998). Furthermore, other studies used similar proving temperatures, ranging from 29°C (Sanz Penella *et al.,* 2008), 30°C (Gujral and Rosell 2004; Lagrain *et al.,* 2008) up to 35°C (Sahlstrom and Brathen 1997; Shah *et al.,* 2006).

The best loaf parameters were obtained for 30 min proving time, which was chosen as optimum proving time. Reduced fermentation time could be explained by the presence of enzymes, which act on dough components. The doughs supplemented with enzymes or improvers reach optimum consistency quicker (Kent and Evers 1994) because of increased substrate availability rate for the yeast, hence, this shortens proving time.

3.5 Straight dough breads with added enzymes

3.5.1 Straight dough 30% Teff breads with single enzyme addition

Cross section of Teff breads with single enzyme addition are presented in Figure 3.8. The enzyme supplemented breads were compared with control 30% Teff bread.

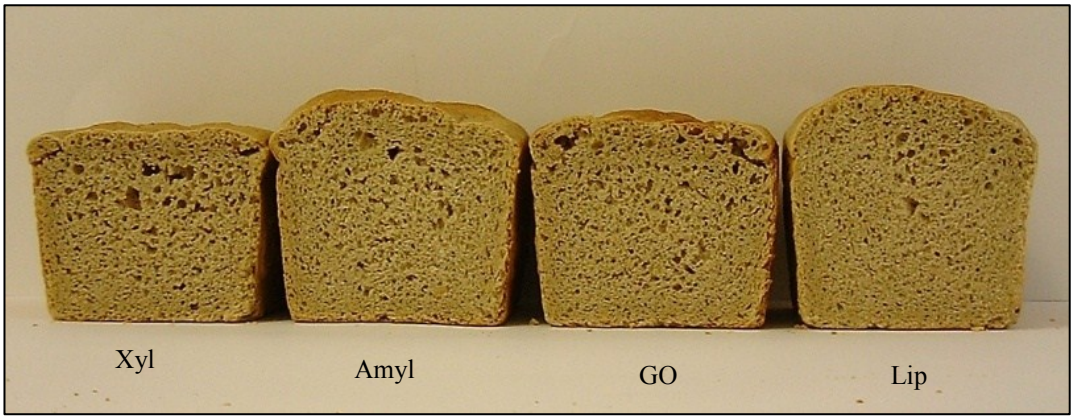

Figure 3.8 Straight dough 30% Teff breads with single enzymes

Addition of single enzymes showed improvements in the physical and structural properties of the bread produced (Table 3.9 and Table 3.10). However, the significant improvements in specific loaf volume and density were only obtained in amylase bread. In terms of crumb cellular structure, there was a significant improvement in slice area of the bread supplemented with amylase and crumb brightness parameter.

Table 3.9 Specific loaf volume and density of straight dough 30% Teff breads with single enzymes

BREAD	Specific loaf volume (ml/g)	Density (g/ml)
Control	2.73±0.08 a	0.37±0.06 a
Xylanase	3.03±0.21 a, b	0.33±0.09 a, b
Amylase	3.15±0.07 b	0.32±0.05 b
GO	3.11±0.15 a, b	0.32±0.06 b
Lipase	3.06±0.08 a,b	0.33±0.04 a,b

Means ±std dev in the same column followed by a different letter are significantly different (P<0.05; One-way independent ANOVA)

Table 3.10 Crumb structure of straight dough 30% Teff breads with single enzymes

SLICE	Slice Area / mm²	Slice Brightness	Number of Cells	Wall Thickness / mm	Cell Diameter / mm	Cell Volume	Number of cells /mm²
Control	6753± 102 a	103± 2.8 a	3739± 212 a	0.502± 0.01 a	2.25± 0.03 a	8.38± 0.3 a	0.554± 0.07 a
Xyl	6441± 156 a	106± 0.7 b	3448± 237 a	0.500± 0.01 a	2.17± 0.16 a	8.03± 0.8 a	0.536± 0.03 a
Amyl	7565± 263 b	110± 2.1 c	3898± 332 a	0.495± 0.02 a	2.23± 0.31 a	8.27± 0.5 a	0.516± 0.05 a
GO	6551± 198 a	104± 1.0 a	3580± 197 a	0.495± 0.01 a	2.17± 0.19 a	8.11± 0.9 a	0.547± 0.03 a
Lip	6694± 129 a	107± 1.5 c	3528± 119 a	0.497± 0.02 a	2.18± 0.29 a	7.95± 1.0 a	0.529± 0.04 a

Means±std dev in the same column followed by a different letter are significantly different (P<0.05; One-way independent ANOVA)

3.5.2 Straight dough 30% Teff breads with added enzyme combinations

Supplementation with combinations of enzymes affected physical (Table 3.11), shelf-life (Figure 3.10), crumb structure (Table 3.12) and sensory (Figure 3.11) properties of straight dough 30% Teff breads produced (Figure 3.9).

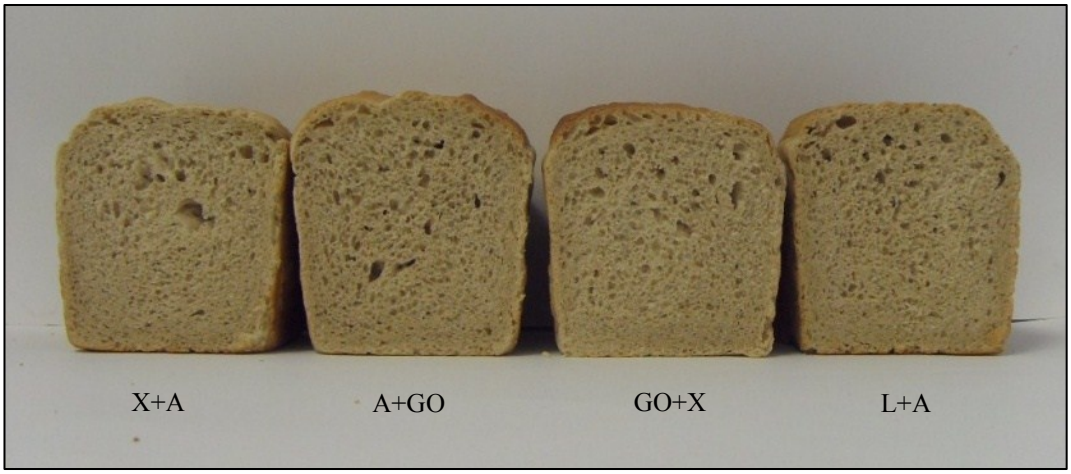

Figure 3.9 Straight dough 30% Teff breads with enzyme combinations

All enzyme combinations significantly improved specific volume and density (Table 3.11), crumb firmness during a shelf-life period (Figure 3.10), crumb cellular structure (Table 3.12) and sensory scores (Figure 3.11); and most of the parameters were comparable to those of white wheat bread.

Addition of all enzyme combination showed significant improvements in specific loaf volume of 30% Teff bread. As a result, the produced breads had similar loaf volume and density to the white wheat bread (3.54ml/g specific loaf volume, 0.28g/ml loaf density).

Table 3.11 Specific loaf volume and density of straight dough 30% Teff breads with enzyme combinations

BREAD	Specific loaf volume (ml/g)	Density (g/ml)
Control	2.73± 0.08 a	0.37± 0.06 a
X+A	3.56± 0.02 c	0.28± 0.01 c
A+GO	3.56± 0.10 c	0.28± 0.08 c
GO+X	3.53± 0.08 c	0.28± 0.04 c
L+A	3.30± 0.08 b	0.30± 0.05 b

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

Combination of enzymes significantly reduced crumb firmness of Teff breads. This trend was observed during the whole 8-day storage period for all of the combinations, with the exception of L+A bread.

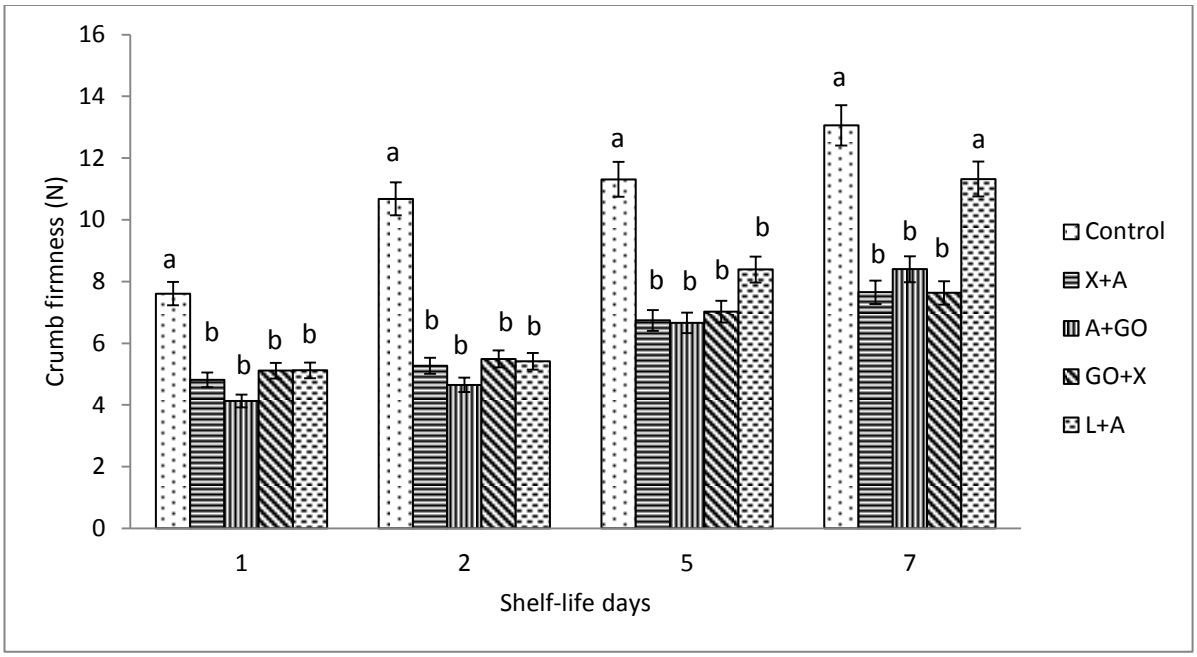

Figure 3.10 Shelf-life of straight dough 30% Teff bread supplemented with enzyme combinations

[a,b,c] Different superscripts indicate that means are significantly different (P<0.05; One-way independent ANOVA)

Enzyme combinations significantly improved some of the crumb cellular structure parameters. The crumb of enzyme supplemented Teff breads had a finer structure with larger number of cells that were smaller in size and that had thinner walls compared to the control bread. This indicates that enzyme addition had a positive effect on crumb cellular structure, which was finer and more uniform.

Table 3.12 Crumb cellular structure of straight dough 30% Teff bread with enzyme combinations

Slice Name	Slice Area / mm²	Slice Brightness	Number of Cells	Wall Thickness / mm	Cell Diameter / mm	Cell Volume	No of cells/mm²
Control	6753± 102 a	103± 2.8 a	3739± 212 a	0.502± 0.01 a	2.25± 0.03 a	8.38± 0.3 a	0.554± 0.07 a
X+A	7418± 318 a	116± 2.3 b	4339± 295 b	0.467± 0.01 b	1.889± 0.01 b	6.42± 0.4 b	0.586± 0.03 a
A+GO	7608± 448 a	117± 2.0 b	4184± 277 b	0.478± 0.01 b	2.055± 0.02 a, b	7.12± 0.7 a, b	0.551± 0.03 a
GO+X	7476± 324 a	118± 1.6 b	4379± 253 b	0.465± 0.01 b	1.891± 0.02 b	6.44± 0.8 b	0.587± 0.04 a
L+ A	7283± 328 a	115± 1.3 b	3982± 294 a	0.486± 0.01 b	2.102± 0.09 a, b	7.41± 0.5 a	0.548± 0.02 a

Means±std dev in the same column followed by a different letter are significantly different (P<0.05; One-way independent ANOVA)

Sensory evaluation results showed higher acceptability scores for enzyme supplemented Teff breads. The greatest improvements were seen with the addition of A+GO combination. The sensory panellist scored this bread significantly higher for textural, flavour and overall acceptability compared to the control bread. Furthermore, the overall acceptability score was similar to that of white wheat bread (6.21 *v* 5.74 for white wheat bread and A+GO 30% Teff bread, respectively).

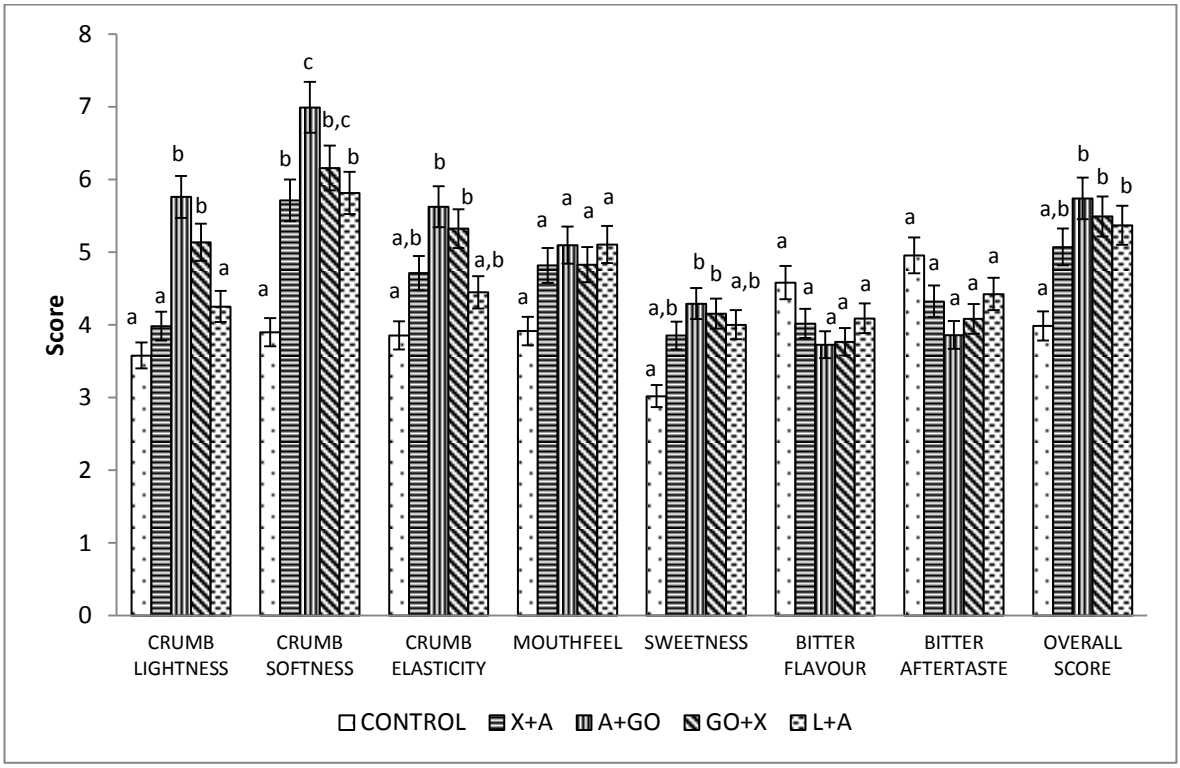

Figure 3.11 Sensory scores for straight dough enzyme supplemented 30% Teff breads
[a,b,c] Different superscripts indicate that means are significantly different ($P<0.05$; One-way independent ANOVA)

In summary, addition of various enzyme combinations showed improved bread quality parameters, such as higher loaf volumes, lower crumb firmness during shelf-life period and a finer and more uniform crumb cellular structure. The greatest improvements were observed in breads supplemented with the gluten strengthening enzyme glucose oxidase and the substrate availability enhancing enzymes amylase and xylanase.

3.6 Sourdough breads

The cross-section view of sourdough breads are shown in Figure 3.12.

Slight differences between LV1 and LV2 starter sourdough breads were observed. Firstly, LV1 starter sourdough breads had more subtle sourdough taste compared to more acidic taste of LV2 starter sourdough bread. This might be explained by the bacteria level in

starter culture. LV1 contains *Lactobacillus casei* and *Lactobacillus brevis* at the levels of 2%, while LV2 contains only *Lactobacillus brevis* at the levels up to 5% (Fermex International Ltd 2004). Lower level of LAB in LV1 would result IN less production of lactic and acetic acids, which explains the milder acidic taste of LV1 sourdough bread.

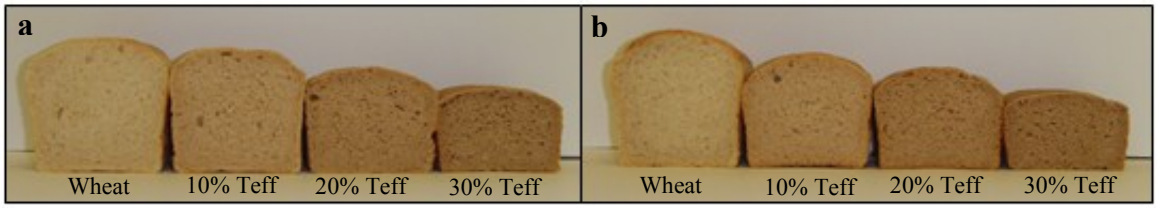

Figure 3.12 Sourdough breads
a- LV1 starter culture, b- LV2 starter culture

Incorporation of Teff into sourdough breadmaking affected physical properties of breads. Both starter cultures (LV1 and LV2) yielded similar quality bread loaves.
Increasing the level of Teff significantly reduced specific volume and increased density.

Table 3.13 Specific loaf volume and density of sourdough breads

Starter culture	BREAD	Specific loaf volume (ml/g)	Density (g/ml)
LV1	**White Wheat**	3.32 ± 0.27 a	0.30 ± 0.02 a
	10% Teff	2.75 ± 0.10 b	0.36 ± 0.02 b
	20% Teff	2.23 ± 0.11 c	0.45 ± 0.09 c
	30% Teff	2.00 ± 0.09 c	0.50 ± 0.15 d
LV2	**White Wheat**	3.15 ± 0.11 a,b	0.32 ± 0.05 a
	10% Teff	2.84 ± 0.19 b	0.35 ± 0.04 b
	20% Teff	2.39 ± 0.09 c	0.42 ± 0.07 c
	30% Teff	1.96 ± 0.18 c	0.51 ± 0.06 d

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

A number of researchers suggest that sourdough may improve loaf volume compared to straight dough breadmaking (Esteve *et al.,* 1994; Hansen and Hansen 1996; Katina *et al.,* 2006a). However, this was not observed in this study, as straight dough breadmaking yielded higher loaf volume and lower densities. This might be firstly explained by the differences in recipes. Straight dough bread recipes contained different proportions of ingredients, as well as additional dough conditioners, such as shortening and improver. Furthermore, the improvements in loaf volume was also not seen in gluten-forming protein

lacking cereal sourdough breads (Moore *et al.,* 2007), which might be a reason why sourdough did not improve loaf volume in Teff breads.

Texture analysis has revealed significant changes in crumb firmness with the increased level of Teff. Incorporation of Teff resulted in a gradual increase in crumb firmness during 8-day bread shelf-life (Figure 3.13). This could be expected as lower specific loaf volume contributes to higher crumb firmness (Axford *et al.,* 1968).

Both starter cultures' breads have shown similar crumb firmness values during 8-day shelf-life period. 30% Teff LV1 sourdough bread had slightly lower crumb firmness than LV2 bread.

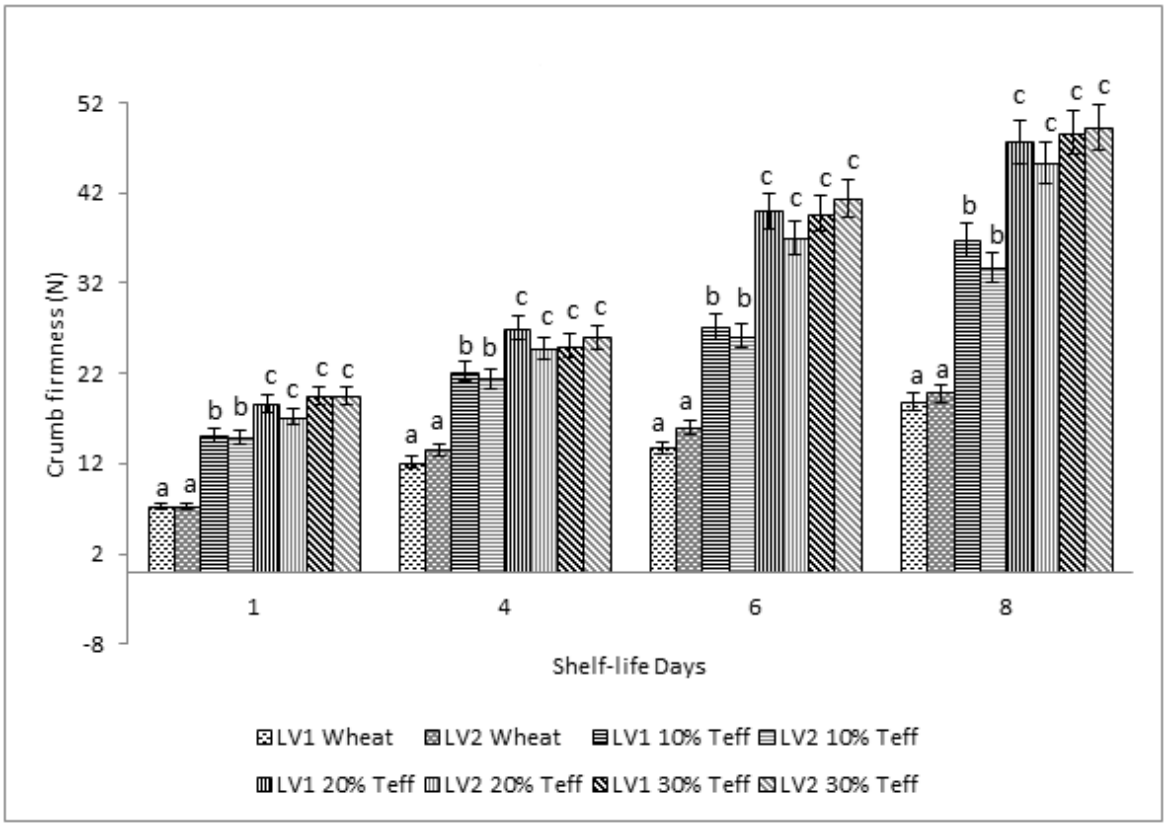

Figure 3.13 Shelf-life of sourdough breads with LV1 and LV2 starters

[a,b,c] Different superscripts indicate that means are significantly different ($P<0.05$; One-way independent ANOVA)

Image analysis revealed changes in crumb cellular properties. Both sourdough starter cultures showed similar patterns in crumb structure (Table 3.14).

Increasing the level of Teff significantly reduced the slice area, crumb brightness, cell diameter and number of cells in sourdough breads. However, there was no significant difference in other crumb structure parameters. Cell wall thickness and cell volumes were not affected by the addition of Teff.

Table 3.14 Crumb cellular structure of sourdough breads

SLICE	Slice Area (mm^2)	Slice Brightness (score)	Number of Cells	Wall Thickness (mm)	Cell Diameter (mm)	Cell Volume (mm^2)	Number of cells (mm^2)
Wheat $_{LV1}$	6845± 146 a	131.1± 1.1 a	4106± 191 a	0.450± 0.001 a	1.60± 0.02 a	4.80± 0.1 a	0.642± 0.01 a
10% Teff $_{LV1}$	6059± 155 c	115.9± 0.3 c	4153± 229 a	0.447± 0.01 a	1.54± 0.11 b	4.92± 0.5 b	0.686± 0.05 b
20% Teff $_{LV1}$	4947± 212 e	99.7± 1.2 e	3474± 140 b	0.449± 0.01 a	1.53± 0.02 b	5.15± 0.1 b	0.702± 0.01 b
30% Teff $_{LV1}$	4229± 137 f	88.6± 0.3 g	3171± 176 b	0.441± 0.01 b	1.44± 0.03 b	4.95± 0.2 a	0.750± 0.02 b
Wheat $_{LV2}$	6390± 195 b	127.5± 0.8 b	4120± 119 a	0.464± 0.01 a	1.71± 0.07 a	5.24± 0.2 a,b	0.602± 0.03 a,c
10% Teff $_{LV2}$	5320± 162 d	110.7± 0.6 d	4117± 187 a	0.434± 0.01 b	1.34± 0.05 b	4.10± 0.2 a	0.775± 0.02 b,c
20% Teff $_{LV2}$	4725± 169 e	96.1± 1.1 f	3792± 181 b	0.434± 0.01 b	1.31± 0.03 b	4.23± 0.2 a	0.802± 0.01 b
30%Teff $_{LV2}$	3951± 175 f	85.7± 1.2 h	3151± 126 b	0.432± 0.01 b	1.35± 0.07 b	4.56± 0.3 a	0.797± 0.02 b

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

LV1 starter culture sourdough bread showed more advantageous physical (Table 3.13), textural (Figure 3.13) and crumb cellular properties (Table 3.14), therefore, it was chosen for enzyme supplementation stage of Teff bread product development.

3.7 Sourdough breadmaking with added enzyme combinations

The breads are presented in Figure 3.14.

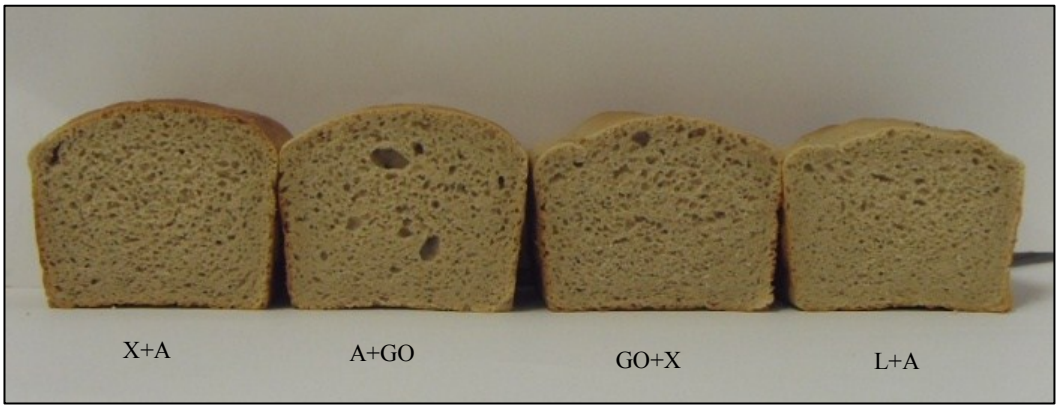

Figure 3.14 LV1 sourdough 30% Teff breads with enzyme combinations

Addition of all enzyme combinations significantly improved loaf volume and density (Table 3.15). A combination of X+A showed the greatest improvement, followed by A+GO, GO+X and L+A. The improvements in X+A combination in sourdough obtained by the present study are in agreement with other researchers who demonstrated similar effects of this combination (Martinez-Anaya *et al*., 1998). L+A combination produced fewer bread quality improvements than any other combinations. This trend was also observed in straight dough 30% Teff breads.

Table 3.15 Physical characteristics of 30% Teff sourdough breads with enzyme combinations

BREAD	Specific loaf volume (ml/g)	Density (g/ml)
Control	2.00 ± 0.09 a	0.50 ± 0.15 a
X+A	2.67 ± 0.10 c	0.37 ± 0.02 c
A+GO	2.61 ± 0.07 c	0.38 ± 0.07 c
GO+X	2.55 ± 0.09 b,c	0.39 ± 0.05 b,c
L+A	2.46 ± 0.05 b	0.41 ± 0.01 b

Means±std dev in the same column followed by a different letter are significantly different (P<0.05; One-way independent ANOVA)

The favourable effect of addition of enzymes was also observed in crumb firmness during shelf-life (Figure 3.15). All enzyme combinations had a significant impact in the shelf-life, however, the greatest improvement was in X+A supplemented bread. On the eight day of

shelf-life the crumb of X+A Teff bread was nearly twice softer when compared to the corresponding control bread. This is in agreement with other authors who used X+A combination for improving loaf volume and crumb firmness in sourdough breads (Martinez-Anaya *et al.,* 1998; Andreu *et al.,* 1999).

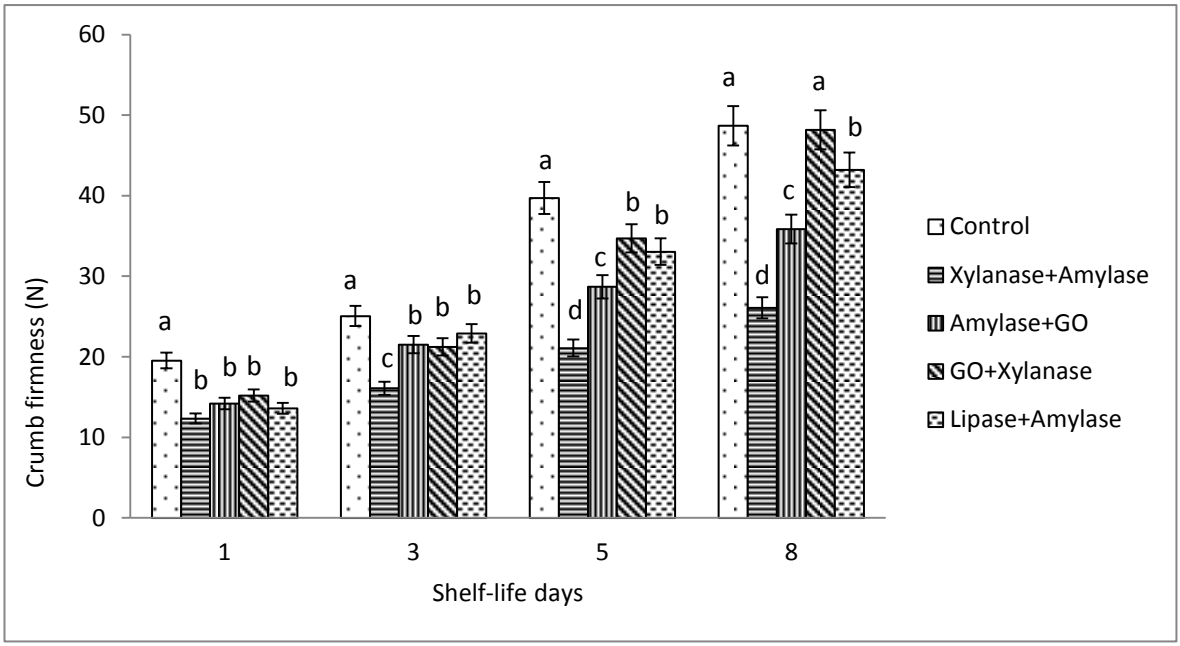

Figure 3.15 Shelf-life of 30% Teff sourdough breads with the addition of enzyme combinations

[a,b,c,d] Different superscripts indicate that means are significantly different (P<0.05; One-way independent ANOVA)

Image analysis revealed changes in cellular crumb structure (Table 3.16). All of the enzyme combinations resulted in significantly larger slice areas compared to the control bread. However, other parameters indicated a coarse structure of the breads crumb: large cell diameter and volume, less cells that have thicker walls. As sourdough with no enzymes showed a fine crumb structure but low bread volume, the addition of enzymes and improvement in loaf volume resulted in an expansion of gas cells in the dough, hence, coarser structure of the crumb. In fact, the finest crumb structure (many small volume cells with thinner walls) was observed in L+A sourdough bread, which yielded the lowest loaf volume of all the enzyme combinations.

Table 3.16 Cellular structure of 30% Teff sourdough breads with enzyme combinations

Slice Name	Slice Area / mm²	Slice Brightness	Number of Cells	Wall Thickness / mm	Cell Diameter / mm	Cell Volume	No of cells /mm²
Control	4229± 137 a	88.6± 0.3 a	3171± 176 a	0.441± 0.01 a	1.44± 0.03 a	4.95± 0.2 a	0.750± 0.02 a
X+A	5743± 102 c	101± 1.0 b	3421± 84 b	0.479± 0.01 b	1.827± 0.08 b	6.58± 0.3 b	0.596± 0.02 b
A+GO	5668± 103 c	101± 1.1 b	3425± 87 b	0.481± 0.01 b	1.794± 0.05 b	6.34± 0.2 b	0.604± 0.01 b
GO+X	5655± 100 c	102± 1.0 b	3531± 126 b	0.474± 0.01 b	1.724± 0.09 b	6.09± 0.4 b	0.624± 0.03 b
L+A	5393± 126 b	105± 1.0 c	3747± 121 c	0.451± 0.01 a	1.557± 0.09 a	5.11± 0.04 a	0.695± 0.02 a

Means±std dev in the same column followed by a different letter are significantly different ($P<0.05$; One-way independent ANOVA)

In summary, findings from the addition of different enzyme combinations to sourdough Teff breads showed improvements in loaf volume and shelf-life parameters. This was especially seen in xylanase and amylase supplemented breads.

Chapter 4 Teff bread development - Discussion

Bread has been a popular staple food for many years. Recent investigations show downward trends in purchases of white bread, yet an increase in wholegrain and ethnic breads (Department for Environment Food and Rural Affairs 2008b). This factor has resulted in the development of baked products incorporating less-utilised and more-nutritious grains. Such cereal products are considered to be of high nutritional value and provide higher levels of important nutrients, including protein, fibre, vitamins and minerals. Therefore, Teff cereal offers a good prospect of improving the nutritional value of wheat bread.

In the present study the first objective was to increase the iron level content of wheat breads by adding Teff (*Eragrostis tef*) wholegrain flour. The second objective was to study the effect of Teff incorporation on textural and sensory properties of breads. Finally a number of enzymes and their combinations were used to improve the quality of Teff bread.

Nutritional composition analysis of wheat and Teff flours used for breadmaking showed that there was no significant difference in their protein and fat content, however, dietary fibre, iron and total antioxidant capacity were significantly higher in Teff flour (Table 3.1). The obtained value of 13.7g/100g of protein content in Teff flour is similar to the literature values, which vary from 9.6g/100g (National Research Council 1996) to 14.6g/100g (Griffith and Castell-Perez 1998). However, it should be noted that Teff is gluten-free flour; therefore, crude protein content value comparison with wheat flour does not take into account the quality of protein and amino acids. Research indicates, Teff contains more adequate essential amino acids balance compared to wheat (Baptist and Perera 1956; Jansen *et al.,* 1962). Hence, similar levels of total protein in Teff and wheat would not represent the quality of the protein.

Teff is whole-grain flour, whilst white wheat is refined flour; hence, significantly lower content of dietary fibre was present in the wheat flour than Teff flour in the present study.

In terms of iron content, Teff flour contained significantly more than wheat flour (Table 3.1). A number of other researchers reported high iron levels in Teff seeds and flour; therefore, the present study confirms these findings. Teff flour contained five times the amount of iron, which indicates there is a possible positive health attributes when Teff is used in bread products. Furthermore, in the UK, white wheat flour is fortified with iron to the level not less than 1.65 mg/100g (The Bread and Flour Regulations 1998), therefore, it

could be suggested that the value of intrinsic iron in the white wheat flour might be even lower.

Teff flour had also significantly higher total antioxidant capacity when compared to white wheat flour (Table 3.1). As Teff flour is whole-grain flour, antioxidant properties are enhanced by the bran particles, which are rich in polyphenol components, present in the flour, hence contributing to the higher antioxidant capacity.

As a result of iron-rich Teff flour incorporation, Teff breads contained significantly more iron compared to the corresponding wheat bread. In fact, on average 30% Teff bread contained more than twice the amount of iron compared to wheat bread. Furthermore, fermentation during breadmaking process is reported to enhance bioavailability of iron further (Poutanen *et al.,* 2009). Ramachandran and Bolodia (1984) demonstrated that fermentation of Teff increased dialysable part of iron from 9% to 24%, hence, increasing bioavailability of iron in food. A similar trend was also observed in another study where fermentation of Teff has resulted in a decrease in phytic acid content, a known inhibitor of non-haem iron absorption, by 72% (Urga *et al.,* 1997). Thus although iron bioavailability in Teff breads were not measured in this study, it is likely that the iron present is more bioavailable.

Another nutritional property of the breads that has been significantly affected by the addition of Teff was total antioxidant capacity. Breads containing 10%, 20% and 30% Teff flour had significantly higher total antioxidant capacity compared with the control wheat bread. Although the research on the total antioxidants levels in Teff is very sparse, some researchers reported the content of phenolic compounds in Teff to be comparable to other wholegrain millets (McDonough and Rooney 1985). Other researchers also improved antioxidant levels in bread when refined wheat flour was substituted with wholegrain barley flour (Holtekjolen *et al.,* 2008) and unhusked buckwheat flour (Lin *et al.,* 2009).

There has been an increasing evidence suggesting a role of antioxidants in prevention of chronic diseases such as coronary heart disease, cancer, diabetes and Alzheimer's disease (Temple 2000). In this study, all Teff breads had significantly higher total antioxidant capacity indicating that if Teff breads were incorporated in the daily diet, they would contribute towards higher antioxidant intake.

In terms of Dietary Reference Values (DRVs) (Table 3.3), Teff breads would contribute to notably higher intakes of dietary iron and similar intakes of protein and fibre if 200g of

bread were consumed daily. The most noticeable difference in contribution between wheat and Teff breads was the dietary iron, which would be notably higher if Teff breads were incorporated as a part of habitual diet. Higher dietary iron intake would be beneficial even more for iron deficiency susceptible population groups, such as children, female adolescents and childbearing age women, elderly, and female athletes. Research indicates that in the UK an average female consumes 10.0 mg of iron a day (Bates *et al.,* 2009), which is significantly lower than recommended nutrient intake (RNI) of 14.8 mg/day (Department of Health 1991). In fact, 200 g daily intake of 30% Teff bread would contribute over 75% of recommended female's intake, which makes Teff bread a rich source of iron. Teff bread would also provide an adequate intake of protein and fibre.

The quality evaluation of breads showed that incorporation of Teff into breadmaking affected final loaf volume, crumb firmness, crumb structure and taste attributes. Similar effects on bread quality were also reported by other authors who used Teff and other wholegrain gluten-free cereal supplementation in breadmaking (Oomah 1983; Salehifar and Shahedi 2007; Ben-Fayed *et al.,* 2008; Mlakar *et al.,* 2008a; Mlakar *et al.,* 2008b; Mohammed *et al.,* 2009).

Farinograph values, such as DDT, DS and DDS, of Teff flour dough showed a decrease in dough quality parameters (Table 3.4). This is probably due to the weakening of the dough by the dilution of the functional gluten-forming proteins; hence, the effect was greatest in 30% Teff dough. This trend was also reported by other researchers (Ben-Fayed *et al.,* 2008; Mohammed *et al.,* 2009). However, WA of Teff doughs increased, which is a positive effect in terms of higher yield of the final product. This was also supported by pasting profile analysis as 30% Teff flour blend had significantly higher peak viscosity, which indicates higher water-binding capacity of this flour blend (Newport Scientific 2001). Increased WA values of Teff supplemented doughs were also reported by other authors (Ben-Fayed *et al.,* 2008; Mohammed *et al.,* 2009). WA was also shown to be increased in wheat doughs supplemented with oat bran and amaranth flour (Krishnan *et al.,* 1987; Mlakar *et al.,* 2008a). Higher bran proportion, which is rich in soluble and insoluble fibres, in wholegrain flours seem to be responsible for increased WA capacity of supplemented doughs, which explains the findings from this study. In terms of flour blends pasting properties, the highest level of Teff flour incorporation show higher starch retrogradation as seen by significantly (P<0.05) higher setback viscosity of Teff flour compared to white wheat flour (Figure 3.2).

In this study, significantly lower specific loaf volumes and poorer crumb structure were also observed in straight dough and sourdough breads with higher substitution levels of Teff flour (Table 3.5, Table 3.6, Table 3.13 and Table 3.14). Teff enriched breads had a more open and coarse crumb structure with fewer cells, which have thicker walls and are larger in size. The most plausible explanation for the structure changes with Teff flour addition would be changes in dough elasticity due to lack of gluten-forming proteins, which would result in poorer gas holding capacity during proving and consequently less uniform crumb cellular structure. This trend was also reported by other researchers who used gluten-free crops for breadmaking (Krishnan *et al.,* 1987; Tosi *et al.,* 2002; Salehifar and Shahedi 2007; Mlakar *et al.,* 2008b). Additionally, lower bread quality of Teff breads could be explained by the disruption of the gluten matrix in the dough by the presence of bran particles in Teff flour. Lower loaf volume and poorer crumb cellular structure was reported in oat supplemented breads due to the large amount of fine bran particles in dough (Zhang and Moore 1999). Similarly to straight dough breads, incorporation of Teff significantly reduced slice area, crumb brightness, cell diameter and number of cells in sourdough breads. However, wall thickness and cell volumes were not affected by the addition of Teff, which was opposite to the results obtained from the straight dough breadmaking trials. This suggests that although volume of Teff breads was significantly reduced, sourdough technology had a favourable effect on some crumb parameters. In fact, some researchers suggest that acidification has a positive effect on bread properties, which may lead to more cohesive crumb and more uniform structure (Esteve *et al.,* 1994). Acidic conditions cause gluten to swell, which is known to increase softness and elasticity of the dough (Schober *et al.,* 2003) and hence, may have a possible favourable effect on crumb structure and uniformity as seen in the present study.

Crumb firmness during 8-day shelf-life period of Teff breads was significantly higher compared to control wheat bread (Figure 3.4). However, it should be noted that after four days shelf-life, crumb firmness of the 10 and 20% Teff breads was not significantly higher than that of the corresponding wheat bread. This indicates a possible positive effect on the rate of crumb firming, when Teff flour is incorporated at the lower addition of 10 to 20%. Research indicates that Teff starches have lower retrogradation tendency than maize or wheat starches (Bultosa and Taylor 2004a), and hence have a possible favourable effect on bread crumb firming rate. The results from this study indicate that addition of lower levels of Teff flour (10-20%) do not have a detrimental effect on crumb firming rate. However, at

the highest level of Teff addition, crumb firmness during shelf-life is negatively affected. This is also in agreement with pasting profile values as 30% Teff flour blend shows a significantly higher setback viscosity, which indicates a higher rate of starch retrogradation during the product's shelf-life period when compared to bread made only from wheat flour.

In the present study bitter flavour and aftertaste (r=-0.62, $P<0.01$) were the main contributors to the lower acceptability rating. Teff flour is wholegrain flour; therefore, higher levels of bran particles might contribute to the bitter taste and aftertaste. Phenolic compounds, found in cereal bran, are considered to be one of the main contributing factors to bitterness. High quantities of these compounds were shown to have a great impact on the perceived bitterness and aftertaste in rye grain (Heinio *et al.,* 2008). Furthermore, Heinio *et al.* (2008) suggested that in particular vanillic and veratric acids were related to cereal and intense aftertaste, whilst pinoresinol and syringic acid were associated with bitterness. Teff cereal contains one of the highest levels of vanillic and syringic acids compared to other millet grains (McDonough and Rooney 1985), which suggests that the bitter flavour and aftertaste in Teff breads observed in the present study was probably due to the high levels of these phenolic compounds. This is also in agreement with other researchers who suggested that bitter flavour of bread is caused by the higher levels of bran particles (Salehifar and Shahedi 2007; Holtekjolen *et al.,* 2008; Mohammed *et al.,* 2009). Teff breads in the present study had significantly higher total antioxidant capacity (due to high levels of phenolic compounds) when compared to the white wheat bread, which suggests that high level of these phenolic components may be the main contributing cause to bitterness in flavour and aftertaste of Teff breads.

Preliminary experimental work in the present study identified a favourable nutritional profile of Teff bread at the addition of 30%. However, poor quality parameters of Teff bread produced highlighted the need for textural improvements.

A number of researchers utilised single and combined enzyme treatments for improving quality of breads (Laurikainen *et al.,* 1998; Katina *et al.,* 2006b). Research suggests that combined use of enzymes may have more notable effects on breadmaking properties, mainly due to the fact that enzymes acting on different flour components can be utilised at once and some of the enzymes can diminish negative effects of one another (Caballero *et al.,* 2007b; Caballero *et al.,* 2007a). This trend was also observed in the present study. Although single enzyme treatments resulted in improvement in bread quality (higher loaf

volume, lower crumb firmness during shelf-life and more uniform crumb structure), more notable changes were observed in combined enzyme breads.

Enzymes and their combinations used in Teff bread development were chosen from the literature search (Literature review section 1.4.1). In straight dough Teff breads, improvements in bread quality and sensory attributes were seen with all enzyme combinations. Nevertheless, A+GO and GO+X were the most successful, closely followed by X+A and L+A, whilst in the sourdough breads X+A and A+GO showed the greatest improvements in bread quality. The improvements observed in the recent study may be explained by the individual and synergistic effects of enzymes and their combinations (Figure 4.1).

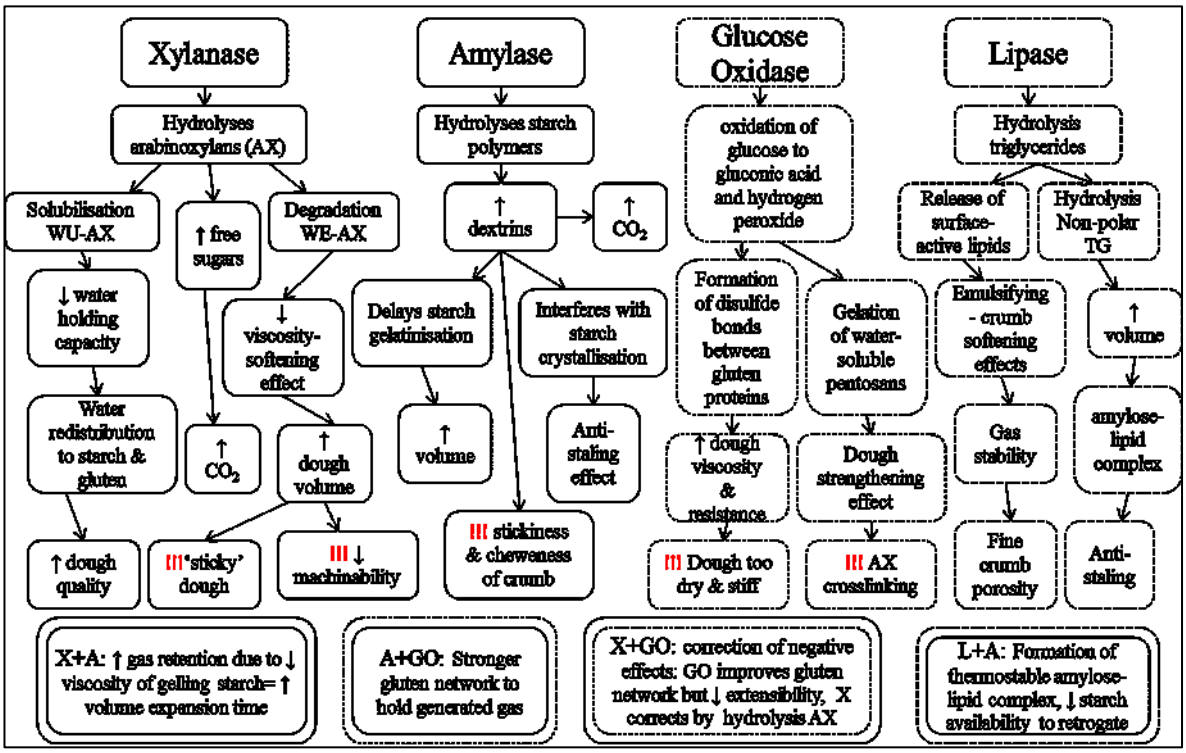

Figure 4.1 Possible mechanisms of enzyme improvement action

Xylanase sobulises non-starch polysaccharides (NSP) arabinoxylans. Therefore, the strongest impact has been seen in whole wheat or bran supplemented doughs, which have higher levels of arabinoxylans (Katina *et al.,* 2006b; Shah *et al.,* 2006). Teff flour contains 1.9% arabinoxylans (Dal Bello and Arendt 2008). This level is comparable to that in wheat flour, which contains 1.5- 2.5% (Courtin and Delcour 2002), however, lower than some other wholegrain flours, such as oats (2.7%) (Dal Bello and Arendt 2008). Therefore, the level of arabinoxylans in 30% Teff flour blend may not have been high enough for the greater effect of xylanase. This explains why single xylanase addition in the present study

produced only a slight improvement in specific volume and density of Teff bread. Nevertheless, a combination of xylanase and amylase (X+A) showed significantly (P<0.05) better bread quality attributes. The positive effect of amylase addition on volume can be explained by the production of fermentable sugars in the dough and thereby higher rate of CO_2 evolution by yeast (Kim *et al.,* 2006). Therefore, the favourable effect of a combination of X+A can be associated with increased dough resilience and increased extensibility due to reduced viscosity of dough by xylanase (Collar *et al.,* 2000) and better gas production by yeast due to increased substrate availability arising from amylase activity (Goesaert *et al.,* 2006). The positive effect on loaf volume and crumb firmness of X+A supplementation was also reported by other researchers (Martinez-Anaya and Jimenez 1997). In the present study, X+A showed the greatest improvements in sourdough Teff bread whilst a lesser extent of improvements were obtained in straight dough bread. This is in agreement with another study by Martinez-Anaya *et al*. (1998) and Andreu *et al.* (1999). Both studies demonstrated that X+A combination improved the loaf volume and shape and decreased the rate of retrogradation in sourdough breads. The favourable effect of X+A combination might be explained by the fact that during sourdough fermentation fibre components in the flour are broken down into the smaller particles (Corsetti and Settanni 2007), therefore increasing the amount of substrate for xylanase action. Katina *et al*. (2006) demonstrated that addition of xylanase into sourdough bread reduced the number of small cell wall particles, which might be attributed to the degradation and solubilisation of arabinoxylans (Gruppen *et al.,* 1993). The positive effect of amylase in X+A combination is also due to its gas producing ability (Cauvain and Chamberlain 1988), which improves loaf volume, and production of dextrins, which interfere in amylopectin retrogradation (Di Cagno *et al.,* 2003; Katina *et al.,* 2006b).

The positive effect of amylase and glucose oxidase (A+GO) combination, which showed one of the greatest improvements for both straight dough and sourdough Teff breads in the present study, can be explained by each enzyme's ability to act on separate functional flour components. Amylase breaks down starch into dextrins, which increases levels of fermentable sugars in the dough (Goesaert *et al.,* 2006) and thereby higher level of CO_2 evolution by yeast. However, increased levels of gas cannot be retained unless dough gluten matrix is strong enough. In this study, there was a significant increase in loaf volume of amylase supplemented Teff bread (Table 3.9), however, the improvements in crumb cellular structure were only marginal (Table 3.10). This suggests that although the generation of additional CO_2 by yeast as a result of increased fermentable sugars release by

amylase provided higher loaf volumes, the dough matrix was not strong enough to produce a more uniform and fine bread crumb structure. This is also supported by the results from A+GO combination Teff bread results. Addition of glucose oxidase has a strengthening effect on dough by formation of additional bonds in gluten network (Bonet *et al.,* 2006). Due to this synergistic effect of A+GO on increased gas production and stronger dough structure, favourable effects on increased loaf volume and decreased density were observed in Teff bread. A+GO combination had one of the the largest improvements in all bread quality parameters, such as loaf volume, density, crumb firmness during shelf-life, and particularly sensory attributes which were comparable to white wheat bread. Similar findings, in terms of improved dough handling properties, ovenspring, volume and texture were reported by other researchers in production of white pan bread with added amylase and glucose oxidase (Haarasilta *et al.,* 1991). Teff bread with A+GO addition scored highest overall acceptability in the taste panel. The favourable effect on flavour, aroma and taste of A+GO Teff bread might have been influenced by increased formation of dextrins and maltose by amylase, which has an effect in Maillard reaction. This would have resulted in flavour component formation, as revealed by sensory test results. A+GO bread had significantly higher scores for sweetness than control bread, which again can be attributed to higher levels of dextrins in the final product. This may also explain the effect of reduced bitter flavour and aftertaste in A+GO supplemented Teff bread as increased sweetness may have masked bitter flavour and aftertaste. This also suggests that amylase in this enzyme combination must have produced more dextrins and simple sugars than yeast have fermented for the bread to have scored high in sensory test for sweetness.

Teff bread supplemented with glucose oxidase and xylanase (GO+X) showed significantly improved bread quality parameters. Several researchers suggested a positive synergistic effect of glucose oxidase and xylanase. The favourable effect was shown to be due to ability of both enzymes to diminish negative effects of each other. GO can limit water holding capacity of dough by gelation of water soluble arabinoxylans, which can make dough dry and stiff (Vemulapalli and Hoseney 1998). Xylanase interferes with this by formation of small arabinoxylan fragments (Primo-Martin *et al.,* 2005). This was observed in the present study, in which single GO supplementation led to restriction of dough expansion, hence resulting lower loaf volume compared to other single enzyme treatments (Table 3.9), whereas GO+X Teff bread had significantly higher specific volume, reduced crumb firmness and good crumb structure. Sensory evaluation showed significantly higher

values for crumb softness, elasticity, sweetness and overall acceptability compared to control 30% Teff bread.

The synergistic effect of A+L was suggested to be due to formation of a more thermostable amylose-lipid complex, which results in less available starch for retrogradation, hence, having positive effect on crumb firmness during shelf-life (Leon *et al.,* 2002). Lipase hydrolysis triglycerides, which has a softening and emulsifying effect on dough. Further to this, lipase exerts a stronger positive effect in dough with added fat (Poulsen *et al.,* 1998), which was used as an ingredient in this study for straight dough Teff bread production but not in sourdough breads. The secondary reaction of lipase is its effect on lipoxygenase, which has been shown to produce a whiter crumb colour (Castello *et al.,* 1999), the effect that was observed in the present study. However, L+A combination has improved bread quality to the lesser extent compared to other enzyme combinations. This was especially the case in sourdough Teff breads. The most plausible explanation would be that sourdough recipe did not contain any added fat, which enhances the potential of lipase (Poulsen *et al.,* 1998), therefore, the effect of lipase was not as strong as seen in the straight dough bread. Furthermore, Teff is a gluten-free cereal; therefore, supplementation with gluten network strengthening enzyme, such as glucose oxidase, had a greater effect on bread quality parameters.

In summary, doughs supplemented with all enzyme combinations significantly improved bread textural properties in straight dough and sourdough 30% Teff breads. The produced breads yielded higher loaf volumes, lower crumb firmness during 8-day storage period, and a finer and more uniform crumb cellular structure. Overall, the combinations of X+A, A+GO and GO+X showed the most noticeable changes in bread textural quality parameters in Teff breads, whilst L+A improved these parameters to a lesser extent. This trend was especially seen in sourdough breads.

In terms of sensory acceptability, although all combinations improved sensory scores, A+GO combination scored the highest for all attributes. The scores were comparable to those of white wheat bread. This suggests that the developed Teff bread was as acceptable as white wheat bread by the consumer panellists.

GO and other substrate availability enhancing enzyme (amylase and xylanase) produced the most acceptable breads, in terms of textural and sensory properties. This suggests that strengthening of gluten network and increasing substrate availability for yeast action were the most important factors in improving Teff breads quality and acceptability.

4.1 Conclusions from the Teff bread development process

Nutritious Teff grain was incorporated into breadmaking. Teff flour replaced white wheat flour at the levels of 0, 10, 20 and 30%. The effect of Teff addition was studied in terms of dough characteristics, bread quality parameters, such as crumb firmness during shelf-life and cellular structure, nutritional properties, including protein, fat, dietary fibre, iron and total antioxidant activity and sensory attributes of breads.

Addition of Teff flour up to 30% positively affected nutritional properties, however, it had a negative impact on texture, crumb cellular structure and the sensory attributes of breads. Supplementation with enzyme mixtures, in particular combinations of gluten strengthening and substrate availability enhancing enzymes, significantly improved the quality parameters and sensory scores of 30% Teff bread. The bread with the highest sensory acceptability scores and quality parameters was selected for the intervention trial. This was a straight dough 30% Teff bread supplemented with amylase and glucose oxidase enzymes.

Consumption of 200g of 30% Teff bread can provide up to around 40% and 50% of the protein requirements for males and females, respectively. Teff bread can also provide 50% of recommended fibre intake and over 75% of the Reference Nutrient Intake (RNI) of iron for adult females. Hence, the consumption of Teff bread will particularly allow consumers to increase their intake of iron as well as provide adequate amount of protein, fat and fibre and antioxidants.

This research has successfully developed nutritious Teff bread with good quality and sensory attributes. Therefore, consumption of iron-rich Teff bread could be another way of improving individual's iron status through a habitual diet.

Chapter 5 Sport and exercise performance and iron status – Literature Review

5.1 Endurance performance parameters

Endurance performance is affected by a number of anatomic and physiological factors. In the broader sports performance context, these include adaptations to the cardiovascular system, innate pulmonary ventilation, muscle composition, substrate availability, nervous control and haematological parameters.

5.1.1 Maximum oxygen uptake

Maximum oxygen uptake (VO_{2max}) refers to the highest rate at which oxygen can be taken up and consumed by the body during exercise, and indicates individual's maximal aerobic capacity (Bassett and Howley 2000). The most important physiological factors involved in VO_{2max} are fuel availability and ventilation (cardiovascular circulation and respiration).

High VO_{2max} values are desired for elite sports performance, values 50-100% greater than those in general population are seen in competitive athletes as a result of physiological adaptation to training (Joyner 1991). VO_{2max} was shown to have a small within-subject variation (2.5%) compared to other physiological measures, such as running economy (3.6%) or lactate threshold (8.7%); and correlated with distance running performance ($r=0.57$, $P<0.05$) (Saunders *et al.,* 2010). This highlights the reliability and objectivity of using VO_{2max} as maximal performance indicator. Nevertheless, researchers argue as to whether VO_{2max} is the best predictor for sports performance because of the narrow range of VO_{2max} and the poor correlation between absolute VO_{2max} and performance results (Bosquet *et al.,* 2002). Despite this, it is generally accepted that a high VO_{2max} is a crucial prerequisite for competitive running.

5.1.2 Anaerobic threshold (ATr)

ATr indicates the transition point of when anaerobic respiration becomes more predominant than during incremental exercise. During an increase in work rate, the O_2 uptake increases. However, a point is reached where aerobic metabolism alone cannot supply enough O_2 for the energy required. The anaerobic metabolism becomes more predominant and as a result lactic acid is released into circulation (McArdle *et al.,* 2010). The point of a steep and non-linear increase in ventilation, blood lactate concentration and CO_2 production is defined as anaerobic threshold (Wassermam *et al.,* 1973). ATr occurs at an exercise level just below VO_{2max}.

The term ‘anaerobic threshold’ is commonly interchangeably used with ‘lactate threshold’ and ‘ventilatory threshold’. This is mainly because of differences in ATr determination methods. An individual’s ATr point can be established by lactate threshold values (abrupt increase in blood lactate with increased work load) (Karlsson and Jacobs 1982), ventilator anaerobic threshold (oxygen consumption VO_2 against pulmonary ventilation V_E) (Reybrouck *et al.,* 1985), and the V-slope (regression of VCO_2 versus VO_2 slope) (Beaver *et al.,* 1986). Although caution must be taken when comparing ATr obtained by different methods, a number of researchers reported no significant difference between the estimation of ATr from blood lactate concentration and gas exchange variables (Caiozzo *et al.,* 1982; Papadopoulos and Del Pozzi 2009).

ATr expressed as $\%VO_{2max}$ is often used as a measure of aerobic endurance (Bosquet *et al.,* 2002), because it is closely linked to the level of VO_{2max} that can be maintained for prolonged exercise durations (Joyner 1991).

5.2 Importance of iron in sports performance

The blood and plasma plays an important role in physical work by facilitating gas transport and exchange, acid buffering and thermoregulation (McArdle *et al.,* 2010). This implies that factors influencing blood and plasma physiological state may exert endurance limiting effects.

Iron is an essential mineral for optimal physical performance. It has a role in oxygen transport and energy production and serves as a functional component of haemoglobin and myoglobin (Suedekum and Dimeff 2005). Additionally, iron is a crucial part of cytochromes, found in the electron transport system during production of ATP, hence, providing energy source for physical movement (Rodenberg and Gustafson 2007). Therefore, in order for metabolism and oxygen transfer to take place efficiently during physical performance, there must be an adequate level of iron in the human body.

5.2.1 Dietary iron

Dietary iron occurs in two forms: haem and non-haem. The haem form of iron refers to iron from animal sources, whilst non-haem iron accounts for all other types of dietary iron.

Haem iron is present within haemoglobin or myoglobin molecules and is released by proteolytic enzymes in the lumen of the stomach and small intestine (Beard and Han 2009). As haem iron absorption does not require binding proteins, it can be as efficient as 40% (Beard and Han 2009). However, it should be noted, that haem iron constitutes only about 10% of all dietary iron (Beard and Tobin 2000).

Non-haem iron is bound to other food components and is usually present in ferric form. In order for it to be used by the body it must be reduced to ferrous iron by either brush border membrane enzymes or dietary reducing agents and transported by the divalent metal transporter into the enterocyte (SACN 2010). Non-haem iron absorption depends on the levels of inhibitors and enhancers, hence, the availability of this form of iron varies greatly from 2 to 20% (Beard and Tobin 2000; Roughead *et al.,* 2002). In the Western diets 50% of total dietary iron comes from grain products (Beard and Han 2009), hence, non-haem iron accounts for the largest proportion of the iron intake for the general population.

5.2.2 Iron metabolism and bioavailability

The human body does not have a direct mechanism of iron excretion, hence, regulation of iron balance is influenced by the current iron status of the individual and the total amount of iron components ingested through the diet; and is overall maintained by the internal homeostasis (Frazer and Anderson 2005). An individual with high iron stores will absorb less iron than an iron depleted person and vice versa. Furthermore, the absorption of iron from the whole diet depends not only on the total amount of iron but also by the type of iron (haem or non-haem iron) and the content of promoters and inhibitors in the diet (Bjorn-Rasmussen *et al.,* 1974).

5.2.3 Iron absorption

Researchers have demonstrated an inverse relationship between iron absorption and serum ferritin (sFer) concentration up to the level of 60 μg/L (Hulten *et al.,* 1995; Hallberg *et al.,* 1997), which suggests that absorption decreases to a level sufficient to cover daily iron losses in order to prevent further iron stores accumulation. It was also reported that absorption of both haem and non-haem iron was 40% in subjects with sFer of 10 μg/L, however, at higher sFer concentration a decrease in iron absorption was observed, especially in non-haem iron (Hallberg *et al.,* 1997). This indicates that regardless of the total dietary iron intake, iron absorption is strongly dependent on overall iron status. In an iron-repleted state the absorption of iron will be reduced, whilst in the iron-depletion state, increased dietary iron absorption is observed. Furthermore, this suggests that in an iron-depletion state, non-haem dietary iron becomes an important source of absorbable iron.

5.2.3.1 Single meal and whole diet iron absorption and bioavailability trials

The amount of iron absorbed from the diet is also dependent on other dietary factors that enhance or inhibit absorption. Iron bioavailability trials showed that high bioavailability diets had an iron absorption of 19.3%, whilst medium bioavailability meals had an absorption of 11.8% in 21 non-anaemic (Hb=13.6 g/dL, sFer=30.4 μg/L) female subjects

(Hulten *et al.,* 1995). The medium bioavailability diet in this trial contained only one third the amount of meat compared to the high bioavailability diet, the breads were made from wholegrain flour and some of the lunch and dinner meals were vegetarian, which resulted in a higher level of phytate. Another study also demonstrated that a much lesser fraction of non-haem iron was absorbed in the diet compared to haem iron (5% *vs* 37%) (Bjorn-Rasmussen *et al.,* 1974), which suggests that diets rich in plant materials and lower in meat may be inadequate to supply dietary iron even if the total amount represents recommended values. The supporting evidence was also provided in another non-haem iron bioavailability single meal trial, where researchers have demonstrated that after controlling for participant's iron status, animal tissue (P=0.0001), phytic acid (P=0.0001) and ascorbic acid (P=0.0441) were all significant biochemical predictors of iron absorption in commonly consumed Western style meals (Reddy *et al.,* 2000).
Overall, researchers demonstrated significant iron availability enhancing effects of ascorbic acid (Hallberg *et al.,* 1989) and meat (Boech *et al.,* 2003), and an inhibitory effect of phytate (Tuntawiroon *et al.,* 1991) and calcium (Hallberg *et al.,* 1993b) in a single meal experiment set up.

The non-haem iron meal designed to maximise iron absorption with the addition of meat and ascorbic acid and showed absorption of 13.5% in the single meal test (same meal without enhancing agents showed only 2.3% absorption), however, mean iron absorption of these meals during 2 week period showed a much lesser difference, as the enhancement diet had iron absorption of 8.0%, whilst the inhibitory diet was 3.2% (Cook *et al.,* 1991). Authors explained the findings as a 'dilution effect' of dietary inhibitors and enhancers by other dietary components in the whole diet. A similar outcome was reported by the study conducted on the effects of ascorbic acid supplementation and iron absorption in typical Western diet in women with low iron stores (Hunt *et al.,* 1994). Even after 5 weeks of 500 mg of ascorbic acid supplementation three times a day, authors did not observe any significant changes in haematological indices. Thus, dietary inhibitors or enhancers influence iron absorption in a single meal by about 16% (Reddy *et al.,* 2000), whilst the effect of these on the iron absorption from the overall diet is less profound.

Overall, research indicates that the amount of dietary iron, the type of iron (haem vs non-haem) and the presence of enhancers or/and inhibitors in the diet affect the bioavailability and absorption of iron. Hence, it appears that the larger the amount of dietary iron consumed, and the greater the proportion of this iron being in the haem form together with

the greater the proportion of dietary enhancers in the diet would result in greater iron absorption. However, this is only observed in individuals with lowered iron status. If the functional iron components and iron stores are at sufficient level, the internal down-regulation mechanisms would decrease the overall iron absorption. Hence, an individual's body iron balance, the amount of total iron in the diet and the form of iron, and finally other components in the diet are all influential determinants of iron absorption.

5.2.4 Iron markers

Several metabolic markers are used to assess iron status of the individual or population. Haemoglobin and red blood cell (RBC) counts represent an indication of the functional iron, whilst serum iron, serum transferrin and serum transferrin receptors are the measures of tissue iron supply; and serum ferritin shows the levels of iron storage in the body (SACN 2010). However, isolated measurements, for instance haemoglobin concentration alone, are unsuitable as a sole indicator of iron status (WHO 2007), as firstly, various markers measure different aspects of iron status parameters, such as storage or functional iron. Secondly, these markers have different sensitivity to health conditions, including inflammation and other micronutrient deficiencies (Clark 2008), hence, relying only on one parameter may 'mask' the true reflection of individual's iron status. In addition to this, in the athletes' population within-subject haemoglobin values can vary up to 20%, suggesting that using only one haematological indice for the determination of iron status in sports performance studies might not be sufficient (Eastwood *et al.,* 2012). Therefore, selecting a wide spectrum of iron markers, i.e. serum transferrin receptor, serum ferritin, serum iron and ratios of these parameters, representing functional, supply and tissue iron seem to be a more sensitive measure for assessing true iron status (Cook *et al.,* 2003).

5.2.4.1 Haemoglobin

Haemoglobin (Hb) is the most widely accepted measure of anaemia at the population level, reflecting functional iron status. The clinical representation of anaemia is set to be at Hb levels below 12g/dl for a general non-pregnant female population (WHO 2001).

Although Hb provides a good measure of advanced iron deficiency and anaemia, it lacks sensitivity in the diagnosis of mild iron deficiency (DeMaeyer and Adiels-Tegman 1985), as functional consequences of iron deficiency can occur with 'normal' Hb values. In addition, because the life span of red blood cells is around 120 days, sufficient time is needed between screenings for changes in Hb concentrations to be detected (Clark 2008). Hence, it could be argued that Hb lacks sensitivity in short-term iron therapy trials.

Most sports performance studies used Hb value, primarily for determining any anaemic state in the subjects. However, other haematological indices are usually employed to determine and monitor overall iron status and changes during intervention period. Indeed, several sports performance enhancement studies reported no changes in Hb values pre and post-treatment (Hinton *et al.,* 2000; Brutsaert *et al.,* 2003; Brownlie *et al.,* 2004), although changes occurred in other haematological indices and sports performance measurements.

5.2.4.2 Serum iron

Serum iron (sFe) is plasma iron bound to the plasma transport protein, transferrin (Fielding 1980) and can be used as an indicator of tissue iron supply. Reference values of 10- 30 μmol/L are considered to be adequate for the general population (British Nutrition Foundation 1995). The disadvantage of using sFe as an indicator of the individual's iron status is a considerable day-to-day variation of sFe values due to release of iron from iron storage reserves (Chatard *et al.,* 1999). Indeed, the daily plasma turnover is much higher compared to total plasma iron pool (35 mg compared to 3 mg/day) (Bothwell *et al.,* 1979). Furthermore, sFe is an acute phase protein reactant (Gibson 2005a) and it shows sensitivity to exercise. Compared to some other iron status related variables, such as serum ferritin and transferrin, sFe was shown to be the most influenced by prolonged exercise in female soccer players and ultra-marathon runners (Fallon 2001; Fallon *et al.,* 2001).

5.2.4.3 Serum ferritin

Serum ferritin (sFer) is a widely accepted measure of iron storage; 1μg of sFer per litre was shown to equate to about 8 mg of storage iron (Walters *et al.,* 1973). sFer is mainly present in the reticuloendothelial cells of the liver, spleen and bone marrow, and is available for the synthesis of haemoglobin and other iron containing enzymes or proteins (Worwood 1980). The representative normal reference ranges for adult females are between 15- 300 μg/L (British Nutrition Foundation 1995), however, cut-off points differ between studies. The cut-off point of 16μg/L was widely used in the European studies (Hallberg *et al.,* 1993a), whilst a lower value of 12 μg/L was chosen by the USA researchers for the general population (Looker *et al.,* 1997). In the sports performance studies using female runners as a population, sFer cut-off points of 12 μg/L (Zhu and Haas 1997), 16 μg/L (Zhu and Haas 1998a; Hinton *et al.,* 2000; Brownlie *et al.,* 2004), 20 μg/L (Lamanca and Haymes 1993; Fogelholm *et al.,* 1994; Friedmann *et al.,* 2000; Brutsaert *et al.,* 2003), 25 μg/L (Fogelholm *et al.,* 1992), 30 μg/L (Telford *et al.,* 1992) and 40 μg/L (Blee *et al.,* 1999) were used. The most plausible explanation for the variation between the chosen cut-off points is the great variation of sFer in the general population and the fact that sFer values of 25-40 μg/L have

been found in subjects with the absence of iron stores (Hallberg *et al.,* 1993a). sFer is a very useful indicator of the true level of iron stores (or absence of them) at an early stages of iron deficiency (ID), however, sFer is a negative acute phase reactant and with conditions, such as inflammation, infection or liver disease, raised sFer are present, thus this may lead to the underestimation of ID in the general population (Worwood 1979). Similarly in athletes, heavy physical loads may induce inflammatory-like responses due to micro-injuries and muscle and joint inflammation reactions (McArdle *et al.,* 2010). Elevated sFer levels were seen after ultra-endurance events (Fallon *et al.,* 1999; Fallon 2001), however, there were no significant changes in sFer after training sessions in elite female soccer, netball players (Fallon *et al.,* 2001) and long-distance runners (Weight *et al.,* 1991a). On balance, research evidence suggests that an acute phase response in relation to elevated sFer levels in athletes may give an inaccurate determination of iron status. However, this is more likely to be caused by ultra-endurance event or training and is not a consequence of exercise at the levels of individual's typical training.

5.2.4.4 Serum Transferrin Receptor

Serum Transferrin receptor (sTsfR) controls the cellular movement of iron within the body according to the iron needs of the cells (Beard and Han 2009). sTsfR has recently become a 'gold standard' as a measure of mild tissue iron deficiency in populations (WHO 2007).

sTsfR is a reliable measure in inflammatory and infectious conditions and has only a small day-to-day variation unlike other haematological indices (Ferguson *et al.,* 1992). sTsfR has been shown to be a stable index of iron status, with an intra-individual coefficient of variation of 5.2%, in exercise-induced acute recovery phase in male athletes (Nikolaidis *et al.,* 2003). Although sTsfR has been described as a new marker of iron status in sports performance research, it may not be a very reliable marker for very low or absent iron storage levels in athletes (Pitsis *et al.,* 2004).

However, sTsfR is a relatively expensive measure and due to a variety of methodological assays there is no uniform unit or reference range for sTsfR (Thorpe *et al.,* 2010), which makes a challenge when comparing different trials. Nevertheless, sTsfR was shown to be a reliable indicator of marginal iron-deficiency in non-anaemic female runners taking part in iron supplementation and sports performance trials (Zhu and Haas 1998b; Hinton *et al.,* 2000; Schumacher *et al.,* 2002b; Brutsaert *et al.,* 2003).

Calculation of the ratio of sTsfR to sFer has been suggested as an outstanding parameter for identification of latent iron-deficiency in the general population (Punnonen *et al.,* 1997) and amongst female athletes (Malczewska *et al.,* 2001).

Overall, sTsfR appears to be a very powerful indication of true iron status; however, as the sensitivity of sTsfR may be compromised in iron-depleted individuals, it seems other haematological indices, such as sFer, should be used in conjunction to reflect iron status in athletes.

5.2.5 Measures of iron status

Determination of iron status can be challenging due to day-to-day individual variations, presence of acute and chronic conditions and the lack of a wider spectrum of measurements (Clark 2008). This is particularly seen in iron deficiency without an anaemia diagnosis. Nevertheless, there have been attempts to categorise reference levels for iron status to indicate iron depletion (Table 5.1).

Table 5.1 Population reference levels for detection of iron depletion in females*

	Normal iron status	Depletion of iron stores	Iron deficiency	Iron deficiency anaemia (IDA)
Haemoglobin (g/dL)	>12	>12	>12	<12
Serum ferritin (µg/L)	>30	<30	<12	<12

Key: * cut-off values taken from SACN (2010) report

5.2.6 Exercise and iron status in female runners

The effects of exercise itself on individual's iron status is a subject of debate in the literature, as some researchers suggest a direct effect of exercise on changes in haematological indices, whilst others characterise these changes as a temporary condition, known as 'sports anaemia' (Tobin and Beard 1997).

The presence of anaemia or more currently depleted iron stores in physically active females and endurance athletes has been a topic of considerable attention over the last few decades. A study evaluating the effects of long-term moderate exercise on the iron status in 62 women observed that 6 months of moderate exercise with no dietary intervention resulted in haemoglobin values decline from 13.8 to 12.8 g/dL, while non-exercising women and exercising women with dietary iron supplementation sustained their haemoglobin levels (Rajaram *et al.,* 1995). Middle- and long-distance runners were found to have significantly lower serum ferritin than controls due to increased iron turnover and red cell destruction (Dufaux *et al.,* 1981). In addition to this, a study of 213 participants showed that an iron depletion state was significantly (P<0.05) more prevalent in habitual female runners compared to the inactive counterparts (Pate *et al.,* 1993). Supporting

evidence was provided by a study of 51 Olympic runners, which showed that although the mean iron intake was 41.9 mg/d, over 35% of the athletes had serum ferritin values below 12 μg/L (Deuster *et al.,* 1986), which indicates an absence of iron stores. Another 2-year observation study revealed that although Hb and sFe did not differ from control subjects, storage iron was either absent or only present in trace levels indicating iron depletion in long-distance female runners (Ehn *et al.,* 1980). This was mainly attributed to increased iron elimination and low dietary iron absorption.

However, a study comparing exercise and control group women during 10 weeks period failed to show any differences in haemoglobin, serum iron, ferritin and TIBC between two groups (Pratt *et al.,* 1996). Supporting evidence was also reported in a cross-sectional study comparing haematological parameters in female runners and non-runners (Balaban *et al.,* 1989). Authors concluded that runners' iron status was similar to that of general population. Other researchers studying elite cross-country runners and skiers concluded that iron status of these athletes were comparable to that of normal population group during a year's follow up trials (Hemmingsson *et al.,* 1991).

5.2.6.1 'Sports anaemia'

The explanation of lower haematological indices in exercising athletes compared to the sedentary counterparts has been suggested to be due to 'sports anaemia' or 'pseudoanaemia' (Suedekum and Dimeff 2005). This is a physiological haemodilution due to increased plasma volume after endurance performance which does not impair exercise capacity. The exercise-induced rise in blood pressure results in this physiological adaptation, expansion of blood plasma by more than 400 ml is observed in some vigorous sports activity (Eichner 1992). Transient 'sports anaemia' is particularly seen in endurance runners (Wu *et al.,* 2004). The 'footstrike' mechanical haemolysis of red blood cells (RBC) during running (Fallon and Bishop 2002; Rietjens *et al.,* 2002; Schumacher *et al.,* 2002a) and oxidative damage to RBC induced by endurance exercise accelerate destruction of RBC (Szygula 1990; Jordan *et al.,* 1998), coupled with expansion of plasma volume, which contributes to lower Hb concentration and compromised iron status (Jerin *et al.,* 2011; Auersperger *et al.,* 2012). Runners experienced a sudden reduction in Hb and red blood cell volume during initial training, however, haematological values returned to normal within 8 weeks of training (Frederickson *et al.,* 1983). Supporting evidence was reported by another study results, which showed compromised initial iron stores, which stabilised after 6 weeks of aerobic exercise in female subjects (Blum *et al.,* 1986). In addition to this, a study investigating haematological indices and iron status in untrained and elite athletes reported that blood and plasma volume values were 35-40% higher in

middle- and long-distance runners when compared to untrained counterparts, yet haemoglobin concentrations were similar (control: Hb 15.3 g/dl *vs* runners: Hb 15.5 g/dl) (Heinicke *et al.,* 2001). This is mainly attributed to the training-induced physiological adaptation, such as the increased haemoglobin mass (control: tHb 913 g *vs* runners: tHb 1011 g) and increased erythropoiesis and red cell turnover (Schmidt *et al.,* 1988; Weight *et al.,* 1991b). The increased turnover rate may be a favourable outcome for the athletes, as the 'young' red blood cells can carry oxygen more efficiently than the older cells (Smith 1995).

Therefore, 'sports anaemia', caused by a physiological adaptation mechanism as a result of increased endurance exercise regime or ultra-endurance events, can show symptoms of iron-deficiency anaemia or anaemia, however, these changes are not an indication of disease. Therefore, it is unlikely that long-term low iron status in an athlete would be caused by this phenomenon, mainly due to the physiological adaptations that occur during long-term habitual training activity.

5.2.6.2 The effects of exercise on iron balance

Several researchers suggested mechanisms by which physical exercise can alter iron balance. Gastrointestinal bleeding due to increased risk of ischemic damage to stomach and intestine following endurance running has been reported to contribute to iron loss (Simons and Shaskan 2005). Quantitative tests of daily haemoglobin concentration loss in runners showed average values of 0.99 mg/g before a race and 3.96 mg/g after a race (Schwartz *et al.,* 1990). Exercise-induced haematuria was also reported to contribute to iron losses, directly, by erythrocyte rupture and damage by foot-strike (Gambrell and Blount 1996). The mean erythrocyte lifespans in athletes were reported to be significantly shorter compared to non-runners (72.4 days *vs* 114 days) (Weight *et al.,* 1991b). Hence, increased erythrocyte turnover is important in order to meet accelerated haematological demands. Supporting evidence was presented by Ehn *et al.* (1980) where long-distance female runners showed much faster elimination of total body iron compared to control subjects (50% iron elimination in 1, 000 days in female runners *vs* 1,300 days in controls).

Iron lost through sweat and heat during exercise can also be an important factor. Sweat iron losses after prolonged exercise of 2 hours represented 3% of Recommended Daily Allowance (RDA) of iron for female cyclists (DeRuisseau *et al.,* 2002), and can increase the loss to 0.08 mg/m^2/hour (Waller and Haymes 1996).

Finally, blood losses through menstrual cycles may put female athletes at an even higher risk of inadequate iron supply. On average an adult female may lose up to 17.5 mg iron per

period, which equates to around 0.5 mg/day (Gropper *et al.,* 2009). Not surprisingly menstrual bleeding can contribute to reduced iron stores, especially with heavy and long cycles. This was suggested to be the main cause of reduced iron stores in a study of 126 female endurance athletes (Malczewska *et al.,* 2000). Although iron blood losses during the menstrual cycle may have an impact on iron status in individual cases, on balance research suggests that the menstrual cycle has very little or no effect on Hb concentrations and plasma volume (de Jonge 2003). Subsequently, there were no changes reported on exercise performance limiting parameters, such as VO_{2max}, oxygen transport or ventilation systems, in female athletes over the menstrual cycle (Oosthuyse and Bosch 2010). In addition to this, menstrual phase (follicular *v* luteal) or menstrual status (regular menstruation *v* amenorrhoeic) did not alter endurance in these athletes (Desouza *et al.,* 1990).

Nevertheless, total iron losses in female endurance runners through gastrointestinal bleeding, exercise-induced haemolysis and haematuria, and menstrual cycle can add up to 2 - 2.3 mg/d (Ehn *et al.,* 1980; Weaver and Rajaram 1992), which not surprisingly may contribute to compromised iron status in these athletes.

5.2.7 Iron requirements for athletes

The reference nutrient intake (RNI) for adult females is 14.8 mg iron per day in the UK (Department of Health 1991) and the recommended dietary allowance in the US is set at 18 mg iron a day (Institute of Medicine Food and Nutrition Board 2001). Whilst additional iron is recommended for pregnant and lactating females, an increased iron allowance is not an official recommendation for female athletes. Although increased loss of several minerals, including iron, from the body during exercise has been well established, there is limited evidence of adverse effect on the body stores (Clarkson and Haymes 1995).

Some authors suggest that iron requirements for endurance female athletes, particularly distance runners, are increased by approximately 70% (Whiting and Barabash 2006). This means that additional 10 mg iron a day should be added to the UK recommended value of 14.8 mg.

Female athletes seem to have increased iron requirements due to iron loss in the gastrointestinal tract, sweat, urine and menstruation, also due to increased haemolysis during endurance training (Suedekum and Dimeff 2005). Furthermore, inadequate dietary iron intake, coupled with limited bioavailability of iron in the diet, may present even a greater risk of iron-deficiency in female athletes. Although anaemia is rare in athletes and the general population, depleted iron stores are common in female athletes, especially runners. Marginal iron deficiency (sFer <20μg/L; Hb>12g/dl) has been reported to be

present in 30- 50% of female runners, more than twice that of a the control population (Lampe *et al.,* 1986a; Risser *et al.,* 1988; Pate *et al.,* 1993; Karamizrak *et al.,* 1996).

5.2.8 Iron intakes in physically active females

Poor iron intake (7-20 mg/day) and low iron stores (sFer 30 μg/L) were reported in a small sample size (n=9) of female marathon runners during 11-week pre-marathon training (Lampe *et al.,* 1986b). In addition, the intakes for most of nutrients, including daily energy, protein and ascorbic acid were near recommended levels, except for iron, indicating that poor dietary iron intake might contribute to compromised iron status in this population. This is in agreement with several other cross-sectional studies investigating the influence of dietary iron sources in female runners (Snyder *et al.,* 1989; Nuviala *et al.,* 1996; Hassapidou and Manstrantoni 2001). Iron intake of 68% of female runners (n=25) in Nuviala *et al.* (1996) study was below 15 mg/day, Hassapidou and Manstrantoni (2001) observed iron intake of 11.4 and 13.8 mg/day during training and the competition season, respectively in middle distance runners, whilst Snyder *et al.* (1989) reported dietary iron intake to be around 14 mg/day with sFer ranging from 6 to 25μg/L, indicating depleted iron stores. Interestingly, authors concluded that vegetarian runners had significantly (P<0.05) lower sFer levels (7.4μg/L) compared to meat eating group (19.8 μg/L), which was mainly attributed to reduced iron bioavailability in vegetarian diet (0.66 mg/day *vs* 0.91 mg/day, P<0.05) as both groups had a similar total iron intake. Similarly another study showed that lean beef consumption had Hb maintenance effects during high intensity training in female runners and control subjects (Pahnke *et al.,* 1999). This highlights the importance not only of adequate total dietary iron intake but also the source of dietary iron. Dietary iron intake in female athletes was reported to be similar to that of general population (runners 11.0 mg/d *vs* controls 10.4 mg/d by Pate *et al*., 1993; athletes 12.2 mg/d v controls 10.8 mg/d by Spodaryk *et al*., 1996), however, serum ferritin and other haematological indices were significantly (P<0.05) lower in athletes than sedentary women (Pate *et al.,* 1993; Spodaryk *et al.,* 1996; Gropper *et al.,* 2006), suggesting the negative effect of exercise on iron status. Indeed, strong associations were reported between haemoglobin and performance tolerance (r=0.74, P<0.001) (Edgerton *et al.,* 1981) as well as serum ferritin levels and endurance time (r=0.74, P<0.05) (Rowland *et al.,* 1988).

On balance, the evidence suggests that the mean intake of iron in female athletes is similar to that of general population which is lower than current recommendations. Furthermore, increased iron demands due to training and poor iron bioavailability in the diet highlights the importance of adequate dietary intakes to achieve the optimum iron status. Good

nutrition to achieve adequate iron balance through dietary means has been suggested as the first line of action in the prevention of iron deficiency in the female athletes (Beard and Tobin 2000).

5.2.9 The effects of poor iron status on endurance performance

Iron deficiency progresses in three stages (Chatard *et al.,* 1999). Firstly, iron stores in reticuloendothelial cells of the liver, spleen and bone marrow are depleted, which is observed as a fall in serum ferritin. The second stage is represented by erythropoiesis, where transport iron is decreased and hence, iron supply to the cells is reduced. This stage is manifested as low serum iron, increased total binding capacity and a decrease in transferrin saturation. The first two stages of iron deficiency are also referred to as pre-anaemic 'latent iron deficiency'. In the last stage of iron deficiency, haemoglobin synthesis falls due to insufficient iron supply, resulting in anaemia (WHO 2007).

It is well documented that anaemia has a detrimental effect on aerobic power and endurance capacity (Calbet *et al.,* 2006). A number of early research investigations into acute reduction of Hb and exercise performance demonstrated that reduction in Hb concentration by 12-13% resulted in reduction in VO_{2max} by 6-10%, whilst endurance time was reduced by 20-30% (Ekblom *et al.,* 1972; Ekblom *et al.,* 1976). Supporting evidence was provided by other researchers, who concluded that anaemia significantly impairs work tolerance and exercise performance as anaemic subjects (Hb=11-11.9 g/dL) had 20% lower exercise performance (Gardner *et al.,* 1977) and significantly increased peak and resting heart rates and post-exercise lactate levels (Gardner *et al.,* 1975) compared to non-anaemic subjects (Hb>13 g/dL). Strong correlations between Hb and workload (r=0.74, $P<0.001$) were reported by Edgerton *et al.* (1981), who showed that blood transfusion increased work tolerance by 83% in anaemic subjects within 24 hours. Similar outcomes were observed in non-anaemic iron-depleted various sports female population. Runners whose haematological indices showed an adequate iron status scored much higher in endurance test. The authors concluded that physical work capacity was significantly ($P<0.01$) correlated with sFer (r=0.55) and transferrin saturation (r=0.53).

5.3 Iron supplementation and sports performance enhancement

Although iron-deficiency anaemia has been shown to have an adverse effect on performance (Gardner *et al.,* 1975; Gardner *et al.,* 1977; Ohira *et al.,* 1979; Edgerton *et al.,* 1981), the evidence of marginal iron deficiency and the effect on athletic performance is conflicting. Whilst some researchers reported a significant improvement in marginally iron-deficient females exercise performance after the correction of iron status (Lamanca

and Haymes 1993; Zhu and Haas 1997; Friedmann *et al.,* 2000; Hinton *et al.,* 2000; Friedmann *et al.,* 2001; Brownlie *et al.,* 2002; Brutsaert *et al.,* 2003; Brownlie *et al.,* 2004), others show no adverse effect of marginal iron depletion on sports performance (Powell and Tucker 1991; Fogelholm *et al.,* 1992; Klingshirn *et al.,* 1992; Telford *et al.,* 1992; Blee *et al.,* 1999; Tsalis *et al.,* 2004; Peeling *et al.,* 2007).
The key findings from iron supplementation studies are presented in Appendix 1.

5.3.1 Pharmacological iron supplementation trials

The first researchers to study iron-depleted but non-anaemic female athletes and the effects of iron supplementation on sports performance reported contrary results. Whilst the study conducted by Rowland *et al.* (1988) showed significant improvements in endurance performance and observed significant correlation ($r=0.74$, $P<0.05$) between improved iron stores and endurance time, other researchers (Matter *et al.,* 1987; Newhouse *et al.,* 1989) reported improvements in iron status without any observed benefits to the athletic performance. Matter *at el.* (1987) only conducted a one week intervention, which might explain the lack of result in such a short duration trial. However, the trial by Newhouse *et al*. (1989) was run over 8 weeks and does not support this argument. The difference in dosages (975 mg, 160 mg, 320 mg ferrous sulphate, respectively) is unlikely to have been a contributing cause, mainly due to the fact that all three studies showed similar improvements in haematological indices. The study by Rowland *et al.* (1988) was conducted during a period of endurance training, which may highlight the importance of iron to optimum performance during a heavy exercise load.

Studies by Powel and Tucker (1991), Telford *et al* (1992) and Fogelholm *et al.* (1992) also disputed the effectiveness of iron supplementation on enhancing athletic performance. However, the cut off point for iron-depletion state was just below 30 μg/L in Telford *et al.* (1992) and Powel and Tucker (1991) studies, therefore, basal iron stores values might have been actually sufficient. Furthermore, the latter study was only run for 2 weeks; hence, short trial time may have had influence on the outcome as well.

Interestingly, studies investigating the effect of iron supplementation on sports performance and adaptation during physical activity indicate positive results (Jensen *et al.,* 1991; Magazanik *et al.,* 1991). Both studies showed that women maintained their iron stores and improved exercise performance, in terms of improved VO_{2max}, during moderate exercise training whilst taking a daily iron supplement of 50-160 mg ferrous sulphate.

Three more recent physical activity trials also generated similar findings. Hinton *et al.* (2000), Brownlie *et al.* (2002 and 2004) demonstrated that 6 weeks of physical training and supplementation at dosages of around 20 mg elemental iron showed significant improved haematological indices and physical activity scores compared to their placebo counterparts. Furthermore, subjects with the most depleted iron stores seem to have benefited most (Brownlie *et al.,* 2004).

Several cross-sectional studies also yielded results suggesting a link between marginal iron deficiency and sports performance (Lamanca and Haymes 1992; Zhu and Haas 1997). Iron-sufficient females had higher VO_{2max} and exercised 14% longer in the treadmill endurance test compared to iron-depleted but non-anaemic subjects. Not all of the most recent and longitudinal studies showed similar results. A study by Tsalis *et al.* (2004) did not observe any haematological or performance differences between iron supplementation and the control groups during 6 months of swimming tests. Another study by Peeling *at al*. (2007) also failed to show any influence of iron supplementation on sports performance even after a dramatic change in sFer (from 19 to 65 μg/L).

Overall, the conflicting evidence suggests that there may be multifactorial effects on iron supplementation and the enhancement of endurance. The pharmacological dosage does not appear to have a lot of influence on achieving improvements in iron status as studies with low iron supplementation showed similar changes in haematological indices to high dose studies in non-anaemic yet iron-depleted female subjects. The duration of iron supplementation may have an effect on improving iron status and hence, may have implications on detecting changes in sports performance as well. On balance the literature evidence suggests that iron-depleted females may experience improved endurance performance parameters with iron supplementation, especially if they are subjected to increased endurance exercise load.

5.3.2 Dietary iron and iron-rich food supplementation trials

Research on dietary iron interventions in female athletes' sports enhancement area is very scarce. Only one longitudinal study on swimmers showed no differences in iron status or performance scores between an iron-rich diet and free choice diet participants during a 6-month period (Tsalis *et al.*, 2004). It should be noted that the study employed iron-repleted participants (Hb>14 g/dL, sFer >30 μg/L), which perhaps explained the lack of effect.

Other studies showed slightly more positive results on dietary intervention and iron status. A four weeks iron-rich diet, which provided 18.2 mg/d, showed significant effects on sFer concentration in rhythmic gymnasts (Ishizaki *et al.,* 2006). However, the study did not assess their performance during the trial and hence, no conclusion can be drawn to any possible effects on performance. A study by Lyle *et al.* (1992) also reported that diet rich in iron, providing 11.8 mg iron/day, was more effective in protecting iron status than were supplements (50 mg ferrous sulphate/day) during 12 weeks of aerobic tests in previous sedentary women. Furthermore, the dietary intervention group showed the highest improvement in exercise performance as well. The most recent dietary intervention and iron status in athletes study by Anschuetz *et al.* (2010) concluded that 4-weeks of dietary advice counselling did not improve overall iron status in athletes. However, the authors suggested that the diet composition, in particular the presence of enhancers of non-haem iron absorption, have a significant influence for iron absorption in this population. The study results revealed that dietary absorbable iron was significantly correlated to sFer ($r=0.9$, $P<0.05$) in female mid-distance runners, highlighting the importance of adequate absorbable iron source (Anschuetz *et al.,* 2010).

The majority of results support the hypothesis of the beneficial effect of dietary and therapeutic iron supplementation on the balance of iron in iron-depleted female athletes (described as having normal Hb levels but depleted iron stores sFer <20 µg/L). Furthermore, evidence indicates a positive relationship between increased iron status and exercise performance during endurance training or adaption period.

5.3.3 Dosages of iron used in supplementation trials

In many physically active females, iron depletion is closely related to inadequate dietary iron intake, as the typical Western diet has a low concentration of iron and is rich in iron absorption inhibitors (Beard and Tobin 2000).

The vast majority of researchers examining iron supplementation in athletes used therapeutic iron supplements in relatively high dosages (100-200 mg elemental Fe/day). Iron supplements offer quick and effective way of changing an individual's compromised iron status (Rodenberg and Gustafson 2007). However, they are also associated with gastrointestinal intolerances, which can reduce participants' compliance (Handin *et al.,* 2003). In addition to this, the absorption of inorganic iron supplements can be as low as 2.87 - 4.38 % (Lyle *et al.,* 1992). Therefore, despite it being a convenient way of

improving the body's iron balance, iron therapy through high dosage supplementation is an expensive treatment and may only show short-term improvement in iron status due to homeostatic mechanism to prevent further enlargement or overload of iron stores (Bezwoda *et al.,* 1979).

Iron status improvement in athletes by dietary means is favoured by the professional bodies, such as the American College of Sports Medicine and the American Dietetic Association (Manore *et al.,* 2000). Iron balance changes in physically active females have been achieved by the modification of participants' diets in a number of studies. This was achieved through increased consumption of iron (Lyle *et al.,* 1992; Anschuetz *et al.,* 2010), through a combination of increased intake of iron-rich foods with absorption enhancers and modifying eating patterns to decrease iron absorption inhibitors or to consume inhibitors between meals (Heath *et al.,* 2001; Patterson *et al.,* 2001). Fogelholm *et al.* (1994) found that treatment of 9 mg Fe/d is enough to correct mild iron deficiency (sFer<20 µg/L, Hb >120g/L) in the general female population but not to enlarge iron stores. Haemoglobin was increased up to 14.2 g/dL after 1 month of supplementation, while sFer was increased from 12-13 µg/L to just over 20 µg/L. Supplementation of 27 mg Fe/d increased sFer up to 30 µg/L, while Hb value were similar to those of 9 mg/d group. Similar findings were reported by Lyle *et al*. (1992) where dietary intervention (advice to consumer and more meat products for 12 weeks, giving a mean total iron intake of 11.8 mg/d) resulted in a significant increase in Hb values from 116 to 124 g/L without any significant changes in other haematological indices. Dietary intervention of 12.4 mg iron per day by modifying eating patterns in order to improve iron status showed improvement in sFer (from 10.3 to 14.0 µg/L), however, very little variation in Hb values (13.16 to 13.22 g/dL) (Heath *et al.,* 2001). Although more research is needed into the effects of dietary modification and improvements in iron status, studies have demonstrated that dietary intervention has the potential to improve haematological indices of women with mild iron deficiency.

In summary, considerable evidence exists to support the negative impact of iron depletion on exercise performance. Despite these well-established consequences of poor iron status in endurance capacity, the prevalence of iron deficiency in female athletes, especially runners, is still high when compared to sedentary counterparts. To date, although promising the research examining the effectiveness of dietary interventions in relation to iron balance and subsequently endurance performance of athletes is limited. Dietary manipulation, through incorporation of iron-rich foods into an athlete's current diet, offers

a less expensive and invasive way of controlling iron balance. Furthermore, the possibility of overdose and side effects is less likely to be experienced with iron-rich food products when compared to therapeutic iron supplements. Finally, obtaining optimum health through good nutrition practices is seen as a more holistic approach that can be maintained long-term.

5.4 Methodological considerations in iron supplementation and sports enhancement research

The most plausible explanation for the conflicting evidence of these intervention studies is the great variation in methodology and techniques used, including sample sizes, exercise protocols, use of haematological markers and their cut-off points, participant selection and control of possible confounders. A number of authors discussed the importance of appropriate design and analysis of these intervention studies (Hopkins *et al.,* 1999; Atkinson and Nevill 2001).

5.4.1 Sampling

The methodological consideration, which is often discussed in exercise studies design issues, is the sample size (Atkinson 2003). Sample sizes vary greatly from 8 to 85 subjects in iron and exercise intervention trials, the most commonly used sample size being 20 to 30 individuals (Appendix 1). A sample size of 30 subjects in each group is considered to result in a sample distribution for the mean that is very close to a normal distribution (Saunders *et al.,* 2003). According to Hopkins *et al.* (1999), a sample size of 65-260 should be used for fully controlled sport performance enhancement studies in order to obtain a precise estimation of the magnitude of the treatment effect. It seems that 30 subjects or more is the desired target sample size sports performance intervention study in order to justify parametric statistical analysis.

5.4.2 Duration of the study

Intervention studies on iron supplementation and performance testing vary in their duration from 2 to 12 weeks, most commonly a 6 to 8 week period was chosen by researchers, although Tsalis *et al.* (2004) carried out an intervention for 6 months. Several intervention studies have shown that iron status can be changed during 2-week supplementation; however, the level of the improvement in the haematological indices was not sufficient to see any change in athletic performance (Schoene *et al.,* 1983; Powell and Tucker 1991; Telford *et al.,* 1992). Therefore, choosing the appropriate length of intervention, which

would be not too long to compromise the compliance but not too short for the treatment effect to take place, is a crucial part of trial design. Several recent studies have shown a significant improvement in both iron and sports performance status of female subjects in a 4 to 6 week intervention period (Hinton *et al.,* 2000; Brownlie *et al.,* 2002; Brownlie *et al.,* 2004), which suggests that such intervention length is sufficient.

5.4.3 Validity, reliability and confounders

Most of quantitative sports performance research studies come under ontological normative paradigm and positivism representation (O'Donoghue 2010). The normative paradigm employs 'single world' principle, which assumes objects to exist independently of the view of different individuals and an 'average human' can be studied by the empirical methods (O'Donoghue 2010). Hence, sampling and analysing the chosen population through the empirical approach, generalisation can be made on the population parameters using quantitative analysis.

The majority of iron supplementation and sports performance enhancement studies try to answer 'applied' questions. As Atkinson and Nevill (2001) defined, applied research investigates factors affecting variables in a 'real-life' setting. In terms of sports performance, whilst 'basic' research tries to underpin theories underlying physiological mechanisms of a particular sports performance phenomenon, 'applied' research in this area usually tests whether proposed ergogenic aid affects sports performance (Atkinson and Nevill 2001).

The nature of the research question is an important factor on the study's validity. The internal validity refers to the control of conditions in order to study isolated variables and is a key criterion for the 'basic' research questions (Newell *et al.,* 2010). Whilst external validity refers to making experimental conditions as close as possible to the usual environment so the results of the 'applied' study could be generalised for the chosen population (Newell *et al.,* 2010). As control over internal validity constrains external validity, the nature of the research determines appropriate validity considerations and optimisation of research conditions (O'Donoghue 2010). Hence, iron supplementation and sports performance studies investigating possible enhancement effects are more concerned with the external validity (making the environment as 'usual' as possible) so the intervention's outcome could be a representation of the 'everyday' result. For instance, researchers account for (by food diaries, physical activity logs, supplement pills counts etc.) rather than control or restrict normal life activities (eating, sleeping, work etc.) so the result of the experiments represents the 'true' value of the intervention.

For research in sports performance field, reliability of measurements such as within-subject variation, change in the mean and retest correlation, is an important factor for trial outcomes, hence, it should be accounted for in the study design (Hopkins 2000). Within-subject variation is the most important type of reliability measure in the experimental study as it estimates the precision of a change in the variables (Hopkins 2000). The smaller the within-subject variation, the easier to notice and measure a change in sports performance and the greater the statistical power of the obtained results (Atkinson and Nevill 2001). Hence, within-subjects variation is used to determine typical error of measurements (typical error= std dev of trials 2-1 of all subjects/√2) (Hopkins *et al.,* 1999). Change in the mean of measurements is in itself a measure of reliability and usually consists of random and systematic change in the mean (Hopkins 2000). Random change in the mean arises from the random error of measurement due to sampling error and can be minimised by selecting a sample size that is large enough for the intervention. Systematic change in the mean is a non-random change and applied to all study participants due to 'learning' or 'training' effect. This can, therefore, be minimised by familiarising subjects with the equipment and protocols. Familiarisation also minimises within-subject variation. Finally, retest correlation is an important measure of reliability and represents how closely measurement values from one trial track the values of replicate trials from individual to individual (Hopkins 2000). All of the reliability values can be assessed prior the intervention by obtaining several test trial results, which can serve as a familiarisation process for participants as well.

Another limitation of sports enhancement studies is accounting for possible confounders. A number of micronutrient supplementation and exercise studies lack control over the assessment of whether the change in iron status is a true effect of supplementation or an adaptive mechanism to the exercise (Akabas and Dolins 2005). For instance, there is a greater increase in blood plasma volume than in Hb as an effect of aerobic exercise (Suedekum and Dimeff 2005). Therefore, measuring differences in blood plasma volume during the trial would account for this confounder and the intervention results would present the actual influence of iron supplementation on changes in sports performance variables rather than the effect of exercise. Furthermore, selecting already regularly physical active women would account for the possible adaptivity element of the trial.

Dietary habits and compliance can be an important confounder. The measurement of the effect of the treatment is difficult to study in isolation as this would decrease external

validity. Therefore, accounting for dietary habits and compliance within the intervention is crucial for applied research. Food records, pill counts and physical activity logs are usually employed by the researchers. A number of performance studies successfully used different dietary habit collection methods, including 24-hour diet recalls (Risser *et al.,* 1988), food frequency questionnaires (FFQ) (Zhu and Haas 1998b; Brutsaert *et al.,* 2003) and 3-day food diaries to estimate dietary intake (Manore *et al.,* 1989; Newhouse *et al.,* 1989 ; Powell and Tucker 1991; Lamanca and Haymes 1992; Lamanca and Haymes 1993; Ishizaki *et al.,* 2006). While a 3-day food diary method results in less errors in food reporting and quantification compared to 24-hour recall or FFQ (Crawford *et al.,* 1994), it can increase drop-out rates as the method very much relies on the degree of motivation of the respondent. Less participant-dependent techniques, such as 24-hour diet recall, can be a good food consumption measure, providing that under- or over-reporting is controlled for (Jonnalagadda *et al.,* 2000).

In conclusion, a number of important methodological issues should be considered and research design optimised to study the effects of intervention on sport and exercise performance enhancement.

5.5 Summary

Evidence suggests that in the UK female population does not consume enough iron in their diet (Bates *et al.,* 2009). This is even more predominant in the female athletes (Gropper *et al.,* 2006), especially as their daily requirement for iron is higher (Whiting and Barabash 2006). It is well documented that adequate iron balance of the female athlete is the important factor for successful training and competition (Beard and Tobin 2000). Therefore, research should focus on increasing iron intake using dietary interventions as a way of improving overall iron status in female athletes and consequently favourably affecting their sport and exercise performance.

Chapter 6 Dietary iron intervention study - Methodology

6.1 Research design – Pilot Study

The aim of the study was to identify any relationships between iron-rich Teff bread consumption, blood iron concentrations and athletic performance. The research design, presented in Figure 6.1, is a pilot intervention study, which investigates some chosen response variables in a single population (Newell *et al.,* 2010). This research approach would come under ontological normative paradigm and positivism representation (O'Donoghue 2010).

Healthy female runners (18-45 years old) were recruited from local running clubs using convenience sampling. All experimental data were collected from each participant at the same time and the day of the week at baseline, midpoint and end.

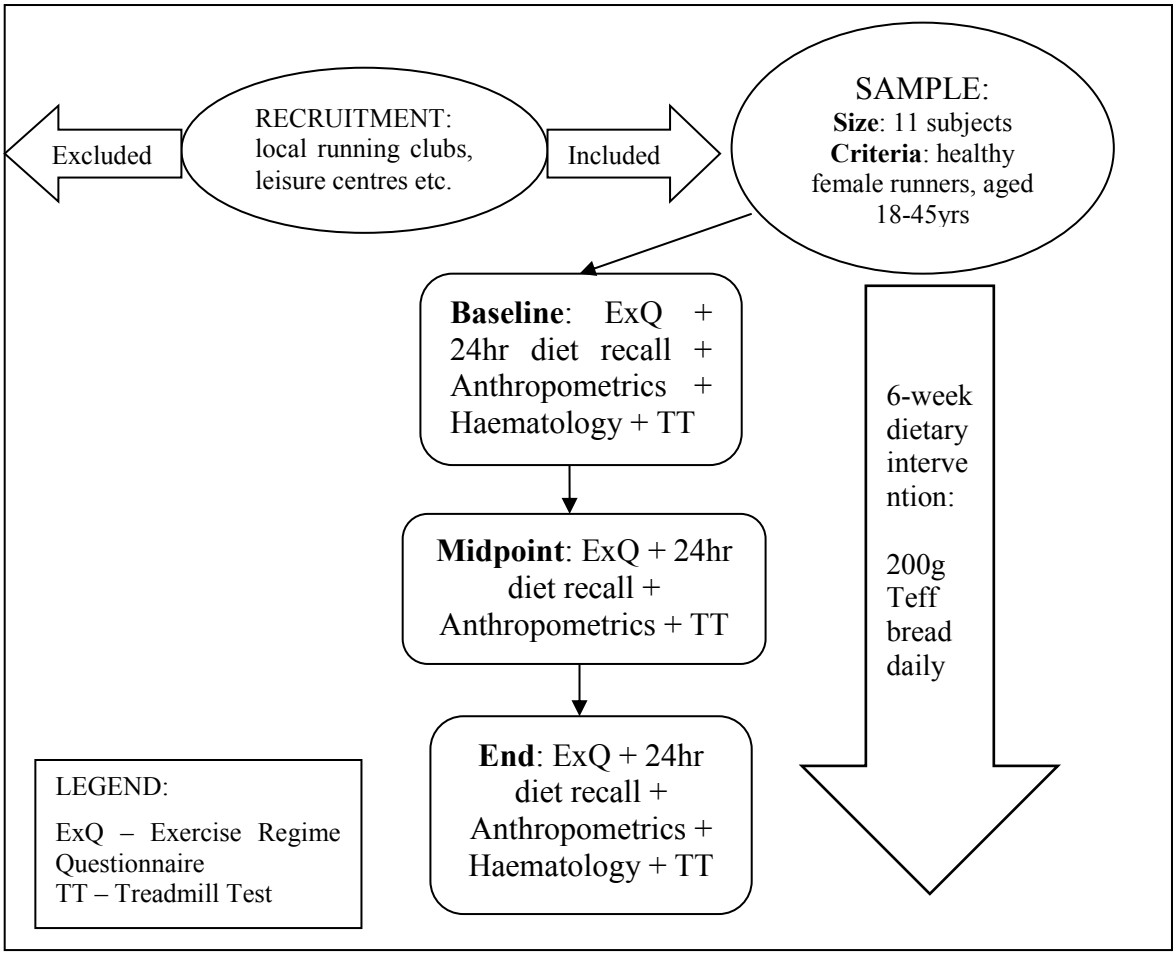

Figure 6.1 Research design for determining associations between iron-rich Teff bread consumption, blood plasma nutrient concentrations and athletic performance

6.2 Ethical considerations

Ethical approval was obtained from The Faculty Ethics Committee; the approved application form is presented in Appendix 10.

Healthy female subjects, aged 18-45 years old, who are habitually engaged in physical activity, were recruited from local running clubs. This population was believed to be able to comfortably perform the treadmill exercise tests and, hence, reduce the ethical issues associated with risks, such as pain and discomfort, as running activity was part of their daily life.

A screening questionnaire (Appendix 2) was administered prior to the start of the study to determine subjects' eligibility to participate in the study in order to account for the ethical issues associated with health and dietary conditions (Table 6.1). For instance, not only subjects who were gluten intolerant were excluded, but also potential participants who did not consume or consumed little bread as part of their diet. It was believed that recruiting participants who do not normally include bread in their habitual diet would have impacted greatly on the dietary habits and would have made it difficult to account for possible dietary confounders.

Potential subjects with chronic or heart conditions or complaints were excluded due to their compromised ability to complete series of treadmill exercise and potential health risk of undertaking such activity.

Psychological aspects of venous blood taking were accounted for as the study did not recruit participants who were uncomfortable with this procedure.

Table 6.1 Inclusion criteria for the intervention study

Inclusion criteria	Justification
Healthy females, aged 18-45 years Habitual physical activity (≥ 6 months; ≥ 30 minutes ≥ 3 times a week) No muscoskeletal problems or recent injury No heart condition or complaints, severe asthma Normal blood pressure Non-smokers	In order to: 1) study desired study population 2) ensure subject's ability to perform time-to-exhaustion treadmill tests 3) account for adaptation to exercise aspect
No current chronic diseases Regular menstrual cycles No recent iron therapy, blood donation or haemophilia No venous blood taking problems: fainting, anxiety No current pregnancy or within past year	In order to: 1) account for possible confounders in iron status determination
No food allergies or intolerances No recent history of eating disorders	In order to: 1) ensure suitability for the dietary intervention

Subjects who were willing to participate and met the study criteria were asked to read the Information sheet (Appendix 3) and sign the Consent form (Appendix 4). The participants had a full understanding of the participation requirements, and an explanation of the purpose of study and experimental design, including measurements frequencies and measurements taken during the study. All subjects were able to withdraw from the study at any time for whatever reason.

The pre-test requirements (Appendix 3) included non-training (or very light training) a day prior testing and no training on the day of testing, consumption of a light carbohydrate meal 2-3 hours before testing, no caffeine and alcohol intake a day before and on the day of the test.

All study testing and measurements were done by appropriately qualified personnel in the designated physiology laboratory at Hollings Faculty under strict supervision of the researchers. Secure changing rooms and showers if needed were available for the participants.

An investigator and one trained observer were present during the anthropometric measurements (weight, height).

Prior to blood taking, the participants signed Phlebotomy consent form (Appendix 11). Blood sampling was carried out using a protocol of *Health and Safety Procedures Manchester Metropolitan University laboratories: Phlebotomy Guidelines*, which complies with the Human Tissue Act, 2004. Blood samples were taken by one trained phlebotomist using Gold-Top Serum Separator Tubes (STT). Blood samples were stored in the appropriate labelled containers and collected by a courier for analysis by The Doctors Laboratory (Manchester, UK). The samples were disposed of by the Doctors Laboratory after analysis.

Study participants were fully informed and familiarised with the treadmill test exercises and blood sampling protocols, frequencies and anthropometric measurements taken during the study. Two trained and certified first aiders were present during the treadmill trials.

All data collected during the study was stored confidentially (password protected databases/spreadsheets) and securely (only accessible to the investigator) in compliance with the Data Protection Act 1998.

6.3 Experimental Protocol

After pre-study screening, all eligible subjects were assigned to a 6-week dietary intervention. During the intervention, study participants were asked to consume approximately 200g of given Teff bread per day and not to change any other of their dietary habits or exercise regime.

All subjects were familiarised with the study's protocol (Appendix 3) a week prior to the intervention and signed the consent form (Appendix 4). The participants had 3 points of contact during the intervention: baseline, midpoint and the end. A number of different tests and measurements (see section 6.3.1) were taken during these appointments. During all three observation points, participants completed a Pre-test questionnaire (Appendix 5), an assisted 24-hour diet recall (Appendix 6), exercise questionnaire (Appendix 7), menstrual cycle log (Appendix 8), were measured for their height and weight and performed a treadmill test. Subjects were also asked to keep a bread consumption log (Appendix 8) in order to record their compliance. During the study, participants gave blood on baseline and on the trial completion.

6.3.1 Measurements

6.3.1.1 Anthropometric measurements

Subjects' heights and weights were recorded at baseline, midpoint and end of the study using a Seca 217 stadiometer (Cranlea, Birmingham, UK) and a Seca 711 personal weighing machine (Cranlea, Birmingham, UK), respectively. The participants were wearing light clothing and no footwear for the measurements.

6.3.1.2 Blood collection and analysis

Iron status was assessed from non-fasted blood samples taken at baseline and completion of the intervention. To limit the possibility of an acute phase response to exercise affecting these results, samples were taken at least 12 hours after last exercise session (Schwellnus 2008). Participants were asked to achieve the same amount of fluid intake during all appointments to ensure similar hydration status, which was assessed by 24-hour recalls.

The following blood indices were analysed: serum ferritin (sFer) as an indicator for iron stores measurement; serum transferrin (sTRF) and serum transferrin receptor (sTsfR) for tissue iron supply determination (SACN 2010). Because these serum iron status indicators are not immediately influenced by food intake (Tobin and Beard 1997) and to ensure participants' study completion rate, the subjects did not fast before having their blood taken.

Serum was prepared from venous bloods by centrifugation after clotting and was stored at -20°C for determination. All haematological analyses were performed at the Doctor's Laboratory- TDL (Manchester/London, UK). Serum ferritin (sFer) was measured by immunometry, using an electrochemiluminescence immunoassay (ECLIA) on Modular Analytics E170 (Roche Diagnostics GmbH, Mannheim, Germany). Serum transferrin (sTRF) was measured by immunoturbidimetric assay; and serum transferrin receptor (sTsfR) by particle-enhanced immunoturbidimetric assay, both using Cobas integra system (Roche Diagnostics GmbH, Mannheim, Germany). All serum samples were analysed concurrently at the completion of the study to eliminate variation in assay conditions.

Additional haematological indices were determined to obtain more comprehensive range of iron status indicators. Total iron binding capacity (TIBC) was calculated using the formula: TIBC (μmol/L) = 25.1 × sTRF (g/L) (Vernet 1993). The sTsfR/log ferritin ratio (sTsfR-F index) was used as an additional marker identification of iron-deficient erythropoiesis.

6.3.1.2.1 Precision and reliability of haematological data

Reproducibility of the test kits was determined using human samples and controls in an internal protocols by the Doctor's Laboratory (Manchester, UK). All analyses were

performed at one point in time, therefore, coefficient of variance (CV) within run (n=84 for sFer, n=20 for sTRF and sTsfR) for the lower and upper range values were used.

CV within run for serum ferritin (sFer) for the lower limit (12.3 µg/L) was reported to be 3.8% whilst the upper limit (392µg/L) was 2.1%.

Serum transferrin (sTRF) CV within run values were 0.86% and 0.77% for the lower (1.35 g/L) and upper (3.36 g/L) range values, respectively.

CV within run for the serum transferrin receptor (sTsfR) lower level (3.17 mg/L) was 0.76% whilst the upper range level (18.9 mg/L) was 1.1%.

6.3.1.2.2 Range and cut-off values for iron markers

Normal physiological ranges for adult female population for the chosen haematological indices were used to determine iron status of study's subjects at the baseline and end of the intervention (see Table 6.2).

Table 6.2 Physiological range values of haematological indices for female population

Haematological marker	Reference interval range in females *	Cut-off value	Indication of iron status
Serum Ferritin (sFer)	13 - 150 µg/L	<12 µg/L	Storage iron depletion
Serum Transferrin (sTRF)	2.0 - 3.6 g/L	>3.3 g/L	Iron-deficient tissue supply
Serum Transferrin Receptor (sTsfR)	1.9 - 4.4 mg/L	>4.4 mg/L	Iron-deficient erythropoiesis
Total iron binding capacity (TIBC)	50 - 70 µmol/L	>72 µmol/L	Depleted iron stores
sTsfR-F index	N/A	>1.5	Functional iron-deficiency

Key: * reference interval ranges taken from haematological kit manufacturer's suggestion and SACN (2010) report

In addition, a number of cut-off values for iron-depletion and iron deficiency states were used. sFer values of <12 µg/L were considered as a state of iron-depletion, hence, an indication of iron deficiency in females (Milman *et al.,* 2003), whereas the values of >30 µg/L were used as repleted-iron stores indication for female population. The sTRF upper cut-off of >3.3 g/L was adopted, which was used by others (Casabellata *et al.,* 2007). Iron status assessment was strengthened with sTsfR-F index values, which indicate the degree

of functional iron-deficient erythropoiesis. The upper threshold value of 1.5 was used (Thomas and Thomas 2002).

6.3.1.3 Exercise performance testing

Trial exercise tests were performed at baseline, midpoint and the end of the study. Exercise tests were performed on a treadmill (Woodway PRO 27) using multi-level 18-min Bruce Protocol (Figure 6.2), which is commonly used to assess athletes' fitness levels (Heyward 2006).

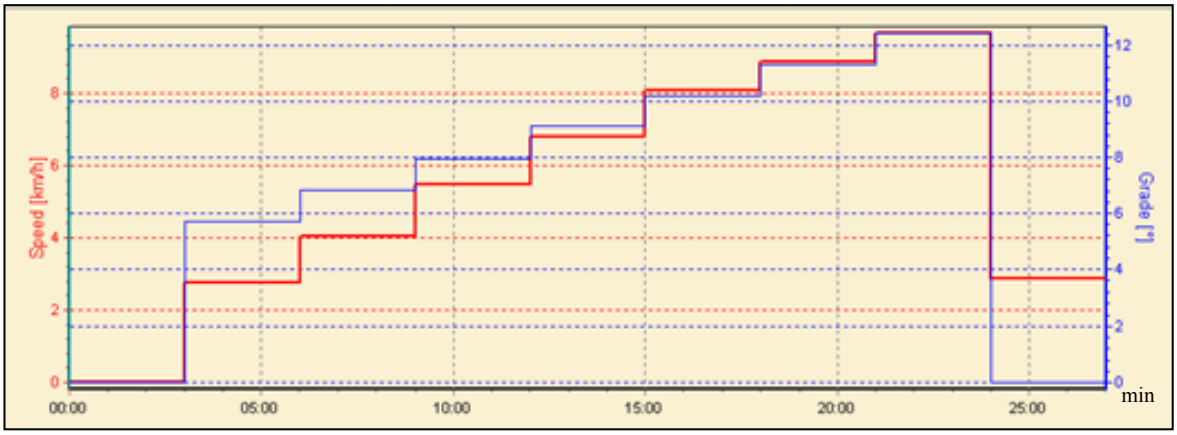

Figure 6.2 Bruce protocol (Metasoft 3.9 Cortex medical, Germany)

Concentration of O_2 and CO_2 in expired air were analysed by breath-by-breath metalyser 3B analyser. Data output from the instrument was analysed by Metasoft 3.9 software (Cortex Medical, Germany) for absolute and relative VO_{2max} at peak, VO_2 at anaerobic threshold (ATr), time ATr reached and time-to-exhaustion.

Reliability of exercise performance measurements was expressed as coefficients of variance (CV) of two familiarisation tests performance by all participants.

The subjects were asked not to perform strenuous exercise 12 hours prior to testing to standardise the exercise protocol across all three-time measurement points and to allow adequate resting period after exercise bouts (Schwellnus 2008). Participants were asked not to consume food or caffeinated beverages 3 hours before the exercise test to avoid possible confounding effect of caffeine as exercise performance enhancer (Sinclair and Geiger 2000).

6.3.1.4 Food intake

Dietary intakes were collected using individual multiple (3X) 24-hour diet recalls (Appendix 6) by interviewing each participant to ensure better validity of this dietary

assessment tool (Montgomery *et al.,* 2005). Dietary intakes were assessed by asking probing questions, particularly on food preparation, portion sizes and whether the reported diet was a representation of subject's usual diet. Individual 24-hour recall record provides accurate reflection on recent subject's diet because of the immediacy of the recall; also little burden to the participant in terms of time allocation; and also 24-hour record is not reported to influence food choice unlike some other dietary recording methods (Thompson and Subar 2008). Nevertheless, 24-hour does not represent long-term dietary intake of an individual (Gibson 2005b). In order to account for this limitation, the present study's recalls were taken at baseline, midpoint and end to ensure more accurate representation of current participants' diet.

The under-reporting was assessed by Goldberg cut-off limits (Goldberg *et al.,* 1991). Total energy in kcal collected by 24-recalls on three separate occasions was tested for under-reporting by applying the following formula: BMR x 1.35. Basal metabolic rate (BMR) for this was obtained by Schofield equations: 18- 29 years BMR = 14.8 x W + 487 SEE; 30-59 years BMR = 8.3 x W + 846 SEE, where W=Body weight (kg), SEE = Standard error of estimation (Schofield 1985).

Dietary data for the intakes of total energy (kcal), carbohydrate (g), sugars (g), total fat (g), saturated fats (g), protein (g), dietary fibre (g), Vitamin C (mg), iron (mg), calcium (mg), zinc (mg), Vitamin A (µg), Thiamin (mg), Riboflavin (mg) and Niacin (mg) were analysed by NetWisp 3.0 (Tinuviel Software, Llanfechell, Anglesey, UK) diet analysis software. Missing food items' nutritional data were manually entered into the software by either locating food composition from product manufacturers' internet sites or the McCance and Widdowson's Composition of Foods integrated dataset (6^{th} Summary, UK Nutrient Databank, 2002, UK).

The percentage of energy derived from each macronutrient (carbohydrate, fat and protein) was calculated by multiplying the grams of each nutrient by the appropriate energy density, and then by dividing by the total energy intake. The recommended intake for macro- and micronutrients were assessed using the Dietary Reference Intakes for adult females (Department of Health 1991).

6.4 Statistical Analysis

Statistical tests were carried out using SPSS 16.0 (SPSS Inc., Chicago, Illinois, US). A significance level of $P<0.05$ was used.

The normality of dependent variables was tested by Shapiro-Wilk test for baseline values for subjects' age, BMI, training regime, iron status parameters, performance scores. The

normal distribution, in terms of skewness and kurtosis, were assessed using 2 X standard deviation for both values (Pallant 2010). Log transformation was applied to variables that were not normally distributed. Boxplots were used to check for possible outliers.

Dietary confounders were assessed using baseline, mid-point and end means values applying repeated measure one-way ANOVA test.

Descriptive statistics and differences in mean values of investigated factors were tested by Pearson's where no account for possible confounders is needed and Partial Correlation with the presence of confounders and Paired-sample t-test to determine the statistical significance effect of pre- and post-intervention. These tests were applied for the haematological and exercise performance data at baseline and end.

Baseline dietary intakes for macro- and micronutrients were compared to current recommendations using One-sample t-test.

Chapter 7 Dietary iron intervention study - Results

7.1 Participants Characteristics

A total of 15 female runners expressed an interest in participating in the study. Participant's eligibility was tested by a screening questionnaire (Appendix 2). The exclusion criteria were: chronic diseases, smoking, recent iron therapy (otherwise 2-week 'washout period' before the study), allergy, raised blood pressure, irregular or missing menstrual cycles, currently pregnant or pregnancy within the past year, current infectious illness, recent blood donation, severe asthma, musculoskeletal problems, recent history of eating disorders.

After a screening process, 4 subjects dropped out due to the exclusion criteria (recent pregnancy, n=1; Diabetes, n=1) and compliance issues (missed their first appointment, n=2).

A total of 11 subjects, aged 20-44 years old, habitually engaged in physical activity for at least the previous 6 months at the level of at least 30 minutes a day 3 or more times a week, were recruited from the local running clubs and leisure centres, successfully completed the study.

Characteristics of the participants are shown in Table 7.1. The mean age of participants was 32 years. The subjects were within the normal BMI range and engaged in regular exercise regime, mostly running.

Table 7.1 Subjects' Characteristics

ID	Age (yrs)	BMI (kg/m^2)	Main physical activity	Inter-vention (number of days)	Exercise regime (min/wk)	Running activity (min/wk)	Baseline VO_{2max} (ml/min/kg)	Comp-liance (bread slices/day)
1	44	20	R (l-d)	35	380	300	46	6.2
2	29	23	R	30	170	110	40	3.0
3	20	22	R, V	38	540	360	38	3.6
4	36	21	R	36	120	60	36	1.4
5	28	22	R, SW	31	120	60	35	2.5
6	39	21	R (l-d)	30	480	480	42	5.0
7	31	25	R	30	160	100	35	5.5
8	26	24	D, R	34	180	30	42	3.5
9	38	27	R	36	200	150	34	2.1
10	33	28	R	36	160	60	33	5.0
11	33	25	R (l-d)	46	120	60	33	5.4
mean	**32**	**23**		**35**	**239**	**161**	**37.6**	**3.9**
std dev	**7**	**2**		**5**	**153**	**150**	**4.3**	**1.6**

*Key: Main physical activity: R-running, R (l-d)- long-distance running, V- volleyball, SW-swimming, D-dancing

7.1.1 Sample size and variables distribution

Although the present study's sample size was small (n=11), it employed stringent inclusion criteria and accounted for possible confounders, including dietary habits and training routine. The statistical tests for normality showed no significant anomalies for subjects' age, BMI, nutrients intakes, exercise and blood iron parameters with the exception of serum transferrin receptor and running activity (Table 7.2). Both parameters were skewed towards lower values. Kurtosis test value show that data was normally distributed with no 'peakedness' or outliers. The fact that most of the variables in iron status, dietary intake and exercise categories showed normal distribution suggests that the present study had overall normal variables distribution and the study population was representative. Furthermore, a number of other iron treatment and sport and exercise enhancement studies used similar sample sizes (Blee *et al.,* 1999; Ishizaki *et al.,* 2006; Peeling *et al.,* 2007).

Table 7.2 Tests for data normality

	Variables	Skewness	Kurtosis	Shapiro-Wilk test significance values
	Age (yrs)	-0.121	0.079	1.0
	BMI (kg/m^2)	0.489	-0.789	0.6
Baseline iron status	Baseline sTRF (g/L)	0.542	-1.251	0.1
	Baseline log sTsfR (mg/L)	0.866	0.012	0.1
	Baseline sFer (µg/L)	0.08	-1.974	0.1
Baseline dietary intakes	Energy (kcal/day)	0.287	-1.478	0.3
	Iron (mg/day)	0.600	-0.870	0.3
	Vitamin C (mg/day)	0.544	-0.335	0.7
	Fibre (g/day)	1.048	1.619	0.4
	Protein (g/day)	0.001	-0.726	1.0
	Calcium (mg/day)	0.435	-1.107	0.3
Baseline exercise regime	Running Activity (min/week)	1.316	0.552	0.01*
	VO_{2max} (ml/min/kg)	0.722	-0.557	0.2
	VO_{2max} at ATr (ml/min/kg)	0.149	-1.481	0.8
	Distance-till-exhaustion (metres)	-0.335	1.392	0.7
	Anaerobic threshold (ATr) reached (min)	-0.945	1.454	0.7

* indicates variables outside normal distribution range.
Normality range for skewness (-1.322 to +1.322), normality range for kurtosis (-2.558 to +2.558).
Abbreviations: sTRF- serum transferrin, sTsfR - serum transferrin receptor, sFer- serum ferritin

7.1.2 Duration and compliance of the intervention

The duration of the intervention varied between 30 to 46 consecutive days (Table 7.1). The main reason for this variation was the fact that some participants were unable to complete a 6-week intervention due to other pre-planned activities. Nevertheless, it was believed that this may have improved subjects' compliance and accounted for possible confounders, such as sudden change in their dietary or exercise habits due to holiday plans or special sport events.

The participants complied with the study's requirements and consumed 125 ± 37 g of bread a day (≈ 4 slices/day) (Table 7.1). This amount of Teff bread provided an average of 7.0 ± 3.3 mg of iron a day and contributed to approximately 38% of total dietary iron intake in this study. Slightly higher treatment of 9 mg Fe/day was employed by Fogelholm *et al*. (1994), which corrected mild iron deficiency in women but not enlarged iron stores.

7.1.3 Measures taken to account for possible confounders

The intakes of energy, fibre, protein and ascorbic acid were unchanged throughout the intervention. These dietary parameters were shown to influence intestinal absorption of dietary iron (Hallberg *et al.,* 1989; Boech *et al.,* 2003), hence, any notable changes in these nutrients may have an impact on the validity of intervention. Because nutrient intakes were similar throughout six week period, the intervention was successful in terms of ensuring control of the possible dietary confounders.

Table 7.3 Nutritional intakes at baseline and the end of the intervention

Dietary factors	Baseline	Midpoint	End	P value*
Energy (kcal/day)	2339±589	2467±507	2401±429	0.8
Protein (g/day)	83±23	89±26	84±15	0.8
Fibre (g/day)	17±6	20±6	22±8	0.3
Vitamin C (mg/d)	89±54	75±78	75±63	0.3
Calcium (mg/day)	1162±499	979±512	799±531	0.2
Meat/poultry/fish (portions/day)	2.5±1.2	2.1±0.9	2.0±0.9	0.4
Coffee/tea intake (servings/day)	2.5±1.6	2.3±1.3	2.5±1.5	0.9

* indicates significant differences between the mean ± std dev values at different intervention time points (P<0.05; One-way repeated measures ANOVA)

Female athletes may practise calorie-restrictive eating behaviours (Weight *et al.,* 1992). In addition to this, food intake under-reporting by the participants may be an issue in estimating accurate dietary habits. However, this is unlikely to have been the case in the current study because all of the participants did not reveal any degree of under-reporting in their food records. All daily energy values as reported by study participants were above the cut-off point for under-reporting (Table 7.4). This suggests that dietary assessment technique (multiple 24-hour recalls by interviews) was adequate for assessing subjects' diets. Literature suggests that 24-hour recall as a method for assessing dietary intake offer less interference with dietary behaviour in contrast to other direct recording methods because the intake recording occurs after foods are consumed (Thompson and Subar 2008). This may also explain why under-reporting was not observed in the present study.

Table 7.4 Dietary under-reporting

Subject ID	Basal metabolic rate[1] (kcal/day)	Goldberg cut-off value[2] (kcal/day)	Energy[3] (kcal/day)
1	1411	1905	2133±133
2	1423	1921	2280±469
3	1413	1908	2783±633
4	1298	1752	2032±366
5	1455	1964	2018±249
6	1204	1625	2351±416
7	1751	2364	2476±490
8	1455	1964	2558±460
9	1484	2003	2441±254
10	1644	2219	2030±394
11	1373	1854	3322±165

[1] BMR calculated according to Schofield equation (Schofield 1985)
[2] Goldberg cut-off value calculated BMR X 1.35 (Goldberg *et al.,* 1991)
[3] Energy represents the mean ± std dev of three 24-hour recalls (baseline, mid-point and end)

7.2 Baseline nutrient intakes

Macronutrients provide energy. Thus, adequate supply and correct balance of macronutrients are essential part of optimum nutrition. In the present study, female runners achieved recommendations for total energy, protein and fibre intakes that were satisfactory, whereas fat consumption was above the recommended values and at the expense of carbohydrate intake, which was significantly lower than the recommendations for optimum athletic performance (Table 7.5). The importance of adequate carbohydrate intake for maintenance and recovery of muscle glycogen stores during training and competition in female runners is well documented (Burke *et al.,* 2007). Yet sub-optimal carbohydrate intakes observed in present study's female runners population corresponds to the findings of other authors (Berning *et al.,* 1991; Papadopoulou *et al.,* 2002; Hinton *et al.,* 2004). Furthermore, the intake of non-milk extrinsic sugars (NMES) was above the recommended value of >10% total energy in the current study. This may further suggest that higher NMES levels would have compromised the intake of complex carbohydrates. Current national and international dietary guidelines suggest increasing complex carbohydrate in the diet for better blood glucose control and high fibre content (Department of Health 2011). For sports performance, complex carbohydrates provide an

important slow-release energy, which is critical for pre- training and competition glycogen stores optimisation and gradual energy release during exercise (Jeukendrup and Williams 2011).

Table 7.5 Comparison of mean daily macronutrient intakes of present study's population and recommended values

Macronutrient	Female runners (n=11)	Recommended intake	P value (One-sample t-test against RNI)
Energy			
kcal	2339±589	2197[1]	NS
kcal/kg BW	35.9	33.8[2]	NS
Carbohydrate			
g/kg	4.7	6 - 10[3]	0.012-0.0005
% energy	52±7	60[3]	0.004
Fat			
% energy	36±10	<33[1]	NS
Saturated fat			
% energy	11±6	<10[1]	NS
Protein			
g/kg	1.3	1.2 - 1.7[3]	NS-0.019
% energy	15±3	15[1]	NS
Fibre			
g	17±5	18[1]	NS
NMES			
% energy	14±7	<10[1]	NS

[1] Recommendations based on EAR/RNI values for women, aged 19-50 years (Department of Health 1991)
[2] Recommendations based on the EARs for energy for women aged 30-59 years, 65 kg and PAL of 1.6 (Department of Health 1991)
[3] Recommendations based on ACSM and ADA joint position statement (Rodriguez *et al.,* 2009)

It is generally agreed that micronutrients required for optimum nutrition in athlete population can be solely achieved by the diet (Rodriguez *et al.,* 2009). In the present study, female runners exceeded the recommendations for Vitamin C, B-group vitamins, calcium and zinc. However, they did not meet recommendations for Vitamin A and showed significantly lower intake of iron compared to national recommendations (Figure 7.1). This is in agreement with most other researchers (Berning *et al.,* 1991; Hassapidou and Manstrantoni 2001), who also reported the mean micronutrient intakes to be above recommended values with the exception of iron.

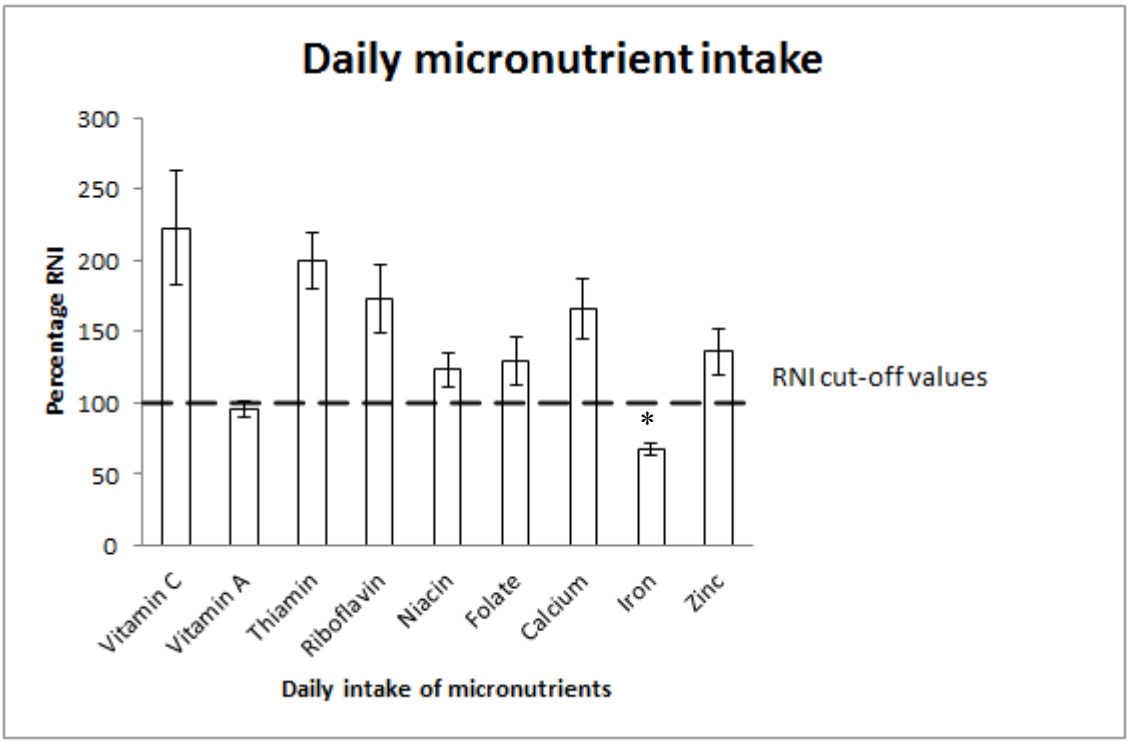

Figure 7.1 Comparison of percentage daily micronutrient intake achieved in female runners' population and reference nutrient intakes

* represent significantly (P<0.001; One-sample t-test) lower observed value compared to that of recommended intake
Abbreviations: RNI- reference nutrient intake

Further to this, the baseline iron intake of the present study's cohort was 10.2 mg/d, providing 70% of recommended daily intake of iron (Department of Health 1991). This agrees with the findings of other authors, who reported iron intakes to be in the range of 11.0-12.2 mg/d for female athletes (Pate *et al.,* 1993; Spodaryk *et al.,* 1996) and is similar to that of the general female population, which is reported to be 10.0 mg/d (Bates *et al.,* 2009). Only 36% of runners reached the estimated average requirement (EAR) of 11.4 mg/day, with 18% of them falling below the lower reference nutrient intake (LRNI) (Figure 7.2), which is similar to the levels (21%) in general female population (Bates *et al.,* 2009).

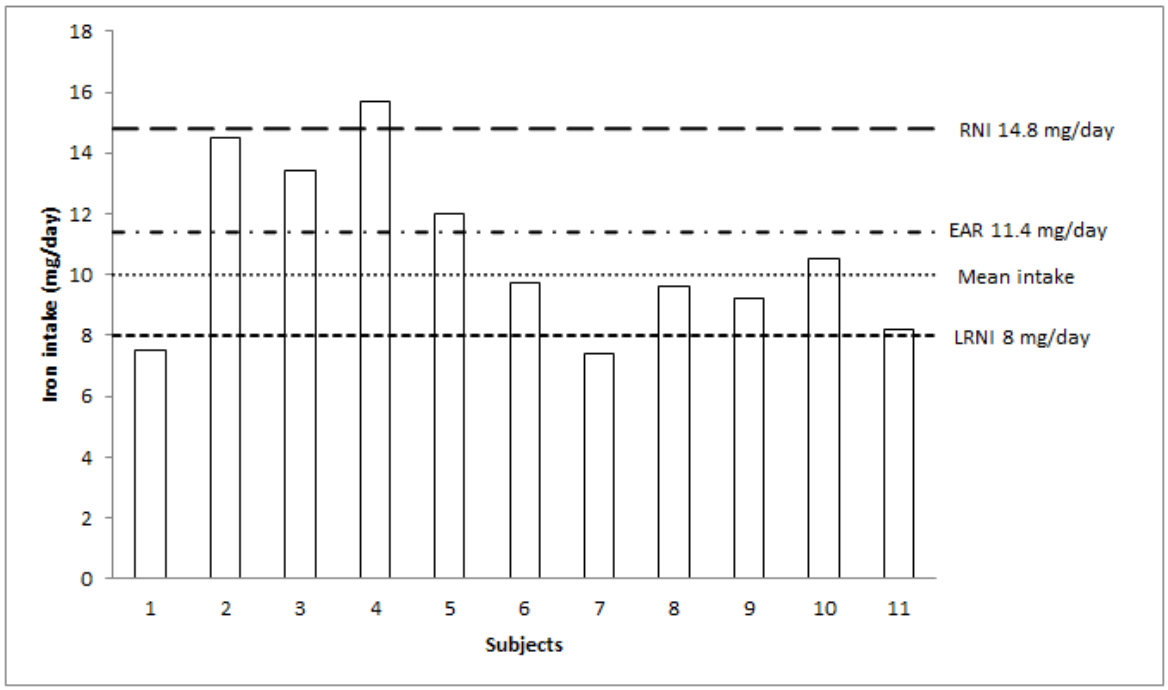

Figure 7.2 Comparison of individual percentage daily dietary iron intake achieved in female runners' population and dietary reference values
Abbreviations: LRNI- lower reference nutrient intake, EAR- estimated average requirements, RNI- reference nutrient intake (Department of Health 1991).

In summary, these results suggest that female runners would benefit from education on the importance of achieving optimum nutrition, in particularly adequate intake of complex carbohydrates and iron.

7.3 Baseline body iron status, diet and training

A number of different iron storage and iron tissue supply haematological indices were tested in order to determine subjects' iron status.

Iron status was assessed by using serum ferritin (sFer) as a measure of total iron stores and serum transferrin receptor (sTsfR), sTsfR to sFer ratio (sTsfR-F index) as an indication of immediately available iron and functional tissue iron deficiency (Cook *et al.,* 2003). Total iron-binding capacity (TIBC) was used as a measure of the maximum amount of iron needed to saturate serum transferrin (sTRF), which is the primary iron-transport protein, hence, enabling to establish adequate iron-tissue supply (British Nutrition Foundation 1995). Table 7.6 shows baseline haematological status female runners.

Table 7.6 Haematological status of female runners in the present study

Subjects	Serum Transferrin g/L	Serum Transferrin Receptor mg/L	Serum Ferritin µg/L	TIBC µmol/L	sTsfR-F index
1	3.85*	3.8	8*	97*	4.2*
2	3.1	2.66	30	78*	1.8*
3	2.61	2.17	44	66	1.3
4	3.35*	1.64	56	84*	0.9
5	2.83	2.58	47	71	1.5
6	3.35*	3.77	8*	84*	4.2*
7	2.79	2.29	22	70	1.7*
8	2.71	2.44	49	68	1.4
9	4.12*	6.64*	5*	103*	9.5*
10	2.54	2.67	53	64	1.5
11	4.08*	7.08*	4*	102*	11.8*
Mean ± std dev	**3.21±0.59**	**3.43±1.81**	**30±21**	**81±15**	**3.6±3.7**
% below the cut-off	**45%**	**18%**	**36%**	**55%**	**55%**

* indicates individual values below the cut-off points (Table 6.2 in the Methods section).

The results showed that 36% of female runners had sFer values below the cut-off value, indicating depleted iron stores. Other iron tissue supply measures (sTRF, TIBC and sTsfR-F index) revealed even a higher degree of functional iron tissue supply deficiency as over half of the study cohort had test values below the cut-off values.

Dietary factors, such as total iron, calcium, protein, Vitamin C, fibre, meat/poultry/fish and coffee/tea intakes, and exercise load were studied for possible correlations with baseline iron status. The findings are presented in Table 7.7 and Table 7.8.

Baseline dietary components showed interesting association with subjects' iron status. Surprisingly, known iron absorption enhancers, including Vitamin C, protein, meat intakes were poorly correlated to any of the haematological indices, whilst inhibitors, such as coffee, showed negative correlations at statistically significant level. Total iron intake was positively correlated with serum transferrin receptor and serum ferritin. This suggests that dietary iron is an important contributing factor to body's iron supply and storage, whilst polyphenol-rich beverages as iron absorption inhibitors show negative impact on iron tissue supply.

Table 7.7 Associations between baseline iron status and diet

Nutrient		Correlations to baseline values: Serum Transferrin g/L	Log Serum Transferrin Receptor mg/L	Serum Ferritin µg/L	TIBC µmol/ L	sTsfR-F index
Iron (mg/d)	r value	-0.35	-0.60	0.66	-0.27	-0.50
	p value	0.29	0.05*	0.028*	0.42	0.12
Calcium (mg/d)	r value	0.11	0.21	-0.35	0.06	0.14
	p value	0.76	0.54	0.30	0.85	0.69
Protein intake	r value	-0.15	-0.15	0.09	-0.14	-0.04
(g/d)	p value	0.66	0.67	0.80	0.69	0.92
Vitamin C (mg/d)	r value	-0.42	-0.50	0.29	-0.40	-0.50
	p value	0.20	0.13	0.40	0.22	0.12
Dietary fibre (g/d)	r value	-0.28	-0.12	0.49	-0.25	-0.04
	p value	0.40	0.73	0.13	0.47	0.90
Meat/ Poultry/	r value	-0.25	-0.33	0.39	-0.21	-0.19
Fish (portions/d)	p value	0.51	0.33	0.24	0.54	0.58
Coffee/ Tea	r value	0.48	0.65	-054	0.47	0.65
(servings/d)	p value	0.14	0.03*	0.09	0.15	0.029*

* indicates a statistically significant correlation ($P<0.05$, Pearson's bivariate correlation)

The current study failed to show any association between training regime and iron status, suggesting that physical activity, at a recreational and habitual level, does not have an adverse effect on overall iron status in female runners.

Table 7.8 Associations between baseline exercise regime and iron status

Exercise parameter		Correlations to baseline values (r values): Serum Transferrin g/L	Log Serum Transferrin Receptor mg/L	Serum Ferritin µg/L	TIBC µmol/ L	sTsfR-F index
Exercise activity (min/week)	r value	-0.05	-0.02	-0.24	-0.05	-0.10
	p value	0.89	0.99	0.49	0.89	0.78
Running activity (min/week)	r value	0.13	0.12	-0.42	0.12	0.02
	p value	0.73	0.72	0.20	0.73	0.96

7.4 Changes in iron status during the intervention

The intake of total dietary iron increased significantly (10.7→18.5 mg/day, $P<0.05$) as a result of the intervention. However, it should be noted that Teff bread provides less absorbable form of non-haem iron. Nevertheless, even though in the form of non-haem iron, the intervention diet provided an average of 18.5 mg of iron a day, the amount which is higher than RNI, hence, a positive dietary iron change.

There were no statistically significant differences observed in sTRF, sTsfR, sFer, TIBC and sTsfR-F index values before and after the intervention in the present study (Figure 7.3 and Figure 7.4). Nevertheless, all of the iron status parameters showed favourable trends. sTRF, sTsfR, TIBC and sTsfR-F index values decreased, which suggest an improvement in iron tissue supply, while sFer increased, suggesting increase in storage iron levels. Furthermore, one of the greatest positive improvements was seen in the sTsfR parameter. This is in agreement with other researchers who suggested sTsfR to be the most reliable and sensitive parameter in determining mild iron tissue deficiency (Zhu and Haas 1998b). Furthermore, favourable changes in iron tissue supply (sTRF, sTsfR) were also supported by within-subject variation data (Table 7.9). Coefficients of variance (CV) for both of these values were considerably below the incremental change, indicating this change was due to the intervention. Nevertheless, this was not observed in iron storage parameter (sFer). CV was 3.8% for the upper reference value and 2.1% for the lower reference value. Because the incremental change in sFer in this cohort was 5.4%, this change must have been notably influenced by the within-subject variation.

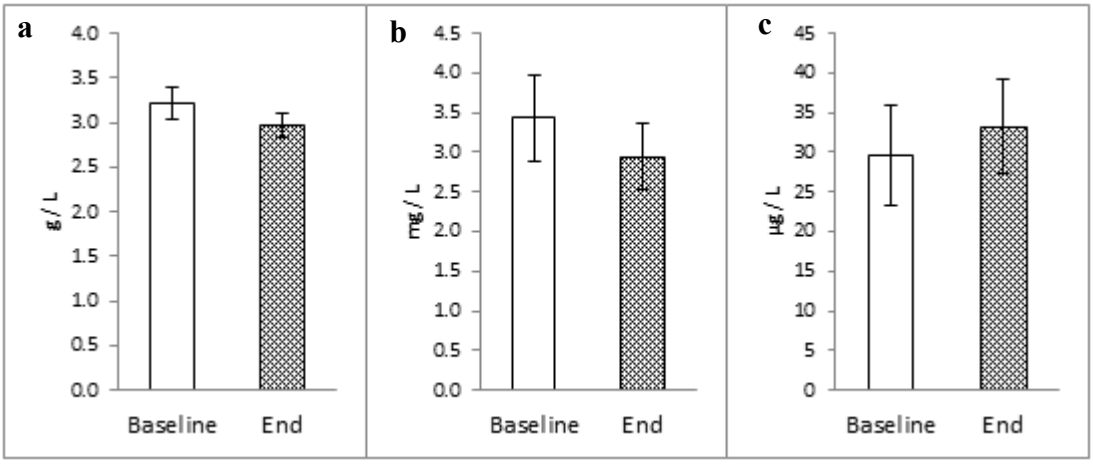

Figure 7.3 Baseline and end values of direct measurement haematological indices (n=11)

a- serum transferrin, **b**- serum transferrin receptor, **c**- serum ferritin

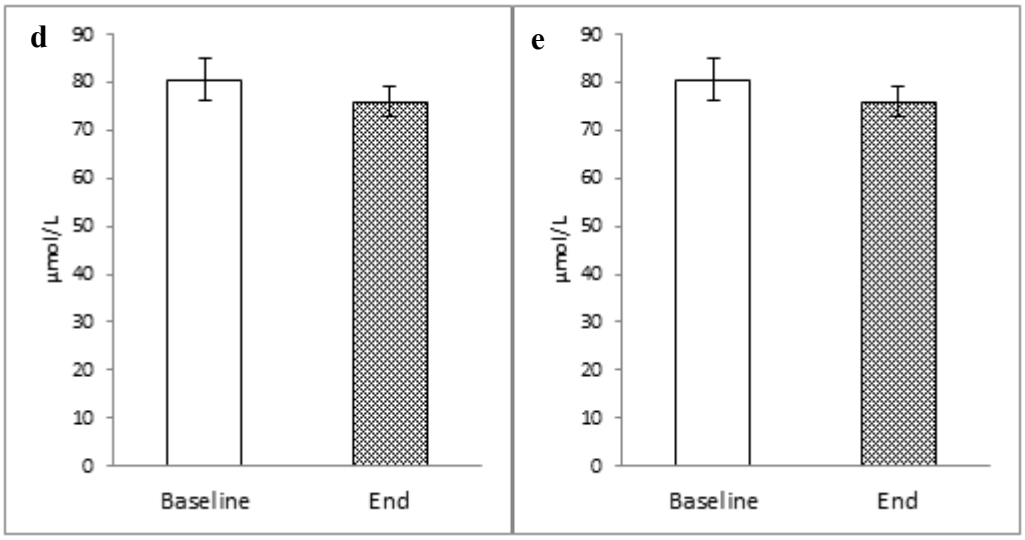

Figure 7.4 Baseline and end values of indirect measurement haematological indices (n=11)

d- TIBC, **e**- sTsfR-F index

Although improvements in iron status were only marginal and not at a statistically significant level, a favourable change in iron status and the iron deficiency levels, as assessed by the haematological cut-off points, were observed (Table 7.9).

The frequency of iron storage depletion, as indicated by changes in serum ferritin (sFer), was reduced by a half by the end of intervention. 18% of study population had sFer readings below the cut-off value of 12 µg/L, suggesting improvements in iron storage status. Lower incidence of inadequate iron tissue supply was also observed. Furthermore, the greatest changes in iron status parameters were evident in serum transferrin receptor (sTsfR) and serum transferrin receptor-serum ferritin index (sTsfR-F index) values. This reiterates the sensitivity and reliability of both parameters to marginal changes in iron status in this population as suggested by other authors as well (Zhu and Haas 1998b; Malczewska *et al.,* 2001).

Table 7.9 Changes in iron status related parameters during the intervention

			Subjects below cut-off value*	
Iron status parameter	**Change during the intervention**	**CV within run** (lower-upper range)	**Baseline**	**End**
Serum Transferrin (g/L)	-5.4%	0.86-0.77 %	45%	18%
Serum Transferrin Receptor (mg/L)	-12.8%	0.76-1.1 %	18%	9%
Serum Ferritin (µg/L)	+5.4%	3.8-2.1 %	36%	18%
TIBC (µmol/L)	-6.2%	N/A	55%	45%
sTsfR-F index	-16.7%	N/A	55%	45%

* indicates individual values below the cut-off points (refer to Table 6.2 in the Methods section).

Additionally, the favourable changes in iron status parameters were associated with baseline haematological indices, the length of intervention and change in dietary iron intake (Table 7.10).

The iron status of already iron-depleted participants showed the greatest improvements in haematological parameters during the intervention. This was indicated by the correlations between changes in tissue iron supply parameters (sTFR and sTsfR) and the highest initial values of these haematological indices, showing inadequate iron supply at baseline.

The increment in iron storage was positively correlated with changes in dietary iron intake (Table 7.10 and Figure 7.5). This suggests that iron stores were enlarged the greatest in runners who increased their dietary iron intake the most. This also indicates that dietary iron was incorporated into body iron stores in female runners with adequate baseline iron stores. Hence, even a marginal increase in dietary iron (from 10.7 to 18.5 mg/day) can provide beneficial effects on storage iron level.

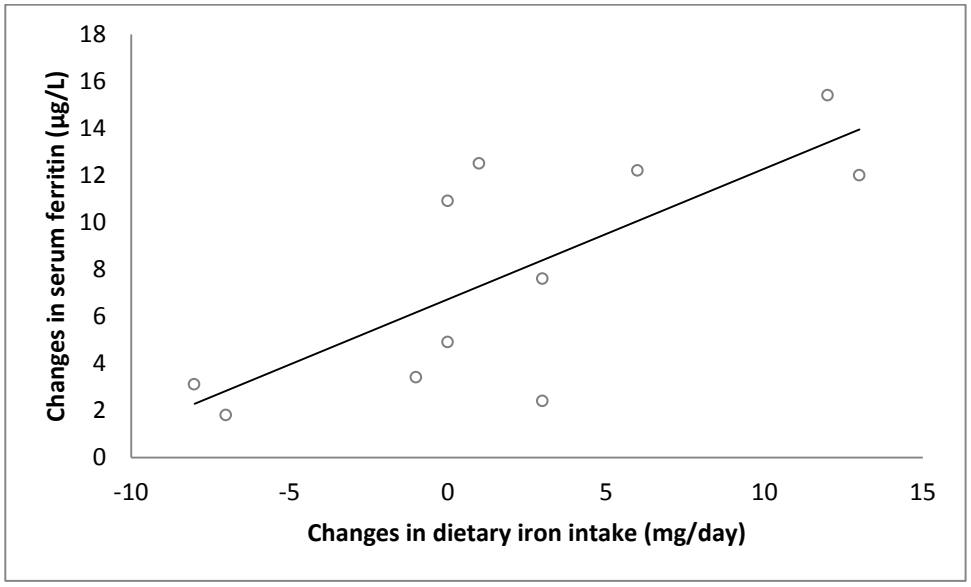

Figure 7.5 Relationship between changes in dietary iron and serum ferritin in the present study's population (r=0.8, *P*<0.05 when controlling for dietary confounders; Partial correlation)

Table 7.10 Relationship between iron status of the study population and other variables

Changes in haematological indices	% change	Correlations with	R value, *P* value
ΔsTRF (g/L)	-5.4	Baseline sTRF	r= - 0.7, *P*<0.05
		Baseline sTsfR	r= - 0.8, *P*<0.01
		Baseline TIBC	r= - 0.7, *P*<0.05
		Baseline sTsfR-F index	r= - 0.8, *P*<0.01
		No of days of intervention[1]	r= - 0.7, *P*<0.05
ΔsTsfR (mg/L)	-12.8	Baseline sTsfR	r= - 0.8, *P*<0.05
		Baseline sTRF	r= - 0.6, *P*<0.05
		Baseline TIBC	r= - 0.6, *P*<0.05
		Baseline sTsfR-F index	r= - 0.8, *P*<0.01
		No of days of intervention[1]	r= - 0.6, *P*<0.05
ΔsFer (μg/L)	+5.4	Δ sTsfR-F index	r= - 0.6, *P*<0.05
		Δ dietary iron intake[2]	r= 0.8, *P*<0.05

[1] partial correlation controlling for corresponding baseline haematological parameter
[2] partial correlation controlling for changes in dietary calcium and vitamin C intake

In summary, favourable changes, even though not statistically significant, in iron status of women regularly engaged in habitual physical activity were observed in the present study. Correlation analysis revealed that the positive increment in tissue iron supply was mostly seen in runners with compromised baseline iron status. Longer intervention exposure for marginally iron-depleted runners was the most beneficial in improving iron tissue supply parameters. Whilst changes in iron storage were not associated with reduced baseline iron status, serum ferritin levels were increased by dietary iron intake, suggesting incorporation of dietary iron in body iron stores.

7.5 Sport & exercise performance tests

7.5.1 Changes in gas exchange and endurance parameters

Pulmonary gas exchange variables, including absolute and relative VO_2 at peak and anaerobic threshold, are used for exercise testing to determine performance potential, aerobic fitness and exercise tolerance (James *et al.,* 2007). Time-to-exhaustion and time-trial tests are commonly administered to assess changes in endurance performance (Newton *et al.,* 2008).

Gas exchange threshold and endurance data are presented in Figures 7.6- 7.9, whilst incremental change of these parameters is presented in Figure 7.10.

VO_{2max} indicates the rate at which oxygen can be taken up and utilised by the body during endurance exercise (Bassett and Howley 2000), and is considered to be one of the most objective measure of physical performance, compared to other parameters, such as time-to-exhaustion, which can be more subjective indication of sports performance (Rodenberg and Gustafson 2007). Absolute (L/min) and relative (ml/min/kg of body weight) VO_{2max} peak values increased by 4.9 and 4.1%, respectively (Figure 7.10), however, at a non-significant level (VO_{2max} values: 2.4→2.5 L/min, P=0.151; 37.6→39.2 ml/min/kg, P=0.096). Furthermore, CV for VO_{2max} peak measurements were 2.3% and 2.1% for absolute and relative VO_{2max} values. This indicates that the change in VO_{2max} peak values as a result of the intervention was only marginal and the change was greatly attributed to within-subject variation of the VO_2 tests performed.

The ATr is commonly used as a submaximal index of aerobic capacity and occurs at an exercise level below VO_{2max} (Wilmore *et al.,* 2008). VO_2 at anaerobic threshold (ATr) point showed a statistically significant increase (VO_2 values: absolute VO_2 incremental increase of 17.7% with CV of 4.6%, 1.7→2.0 L/min, P<0.0005; relative VO_2 incremental increase of 16.8% with CV of 4.9%, 27.0→31.5 ml/min/kg, P<0.0005). Consequently, anaerobic threshold point time significantly increased when baseline and end values were compared (Figure 7.8; 10.5→14.6 minutes with CV 8.2%, P<0.0005).

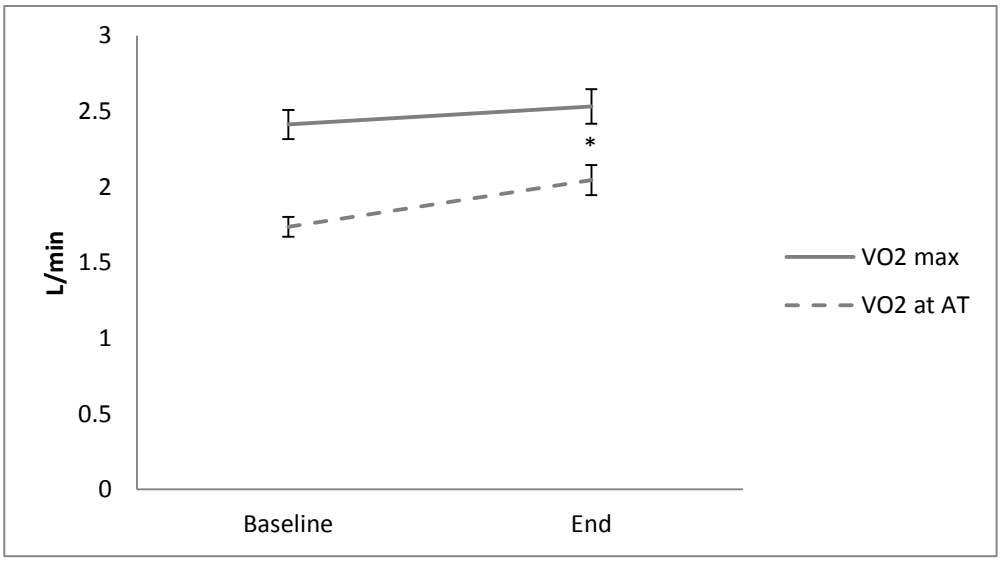

Figure 7.6 Changes in absolute VO_{2max} values before and after intervention (n=11)
* indicates significant difference between baseline and end values (P<0.0005; Paired-samples t-test)

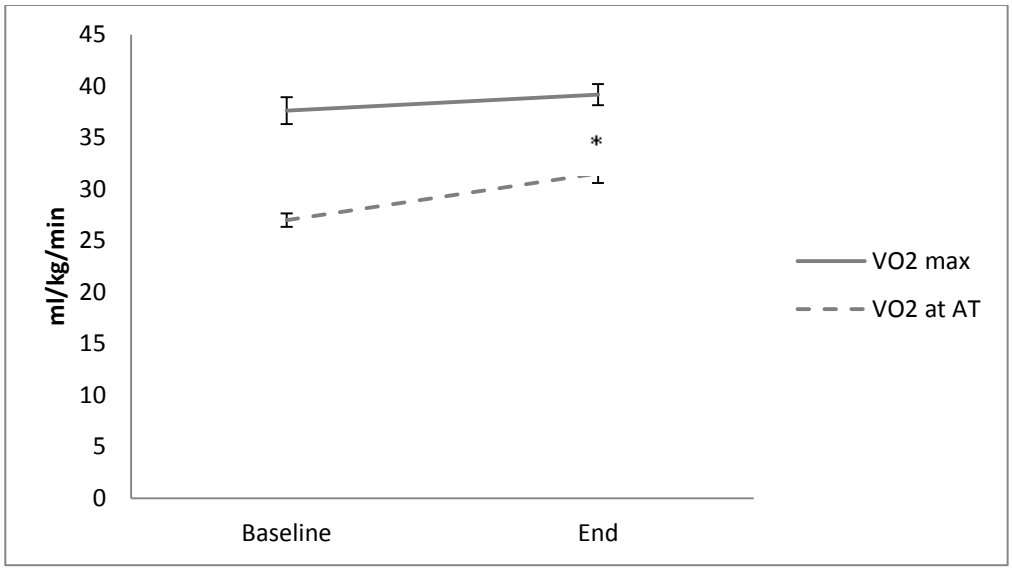

Figure 7.7 Changes in relative VO_{2max} values before and after intervention (n=11)
* indicates significant difference between baseline and end values (P<0.0005; Paired-samples t-test)

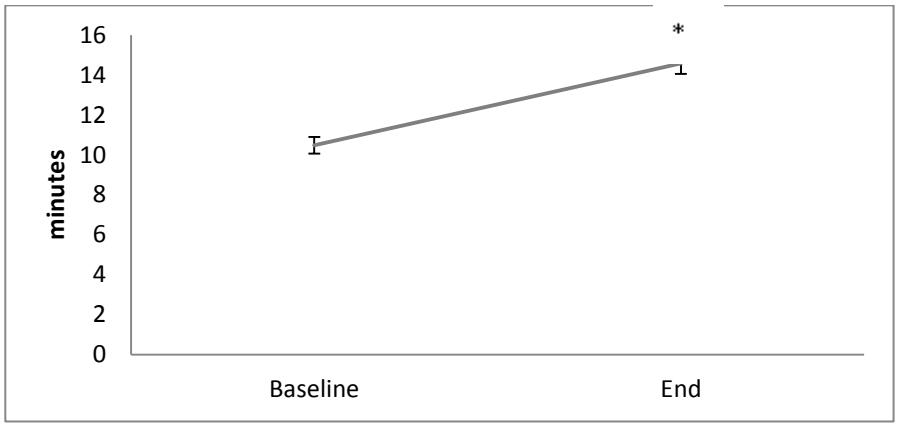

Figure 7.8 Changes in anaerobic threshold (ATr) reached time before and after intervention (n=11)
* indicates significant difference between baseline and end values (P<0.0005; Paired-samples t-test)

Time-to-exhaustion is a frequently used measure for endurance performance. Athletes are asked to perform to exhaustion at specific submaximal exercise intensities until they are not able to maintain the required speed or power (Newton *et al.,* 2008). In the present study, time-to-exhaustion was part of progressive exercise testing protocol where the exercise intensity is incremental under specific time sets; hence the workload was not kept constant.

The results from the current study show that time-to-exhaustion significantly increased during the intervention period (incremental change of 24.5% with CV of 4.3%, 15.6→19.4 minutes, P<0.0005). Although possible confounders influencing the favourable outcome of this study's findings, especially 'learning effect', should not be overlooked, an incremental

change in anaerobic threshold times supports these favourable changes in time-to-exhaustion results.

Indeed, incremental changes in performance measures show that endurance variables (ATr time reached at and time-to-exhaustion) showed highest improvements in this cohort (Figure 7.10). Furthermore, improvements in endurance performance parameters, including VO_{2max} and time-to-exhaustion, were reported in other placebo-controlled iron intervention trials (Friedmann *et al.,* 2000; Hinton *et al.,* 2000). Similarly to the findings from the present study, both of the latter authors observed favourable changes in aerobic and endurance performance with improved iron status.

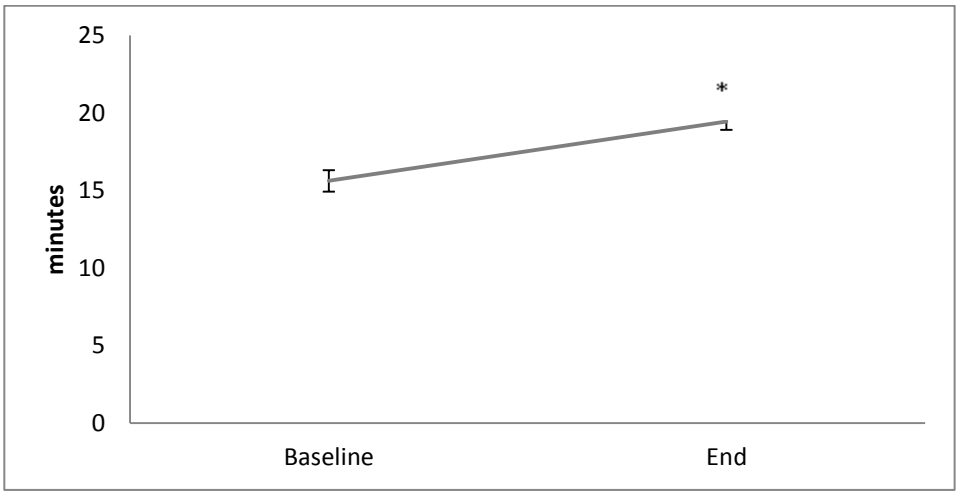

Figure 7.9 Changes in time-to-exhaustion before and after intervention (n=11)
* indicates significant difference between baseline and end values (P<0.0005; Paired-samples t-test)

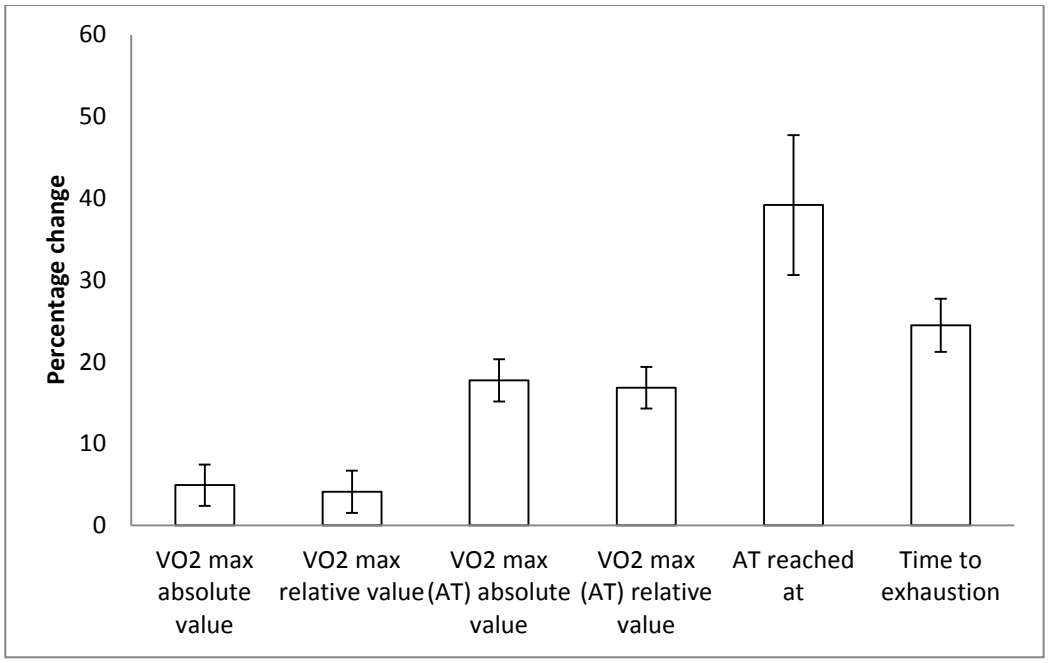

Figure 7.10 Incremental changes in sports performance parameters (n=11)

Overall, the findings from the current study show that maximal performance values (absolute and relative VO_{2max}) improved marginally during the intervention in female runners' population, which was mostly contributed to the within-subject variation. However, gas exchange parameters (absolute and relative VO_2) at anaerobic threshold point showed that runners significantly improved their aerobic capacity, seen as an increase in VO_2, and maintained endurance activity for significantly longer periods before reaching anaerobic transition point, seen as an increase in time ATr point was reached. This was also strengthened by time-to-exhaustion findings indicating improvements in endurance performance.

7.5.2 Relationship between changes in iron status, sports performance and dietary iron

Supporting correlations between changes in haematological parameters and sports performance values were observed (Table 7.11). This suggests that the outcomes of the present study were due to dietary intervention.

Oxygen uptake parameters (absolute and relative VO_2) at anaerobic threshold point were significantly correlated to iron storage, serum ferritin, but not to any of the tissue iron supply indicators.

Improvement in endurance performance (time-to-exhaustion) was strongly associated with favourable changes in iron tissue supply indicators (serum transferrin, serum transferrin receptor, TIBC and serum transferrin/serum ferritin index) in the present study.

Table 7.11 Relationship between performance parameters of the study population and iron status variables

Changes in performance parameters	% change	Correlations with	R value, *P* value, Pearson's bivariate correlations
Δ absolute VO_{2max} at ATr (L/min)	+17.7%	Δ sFer	r= 0.7, $P<0.05$
Δ relative VO_{2max} at ATr (ml/min/kg)	+16.8%	Δ sFer	r= 0.7, $P<0.05$
Δ time-to-exhaustion	+24.5%	Δ sTRF	r= - 0.8, $P<0.005$
		Δ sTsfR	r= - 0.7, $P<0.05$
		Δ TIBC	r= - 0.8, $P<0.005$
		Δ sTsfR-F index	r= -0.7, $P<0.05$

In summary, the findings from the present study indicate positive relationships between dietary iron intervention and sports performance results, including maximal aerobic (VO_2 at peak and anaerobic threshold) and endurance performance parameters (time-to-exhaustion). Although some of the improvements may have been attributed to the limitations in study design and possible confounders, some significant correlations between haematological changes and sports performance findings suggests that improved athletic performance was associated with dietary iron intervention and subsequently with marginally improved iron status in this female runners' population.

Chapter 8 Dietary intervention study - Discussion

Adequate nutrition is critically important for achieving optimal sports performance. Like all athletes, female runners require a nutritionally balanced diet to maintain daily activities and also successful training regime and competition.

Despite the increased interest in nutrition amongst athletes and well documented importance of a balanced diet in athletic performance, research suggests that many athletes might be consuming diets less than optimal (Papadopoulou *et al.,* 2002; Hinton *et al.,* 2004). This trend was also observed in the present study. The runners' diets provided inadequate amounts of carbohydrate, whilst their intake of total fat, saturated fat and NMES were above the national recommendations (Table 7.5). Most of the micronutrient intakes were observed to be above reference nutrient intake (RNI), with the exception of Vitamin A and iron (Figure 7.1). The baseline iron intake of the present study's cohort provided only 70% of RNI for iron (Figure 7.2). This trend is in agreement with most of other researchers (Berning *et al.,* 1991; Hassapidou and Manstrantoni 2001), who also reported the majority of macro- and micronutrient intakes to be above recommended values with the exception of iron. The mean intake of iron in this study was 10.7 ± 2.7 mg/d. This corresponds with other authors' reported iron intakes of 11.0-12.2 mg/d for female athletes' population (Pate *et al.,* 1993; Spodaryk *et al.,* 1996) and in fact is similar to that of the general female population, which is reported to be at around 10.0 mg/d (Bates *et al.,* 2009). In addition to this, the present study found that only 36% of runners reached estimated average requirement (EAR) of 11.4 mg/day, with 18% of them falling below the lower reference nutrient intake (LRNI) (Figure 7.2). This is similar to the levels (21%) reported for the general female population (Bates *et al.,* 2009). Taking into account that the iron requirement for female runners may be higher than general population (Whiting and Barabash 2006), the baseline observations from the current study indicate that dietary iron intakes in this population are far from adequate.

Baseline iron status of female runners in the present study showed similar trends reported by other authors. It is generally agreed that serum ferritin (sFer) is the index of iron stores in healthy subjects (Beard and Han 2009). Plasma sFer levels are strongly correlated with iron stores in the bone marrow (Cook *et al.,* 2003), hence, low levels of sFer indicate iron deficiency. The physiological range of sFer in adult females is 15 to 200 μg/L (SACN 2010). Due to this wide inter-individual variation, the lower limit of sFer for indication of iron deficiency is not well established. The lower limit varies over a wide range from 12 to 30 μg/L in iron supplementation and sports performance studies. The analysis of sFer in

the present study used the value of 12 µg/L, the cut-off point for indication for the absence of marrow iron stores and completely depleted iron stores, usually considered as iron deficiency state in females (Milman *et al.,* 2003; Rodenberg and Gustafson 2007).

Baseline iron status results showed that 36% of all participants had sFer levels lower than 12 µg/L, with the lowest value observed being 4 µg/L, indicating very low or absent iron stores. Similar findings were reported by other authors, who found out 20-50% of female runners had marginal iron deficiency (Table 8.1), which is more than twice that of the general population (Lampe *et al.,* 1986b; Risser *et al.,* 1988; Pate *et al.,* 1993; Sinclair and Hinton 2005). Thus, current national recommendations for dietary iron may not be sufficient for the maintenance of normal iron status in female runners. In the present study, there were no cases of elevated sFer, the highest value found in this female runners' group was 56 µg/L, which was well below the midpoint of normal physiological range of 13-150 µg/L (SACN 2010). The mean sFer concentration in the present study's population at baseline was 30±21 µg/L, which indicates adequate iron stores. This is in agreement with similar smaller sample size (Ostojic and Ahmetovic 2008) and large sample athlete population studies (Pate *et al.,* 1993; Di Santolo *et al.,* 2008). Nevertheless, due to a high standard deviation value and the proportion of participants below the cut-off level of 12 µg/L, it should be noted that there was a wide spread of different iron stores levels even within this small sample size. This highlights a great degree of between-subject variability within female runners' population and may explain the vast majority of conflicting results obtained by the iron therapy studies (Literature review section 5.3).

Table 8.1 Comparison of mean serum ferritin values and iron depletion levels in female athletes

Studies	Participants	Mean sFer (µg/L)	Cut-off value (µg/L)	Iron depletion
Present study	Distance runners (n=11)	30±21	<12	36%
Woolf *et al.* (2009)	Highly active females (n=28)	32±28	<12	21%
Ostojic & Ahmetovic (2008)	Distance runners (n=15)	27±12	<12	20%
Di Santolo *et al*. (2008)	Various sports athletes (n=70)	24±17	<12	27%
Gropper *et al*. (2006)	Cross-country runners (n=9)	38±38	<12	22%
Spodaryk *et al.* (1996)	Various sports athletes (n=40)	40±11	<20	20%
Pate *et al.* (1993)	Distance runners (n=111)	26±21	<20	50%

Serum transferrin receptor (sTsfR) and sTsfR to sFer ratio (sTsfR-F index) are quantitative measures of tissue iron deficiency and functional iron deficiency, respectively (Skikne *et*

al., 1990). However, due to lack of international agreement over the cut-off values for these parameters, determination of iron deficiency levels on a population scale is challenging. This is reflected in the current study's findings. According the cut-off value for sTsfR, only 18% of current study's participants showed iron-deficient erythropoiesis, whilst sTsfR-F index values showed that more than half of the runners had functional iron-deficiency.

One of the most frequently suggested reasons in the literature for low iron status in physically active female population is inadequate habitual dietary iron intake (Rodenberg and Gustafson 2007). Both compromised iron status in a third of runners and inadequate dietary iron intakes were observed in the current study, and consequently there was a significant positive correlation between low total dietary intake and low serum ferritin and elevated serum transferrin receptor values at baseline (Table 7.7). Whilst a number of studies do not support this observation (Pate *et al.,* 1993; Telford *et al.,* 1993; Malczewska *et al.,* 2000), there is some evidence to suggest that dietary iron may influence body iron stores (Lamanca and Haymes 1992; Pynaert *et al.,* 2007). A large scale study (n=641) by Pynaert *et al.* (2007) indicated that there was a significant difference lowest *vs* highest quartile of dietary iron intake and serum ferritin in females subjects, however, as the increase in iron stores was not gradual throughout quartiles, the authors highlighted the need for further research to strengthen this association. Supporting evidence was reported by Lamanca and Haymes (1992). The authors investigated the relationship between dietary iron intake and serum ferritin levels in active women (n=16). Ferritin concentration was significantly related to absorbable iron (r=0.72, $P<0.05$) and total iron (r=0.70, $P<0.05$) intake, the observation also seen in the present study. However, Malczewska *et al.* (2000) study findings showed that the principal cause of iron deficiency in female athletes was menstrual blood loss, whilst low dietary iron intake contributed to much lesser extent, contrary to the observations in the control population. In the present study, runners did not report any irregularities in their menstrual cycle, whilst Malczewska *et al.* (2000) observed significantly higher values for the intensity and duration of menses in iron deficient athletes when compared to iron-repleted female athletes. This may explain lack of agreement between the studies.

Dietary factors considered to critically enhance iron bioavailability, hence, potentially iron status, include Vitamin C, total protein, meat/poultry/fish intake (Reddy *et al.,* 2000). In the present study, these dietary components were found to bear no relationship to iron

status. This is contrary to the findings of Spodaryk and colleagues (1996), who reported strong associations between animal proteins, meat and fish intake and serum ferritin in 40 female athletes. The percentage of energy derived from proteins was similar in the study undertaken and that of Spodaryk *et al.* (1996) (16% *v* 14%, respectively). Hence, the most plausible explanation would be a cohort effect. Nevertheless, the current study's observed lack of relationship between Vitamin C, dietary fibre and animal protein sources is comparable with the study of Pate *et al*. (1993). The latter study employed participants with slightly lower storage iron balance (sFer 25.6±21.1 µg/L) compared to Spodaryk *et al.* (1996) (sFer 39.9±21.1 µg/L). In the present study, the mean sFer was 30±21 µg/L, which possibly explains why the findings were comparable to those of Pate *et al*. (1993). It should also be noted that the lack of relationship between dietary iron absorption enhancers and increased iron status as seen in the current study, could be attributed to the internal iron homeostasis 'masking' effect (Bjorn-Rasmussen *et al.,* 1974). This is also in agreement with iron bioavailability trials, as discussed in section 5.2.3.1, which suggest that although iron availability promoters affect overall iron absorption, the effect of these in the whole diet is negligible due to the 'dilution' effect. In addition to this, although at a statistically non-significant level, correlations in the current study showed a similarity to other studies (Spodaryk *et al.,* 1996; Malczewska *et al.,* 2000) as factors such as animal protein and Vitamin C enhanced iron absorption.

Unlike iron absorption enhancers, dietary inhibitors showed statistically significant associations with iron status in this study. Polyphenols-rich beverages, such as coffee and tea, are all known to be iron absorption inhibitors (Tuntawiroon *et al.,* 1991; Hallberg *et al.,* 1993b). In the present study, higher coffee and tea intakes showed associations with reduced iron tissue supply (Table 7.7). This is in agreement with another study, in which a significant influence on iron balance in female runners was reported (Pate *et al.,* 1993). Malczewska *et al.* (2000) suggests high calcium intake to be one of the attributing factors to reduced iron status, contrary to the observation seen in the present study, even though calcium intake was well above the recommended intake (refer to section 7.2). One plausible explanation would be the discrepancy in calcium intake in athletic population with iron deficiency and normal iron stores as reported by Malczewska *et al.* (2000). In their study iron deficient female athletes consumed significantly higher levels of calcium compared with the athletes' normal iron stores (1066 mg/day *v* 785 mg/day, respectively, $P<0.01$). This explains strong association observed between increased calcium intake and reduced overall iron status. However, the present study participants reported daily calcium

intake of 1161 mg, which is above that observed by Malczewska *et al.* (2000). Furthermore, the lack of association between some known dietary iron absorption enhancers and inhibitors in the present study may also be explained by methodological factors, especially relatively small samples size, no reference population and reliability of 24-hours recall to represent a true habitual diet.

Interestingly, when only iron-depleted (sFer <12 μg/L) subjects (n=4) were included into analysis, no statistically significant correlations were observed between iron status and dietary components. Although with such a small sample sub-group, it is difficult to draw conclusions, this may suggest the importance of an individual's current iron stores in overall iron status (Hulten *et al.,* 1995).

The effects of exercise on body iron stores are controversial. A number of authors reported lower iron stores in female athletes (Gropper *et al.,* 2006) compared to sedentary controls (Woolf *et al.,* 2009). This was also supported by the relationship between increased physical training and lowered iron status (Pate *et al.,* 1993). However, others did not confirm this observation and suggest a lack of relationship between compromised iron status and increased training in female athletes (Malczewska *et al.,* 2000; Ostojic and Ahmetovic 2008).

In this study, there was no association between training regime and any of the haematological indices as revealed by correlation analysis. Such observations may have been influenced by the limitations of a small sample size and heterogeneous cohort. Participants in the present study were engaged in similar amounts of exercise and running activity (exercise activity 239±153 min/week; of which running activity 161±150 min/week; n=11) when compared to a much larger study (running activity 167.3±123.9 minutes/week; n=111) (Pate *et al.,* 1993). However, Pate *et al.* (1993) sample showed similar running activity time to the current study's cohort, but the authors observed a weak, statistically significant trend of increased physical activity and lowered iron status (r= - 0.2; $P<0.05$), which was not the case in this study. Taking into account that the present study sample was considerably smaller, the between-subject variation is much greater, which may explain why the statistical significance was not achieved. In fact, the trend of reduced serum ferritin levels and running activity was the strongest observed association (r= - 0.41, NS) in this study, which again suggests that a larger sample size might have shown a significant trend (Table 7.8). Another plausible explanation of the lack of relationship between exercise and iron status might be the level of training regime. The

subjects in the current were not at elite sport level, hence, would not have engaged in ultra-endurance training, which may have a greater impact on iron status (Candau *et al.,* 1992).

As a result of the intervention, the dietary iron intake increased to 18.5 ± 3 mg/d by the end of this study. However, only marginal improvements in iron status were observed (Figure 7.3 and Figure 7.4). The lack of significant change in iron status in the current study may be attributed to several factors.

One plausible explanation would be that mean serum ferritin baseline value showed iron-repletion state (sFer 29.64 ± 20.9 µg/L). Researchers have demonstrated an inverse relationship between iron absorption and serum ferritin (Hulten *et al.,* 1995). Hence, it may be possible that the subjects in this study had sufficient iron stores at baseline, which would explain the lack of significant effect of increased dietary iron intake on overall iron status. The majority of other iron therapy research studies recruited iron-depleted female runners (sFer<20 µg/L) and used pharmacological dosages of iron supplementation, hence, it is difficult to compare them to the findings from current study. However, a longitudinal study by Tsalis *et al.* (2004) demonstrated no change in sFer levels of swimmers after 5 months of dietary intervention (26 mg/day) and a decrease in sFer after 6 months. The participants in the study carried out by Tsalis *et al.* (2004) showed similar iron storage values to the present study (sFer>30 µg/L). Hence, similar baseline iron status of these studies cohorts may explain the lack of iron therapy effect in both studies.

Another reason for the lack of notable changes in iron status might be due to the dietary iron bioavailability. However, researchers suggest that around two thirds of dietary non-haem iron was incorporated into the red blood cells (RBC) after 2 weeks of consumption in participants with sufficient iron stores (Roughead *et al.,* 2002). Therefore, non-haem iron derived from an iron-rich bread product in this study during 6-week intervention period would have been sufficient for the iron uptake and utilisation. Nevertheless, cereal products provide a less absorbable form of non-haem iron compared to animal sources, which contain haem iron (Hallberg 1981). Therefore, the lack of the effect on iron status suggests lower iron bioavailability in Teff bread or/and presence of iron absorption inhibitors in Teff bread meal composition in the present study. Indeed, another recent study reported significant correlations between absorbable iron in the diet and iron status in distance runners (Anschuetz *et al.,* 2010). Authors concluded that non-haem iron absorption is strongly influenced by meal composition, especially in the presence of iron absorption enhancers, and consequently can impact overall iron status.

Finally, the increase in dietary iron intake by 7 mg/day might not have been sufficient to see notable changes in iron status, especially as most studies showing iron status improvements used therapeutic supplementation dosages (100 mg elemental Fe/day) (Lamanca and Haymes 1993; Friedmann *et al.,* 2000). Ishizaki *et al*. (2006) suggested that 4-week dietary intervention of 15 mg of iron a day increased the activity of δ-ALAD, an enzyme participating in red blood cells (RBC) turnover; however, this did not increase any other haematological indices. The dosage of dietary iron supplementation in the present study (mean of 7 mg/day from Teff bread, total intake of 18.5 mg Fe/day) is similar to the total dietary treatment of 15 mg/day by Ishizaki *et al*. (2006). However, perhaps this increase in dietary iron may have not been sufficient enough to increase subjects' iron status notably. Fogelholm *et al*. (1994) compared different iron therapy dosages in iron-depleted female subjects (sFer <20μg/L; Hb >12g/dl) over a period of 4 weeks. The dosage of 27 mg/day significantly improved sFer levels, while 9 mg/day corrected initial low sFer values but did not enlarge iron stores. Although the researchers did not report dietary iron consumption of the participants, it is likely that the present study's total iron intake of 18.5 mg/day is at a similar level of Fogelholm's *et al.* (1994) 9 mg/day treatment group. In addition, the mean sFer levels of the present study's subjects were ≈30 μg/L, which is higher than that of the iron-depleted population in the study by Fogelholm *et al.* (1994).

Despite the lack of significant changes in haematological indices, the study observed some important correlations between baseline iron status and favourable outcomes of the intervention. The iron status of iron-depleted participants showed the greatest improvements in haematological parameters during the intervention (Table 7.10). This was indicated by the correlations between changes in tissue iron supply parameters (sTFR and sTsfR) and the highest initial values of these haematological indices, showing inadequate iron supply at baseline. This can be explained by the homeostatic body iron metabolism control as the absorption of dietary iron is increased with compromised iron status (Frazer and Anderson 2005; Beard and Han 2009). This agrees with the findings of the present study in which it was found that the greatest improvements in iron status were seen in the runners with low initial iron supply.

The increment in iron storage was positively correlated with changes in dietary iron intake, even when controlling for initial iron status (Table 7.10). This suggests that iron stores were increased more in runners, who increased their dietary iron intake the most regardless of their baseline iron status. This also indicates that dietary iron was incorporated into body iron stores. Hence, even a marginal increase in dietary iron (from 10.7 to 18.5 mg/day) can provide beneficial effects on storage iron level.

Furthermore, the favourable changes in these iron supply indicators were also associated with the prolonged intervention. It is debatable how long dietary iron intervention should take place in order to correct or improve the iron status of physically active females. Whilst some therapeutic iron supplementation studies showed significant improvements after 2-week treatment (Powell and Tucker 1991; Telford *et al.,* 1992), it is not clear whether the same length of dietary intervention would provide similar results. Ishizaki *et al*. (2006) suggested that 4-week dietary intervention of 15 mg of iron a day increased the activity of δ-ALAD, an enzyme participating in red blood cells (RBC) turnover, however, this did not increase any other haematological indices.

Iron is important for both oxygen transport and mitochondrial energy production; hence, not surprisingly an adequate iron status is essential for aerobic fitness and endurance physical activities (Suedekum and Dimeff 2005). There is a considerable debate in the literature as to whether iron supplementation can improve sport and exercise performance (Table 8.2).

Table 8.2 Comparison of iron supplementation and exercise performance studies

Study	Subjects	Protocol	Findings
Present study Dietary intervention	11 habitual runners, aged 20-44y, BMI 23	**Study design:** 6-wk intervention (18.5mg Fe/d) **Dietary assessment:** Multiple 24-hr recalls **Performance assessment**: Treadmill tests	NS improvements in iron status. SIG correlations between ↑ dietary iron and ↑ sFer ($r=0.8$, $P<0.05$). NS improvements in VO_{2max}, but SIG in time-to-exhaustion.
Peeling *et al.* (2007) Intramuscular injection randomised control trial	16 physically active females, recruited from running, cycling & triathlon clubs, aged 15-40y	**Study design:** 20-day intervention IG: 5x2ml (100mg elemental Fe) intramuscular injection for 10 days C: placebo group **Dietary assessment:** 4-day FD **Performance assessment**: Cycle ergometer / treadmill tests	Fe injections SIG increased sFer (faster rate than oral supplementation). NS improvement in VO_{2max} after intramuscular Fe injection for 10 days. Time-to-exhaustion at VO_{2max} workload SIG increased in IG group, but NS difference between groups.
Ishizaki *et al.* (2006) Dietary intervention	8 collegiate rhythmic gymnasts, aged 18-19y, BMI 19.7	**Study design:** 4-wk intervention in 2yr cohorts: -1st yr self-selected diets -2nd yr fixed diet for 4 wks (15mg Fe/day) **Dietary assessment:** 3-day FD 3 times in 1st / 2nd yr at baseline, 4,8 wks **Performance assessment**: NONE	SIG increased sFer and δ-ALAD activity (responsible for RBC turnover) after diet intervention. No effect on other parameters or iron depletion. Intervention during weight loss period- confounder to show true effect? ↓Haptoglobin=footstrike haemolysis.

Tsalis *et al.* (2004) Supplementation and dietary randomised control trial	21 male and 21 female swimmers, iron-repleted (sFer >30 µg/L, Hb>12 g/dl) aged 12-17y BMI 20	**Study design:** 6-month intervention A: Fe supplement (47mg/d) B: diet rich in Fe (26mg/d) C: placebo **Dietary assessment:** Records of daily food intake **Performance assessment**: Swimming tests	NS differences in iron status or performance among 3 groups. Improvements in performance-due to adaptations to program? Failure of high Fe intake to change Fe status in Fe depleted athletes- possible homeostatic mechanism?
Friedmann *et al.* (2000) Supplementation randomised control trial	23 female athletes, iron-depleted nonanaemic (sFer <20 µg/L, Hb>12 g/dl), aged 14-18y	12-wk intervention IG:100mg/d elemental Fe X 2/d B. placebo Diets not assessed *<u>*Treadmill Medbo-test*</u>s	sFer and VO_{2max} increased significantly in IG but not in C group. Fe supplementation in iron-deficient female athletes improves aerobic performance.
Zhu & Haas (1997) Cross-sectional observation study	30 females (15 iron-depleted (sFer< 12ug/L, Hb >120 g/L); 15- control) aged 19-36y, physically active	**Study design:** Cross-sectional ID- iron deficient group C- control group **Dietary assessment:** FFQ focused iron-rich foods **Performance assessment**: Cycle ergometer test	ID group had lower VO_{2max} than C, when controlled for PA and fat-free mass (r=-0.367,P=0.011). VO_{2max} associated with sFer when controlled for PA and free-mass (r=0.184, P<0.05), but not affected by oxygen-transport capacity.
LaManca & Haymes (1993) Randomised control trial	*20 female athletes, iron-depleted (sFer <20 µg/L), aged 19-35y	**Study design:** 8-wk intervention IG: Fe (50mg X 2/d elemental Fe) supplement C: placebo **Dietary assessment:** 3-day FD **Performance assessment**: Cycle ergometer tests	SIG VO_{2max} improvement in IG compared to C- due to decrease in C value? Endurance time to exhaustion increased by 38% in IG but NS between groups.
Lyle *at al.* (1992) Supplementation and dietary randomised control trial	60 exercising (2.3-2.5 d/wk) females, previously sedentary, iron-repleted (sFer >20 µg/L, Hb>12 g/dl) aged 18-19y	**Study design:** 12-wk intervention A: Fe 50mg+ low diet Fe B: Fe 10mg+low diet Fe C: placebo D: meat diet (18mg) **Dietary assessment:** 7-day FD **Performance assessment**: Walking and treadmill tests	Moderate aerobic exercise compromised iron status. Meat diet was more effective in protecting Hb and Fer status than were iron supplements.
Klingshirn *et al.* (1992) Supplementation randomised control trial	18 female runners (sFer <20 µg/L, Hb >12 g/dL), aged 22-39y	**Study design:** 8-wk intervention IG:50mg elemental Fe X 2/d C: placebo matched for time-to-exhaustion **Dietary assessment:** Questionnaire **Performance assessment**: Treadmill tests	8 week Fe supplementation improved Fe indices in iron-depleted runners. Improved Fe status did not improve performance.

Abbreviations: PA- physical activity, SIG – significantly, NS- non significantly, IG- intervention group, C- placebo group, FD- food diary, FFQ- food frequency questionnaire

Although iron-deficiency anaemia has been shown to have an adverse effect on performance (Ohira *et al.,* 1979; Edgerton *et al.,* 1981), the evidence of marginal iron deficiency and the effect on athletic performance is conflicting. Whilst some researchers

reported significant improvements in sport and exercise performance in marginally iron-deficient females after the correction of iron status using iron supplements (Lamanca and Haymes 1993; Zhu and Haas 1997; Friedmann *et al.,* 2000), others show no considerable improvement or an adverse effect of marginal iron depletion on the performance (Klingshirn *et al.,* 1992; Peeling *et al.,* 2007).

The research on dietary iron interventions in sport and exercise performance in female athletes is very scarce. A longitudinal study conducted on swimmers investigated iron status and exercise performance during a 6 month period (Tsalis *et al.,* 2004). The authors observed no differences in iron status or performance scores between iron-rich diet and free choice diet participants. However, a four weeks iron-rich diet, which provided 18.2 mg/d, showed significant effects on sFer concentration in rhythmic gymnasts (Ishizaki *et al.,* 2006). Another study by Lyle *et al.* (1992) reported that a diet rich in iron, providing 11.8 mg iron/day, was more effective in protecting iron status than were supplements (50 mg ferrous sulphate/day) during 12 weeks of aerobic tests in previously sedentary women. Additionally, the dietary intervention group also showed the highest improvement in their exercise tests. The most recent study on dietary intervention and iron status in athletes study concluded that 4-week dietary advice counselling did not improve overall iron status (Anschuetz *et al.,* 2010). However, the authors suggested that diet composition, in particularly the presence of enhancers of non-haem iron absorption, have a significant influence for iron absorption in this population.

The present study aimed to explore dietary iron intervention by the means of a staple food product and the effects of that on iron status and exercise performance in female runners. The findings showed a non-significant change in VO_{2max}, whilst VO_2 at the anaerobic threshold and time-to-exhaustion improved significantly (Figures 7.6- 7.10).

Marginal changes in VO_{2max} post-intervention in regularly physically active female participants observed in this study are in agreement with other similar sample size studies of female runners (Klingshirn *et al.,* 1992; Peeling *et al.,* 2007). These authors also reported a non-significant change in VO_{2max} after iron supplementation despite significant improvements in other performance parameters in the intervention groups. As maximal oxygen uptake (VO_{2max}) is largely dependent on training adaptations to the cardiovascular system and innate pulmonary ventilation (McArdle *et al.,* 2010), it may not be as sensitive to change in well-trained individuals. This is why perhaps in the present study, the changes in VO_{2max} in already regular physical activity engaged female participants were only marginally affected.

Nevertheless, VO_2 at anaerobic threshold (ATr) point showed a statistically significant increase. The ATr point indicates a high share of carbohydrates as main fuel and results in increased demands on blood glucose and muscle glycogen; built-up of lactate in the blood occurs and exhaustion is seen (Mougios 2006). Therefore, the concept of anaerobic threshold refers to the transition of aerobic to anaerobic point during endurance physical activity. The ability of an individual to obtain and sustain a high oxygen uptake and delay the onset of anaerobic respiration is one of the most physiological significant parameters in achieving efficient maximal performance (Joyner and Coyle 2008). Hence, the observations from the current study suggest that although maximal parameters of athletic performance efficiency (i.e. VO_{2max}) did not show significant improvements, pulmonary gas exchange variables (i.e. VO_2 at ATr) show favourable outcome in enhanced aerobic capacity in this female runners population group.

In addition to this, time-to-exhaustion, the variable frequently used as endurance performance measure (Newton *et al.,* 2008), significantly increased in the post-intervention test. This is in agreement with several iron supplementation and performance enhancement studies reporting notable increases in time-to-exhaustion values in the intervention group (Lamanca and Haymes 1993; Peeling *et al.,* 2007). However, when compared to the placebo groups, these changes were not significant (Lamanca and Haymes 1993; Peeling *et al.,* 2007). This suggests that there may be other confounding variables, which contributed to the change in endurance performance in both control and intervention groups. Therefore, due to the nature of the study design in the present study, it may be questioned whether these changes have occurred as a result of the dietary intervention, or as a result of other confounders. The absence of control group is the main limitation in this type of study. Furthermore, possible confounders influencing the favourable outcome of this study's findings, especially 'learning effect', should not be overlooked. It is well documented that participants perform the second trial better than the first, solely because they have 'learnt' from the first attempt (Hopkins 2000). However, the present study's participants were familiarised with exercise tests prior the intervention and researchers accounted for possible dietary and training confounders, therefore, this would suggest that the latter would not have contributed greatly to the findings. This is also supported by within-subject variation data for exercise tests obtained from the familiarisation trials. Coefficients of variance for absolute and relative VO_2 values at anaerobic threshold point and time-to-exhaustion values were notably lower than the incremental change in these parameters. This suggests that participants were adequately familiarised with the exercise protocol.

Nevertheless, the marginal change in maximal VO_{2max} was mostly attributed to the within-subject variance and not the intervention.

Some further supporting correlations between changes in haematological parameters and exercise performance values were observed (Table 7.11), suggesting that there may be a trend between improved overall iron status and exercise performance parameters.

Oxygen uptake parameters (absolute and relative VO_2) at the anaerobic threshold point were significantly (P<0.05) correlated to iron storage, serum ferritin, but not to any of the tissue iron supply indicators. This indicates that those runners who increased their iron storage during the intervention, also improved their VO_2 (ATr) values the most. Although most of research studies report that oxygen uptake and aerobic power is not influenced by iron status if haemoglobin levels are above 12 g/dL (Newhouse *et al.,* 1989; Lamanca and Haymes 1992), the relationship between depleted iron stores (sFer < 12µg/L) and reduced VO_{2max} was observed in a cross-sectional physically active female population (Zhu and Haas 1997). Reduced iron stores may indicate reduced oxygen-transport capacity due to insufficient iron delivery to bone marrow for erythropoiesis (Beard and Han 2009), hence, this may explain the association between increased iron stores and the improvement in submaximal VO_2 in the present study. In addition, increased iron storage was strongly correlated with the change in dietary iron (r= 0.8, P<0.05, controlling for Vitamin C and Calcium intake- Table 7.10). This again highlights a possible relationship between improved VO_2 (ATr) as a result of dietary iron intervention.

Improvement in endurance performance (time-to-exhaustion) was strongly correlated with favourable changes in iron tissue supply indicators in the present study. Taking into account limitations of research study design previously discussed, and considering the fact that the positive changes in haematological indices were associated with compromised baseline iron status in female runners, it may be that the correction of iron status had a direct effect on this athletic performance parameter. This observation is also supported by a number of iron therapy and performance enhancement studies which suggest that even non-significant improvements in tissue iron supply (sTsfR) prevent further iron status decline and subsequently improve aerobic performance or aerobic adaption to exercise in female subjects with initial tissue-iron deficiency (Brutsaert *et al.,* 2003; Brownlie *et al.,* 2004).

8.1 Intervention study conclusions

Dietary intervention study successfully incorporated Teff bread product into daily diets of female runners. As a result of this, habitual iron intake was significantly increased. Teff bread has contributed to 38% of total dietary iron intake throughout the intervention.

Initial blood iron indices showed that 36% of participants had depleted iron stores. Correlation analysis showed that reduced iron status was not associated with exercise regime or running activity. However, compromised iron status was associated with some dietary components. Total iron intake showed a positive relationship with increased iron stores, while polyphenol-rich beverages had a negative impact on iron storage and iron tissue supply. No other dietary iron absorption promoters or inhibitors showed significant correlations with iron status parameters.

Final haematological results revealed only marginal improvements in overall iron status. Nevertheless, only 18% of runners showed iron-depleted stores, which is an improvement when compared to the baseline result. In addition to this, correlation analysis found some further relationships. Firstly, the greatest improvements in iron status parameters were associated with compromised initial iron tissue supply and prolonged intervention. Further to this, enlarged iron stores were strongly correlated with the increase in dietary iron. This association stayed significant even when controlling for baseline iron status.

Female runners significantly improved their gas exchange parameters at anaerobic threshold point and time-to-exhaustion. There was also an increase in maximal oxygen uptake at peak, however, at a statistically non-significant level and mostly attributed to within-subject variation. Further to this, the improvements in anaerobic threshold parameters were positively correlated with enlarged overall iron stores, whilst endurance performance was linked to the improved iron tissue supply.

On balance, the findings from the intervention study show that Teff could be incorporated into an individual's diet in order to increase one's habitual iron intake. This would particularly benefit individuals with already compromised iron status. Improvements in iron status may also improve endurance sport and exercise performance.

Chapter 9 Research limitations

The limitations of this research include the generalisation of data using a small sample size of representable population, high variability of participants' characteristics, issues with accuracy and reliability of habitual diet records, limited food items availability on dietary software program, noncompliance to intervention duration and no control group.

The data cannot be generalised to all female runners. The current study made an attempt to explore iron status changes and athletic performance during dietary iron intervention in physically active females, who are habitually engaged in running activity. However, a small sample size raises results validity and statistical power issues. Nevertheless, the investigator believes that stringent recruitment criteria, monitoring of a number of anthropometric, physiological and dietary factors and statistical 'normality' of the sample baseline variables overcome these validity issues.

According to Hopkins *et al*. (1999), a sample size of 65- 260 should be used for sport performance enhancement fully controlled studies in order to obtain a precise estimation of the magnitude of the treatment effect. It generally accepted that 30 subjects or more is the desired target sample size sports performance intervention study in order to justify parametric statistical analysis (Saunders *et al.,* 2003). The present study recruited 15 female participants of which, due to inclusion/exclusion criteria and injury, 11 successfully completed the intervention. Although this sample size presents a strong study design limitation, taking into account constraints of trial facilities and resources and time availability of participants, it seemed be unrealistic to recruit much larger size sample group. Furthermore, it is believed those stringent inclusion criteria, high participants' retention and compliance rates present favourable aspects of this small size sampling.

The findings from exercise performance tests in the present study show favourable changes, particularly in endurance parameters including oxygen uptake at anaerobic threshold value and time-to-exhaustion. However, it is questionable whether these changes have occurred due to the dietary intervention or other confounding variables. The absence of control group is a limitation in this type of study, especially as most of iron supplementation trials observed improvements in endurance performance in both intervention and control groups (Klingshirn *et al.,* 1992; Lamanca and Haymes 1993; Peeling *et al.,* 2007). It could also be argued that the positive effects of intervention on sports performance values were due to the 'learning effect' (Hopkins 2000). And although

this cannot be dismissed, the study participants were familiarised with the procedures and were not aware of any of the performance parameters throughout the intervention.

Another limitation of a majority sport and exercise performance improvement studies is accounting for possible confounders. A number of micronutrient supplementation and exercise studies lack control over assessment of whether the change in iron status is a true effect of supplementation or an adaptive mechanism to the exercise (Akabas and Dolins 2005). For instance, there is a greater increase in blood plasma volume than in Hb as an effect of aerobic exercise (Suedekum and Dimeff 2005). Therefore, measuring differences in blood plasma volume during the trial would account for this confounder and the intervention results would present the actual influence of iron supplementation on changes in sports performance variables rather than the effect of exercise. Furthermore, selecting already regularly physically active women would account for the possible adaptation element of the trial. The present study employed physically active females who were engaged in regular exercise regime for at least the last 6 months prior the intervention. Nevertheless, most of the participants were in the training period during the intervention, hence, their exercise regime marginally increased. Although the change of exercise was not significant and accounted for as a possible confounder in statistical analysis, it cannot be assumed that increased exercise stress would not have influenced the effect of dietary iron supplementation on the iron-related parameters.

In addition, it was not possible to control for the effects of menstrual blood loss during the conduct of the study, which is a strong confounder in female athletes iron status (Malczewska *et al.,* 2000). The screening process ensured that all of the subjects were absent of menstrual irregularities and heavy bleeding. Furthermore, all blood samples, which were taken at baseline, did not coincide with menstrual period for any of the runners. However, due to variability in compliance of study's duration (30-46 days), some participants (n=2) were on their menstrual cycle at the end of the trial. This could have affected the final iron status parameters, hence, reducing the effect of dietary iron intervention.

Dietary habits and compliance can be an important confounder. The measurement of effects of the treatment is difficult to study in isolation as this would decrease external validity. Therefore, accounting for dietary habits and intervention compliance is crucial for applied research. Food records, pill counts and physical activity logs are usually employed

by the researchers. A number of performance studies successfully used different dietary habits collection methods, including a 24-hour diet recalls (Risser *et al.,* 1988), food frequency questionnaires (FFQ) (Zhu and Haas 1998b; Brutsaert *et al.,* 2003) and a 3-day food diary to estimate dietary intake (Manore *et al.,* 1989; Newhouse *et al.,* 1989; Powell and Tucker 1991; Lamanca and Haymes 1992; Lamanca and Haymes 1993; Ishizaki *et al.,* 2006). While a 3-day food diary method results in less errors in food reporting and quantification compared to 24-hour recall or FFQ (Crawford *et al.,* 1994), it can increase drop-out rates as the method very much relies on the degree of motivation of the respondent. Less participant-dependent techniques, such as 24-hour diet recall, can be a good food consumption tool, providing under- or over-reporting is controlled for (Jonnalagadda *et al.,* 2000). In the present study, 24-hour recalls were administered by the investigator through the 'prompting' interview technique, which is reported to reduce the variances of nutrients and possibly improve the accuracy of 24-hour dietary recall method (Cullen *et al.,* 2004). In addition to this, it was acknowledged that it was not appropriate to use data from a single 24-hour recall to characterise an individual's usual diet, therefore, baseline dietary intake was assessed by multiple repeats (at familiarisation and baseline visits).

In summary, whilst the recent intervention presents a number of limitations, which may have an impact on the validity of the results, methodological considerations and supportive correlation results show the trend between improved iron status and exercise performance parameters as a result of dietary intervention in this study sample of female runners.

Chapter 10 Conclusions, recommendations and future research

Incorporation of less-utilised and nutritious grains is still a new but developing research area. Wholegrain products provide higher levels of important nutrients such as protein, fibre, vitamins and minerals. Consumers seek healthier staple food alternatives, which has led to the development of new cereal products by the food industry. Incorporating less-utilised grains into traditional cereal products presents challenges to the food industry.

The research to date (Mengesha 1966; Bultosa 2007) has identified a favourable nutritional profile of Teff cereal. Cereal products, in particular bread, are the main contributors to the mean proportional iron intake in general female (Pynaert *et al*., 2007) and female athletes population (Spodaryk *et al*., 1996) when compared to any other food groups. Therefore, incorporation of iron-rich Teff bread into a daily diet of female athletes may be one of the ways to improve their haematological indices and possibly aid their sport and exercise performance.

This research study aimed to develop a novel Teff bread product with high levels of dietary iron and consumer acceptable quality and sensory attributes. Further investigations were completed to explore the associations between Teff consumption, body iron status and sports performance in female adult subjects, who are habitually engaged in exercise activity, particularly running.

The initial results indicated that addition of Teff up to 30% (flour weight) into breadmaking formulation has significantly ($P<0.05$) increased dietary iron levels (2.39 mg/100g wheat bread *v* 5.62 mg/100g Teff bread). However, Teff breads had significantly lower volume, higher crumb firmness during 8-day shelf-life period and was scored significantly less acceptable by the taste panel, which was significantly correlated to bitter flavour and aftertaste ($r= - 0.62$; $P<0.01$). The study identified a favourable nutritional profile of Teff breads and the need of textural and sensory improvements for the Teff bread.

A number of different enzymes, including xylanase, amylase, glucose oxidase and lipase, were used during product development stage to overcome these problems. Both straight dough and sourdough breads with added enzymes resulted in significant ($P<0.05$) improvements in volume, crumb firmness, crumb cellular structure and taste properties.

The best overall textural and sensory properties of Teff bread were observed with the addition of gluten strengthening (glucose oxidase) and substrate generation for better dough gassing (amylase) enzyme combinations. Teff bread supplemented with amylase and glucose oxidase had higher loaf volume, lower crumb firmness during shelf-life and improved sensory characteristics. Improved shelf-life was mainly due to a decrease in crumb firmness, whilst favourable sensory changes were due to decreased bread loaf density, hence, less intense bitter flavour and aftertaste. Overall, the developed Teff bread product was comparable in textural and sensory properties to white wheat bread, the staple food of the nation.

Teff grain was successfully incorporated into a consumer acceptable bread product with favourable nutritional profile and improved texture and taste attributes. The obtained iron-rich Teff bread was used in the intervention study, which aimed to explore dietary iron intervention by the means of a staple food product in a female runners' population.

A sample of healthy female runners was recruited for the study. Female runners do generally meet all macro- and micro-nutrient requirements or the intakes are found to be above the recommended values with the exception of iron (Hassapidou and Manstrantoni 2001). This observation was also confirmed by the present study. Baseline dietary results revealed that runners diet was inadequate in dietary iron (10.7±2.8 mg/day compared to 14.8/g/day recommendation, $P<0.001$). Moreover, it is generally accepted that poor dietary habits can lead to compromised iron status and subsequently impair physical performance. Sports nutrition advice on iron is to obtain it through the diet, however, most athletes chose the option of iron supplements (Nakanishi *et al.,* 2003; Herbold *et al.,* 2004). Furthermore, the importance of dietary intervention to improve iron status of female distance runners is highlighted by several researchers (Beard and Tobin 2000; Burke *et al.,* 2007), and yet most iron intervention studies employ pharmacological iron supplementation strategies.

In the present study, over a third of participants had depleted iron stores (sFer <12 µg/L) at baseline. Furthermore, partial correlation analysis revealed that poor dietary iron intake was independently associated with depleted iron stores.

Eleven female runners completed a 6 week intervention trial. Participants consumed and average of 4 slices of Teff bread (125±37 g) a day. As a result, their total iron intake

significantly increased (from 10.7 to 18.5 mg/day, $P<0.0005$). Teff bread contributed to the 38% of the total iron intake.

Intervention findings showed a trend of improved iron status of female runners (sTRF ↓ 5.4%, CV 0.77-0.86%, sTsfR ↓12.8%, CV 0.76-1.1%, sFer ↑5.4%, CV 2.1-3.8%, TIBC ↓6.2% and sTsfR-F index ↓16.8%), however, these changes were not statistically significant. Whilst the favourable changes in sTRF and sTsfR could be attributed to the dietary intervention due to low within-subject variation, improvement of sFer was mainly associated with intrinsic error. This indicates that the trend of improved iron status was only achieved for iron tissue supply and not increased iron storage as a result of this dietary intervention. Nevertheless, the favourable changes in iron storage (sFer) were strongly and significantly correlated with the change in dietary iron, whilst the improvements in iron tissue supply values (sTRF, sTsfR, TIBC and sTsfR-F index) were correlated with prolonged intervention. All of the favourable changes in iron status indicators were correlated with low baseline iron status.

On balance, the positive change in iron status seems to be heavily influenced by compromised initial body iron balance, which can be explained by the homeostatic iron regulation mechanism (Beard and Han 2009). Nevertheless, the improvements in iron storage and iron tissue supply were associated with increased dietary iron intake and prolonged iron intervention, respectively, even when controlling for baseline iron status. Hence, these associations provide evidence that dietary iron was incorporated into iron stores in female runners as a result of the intervention.

In terms of exercise performance, maximal oxygen uptake improved marginally which was mainly attributed to the intrinsic error (VO_{2max} absolute value by 5%, relative by 4%, CV 2%). However, the gas exchange values showed significant improvements at anaerobic threshold point (VO_2 absolute value by 18%, relative by 17%, CV 5%), whilst endurance parameters showed the greatest improvements (AT time reached by 39%, CV 8%; time-to-exhaustion by 24%, CV 4%). Although the latter parameters may have been influenced by the repeated test measures, some statistically significant correlations suggest that the change in sports performance was due to the changes in iron status. Gas exchange parameters were linked to the increased storage iron, whilst endurance parameters were associated with improved iron tissue supply.

The most important contributors to the total iron intake in general female population n=621) were reported to be cereals and cereal products, accounting for 31% of daily iron, in particular bread (19%) (Pynaert *et al.,* 2007). The present study showed that Teff bread contributed to 38% of daily dietary iron during the intervention. Taken into account the current dietary guidelines on increasing consumption of cereals, fruit and vegetables (Department of Health 2011); and the importance of cereal products in providing large proportion of daily iron, it seems that encouraging female athletes to increase cereal and cereal products consumption is justifiable.

It remains to be further researched if dietary intervention through a staple food product can significantly improve iron status and consequently sport and exercise performance. Nevertheless, taking into account research design and sample size limitations, the current study findings show positive trend of marginally improved iron status and exercise performance as a result of Teff bread dietary intervention.

Literature suggests that dietary iron intervention should involve strategies to increase total iron intake and the bioavailability of this iron (Burke *et al.,* 2007). The current study explored the effects on iron status through incorporating a staple food into diet in order to increase daily iron intake in female runners. It was concluded that an increase in dietary iron approximately by 8 mg a day in this population showed favourable effects on the overall iron status and exercise performance.

Future research is needed to elaborate and confirm this association, in particularly by using a randomised control trial design and a larger sample size population. A sample size of 30 subjects in each group (intervention and control) would be advisable. In the present study, although statistically normally distributed, participants' haematological data varied greatly. Therefore, obtaining a larger sample size would result in a sample distribution for the mean that is closer to normal distribution (Saunders *et al.,* 2003). A control group would also ensure that the outcome of the study is a 'true' representation of the intervention.

Furthermore, the bioavailability of dietary iron has not been looked at the experimental level in the present study; hence, further work on this aspect is recommended. Thus future research should focus on the improvements in bioavailability of iron in Teff bread or within consumed meals. Cereal products, including Teff bread in this study, are a good source of non-haem iron, other minerals, vitamins, carbohydrates and fibre. Firstly, non-

haem iron is less bioavailable (Hallberg 1981). In addition, cereals contain phytates that are known iron absorption inhibitors. One of the suggestions for improving iron bioavailability in Teff bread would be to optimise processing conditions towards the highest phytate degradation (temperature, pH for optimum phytase activity) and/or adding exogenous phytase to facilitate phytate breakdown (Frolich 2001).

In addition to this, the absorption of non-haem iron, which seems to be the main contribution to total iron intake in the Western countries (Hallberg 1981; Pynaert *et al.,* 2007), can be improved by altering meal composition. For instance, cereal product could be consumed with fruit and vegetables for Vitamin C enhancing properties. Also, the intake of iron absorption inhibitors, i.e. tannins in tea or coffee, calcium in milk, could be decreased or at least avoided in the same meal. In terms of Teff bread developed as part of this research, several meal composition options could be suggested. Iron absorption in Teff bread could be improved by adding good protein and Vitamin C sources, i.e. ham and cheese or tuna Teff sandwich with tomato and peppers. Further investigations could establish *in vitro* iron bioavailability in Teff bread, whilst *in vivo* trials could be used to determine iron bioavailability in single meals or the whole diet.

To conclude, this research provides additional knowledge on the nutritional benefits and textural and sensory challenges of Teff grain incorporation into a consumer acceptable bread product. Therefore, the obtained data contributes to new knowledge in baked product development using Teff grain and resolving quality issues affecting the final product's acceptability.

To author's knowledge, this is the first time that a dietary iron intervention was achieved by incorporating iron-rich staple food alternative in female runners population. An intervention study findings led to new knowledge of the associations between increased dietary levels of iron through a staple food, iron status and the contributing trends of that to exercise performance. Furthermore, the study identified current study research limitations and outlined important future research directions in improving total dietary iron availability in a cereal product or through the modification of meal composition.

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Appendix 1 Comparison of iron supplementation and exercise performance studies

Study & final intervention result	**Subjects:** number, gender, age, characteristics	**Duration & groups**	**Dietary intake/ assessment**	**Performance protocol/ Biochemical assessment**	**Haematological indices at baseline-end**	**Performance at baseline-end**	**Outcomes and results**
Peeling *et al.* (2007) **NO**	*16 females, from running, cycling & triathlon clubs, aged 15-40y height 161-170 cm weight 50-66kg	20-day intervention *IG: 5 X 2ml (100mg elemental Fe) intramuscular injection for 10 days *Placebo (C)	*4-day food diary Fe intake: -IG: 14.1mg/d -C: 14.4 mg/d *7-day exercise diary -IG 132 min/d -C 119 min/d	*<u>*Cycle ergometer*</u> (n=2 in IG, 2 in C) *Treadmill (n=6 in IG, 6 in C) at baseline and after 20 days *Blood at baseline, 20 days and 28 days	*IG group: -sFer: 19→65 µg/L (P<0.01 from baseline + C group) - sFe, sTRF, TS, sTsfR, Hb- NS *Placebo group: -No change in all -sFer: 30→33 µg/L	*VO_{2max}: IG: 2.87-2.87 L/min C:2.92-2.85L/min *Total time: IG:7.35→7.13min *Time to exhaustion: IG: 2.77-3.36min (P<0.05) C:3.02-3.22 (NS)	*Fe injections sig. increased sFer (faster rate than oral supplementation). *NS improvement in VO_{2max} after intramuscular Fe injection for 10 days *Time-to-exhaustion at VO_{2max} workload sig increase in IG group, but no sig. difference between groups.
Ishizaki *et al.* (2006) **MAYBE**	*8 collegiate rhythmic gymnasts, aged 18-19y, BMI 19.7	*4wks intervention in 2yr cohort: -1st yr self-selected diets -2nd yr fixed diet for 4 wks (15mg Fe)	*3-day FD 3times in 1st / 2nd yr at baseline, 4, 8 wks *Iron intake: -Base: 7.3mg/d -Intervention: 18.2mg/d	*Not assessed	*After 4 wks: -RBC: 491→424X10^4/µl -Hb: 13.6→11.9g/dL -Hpt: 41.5→35.0 (P<0.05) -sFer: 16→24 µg/L (P<0.05) -sFe: 58→55 µg/L	*Sig increased sFer conc & δ-ALAD activity (responsible for RBC turnover) after diet intervention *No effect on other parameters or iron depletion *Intervention during weight loss period-confounder to show true effect? *↓Haptoglobin=footstrike haemolysis	
Tsalis *et al.* (2004) **NO** *Combined groups together for analysis as parameters NS different between	*21 male and 21 female swimmers, aged 12-17y BMI 20 Body fat 14-16%	*6 months A: Fe supplement (47mg/d) B: diet rich in Fe (26mg/d) C: control	*Records of daily food intake *Iron intake: A: 58mg/d B: 26mg/d C: 12mg/d	*Protocol: moderate intensity→ high intensity→ tapering *<u>*Swimming tests*</u> *Blood analysis: Base-mid-end	*NS difference between groups: RBC: 4.8-5.0-4.9 M/µL PCV: 41.0-45.6-42.4% Hb: 14.0-14.6-14.4 g/dL sFe: 83-69-91-75 µg/dl TIBC: 33.7-31.1-30.7 µg/L TS: 31.8-32.1-27.6 µg/L	*NS difference between groups: -Performance at 2000, 800, 200 m swim & 25m sprint in water increased sig. (P<0.01)	*No sig. differences in iron status or performance among 3 groups *Improvements in performance-due to adaptations to program? *Failure of high Fe intake to change Fe status-homeostatic mechanism?

Brownlie *et al.* (2004) **YES**	*41 females, iron-depleted non-anaemic (sFer< 16µg/L, Hb >120 g/L), untrained, aged 18-33y	*6wks intervention: IG: 8mg elemental Fe X 2/d + juice C: placebo *For analysis stratified to normal baseline sTsfR (A <8.0mg/L) & elevated sTsfR (B)	*Pre-study 4-day FD: Baseline Fe intake: -IG: 14.4mg/d -C: 14.8mg/d *Pre-study habitual PA assessed by FQ	*4wks training starting on 3rd wk: 5d/wk on ergometer *<u>*Cycle ergometer tests*</u> at start & end. *Bloods: start, 3wks& end	*Hb, sTsfR, TIBC- NS *sFer: IG 10.38→14.52 µg/L (P<0.05 from C 8.07→8.11 µg/L) *sFe: IG 12.2→19.4 µmol/L (P<0.05 from C 13.4→12.2 µmol/L) *TS: 18.7→31.9 % (P<0.05 from C 20.9→22.0%)	-TT: ↓ in IG B at 15km compared to C (P<0.05) -Work rate & %VO_{2max}: ↑ in B IG (P<0.05 compared to C at 15km)	*Subjects with elevated sTsfR (iron stores depletion) after Fe therapy showed greater improvement in time, work rate & %VO_2= ↑adaptation to aerobic training. *Trend not seen in normal iron tissue subjects= no effect on aerobic adaptation.
Brutsaert *et al.* (2003) **YES**	*20 females, iron-depleted nonanaemic (sFer <20 µg/L, Hb >11 g/dL), aged 27-32y, untrained	6 wks intervention: *IG: 10mg elemental Fe X 2/day, instructed to consume with citrus juice *C: placebo	*FFQ a month before study *24-h physical activity recall *Baseline work rate or time length for muscle fatigue- similar between groups	*<u>*Knee-extension exercise*</u> protocol to measure progressive muscle fatigue: Maximal voluntary contractions (MVC)	*IG: Hb, sFer, sTsfR, TIBC- improved but NS sFe: 11.3→22.7 µmol/L (P<0.01) TS: 19.9→39.6 % (P<0.05) *C: Hb, sFer, sFe, TIBC, TS- no change sTsfR: 4.52→6.30mg/L (P<0.05)	*Sig (P<0.01) 26.5% improvement MVC_{end} in IG 6 mins into protocol. No NS change in C group.	*Changes in sTsfR response (P<0.01) (IG: ↓ 0.8, C:↑ 1.78 mg/L) show prevention of Fe depletion rather than improved tissue Fe status. *Fe status improved sig after 6 weeks of oral supplementation *Fe improved muscle fatigue resistance.
Brownlie *et al.* (2002) **YES**	*41 females, iron-depleted non-anaemic (sFer<16 µg/L, Hb >12 g/dL), untrained, aged 18-33y, weight 57-60kg	6 wks PA trial: *IG: 8mg elemental Fe X 2/day with citrus juice *C: placebo *Baseline Fe intake: -IG: 14.7mg/d -C: 14.8mg/d	*Pre-study 4-day FD + 3-day FD before 3rd wk training regime (latter not analysed) *Pre-study habitual PA assessed by FQ	*4wks training starting on 3rd wk: 5 d/wk on ergometer. *<u>*Cycle ergometer tests*</u> at baseline and end. *Bloods: baseline, 3wks, end	*IG: Hb, sTsfR, TIBC- improved, but NS Hct: 38.5→3.98 % sFer: 10.38-14.52µg/L sFe: 12.2→19.4µmol/L TS: 18.7→31.9% (P<0.05) *C: Hb, Hct, sTsfR, TIBC- NS changes sFer: 8.07→8.11µg/L sFe: 13.4→12.2µmol/L TS: 20.9→22.0% (P<0.05 from Intervention group)	*Absolute VO_{2max} -IG ↑ 0.37 L/min -C ↑ 0.17 L/min *Relative VO_{2max} -IG ↑ 8.54 ml*kg/fat free mass*min -C ↑ 3.5 *Both parameters sig (P<0.05) higher in IG compared to C group	*Fe supplementation enhances aerobic adaptation in iron-depleted women. *The greatest VO_{2max} improvement in subjects with overt tissue-iron deficiency (high sTsfR at baseline). *↑ in performance- due to changes iron status & fitness.

Hinton *et al.* (2000) **YES**	*42 females, iron-depleted non-anaemic (sFer<16 µg/, Hb >12 g/dL), aged 18-33y, untrained	6 wks PA trial: *IG: 10mg elemental Fe X 2/day with citrus juice *C: placebo	*Pre-study 4-day FD *Pre-study habitual PA assessed by FQ	*4wks training starting on 3rd wk: 5d/wk on ergometer. * *15-km cycle ergometer tests* at baseline and end. *Bloods: start, 3wks, end.	*Hb, Hct, TIBC-NS IG: sFer: 10.38→14.52 µg/L sTsfR: 7.92→6.78 mg/L sFe: 12.2→19.4µmol/L TS:18.7→31.9% *C: sFer: 8.07→8.11µg/L sTsfR:7.94→7.93 mg/L sFe: 13.4→12.2 µmol/L -End sig. different (P<0.05) between groups.	*15-km time: IG ↓3.4min, C↓ 1.6min (P<0.05) *VO_{2max} IG↑ 0.37 L/min, C 0.17(NS) *TT RER, TT WR ↑ in both (P<0.05) *TT VO_2 ↑ in IG only (P<0.05)	*Post-test VO_{2max}, RER, work rate and time-to-complete improved in both groups=effects of 4wk training regime. *IG ↑ more in time-to-finish, VO_{2max} than C. *IG ↓ %VO_{2max}, ↑ O_2 consumption but not C group.
Friedmann *et al.* (2001) **YES**	*40 athletes (23F, 17M), aged 13-25y (sFer <20 µg/L, Hb >13.5male, 11.7g/dL female)	12wks intervention IG: 100mg elemental Fe X 2/d + 200ml orange juice C: placebo	*Not assessed	*Treadmill Medbo-test at baseline & end. *Blood samples at baseline & end.	*sFer: IG: 15.8→35.9 µg/L (P<0.001 compared to C: 14.3→12.9 µg/L). *sFe, TS, Hb, Hct, RBC-NS in both groups	*VO_2: IG 3334 → 3445 ml/min (P<0.01) C 3210→3219 ml/min (NS) *Time-to-exhaustion: IG 156→171 (P<0.05 compared to C 134→140min	*12wk Fe treatment in iron-depleted athletes ↑ sFer to normal values (35.9µg/L). *RBC volume or erythropoiesis- unchanged *VO_2 & O_2 consumption ↑ in IG=↑ maxima; aerobic capacity=improved performance.
Friedmann *et al.* (2000) **YES**	*23 female athletes, iron-depleted nonanaemic (sFer <20 µg/L, Hb>12 g/dl), aged 14-18y	12 wks intervention -IG:100mg/d elemental Fe X 2/d B. placebo	*Not assessed	*Treadmill Medbo-test at baseline & end. *Blood samples at baseline & end.	*sFer: IG:16.6→40.1µg/L (P<0.05) C: 14.3→12.9µg/L *RBC volume did not change in either groups	*VO_{2max} and O2 consumption sig (P<0.05) ↑ only in IG: 2.81→2.92 L/min *Maximal lactate not changed	*sFer and VO_{2max} increased significantly in IG but not in C group. * Fe supplementation in iron-deficient female athletes improves aerobic performance.
DeRuisseau *et al.* (1999) **MAYBE**	*11 physically active females	*Cross-sectional A: Fe depleted group (ID) (sFer<20 µg/L) B: normal iron group (C)	*Not assessed	*Cycle ergometer time-trial test	*sFer: ID group: 13.7µg/L C group: 37.3µg/L (P<0.05)	*Relative mean VO_2 peak: IG:38.3ml/kg/min C: 43.4 ml/kg/min *No difference in endurance test.	*sFer significantly lower in the ID group. *Correlation between sFer and VO_2peak (r=0.75) *No effect on endurance but VO_2 impaired.

Blee *et al.* (1999) **NO**	*15 elite female netballers, iron-depleted nonanaemic (sFer <40µg/L, Hb>12.5g/dL), aged 16-22y	*10-day intervention: -IG: 5 X 2ml intramuscular injections (total 500mg elemental Fe/d) -C: placebo	*4-day FD -Fe intake: IG: 17.08mg/d C: 18.41mg/d -Vit C intake (P<0.05): IG: 204.72mg/d C: 167.74mg/d	* *Routine tests*: -vertical jump -power&sprint tests -ergometer -shuttle run *Bloods: start, 5-day, 10-day	*sFe, sTRF, TS, Hb- NS changes in both groups *sFer (P<0.05 between groups): -IG: 22→61µg/L -C: 26→29µg/L	*No changes in 10 sec power, 5 X 6 sec sprints, vertical jump or shuttle run tests between groups or baseline-end interactions	*Rapid ↑ sFer in 5days. *No effect on aerobic or endurance capacity. *Iron-depletion criteria sFer >40µg/L might fall into a 'normal' range, as both groups fall under (IG- 22 µg/L, C- 26 µg/L).
Zhu & Haas (1998) **YES, MAYBE**	*37 females, aged 19-36y, iron-depleted nonanaemic (sFer <16 µg/L, Hb >12 g/dL)	*8wks intervention: -IG: 45 mg/d elemental Fe -C: placebo	*Baseline 4-day FD, Fe intake: *Pre-study habitual PA assessed by FQ	**15-km cycle ergometer trial* *Bloods: baseline, 4wks, end	*Hct, sFe, TIBC, TS- NS changes in both groups *sFer: IG- 14.3→36.9 µg/L , C- 12.2→16.2µg/L (P<0.05 between groups) *Hb: NS in IG ;C-137.3 →132.2 g/L (P<0.05); * sTsfR: IG-6.40→4.51, C- 6.01→5.83mg/L (P<0.05 between groups)	*15-km: NS * VO_2peak L/min, TT VO_2 L/min, TT RER ↑ (P<0.05) in both. *TT energy expenditure kJ/min & TT%VO_2peak% better in IG (P<0.05)	*8wk supplementation replenished iron stores in iron-depleted subjects (↑sFer, ↓ sTsfR) & prevented Hb fall (unlike ↓ in C group). *Lower energy (2.0kJ/min) and lower %VO_2peak (5.1%) compared to C group (P<0.05) = more energetically efficient. *No effect on 15-km time.
Zhu & Haas (1997) **YES**	*30 females (15 iron-depleted (sFer< 12ug/L, Hb >120 g/L); 15-control) aged 19-36y, physically active	*Cross-sectional: -screening -blood sampling in 2wks -once Physical performance	*FFQ focused iron-rich foods -Iron intake: ID: 14.6 mg/d C: 10.8 mg/d *PA level- 14d recall & FQ	**Cycle ergometer test* for VO_{2max} and σ-efficiency (relationship between VO_2, work rate & O_2) - indication of O_2-trainsport capacity.	*Hb: ID-136, C-145 g/L (P<0.05) *Hct: ID-41%,C-42% (NS) *sFer: ID-4.0, C-31.3µg/L (P<0.05) *TIBC: ID- 83.5, C-65.6 µmol/L (P<0.05) *sFe: ID-14.3, C-16.5µmol/L (NS) *TS:ID-16.0,C-25.3%(NS)	*Correlations between VO_{2max} : -PA (r=0.569, P=0.004) -Fat-free mass (r=0.339, P=0.067) *VO_{2max}/fat free mass: ID-46.3, C-53.2 ml*min/kg (P<0.05)	*ID group had lower VO_{2max} than C, when controlled for PA and fat-free mass (r=-0.367, P=0.011). * VO_{2max} associated with sFer when controlled for PA and free-mass (r=0.184, P<0.05), but not affected by oxygen-transport capacity.
Fogelholm *et al.* (1994)	*64 females (sFer <20 µg/L, Hb >12 g/dL) aged 36-40y	*6months trial: A. Fe- 9mg/d (n=18) B. Fe- 27mg/d (n=19) C. placebo (n=27)	*FFQ focused on tea/coffee, fruit & veg, fruit juice, meat & liver	*Blood samples at baseline, 1, 3, 6months. *Performance not assessed.	*sFer: A. ↑by 45; B. ↑ by 112; C. ↑by 15µg/L (P=0.0003 group effect) *Hb: A.↑by 6; B.↑ by 6 µg/L C.no change (P=0.02)	*Treatment of 9mg & 27mg Fe/d (11% haem & 89% inorganic Fe) ↑ sFer & Hb in 1 month. sFer remained stable after that (homeostatic status?). *9mg enough to correct mild iron deficiency but no enlarge Fe stores.	

LaManca & Haymes (1993) **YES**	*20 female athletes, iron-depleted (sFer <20µg/L) aged 19-35y	*8 wks intervention A. Fe (50mg X 2/d elemental Fe) supplement (IG) B. placebo (C)	*3-day FD -baseline Fe intake: IG: 14.4mg/d C: 18.7mg/d *PA Questionnaire	*<u>*Cycle ergometer test*</u> – baseline, end *2d before tests: given diets (CHO-70%, Fat-20%, Protein-10%)	*IG: Hb 12.8→14.1g/dL (P<0.05); TIBC 366.2→293.8 µg/dL (P<0.05); sFer 10.8→ 22.5 µg/L (P<0.05 from C (11.2→14.3µg/L) *No changes in C group	*VO_{2max} IG 40.19→41.72 ml/kg*min (P<0.05 from C 41.24→39.48) *Test at exhaustion- NS	*Sig improvement in IG VO_{2max} compared to C- due to decrease in C value? *Endurance time to exhaustion increased by 38% in IG but NS compared to C.
Lyle *et al.* (1992) **MAYBE**	*60 females, previously sedentary Aged 18-19y, Height 162-164cm, weight 61-69.5kg, exercise 2.3-2.5d/wk	*12 wks aerobic test A: Fe 50mg+ low diet Fe B: Fe 10mg+low diet Fe C: control D: Meat diet (18mg)	*12-wk dietary records (only 2nd wk 7-day FD analysed) *Iron intake: A.58mg/d B.17.5mg/d C.8.0mg/d D.11.8mg/d	*Iron status at baseline, 4, 8, 12-wk *<u>*Walking and treadmill tests*</u> at baseline and after 12-wks	*After 12wks Meat group Hb sig. higher. **A**. TIBC: 68.5-62.5 µmol/L (P<0.05) **B**. NS changes **C**. TS: 31.5→21.8% sFe: 22.6→17.7µg/L sFer:22.2→12.7µg/L **D**. Hb: 116→12.4g/dL Hct: 37.5- 39.4% (P<0.05)	Max O_2 uptake improvements: A. 18% (41.4) B. 18% (42.7) C. 10% (39.7) D. 21% (43.2)	*Moderate aerobic exercise compromise iron status. *Meat diet was more effective in protecting Hb and Fer status than were iron supplements. *Fe supplements absorption 4.38-2.87%
LaManca & Haymes (1992) **MAYBE**	*16 female recreational athletes (>30 min/d X >3/wk aerobic exercise), aged 21-35y	*Cross-sectional A. low sFer (<12 µg/L) group B. normal sFer (>26 µg/L) group	*3-day FD Fe intake: A.15.2 mg/d B.25.9 mg/d (NS) *2 exercise tests on 2 separate occasions	*<u>*Cycle ergometer test*</u> for VO_2max and endurance tests *Blood sample	*Hct, Hb- NS changes. *sFe: A. 131.5, B. 173.6 µg/dL (P<0.05) *TIBC: A. 393.8, B. 305.0 µg/dL (P<0.05) *TS: A. 34.6, B. 58.2% *sFer: A. 5.9, B. 44.4 µg/L (P<0.05)	*VO_2: A.34.4, B. 34.8 ml/kg/min *Exhaustion time: A.23.2 min B.27.0 min	*VO_2max did not differ sig. between groups. *Normal sFer group exercised 14% longer during endurance test *sFer related to absorbable Fe (r=0.72) & total Fe in diet (r=0.70).
Fogelholm *et al.* (1992) **NO**	*31 female athletes, aged 17-31y, iron-depleted nonanaemic (sFer <25 µg/L, Hb >12 g/dL)	*8wks intervention: IG: 100mg ferrous sulphate + vit C/d C: placebo	*FFQ at the end of study- no difference between groups	*<u>*Cycle ergometer test*</u> at baseline, end *Blood tests at baseline and end	*sFer: IG 14→26µg/L (P<0.005 from C 14→11 µg/L) *Hb: IG 13.7→13.9g/dL (P<0.005 from C 13.5→12.8g/L) *Hct: IG 42→42.9% (P<0.005 from C 41→39%)	*VO_{2max}: -IG: 47.0→45.7 ml/kg*min -C: 45.3→45.3 (NS in both groups or between groups)	* Iron supplementation increased iron status in iron-depleted athletes. *Supplementation did not affect performance. *sFer increase up to 26 µg/L enough for effect?

Klingshirn *et al.* (1992) **NO**	*18 female runners (sFer <20 µg/L, Hb >12 g/dL) aged 22-39y	*8wks intervention: IG:50mg elemental Fe X 2/d C: placebo matched for time-to-exhaustion	*Q for diet, exercise habits, supplementation. *Exercise logs for miles run/d	*<u>*Treadmill test*</u> at baseline and after 8 wks	*Hb, Hct, sFe, TS- NS *TIBC: IG 318→275, C 324→328 µg/dl (P<0.05) *sFer: IG 11.6→23.4, 12.0→15.8 µg/L (P<0.05)	*Time-to-exhaustion: IG↑ 25.5%, C↑22.2% *VO_{max}: NS in both groups	*8wk Fe supplementation ↑ Fe indices in iron-depleted runners. *↑ Fe status does not improve performance.
Telford *et al.* (1992) **NO**	*31 athletes (18F, 13M), of which 15 IG (sFer<30 µg/L)	*2wks intervention: IG: 100-300mg/d elemental Fe C: placebo	*Both groups counselled by dietitian.	*<u>*Cycle ergometer*</u> test after sFer ↑ by 15ng/ml in IG	*sFer: IG 19.8→46.3 µg/L (P<0.05 from C 83.3→94.1 µg/L) *sFe, Hb, TIBC, TS- NS	*10sec work capacity (Males): IG ↑ 0.8, C 0.33kj (P<0.05); NS in Females	*sFer ↑ but not most of performance measures. *Value of 46.3 ng/ml too low for ↑ performance?
Lukaski *et al.* (1991) **YES**	*11 females, aged 22-36y, (Hb 13.4 g/dL; sFer 26 µg/L)	*180 days trial: -Fe depletion to 8.5 µg/L by diet, phlebotomy & menses (67-88d) -Fe repletion by diet (17.3 mg/d) + supplements (50.3 mg/d)	*Constant weight diets on a 3-d rotation menu given by investigators	*<u>*Cycle ergometer*</u> test at baseline, depletion & repletion *Blood samples	*sFer: 26→6→10 µg/L *Hb: 13.4→12.0→12.6 g/dL *Hct: 38.5→35.5→36.4% All parameters sig. different from depletion (P<0.05)	*TT, VO_{2max}- NS *VCO_{2max}: 1.91→2.11→2.02 L/min (P<0.05) *VO_{2total}: 17.1→15.8→17.4 L (P<0.05)	*Fe depletion- No influence on O_2 carrying capacity- no changes in cardiovascular parameters. *Sig effect on glycolytic metabolism- ↓rate O_2 utilisation, ↓energy output.
Powel & Tucker (1991) **NO**	*10 non-anaemic female runners (40-50 miles/wk), aged 18-22y	*2 X 2wks intervention: IG: 130mg elemental Fe/d C. placebo !!! groups reversed after 2 wks	*3-day FD Fe intake: 13 mg/d *subjects past & current training history	*Pre-test, Post-test, single-blind crossover design *<u>*Treadmill test*</u> at baseline and after 2 wks	*sFer: 29.1→IG 51.6, C→44.1 µg/L *TIBC: 241→IG 270, C→255 µg/dL *sFe: 71→IG 60, C→ 67 µg/dL * sTRF: 29.1→IG 22.4, C→25.9 %	*VO_2: 52.5→IG 51.4, C→53.6 ml/kg/min *RER(respiratory exchange ratio): 0.90→IG 0.97, C→0.95	* No significant changes in blood Fe indices or performance in both groups after 2 wks of supplementation. *No diff. in Fe status- short (2wks) trial time/ normal sFer baseline values (29.1 µg/L)?
Jensen *et al.* (1991) **YES**	*13 sedentary females, aged 18-25y	*12wks PA trial: IG:50mg ferrous sulphate X 1/d *C: placebo	*Baseline & end 3-day FD Fe intake: IG 65.5 C 8.9mg/d (wk12) *Weekly exercise training dairy	*Training: 3-5 d/wk,15-60min sessions *Performance test at start & end	*NS changes in Hb, Hct, sFe, TIBC, TS in both groups (but improved in IG compared to C group) *sFer: IG 29→42 µg/dL, C 26→17 µg/dL (P<0.05)	*VO_2: IG: 33.8→38.0 L/min (P<0.05) C: 27.8→29.3 L/min (NS)	*12wks moderate exercise decreased iron stores. *IG maintained Fe stores while placebo group ↓ *VO_2 of IG improved 13.5% while C only 5.4%

Magazanik *et al.* (1991) **YES, MAYBE**	*28 females, untrained, aged 19	*7wks PA trial: IG: 160mg ferrous sulphate X 1/d *C: placebo	*Estimated daily diet from food charts: Fe intake 15 mg/d	*Training: 5-6h intensive PA X 6d/wk *<u>*Treadmill test*</u> at start, mid, end	*Hb: IG 12.4→13.5 g/dL ($P<0.05$), C 12.5→12.8 g/dL *PCV: IG 39→43, C 39→41% ($P<0.05$ between groups)	*VO_{2max} higher ($P<0.025$) in IG on day 21 but NS by day 42.	* VO_{2max} and Hb correlated in IG ($r=0.609$, $P<0.01$). *Supplementary Fe most effective during early stages of intensive training.
Manore *et al.* (1989)	*10 females runners, aged 27-41y, 6.1hrs/wk PA	*9 wks observation study during training period	*3-day FD at wks 1, 4 & 9. -Fe intake: 13.7→ 11.6→10.5mg/d	*Blood samples at wks 1, 4 & 9.	*Hb, Hct, RBC, sFe, TIBC, TS, sFer- NS changes during 9 wks	Not assessed	*Correlation between sFer & miles run ($r=-0.66$, $P<0.03$) *50-60% runners iron deficient.
Newhouse *et al.* (1989) **NO**	*40 female recreational runners (3Xwk >120min) (sFer<20 µg/L, Hb >12 g/dL), aged 18-40y	*8wks intervention: IG: 320mg ferrous sulphate (=100mg elemental Fe)X2/d C:placebo	*3-day FD at baseline & end -Fe intake: 11.8→11.0 mg/d *Training record *Menstrual blood loss	*<u>*Cycle ergometer*</u> Wingate power test: start&end *<u>*Treadmill*</u> anaerobic speed test: start&end	*Hb, TS, UIBC- NS changes *sFer: IG 12.4→37.7, C 12.2→17.2 µg/L ($P<0.05$) *sFe: 79.8→98.5, C 84.1→70.5 µg/dL ($P<0.05$)	*VO_{2max}: IG 51.3→52.7 C 50.6→50.6 ml/kg*min (NS) *Anaerobic speed test- NS	*↑ in sFer in IG did not sig enhance performance. *Final sFer level (37.7ng/ml) may not be sufficient to show improvement? (sFer<50, still show iron-deficiency?)
Rowland *et al.* (1988) **YES**	*13 adolescent female runners (sFer <20 µg/L, Hb >12 g/dL) during training period	*8wks intervention (4wks control period- to adjust to treadmill test) IG: 975mg ferrous sulfate/d C: placebo	*No dietary assessment *Records for training mileage	*<u>*Treadmill test*</u> at baseline, 4wk & end *Blood at baseline, 4wk & end	*Hb- no changes *sFer: IG 13.9→8.9→26.6 µg/L C 14.3→10.7→8.6 µg/L ($P<0.05$ between groups & time)	*Endurance time: IG 16.59→17.56 min C 16.78→16.39 min ($P<0.05$ between groups & time) *VO_2 – NS in both groups	*Fe therapy improved sFer during competitive season. *Correlation between sFer & endurance time ($r=0.74$, $P<0.05$) *↑endurance but not gas exchange/cardiac measures.
Risser *et al.* (1988) **MAYBE**	*100 female athletes, aged 18-21y *66 non-athletes	*Training season intervention: IG: iron-depleted athletes (65mgFe) C: placebo	*24h diet recall *Q, menses, tablets mood by subjects & coaches.	*10-point Likert scale satisfaction Q on performance at start & end	*31% athletes iron deficient (sFer<12ng/ml, TS<16%) *After treatment 64% IG athletes ↑ their iron status.	*Iron-deficient athletes considered their performance to be worse ($P<0.05$)	*Fe treatment ↑iron status. *↑iron status in IG=↑ performance satisfaction (3.11→6.33) *Subjective assessment?

Matter *et al.* (1987) **NO**	*85 female marathon runners	*1wk intervention: IG:50mg elemental Fe/d + 5mg/d folate C: placebo	*Not assessed	*<u>*Treadmill test*</u> Baseline, 1wk & 10wks	*sFer: -IG: 29.4→59.8 µg/L (P<0.05) -C: 302.5→48.3 µg/L (P<0.05)	*VO_{2max}, time-to-exhaustion, running speed, $lactate_{peak}$- NS	*Iron-deficiency and iron-treatment did not influence maximal exercise performace.
Lampe *et al.* (1986)	*22 adult runners (16F, 6M) sFer <20 µg/L	*8wks intervention: ID: 120mg ferrous sulphate/d	*3-day FD RDA Fe intake achieved: -F: 63% -M: 100%	*Performance not assessed *Blood samples at baseline & end.	*sFer: ID: F 14.9→26 µg/L (P<0.05) M 18→29 µg/L (P<0.05) *MCHC(mean cell Hb conc): F 33.8→34.2ng/ml (P<0.05)	*Not assessed	*Low sFer prevalent in female runners (32%, 24 out of 75). *Fe treatment ↑ sFer in iron-depleted F & M.
Schoene *et al.* (1983) **MAYBE**	*15 female athletes participating in running, cycling, golf, basketball or tennis, aged 19-35	*2wks intervention: ID: iron-depleted group (n=9)-300mg ferrous sulphate/d C: placebo (n=6) *Groups reversed after 2 wks	*No dietary assessment	*<u>*Cycle ergometer*</u> at baseline & end *Blood samples at baseline & end	*Hb: ID 12.2→12.7 C 13.8→ 14.3 g/dL *sFer: ID 10.0→ 22.1 C 19.8→13.7 µg/L (P<0.005) *TS: ID 12.1→32.8% C 25.3→27.2% (P<0.05)	*VO_{2max}: ID 2.73→2.76 L/min C 2.71→ 2.69 L/min (NS) *Lactate max: ID 10.3→8.4 mmol/L (P<0.05) C 8.3→8.5 mmol/L (NS)	*Fe treatment sig ↑iron status & reduced lactate after exhaustion exercise = possible improvement in muscle oxidative metabolism? *No effect on VO_{2max}=need for prolonged endurance protocol?
Hegenauer *et al.* (1983)	15 females, previously sedentary, Hb >12g/dl, aged 18-37y	*8wks PA trial: A. 9 mg Fe/d B. 18mg Fe/d C: placebo	*7-day dietary recall with dietitian at baseline & wk8. -Iron intake: 50-60% RDA	*Fitness program: -HR (220-age)X 0.85 -Exercise: 4/wk, increasing to 45min *Treadmill test *Blood at start, 5wks & end	*NS between groups. *Overall results: -Hb: 14.4→13.7→14.1g/dL -Hct: 40.2→39.6→40.5 % -sFer: 28.5→22.4→24.1 µg/L	*Not assessed	*Reduction in Hb after 5 wks of exercise+ haemodilution due to exercise. *Fe supplementation did not affect iron status in non-depleted subjects.
Plowman & McSwegin (1981)	*42 females (14 control, 28 runners), aged 14-22y	*12wk intervention A. 500mg Vit C B.234mg Fe + 450mg Vit C C. control-untrained	*No dietary assessment *Weekly training mileage log *Menstrual log	*Performance not assessed *Blood samples at baseline & end.	*Hb: A.12.94→13.90 g/dL (NS) B. 13.06→14.20 g/dL (P<0.05) C. 13.44→13.50 g/dL (NS)	*Not assessed	*Iron supplemented group showed a sig greater Hb level after 12wks treatment compared to other groups.

Edgerton *et al.* (1981) **YES**	*31 adults (28 females, 3 males) from general Sri Lanka's population, aged 31-46y	*2 wks intervention (except group A-cross-sectional): A. normal group (n=11) (Hb>13g/100ml) B. moderately anaemic (Hb=6.3) (5)-iron dextran injection single dose C. anaemic (Hb=5.6) (n=8)-Fe dextran injection single dose D. severely anaemic (Hb=3.5) (n=7) -570ml blood transfusion		*Progressive 12-min <u>*Treadmill test*</u> for maximum work load (max speed & grade attained on the treadmill) : -D. pre & post transfusion, -B, C post the treatment only -A. once *No dietary assessment.		*Max work load: -A.12 (P<0.01 compared to other groups) -B. 8 km/h -C. 7 km/h -D. 3.5→6.5 (P<0.05 compared to pre-treatment)	*Strong correlation between Hb & work tolerance (r=0.74, P<0.001). *Anaemia impairs work performance capacity. *Blood transfusion ↑ work tolerance by 83% within 24hrs of transfusion.
Ohira *et al.* (1979) **YES**	*20 adults (14 females, 6 males), aged 21-72y	*16-d intervention: IG: 30-50ml Fe dextran injection (n=10) C: placebo (n=10)	*No dietary assessment	*Progressive 18-min <u>*Treadmill Test*</u> before, day 4, 8, 12 &16.	*Hb: IG 6.6→8.4 (P<0.01) C 8.0-→8.1 g/dL (NS) *sFe: IG 0.51mg/L C 0.67mg/L	*Max work load: -IG 2.6→5.6 km/h (P<0.01) -C 8→8km/h (NS)	*Hb & max work time ↑ within 4d & continue to ↑ up to 16d (P<0.01). *Heart rates↓ in IG (P<0.001)
Gardner *et al.* (1977) **YES**	*75 females, aged 22-65y, untrained (Hb 6.1-15.9 g/100ml)	*Cross-sectional: 8 groups according to their Hb v values, 6 to13.9 at 1ml intervals	*No dietary assessment	*Progressive 18-min Treadmill Test	*Work time: -lowest Hb 10.4min, highest Hb 18min (P<0.001) *HR at 4.77km/h speed: -lowest Hb 175 beats/min, -highest Hb 130beats/min (P<0.01) *$Lactate_{max}$: -lowest Hb 4.9umol/ml, -highest Hb 2.7umol/ml (P<0.001)		*Anaemic subjects have lower work tolerance than normal Hb subjects. *Subjects with Hb 11-11.9 have 20% decrease in performance to >13g/dL
Gardner *et al.* (1975)	*29 adults (16F, 13M) (Hb 4-12g/dl), aged 17-46y	*80-d intervention: -IG: intramuscular injections of Fe dextran -C: placebo	Not assessed.	*Performance testing: -handgrip dynamometer -5-min 40com step test	*Hb: IG F 7.7→12.4 (P<0.05), M 7.1→14.0 C F 8.1→8.4 (NS), M 7.7→7.4 g/dL (NS) *Hct: IG F 27→37.5% (P<0.05), M25→42% (P<0.05); C F 27→28.2% (NS), M 27→26.8% (NS)	*Handgrip- NS *HR_{peak}: IG F 152→123 beats/min (P<0.05)C 154 → 144 beats/min *Lactate: IG F 1.18; C F 5.3 mmol/L (P<0.05)	*Anaemic subjects had ↑ $HR_{peak\ \&\ resting,}$ ↑$lactate_{post}$. *15% more O_2 was delivered per pulse in IG. *Performance requiring high O_2 delivery is significantly affected by Hb levels.

*Abbreviations: **YES**- study showed a significant improvement in performance after iron supplementation; **NO**- study did not show a significant improvement in performance after iron supplementation; **MAYBE**- study suggest a possible partial improvement in performance after iron supplementation
Hb- haemoglobin, **RBC**- red blood cells, **PCV**- packed cell volume, **sFe**-serum iron, **TS**- transferrin saturation, **sTRF** – serum transferrin, **sTsfR** - serum transferrin receptor, **sFer**- serum ferritin, **TIBC**- total iron binding capacity, **Ht**- haptoglobin, **Hct**- haematocrit.

Appendix 2 Screening Questionnaire

ID No:	
Please complete the following questionnaire. **Any information contained herein will be treated as confidential.** Please circle answers where relevant	
PERSONAL	Name:_____ Age:_____ E-mail:_____Telephone:_____
PHYSICA L ACTIVITY	How would you describe your present level of activity? Sedentary / Moderately active / Active / Highly active
	In terms of fitness, how would you describe your present level of fitness? Sedentary / Moderately fit / Trained / Highly trained
	How long have you been engaged in current exercise regime? For the last 3 months / 6 months / > 1year / > 3 years
	How often do you engage in physical activity? Never / 1-2 times a week / 3-6 times a week / Everyday
	How long do you exercise per session? Up to 30 mins / Up to 1 hour / Up to 2 hours / Over 2 hours
	Have you had any cause to suspend your normal physical activity regime for the last two weeks? Yes / No If YES, please give details:
DIET	Are you a vegetarian? Yes/ No
	Do you have any food allergies or intolerances? Yes/ No If YES, please specify:
	How often do you consume bread as a part of diet? Never / 1-2 times a week / 3-6 times a week / Everyday
LIFESTYLE	Smoking habits: Currently non-smoker Yes/ No If YES: A previous smoker Yes: How many cigarettes per day:_____ An occasional smoker Yes: How many cigarettes per day:_____ A regular smoker Yes: How many cigarettes per day:_____

	Consumption of alcohol:	Do you drink alcoholic drinks	Yes / No
	If YES do you:	have the occasional drink?	Yes / No
		have a drink a day?	Yes / No
		have more than one a day?	Yes / No
HEALTH	Have you had to consult your GP within last six months?		Yes / No
	If YES, please give details why:		
	Are you presently taking any form of medication or supplements?		Yes / No
	If YES, please give details (brand, frequency, dosages):		
HEALTH	Do you currently suffer from:	Diabetes	Yes / No
		Raised blood pressure	Yes / No
		Asthma	Yes / No
		Bronchitis	Yes / No
		Any form of heart complaint	Yes / No
		Muscle or joint injury	Yes / No
		An infectious illness	Yes / No
		Allergies	Yes / No
		Other:……....	Yes/No
	Are you currently pregnant or have been pregnant within the past year?		Yes / No
	Have you recently donated blood (within previous 30 days)?		Yes / No
	What is your current menstrual status?		
	No menstruation / Irregular menstruation / Regular menstruation		
	When was your last menstrual cycle?		
	_____		
	Do you currently take and oral contraception?		
	To your knowledge is there anything that may prevent you from successfully completing the tests in this study that have been outlined to you?		
	Yes / No		

Appendix 3 Study Information Sheet

Project title

The effects of dietary iron on runners' iron status and sports performance

Thank you for showing an interest in this project. Please read all the information carefully. If you decide to take part, you will be given detailed instructions and will be asked to sign a consent form.

You do not have to take part. If you decide that you do not want to participate, there will be no disadvantage to you.

What are the aims of the project?

The main aims of the project are:

To evaluate the effects of iron-rich bread on an individual's iron status

To evaluate the effects of iron-rich bread on the individual's sports performance

What will you be asked to do?

Procedures

Anthropometric measurements. At the beginning and end of the study some physical measurements will be taken, such as weight and height. This will take few minutes.

Treadmill test. You will be asked to perform an 18-minute treadmill test using the Bruce protocol. Your aerobic capacity and heart rate will be continually monitored by the appropriate electronic equipment. The initial workload of treadmill test is fairly light and then increases gradually. If at any point of the trial you cannot sustain the work rate, you can finish it by pulling the strap around your chest. As a safety feature, the trial would cease automatically once your heart rate reaches 200 beats/minute.

You will perform treadmill tests on weeks 1, 3 and 6 of the study. When you come to do your first test, you will be familiarised with the procedures so feel comfortable in performing it.

Blood samples. The blood will be drawn by putting a needle into a vein in your arm. This will take about five minutes. You will be asked to give a blood sample twice during the study, at screening (week 1) and end (week 6).

Bread consumption. Your will be asked to consume 5 slices of bread everyday for 6 weeks. Bread will be prepared in the University bakeries and will be supplied to you on weeks 1 and 3 when you come to do your treadmill test. To keep the breads fresh, we would advise you to freeze them.

Food Intake and Exercise Log. You will be asked to fill in your 24-hour diet & exercise recall when you come in for the treadmill test on weeks 1, 3 and 6.

Risks and discomfort

There might be some risk and discomfort associated with performing treadmill trials. To reduce possible risks, you will be familiarised with the test beforehand and there will be 2 trained and certified first-aiders during all of the tests.

During blood taking, the needle stick may hurt, and there might be a small risk of bruising, and a very rare risk of infection. To minimise this, a fully trained phlebotomist will be taking the blood samples during the trial.

If you experience pain or discomfort, please tell the researcher immediately.

Can you stop taking part?

You can change your mind and decide not to take part at any time.

What information will be collected, and how will it be used?

Data on blood iron status, anthropometric measurements and treadmill performance scores will be collected and used solely for this project. The data will be stored securely and only accessible to the investigator.

The results of this project may be published, but the information will not be linked to any specific person.

Guidelines for Performers

To ensure everything runs smoothly, please try to adhere to the following guidelines:

- Make sure you are familiar with all the tests to be performed and understand what will be required of you on the day.
- You should ensure that no training is performed on the day of testing and the day before should ideally be a non-training (or very light training) day.
- Please wear light and comfortable clothing. Also, please bring the necessary shower needs and a change of clothing. Secure changing facilities will be provided to you.
- Ensure you consume a light carbohydrate meal (e.g. 2 slices of toast) 2-3 hours before testing. Only water should be consumed after this time.
- You should avoid smoking or drinking alcohol on the day of the test (and preferably the day before).
- Avoid drinking tea or coffee on the day of the treadmill trial.

Follow up

You can ask questions about the project at any time.

Appendix 4 Informed Consent Form

Project title

The effects of dietary iron on runners' iron status and sports performance

Thank you for showing an interest in this project. Please read all the information carefully.

If you decide to take part, please sign this form.

You do not have to take part. If you decide that you do not want to participate, there will be no disadvantage to you.

You can ask questions about the project at any time.

What are the aims of the project?

The main aims of the project are:

To evaluate the effects of dietary iron on an individual's iron status

To evaluate the effects of dietary iron on the individual's sports performance

If you consent to participate in this study, please sign next to each statement.

Statement by participant	**Please initial**
I have volunteered to take part in this project	
I have read *Information Sheet* provided and understand what I will be required to do	
I know I can stop taking part at any time	
I am satisfied that the results will be stored securely	
I know that the results may be published, but they will not be linked to me	
I am aware of all possible risks and discomfort	
I agree to inform the researcher immediately if I am in pain, or if I feel uncomfortable	
I have had the chance to ask questions	

I have read this form and I understand it. I agree to take part in the project titled: 'The *effects of dietary iron on runners' iron status and sports performance'*.

Participant:_____ Date:_____

Signature:_____

Investigator::_____ Date:_____

Signature:_____

Appendix 5 Pre-test Questionnaire

ID No:_____ Test Date:___/___/____

Emergency

In case of emergency, please notify:

Name:_____ Relationship:_____

Telephone:_____

Health condition

I have recently experienced or at present experience:

☐ Heart conditions

☐ High blood pressure

☐ Diabetes, goitre, or any gland disease

☐ Tuberculosis, asthma or any lung disease or respiratory disorder

☐ Epilepsy

☐ Disease of the blood (anaemia), easy bruising or bleeding tendency (haemophilia)

☐ Allergies

☐ Difficulties with eye(s) or vision

☐ Difficulties with nose or throat

☐ Problems with hearing

☐ Headaches, dizziness, weakness, fainting, a problem with coordination or balance

☐ Cough, shortness of breath, chest pain, dizziness or palpitation with exercise

☐ Poor appetite, vomiting, abdominal pain, abnormal bowel habits (diarrhoea, bleeding)

☐ Problems with the muscle, bones or joints (e.g. stiffness, swelling, pain)

☐ Problems with skin (e.g. sores, rashes, itchy or burning sensations ect.)

☐ Menstrual irregularities or problems (e.g. severe period pains, missed periods, heavy bleeding etc.)

☐ Other symptoms- please list:_____

Signature (Participant)_____ Date:___/___/____

Signature (Investigator)_____ Date:___/___/____

Appendix 6 A sample of 24-hour dietary recall

Record the time, the type of food, serving size (large bowl, tbsp, tsp, oz, grams, cup) and method of preparation (baked, fried, boiled).

Taking supplements? YES / NO

If YES, please state what:_____

What did you eat and drink yesterday? Please be thorough.

Time	Food & Beverages consumed. Describe in detail. List one food per line	Amount eaten
Dinner 7 PM	Jacket potato with tuna mayo & bowl salad (lettuce, cucumber, tomato) & glass of orange juice	Medium size potato, a tin of tuna + 2tbsp mayo
	Teff bread (toasted) with butter & apricot jam	2 slices
	Black tea with sugar & semi-skimmed milk	1 mug
Breakfast 10 AM	Teff bread (toasted) with peanut butter & strawberry jam	2 slices
	with semi-skimmed milk	½ cup
	Banana	1 medium size
	Decaff coffee with semi-skimmed milk & sugar	1 mug, 1tsp
Snack 11.30AM	Sainsbury's Nuts & Fruits mix	2 handfuls
12.30PM	Sandwich: 2 slices Teff bread, butter, 2 slices gammon, lettuce, cucumber	
	Snickers	1 bar
	Walker Ready Salted crisps	1 pack
	Diet Coke	1 can
2 PM	Activia rhubarb yogurt	1 standard pot
	apple	1 medium
	Black tea with semi-skimmed milk & sugar	2 cups, 2tsps
Snack	Teff bread with Philadelphia light cheese	1 slice
	Apple juice	2 glasses
Dinner 7 PM	Pasta Bolognese (spaghetti, beef mince (200g), onions, bell peppers, mushrooms, tomato sauce)	1 medium serving (400g)
	Piece of lemon cheesecake (Sainsbury's)	1 piece (80g)

What did you eat and drink today?

2 slices Teff bread with butter & 1 glass of water

Is this your typical daily diet? If Not, explain why not:

Appendix 7 Exercise Log

Describe you lasts 3 days of training/exercise sessions

Time	Exercise or training activity	Duration	Intensity
Yesterday	Jogging in the park	45 mins	Moderate
	Gym session (treadmill, rowing machine)	60 mins	Hard
Today			
Yesterday			
Two days ago			

How many hours ago did you last exercise?_____

How fatigued are you today?

□ Not at all □ Slightly □ Moderately □ Extremely

Appendix 8 Menstrual Log

Record your menstrual cycles

Start date	End date	Intensity
15/01/11	20/01/11	Heavy for the first 2 days, light the rest

Appendix 9 Bread Consumption Log

Please record the number of slices consumed each day

Day	Number of slices eaten
3rd March Thursday	
4th March Friday	
5th March Saturday	
6th March Sunday	
7th March Monday	
8th March Tuesday	
9th March Wednesday	
10th March Thursday	
11th March Friday	
12th March Saturday	
13th March Sunday	
14th March Monday	
15th March Tuesday	
16th March Wednesday	
17th March Thursday	

18th March Friday	
19th March Saturday	
20th March Sunday	
21st March Monday	
22nd March Tuesday	
23rd March Wednesday	
24th March Thursday	
25th March Friday	
26th March Saturday	

Appendix 10 Ethical Issues Consideration in Ethics Approval Application

DETAILS OF THE PROJECT

Title: The effects of dietary iron on female runners' iron status and sports performance

Description of Project:

Female athletes, especially runners, are at greater risk of iron deficiency due to increased iron loss in the gastrointestinal tract, sweat, urine and menstruation, also due to increased haemolysis during endurance training. Furthermore, inadequate dietary iron intake, coupled with limited bioavailability of iron in the diet, may present even a greater risk of iron-deficiency in female athletes. Although anaemia is rare in athlete and general population, depleted iron stores are common in female athletes, especially runners. Researchers reported marginal iron deficiency (defined as Serum ferritin <20 μg/L; Haemoglobin >12g/dl) to be present in between 30- 50% of female runners, more than twice compared to control population (Woolf *et al*., 2009).

Good nutrition to achieve adequate iron balance has been suggested as the first line of action in the prevention of iron deficiency in the female athletes' population (Beard and Tobin, 2000).

Research indicates that modification of staple foods, such bread or pasta, to healthier alternatives is perceived to be more beneficial than improvements of hedonistic foods, such as biscuits (Dean *et al*., 2007). Furthermore, recent study concluded that bread is one of the most preferred foods and the major contributor to energy, carbohydrate and fibre intake for high-levels athletes (Iglesias-Gutierrez *et al*., 2008). Hence, modifying dietary intake of iron through a staple food offers a good opportunity to improve iron status of physically active females.

Consumer acceptable and iron-rich Teff bread was developed in the first phase of this research. The aim of this study is to identify any relationships between Teff bread consumption, blood plasma iron concentrations and athletic performance.

The research design employs 6-week intervention trial. Potential participants will be recruited from local running clubs. Initial pre-study screening will involve screening questionnaire involving inclusion criteria. Eligible participants will be asked then to complete exercise & dietary frequency questionnaires and body composition measurements. During 6-week period, participants will be asked to consume 5 slices of

Teff bread (200g) a day provided by the study investigators. Subjects will be asked to give blood on baseline and trial completion for iron status analysis. Participants will also perform treadmill tests at baseline, midpoint (week 3) and trial completion to determine any changes in their performance capabilities. Subjects will be asked to complete 24-hour Food recall and exercise log at baseline, midpoint and completion in order to account for their dietary habits and exercise regimes.

References

Beard, J. and Tobin, B. (2000) 'Iron status and exercise'. American Journal of Clinical Nutrition, Vol. 72, Issue 2, pp. 594s-597s.

Dean, M., Shepherd, R., Arvola, A., Vassallo, M., Winkelmann, M., Claupein, E., Lahteenmaki, L., Raats, M.M. and Saba, A. (2007) 'Consumer perceptions of healthy cereal products and production methods'. Journal of Cereal Science, Vol. 46, Issue 3, pp. 188-196.

Iglesias-Gutierrez, E., Garcia-Roves, P.M., Garcia, A. and Patterson, A.M. (2008) 'High-level athletes' dietary intake food preferences do not influence adolescent'. Appetite, Vol. 50, Issue 2-3, pp. 536-543.

Woolf, K., Thomas, M.M.S., Hahn, N., Vaughan, L.A., Carlson, A.G. and Hinton, P. (2009) 'Iron Status in Highly Active and Sedentary Young Women'. International Journal of Sport Nutrition and Exercise Metabolism, Vol. 19, Issue 5, pp. 519-535.

Are you going to use a questionnaire? YES/~~NO/N/A~~

Please find attached:

Screening Questionnaire for screening purposes

Pre-Test Questionnaire

Duration of project: 6 weeks

DETAILS OF PARTICIPANTS

How many?

Up to 15 eligible participants.

Age: 18-45 years old

Sex: Females

How will they be recruited?

The subjects will be recruited from local running clubs by directly contacting the clubs and runners themselves.

Status of participants:

General public females, habitually involved in running activity

Inclusion and exclusion from the project:

Inclusion criteria: Healthy female subjects, aged 18-40 years old, habitually engaged in physical activity for at least previous 6 months at the level of at least 30 mins a day 3 or more times a week as used by LaManca et al. (1992) in order for them comfortably to perform treadmill exercise tests.

Exclusion criteria: - Food allergies, i.e. gluten intolerance (because treatment group would consume gluten containing breads)

- Heart conditions or complains, severe asthma, musculoskeletal problems (compromises participants' ability to complete series of treadmill exercise)
- Haemophilia, recent blood donation (subjects will be giving blood samples)
- Irregular or missing menstrual cycles
- Current pregnant or pregnancy within the past year
- Recent history of eating disorders
- Other medical problems that would negatively affect ability physically perform: diabetes, epilepsy
- Other medical problems that would affect blood donation: current incidence of haematoma, inflammation, current infectious illness
- Other problems: in the past during blood taking a person has fainted or suffered anxiety attack in relation to needles or blood.

Payment to volunteers:

Participants will not gain any financial incentives.

Study information:

Have you provided a study information sheet for the participants? YES~~/NO/N/A~~

Please find attached:

Informed Consent, Information Sheet and Phlebotomy Consent

Consent:

Have you produced a written consent form for the participants to sign for your records? YES~~/NO/N/A~~

Please find attached:

Informed Consent and Phlebotomy Consent

RISKS AND HAZARDS

Are there any risks to the researcher and/or participants?

Treadmill Test. Participants will be asked to perform an 18-minute treadmill test using standard Bruce protocol (gradual increasing speed and incline running test). There is a small risk of injury during running on a treadmill.

Blood samples. Participants will be asked to give blood sample at screening and trial completion. The might be some discomfort and pain as the needle stick may hurt; there might be a small risk of bruising and a very rare risk of infection.

State precautions to minimise the risks and possible adverse events:

Treadmill Test. Participants will be familiarised with the procedures prior the trials. They will be explained on how to do the test and will be given two trial tests to complete. There will be two certified and trained first aiders during all of the tests to account for any injuries. There will be mattress placed by the end of treadmill track, in case the participant falls off the treadmill. The Bruce Protocol itself has safety precautions (once the subject's heart rate goes up to 200 beats/minute, the trial is ceased; a subject also has the ability to stop the test at any time by pulling the stripe of the chest).

Changing facilities will be provided at the campus, where participants can change, shower and securely store their belongings.

Water will be available during the trials in the physiology room.

Blood samples. A certified and trained phlebotomist will be taking blood samples using appropriate equipment (Bunzl, London) to avoid risks and discomfort.

The blood samples will be stored in the appropriate labelled containers. All samples will be discarded after the analysis. All the procedures therefore, would comply with Human Tissue Act (2004).

What discomfort (physical or psychological) danger or interference with normal activities might be suffered by the researcher and/or participant(s)? State precautions which will be taken to minimise them:

Blood samples. Taking of blood samples may cause some discomfort to the participants. To minimise this, a fully trained phlebotomist will be taking blood samples during the trial. Study participants will be fully informed about the frequency, duration (twice: beginning

and completion of study, approximately 5 minutes each time) and the procedures involved during this (refer to *Information Sheet, Informed Consent and Phlebotomy Consent*).

PLEASE DESCRIBE ANY ETHICAL ISSUES RAISED AND HOW YOU INTEND TO ADDRESS THESE:

Ethical issues associated with participation:
-Written Information Sheet and familiarising session by the investigator will be provided prior the study. During this session, all participants will be clearly informed about the purpose of the study, what they will be asked to do if they agree to participate in the study, what data will be collected and how it will be used.
-Subjects who are willing to participate and meet study criteria will be asked to read and sign the consent form.
-Withdrawal/termination: subjects will be able to withdraw from the study at any time for whatever reasons.

Procedures of the study and ethical considerations

- Anthropometric measurements. Taking some physical measurements (height, weight) may present some ethical issues. To account for them, firstly all participants will be informed about the exact measurements and procedures this involves. Also, an investigator and one other team member will be present during taking of measurements.

-Blood Taking. All subjects will sign the consent as required by Human Tissue Act, 2004. Blood samples will be only taken by trained phlebotomist. Blood samples will be stored in appropriately labelled containers, analysed by the investigator in the University laboratories and discarded after analysis as required by Human Tissue Act, 2004.

-Treadmill Test Procedures
Study subjects will be fully informed about the treadmill test's exercises, frequencies and measurements taken during the study. All subjects will be asked to sign the consent.
Study subjects will perform treadmill test in the designated study room at Hollings Faculty under strict supervision by the study conductors.

-Dietary intake/ Bread consumption

Participants will be given Teff bread baked at Hollings bakeries at the beginning of each week. Participants will be asked to consume 200g of bread a day (5 slices). Suggestions and examples on how to achieve this number of slices will be given to each participant.
Subjects will be asked to record their 24-hour Food intake at baseline, midpoint & end of the study. A sample of 24-hour food record will be given for explanatory purposes.

-Follow-up and support

-Subjects will be able to contact study conductors by telephone, e-mail or arrange a meeting if needed.

-Storage of research data

Study data will be stored confidentially (password protected databases/spreadsheets) and securely (only accessible to the investigator) in compliance with the Data Protection Act 1998. All data related to participants (physical measurements, blood test results, treadmill scores, nutritional analysis) will be only used solely for this project and the information will not be linked to any specific person.
Participants will be provided with the opportunity to request personal analysis of their diet and any changes in performance or iron status during the trial.

SAFEGUARDS /PROCEDURAL COMPLIANCE

Confidentiality:

Indicate what steps will be taken to safeguard the confidentiality of participant records. If the data is to be computerised, it will be necessary to ensure compliance with the requirements of the Data Protection Act.

If you are intending to make any kind of audio or visual recordings of the participants, please answer the following questions:

a. How long will the recordings be retained and how will they be stored? N/A

b. How will they be destroyed at the end of the project? N/A

c. What further use, if any, do you intend to make of the recordings? N/A

6.2 Human Tissue Act:

The Human Tissue Act came into force in November 2004, and requires appropriate consent for, and regulates the removal, storage and use of all human tissue.

a. Does your project involve taking tissue samples, e.g., blood, urine, hair, etc., from human subjects? YES~~/NO/N/A~~
b. Will this be discarded when the project is terminated? YES~~/NO/N/A~~
If NO – Explain how the samples will be placed into a tissue bank under the Human Tissue Act regulations:

6.3 Insurance:

The University holds insurance policies that will cover claims for negligence arising from the conduct of the University's normal business, which includes research carried out by staff and by undergraduate and postgraduate students as part of their courses. This does not extend to clinical negligence. There are no arrangements to provide indemnity and/or compensation in the event of claims for non-negligent harm.

Will the proposed project result in you undertaking any activity that would not be considered as normal University business? If so, please detail below:
6.4 Notification of Adverse Events (e.g., negative reaction, counsellor, etc.):
(Indicate precautions taken to avoid adverse reactions.)

Some possible adverse events (e.g. food intolerance, increased risk of injury etc.) will be accounted by the screening process:
E.g. subjects with gluten intolerance will not participate as they cannot consume Teff bread
E.g. subjects in good health (no current injuries, illnesses or other conditions) so that participants are not in greater risk of injury or adverse reaction of undertaking physical activity.
All participants will be asked verbally and in written (*Information Sheet, Informed Consent and Phlebotomy Consent*) to tell the researcher immediately if they experience any pain or discomfort associated with the trial.

Appendix 11 Phlebotomy Consent Form

ID No:	Date:
Please circle answers where relevant	
Age:_____ Height(cm):_____Weight (kg)_____	
Your blood test will be carried out by a trained phlebotomist. However, before we take your blood sample we need to check your medical history and obtain your consent. Please inform the investigator of any of the following:	
Current or recent infection Yes / No	
Any device in situ Yes / No	
Past or current history of mastectomy Yes / No	
Cardiovascular incidents of stroke Yes / No	
Have you fainted in the past when phlebotomy has been performed? Yes / No	
Do you suffer from anxiety attack in relation to needles or blood? Yes / No	
Any other complication? E.g. allergies Yes / No Please state:……..	
Please talk to the investigator about any concerns you may have. If you prefer to lie down during the procedure or if from previous experience of phlebotomy you feel you know which veins are usually reliable, please let the phlebotomist know.	
Please sing below if you are happy to proceed with the trial.	
Signature (participant)	Date:
Signature (investigator)	Date: