UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXV

S.S.D: AGR/15 - Scienze e Tecnologie Alimentari

Impact of food processing on tannins and phytic acid activities in sorghum porridge: integrated *in vitro* and analytical approaches

Candidate: Ilaria Proietti
Matr. n.: 3810667

Academic Year 2011/2012

UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXV
S.S.D: AGR/15

Impact of food processing on tannins and phytic acid activities in sorghum porridge: integrated *in vitro* and analytical approaches

Coordinator: Ch.mo Prof. Romeo Astorri

____

Candidate: Ilaria Proietti
Matriculation n. : 3810667

Tutors: Prof. Pier Sandro Cocconcelli
Dr. Alberto Mantovani

Academic Year 2011/2012

Table of contents

Summary 11

Introduction 15

Objectives 17

Background 19

Sorghum 19

Sorghum preparations 20

Nutritional profile 21

Nutritional Concerns 24

Tannins 25

Tannins in plants foods 28

Biological activities of tannins 29

Phytic acid 33

Biological activities of phytates 34

Beneficial effects of phytates 36

Influence of landrace on nutritional characteristics 37

Impact of sorghum processing on nutritional characteristics 38

Cooking 39

Fermentation 41

Project design 47

Pilot study (Manuscript 1) 48

In vitro analysis (Manuscript 2) 48

Content of AN and Trace Elements (Manuscript 3) 49

Bioaccessibility and Bioavailability of trace elements (Manuscripts 2-3) 50

Materials and Methods 53

Materials 53

Sorghum samples 53

Treatments 53

Subdivision of samples according to treatment 54
In vitro methods 54
Standard substances 54
In vitro gastrointestinal digestion 55
Cell line and culture conditions 55
Growth inhibition assay and working concentration determination 56
Cell line treatments 57
Study of functional markers 58
Data analysis and statistics 60
Analytical methods 61
Standard substances 61
Determination of iron binding phenolic groups 61
Determination of phytate content 62
Determination of phytase activity 62
Determination of total iron and zinc content 63
Data analysis and statistics 63
Bioavailability and bioaccessibility of trace elements 64
Molar ratios of phytate to iron and zinc 64
Study of algorithms 64
Results 67
In vitro analysis 67
Quantization of total protein content 67
Antioxidant capacity 70
Content of AN and Trace Elements 72
Content of iron binding phenolic groups 72
Phytate content and phytase activity 75
Iron and zinc content 81
Bioavailability and bioaccessibility of trace elements 83
Molar ratios of phytate to iron and zinc 83
Study of algorithms 86

Discussion & future perspectives **87**
In vitro analysis 88
Quantization of total protein content 88
Antioxidant capacity 90
Content of AN and Trace Elements 91
Content of iron binding phenolic groups 91
Phytate content and phytase activity 92
Iron and zinc content 95
Bioavailability and bioaccessibility of trace elements 96
Molar ratios of phytate to iron and zinc 96
Study of algorithm 97
Effect of variety 98
Effect of treatments 98
Future perspectives 99
Conclusions **103**
References **105**

List of Tables

Table 1. Nutritional composition of sorghum 22

Table 2. Nutritional composition of sorghum and other cereals 23

Table 3. Amino acid content of cereals 24

Table 4. Synoptic table of positive and negative effects of cooking 41

Table 5. Synoptic table of positive and negative effects of fermentation 42

Table 6. Effect of variety and food process on total protein content of DLD-1 cells exposed to sorghum 69

Table 7. Effect of variety and food process on GPx activity of DLD-1 cells exposed to sorghum 72

Table 8. Effect of treatments on iron binding phenolic groups in samples of sorghum varieties 75

Table 9. Effect of treatment on phytate content in samples of sorghum varieties 78

Table 10. Effect of fermentation on phytase activity within each tested sorghum variety 80

Table 11. IP6/Fe and IP6/Zn molar ratios in samples of sorghum varieties 85

Table 12. Percentage (%) of bioavailable iron in 50g of DM 86

List of Figures

Figure 1. Structures of condensed tannin monomers (a) and hydrolysable tannic acid (b) 27

Figure 2. Structure of phytic acid 33

Figure 3. Formation of complexes of phytic acid with metal cations and protein residues 34

Figure 4. Total protein content of cells exposed to different sorghum varieties 67

Figure 5. Total protein content of cells exposed to samples undergoing different 68

Figure 6. GPx activity of cells exposed to different sorghum varieties 70

Figure 7. GPx activity of cells exposed to sorghum samples undergoing different 71

Figure 8. Content of iron binding groups in porridges of different sorghum varieties 73

Figure 9. Content of iron binding groups in sorghum varieties undergoing different treatments 74

Figure 10. Phytate content in porridges of sorghum varieties 76

Figure 11. Phytates content in sorghum varieties undergoing different treatments 77

Figure 12. Phytase activity in the flour of sorghum varieties 79

Figure 13. Phytase activity in fermented slurry (*f*) of sorghum varieties 80

Figure 14. Iron and zinc content in slurries (*s*) of different sorghum varieties 81

Figure 15. IP6/Fe and IP6/Zn molar ratios in samples of sorghum varieties 83

Figure 16. IP6/Fe and IP6/Zn molar ratios in sorghum varieties undergoing different treatments 84

Aknowledgments

It is a great pleasure to thank everyone who helped me write my dissertation. Completing a PhD is a challenge, and I would not have been able to complete this journey without the aid and support of countless people over the past three years.

First and foremost, I owe sincere and earnest thankfulness to Professor Pier Sandro Cocconcelli who welcomed me to his research group in the Department of Microbiology, whose expertise, understanding, and patience, added considerably to my graduate experience. Many thanks for giving utmost freedom to perform experiments in your laboratory and also for granting ample time to complete my thesis.

In addition, I would like to express all my gratitude to Dr Alberto Mantovani. To work with you has been a real pleasure to me, with heaps of fun and great moments. You have been a steady influence throughout my Ph.D.; you have oriented and supported me with promptness and care, and have always been patient and encouraging in times of difficulties; you have listened to my ideas and discussions, always with the ability to get out the best from them. Your ability to select and to approach compelling research problems, your high scientific writing ability and attention to details set an example.

Furthermore, I am very grateful to my external reviewers: Professor Lubomir Leng and Professor Mikolaj Gralak, for their availability, time, interest and helpful comments, despite the really short notice.

During this research period, many colleagues provided valuable assistance and information that helped organize my experiments and data collection and I am grateful to all of them for their support. In particular, I would like to thank

Sabrina Tait, for her assistance with in vitro assay and with the statistical analysis, and all the members of Francesco Cubadda's research group for their availability and assistance in the simulated gastrointestinal digestion. Moreover, I would like to extend my gratitude to all the numerous people who contribute in making the TAV such a stimulating and lively place.

I extend my hearty thanks to Marisa, Francesca and, in general, to the entire Institute of Microbiology of the Università Cattolica del Sacro Cuore of Piacenza. I want to thank the entire laboratory and Department staff for their support and for providing a pleasant and productive working atmosphere. Thank you also to the secretary of the Doctoral School Agrisystem, Roberta Tedesco, for her prompt communication, availability and attention to details.

Thank you as well to the people who provided me with the different sorghum samples: thank you to Dr Stefano Amaducci, Valentina e Francesca, Edgar Atoe (Nigeria), Tine Djenaba (Senegal).

In addition, I am obliged to many of the fellows I met at the ISS during my journey, who supported me and for all the emotional support, comradeship, entertainment, and caring they provided and that became a part of my life. I have been very privileged to have known and to collaborate with many great people who became friends over the last years. I learned a lot from you about life, research, how to tackle new problems and also how to solve them. Special thanks to Laura, Bruno, Daniela, Candida, Gabriele, Daniele, Rachel and Meire, thank you for having shared with me the alternating fortunes of the PhD student life, thank you for the fun and the encouraging discussions; you have been an invaluable support, during all these years.

Montpellier: quelle belle aventure …

J'exprime mes profonds remerciements à George-André Simon, sans lui cette expérience n'aurait pas pu exister, merci beaucoup George pour ta grande disponibilité et générosité.

Je tiens aussi à exprimer mes plus vifs remerciements à Jean-Pierre et Claire Mouquet-Rivier, pour m'avoir offert l'opportunité d'aller faire une partie de mes recherches dans leur division et m'avoir donné la possibilité de faire les dosage des tannins et phytates et des minéraux et aussi pour avoir rédigé ensemble un article sur les résultats obtenus. Merci également pour les échanges que nous avons eu au sujet des facteurs antinutritionnels et des minéraux en trace.

Ensuite je tiens à remercier tous les membres de l'équipe UMR 204 « Prévention des Malnutritions et des Pathologies associées » (Nutripass), IRD, pour m'avoir fait profiter de leurs connaissances sur les thématiques abordées et les analyses que j'ai réalisé. Voilà une très belle équipe ! Encore merci à vous pour la votre grande disponibilité, vos précieux conseils ainsi que votre bonne humeur. En particulier je remercie Christian Picq (le maître), Isabelle Rochette et Kaleab pour leur assistance technique sans laquelle les manips n'auraient pas pu être possibles.

Enfin, j'exprime toute ma gratitude à mes « coco » : ma Chère (Krishna), mon Coco (Christian) et Calib (Kaleab), mais aussi à Hama, Cécile et Youna . Merci d'avoir été les confidents, les camarades et les complices de mes plus beaux moments. Merci à vous tous, ce fut comme se sentir en famille !

I would also like to thank David for his love and encouragement. Thank you for your support when I have needed it the most. Thank you with all my heart!

Finally, I would like to thank my mom, dad, brother and sister for their infinite support throughout everything. Per essere stati sempre presenti e per non avermi mai fatto mancare il vostro incoraggiamento: grazie!

Summary

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important grain corn in the world agricultural economy and represents an important staple food for the populations of many developing countries. It is estimated that the cereal is part of the diet of more than 300 million people, representing a major source of energy and nutrients. Nevertheless, the nutritional value of sorghum is impaired by the activity of endogenous antinutrients (AN), such as phenolic compounds (mainly tannins) and phytic acid, which inhibit iron and zinc absorption and digestibility of proteins by forming insoluble complexes in the gastrointestinal tract.

The aim of the present thesis was to investigate the impact of both variety and food processing (fermentation and cooking) on the amount and activities of the main AN as well as on the content and bioaccessibility and bioavailability of iron and zinc in two main traditional types of sorghum meals: the porridge and the fermented slurry.

An innovative comprehensive approach based on three different investigation methods has been performed, namely *in vitro* assay (line DLD-1 from human colon adenocarcinoma), quantitative analysis, and mathematical models. The modulation of *in vitro* functional markers after *in vitro* gastrointestinal digestion (total protein content and GPx activity of exposed cells), the content of iron binding phenolic groups (tannins) and phytates, the phytase activity as well as the amount and the bioaccessibility and bioavailability of iron and zinc were investigated in ferment and unfermented slurries and in porridges undergoing cooking with or without fermentation from three African (Nigeria, Senegal and Burkina Faso) and two Italian sorghum varieties.

Results showed that the amount of AN, content and bioaccessibility and bioavailability of trace elements content, total protein content of cells as well as the effect of process (mainly fermentation) were modulated by variety. There is, indeed, a significant difference among traditional African varieties with regard to aforementioned parameters. The two Italian varieties showed an overall poor nutritional profile.

Results also showed that fermentation and cooking have different impact on the tested parameters. In the different varieties, fermentation decreased iron-binding phenolic groups until 49% and phytate content until 72% as well as increased phytase activity 3.4 to 16.4 fold, leading to enhancement of iron and zinc estimated bioavailability. On the other hand, fermentation process significantly decreased also the total protein content and GPx activity of to cells. Cooking alone had almost no effect on the content of AN and the two trace elements. Similarly, it showed no impact on the GPx activity of cells exposed to cooked sorghum samples, whereas it significantly decreased cells total protein content. It cannot be excluded that fermentation and cooking may develop compounds able to inhibit protein synthesis and/or enzymatic functions of cells *in vitro*. Interestingly, most of the tested parameters showed further modulation effects of the variety on food process.

Finally, the combined treatments of fermentation and cooking resulted to be the best process, showing the positive effect of cooking in the *in vitro* analysis (no impact on the cells GPx activity) and of fermentation in the quantitative analysis of AN and bioaccessibility and bioavailability of iron and zinc (reduction of the amount of both AN and increase of the two trace elements estimated bioaccessibility and bioavailability).

The lowest AN content and the highest trace minerals bioaccessibility and bioavailability were shown by fermented (also with cooking) Senegal landrace, whereas the Italian varieties showed the worst results.

The results indicate that selection of traditional varieties and appropriate processing methods can be a promising way to significantly improve sorghum nutritional value at the household level.

Introduction

Sorghum is the fifth most important grain corn in the world agricultural economy after wheat, rice and barley. It is mainly grown in semi-arid areas of the world, characterized by high drought and low rainfall, climate conditions poorly favourable to the production of other cultivars. In industrialized countries, sorghum is mainly grown for the production of animal feed (forage or grain) or biofuel. On the other hand, in Africa and Asia, sorghum is grown primarily for human consumption. It is estimated that sorghum is part of the staple diet of more than 300 million people in developing countries, representing a major source of energy and nutrients. Sorghum is a valuable staple food, for its content of protein, pro-vitamins (carotenoids) and vitamins, fat-soluble (D, E and K) and of B group (except for B12) and minerals, such as iron, phosphorus and zinc.

The nutritional value of sorghum is, nevertheless, impaired by the activity of endogenous AN, such as phenolic compounds (mainly condensed tannins) and phytic acid. The concentration of tannins and phytic acid greatly varies among different cultivars, as well as among plants belonging to the same cultivar. The phytic acid concentration can range 2.5-22 mg/g and that of tannins 0.8-70 mg/g, expressed as catechin equivalent (Elkhalifa and El Tinay, 1994; Kunene *et al.* 1999; Matuschek E., 2005; McMillan *et al*. 2007). These sorghum AN interact negatively with the bioaccessibility of essential elements, in particular iron and zinc; moreover, tannins, reduce the digestibility of proteins. The general mechanism involves the formation of insoluble complexes at physiological pH, due to the ability of phytic acid and tannins to bind proteins and divalent cations. The tannins are also able to bind human gut enzymes involved in the cereal digestion. As a consequence, the AN presence reduces me-

tabolizable energy and amino acid availability, as well as elicits an unbalanced intake of essential elements. The impaired bioavailability of these micronutrients may cause severe metabolic disorders, especially in vulnerable groups of population. Consequently, improving the nutritional value of sorghum would improve their nutritional status.

Sorghum, mainly fermented, is the basic ingredient of many recipes in less developed and emerging countries (e.g. Botswana, Ethiopia, Kenya, Nigeria, Tanzania), such as couscous, porridge and beer. The porridge, in particular, is a staple component of the traditional diet, especially breakfast, for adult as well as young children; indeed it is used as weaning food. For its importance and wide use in developing countries, sorghum porridge represents the main focus of this research project.

The available literature reports that food processes might influence both nutrient and AN content and activity. In particular, fermentation appears to reduce the presence of AN, thus increasing both the bioaccessibility and bioavailability of iron and zinc; on the other hand, cooking may reduce protein digestibility. Nevertheless, these data were obtained through chemical analyses; the net effect of sorghum as whole food upon fermentation and/or cooking has never been investigated in biological systems. Moreover, the study of the effects of a food *in toto* is still poorly developed as well as the combined modulation of such effects by different factors, such as cultivar and process. An interesting approach would be, therefore, to evaluate the impact of both sorghum variety and processing on the main AN through a comprehensive analytical and *in vitro* strategy.

Objectives

The main objective of the present thesis was to evaluate the impact of the two principal traditional processes (cooking and fermentation) on tannins and phytic acid activities in sorghum porridges and their effects in a biological system.

In order to achieve this purpose, investigations were carried out with the following aims:

1) the comparison of different varieties of sorghum (from Italy and three African countries), conducted on the porridge before and after *in vitro* gastrointestinal digestion, by:

1.a) analysing the biological effects (total protein content and the modulation of antioxidant capacity) of the matrices using an *in vitro* model (line DLD-1 from human colon adenocarcinoma);

1.b) determining tannins, phytate and selected trace minerals content in sorghum samples before and after food processing (fermentation and/or cooking) through analytical approaches;

1.c) estimating the bioaccessibility and bioavailability of iron and zinc in sorghum, before and after food processing, through two mathematical models.

2) the study of AN activities in the porridge in the absence and presence of fermentation and/or cooking, representing the food processes suitable for the production of the main porridge types, as well as other sorghum products, in developing countries. The objective was to determine whether such

food processes influence the concentration and activity of AN and the bio-accessibility and bioavailability of iron and zinc, by using the same *in vitro* approach (cell line DLD-1), the quantitative analysis and estimated bioaccessibility and bioavailability of iron and zinc as in 1.b) and 1.c), respectively.

Background

Sorghum

Sorghum belongs to the grass family of *Graminea*. In 1974 Moench established the genus Sorghum and grouped all the species together under the name Sorghum bicolor (Dicko *et al*., 2006) which includes five basic races (bicolor, guinea, caudatum, kafir and durra) and ten intermediate races. Sorghum is characterized by notable genetic variability: more than 30,000 selections in the world genetic collections exist (Assefa *et al*., 2010).

Sorghum is the fifth largest most important cereal, after wheat, maize, rice and barley in the world and the second (after maize) in sub-Saharan Africa (Taylor, 2003). In 2007, the global area cropped with sorghum was 43.8 million hectares (Prasad and Staggenborg, 2009) and in 2010 the worldwide production was 59.51 million metric tons; with Nigeria, USA, Mexico and India representing the largest producers (USDA, 2010). Nearly 55 per cent of world's sorghum cultivation areas are in Africa, representing one-third of the world crop production: about 20 million metric tons per annum (Taylor, 2003).

Sorghum is a real versatile crop: it can be used both for human and animal feeding, in the manufacture of potable alcohol and as bioenergy crop, for production of biomass. In the United States, for instance, sorghum is primarily used for animal feed and, secondarily, in food and ethanol production, while in Africa, sorghum is mainly destined to human consumption and represents a basic staple food for many rural communities, especially in drought prone areas, characterized by shallow and heavy clay soils (Du Plessis, 2008).

Sorghum can be cultivated over a wide range of ecological conditions than the majority of the food crops: it is well adapted to both temperate and tropical zones (BOSTID, 1996). Moreover, it can grow in all Africa's climatic zones, be-

ing drought-resistant and able to withstand periods of water-logging (Taylor, 2003).

In Africa, sorghum is a subsistence food crop for many indigent people and it is considered the viable food grain for many of the world's most food insecure people. Together with millet, it represents the major source of energy and protein for about one billion people in the semi-arid region of tropics and it is part of the staple diet of more than 300 million people in developing countries (Taylor, 2003).

Sorghum is an important crop due to its large number of utilizations, besides human food: the whole plant is often used as forage, silage or hay, while the stems for building, weaving or firewood. Stems of some varieties are also use as biomass for biogas production or processed for sugar and syrup. Other industrial products, like vegetable oil, adhesives, waxes or dyes are obtained by sorghum processing. Finally, the seeds are used in the livestock sector, as feed for poultry, cattle and swine (BOSTID, 1996).

Sorghum preparations

A wide variety of traditional food products are produced from sorghum. The cereal is indeed incredibly versatile and can be prepared in a great number of way: some types are boiled like rice, some "malted" for beer production, some baked into flatbreads or cracked for porridge preparation, etc. Sorghum, mainly fermented, is the basic ingredient of many recipes in less developed and emerging countries (e.g. Senegal, Ethiopia, Burkina Faso, Nigeria), such as couscous, porridge and beer. Traditional recipes include porridges, such as *ogi* (Nigeria, Benin) and *mahewu* (East African Countries), *bogobe* (Ghana, Botswana) typical breads like *injera* and *kisra* (Ethiopia, Sudan), dumplings such as

kenkey (Ghana, Botswana) and alcoholic beverages such as *merissa* (Zambia), *seketeh* (Sudan) and *kishk* (Ethiopia) (FAO, 1999b).

Fermentation, mainly performed by lactic acid bacteria, generally of the genus Lactobacilli, is critically important, not only for the appreciated taste, but also with regard to the shelf-life and safety of food. The lactic acid produced during fermentation determines the reduction of food pH, which prevents or reduces the proliferation of microorganisms and, as a consequence, food spoilage and microbiological risks. Moreover, other than those related to food safety, other nutritional advantages of lactic acid fermentation have been stated: fermentation has been demonstrated to increase *in vitro* carbohydrate and minerals availability and starch ad protein digestibility (Moneim *et al.,* 1994; Taylor, 2003; Elkhalifa *et al*., 2004).

Among fermented food, the porridge, in particular, is a staple component of the traditional diet, especially breakfast, for adult as well as young children; indeed it is used as weaning food.

For its importance and wide use in developing countries, sorghum porridge represents the main focus of this research project.

Nutritional profile

Sorghum kernel is composed of three main parts: pericarp (seed coat), embryo (germ) and endosperm (storage tissue). The proportion among the parts varies, but the majority of kernels are constituted by 6 percent pericarp, 10 percent embryo and 84 percent endosperm. Sorghum kernel is similar to wholegrain, maize or wheat kernel and is made up by about 70 percent carbohydrate, 11 percent protein, 3 percent fat, 2 percent fiber, and 1.5 percent ash (FAO, 1995). The protein content of sorghum is usually 11-13%, but it is quite variable and can range from 7 to 15 percent (BOSTID, 1996, Dendy, 1995).

Table 1. Nutritional composition of sorghum

Main Components		**Essential Amino Acids** (g/16 g N)	
Edible portion (g)	100	Cystine	1.3
Moisture (g)	9	Isoleucine	4.0
Food energy (Kc)	356	Leucine	13.5
Carbohydrate (g)	71	Lysine	2.1
Protein (g)	12.0	Methionine	1.3
Fat (g)	3.4	Phenylalanine	4.9
Fiber (g)	2.0	Threonine	3.3
Dietary Fiber (g)	8.3	Tryptophan	1.0
Ash (g)	2.0	Tyrosine	3.1
		Valine	5.0
Micronutrients			
Vitamin A (RE)	21		
Thiamin (mg)	0.35		
Riboflavin (mg)	0.14		
Niacin (mg)	2.8		
Vitamin B6 (mg)	0.5		
Biotin (μg)	7		
Pantothenic acid (mg)	1.0		
Vitamin C (mg)	0		
Calcium (mg)	21		
Chloride (mg)	57		
Copper (mg)	1.8		
Iodine (μg)	29		
Iron (mg)	5.7		
Magnesium (mg)	140		
Phosphorus (mg)	368		
Potassium (mg)	220		
Sodium (mg)	19		

Adapted from BOSTID, 1996

However, like other cereals sorghum proteins are deficient in the essential amino acid lysine as well as in the sulphur-containing amino acids. In addition, it is reported to be a good source of more than 20 micronutrients as it is an important source of minerals, such as phosphorus, potassium, iron and zinc

and vitamins, both those of B group (except for B12) and fat-soluble vitamins (A, D, E and K) (Table 1).

In composition, sorghum grain compares favourably with some other cereals: it has similar protein content to wheat, but higher than maize and rice; as for iron content, sorghum is higher than wheat, maize and rice, but lower than millet. Concerning calcium, it has similar content to maize but lower than wheat, millet and rice (Table 2).

Table 2. Nutritional composition of sorghum and other cereals

Cereal	Protein (g)	Fat (g)	Crude fibre (g)	Carbohydrate (g)	Energy (kcal)	Ca (mg)	Fe (mg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)
Sorghum	10.4	3.1	2.0	70.7	329	25	5.4	0.38	0.15	4.3
Rice (brown)	7.9	2.7	1.0	76.0	362	33	1.8	0.41	0.04	4.3
Wheat	11.6	2.0	2.0	71.0	348	30	3.5	0.41	0.10	5.1
Maize	9.2	4.6	2.8	73.0	358	26	2.7	0.38	0.20	3.6
Pearl millet	11.8	4.8	2.3	67.0	363	42	11.0	0.38	0.21	2.8

Data are expressed per 100 g edible portion; 12% moisture. Source: FAO, 1995

The essential amino acid composition of sorghum is comparable to other cereals like maize or wheat, due to the limited content of threonine, arginine and, especially, lysine (Table 3).

Table 3. Amino acid content of cereals

Amino Acid	Sorghum	Rice (brown)	Wheat	Maize	Pearl Millet	Barley	Oats	Rye
Tryptofan	1.12	1.08	1.24	0.61	2.18	1.25	1.29	1.13
Threonine	3.58	3.92	2.88	3.98	4.00	3.38	3.31	3.70
Isoleucine	5.44	4.69	4.34	4.62	5.57	4.26	5.16	4.26
Leucine	16.06	8.61	6.71	12.96	15.32	6.95	7.50	6.72
Lysine	2.72	3.95	2.82	2.88	3.36	3.38	3.67	4.08
Methionine	1.73	1.80	1.29	1.86	2.37	1.44	1.47	1.58
Cystine	1.66	1.36	2.19	1.30	1.33	2.01	2.18	1.99
Phenylalanine	4.97	5.03	4.94	4.54	4.44	5.16	5.34	4.72
Tyrosine	2.75	4.57	3.74	6.11	…	3.64	3.69	3.22
Valine	5.71	6.99	4.63	5.10	5.98	5.02	5.95	5.21
Arginine	3.79	5.76	4.79	3.52	4.60	5.15	6.58	4.88
Histidine	1.92	1.68	2.04	2.06	2.11	1.87	1.84	2.28
Alanine	…	3.56	3.50	9.95	…	4.60	6.11	…
Aspartic Acid	…	4.72	5.46	12.42	…	5.56	4.13	…
Glutamic Acid	21.92	13.69	31.25	17.65	…	22.35	20.14	21.26
Glycine	…	6.84	6.11	3.39	…	4.55	4.55	…
Proline	…	4.84	10.44	8.35	…	9.02	5.70	…
Serine	5.05	5.08	4.61	5.65	…	4.65	4.00	4.13

Data are expressed as % amino acid in the protein. Adapted from NIIR, 2006

Due to the lack of gluten, sorghum is considered suitable for people with coeliac disease (Kasarda, 2001; Ciacci *et al.*, 2007; Schober *et al.*, 2007). Sorghum might therefore provide a good basis for gluten-free products, such as cookies and bread. Nevertheless, gluten-free breads in general require a different and more complicated technology, due to the lack of a gluten network, with respect to gluten breads (Schober *et al.*, 2007).

Nutritional Concerns

The good nutritional value of sorghum is, nevertheless, impaired by the activity of phenolic compounds (mainly condensed tannins) and phytic acid. The sorghum AN interact negatively with the bioaccessibility of essential elements, in particular iron and zinc; moreover, tannins reduce the digestibility of proteins. The general mechanism involves the formation of insoluble complexes at physiological pH, due to the ability of phytic acid and tannins to bind proteins and divalent cations. The tannins are also able to bind human gut enzymes involved in the cereal digestion. As a consequence, the AN presence reduces metabolizable energy and amino acid availability as well as elicits an unbalanced intake of essential elements.

Tannins

The name 'tannin' derived from the French 'tanin' (tanning substance) and represents a group of polyphenolic secondary metabolites produced by many plants.

Tannin are found in almost every part of the plant, including bark, wood, leaves, fruit, roots and seed and it is assumed that their principal biological role in the plant is related to protection against infection, insects, or animal herbivory. They provide plants with resistance to predators and pathogens, by acting as phytoalexins (antimicrobial substances synthesized by plants as defensive response) and increasing the astringency of food to make it unpalatable, and protect crops from preharvest seed germination and plague (Haslam, 1989; Porter, 1989; Bravo, 1998).

In nature, tannins are found in many different families of the higher plants, while they have not yet been isolated from lower plants such as algae, or from the animal kingdom, and are, therefore, an integral part of the human diet (Bravo, 1998).

Their average molecular weight ranges between 500 and 3000 Daltons, but molecular weight as high as 30000 Daltons have been also reported (Bravo, 1998; Haslam, 1998).

Due to their enormous variety of structures, the nomenclature of tannins is often improper and full of erroneous interpretation. Their classification is mainly based on specific structural characteristics and chemical properties, but not all substances with tanning properties can be called tannins and, on the other hand tannins without tanning properties are classified as tannins for their structural characteristics (Khanbabaee and van Ree, 2001).

Several authors espoused the classical definition of tannins formulated in 1920 by Freudenberg. According to him, tannins can be divided into condensed tannins (proanthocyanidins) and hydrolysable tannins (gallo- and ellagitannins).

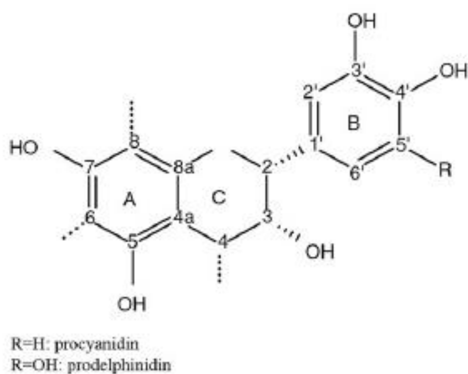
The former are oligomeric and polymeric proanthocyanidins consisting of flavan-3-ol (catechin, epicatechin, etc.) monomers, flavan-3,4-diols (leucoanthocyanidins), or a combination of both, linked through carbon–carbon bonds (Fig.1a). Proanthocyanidins are polymeric flavonoids: a group of metabolites based on a heterocyclic ring system derived from phenylalanine and polyketide biosynthesis. Proanthocyanidins are substances yielding anthocyanidin pigments upon oxidative cleavage in alcoholic solutions (e.g. via acid butanol chemistry) (Hagerman, 2002).

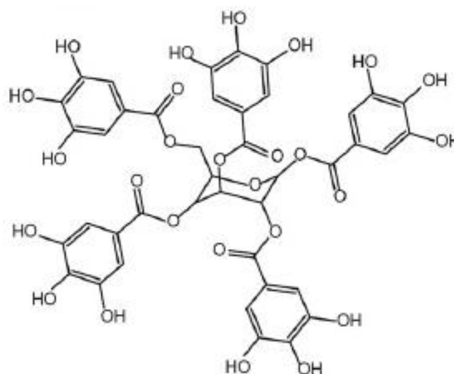
The hydrolysable tannins consist of galloyl acids and/or hexahydroxydiphenic acids esterified to a central polyolcore (usually glucose) (Fig.1b) and are divided into gallotannins, which include also meta-depsides, and ellagitannins. The latter are formed from gallotannins by the oxidative coupling of at least two galloyl units. As indicated by their name, they are easily hydrolysed by treatment with acid, alkali, enzymes or hot water, liberating phenolic acids.

Tannic acid is the best-known gallotannin (hydrolyzable tannins), consisting of a pentagalloyl glucose molecule which can further esterify with other five units of gallic acid.

Figure 1. Structures of condensed tannin monomers (a) and hydrolysable tannic acid (b)

a. condensed tannin monomer **b. tannic acid**





Adapted from Nierop *et al.* (2006)

Tannins in plants foods

Due to their widespread occurrence, tannins are an integral part of our daily diet. They are, indeed, almost ubiquitous in plant foods and their content may be particularly high in vegetables, cereals, legumes, fruits, nuts and beverages, such as tea, wine, cocoa, etc.

Their levels vary greatly even between cultivars of the same species, depending on both genetic and environmental factors, but also germination, storage and degree of ripeness (Bravo, 1998). Tannins are to some extent responsible for the sensory and nutritional quality of plant foods; e.g. the bitterness and astringency of foods and beverages are often linked to the content of tannins, because of their interaction with salivary proteins. Anthocyanidin pigments, for example, are responsible for the astringent taste of fruit and wines and also for the wide array of colours, like pink, red, violet, and blue in flowers, leaves, fruits and wines.

All sorghum varieties contain polyphenolic secondary metabolites, including phenolic acid and flavonoids, and varieties with a pigmented testa, *B1_B2*_genes, also contain condensed tannins (Hahn *et al.,* 1984; Waniska and Rooney, 2000). Sorghum containing condensed tannins are diffused in almost all wild sorghum plants, whereas both tannin and non-tannin types are naturally present in cultivated sorghums (Yuye *et al*., 2012).

The concentration of tannins greatly varies among different cultivars, as well as among plants belonging to the same cultivar, and values of 0.8-70 mg/g, expressed as catechin equivalent, have been reported (Kunene *et al.* 1999 Matuschek, 2005; McMillan *et al.* 2007). Sorghums are classified in different types, depending on tannins content: type I, no tannins; type II, tannins in pigmented testa; type III, tannins in pigmented testa and in the pericarp (Richard and Wayne, 2001). Sorghum with high tannin content is also called brown sor-

ghum, even though the colour of pericarp is not brown. Tannins are the most abundant phenolic compound in brown sorghum.

During maturation, the brown-sorghum grain develops astringency which protects the grain against insects, bird and fungi; this characteristic is vital in pest-ridden and arid regions of the world, where other crops fail (Butler, 1990; FAO, 1995). Interestingly, tannins have also been found in other grains, such as millets and barley, but not in major cereals, such as wheat, rice and maize (Dykes *et al*., 2007).

Biological activities of tannins

Tannins have different biological effects in human and animal nutrition because of their ability to chelate metal ions, form complexes with macromolecules and to act as antioxidants. Due to varied biological roles and the large amount of different structures of tannins, it is difficult to set relationships between their structure and the biological activity (Bravo, 1998; Hagerman, 2002).

- <u>Role in the bioaccessibility and bioavailability of trace minerals</u>

Bioavailability is defined as the proportion of a substance that is digested, absorbed, and utilized in normal metabolism (Palafox-Carlos *et al*., 2011); bioaccessibility is defined as the amount of an ingested nutrient that is available for absorption in the gut after digestion (Hedren *et al.*, 2002); thus, the bioavailability is expected to depend on the bioaccessibility, as well as on other factors.Tannins affect the absorption of trace minerals by forming insoluble complexes in the gastrointestinal tract. The formation of complexes involves the phenolic hydroxylic and carboxylic groups present in tannins which show high affinity for metal ions such as iron, zinc and calcium, thus interfering with their

intestinal absorption (Brune *et al.*, 1989; Bravo, 1998, Hallberg and Hulthén, 2000).

It is widely believed that metals chelated by tannins are not bioavailable for the organism, thus a diet based on consumption of large quantities of tannin-rich food, such as sorghum, is associated with minerals deficiency diseases, such as iron-deficiency anaemia (Baynes and Bothwell, 1990; Mamiro *et al.*, 2005). Several studies have reported that the inhibitory effect of phenolic compounds is dose dependent and both low- and high-molecular weight phenols are able to render metals unavailable for absorption (Brune *et al.*, 1989; Hurrel *et al.*, 1999; Larysse *et al.*, 2000).

It has been demonstrated that phenolic compounds with vicinal hydroxyl groups, such as *ortho*-dihydroxyl or thrihydroxyl groups, form stable complexes with both ferric iron (Fe^{3+}) and ferrous iron (Fe^{2+}), even though the latter results to be less stable (Slabbert, 1992; Scalbert *et al.*, 1999; Santos-Buelga and Scalbert, 2000). The iron-phenolic compound ratio differs, partly depending on pH, but 1:1 complexes are commonly formed (Slabbert, 1992). Nevertheless, other authors have found different ratios, such as 1:2 when iron complexes with gallocatechin or galloylated units or 1:3 with catechin units (Marmolle *et al.*, 1997; Jovanovic *et al.*, 1998).

- <u>Influence on the digestibility of macronutrients</u>

Tannins are able to form complexes also with numerous types of molecules, including proteins, carbohydrates and enzymes involved in their digestion, polysaccharides and bacterial cell membranes.

Although most polyphenolic compounds have the capacity to bind and precipitate protein, highly polymerized tannins result to be the most effective precipitators of proteins, determining their reduced digestibility and a subse-

quent increase in faecal nitrogen excretion (Bravo, 1998). Tannin- protein interactions are specific and dependent on both protein and tannin structure: proteins of large molecular size, characterized by open and flexible structures and rich in proline and tannins of high molecular weight and with high conformational mobility are believed to favour strong complexes (Hoffman, 2003). In addition, the interaction is influenced also by other factors, such as temperature, pH and tannin-protein ratio (Hagerman, 1998). Complexes between tannins and proteins are formed by hydrogen bonds with the protein's carboxyl group, with less frequent contribution of covalent or ionic bonds (Hagerman, 1992; Bennick, 2002).

Different studies have shown that precipitation of tannin-protein complexes is pH-dependent and is at highest level at pH values close to the protein isoelectric point (Hagerman *et al.*, 1978; Bennick, 2002). In the interaction with proteins, both soluble and insoluble complexes are formed, depending on the size and concentration of both molecules. When proteins are in excess, due to fewer tannin binding sites per each protein, soluble complexes are favoured; conversely, when tannins are in excess, and form a hydrophobic outer layer in the complex surface, insoluble complexes are constituted (Van Buren and Robinson, 1969; Hagerman, 1992).

Moreover, tannins are able to bind other endogenous proteins, such as digestive enzymes, and inhibit them. Likewise, they can form complexes with other macronutrients, such as carbohydrates, polysaccharides and lipids, and affect the glycemic and insulinemic responses and faecal fat excretion (Bravo, 1998).

- Antioxidant Activity

Even though the ability of tannins to chelate trace minerals is related to negative effects in some circumstances (especially in poor varied diets and in condition of minerals deficiency), it can result beneficial in others. The positive properties of tannins are partly dependent on their ability to be absorbed and metabolized in the organism (Matuschek, 2005).

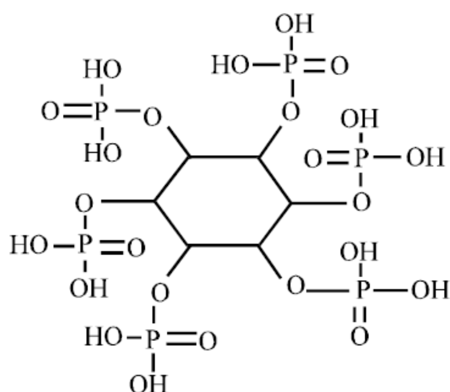
Recent studies demonstrated their role as antioxidant, scavengers of free radicals and their implication in the prevention of pathologies such as cardiovascular diseases and cancer (Shahidi and Wanasundara, 1992; Bravo, 1998; Hagerman *et al*., 1998). The antioxidant activity of tannins can be related to their favorable redox potential and their relative stability of the aryloxy radical and free or protein-complexed condensed and hydrolyzed tannins are demonstrated to be more effective in scavenging radicals than small phenolic compounds (Hagerman, 2002). Chelation of metals copper and iron, potential initiators of hydroxyl radical production by the Fenton and Haber-Weiss reactions, is one of the ways polyphenols exert their antioxidant activity. Phenolic compounds interfere with the oxidation of molecules by donating a hydrogen atom to radicals (Bravo, 1998).

Nevertheless, under certain conditions, such as high concentrations of phenolic antioxidants, high pH and presence of iron, they can exert a prooxidative effect in Fenton-driven systems, by initiating an auto-oxidative process (Aruoma *et al*., 1993; Bravo, 1998, Hagerman, 2002).

Phytic acid

Phytic acid (PA) is the primary storage compound of phosphorus in cereals, legumes, nuts and oil seeds, accounts for up to 90% of total phosphorous content and contribute as much as 1.5% to the seed dry weight (Lott *et al*., 2009). PA is also named inositol hexaphosphate (IP6), or phytate as a salt, its molecular formula is $C_6H_{18}O_{24}P_6$ and its structure is shown in Figure 2 (Coulibaly *et al*., 2011).

Figure 2. Structure of phytic acid



Source: Coulibaly *et al*., 2011

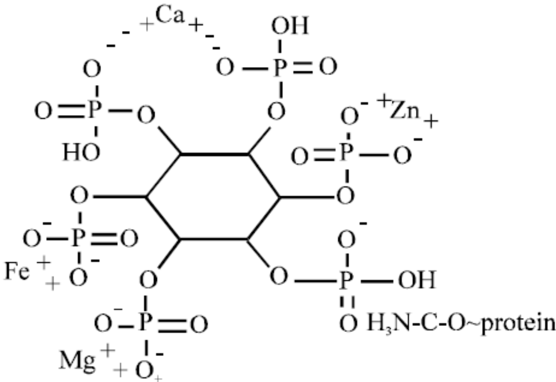
Its principal functions in seeds are storage of phosphates as source of energy and antioxidant for the germinating seed (Raboy, 2003; Bohn *et al*., 2008). PA biosynthesis starts after flowering and it accumulates during maturation until desiccation of plant seed and grains. Phytate is stored in protein storage vacuoles as globoids mainly located in the aleurone cell layer (in sorghum, wheat and barley) or in the embryo (in maize) of the seed. The amount of PA in plants is very variable and, presumably, it depends on growing conditions and harvesting techniques (Okazaki *et al*., 2005, Coulibaly *et al*., 2011).

Nevertheless, phosphorus in PA is mostly not biologically available to monogastric animals, due to insufficient degradation capabilities in their gastrointestinal tract under the pH conditions of the small intestine (Minihane and Rimbach, 2002; Bohn *et al*., 2008; Coulibaly *et al*., 2011).

Biological activities of phytates

Like tannins, also PA has the ability to chelate metal cations, primarily iron, zinc, calcium, as well as proteins and digestive enzyme, such as pepsin, amylase and trypsin. The creation of insoluble complexes with metals and proteins determines their unavailability as nutritional factors and can lead to deficiencies in population where staple food like sorghum, wheat and maize represent the principal source of nutrition. At a low pH below the isoelectic point of proteins, positively charged terminal amino groups like lysyl, histidyl and arginyl can form complexes with negatively charged phytate anion. In normal steric condition, each phytate anion can interact with two charged groups of protein (Figure 3) (Coulibaly *et al*., 2011).

Figure 3. Formation of complexes of phytic acid with metal cations and protein residues



Source: Coulibaly *et al*., 2011

As aforementioned, phytates form strong complexes also with metals. The strong chelating effect of the phosphate groups of PA with divalent mineral cations is probably due to the high affinity of them for inositol phosphates of PA and the strength of binding becomes progressively lower and, conversely, the solubility higher, for the lower inositol phosphates (Harland and Morris, 1995; Persson *et al*., 1998). PA molecule contains 12 dissociable hydrogens; in conditions of excess of phytic acid, formation of soluble complexes between PA and metal cations of ratio 1:1 predominates; vice versa, when metal cations are in excess, insoluble phytates with stoichiometry between 1:2 and 1:5 are formed (Torres *et al*., 2005 Bohn *et al*., 2008).

In cereals mostly used form preparing weaning food for children, like sorghum, phytates were revealed to have a direct correlation with poor iron and zinc status, commonly seen after 6 months of age in low-income countries (Gibson 1994, Abdel-Rahim and El-Beltagi, 2010). Another study carried out in Malawi reported a correlation between a high intake of phytates and the poor iron and zinc status in preschool children (Lind *et al*., 2003).

The reduction of iron absorption by phytates in human subjects has been demonstrated to be dose-dependent and even a small amount of phytates in food can markedly reduce it (Hallberg *et al*., 1989). This dose dependency has been confirmed by Siegenberg *et al*. (1991), who also found that the inhibition of PA on iron absorption can be reduced by ascorbic acid.

In order to reduce the binding property of phytates, and thus increase the bioaccessibility and bioavailability of minerals and proteins, phytates should be in the lowest amount as possible, ideally 25 mg or less per 100 g or about 0.035% of ingested food (Onomi *et al*., 2004). Alternatively, according to Hurrell (2004), in order to improve iron absorption, the phytates:iron molar ratio (Phy/Fe) should be below 1:1 and preferably 0.4:1. However, in composite

meals containing ingredients enhancing iron absorption such as ascorbic acid or animal protein, a phytates:iron molar ratio < 6:1 is proposed (Hurrell and Egli, 2010).

Additional studies have shown the ability of PA to inhibit also other minerals, such as zinc (Navert *et al,* 1985; Gibson, 2006) calcium (Weaver *et al,* 1991) as well as magnesium (Bohn *et al*, 2004) absorption. Concerning phytates:zinc molar ratio (Phy/Zn), Gibson (2006) proposed different ranges, based on absorption studies in humans: a ratio <5 has been associated with high bioavailability, 5 to 15 with moderate bioavailability and >15 with low zinc bioavailability corresponding to around 50%, 30% or 15% of total zinc, respectively. Thus, according to these authors, Phy/Fe and Phy/Zn molar ratios can be used as indices to estimate bioavailability of these minerals.

In literature, two main processes able to reduce the inhibitory effect of PA on mineral absorption are suggested: the mechanical removal of PA by processes such as extraction or milling and the enzymatic degradation of PA, by activation of endogenous or addition of exogenous phytases (Hurrell, 2004).

Beneficial effects of phytates

Although the numerous adverse effects of its inadequate intake, studies have been reported that PA can also have several beneficial properties. Numerous recent studies have demonstrated the ability of phytates to act as an antioxidant, by binding iron ions in solution, and thereby prevent ferric irons from participating to the generation of the hydroxyl radical ·OH via the Fenton reaction (Graf *et al.,* 1987; Midorikawa *et al*., 2001; Bohn *et al.*, 2008). PA is also implicated in the delay of glucose absorption (Lee *et al*., 2006; Yoon *et al*., 1983) decrease of cholesterol and triglycerides in plasma (Katayama, 1995 Onomi *et al*., 2004), reduction of kidney stone formation (Grases *et al.*, 1998; Selvam, 2002), as well as reduction of the bioavailability and, therefore, toxicity of heavy metals

such as lead (Rimbach *et al.*, 1996) and cadmium (Rimbach and Pallauf, 1997). Moreover, tests *in vitro* have also revealed positive effects of PA against carcinogenesis, by reducing cell proliferation in different cell lines, such as human mammary cancer cells (Shamsuddin, 1995), and enhancing the immune system (Bohn *et al*., 2008).

Due to its potential favourable effects, the setting of Recommended Daily Intakes (RDI) of PA has been suggested: nonetheless, these values could be quite variable among countries as well as among different population groups (Coulibaly *et al*., 2011), depending on the age, physiological and nutritional status. These suggested RDI should not be exceeded: however, vulnerable groups of population, including children under six, pregnant women and people suffering from macro- and/or micronutrient deficiencies, are recommended to ingest lower amount of PA (Coulibaly *et al*., 2011). For such groups, a varied diet and/or adequate food processing could be exploited for reducing PA's activity.

Influence of landrace on nutritional characteristics

A landrace, o traditional variety, is a plant population with a limited range of genetic variation, which is adapted to local agro-climatic conditions and which has been generated, selected, named and maintained by traditional farmers (Teshome *et al*., 1999).

As many nutritional characteristics of plants are strictly dependent by local agro-climatic conditions, it is worthwhile to hypothesize that a large difference in the amount of nutrients as well as AN profile among landraces exist.

The chemical composition of cereal grains has been reported to vary widely, depending on environment, soil, and variety (FAO, 1966; Bryden *et al*., 2009; Mutayoba *et al*., 2011). Kayodé *et al*., (2007) pointed out that, with regard to tra-

ditional sorghum varieties, iron and zinc content are more influenced by the environmental component (e.g. field location) than by genetic variation.

Furthermore, there is a clear difference among traditional varieties also with regard to AN content. A marked influence of the soil, pesticides and other chemicals and environmental conditions on the amount of both phenolic coumpounds and phytates has been shown (Parr and Bolwell, 2000; Tomas-Barberan and Espín, 2001; Dykes *et al.*, 2005; Godoy *et al.*, 2005; Soetan and Oyewole, 2009).

Impact of sorghum processing on nutritional characteristics

The nutritional value of sorghum can be improved through appropriate processing methods. Processing can ameliorate the food, by either inactivating, destroying or removing toxins or AN without any change in the nutritive value and acceptability of the food product (Waliyar *et al.* 2007). Several processing methods have been reported to significantly reduce AN concentrations prior to consumption (Ikemefuna *et al.*, 1991; FAO, 1995; Mahgoub and Elhag, 1998; Abdelhaleem *et al.*, 2008; Neelam, 2012). Nevertheless, together with reducing AN, food processing can determine a reduction of nutrients as well. Milling of cereals, for instance, removes large amount of PA, but this treatment removes at the same time the majority of the minerals and dietary fibres of the food (Bohn *et al.*, 2008). Similarly, soaking or extracting in aqueous solutions can remove up to 75% of the PA, but loss of minerals, vitamins and water-extractable proteins also occurs (Hurrell, 2004; Claver *et al.*, 2010). Afify *et al.* (2011) reported that soaking sorghum for 20 hours in distilled water reduces phytate content by 32.4%, but also zinc by about 30% and iron reduced by between 28.16 and 40.06%. Similar results have been stated also by Lestienne *et al.* (2005), who reported a loss of about 40% of iron and 30% of zinc during soaking.

A number of studies reported that the content of specific AN is significantly modulated by fermentation and cooking, such as the reduction of phytate and tannins content during fermentation and protein digestibility during cooking (Marfo *et al*., 1990; Abdelhaleem *et al.,* 2008; Mohammed *et al*., 2010). Moreover, since in Africa sorghum porridges are usually prepared by cooking slurry of fermented or unfermented flour in boiling water (Rooney *et al*., 1986; Asante, 1995), the role of fermentation and cooking in reducing the content and/or the biological activity of AN are therefore considered.

Cooking

Cooking, especially in boiling water, is one of the most common household practices of sorghum processing. In addition to make food appetizing and safe, cooking has been demonstrated to reduce almost all heat-labile antinutritional as well as nutritional factors.

Reduction in AN content during various cooking times has been reported in many studies (Ikemefuna *et al*., 1991; Alonso *et al.,* 1998; Alonso *et al.,* 2000; Abdelhaleem *et al*., 2008). Cooking has been shown in reducing phenolic content in sorghum by 28-31% (Abdelhaleem *et al*., 2008) and 38-70% when in combination with other processes, like grinding and sieving (Kayodé *et al*., 2007). It has been suggested that the decrease in phenolic compound content by cooking may be caused by the leaching out of this compound into the water (Barroga *et al.,* 1985; Osman, 2007), or by reduced extractability as a result of a change in chemical reactivity (Reddy *et al.,* 1985; Kataria *et al*., 1989).

Incongruous data exists on the impact of cooking on phytate content of sorghum. Kayodé *et al*. (2007) reported that cooking did not affect phytate content. On the other hand, other authors observed a decrease in phytate content when sorghum was cooked (Clydesdale and Camire, 1983; Marfo *et al*., 1990; Mahgoub and Elhag, 1998; Abdelhaleem *et al*., 2008). Generally, cooking has

found to have modest reducing effect on phytate levels, which commonly ranged from 8.6-10% (Abdelhaleem *et al*., 2008) to 16% (Marfo *et al*., 1990) and Clydesdale and Camire (1983) reported that cooking reduced the mineral-binding potential of the naturally present phytate. However, most investigators reported that cooking had a lesser reductive effect on phytate levels compared with fermentation.

As previously stated, cooking determines also the reduction of important nutrients. Some B-group vitamins, like riboflavin and vitamin B-6, are lost and the concentrations of lysine, tryptophan, and total aromatic and sulfur-containing amino acids decreased in the cooking process (Soetan and Oyewole, 2009). Moreover, a fall in *in vitro* protein digestibility during cooking has been copiously documented (Hamaker *et al*., 1986; Klopfesntein and Hoseney, 1995; Duodu *et al*., 2002; Taylor and Taylor, 2002; Abdelhaleem, *et al*, 2008).

Condensed tannins have been implicated in the reduction of protein digestibility in sorghum during cooking (Chibber *et al.,* 1980; Elkhalifa and El Tinay, 1994). Alternatively, a number of authors, including Hamaker *et al.,* 1987; Duodu *et al*., 2002, 2003; Oria *et al.,* 1995, suggested that cooking process reduces digestibility of proteins by determining a conformational change in proteins that could facilitate formation of disulfide-linked polymers, which are less susceptible to the attack of enzymes.

The positive and negative effects of cooking are summarised in the Table 4

Table 4. Synoptic table of positive and negative effects of cooking

Positive Effects	Negative Effects
Make food appetizing and safe	Reduction of some B-group vitamins
Reduction of phenolic content	Decrease of certain amino acids
Decrease in phytate content*	Fall in *in vitro* protein digestibility

*observed only by some authors

Fermentation

Fermentation is one ancient technology used for the improvement and preservation of food. A large variety of fermented foods is still used today in developed as well as developing countries: in households, small-scale food industries and big enterprises (Motarjemi and Nout, 1996). Fermented foods form a major part of the diets of many populations all over the world and are widely used as weaning foods. In addition to nutritional benefits, fermentation has a big safety and economic importance as a method of food preservation, especially in many low-income countries where preservation techniques are improper due to lack of adequate facilities and resources (Motarjemi and Nout, 1996; FAO, 1998). Traditionally, fermentation is widely used for processing sorghum (Boling & Eisener, 1982; Novellie, 1982; Sooliman, 1993). Fermented sorghum is indeed the basic ingredient of many recipes in less developed and emerging countries, such as couscous, porridge and beer, and is particularly important also as weaning food for infants (FAO, 1999a, Faber *et al.,* 2001; Osungbaro, 2009). The microorganisms involved in spontaneous fermentation of sorghum include lactic acid bacteria and yeasts, such as *Lactobacillus spp, Acetobacter spp.* and *Saccharomyces cervisiae* (Vieira-Dalodé *et al.,* 2007; Tsaousi et al., 2008; Nout, 2009; Afolayan *et al*., 2010; Nwachukwu *et al.,* 2010).

There is considerable evidence showing that fermentation brings biological, chemical as well as nutritional benefits (Table 5).

Table 5. Synoptic table of positive and negative effects of fermentation

Positive Effects	**Negative Effects**
Improvement and preservation of food	Reduction of some hydrolyzed elements for metabolic activities
Reduction of phenolic content	
Decrease in phytate content	
Inhibition of growth and toxin production of numerous pathogenic bacteria	
Improvement of the availability of certain B-group vitamins and trace minerals	
Improvement of the *in vitro* protein and starch digestibility	

Lactic acid fermentation inhibits growth and toxin production of numerous pathogenic bacteria, by competition, reduction of pH and production of antimicrobial factors, including lactic acid. Moreover, fermentation contributes to confer quality and desirable sensorial properties as well as reduce some naturally-occurring toxic compounds, such as cyanide levels in cassava (Motarjemi and Nout, 1996). Another important function of fermentation is the change in nutrient composition; for example by decreasing the level of carbohydrates, as the principal energy source of fermenting microorganisms, and improving the availability of certain B-group vitamins and *in vitro* protein and starch digestibility (Hassan and El Tinay, 1995). Fermentation also influences the levels of reducing sugars in sprouted seeds and causes increases in lipids, maybe due to increased activity of lipolytic enzymes which produced more free fatty acids

(Soetan and Oyewole, 2009). Finally, one of the most important properties of fermentation is the ability to reduce the activity of AN, mainly tannins and phytates.

Reduced tannin content upon fermentation was observed by many investigators (Ikemefuna *et al.* 1991; Antony and Chandra, 1998; Osman, 2004; Towo *et al.*, 2006; Abdelhaleem *et al.,* 2008; Svensson *et al.*, 2010; Schons *et al*., 2011): the decrease has been associated to microbial activity during fermentation (Dhankher and Chauhan, 1987; Ikemefuna *et al.* 1991; El Hag *et al.*, 2002) or to abstraction of hydride ions and rearrangement of the phenolic structures due to the acidic environment (Towo *et al.*, 2006). The reduction of tannin content due to fermentation is variable and in several cases notable: Osman (2007) and Abdel-Rahman (2000) reported a reduction in tannin in three different sorghum varieties ranged from 15 to 35% and 52.7 - 56.9%, respectively. A higher tannin reduction during natural fermentation has been found by Abdelhaleem *et al.* (2008) and Hassan and El Tinay (1995) who reported a reduction in tannin content of 60-70%, respectively, and Elkhalifa and El Tinay (1994) reached an even decrease of 92% in tannin content through fermentation.

The important effect of fermentation in the reduction of phytates in sorghum is thoroughly documented by many authors, including Ikemefuna *et al*. (1991), Idris *et al.* (2005), Towo *et al*. (2006); Osman (2007), Abdelhaleem *et al*. (2008), and Abdelseed *et al. (*2011). The rate of decrease is different among varieties and it strongly depends on time of fermentation: as the period of fermentation increases, a decrease in phytic acid content occurs. Data reported by different studies indicate that the decrease of PA during fermentation ranges from about 68% in Mohammed *et al*. (2010) 16h fermentation (Abdelhaleem *et al.*, 2008), to 72% in 36h fermentation (Kruger *et al*., 2012). Even though 16h fermentation is a little time span, the difference in terms of results with a prolonged

fermentation is negligible. The degradation of phytate acquires nutritional importance because as the mineral binding strength of phytate decreases, the bioavailability of proteins and minerals could increase. A number of recent studies have reported that the reduction of phytate content of foods may have beneficial impact, in particular, on iron availability (Minihane and Rimbach, 2002; Sandberg and Andlid, 2002; Osman, 2004; Lestienne *et al*., 2005).

It has been suggested that the decrease of phytate during fermentation might be due to the action of fermenting microorganisms able to hydrolyze phytate into lower inositol phosphates: inositol mono-, bi-, tri-, tetra- and pentaphosphates (IP1, IP2, IP3, IP4 and IP5, respectively). The reduction in pH of fermented foods, caused by the production of various organic acids (e.g. lactic acid, citric acid and acetic acid), favours the activity of the enzyme phytase (endogenous or from lactic acid bacteria) able to dephosphorylate phytate effectively (Marfo *et al*., 1990; Sandberg and Andlid, 2002; Reale *et al*., 2007; Abdelseed *et al*., 2011).

Different cereals have different phytase activity: wheat, barley and rye all have high phytase activity, whereas sorghum, maize and millet have low basal phytase activity, but it increases rapidly after adequate processing (Egli *et al*., 2002). When yeasts are present, they may also contribute to phytase activity (Lambrechts *et al*., 1992). Certain yeasts, such as *Saccharomyces cerevisiae*, *Saccharomyces pastorianus* and *Candida krusei,* were found to have an important phytase activity in grain-based food and beer (Nuobariene *et al*., 2011).

The combination of coking and fermentation has been demonstrated to improve the nutrient profile of food and reduce AN to higher levels than any other processing methods analyzed (Ikemefuna *et al*., 1991; Chavan *et al*., 2006). Abdelhaleem *et al*., (2008) found that cooking of slurry after short-term (16 h)

fermentation further reduced tannin content by 63-76% and phytate content by 66-68%, to a higher extent than fermentation and cooking processes alone. Other authors stated that the combination of fermentation and cooking (either fermentation before and after cooking) improves also *in vitro* protein digestibility when compared with cooking alone (Taylor and Taylor, 2002; Fadlallah *et al*., 2010). According to these authors, fermentation seems to partially reduce the adverse effect of cooking on sorghum protein digestibility.

Project design

The available literature reports that sorghum nutritional characteristics, including the content of some AN, may be related to the variety (Awadelkareem *et al.*, 2009) and/or be modulated by processing methods (Marfo *et al.*, 1990; Abdelhaleem *et al.*, 2008; Mohammed *et al.*, 2010). Nevertheless, these findings result from the dosage of the investigated analytes; the net effect of whole sorghum upon fermentation and/or cooking has never been investigated in biological systems. In fact, the study of the effects of a food in its entirety is a topic still poorly developed. Moreover, only limited data exist on the integrated and comparative assessment of the role of variety and main traditional processing methods on the sorghum nutritional characteristics.

The aim of this study was therefore to examine the impact of both food processing and variety on tannins, phytic acid and AN-related activities in sorghum porridge, trough *in vitro* and analytical approaches.

The *in vitro* analysis allowed assessing the biological effects of whole sorghum, resulting from the combination of AN and nutrient content; the *in vitro* model used has been selected as representative of the human intestine epithelial cell, namely the cell line DLD-1. These results have been integrated with the determination of the AN content in order to determine whether biological activities are related to quantitative variations of specific AN and to support understanding of any combined (additive, antagonistic, etc.) effects. Moreover, since AN dosage has been performed in sorghum porridge before and after fermentation and cooking, the effect of the two food processes on the content and activity of specific AN has been evaluated. In addition, the modulating effects of the variety on both the aforementioned effects have also been evaluat-

ed. Finally, the estimation of iron and zinc bioaccessibility and bioavailability has been performed through two mathematical models.

The work started with a pilot study in Ouagadougou (Burkina Faso) and continued with the development of specific *in vitro* and analytical methods.

Pilot study (Manuscript 1)

At the beginning of the research project, a pilot study was conducted in Ouagadougou, capital of Burkina Faso, in collaboration with the University of Ouagadougou. This study has allowed the collection of samples as well as to gain direct knowledge about technologies, processes and traditional consumption of sorghum porridge through an investigation to local markets and benches of street food vendors. The findings of the pilot study have been used for the preparation and development of the entire project as well as in the drafting of the Manuscript 1.

In vitro analysis (Manuscript 2)

The research activity involving the *in vitro* methods has been carried out at the Food and Veterinary Toxicology Unit within the Dept. of Veterinary Public Health and Food Safety of the Italian National Institute of Health (ISS).

In order to analyse the biological effects of the matrices on the cell line, the assessment of the antioxidant capacity and the quantization of total protein content have been performed.

Prior the evaluation of AN biological activities of porridges in an *in vitro* model, an *in vitro* gastrointestinal digestion on each sample was conducted, in order to simulate the digestion processes in the human gastrointestinal tract.

The DLD-1cells have been then exposed to soluble extracts. The biological activities are assessed by:

1) preliminary determination of exposure conditions devoid of cytotoxicity, through the analysis of cell respiration and proliferation by colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS Assay);
2) the study of functional markers able to detect biological activities of AN in the lysed cells, namely:

   - antioxidant capacity through the assessment of the Glutathione Peroxidase modulation by ELISA assay;

   - quantization of total protein content by Bicinchoninic Acid method (BCA method).

Content of AN and Trace Elements (Manuscript 3)

The dosage of AN and trace elements has been performed at the Dept. of Prevention of Malnutrition and Related Pathologies ("Prévention des Malnutritions et des Pathologies Associées"- Nutripass) within the Institut de Recherche pour le Développement (IRD) at Montpellier, France.

AN and trace minerals were measured in sorghum porridges upon different preparation processes, in order to determine modification in their content and a potential relationship between quantitative variations and AN biological activities

The content of AN has been evaluated by:

1) The determination of iron binding phenolic groups in tannins, namely catechol and galloyl groups, in all samples by the modified ferric ammonium sulfate (FAS) method by Brune *et al*. (1991)

2) The determination of phytate content according to the method of Talamond *et al.* (1998) and Lestienne *et al.* (2005)

The total amount of calcium, iron and zinc in the samples has been determined by the method described in Hama *et al.* (2011) followed by analysis with Atomic Absorption Spectrophotometer.

Bioaccessibility and Bioavailability of trace elements (Manuscripts 2-3)

Over the years, the term bioavailability has known many interpretations as consensus on its definition has been lacking among researchers (Hurrell, 2002; Versantvoort *et al.,* 2004). The term bioavailability, indeed, has been often confused with bioaccessibility.

The bioavailability of a compound has been suggested to result from the release of the compound from the matrix into digestive juices in the gastrointestinal tract (bioaccessibility), the transport across the intestinal epithelium into the portal vena (absorption) and its metabolism (Versantvoort *et al.,* 2004). In the present thesis, the term bioavailability of trace elements is used to describe the amount of trace elements available for metabolic use, after a process that simulates the human digestion system.

The bioavailability of trace elements has been assessed through the molar ratios of phytate to iron and zinc and the iron bioaccessibility through the study of algorithms (limited to iron).

Molar ratios of phytate to iron and zinc have been suggested in order to estimate the bioavailability of these two trace elements by Davies and Olpin (1979), Saha *et al*. (1994) and Hurrell and Egli (2010). The study of algorithms in order to predict iron absorption has been suggested by Hallberg and Hulthén (2000). The proposed algorithm would predict the effect of factors able to influ-

ence (both positively and negatively) heme- and non heme- iron absorption from meals and diets. For this thesis, the algorithms considered the main factors relevant to sorghum, namely phytates, tannins and calcium.

Materials and Methods

Materials

Sorghum samples

Five different sorghum varieties were investigated: three varieties from Africa and two from Italy. The grains from Africa were three traditional varieties (landraces) purchased from local markets in Lagos (Nigeria - N), Dakar (Senegal - S) and Ouagadougou (Burkina Faso - B) in February-April 2011. The two Italian grains varieties were provided by the Institute of Agronomy, Genetics and Field Crops of the Università Cattolica del Sacro Cuore, Piacenza (Italy). One variety, namely Aralba (Ar), is a sorghum genotype for grain yield, while the other variety, ABF 306 (ABF), is a biomass sorghum hybrid selected by the Institute of Genetics of the Università Cattolica del Sacro Cuore.

The grains of each variety were cleaned from damaged seeds and extraneous materials and then milled with an automatic pestle mill with internal parts made in agate (model RM100 by Retsch GmbH & Co. Haan, Germany), to obtain a fine flour.

Treatments

For each variety, slurry was obtained by mixing 100 g of the flour with 200 mL of water.

Fermentation and cooking of the porridges in this study were based on traditional practices.

- Fermentation

The slurry was covered and kept for 72 hours at room temperature and away from light for spontaneous fermentation to occur. The pH reduction to an average value of 4.2 was a sign of occurred fermentation.

- Cooking

The slurry was further added with 100 mL water and cooked with constant stirring for 10 min at approximately 95 °C to obtain porridges.

Subdivision of samples according to treatment

For each variety of sorghum, four samples were so obtained, namely:

fc (fermented and cooked porridge),

c (unfermented cooked porridge),

f (fermented slurry), and

s (slurry).

for a total of 20 samples. Samples were stored at - 80 °C prior analysis.

In vitro methods

Standard substances

Porcine enzymes: pepsin (EC 3.4.23.1) and pancreatin (EC 3.1.1.3), bile salts and alpha-amylase (EC 3.2.1.1) from *Bacillus subtilis*, as well as phytic acid and catechin were supplied from Sigma-Aldrich (Steinheim, Germany). The Glutathione Peroxidase Cellular Activity Assay Kit was purchased from Biovision (Mountain View, CA, US) and the Pierce BCA Protein Assay from Thermo Scientific (Rockford, IL).

In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was performed according to the method of Crews *et al*. (1996) with small modifications. Briefly, 5mL of gastric juice (1% w/v pepsin in 0.15 M NaCl, pH=2) were added to 1g sample and then incubated in a shaking water bath at 37°C for 4 h to allow pepsin digestion. Procedural blanks were run in parallel. Prior the intestinal digestion, the pH of the gastric digest was adjusted to 6.8 by addition of $NaHCO_3$ 2 M. Then, 5 mL of intestinal juice (3% w/v pancreatin, 1.5% w/v amylase, 1% w/v bile salts in 0.15M NaCl) were added to each sample and incubation was continued for further 4 h. Samples were then centrifuged at 8000 × g for 30 min at 4°C and the supernatant was filtered through a 0.45 μm-pore-diam. membrane filters. The supernatants (soluble fraction) were stored at -80 °C until use in cell assays.

Cell line and culture conditions

The cell line DLD-1, derived from a human colorectal adenocarcinoma, was chosen as *in vitro* model for the human gut epithelium.

DLD-1 was kindly obtained from Prof. Maria Marino (Università Roma Tre, Roma), through the collaboration established with the Food and Veterinary Toxicology Unit of the ISS.

Cells were cultured in RPMI 1640 medium without phenol red (Gibco, LifeTechnologies, Paisley, UK), supplemented with 10% Fetal Bovine Serum (Gibco), 2mM-glutamine (Gibco), 100 U/ml penicillin and 100g/ml streptomycin (Gibco). Cells were maintained in a humidified Steri-Cult 200 Incubator (Forma Scientific, Marietta, OH, USA) at 37°C and 5% CO_2.

Growth inhibition assay and working concentration determination

As a preliminary step, all samples (*fc, c, f, s*) were tested at different concentrations on cells, in order to determine the most suitable concentration to be used on the selected cell line, allowing to study AN-relevant activities in the absence of effects on cell viability.

Test concentrations of samples were chosen by referring to the mean concentration of sorghum used for cell treatment, reported in a previous study by Rafi *et al.* (2008), as 0.5 mg/ml, utilized as a standard solution. The maximum concentration to be used in cell cultures and obtainable by filtering with 0.22 μm filters without losing material onto the filter, was equal to 1/25 of standard solution. Such filtered solutions were utilized to prepare other sequential two-fold diluted solutions ranging from 1/25 (20 μl/ml) to 1/200 (2.5 μl/ml) of standard solution.

Cytotoxicity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) inhibition assay (MTS-based CellTiter 96® AQueous Assay, Promega, Madison, WI, USA).

Such assay is a colorimetric method for determining the number of viable cells in proliferation stage and it is based on the ability of mitochondrial dehydrogenases in viable cells to convert the MTS tetrazolium compound into formazan; conversion into formazan is directly proportional to the number of living cells in culture and, being colored, it can be detected by spectrophotometric reading.

DLD-1 were plated at 5000 cells per well in RPMI 1640 medium in a 96 well flat bottomed multiwells and incubated overnight in a humidified incubator at 37 °C to permit cell adhesion. All treatments were performed in triplicate, incubating the cells for 72h at 37 °C with the different diluted solutions of each

standard and sample. The 72h treatment allowed to assess effects upon a chronic *in vitro* exposure.

At the end, 20µl of the CellTiter 96® AQueous reagent were added to culture wells incubating for 2h at 37 °C. Cell viability was determined by reading absorbance at 490 nm by a Victor 3 Multilabel Reader (PerkinElmer, Waltham, MA, USA).

The cytotoxicity dose-response curve showed that all dilutions, except for the 1/200 one, affected cell proliferation. Accordingly, 1/200 dilution was selected as working dilution, thus adopted as suitable test concentration for all samples.

Cell line treatments

DLD-1 cells were treated in a culture flask with 175 µL soluble fractions of samples, after *in vitro* gastrointestinal digestion, in 35 mL medium (1/200 ratio), or with the medium alone as control, and incubated for 72h at 37 °C.

Treatments were performed in triplicate in order to obtain three biological replicas. After 72h, DLD-1 medium supernatants were collected and stored at -80 °C, whereas DLD-1 monolayers were trypsinized, counted and stored at -80 °C until use.

Study of functional markers

- Antioxidant capacity through the assessment of the Glutathione Peroxidase

Glutathione Peroxidase (GPx) family of enzymes (EC 1.11.1.9) plays important roles in the protection of organisms from oxidative damage. To assess any modulation in the activity of GPx exerted by each sample, we used the Glutathione Peroxidase Activity Assay (Biovision, Mountain View, CA, US), a colorimetric assay based on the ability of GPx to convert reduced glutathione (GSH) to oxidized glutathione (GSSG), while reducing Cumene Hydroperoxide to its corresponding alcohol. The generated GSSG is reduced to GSH by Glutathione Reductase (GR), with consumption of NADPH. The decrease of NADPH is proportional to GPx activity and it is easily measured at 340 nm.

DLD-1 cell monolayers (about 10^6 cells) were homogenized in 0.2 ml cold Assay Buffer, centrifuged at 10,000 x g for 15 min at 4 °C, collecting the supernatant. 2µl of each sample was dispensed in duplicate in a 96 well flat bottomed multiwell, diluting to 100µl with the assay buffer. For each well, 40 µL of Reaction Mix, constituted by Assay Buffer, NADPH solution, GR solution and GSH solution, was added. After incubating the cells for 15 minutes, in order to deplete all GSSG, 10 µL of Cumene Hydroperoxide was added to start GPx reaction. Absorbance was read at 340 nm (T1) and again after incubating the reaction at 25 °C for 5 min (T2) or longer when GPx activity was low.

GPx activity was obtained by the equation:

$$\text{GPx Activity} = \frac{(B - B^0)}{(T2 - T1) \times V} \times \text{Sample dilution (nmol/min/ml = mU/ml)}$$

Where:

B is the NADPH amount that was decreased between T1 and T2 (in nmol)

B^0 is the background change (without Cumene Hydroperoxide) between T1 and T2

T1 is the time of first reading (A1) (in min)

T2 is the time of second reading (A2) (in min)

V is the pretreated sample volume added into the reaction well (in ml)

One unit of GPx activity is defined as the amount of enzyme that causes the oxidation of 1.0 μmol of NADPH to $NADP^+$ per minute at 25°C.

- Quantization of total protein content by BCA method

The Pierce BCA Protein Assay (Thermo, Rockford, IL) is a colorimetric assay for the detection and quantisation of total proteins. This method is based on the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing BCA. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. The absorbance, nearly linear with increasing protein concentrations, was read at 570 nm.

DLD-1 cell monolayers were exposed to a solution containing ice cold RIPA Buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate) and the PMSF (phenylmethylsulfonyl fluoride, an inhibitor of protease) in a 100:1 ratio.

Into a 96 well flat bottomed multiwell, 25 μl of each standard and sample biological replica was added to 200 μl of the Working Reagent to each well. The

Working Reagent was constituted by 50 parts of BCA Reagent A (Sodium Carbonate, Sodium Bicarbonate, Bicinchoninic Acid and Sodium Tartrate in 0.1 M NaOH) with 1 part of BCA Reagent B (containing 4% Copper Sulfate).

After incubation at 37°C for 30 minutes and cooling to room temperature, the absorbance was read at 570 nm.

Data analysis and statistics

For all analyses, the results are reported as the mean of triplicate determinations.

Since the small sample size cannot guarantee that results reflect those of the whole population, a distribution-independent non-parametric Wilcoxon Rank Sum test was applied to compare statistical differences among samples. A p-value < 0.05 was set as significance level. Statistical analysis was performed by using the JMP 9.0 statistical software (SAS Institute Inc.).

Analytical methods

Prior analysis, frozen samples were lyophilized for 48 hours at –40 °C in a freeze dryer Model Alpha 1-2 LD plus (Christ, Germany) and the resulted samples used for subsequent analysis.

Dry matter (DM) contents were determined by oven drying at 105 °C to constant weight.

Standard substances

Phytic acid and catechin were purchased from Sigma-Aldrich, (Stockholm, Sweden) and tannic acid was obtained from Merck.

Determination of iron binding phenolic groups

The content of iron binding phenolic groups, i.e. catechol (ortho-dihydroxyl) and galloyl (trihydroxyl) groups was determined with the modified ferric ammonium sulfate (FAS) method according to Brune *et al*. (1991) using a solution of 50% dimethylformamide (pH 4.4) as extraction solvent. The method was developed in order to determine iron binding phenolic groups of tannins in foods, which was of specific interest in this study.

Catechol and galloyl groups form iron complexes with FAS which can be read spectrophotometrically at two wavelengths: 578 nm and 680 nm, corresponding to the absorbance maxima of iron-galloyl and iron-catechol, respectively. The amount of the catechol and galloyl groups was calculated against standard curves of catechin and tannic acid, respectively, at the two wavelengths. Catechin and tannic acid were used as standards of catechol and galloyl groups, respectively. Catechols are expressed as mg of catechin equivalents (CE)/100g DM and galloyls as mg of tannic acid equivalents (TAE)/100g DM.

Determination of phytate content

The determination of phytate content was performed according to the method of Talamond *et al*. (1998) with modifications: 0.2 g sample was extracted with 10 ml 0.5M HCl during stirring for 6 minutes in boiling water, followed by centrifugation at 4500 *g* for 20 min at 4°C. The supernatant was dried in a Speed-Vac (JOUAN RC 10-10, Saint Herblain, France) vacuum centrifuge at 45°C and the residue was reconstituted in 200 μL of Milli-Q water. After filtration through 0.2 μm pore-diameter filters, the filtrate was diluted 25x and phytate content was estimated by determination of myo-inositol-hexaphosphate (IP6) by high performance anion-exchange chromatography (Dionex AS50, using an AS-11 pre-column and column kit (Dionex, Sunnyvale, USA) according to the method detailed in Lestienne *et al*. (2005).

Determination of phytase activity

Phytase activity was determined only in the flour and fermented slurries, as endogenous phytases are deactivated by heat (Ryden and Selvendran, 1993) and activated when flour was suspended in water (Frias *et al.,* 2003). The activity was evaluated through spectrophotometry, by determining phosphorous release during incubation of phytase extract in a sodium phytate solution (pH 5.6) at a wavelength of 405 nm. Phytase extraction was performed from 2g of samples according to the method of Konietzny *et al*. (1995) with buffer modification (0.1 M acetate buffer, pH 5.6). Samples were stirred for 2 h at 4°C and centrifuged at 10,000 *g* for 30 min at the same temperature. After eliminating phytates and free phosphorus from the supernatants by utilization of an AG 1-X8 anion resin (Bio-Rad Laboratories, Richmond, CA), 3 ml of resulted enzyme extracts were incubated for 1h at 50°C with 2 ml of 2.5 mM sodium phytate so-

lution and 9 ml of 0.1 M acetate buffer at pH 5.6. Phosphorous released by phytase activity was measured according to the method of Heinonen and Lahti, (1981), where 1 phytase unit (PU) is equivalent to the enzymatic activity that liberate 1μM of phosphorus from phytic acid per minute, according to a standard range prepared with 5 mM KH_2PO_4 solution.

Determination of total iron and zinc content

Total iron and zinc contents were determined according to the method described in Hama *et al*, 2011. The two trace minerals were extracted with a closed-vessel microwave digestion system (ETHOS-1, Milestone, Italy), from about 0.4 g of flour in a 7:1 nitric acid/hydrogen peroxide mixture, and digested in a microwave oven at 1200 W power for 30 min.

Iron and zinc content were analysed with an Atomic Absorption Spectrophotometer (Perkin-Elmer Analyst 800) with a deuterium background corrector and identified by air–acetylene flame.

The iron:zinc molar ratio (Fe/Zn) was calculated as relevant indicator of zinc bioavailability (Solomons and Jacob, 1981).

Data analysis and statistics

For all analyses, the results are reported as the mean of triplicate determinations. All values were calculated per 100 g DM. The approximate DM content in the different treatment samples were as follows: *fc* and *c* = 16%, *f* = 19 % and *r* = 30%.

Data were subjected to analysis of variance (ANOVA) and Tukey's HSD multiple range test was used to compare means at the 5% significance level (Wilkinson, 1990), using the JMP 9.0 statistical software (SAS Institute Inc.).

Bioavailability and bioaccessibility of trace elements

Molar ratios of phytate to iron and zinc

The levels of phytic acid, iron and zinc have been converted in mole by using a molecular mass unit of 660 for phytic acid and an atomic mass unit of 56 and 65 for iron and zinc, respectively. Molar ratios of phytate to iron (Phy/Fe) and phytate to zinc (Phy/Zn) were then calculated.

Study of algorithms

Among the parameters considered in the algorithm proposed by Hallberg and Hulthén (2000) to predict dietary iron absorption, only the parameters contained in sorghum are taken into account in this thesis, namely phytates, tannins and calcium.

The algorithm contains the value for iron absorption (relative to about 40% of the absorption of the reference dose of iron) from a single basal meal (low-extraction (40%) flour) without taking into account components known to inhibit or enhance iron absorption. The mean absorption of iron, adjusted to 40% reference dose absorption, was 22.1. This basal value was then multiplied by factors present in the meal able to influence iron absorption, such as phytate, tannins and calcium, according to the following formula:

Abs Fe (%) = 22.1 * f IP6 * f tannin * f Ca

where:

f IP6 = 10^[-0.30*log(1+phytate-P)]

f tannin = 10^[0.4515-0.715*log tannic acid(mg)

$$f\ Ca = 0.4081+[0.5919/(1+10^{-(2.022-\log(Ca+1))*2.919})]$$

In the computation of f IP6, 1mg of phytate-P= 3.53mg phytic acid

The study of the algorithm has been reported to 50g of DM as it corresponds to about 250g of porridge, assumed as the mean quantity likely to be ingested by an adult in a meal.

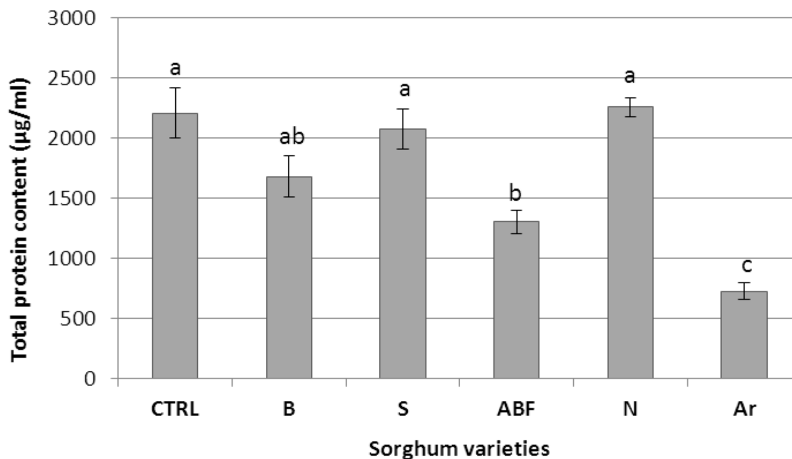
Results

In vitro analysis

Quantization of total protein content

Results showed a significant difference among varieties. Overall, the cells exposed to samples from the two Italian cultivar (Ar and ABF) showed a significantly lower total protein content than those exposed to African samples (N, S and B) and the control (CTRL) cells (Figure 4). In particular, cells exposed to Ar showed a lowest total protein content, significant different to cells exposed to all other varieties; cells exposed to ABF showed a reduced total protein content with respect to the CTRL cells and those exposed to N, S and also to Ar. No significant difference has been shown between cells exposed to ABF and B and among cells exposed to the African varieties (Figure 4).

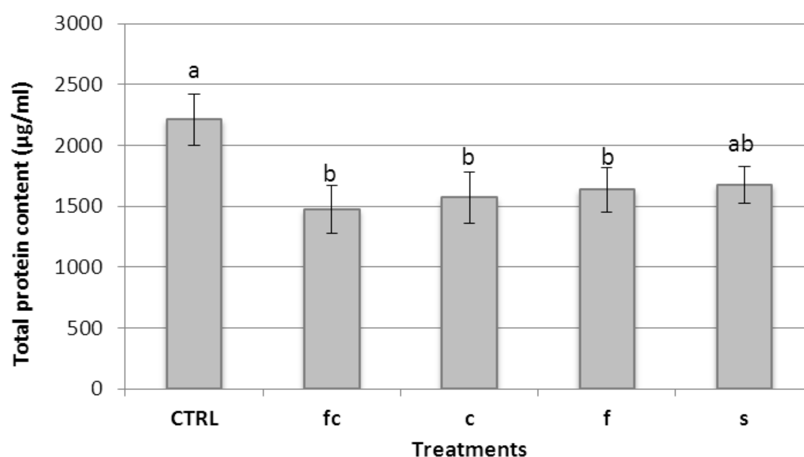
Figure 4. Total protein content of cells exposed to different sorghum varieties



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Wilkoxon test (n = 20); bars indicate SD.

Fermentation and/or cooking both reduced the protein content as compared to the CTRL cells; the slurry (*s*)-exposed cells were not significantly different from either CTRL cells or cells exposed to processed sorghum (Figure 5).

Figure 5. Total protein content of cells exposed to samples undergoing different treatments



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Wilkoxon test (n = 20); bars indicate SD.

Across varieties, either fermentation and/or cooking of sorghum samples significantly reduced the protein content of exposed cells as compared to control cells (Table 6).

Analysis of processing effects within the same sorghum variety showed that only DLD-1 exposed to B samples were significantly different in total protein content among treatments. In particular, cells exposed to cooked porridge, with (*fc*) and without fermentation (*c*), had total protein content significantly lower than slurry (*s*) and fermented slurry (*f*). On the other hand, comparisons among different varieties across the same treatment did not reveal any statistical difference (Table 6).

Table 6 . Effect of variety and food process on total protein content of DLD-1 cells exposed to sorghum

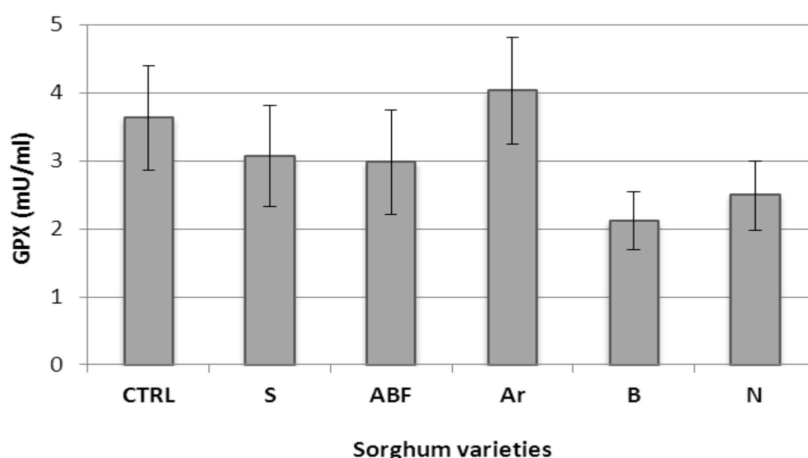
Treatment		Variety (μg/ml)					
	Across varieties	CTRL	N	S	B	Ar	ABF
Across processes		2212±211^{A}	2260±78.9^{A}	2074±167^{A}	1683±170^{AB}	729±71.6^{C}	1307±98.4^{B}
CTRL	2212±211^{a}						
s	1674±153^{ab}		2300±182	1469±113	2170±116^{a}	952±272	1221±477
c	1569±210^{b}		2079±331	2489±70	1137±22^{b}	548±267	1393±436
f	1634±179^{b}		2429±102	1685±382	2238±242^{a}	707±148	1317±342
fc	1470±194^{b}		2304±316	1804±599	1189±453^{b}	709±215	1296±287

Mean values ± SEM of three replicates (n = 20). Values followed by different small letters in the same column, and capital letter in the same row, are significantly different, as assessed by Wilkoxon test. Comparisons were made among treatments within the same variety.

Antioxidant capacity

Varieties did not significantly influence the GPx activity of cells. There was no significant difference in the GPx activity neither among cells exposed to different sorghum varieties nor with respect to the CTRL cells (Figure 6).

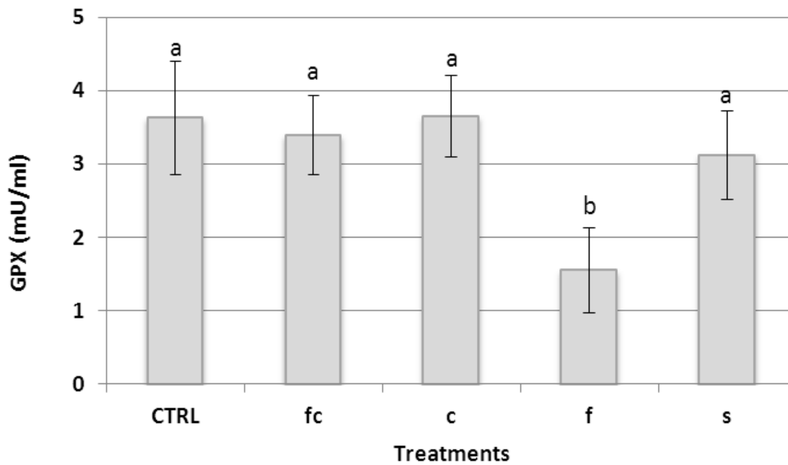
Figure 6. GPx activity of cells exposed to different sorghum varieties



Mean values ± SD of three replicates (n = 20). Bars indicate SD.

There was no significant difference in the GPx activity also between cells exposed to cooking, both alone (*c*) and with fermentation (*fc*), and CTRL cells. On the contrary, fermentation process significantly influenced the activity of GPx in cells. Cells exposed to fermented samples (*f*) showed, indeed, a reduction in the GPx activity with respect to cooked samples (*c, fc*), slurries (*s*) and the CTRL cells (Figure 7).

Figure 7. GPx activity of cells exposed to sorghum samples undergoing different treatments



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Wilkoxon test (n = 20); bars indicate SD.

When considering the effect of processes on GPx activity in DLD-1 cells within the same sorghum variety, only ABF fermented slurry (*f*) induced a significant reduction in GPx activity, being almost null, with respect to cooked porridges (both *c* and *fc*). On the other hand, no statistical difference was found among DLD-1 cells exposed to different varieties across the same treatment (Table 7).

Table 7. Effect of variety and food process on GPx activity of DLD-1 cells exposed to sorghum

Treatment		Varieties (mU/ml)					
	Across varieties	CTRL	N	S	B	Ar	ABF
Across processes		3.6 ± 0.8	2.5 ± 0.5	3.1 ± 0.7	2.1 ± 0.4	4.0 ± 0.8	3.0 ± 0.8
CTRL	3.6 ± 0.8[a]						
s	3.1 ± 0.6[a]		3.6 ± 2.3	1.2 ± 1.1	2.9 ± 1.5	4.8 ± 3.8	2.5 ± 0.6[ab]
c	3.6 ± 0.5[a]		3.8 ± 0.9	4.3 ± 1.1	3.3 ± 1.5	2.0 ± 3.1	5.0 ± 2.5[a]
f	1.5 ± 0.6[b]		0.9 ± 1.0	2.5 ± 3.1	0.7 ± 0.3	4.2 ± 1.7	0.00 ± 1.9[b]
fc	3.4 ± 0.5[a]		1.6 ± 0.6	4.3 ± 2.1	1.6 ± 0.9	5.1 ± 1.9	4.6 ± 1.6[a]

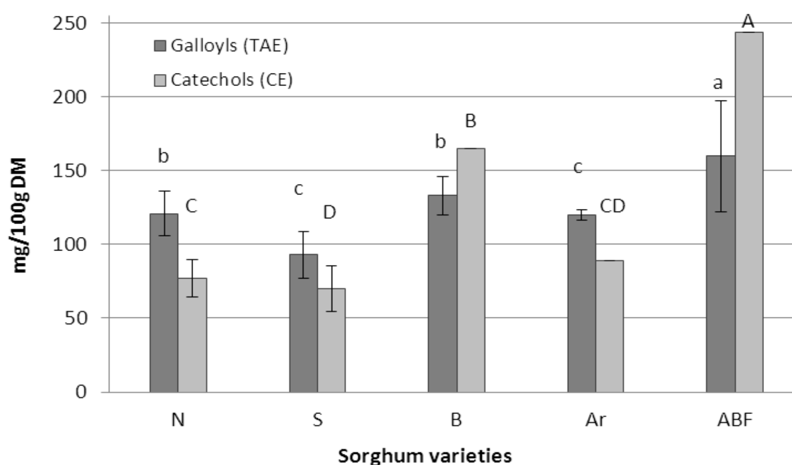
Mean values ± SEM of three replicates (n = 20). Values followed by different letters in the same column significantly differ ($p \leq 0.05$), as assessed by Wilkoxon test.

Content of AN and Trace Elements

Content of iron binding phenolic groups

Varieties significantly influenced the amount of both catechol and galloyl groups. S showed the lowest content of both iron binding phenolic groups, significantly ($p \leq 0.05$) different from ABF, B and N. Similarly, Ar showed both catechol and galloyl groups significantly lower than ABF and B (Figure 8).

Figure 8. Content of iron binding groups in porridges of different sorghum varieties



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD. Comparisons were made among varieties within each phenolic group (galloyls and catechols).

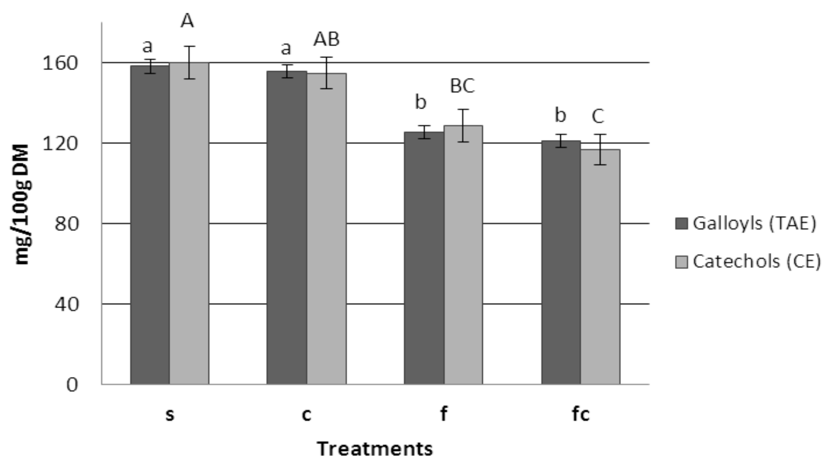
On the contrary, ABF showed the highest content of both catechol and galloyl groups, significantly different from all other groups. The content of catechols in N and Ar was significantly lower than in ABF and B; these two varieties showed also a content of galloyls significantly lower than ABF.

Also process significantly influenced the content of both catechol and galloyl groups in sorghum samples.

Fermentation, alone (*f*) and with cooking (*fc*), significantly reduced the amount of galloyl groups with respect to slurries (*s*) and cooking alone (*c*).

Regarding catechol groups, fermented slurries (*f*) and fermented and cooked porridges (*fc*) showed a significantly lower content than slurries (*s*); (*fc*) chatechol content was also significantly lower than in (*c*), whereas no significant difference was observed between (*f*) and (*c*) (Figure 9).

Figure 9. Content of iron binding groups in sorghum varieties undergoing different treatments



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD. Comparisons were made among treatments within each phenolic group (galloyls and catechols)

The effects of treatment on catechol and galloyl groups within the same sorghum variety are presented in Table 8.

Table 8. Effect of treatments on iron binding phenolic groups in samples of sorghum varieties

	Galloyls (mg TAE/100g DM)				
Treatments	Varieties				
	N	S	B	Ar	ABF
s	159 ± 13[a,b]	129 ± 10[a]	172 ± 6[a]	137 ± 3[a]	194 ± 5[a]
c	186 ± 14[a]	116 ± 0.3[a]	168 ± 3[a]	127 ± 5[a,b]	182 ± 14[a,b]
f	121 ± 6[c]	93 ± 5.0[a]	133 ± 14[b]	120 ± 6[b,c]	160 ± 15[b,c]
fc	126 ± 14[b,c]	106 ± 26[a]	133 ± 0.3[b]	111 ± 8[c]	131 ± 14[c]
	Catechols (mg CE/100g DM)				
Treatments	Varieties				
	N	S	B	Ar	ABF
s	151 ± 60[a]	81 ± 11[a,b]	221 ± 24[a]	94 ± 9[a,b]	254 ± 7[a]
c	179 ± 41[a]	56 ± 13[c]	209 ± 11[a,b]	104 ± 19[a]	227 ± 16[a,b]
f	77 ± 7.0[a]	70 ± 8 [a,b,c]	165 ± 17[b,c]	89 ± 11[a,b]	244 ± 8[a,b]
fc	95 ± 23[a]	62 ± 12[b,c]	149 ± 23[c]	67 ± 11[b]	212 ± 23[b]

Mean values ± SD of three replicates (n = 20). Values followed by different letters in the same column significantly differ ($p \leq 0.05$), as assessed by Tukey's test. Comparisons were made among treatments within the same variety.

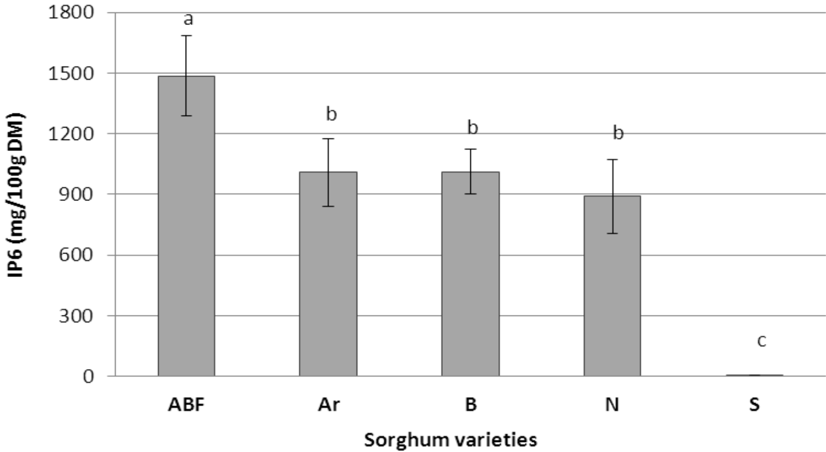
Compared to slurries (*s*), the fermented slurries (*f*) showed a significant reduction of galloyl groups in all varieties except for S, whereas after cooking, the reduction remained significant in B, Ar and ABF. Catechol groups appeared somewhat less affected by treatment: fermented slurries *(f)* significantly reduced the content in B only, and fermented and cooked porridges (*fc)* in B and ABF.

Phytate content and phytase activity

Variety significantly ($p \leq 0.05$) influenced also the content of phytates. ABF and S showed the highest and lowest phytate content, respectively, both significantly different from all other varieties; indeed, the phytate content in S was 50

to 100-fold lower than the other varieties. No other significant differences were observed (Figure 10).

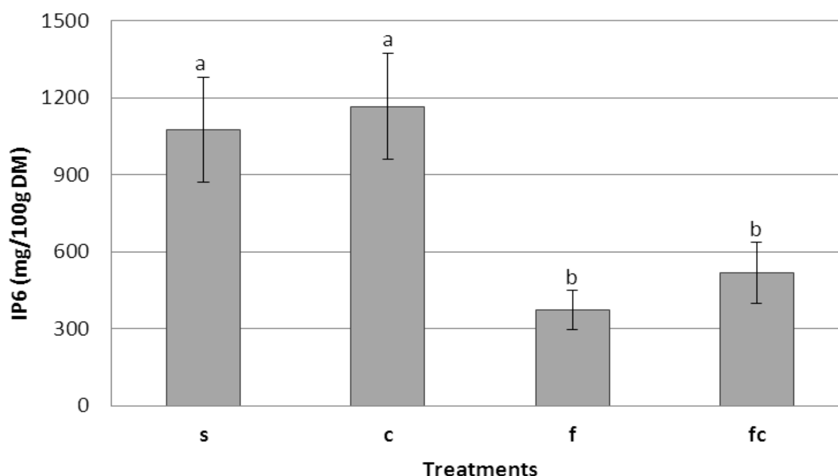
Figure 10. Phytate content in porridges of sorghum varieties



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD.

Fermentation significantly influenced the content of phytates in samples. Fermentation, alone and associated to cooking (*f* and *fc*), significantly ($p \leq 0.05$) reduced the phytate content with respect to cooking alone (*c*) and slurries (*s*) (Figure 11).

Figure 11. Phytates content in sorghum varieties undergoing different treatments



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD. Comparisons were made among treatments in all varieties.

Fermented slurries (*f*) and fermented and cooked porridges (*fc*) of all varieties, with the exception of S, showed a significantly reduction of phytate content. However, the phytate content in S was already very low in slurry. In cooked porridges (*c*) there was no significant difference with respect to slurries (*s*), with exception of N which showed a significant increase in phytate content. A slight but not significant increase was also observed for B and Ar (Table 9).

Table 9. Effect of treatment on phytate content in samples of sorghum varieties

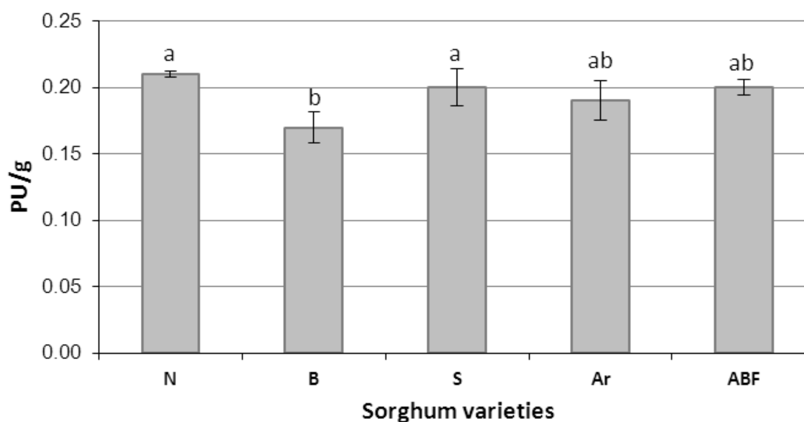
Samples	IP6 (mg/100g DM)
N	
s	1071 ± 0.1[b]
c	1369 ± 0.1[a]
f	302 ± 0.0[c]
fc	252 ± 0.0[c]
S	
s	5.2 ± 0.0[a]
c	4.3 ± 0.0[a]
f	2.5 ±0.0[a]
fc	3.7 ± 0.0[a]
B	
s	1176 ± 0.1[a]
c	1211 ± 0.0[a]
f	490 ± 0.0[c]
fc	871 ± 0.0[b]
Ar	
s	1224 ± 0.0[a]
c	1396 ± 0.1[a]
f	361 ± 0.0[b]
fc	501 ± 0.0[b]
ABF	
s	1900 ± 0.0[a]
c	1848 ± 0.0[a]
f	710 ± 0.0[c]
fc	962 ± 0.0[b]

Mean values ± SD of three replicates (n = 20). Values followed by different letters are significant different ($p \leq 0.05$), as assessed by Tukey's test. Comparisons were made among treatments within the same variety

Flour phytase activity ranged from 0.17 to 0.21 PU/g in the different varieties; N and S showed the highest values, significantly ($p \leq 0.05$) different from

the lowest value found in B. No significant differences have been found among Ar and ABF and the other varieties (Figure 12).

Figure 12. Phytase activity in the flour of sorghum varieties

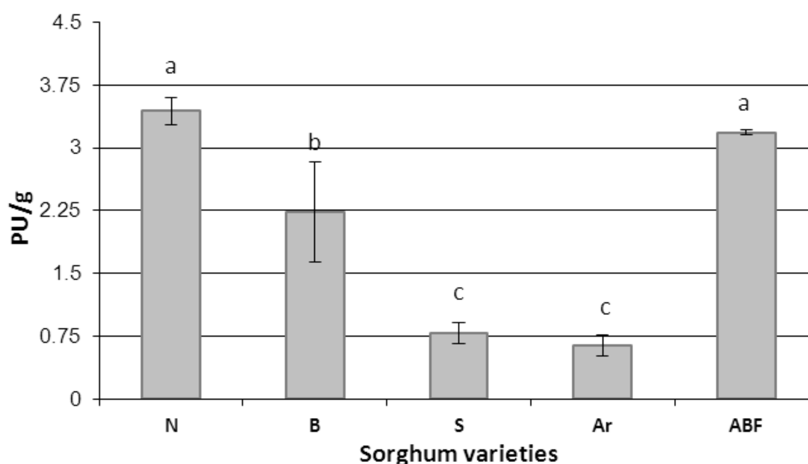


Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD.

In fermented slurries (*f*), phytase activity ranged from 0.64 to 3.44 PU/g DM: compared to the flour, a significant increase ($p \leq 0.01$) was found in all varieties (Table 10).

Fermented samples of N and ABF varieties showed the highest values, significantly different ($p \leq 0.05$) from all other varieties, while in S and Ar the phytase activity was significantly lower than in the other varieties (Figure 13).

Figure 13. Phytase activity in fermented slurry (*f*) of sorghum varieties



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD.

Phytase activity in the flour and in fermented porridge of each tested sorghum variety is reported in Table 10. Data showed that fermentation significantly increased the enzymatic activity of phytase with respect of the flours in all varieties.

Table 10. Effect of fermentation on phytase activity within each tested sorghum variety

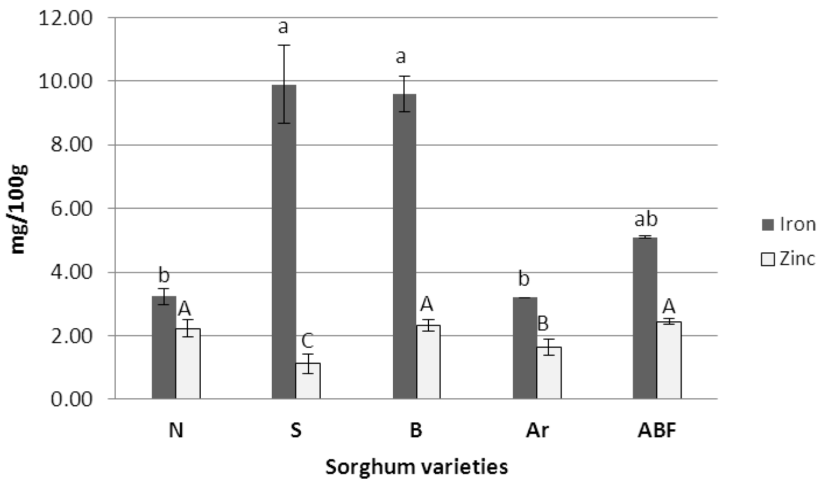
	Sorghum varieties (PU/g DM)				
	N	S	B	Ar	ABF
flour	0.21 ± 0.0^{a}	0.21 ± 0.0^{a}	0.17 ± 0.0^{a}	0.19 ± 0.0^{a}	0.20 ± 0.0^{a}
f	3.44 ± 0.2^{b}	0.79 ± 0.1^{b}	2.23 ± 0.6^{b}	0.64 ± 0.1^{b}	3.18 ± 0.0^{b}

Mean values ± SD of three replicates (n = 20). Values followed by different letters are significant different ($p \leq 0.05$), as assessed by Tukey's test. Comparisons were made among treatments within the same variety

Iron and zinc content

The iron content in slurries (*s*) ranged from 3.2 to 9.9 mg/100g DM. S showed the highest content, significantly ($p \leq 0.05$) different compared to all other varieties, and Ar the lowest, three times less than S and significantly ($p \leq 0.05$) different from ABF, Ar and S (Figure 14).

Figure 14. Iron and zinc content in slurries (*s*) of different sorghum varieties



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20); bars indicate SD. Comparisons were made among varieties for the same mineral (iron and zinc)

Zinc content in slurries (*s*) ranged from 1.1 to 2.5 mg/100g DM, with a mean value of 1.87 mg/100g DM. With regard to zinc, S showed the lowest content, significantly different ($p \leq 0.05$) compared to ABF, N and B. ABF showed the highest content, more than two times higher than S and significantly ($p \leq 0.05$) different from B, Ar and S (Figure 14).

Processing of the samples had no significant effect ($p \leq 0.05$) neither on the iron nor on the zinc content (data not shown).

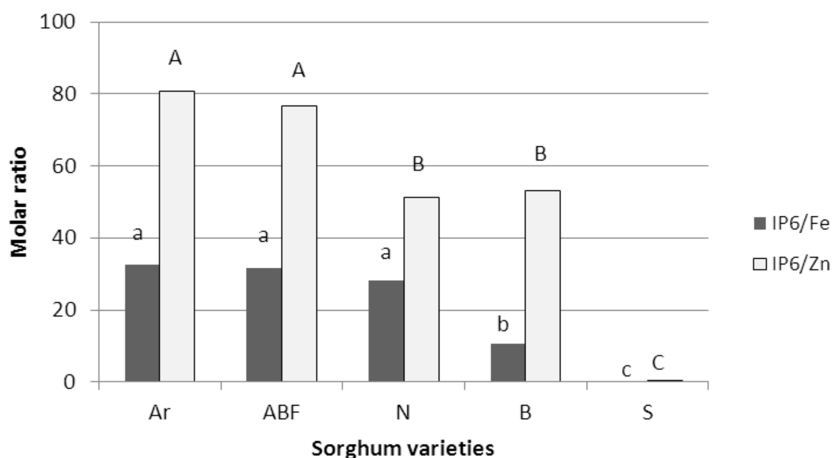
Among varieties, the Fe/Zn ratio ranged from 1.4 (N) to 8.8 (S); Ar and ABF showed a 2 ratio and B a 4.1 ratio.

Bioavailability and bioaccessibility of trace elements

Molar ratios of phytate to iron and zinc

Variety significantly ($p \leq 0.05$) influenced both IP6/Fe and IP6/Zn molar ratios. In the different varieties, IP6/Fe molar ratio ranged from 32.5 to 10.4 while IP6/Zn molar ratio ranged from 80.8 to 51.3; S was an exception, with very low molar ratios related to the low phytate content, (0.045 and 0.47 for IP6/Fe and IP6/Zn, respectively), significantly different from all other varieties. Ar and ABF showed the highest ratios, both significantly different from B and S as regards IP6/Fe, and from N, B and S as regards IP6/Zn (Figure 15).

Figure 15. IP6/Fe and IP6/Zn molar ratios in samples of sorghum varieties

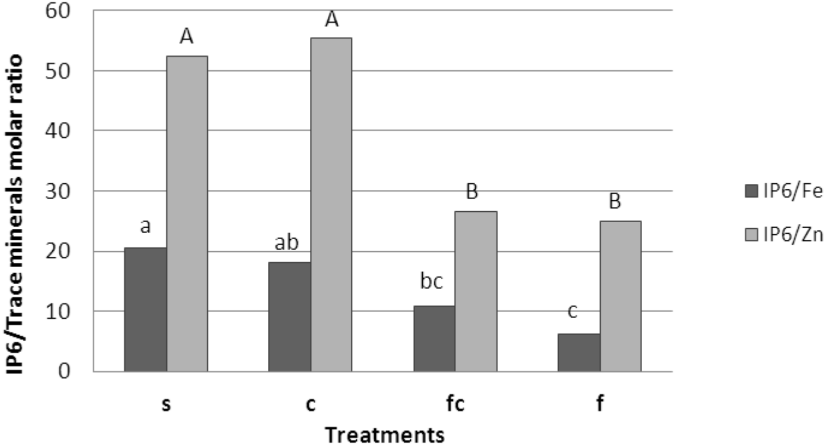


Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20). Comparisons were made among varieties for the same ratio (IP6/Fe and IP6/Zn)

Cooking did not significantly influence molar ratios of phytates and trace elements, whereas fermentation, alone (*f*) and with cooking (*fc*), significantly

reduced IP6/Zn molar ratio with respect to slurries (*s*) and cooking (*c*) and IP6/Fe molar ratio with respect to slurries (*s*) (Figure16).

Figure 16. IP6/Fe and IP6/Zn molar ratios in sorghum varieties undergoing different treatments



Columns with different letters are significant different ($p \leq 0.05$), as assessed by Tukey's HSD multiple range test (n = 20). Comparisons were made among varieties for the same molar ratio (IP6/Fe and IP6/Zn)

In all varieties, fermented slurries (*f*) showed the lowest molar ratios. With the exception of S, IP6/Fe molar ratio in the slurries (*s*) of different varieties ranged from 10.4 to 32.5 and in fermented slurries (*f*) ranged from 4.5 to 12.6. As for IP6/Zn molar ratio, the slurries (*s*) of different varieties ranged from 51.3 to 80.8, while in fermented slurries (*f*) N showed a lower value (8.5) whereas the other three varieties ranged 21.2 to 61.3 (Table 11).

Table 11. IP6/Fe and IP6/Zn molar ratios in samples of sorghum varieties

Variety	**IP6/Fe (mmol)**				**IP6/Zn (mmol)**			
	s	c	*f*	*fc*	*s*	c	*f*	*fc*
N	28.2	7.8	4.7	7.9	51.3	64.0	8.5	16.8
S	0.05	0.02	0.02	0.03	0.47	0.36	0.47	0.27
B	10.4	10.5	4.5	22.5	53.0	52.9	33.4	55.9
Ar	32.5	40.5	12.6	11.7	80.8	88.9	61.3	34.9
ABF	31,7	31.6	9.0	11.9	76.6	70.9	21.2	24.5

Values expressed in mole by using a molecular mass unit of 660 for phytic acid and an atomic mass unit of 56 and 65 for iron and zinc, respectively (n = 20).

Both IP6/Fe and IP6/Zn molar ratios were affected by fermentation in all varieties, and the percentage of reduction of fermented slurries (*f*) with respect to slurries (*s*) ranged from 58 and 84% and from 24 to 83%, respectively.

The IP6/Fe molar ratio reached a value < 6 only in fermented slurries (*f*) of S, B and N varieties (0.02, 4.48 and 4.71 respectively) and only fermented slurries (*f*) from S and N showed IP6/Zn molar ratio < 15.

Study of algorithms

Results from the algorithm showed little difference in iron bioaccessibility, expressed as percentage, among varieties except for S. The variety from Senegal showed, indeed, a an approximate 3-fold increase of iron bioaccessibility in all samples, with respect to other varieties.

The findings also showed that fermentation, alone (*f*) and with cooking (*fc*), was able to improve the bioaccessibility of iron with respect to slurries, whereas cooking had no susbstantial impact (Table 12). The increase of bioaccessibility in fermented slurries (*f*), with respect to the respective slurries (*s*), differed among varieties and ranged from 25% to 45%, where S showed the lowest increase and N the highest. With respect to slurries (*s*), also fermented and cooked porridges (*fc*) showed an increase of the iron bioaccessibility, even though lower than fermented slurries (*s*); this ranged from 14% (S) to 45% (N).

Table 12. Percentage (%) of bioavailable iron in 50g of DM

Variety	***fc***	***c***	***F***	***s***
N	1,1	0,5	1,1	0,6
B	0,7	0,6	0,9	0,6
S	3,2	3,0	3,6	2,7
Ar	1,0	0,7	1,0	0,6
ABF	0,7	0,5	0,7	0,4

Discussion & future perspectives

The present study was undertaken to evaluate the impact of variety and the two principal traditional processes (cooking and fermentation) on tannins and phytic acid activities in sorghum and the related modulation of effects in a biological system.

In order to achieve this purpose and perform a comprehensive analysis of sorghum as "whole food", an *ad-hoc* model based on a combination of established and novel approaches has been set. This model includes the use of three different investigation approaches: i) an *in vitro* assay, ii) quantitative determinations by established chemical analytical methods and iii) mathematical models, by using both established and emerging approaches.

The *in vitro* method represents an innovative approach as it implies the analysis in a biological system (model for the human gut epithelium) of effects of sorghum in its entirety after *in vitro* gastrointestinal digestion. Indeed, *in vitro* investigation of a food commodity *in toto* is still receiving limited attention.

Another innovative aspect lies in the effort to integrate and compare data obtained from the three different investigation approaches in the analysis of the role of variety and main traditional processing methods on the sorghum nutritional characteristics.

In the following section results are discussed first by taking into account each parameter and then analysing the comprehensive effects of variety and food process.

In vitro analysis

Quantization of total protein content

Results show that total protein content of cells exposed to different varieties differs. In particular, cells exposed to the two Italian varieties, namely ABF and Ar, show a significant reduction in the total protein content with respect to the CTRL cells and Ar also to the cells exposed to all the African varieties (B, S and N). These results outline the influence of variety, including the importance of pedoclimatic parameters, in the modulation of biological activity of sorghum *in vitro*.

Concerning the different treatments, both fermentation and cooking determine the reduction of the cell protein content as compared to the CTRL cells. Overall, no differences were observed among fermented samples and cooked samples (*f*, *c*, *fc*) has been observed. When analysing the effect of treatment within each variety, a significant difference is observed only in the sorghum variety from Burkina Faso (B), where cells exposed to cooked porridge, with (*fc*) and without fermentation (*c*) show total protein content significantly lower than slurry (*s*) and fermented slurry (*f*) exposed cells.

The decrease of total protein content in cells exposed to sorghum samples can be linked to a reduced bioaccessibility of proteins from the culture medium, due to the antinutrient effect of tannins (Butler, 1990) as well as of phytates (Morales *et al.*, 2011). On the other hand, it cannot be excluded a direct effect of specific sorghum components on the synthesis and metabolism of cell cycle proteins, which might result in a modulation of cellular protein levels (see, e.g. Vucenik *et al.*, 2005). In order to explain food processing effects *in vitro*, it might be hypothesized that fermentation and/or cooking induce the formation of tannin and/or phytate degradation products, and/or other process by-products, which may inhibit protein synthesis. Finally, since varieties of sorghum differ

in amino acid composition and fermentation determines the change in sorghum protein solubility (Afify *et al.*, 2012), it might not be ruled out altogether that a different composition of essential aminoacids would affect total protein content of cells exposed to sorghum samples. Nevertheless, this appears rather unlikely due to the high dilution of the soluble fractions of samples in the culture medium (1/200 ratio); thus, sorghum proteins would represent only a minor fraction of total medium proteins.

Net effects of processed whole foods are an up-to-date issue, in particular for foods of vegetable origin that are rich of bioactive substances: one recent example is the antioxidant/anti-inflammatory action elicited by a polyphenol-rich beverage (Nemzer *et al.*, 2011). Apparently, only a limited correlation exists between the modulation by variety and by food process on the total protein content *in vitro*.

Antioxidant capacity

In contrast to the results observed for total protein content, the influence of variety on the GPx activity of cells exposed to sorghum samples is negligible. In fact, there is not a significant difference between GPx activity of CTRL cells and those exposed to sorghum varieties.

No difference in the GPx activity has been also shown between cells exposed to cooked samples (*c, fc*) and CTRL cells. On the other hand, fermentation process significantly influences the activity of GPx in cells. Cells exposed to fermented samples (*f*) show, indeed, a reduction in the GPx activity with respect to cells exposed to all the other samples and the CTRL cells.

These results suggest that cooking does not influence the GPx activity of cells whereas fermentation greatly affects it. Nevertheless, when analyzing the effect of treatment within each variety, only ABF shows a difference among the treatments. In particular, only cells exposed to ABF fermented slurry (*f*) exhibit a marked reduction, indeed to almost null, of the GPx activity. Thus, the general reducing effect of fermentation on GPx activity of cells can be affected by the ABF results.

In general, reduced GPx activity in cells might be attributed to the diminished oxidative stress in cells or to the reduced GPx enzymatic functions. In this case the interpretation of results is not straightforward. Fermentation, indeed, was found to reduce both the tested AN and to increase the bioaccessibility and bioavailability of iron and zinc (see below for detailed discussion); nevertheless, fermented samples also showed a significant reduction of cells total protein content. Therefore, considering the overall poor nutritional profile showed by ABF variety, it cannot be excluded that the inhibition of GPx activity of cells is due to the development of compounds during fermentation acting as GPX inhibitors.

Content of AN and Trace Elements

Content of iron binding phenolic groups

The phenolic compounds in samples of different sorghum varieties have been assayed as the total amount of galloyl and catechol groups, because of their strong capacity to bind iron (Brune *et al.*, 1989; Hallberg and Hulthén, 2000; Santos-Buelga and Scalbert, 2000).

The analysis of iron-binding phenolic groups shows a marked influence of the variety in the selected sorghum varieties of this study, that results to be consistent with previous findings in other varieties (Parr and Bolwell, 2000; Tomas-Barberan and Espín, 2001; Dykes *et al.*, 2005).

Like variety, also fermentation is able to significantly influence the amount of both galloyl and catechol groups. Indeed, associated or not to cooking, fermentation results in a remarkable reduction of the amount of both iron binding phenolic groups in all varieties. Reduced phenolic content upon fermentation was also observed in previous papers (Antony and Chandra, 1998; Towo *et al.*, 2006; Svensson *et al.*, 2010; Schons *et al.*, 2011) and different explanations have been proposed. The decrease may be related to microbial activity during fermentation (Dhankher and Chauhan, 1987; El Hag *et al.,* 2002) or to abstraction of hydride ions and rearrangement of the phenolic structures due to the acidic environment (Towo *et al.*, 2006). It is furthermore suggested that fermentation process results in a reduced extractability of phenolic compounds due to self-polymerization and/or interactons with macromolecules (Beta *et al.*, 2000).

On the other hand, no significant decrease could be seen when porridges were only cooked. The lack of effect of cooking in phenolic compounds is in contrast with previous findings, which report that also cooking contribute to lower amounts of phenolic compounds. Our results may suggest that, in order to reduce AN, a longer cooking time might be needed than that used in the tra-

ditional recipes we followed in this study, as suggested by Omeje (1999) and Kaankuka *et al*. (2000). The reduction in phenolic content upon longer cooking time can be caused by thermal degradation, condensation reactions, changes in chemical reactivity or formation of complexes with other food components, such as proteins (Barroga *et al*., 1985; Ekpenyong, 1985; Matuschek, 2005).

Data here reported also demonstrate that different variety determines different reduction rates of iron binding phenolic groups during fermentation, with and without cooking. Varieties with the highest content of phenolic groups (B and ABF), indeed, show also the highest reduction rates.

The two phenolic groups have been reported to have different iron binding capacities *in vitro* (Jovanovic *et al*., 1998). Brune *et al.* (1989) suggested, for instance, that galloyl groups have a greater inhibitory effect on iron absorption, whereas Samman *et al*. (2001) reported no difference in the effect of foods containing galloyl or catechol groups. Nevertheless, the results of the present study show that fermentation, alone or followed by cooking, significantly reduces both galloyl and catechol groups to an overall comparable extent.

In conclusion, the content of both galloyl and catechol groups significantly differs among varieties and it is further modulated by fermentation, especially if associated to cooking.

Phytate content and phytase activity

Results demonstrate that variety significantly affects also the amount of phytates in sorghum. An interesting result has been observed for the variety from Senegal (S), showing a very low content of phytates, greatly different from all other varieties. The extremely low amount of phytates showed by S is preferable in case of monotonous diets or for vulnerable groups of population (p.27).

Moreover, also process can influence the amount of phytates. Reduction of the phytate content in fermented samples was consistent with previous reports on other sorghum varieties (Marfo *et al.,* 1990; Mahgoub and Elhag, 1998; Egli *et al.*, 2003; Idris *et al.,* 2005; Kayodé *et al.,* 2007; Towo *et al.,* 2006; Proulx and Reddy, 2007). The decrease of phytate content by fermentation can be explained by the enzymatic activity of both cereal and microbial phytases (Sandberg and Svanberg, 1991; Abdelhaleem *et al.,* 2008), which hydrolyse phytate (IP6) into inorganic phosphorus (Pi), inositol or *myo*-inositol phosphates (IP5 to IP1) (Lestienne *et al.,* 2005).

On the contrary, the results show that cooking has no significant effect also on phytate content. The present findings are in accordance with previous studies on sorghum by Kayodé *et al*. (2007) as well as with previous studies on other food crops, e.g., yam and rye flour (Fretzdroff and Weiper, 1986; Wanasundera and Ravindran, 1992). On the other hand, other authors reported that cooking of sorghum may induce a degradation of phytates (Marfo *et al.,* 1990; Mahgoub and Elhag, 1998; Idris *et al,* 2005; Towo 2006). However, in these studies the processing methods involved a markedly longer cooking time than the present study; for instance, Marfo *et al*.,(1990). Omeje (1999) and Kaankuka *et al.* (2000), reported that a cooking time of about 30 min resulted in a higher reduction of different AN, including phytates, with respect to shorter cooking times. Accordingly, the reason why cooking has not affected phytate content in this study could be attributed to the relatively short (10 min.) cooking time, as in the traditional recipes we referred to.

It might be worth noting that in a single African variety, N, cooking determines an increase of the phytate content with respect to the slurry. In this regard, it might be worth noting that Lestienne *et al.* (2005) also found an increase

of IP6 in cooked soybean flour. Thus, the occurrence of similar events in N variety might not be ruled out altogether.

As a whole, variety as well as fermentation significantly influences phytate content in sorghum.

Compared to other cereals, sorghum has low phytase activities (Egli *et al.*, 2003). Data here reported show that in all sorghum flours phytase activity is, indeed, low (0.17-0.21 PU/g DM) and comparable to values reported in the literature (Egli *et al.*, 2003). The findings show that also the amount of phytase activity was significantly influenced by both the variety and fermentation. Fermentation determines a significant effect of the enzymatic activity. Fermented slurries show a significant increase of phytase activity with respect to flours, in all sorghum varieties. The generally increased activity of phytase upon fermentation may be related to an augmentation of enzyme production due to microbial growth (yeasts and lactic acid bacteria): indeed, several yeasts and *Lactobacillus* species are recognized as phytase producers in fermented grain-based products (De Angelis *et al*., 2003). Indeed, cereal phytases have a pH optimum at around 5.0 (Sandberg and Svanberg, 1991; Greiner *et al.*, 2000), thus, during fermentation phytase may increase its enzymatic activity.

Interestingly, the results show a lack of linearity among phytase activity in flour and in fermented slurry within the same variety. For instance, S stands out as a variety with low amount of phytates and high phytase activity in unprocessed samples. However, S seems to be weakly susceptible to the increase of phytase activity in fermented porridge. With the exception of S, varieties show no readily apparent relationship between the concentration of phytates and the phytase activity in the unprocessed samples, as well as between the reduction of phytate content and the increment of phytase activity in fermented slurries.

In conclusion, the traditional fermentation process shows a higher impact than variety on the increase of phytase activity in sorghum.

Iron and zinc content

The results of quantitative analysis on iron and zinc content show that the amount of these essential elements is influenced neither by fermentation, nor by cooking, whereas it showed consistent linkage to variety. Processing of the samples has, indeed, no significant effect either on the iron or on the zinc content.

On the contrary, significant differences are observed among varieties. Two African landraces (S and B) show the highest content of iron. However, S shows the lowest amount of zinc. Therefore, the contents of iron and zinc do not show homogeneous patterns in the different varieties.

Iron and zinc are essential for the normal physiological function of the organism. Nevertheless, an adequate ratio Fe:Zn has reported to be equally important, due to the inhibitory effect of iron on zinc absorption. Upon direct oral administration of inorganic Fe and Zn, a Fe/Zn ratio ≥ 2 has been reported to significantly inhibit zinc absorption (Solomons and Jacob, 1981); on the other hand, no significant negative effect on zinc absorption was found when a Fe/Zn molar ratio up to 10 was provided through a meal (Valberg *et al.*, 1984; Fairweather-Tait *et al.*, 1995). Thus, the Fe/Zn ratios found in the porridges from the present varieties may not impair zinc absorption.

Bioavailability and bioaccessibility of trace elements

Molar ratios of phytate to iron and zinc

The molar ratios of phytate (IP6) to iron and phytate to zinc have been calculated in order to estimate the bioavailability of the two trace elements as proposed by Hurrell and Egli (2010) (IP6/Fe) and Gibson (2006) (IP6/Zn).

The findings outline the importance of both variety and fermentation on the estimated bioavailability of iron and zinc. On the other hand, cooking does not significantly affect phytate to iron and phytate to zinc molar ratios. Indeed, since iron and zinc contents were not influenced by processing, molar ratio variation mirrored the influence of sample treatment on IP6.

According to Hurrell and Egli (2010) and Gibson (2006), IP6/Fe and IP6/Zn molar ratios of all varieties, except for S, are too high to allow an adequate bioavailability of iron and zinc. Indeed, according to the authors, the IP6/Fe ratio should be preferably less than 0.4 and the IP6/Zn molar ratio < 15 to significantly improve the two minerals absorption. Concerning the IP6/Fe molar ratio, only S shows an optimum value; nevertheless, in case of composite meals containing ingredients enhancing iron absorption such as vitamin C or animal protein, it is acceptable an IP6/Fe ratio less than 6, as we observed in fermented slurries (*f*) of B and N. Consequently, fermented slurries (*f*) of varieties from Burkina Faso, Nigeria and, particularly, Senegal (the three African varieties) are those which present a highest iron bioavailability.

Regarding the IP6/Zn molar ratio, only fermented slurries from S and N showed IP6/Zn molar ratio < 15, namely 0.47 in S and 8.5 in N, according to Gibson (2006) indicating a high and moderate zinc bioavailability, respectively. In the other varieties IP6/Zn molar ratio markedly exceeded 15, suggesting a very low Zn availability. Similarly to the results on iron bioavailability, those

related to zinc bioavailability also indicate that varieties from Nigeria and Senegal (two African varieties) show the highest zinc bioavailability.

Study of algorithm

The algorithm here utilized has been devised by Hallberg and Hulthén (2000) in order to predict dietary iron absorption from a meal, through a mathematical model. The algorithm has been used to assess the effect of factors able to influence heme- and non heme- iron absorption from sorghum samples.

Even though Hallberg and Hulthén (2000) consider to estimate bioavailability through the algorithm, in this study, the algorithm results are retained as indicators of bioaccessibility, since bioavailability of trace elements is affected by a number of factors not included in the study of algorithm, such as dietary, luminal and systemic factors (Miret *et al*., 2003; Hunt, 2005).

Results show that variety remarkably influences the estimated bioaccessibility of iron for the human organism. In particular, S (the variety from Senegal) shows an approximate 3-fold higher iron bioaccessibility in all treatments than all the other varieties, which show instead similar values.

Concerning the effect of food process, results from the study of algorithm are in accordance with those observed in the analytical methods. Fermentation has been shown to decrease the amount of both iron binding phenolic compounds and phytates and the application of algorithm confirms that fermentation process is able to increase the bioaccessibility of iron of 34-77%.

Finally, this study showed that findings from a consolidates approach (the molar ratios of phytate to iron and to zinc) and from an innovative approach (study of algorithm) are pretty consistent.

Effect of variety

Results reported in this thesis show that variety significantly influences the content of the AN as well as of iron and zinc and estimated bioaccessibility and bioavailability; in particular, different varieties show significant variations as regards the content of both iron binding phenolic groups and phytates. Concerning the impact of variety in a biological system, it has been found that variety influences the content of total protein of exposed cells, whereas it does not affect the activity of GPx in the present experimental conditions.

Finally, the overall better profile of African compared to Italian varieties and the differences among African varieties coming from diverse areas suggest that the impact of variety on the tested parameters is attributable to both genotype and pedoclimatic factors. Indeed, as previously reported (p. 32), many nutritional characteristics of plants are dependent by local agro-climatic conditions; as a consequence, it is worthwhile to hypothesize that not only AN and minerals content, but also the effects on *in vitro* cells are influenced by the intrinsic characteristic of each variety adapted to the local environment (landraces).

Effect of treatments

Generally, findings here reported show that food processing is able to influence both tannins and phytic acid amount and biological activities in sorghum porridge.

In particular, cooking, like fermentation, decreases the total protein content of cells exposed to cooked sorghum samples, but it does not affect cells GPx activity. Similarly, neither the amount of iron binding phenolic group nor

phytates are influenced by cooking alone. On the contrary, cooking associated with fermentation elicits overall comparable effect as fermentation alone.

Fermentation process shows to have a two-way effect. A negative impact was observed on physiological cells functions, as regards both the total protein content and the GPx activity of cells exposed to fermented sorghum samples. On the other hand, fermentation has a positive impact on the content of AN, as it significantly reduces both iron binding phenolic compounds and phytates content, and increases the estimated bioaccessibility and bioavailability of iron and zinc. Moreover, the effect of fermentation results to be also modulated by the variety. It cannot be excluded that the different results observed in the two approaches: the *in vitro* and analytical approach, are linked to the different investigation aspects, resulting in the biological effects and the quantitative analysis, respectively. The effects observed in the *in vitro* models are correlated to the overall effects of the food, including the bioaccessibility of AN and the production of by-products, while quantitative analysis represent established methods giving punctual data; this is the reason why they need to be integrated.

Future perspectives

One of the main aims of this work was to evaluate the effects of a food *in toto*, namely sorghum porridges and fermented slurries, in a biological system. The present study has a proof-of-concept character, and involves a novel, integrated approach; thus, five sorghum varieties of different origin have been investigated. The experiments cannot rule out altogether the possibility of a modulation of the observed effects by certain common sorghum contaminants, such as arsenic, cadmium and mycotoxins (mainly aflatoxins). However, the consistent trends identified by our results may indicate that the role of contaminants, if any, was not a major one.

In order to expand this study, it would be interesting, therefore:

- to increase the number and the diversity of geographical origin of varieties to be tested, in order to increase the data base and to validate the approach;
- to increase the study of functional markers able to detect biological activities of AN *in vitro*, both on medium supernatants and on lysed cells, such as enzyme activities, cell metabolites or metallothionein, as markers of intracellular bioaccessibility of trace elements;
- to detect the presence of main sorghum contaminants, such as arsenic, cadmium and mycotoxins, in order to evaluate their potential impact on some *in vitro* parameters, such as cells protein synthesis and/or antioxidant activity;
- to perform additional quantitative analysis on AN and iron and zinc, such as the molecular fractions of iron and zinc in the soluble extract of the *in vitro* digestion after sorghum exposure, in order to evaluate bioaccessibility.

Since the *in vitro* (activities) and quantitative analysis (amount) of iron binding phenolic groups and phytates, appear to have independent results, it would be of importance to make detailed studies on the products formed during fermentation and cooking. Interesting aspects include the antinutrient activities of lower inositol phosphates (products of the phytate hydrolysis) and tannin degradation products.

The results of this work clearly show that fermentation, associated or not to cooking, significantly decreases the amount of iron binding phenolic groups and tannins and increases the estimated bioaccessibility and bioavailability of iron and zinc. It would be interesting to investigate whether the reduction of

AN and therefore the increased bioavailability of iron and zinc observed can be successfully achieved at the household level through selection of varieties and adequate processes as well as investigate the effect on human absorption. This could be assessed through a pilot human trial, by testing blood level of iron and zinc.

Conclusions

Results showed that both variety and process are able to modulate different tested parameters.

Data demonstrated that variety strongly influences the majority of the tested parameters, namely the total protein content of cells exposed to different sorghum samples, the amount of both tested AN and trace minerals analyzed and the estimated bioaccessibility and bioavailability of iron and zinc. Only the GPx activity of cells exposed to sorghum samples was not affected by variety. There is a clear difference among traditional varieties with regard to AN content and activity. The variety from Senegal showed the most promising nutritional profile, according to AN content, IP6/Fe and IP6/Zn molar ratios, iron content, bioaccessibility and bioavailability of trace elements. On the other hand, the two Italian varieties (ABF and Ar) overall showed a poor nutritional profile in terms of mineral bioaccessibility and bioavailability, by showing the lowest IP6/Fe and IP6/Zn molar ratios and the higher amount of AN, as well as a significant reduction of the total protein content of cells.

Data also show that fermentation had a markedly greater impact than cooking on AN amount and activities, whereas both had no impact on the minerals content. Fermentation, alone and with cooking, reduced AN content and enhanced, consequently, the estimated bioavailability of iron and zinc. On the other hand, it showed a negative impact on both functional markers *in vitro*. Cooking alone reduced the total protein content of exposed cells, like fermentation, as the only significant effect.

Interestingly, the combination of fermentation and cooking results to have the best overall results, as it showed the positive effect of cooking in the *in vitro* analysis (no impact on the cells GPx activity) and of fermentation in the quanti-

tative analysis of AN and bioavailability of iron and zinc (reduction of the amount of both AN and increase of the two trace elements estimated bioavailability). Finally, most tested parameters showed further modulation effects of the variety on food process.

The results outline the importance of sorghum landraces characterization for assessing the impact of traditional processes on the content and the activity of AN and the importance of applying appropriate processes at household level, such as the combination of cooking and fermentation, in order to improve the nutritional characteristics of a sorghum meal.

References

Abdelhaleem, W. H., El Tinay, A. H., Mustafa A.I. & Babiker, E.E., 2008. Effect of fermentation, malt-pretreatment and cooking on antinutritional factors and protein digestibility of sorghum cultivars. *Pakistan Journal of Nutrition*, 7, 335-341.

Abdel-Rahman, I.E., 2000. Microbiological and biochemical change during fermentation of sorghum. M.Sc. Thesis, Faculty of Agriculture. University of Khartoum, Sudan.

Abdel-Rahim, E.A., El-Beltagi, H.S., 2010. Constituents of apple, parsley and lentil edible plants and their therapy treatments for blood picture as well as liver and kidneys functions against lipidemic disease. *EJEAF* Che 9: 1117–1127.

Abdelseed, B.H., Abdelwahab, H.A., Abu ElGasim, A.Y., Isam, A.M. & Babiker, E.E., 2011. Some nutritional attributes of selected newly developed lines of sorghum after fermentation. *Journal of Agricultural Science and Technology*, 13, 399–409.

Afify, A.E.-M.M.R., El-Beltagi, H.S., Abd El-Salam, S.M., Omran, A.A., 2011. Bioavailability of Iron, Zinc, Phytate and Phytase Activity during Soaking and Germination of White Sorghum Varieties. *PLoS ONE* 6(10): e25512.

Afify, A.E.-M.M.R., El-Beltagi, H.S., Abd El-Salam, S.M., Omran, A.A., 2012. Protein Solubility, Digestibility and Fractionation after Germination of Sorghum Varieties. *PLoS ONE* 7(2): e31154.

Afolayan, M.O., M. Afolayan and J.N. Abuah, 2010. An investigation into sorghum based ogi (Ogi-Baba) storage characteristics. *Advanced Journal of Food Science and Technology*, 2: 72-78.

Alonso, R., Orue, C., Marzo, F., 1998. Effect of extrusion and conventional

processing methods on protein and antinutritional factor content in pea seeds. *Food Chemistry*, 63: 505-512.

Alonso, R., Aguirre, A. and Marzo, F., 2000. Effect of traditional processing methods on antinutritionals and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry,* 68: 159-165.

Antony, U. and Chandra, T. S., 1998. Antinutrient reduction and enhancement in protein, starch, and mineral availability in fermented flour of finger millet (*Eleusine coracana*). *Journal of Agriculture and Food Chemistry,* 46:2578–2582.

Aruoma, O.I., Murcia, A., Butler, J. and Halliwell, B., 1993. Evaluation of the Antioxidant and Prooxidant Actions of Gallic Acid and Its Derivatives. *Journal of Agriculture and Food Chemistry,* 41:1880-1885.

Asante, S.A., 1995. Sorghum quality and utilization. *African Crop Science Journal*, 3:231-240.

Assefa, Y., Staggenborg, S.A. and Prasad, P.V.V., 2010. Grain sorghum water requirement and responses to drought stress: A review. Online. *Crop Management* doi: 10.1094/CM-20101-1109-01-RV.

Barroga, C.F., Laurena, A.C. and Mendoza, E.M.T., 1985. Polyphenols in mung bean (*Vigna radiata* (L.) Wilczek): determination and removal. *Journal of Agriculture and Food Chemistry*, 33: 1006-1009.

Baynes, R.D. and Bothwell, T.H., 1990. Iron deficiency. *Annual Review of Nutrition,* 10:133-148.

Bennick, A., 2002. Interaction of Plant Polyphenols with Salivary Proteins. *Critical Reviews in Oral Biology & Medicine,* 13: 184-196

Beta, T., Rooney, L.W., Marovatsanga, L.T. and Taylor, J.R.N., 2000. Effect of chemical treatments on polyphenols and malt quality in sorghum. *Journal of Cereal Science*, 31: 295-302.

Bohn, T., Davidsson, L., Walczyk, T. and Hurrell, R.F., 2004. Phytic acid added to white-wheat bread inhibits fractional apparent magnesium absorption in humans. *American Journal of Clinical Nutrition*, 79: 418-423.

Bohn, L., Meyer, A. and Rasmussen, S., 2008. Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *Journal of Zhejiang University Science, B* 9: 165–191.

BOSTID (Board on Science and Technology for International Development National Research Council), 1996. *Lost Crops of Africa, volume I: Grains*. National Academy Press, Washington, D.C.

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance, *Nutrition Review,* 56: 317-333.

Brune, M., Rossander, L. and Hallberg, L., 1989. Iron absorption and phenolic compounds: Importance of different phenolic structures. *European Journal of Clinical Nutrition*. 43: 547-557.

Brune M., Halberg, L. and Skanberg, A. B, 1991. Determination of iron-binding phenolic groups in foods. *Journal of Food Science*, 56, 128-131.

Bryden, W.L., Selle, P.H., Cadogan, D.J., Li, D., Muller, N.D., Jordan, D.R., Gidley, M.J. and Hamilton, W.D., 2009. A review of the nutritive value of sorghum for broilers. *RIDRC Puplication* No 09/077, 57.

Butler, G. L., 1990. Tannins and other phenols: Effects on sorghum production and utilization. *INTSORMIL* Annual Report 140-144. A technical research report of the grain sorghum/pearl millet collaborative research support program (CRSP).

Chavan, U.D., Chavan, J.K. and Kadam, S.S., 2006. Effect of fermentation on soluble proteins and *in vitro* protein digestibility of sorghum, green gram and sorghum-green gram blends. *Journal of Food Science*, 53:1574-1575.

Chibber, B.A.K., Mertz, E.T. and Axtell, J.O., 1980. *In vitro* digestibility of high

tannin sorghum at different stages of dehulling. *Journal of Agricultural and Food Chemistry*, 28:160-161.

Ciacci, C., Maiuri, L., Caporaso, N., Bucci, C., Del Giudice, L., Massardo, D.R., Pontieri, P., Di Fonzo, N., Bean, S.R., Ioerger, B. and Londei, M., 2007. Celiac disease: *In vitro* and *in vivo* safety and palatability of wheat-free sorghum food products. *Clinical Nutrition*, 26(6)799-805.

Claver, I.P., Zhang, H., Li, Q., Zhu, K. and Zhou, H., 2010. Impact of the Soak and the Malt on the Physicochemical Properties of the Sorghum Starches. *International Journal of Molecular Sciences*, 11:3002-3015.

Clydesdale, F. M. and Camire, D. I. 1983. Effect of pH and Heat on the Binding of Iron, Calcium, Magnesium and Zinc and the Loss of Phytic Acid on Soy Flour. *Journal of Food Science*, 48:1271-1274.

Coulibaly, A., Kouakou, B. and Chen, J., 2011. Phytic Acid in Cereal Grains: Structure, Healthy or Harmful Ways to Reduce Phytic Acid in Cereal Grains and Their Effects on Nutritional Quality. *American Journal of Plant and Fertilization Technology*, 1:1-22.

Crews, H.M., Clarke, P.A, Lewis, D.J, Owen, L.M., Strutt, P.R. and Izquierdo, A., 1996. Investigation of selenium speciation in *in-vitro* gastrointestinal extracts of cooked cod by high performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray mass spectrometry. *Journal of Analytical Atomic Spectrometry*, 11:1177–1182.

Davies, N. T. and Olpin, S., 1979. Studies on the phytate:zinc molar content in diet as a determinant of Zn availability to young rats. *British Journal of Nutrition*, 41:591–603.

De Angelis, M., Gallo, G., Corbo, M.R., McSweeney, P.L.H., Faccia, M., Giovine, M. and Gobbetti, M., 2003. Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus*

sanfranciscensis CB1. *International Journal of Food Microbiology*, 87:259-270.

Dendy, D.A.V., 1995. Sorghum and Millets Chemistry and Technology. 406 pp. USA, St. Paul, Minnesota, American Association of Cereal Chemists, Inc.

Dhankher, N. and Chauhan, B.M., 1987. Effect of temperature and fermentation time on phytic acid and polyphenol content of rabadi, a fermented pearl millet food. *Journal of Food Science*, 52, 828–829.

Dicko, M.H., Gruppen ,H., Traore, A.S., Voragen, A.G.J. and van Berkel, W.J.H., 2006. "Review: Sorghum grain as human food in Africa: relevance of starch content and amylase activities. *African Journal of Biotechnology,* 5: 384-395.

Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S. and Taylor, J.R.N., 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *Journal of Cereal Science,* 35:161–174.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. *Journal of Cereal Science,* 38 :117–131.

Du Plessis, J., 2008. Sorghum production. www.nda.agric.za/publications

Dykes, L., Rooney, L. W., Waniska, R. D. and Rooney, W. L., 2005. Phenolic Compounds and Antioxidant Activity of Sorghum Grains of Varying Genotypes. *Journal of Agricultural and Food Chemistry*, 53:6813-6818.

Dykes, L. and Rooney, L.W., 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World,* 52:105–111.

Egli, I., Davidsson, L., Juillerat, M.A., Barclay, D. and Hurrell, R.F., 2002. The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. *Journal of Food Science*, 67(9):3484-3488.

Ekpenyong, T.E., 1985. Effect of cooking on polyphenolics content of some Nigerian legumes and cereals. *Nutrition Reports International*, 31: 561-565.

El Hag, M.E., El Tinay, A.H. and Yousif, N.E., 2002. Effect of fermentation and

dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chemistry*, 77:193–196.

Elkhalifa, A.O. and El Tinay, A.H. ,1994. Effect of fermentation on protein ractions and tannin content of low and high tannin cultivars of sorghum. *Food Chemistry*, 49:265–269.

Elkhalifa, A.E., Schiffler, B. and Bernhard, R., 2004. Effect of fermentation on the starch digestibility, resistant starch and some physicochemical properties of sorghum flour. *Die Nahrung*, 48(2):91-4.

Faber, M., Jogessar, V.B. and Benade, A.J.S., 2001. Nutritional status and dietary intakes of children aged 2-5 years and their caregivers in a rural South African community. *International Journal of Food Science and Nutrition*, 52:401-411.

Fadlallah, O.E., El Tinay, A.H. and Babiker, E.E., 2010. Biochemical Characteristics of Sorghum Flour Fermented and/or Supplemented with Chickpea Flour. *International Journal of Biological and Life Sciences*, 6:1.

Fairweather-Tait, S. J., Wharf, S. G. and Fox, T. E, 1995. Zinc absorption in infants fed iron-fortified weaning food. *American Journal of Clinical Nutrition*, 62, 785-789.

FAO, 1966. Food and Agricultural Organization of the United Nations. FAO Production Year Book, Rome.

FAO, 1995. Sorghum and millets in human nutrition. *FAO Food and Nutrition Series*, No. 27. Food and Agriculture Organization of the United Nation, Rome.

FAO, 1998. Fermented fruits and vegetables: A global perspective. *Agricultural Services Bulletin* No. 134. Food and Agriculture Organization of the United Nations Rome 1998.

FAO, 1999a. Cereal Fermentations in African Countries. Food and Agriculture

Organization of the United Nations, Rome.

FAO, 1999b. Fermented Cereals. A Global Perspective. FAO *Agricultural Services Bulletin* No. 138. Food and Agriculture Organization of the United Nations, Rome.

Fretzdroff, B. and Weiper, D., 1986. Phytic acid in cereals: phytase in rye and rye products. *Zeitschrilt fur Lebensmittel Untersuchung und Forschung* 82:287-293.

Frias, J., Doblado, R., Antezana, J.R. and Vidal-Valverde, C., 2003. Inositol phosphate degradation by the action of phytase enzyme in legume seeds.
Food Chemistry, 81(2):233-239.

Gibson, R.S., 1994. Zinc nutrition in developing countries. *Nutrition Research Reviews,* 7: 151–173.

Gibson, R.S., 2006. Zinc: The missing link in combating micronutrient malnutrition in developing countries. *Proceedings of the Nutrition Society*, 65:51–60.

Godoy, S., Chicco, C., Meschy, F. and Requena, F., 2005. Phytate phosphorus and phytase activities of animal feed ingredients. *Interciencia*, 30:24-28.

Graf, E., Empson, K.L. and Eaton, J.W., 1987. Phytic acid. A natural antioxidant. *Journal of Biological Chemistry,* 262:11647–11650.

Grases, F., Garcia-Ferragut, L. and Costa-Bauza, A., 1998. Development of calcium oxalate crystals on urothelium: Effect of free radicals. *Nephron*. 78(3):296–301.

Greiner, R., Jany, K.D. and Larsson Alminger M., 2000. Identification and propertiesof *myo*-inositol hexakisphosphate phosphohydrolases (phytases) from barley (*Hordeum vulgare*). *Journal of Cereal Science*, 31:127-139.

Hagerman, A.E. and Butler, L.G., 1978. Protein precipitation method for the quantitative determination of tannins. *Journal of Agriculture and Food*

Chemistry, 26:809–812.

Hagerman, A.E., 1992. Tannin-protein interaction. In: Ho CT, Lee CY, Huang MT, eds. *Phenolic compounds in food and their effects on health, I: analysis, occurrence, and chemistry*. ACS Symposium Series 506, 236-247. Washington: American Chemical Society.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W. and Riechel, T.L., 1998. High molecular-weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agriculture and Food Chemistry*, 46:1887-92.

Hagerman, A.E., 2002. Tannin Chemistry. Available on: http://www.users.muohio.edu/hagermae/. Accessed March 2012.

Hahn, D.H., Rooney, L.W. and Earp, C.F., 1984. Tannins and phenol in sorghum. *Cereal Foods World*, 29:776-779.

Hallberg, L., Brune, M. and Rossander, L., 1989. Iron absorption in man: ascorbic acid and dose-dependent inhibition by phytate. *American Journal of Clinical Nutrition*, 49:140-144.

Hallberg, L. and Hulthén, L., 2000. Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. *American Journal of Clinical Nutrition*, 71(5):1147-1160.

Hama, F., Icard-Vernière, C., Guyot, J-P, Picq, C., Diawara, B. and Mouquet-Rivier, C., 2011. Changes in micro- and macronutrient composition of pearl millet and white sorghum during in field versus laboratory decortication. *Journal of Cereal Science*, 54:425-433.

Hamaker, B.R., Kirleis, A.W., Mertz, E.T. and Axtell, J.D, 1986. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. *Journal of Agricultural and Food Chemistry*, 34:647-649.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D. and Mertz, E.T., 1987.

Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences of the United States of America*, 84:626–628.

Harland, B.F. and Morris, E.R., 1995. Phytate: A good or a bad food component? *Nutrition Research*, 15:733-754.

Haslam, E., 1989. Plant Polyphenols – Vegetable Tannins Revisited –Chemistry and Pharmacology of Natural Products, *Cambridge University Press*: Cambridge, UK.

Haslam, E., 1998. Practical polyphenolics: from structure to molecular recognition and physiological function. *Cambridge University Press*: Cambridge, UK.

Hassan, I. A. G. and El Tinay, A. H., 1995. Effect of Fermentation on Tannin Content and in vitro Protein Digestibility of Two Sorghum Cultivars. *Food Chem*istry, 53:149-151.

Hedren, E., Diaz, V. and Svanberg, U., 2002. Estimation of carotenoid accessibility from carrots determined by an in vitro digestion method. *European Journal* of *Clinical Nutrition*, 56(5):425–30.

Hoffman, D., 2003. Medical Herbalism. Rochester, Vt: Healing Arts Press.

Hunt, J.R., 2005. Dietary and physiological factors that affect the absorption and bioavailability of iron. *International Journal for Vitamin and Nutrition Research*, 75:375–384.

Hurrell R, 2002. Bioavailability - a time for reflection. *International Journal of Vitamin and Nutrition Research*, 72:5-6.

Hurrell RF, 2004. Phytic acid degradation as a means of improving iron absorption. *International Journal for Vitamin and Nutrition Research*, 74(6):445–452.

Hurrell, R. and Egli, I., 2010. Iron bioavailability and dietary reference values.

American Journal of Clinical Nutrition, 91:1461S–1467S.

Idris, W.H., Hassan, A.B., Babiker, E.E.and El Tinay, A.H., 2005. Effect of malt pretreatment on antinutritional factors and HCl extractability of minerals of sorghum cultivars. *Pakistan Journal of Nutrition*, 4:396-401.

Ikemefuna, Obizoba, C., andAtii, J.V., 1991. Effect of soaking, sprouting, fermentation and cooking on nutrient composition and some anti-nutritional factors of sorghum (*Guinesia*) seeds. *Plant Foods for Human Nutrition* (Formerly Qualitas Plantarum), 45:23-34.

Jovanovic, S.V., Simic, M.G., Steenken, S. and Hara, Y., 1998. Iron complexes of gallocatechins. Antioxidant action or iron regulation? *Journal of the Chemical Society Perkin Transactions, 2:*2365-2369.

Kaankuka, F.G., Balogun, T.F., Yaakugh, I.D.I., 2000. Effect of duration of cooking raw fullfat soyabeans on dry matter digestibility and energy utilization by young pigs. *Proceedings of the 27th Annual Conference of the Nigeria Society of Animal Production* (NSAP) pp.191-193.

Kasarda, D.D., 2001. Grains in relation to celiac disease. *Cereal Foods World*, 46:209-210.

Kataria, A., Chauhan, B.M. and Punia, D., 1989. Antinutrients and protein digestibility (*in vitro*) of mung bean as affected by domestic processing and cooking. *Journal of Food Chemistry,* 32:9-17.

Katayama, T., 1995. Effect of dietary sodium phytate on the hepatic and serum levels of lipids and on the hepatic activities of NADPH-generating enzymes in rats fed on sucrose. *Bioscience and Biotechnological Biochemistry*, 59:1159–1160.

Kayodé, A.P.P., Linnemann, A.R., Nout, M.J.R. and Van Boekel, M.A.J.S., 2007. Impact of sorghum processing on phytate, phenolic compounds and *in vitro* solubility of iron and zinc in thick porridges. *Journal of the Science of*

Food and Agriculture, 87:832–838.

Khanbabaee, K. and van Ree, T., 2001. Tannins: Classification and Definition. *Natural Product Reports*, 18:641-649.

Klopfesntein, C.F. and R.C. Hoseney, 1995. Nutritional properties of sorghum and millets. In: *Sorghum and Millets: Chemistry, Technology* (edited by D.A.V. Dendy) St Paul, MN: Am. Assoc. Cereal Chemists., pp:125-168.

Konietzny, U., Greiner, R. and Jany, K.L.D., 1995. Purification and characterization of a phytase from spelt. *Journal of Food Biochemistry*, 18:165–183.

Kruger, J., Taylor, J.R.N. and Oelofse, A., 2012. Effects of reducing phytate content in sorghum through genetic modification and fermentation on in vitro iron availability in whole grain porridges. *Food Chemistry*, 131:220–224.

Kunene, N.F., Hastings, J.W. and von Holy, A., 1999. Bacterial populations associated with a sorghum-based fermented weaning cereal. *International Journal of Food Microbiology*, 49:75–83.

Lambrechts, C., Boze H., Moulin G. and Galzy P., 1992. Utilization of phytate by some yeast. *Biotechnology Letters,* 14:61-66.

Larysse, M.G.C., Solano, M.N., Baròn, M.A., Arguello, F., Llovera, D., Ramirez, J., Leets, I. and Tropper, E., 2000. Iron bioavailability in humans from breakfast enriched with iron bis-glycine chelate, phytates and polyphenols. *Journal of Nutrition*, 130:2195-2199.

Lee, S.H., Park, H.J., Chun, H.K., Cho, S.Y., Cho, S.M., Lillehoj, H.S., 2006. Dietary phytic acid lowers the blood glucose level in diabetic KK mice. *Nutrition Research*, 26(9):474–479.

Lestienne, I., Icard-Vernière, C. , Mouquet, C. , Picq, C. , Trèche, S. ,2005. Effects of soaking whole cereal and legume seeds on iron, zinc and phytate

contents. *Food Chemistry*, 89:421-425.

Lind, T., Lönnerdal, B., Persson, L., Stenlund, H., Tennefors, C. and Hernell, O., 2003. Effects of weaning cereals with different phytate contents on hemoglobin, iron stores, and serum zinc: a randomized intervention in infants from 6 to 12 mo of age1–3. *Journal of Clinical Nutrition*, 78:168–175.

Lott, J.N.A., Bojarski, M., Kolasa, J., Batten, G.D. and Campbell, L.C., 2009. A review of the phosphorus content of dry cereal and legume crops of the world. *International Journal of Agricultural Resources, Governance and Ecology*. 8, 351–370.

Mahgoub, S.E.O., Elhag, S.A., 1998. Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. *Food Chemistry,* 61:77–80.

Mamiro, P.S., Kolsteren, P., Roberfroid, D., Tatala, S., Opsomer, A.S. and Van Camp, J.H., 2005. Feeding practices and factors contributing to wasting, stunting, and iron-deficiency anemia among 3-23-month old children in Kilosa district, rural Tanzania. *Journal of Health, Population and Nutrition,* 23:222-230.

Marfo, E.K., Simpson, B.K., Idowu, J.S. and Oke, O.L., 1990. Effect of local food processing on phytate levels in cassava, cocoyam, yam, maize, sorhum, rice, cowpea and soybean. *Journal of Agricultural and Food Chemistry*, 38:1580–1585.

Marmolle, F., Leize, E., Mila, I., Van Dorsselaer, A., Scalbert, A. and Albercht-Gary, A.M., 1997. Polyphenol Metallic Complexes: Characterization by Electrospray Mass Spectrometric and Spectrophotometric Methods. *Analusis*, 25:M53-55.

Matuschek, E., 2005. *In vitro* availability of iron in high-tannin sorghum. Effects of Enzymatic Oxidation of Phenolic Compounds. Chalmers University of

Technology, Goteborg, Suecia, pp 25-54.

McMillan, B., Riggs, D.R., Jackson, B.J., Cunningham, C. and McFadden, D.W., 2007. Dietary Influence on Pancreatic Cancer Growth by Catechin and Inositol Hexaphosphate. *Journal of Surgical Research*, 141(1):115-9.

Midorikawa, K., Murata, M., Oikawa, S., Hiraku, Y. and Kawanishi, S., 2001. Protective effect of phytic acid on oxidative DNA damage with reference to cancer chemoprevention. *Biochemistry and Biophysics Research Communications*, 288:552–557.

Minihane, A.M. and Rimbach, G., 2002. Iron absorption and the iron binding and anti-oxidantproperties of phytic acid. *International Journal of Food Science and Technology*, 37:741–748.

Miret, S., Simpson, R.J., McKie, A.T., 2003. Physiology and molecular biology of dietary iron absorption. *Annual Review of Nutrition*, 23:283-301.

Mohammed, N.A., Mohamed I.A. and Babiker, E.E, 2010. Nutritional Evaluation of Sorghum Flour (Sorghum bicolor L. Moench) During Processing of Injera. *International Journal of Biological and Life Sciences*, 6:35-39.

Moneim, A., El Khalifa, O., El Tinay, A.H., 1994. Effect of fermentation and germination on the in vitro protein digestibility of low and high tannin cultivars of sorghum. *Food Chemistry*, 54:147-150.

Morales G.A., Moyano, F.J., Marquez, L., 2011. *In vitro* assessment of the effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. *Animal Feed Science and Technology,* 170:209–221.

Motarjemi, Y. and Nout, M.J., 1996. Food fermentation: a safety and nutritional assessment. Joint FAO/WHO Workshop on Assessment of Fermentation as a Household Technology for Improving Food Safety. *Bulletin of the World Health Organization,* 74(6): 553–559.

Mutayoba, S.K., Dierenfeld, E., Mercedes, V.A., Frances, Y. and Knight, C.D, 2011. Determination of Chemical Composition and Ant-nutritive Components for Tanzanian Locally Available Poultry Feed Ingredients. *International Journal of Poultry Science,* 10 (5): 350-357.

Navert, B., Sandstrom, B. and Cederblad, A., 1985. Reduction of the Phytate Content of Bran by Leavening in Bread and Its Effect on Zinc-Absorption in Man. *British Journal of Nutrition*, 53:47-53.

Neelam, Y., Alka, S. and Shruti, S., 2012. Effect of fermentation on physicochemical properties and *in vitro* starch and protein digestibility of selected cereals. *International Journal of Agriculture and Food Science,* 2(3):66-70.

Nemzer, B.V., Rodriguez, L.C., Hammond, L., Di silvestro, R., Hunter, J.M. and Pietrzkowski, Z., 2011. Acute reduction of serum 8-iso-PGF2-alpha and advanced oxidation protein products *in vivo* by a polyphenol-rich beverage; a pilot clinical study with phytochemical and *in vitro* antioxidant characterization. *Nutrition Journal*, 10:67.

Nierop, K.G.J., Verstraten, J.M., Tietema, J., Westerveld, J.W. and Wartenbergh, P.E., 2006. Short- and long-term tannin-induced carbon, nitrogen and phosphorusdynamics in Corsican pine litter. *Biogeochemistry,* 79:275–296.

NIIR (Board of Consultants & Engineers), 2006. Wheat, Rice, Corn, Oat, Barley and Sorghum Processing Handbook (Cereal Food Technology). *Asia Pacific Business Press Inc*. pp 464.

Nout, M.J.R, 2009. Rich nutrition from the poorest – Cereal fermentations in Africa and Asia. *Food Microbiology*, 26:685–692.

Nuobariene, L., Hansen, A.S., Jespersen, L. and Arneborg, N., 2011. Phytase-active yeasts from grain-based food and beer. *Journal of Applied Microbiology*, 110(6):370–1380.

Nwachukwu, E., Achi, O.K. and Ijeoma, I.O., 2010. Lactic acid bacteria in fermentation of cereals for the production of indigenous Nigerian foods. *African Journal of Food Science and Technology,* 1(2): 021-026.

Omeje, S.K., 1999. Issues in Animal science. *Raykennedy Scientific Publication*, Enugu, Nigeria.

Onomi, S., Okazaki, Y. and Katayama, T., 2004. Effect of dietary level of phytic acid on hepatic and serum lipid status in rats fed a high-sucrose diet. *Bioscience, Biotechnology, and Biochemistry,* 68(6):1379-1381.

Oria, M.P., Hamaker, B.R. and Shull, J.M., 1995. Resistance of sorghum a-, b- and gkafirins to pepsin digestion. *Journal of Agricultural and Food Chemistry,* 43:2148–2152.

Osman, A.M., 2007. Effect of different processing methods on nutrient composition, anti-nutritional factors and in vitro protein digestibility on Dolichos lablab bean (*Lablab purpureus* (L) Sweet). *Pakistan Journal of Nutrition,* 6(4): 299-303.

Palafox-Carlos, H., Ayala-Zavala, J.F. and González-Aguilar, G.A., 2011. The Role of Dietary Fiber in the Bioaccessibility and Bioavailability of Fruit and Vegetable Antioxidants. *Journal of food science*, 76(1): R6–R15.

Parr, A.J. and Bolwell, G.P., 2000. Phenols in plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture*, 80:985–1012.

Persson, H., Turk, M., Nyman, M. and Sandberg, A.S., 1998. Binding of Cu^{2+}, Zn^{2+} and Cd^{2+} to inositol tri-, tetra-, penta, and hexaphosphates. *Journal of Agricultural and Food Chemistry,* 46(8):3194–3200.

Porter, L.J., 1989. Methods in Plant Biochemistry-Plant Phenolics, Series ed P. M. Dey and J. B. Harborne, Academic Press, London, vol. 1, p. 389.

Prasad, P.V.V. and Staggenborg, S.A., 2009. Growth and production of sorghum

and millets. In soils, plant growth and crop production –Volume II. In: *Encyclopedia of life Support Systems*, Eolss Publishers, Oxford, UK.

Raboy, V., 2003. *Myo*-Inositol-1,2,3,4,5,6-hexakisphosphate. *Phytochemistry*, 64(6):1033–1043.

Rafi, M.M., Hartman, T.G., Das, Y. T., Shafaie, Y. and Hargreaves, J., 2008. Methods and compositions for preventing or treating various diseases or conditions in a patient with an alcohol extractable fraction of Sorghum bicolour grain. *US Patent Application Publication*. Pub. No.: US 2008/0241282 A1.

Reale, A., Konietzny, U., Coppola, R., Sorrentino, E. and Greiner, R., 2007. The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. *Journal of Agriculture Food Chemistry*, 55:2993-2997.

Reddy, N.R., Pierson, M.D., Sathe, S.K. and Salunkhe, P., 1985. Dry bean tannin: A Review of nutritional implication. *Journal of the American Oil Chemists' Society*, 62: 451-549.

Richard, A.F. and Wayne, S.C., 2001. Sorghum: Origin, History, Technology, and Production. In: *Crop Science*. Editor: C. Wayne Smith. John Wiley And Sons Ltd (United States),

Rimbach, G., Pallauf, J. and Walz, O.P., 1996. Effect of microbial phytase on cadmium accumulation in pigs. *Archives of Animal Nutrition*, 49:279–286.

Rimbach, G. and Pallauf, J., 1997. Cadmium accumulation, zinc status, and mineral bioavailability of growing rats fed diets high in zinc with increasing amounts of phytic acid. *Biological Trace Element Research*, 57:59–70.

Rooney, L.W., Kirleis, A.W. and Murty, D.S., 1986. Traditional foods from sorghum: their production, evaluation and nutritional value. In: Advances in Cereal Science and Technology, Vol. 8. Pomeranz, Y. (Ed.), pp. 317-353.

ICRISAT, American Association of Cereal Chemistry, St. Paul, MN, USA.

Ryden, P. and Selvendran, R.R., 1993. Phytic acid: properties and determination. In *Encyclopaedia of food science, food technology and nutrition* (edited by R. Macrae, R. K. Robinson & M. J. Sadler). Pp. 3582–3587 . London: Academic Press.

Saha, P. R., Weaver, C. M. and Mason, A. C., 1994. Mineral bioavailability in rats from intrinsically labeled whole wheat flour of various phytate levels. *Journal of Agricultural and Food Chemistry*, 42:2531−2535.

Samman, S., Sandström, B., Bjørndal Toft M., Bukhave, K., Jensen, M., Sørensen, S.S. and Hansen, M., 2001. Green tea or rosemary extract added to foods reduces nonheme-iron absorption. *American Journal of Clinical Nutrition*, 73:607-612.

Sandberg, A.S. and Svanberg U., 1991. Phytate hydrolysis by phytase in cereals: effects on *in vitro* estimation of iron availability. *Journal of Food Science*, 56:1330-1333.

Sandberg, A.S. and Andlid, T., 2002. Phytogenic and microbial phytases in human nutrition. *International Journal of Food Science & Technology*, 37:823-833.

Santos-Buelga, C. and Scalbert, A., 2000. Proanthocyanidins and tannin-like compounds: nature, occurrence, dietary intake and effects on nutrition and health. *Journal of Food Science and Agriculture*, 80:1094-1117.

Scalbert, A., Mila, I., Expert, D., Marmolle, F., Albrecht, A.M., Hurrell, R., Huneau, J.F. and Tomé, D., 1999. Polyphenols, metal ion complexation and biological consequences. *Basic Life Sciences*, 66:545-54.

Schober, T.J., Bean, S.R. and Boyle, D.L., 2007. Gluten-Free Sorghum Bread Improved by Sourdough Fermentation: Biochemical, Rheological, and Microstructural Background. *Journal of Agricultural and Food Chemistry*,

55:5137-5146.

Schons, P.F., Ries, E.F., Battestin, V. and Macedo, G.A., 2011. Effect of enzymatic treatment on tannins and phytate in sorghum (Sorghum bicolor) and its nutritional study in rats. *International Journal of Food Science & Technology*, 46:1253–1258.

Selvam, R., 2002. Calcium oxalate stone disease: Role of lipid peroxidation and antioxidants. *Urological Research,* 30(1):35–47.

Shahidi, F. and Wanasundara, P.K.J.P.D., 1992. Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition,* 32: 67–103.

Shamsuddin, A.M., 1995. Inositol phosphates have novel anticancer function. *Journal of Nutrition,* 125:725S–732S.

Siegenberg, D., Baynes, R.D., Bothwell, T.H., Macfarlane, B.J., Lamparelli, R.D., Car, N.G., MacPhail, P., Schmidt, U., Tal, A. and Mayet, F., 1991. Ascorbic-Acid Prevents the Dose-Dependent Inhibitory Effects of Polyphenols and Phytates on Nonheme-Iron Absorption. *American Journal of Clinical Nutrition,* 53:537-541.

Slabbert, N., 1992. Complexation of condensed tannins with metal ions, in R. W. Hemingway and P. E. Laks (eds), *Plant Polyphenols*, Plenum Press, New York, pp 421-436.

Soetan, K.O. and Oyewole, O.E, 2009. The need for adequate processing to reduce the antinutritional factors in plants used as human foods and animal feeds: A review. *African Journal of Food Science,* 3 (9):223-232.

Solomons, N.W. and Jacob, R.A., 1981. Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. *American Journal of Clinical Nutrition*, 34:475-482.

Svensson, L., Sekwati-Monang, B., Lutz, D.L., Schieber, A. and Gänzle, M.G., 2010. Phenolic Acids and Flavonoids in Nonfermented and Fermented Red

Sorghum (Sorghum bicolor (L.) Moench). *Journal of Agricultural and Food Chemistry*, 58:9214-9220.

Talamond, P., Gallon, G. and Trèche, S., 1998. Rapid and sensitive liquid chromatographic method using a conductivity detector for the determination of phytic acid in food. *Journal of Chromatography A*, 805:143–147.

Taylor, J. and Taylor, J.R.N., 2002. Alleviation of the adverse effect of cooking on sorghum protein digestibility through fermentation in traditional African porridges. *International Journal of Food Science and Technology*, 37:129-137.

Taylor, J.R.N., 2003. Overview: Importance of Sorghum in Africa. Paper 1 in: AFRIPRO, Workshop on the Proteins of Sorghum and Milets: Enhancing Nutritional and Functional Properties for Africa. P.S. Belton and J.N.R. Taylor, eds. Available on: http://www.afripro.org.uk

Teshome, A., Fahrig, L., Torrance, J.K., Lambert, J.D., Arnason, T.J. and Baum, B.R., 1999. Maintenance of sorghum (*Sorghum bicolor*, Poaceae) landrace diversity by farmers' selection in Ethiopia. *Economic Botany*, 53: 79–88.

Tomas-Barberan, F., Espín, J.C., 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture*, 81:853−876.

Torres, J., Domínguez, S., Cerdá, M.F., Obal, G., Mederos, A., Irvine, R.F., Díaz, A. and Kremer, C., 2005. Solution behaviour of myo-inositol hexakisphosphate in the presence of multivalent cations. Prediction of a neutral pentamagnesium species under cytosolic/nuclear conditions. *Journal of Inorganic Biochemistry*, 99(3):828-40.

Towo, E., Matuschek, E. and Svanberg, U., 2006. Fermentation and enzyme treatment of tannin sorghum gruels: effects on phenolic compounds,

phytate and in vitro accessible iron. *Food Chemistry*, 94:369–376.

Tsaousi, K., Dimitrellou, D. and Koutinas, A.A., 2008. Low-temperature thermal drying of *Saccharomyces cerevisiae* starter culture for food production. *Food Chemistry*, 110:547–553.

USDA, Grain: World Markets and Trade. October 2010. www.Grains.org/index.php?option=Com_content&view=article&id=74&Itemid=120

Valberg, S. L., Flanagan, P. R. and Chamberlain, M., 1984. Effect of iron, tin, and copper on zinc absorption in humans. *American Journal of Clinical Nutrition*, 40:536-541.

Van Buren, J.P. and Robinson, W.B., 1969. Formation of complexes between protein and tannic acid. *Journal of Agricultural and Food Chemistry, 17* (4):772-777.

Versantvoort, C.H.M., Van de Kamp, E. and Rompelberg, C.J.M., 2004. Development and applicability of an *in vitro* digestion model in assessing the bioaccessibility of contaminants from food. Report no. 320102002, National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

Vieira-Dalodé, G., Jespersen, L., Hounhouigan, J., Moller, P.L., Nago, C.M. and Jakobsen, M., 2007. Lactic acid bacteria and yeasts associated with gowé production from sorghum in Bénin. *Journal of Applied Microbiology,* 103(2):342-9.

Vucenik I., Ramakrishna G., Tantivejkul K., Anderson L.M., Ramljak D., 2005. Inositol hexaphosphate (IP6) blocks proliferation of human breast cancer cells through a PKCδ-dependent increase in p27Kip1 and decrease in retinoblastoma protein (pRb) phosphorylation. *Breast Cancer Research and Treatment*, 91:35-45.

Waliyar, F., Ravinder, R.C., Alur, A.S., Reddy, B.V.S, Reddy, A.R., Rai, K.N. and Gowda, C.L.L., 2007. Management of Grain Mold and Mycotoxins in Sorghum. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Wanasundera, J.P.D. and Ravindran, G., 1992. Effect of cooking on the nutrient and antinutrient contents of yam tubers (*Dioscorea alata* and *Dioscorea esculenta*). *Food Chemistry* 45:247-250.

Waniska, R. D. and Rooney, L. W. 2000. Structure and chemistry of the sorghum caryopsis. John Wiley & Sons, Inc: New York.

Weaver, C.M., Heaney, R.P., Martin, B.R. and Fitzsimmons, M.L., 1991. Human Calcium-Absorption from Whole-Wheat Products. *Journal of Nutrition*, 121: 1769-1775.

Wilkinson, L., 1990. SYSTAT: The system for statistics. IL, Evanton: SYSTAT Inc.

Yoon, J.H., Thompson, L.U. and Jenkins, D.J., 1983. The effect of phytic acid on in vitro rate of starch digestibility and blood glucose response. *American Journal of Clinical Nutrition*, 38:835–842.

Yuye, W., Xianran, L., Wenwen, X., Chengsong, Z., Zhongwei, L., Yun, W., Jiarui, L., Satchidanand, P., Dustan, D.R., Guihua, B., Ming, L.W., Harold, N. T., Scott, R.B., Mitchell, R.T., Tesfaye, T.T. and Jianming, Y., 2012. Presence of tannins in sorghum grains is conditioned by different natural alleles of *Tannin1. Proceedings of the National Academy of Sciences of the United States of America*.

Paper I

Cameroon-Nigeria-Italy scientific cooperation: veterinary public health and sustainable food safety to promote “one health/one prevention”

Edited by Chiara Frazzoli (a), Emmanuel Acha Asongalem (b)
and Orish Ebere Orisakwe (c)
(a) Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Italy
(b) Faculty of Health Sciences, University of Buea, Cameroon
(c) Faculty of Pharmacy, University of Port Harcourt, Nigeria

HOW TO IMPROVE SAFETY AND NUTRITIONAL SECURITY OF RAW INGREDIENTS: THE CASE OF SORGHUM

Ilaria Proietti
Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Italy

Introduction

For years advancing technology allowed to raise land productivity all around the world. As a consequence, since 1950 the world as a whole has nearly tripled land productivity (1). Unfortunately, the steady growth of productivity is coming to an end while, on the other hand, the world population has been growing exponentially. As a result, and due to recent changes in the environment, food demand is more and more difficult to be met and one of major challenges facing the world is to meet the nutritional needs of more vulnerable groups. In order to meet the enhancing food requirement, innovative and sustainable measures are needed.

It is believed that the issues of food and nutrition security and health in developing countries can be addressed relying on indigenous crops (2). Indeed, with a soaring food crisis and changing weather patterns, the need to diversify the crop production to other less common varieties, such as local and indigenous crops like millets and sorghum, is real. Sorghum is one of the main traditional crops grown in numerous developing countries; it is a basic staple food for many rural communities, especially in drought prone areas of Africa, where it represents an important subsistence crop for many households. Sorghum has an important role in mitigating food insecurity, in particular of the low-income groups, which are the most affected by the lack of food and nutritional security, as it is widespread, nutritious, easy to grow and well adapted to local climate.

On the other hand, the nutritional value of sorghum is affected by inherent factors influencing its utilization, including Anti-Nutritional Factors (ANFs), able to bind proteins and divalent cations and interfere with their absorption, and mycotoxins, fungi secondary metabolites with adverse health effect on human health. Nevertheless, specific pre- and post-harvesting good practices and adequate food processing can be implemented in order to decrease the amount and activity of ANFs and mycotoxins and improve the food and nutritional quality of sorghum.

This study was supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

Nutritive and anti-nutritive properties of sorghum

Sorghum is the fifth most important cereal corn grown in the world agricultural economy after wheat, rice and barley. It is mainly grown in semi-arid areas of the world, characterized by high drought and low rainfall, climate conditions poorly favourable to the production of other cultivars. In industrialized countries, sorghum is mainly grown for the production of animal feed (forage or grain) or biofuel and more recently, for the lack of gluten content, it has been proposed as suitable food for coeliac people. On the other hand, in Africa, Asia and more recently in Latin America, sorghum production

has increate steadily and is grown primarily for human consumption. Only Africa produces around 20 million tons of sorghum per year, a third of the world total (2).

It is estimated that sorghum is part of the staple diet of more than 500 million people in more than 30 countries of the semi-arid tropics, representing a major source of energy and nutrients (2). The sorghum kernel contains about 70% carbohydrate and 12% protein – similar content to that of wheat and maize - but the grain has more vitamin B than maize (2). Indeed, sorghum has a good content of protein and vitamins, mainly those of B group (except for B12) and fat-soluble vitamins (A, D, E and K), compared to other cereals. In addition, it is considered an important source of minerals, as it is rich in phosphorus, potassium, iron and zinc.

The nutritional value of sorghum is, nevertheless, impaired by the activity of endogenous factors, namely ANFs, such as phenolic compounds (mainly condensed tannins) and phytic acid, and mycotoxins. From an agronomical point of view, the presence of ANFs is associated with diminished pre- and post-harvest losses due to bird predation and storage pests, respectively. On the other hand, these sorghum ANFs interact negatively with the bioaccessibility of essential elements, in particular iron and zinc, and the digestibility of proteins. The general mechanism involves the formation of insoluble complexes at physiological pH, due to the ability of phytic acid and tannins to bind proteins and divalent cations. The concentration of tannins and phytic acid greatly varies among different cultivars, as well as among plants belonging to the same cultivar. The phytic acid concentration can range from 2.5 to 22 mg/g and that of tannins from 0.8 to 55 mg/g, expressed as catechin equivalent (4). This range in the amount depends on several factors, such as sorghum variety, growing conditions, harvesting techniques and processing methods. Moreover, it has been demonstrated that the diversity and severity of adverse effects of ANFs are greater in tropical than in temperate climates (5).

Phytic acid is the storage form of phosphorus bound to inositol in the fiber of raw whole grain. It accounts for 50-80% of the total phosphorus (6). Although sorghum is relatively rich in phosphorus, phytate-phosphorus is less nutritionally available since the phytate is not quantitatively hydrolyzable in human gut (7). Another ability of phytic acid is to form binary protein-phytate complexes under acidic pH conditions (8) and the effects of this interaction can be detrimental for malnourished people. Also tannins have the ability to interact with proteins and complex a substantial proportion of them (9,10). It has been demonstrated that an amount of tannins of 1.9 g/kg is able to reduce real ileal digestibility of 16 aminoacids by nearly 9.8% in pigs, while 13.6 g/kg of phytate can reduce apparent ileal digestibility of 18 aminoacids by around 3.6% (11). Tannins and phytic acid are also able to bind enzymes in the gastrointestinal tract, thereby inhibiting their activity: glucosidase activity,α-amylase, trypsin and lipase activity (12-14).

Furthermore, sorghum, so as many other cereal plants, may be infected with mycotoxin producing fungi at all levels of the food chain: from production to processing and also in the supply chain. Mycotoxins represent one of the heaviest burdens, particularly for developing countries, both in terms of health implications and economic losses. Among the several mycotoxins, aflatoxins and fumonisins produced by *Aspergillus* and *Fusarium*, respectively, are a major problem in sorghum grains. Mycotoxins are potent carcinogens and can interfere with the immune system functioning and the normal human homeostasis. Moreover, mycotoxins or its metabolites pass into milk, eggs and other organs when animals are fed with contaminated grain, thus entering the food chain. The ingestion may be critical especially in vulnerable and weakened human bod-

ies. For example, children exposed to aflatoxin may become stunted, underweight and more susceptible to infections. A study in West Africa shows a strong correlation among stunted growth in children and exposure to aflatoxin (15). Hendrickse states that the mycotoxins cause kwashiorkor by damaging the liver, which becomes unable to produce albumin and low levels of the protein lead to the disease (16).

Effective practices for reducing the amount/activity of adverse endogenous factors

Factors enhancing the likelihood of ANFs and mycotoxins are numerous and include extreme environmental conditions, inadequate practices and the absence of adequate monitoring and control system (17). As a consequence, a number of preventive and corrective measures able to reduce the risk of ANFs and/or mycotoxins in sorghum grains exist.

The available literature (18-20) reports that food preparation and/or processing influence ANF content and activity. In particular, wet processing (including soaking, germination and fermentation) leads to a decrease of phytic acid and increase of solubility of minerals in foods. A study by Mahgoub and Elhag states that the soaking of sorghum flour at room temperature reduces phytic acid by 16-21%, germination for four days by 68-87%, while accelerated fermentation by 60% (21). Decreased content of phytic acid and tannins after fermentation or germination is mostly due to the activity of enzymes, while in soaking to a combination of diffusion and enzymatic action (21-24). Many authors state, in particular, that enzymatic hydrolysis of phytic acid during fermentation is caused both by endogenous and microbial phytase (mainly lactic acid bacteria phytase) (24-26). Another practical and effective process able to reduce tannins in sorghum grain is the treatment with wood-ash slurry. The action is probably caused by its alkali content able to detoxify tannins (27).

While for the reduction of ANF risk adequate post-harvesting practices are required, for mycotoxins control, both good pre- and post- harvesting practices are needed. Prevention is the best practice to improve the safety of vegetable and animal product from mycotoxins contamination. Adequate good practices should be applied in every phase of the food chain: harvesting, processing, storage and distribution in order to carry out an ideal integrated risk management. Examples of pre-harvest measures include the selection of cultivars less susceptible to grain moulds and good crop management practices, such as crop rotation and right harvesting time (28); while, post-harvesting practices include the good management practices in order to avoid grain damage during threshing and from insect damage during storage, the selection of not mouldy and damaged panicles, the use of certain feed additives like toxin adsorbents (28, 29). More recently, different physical and chemical methods have been proposed for their ability of detoxifying mycotoxins from food constituents. Biotechnological methods based on microbial fermentation, to detoxify contaminated grains, or addition of binding agents such as bentonite clay, aluminosilicates, activated charcoal and bacteria, to absorb the mycotoxins, have been investigated (30,31). Mycotoxin binders represent an attractive short-term solution to the challenge of mycotoxin-contaminated food, while a better solution would be the achievement of improved quality control and good practices.

In conclusion, an adequate combination of good pre- and post-harvest practices are the successful strategy for minimizing the risk of adverse endogenous factors, such as ANFs and mycotoxins and improve the safety and nutritional quality of sorghum.

Conclusion

Given the important role of sorghum in the diet as a source of both macro- and micronutrients for many low-income people, an improvement of its safety and nutritional quality is needed. Moreover, because of its ample utilization both as feed and, more recently, as food, such improvement would have positive consequences also for more developed countries.

Sorghum grains offer a good nutritional value which is, nevertheless, partially impaired by the activity of inherent adverse factors such as ANFs and mycotoxins. In order to reduce their content and/or activities, and so increase the bioavailability of proteins and zinc and iron, a number of measures are available. The most effective ones are the implementation of good practices throughout the food chain. Good practices are applicable both in pre- and post-harvest phase and allow preventing and / or minimizing the risk of adverse endogenous factors activity. In this regard, it is necessary to assess and control, along the sorghum production chain, the phases considered critical for ANFs and mycotoxins. Only with an integrated assessment and management of risks the improvement of nutritional value of sorghum could be possible.

In addition, through cheap and easy to use methods the content and/or activity of ANFs can be drastically reduced and its nutritional quality improved. As the sorghum is mainly used as staple food in developing countries, its improved quality would allow reducing the risks for more vulnerable population groups, such as the infants (as sorghum porridge is largely used as weaning food), pregnant women and, more in general, undernourished people. The safety assessment of sorghum products has an important value especially for pregnant women, in order to «*minimize also adverse health impact on future generation*», according to the “sustainable food safety” approach (32).

In conclusion, the enhanced food safety and nutritional quality of sorghum will have a valuable impact also for the food security. Moreover, it is important to raise awareness of the sorghum as valuable indigenous crop and spread the optimal food management and processes, with the aim of combating food shortages and also revitalize local culinary traditions.

References

1. Brown RL. *Eco-economy: building an economy for the earth.* New York: WW Norton & Co.; 2001.

2. Worldwatch Institute. *State of the world. Innovations that nourish the planet*. Washington, DC: Worldwatch Institute; 2011.

3. Henley EC. *Sorghum: an ancient, healthy and nutritious old world cereal*. St.Louis, Missouri, JPZ Consulting Group: 2010.

4. Radhakrishnan MR, Sivaprasad J. Tannin content of sorghum varieties and their role in iron bioavailability. *J Agric Food Chem* 1980;28(1): 55-7.

5. Kumar R, D'Mello JPF. Anti-nutritional factors in forage legumes. In: D'Mello JPF, Devendra C (Ed.). *Tropical legumes in animal nutrition.* Wallingford, UK: CAB International; 1995. p. 95-133.

6. Coulibaly A, Kouakou B, Chen J. Phytic acid in cereal grains: structure, health or harmful ways to reduce phytic acid in cereal grains and their effect on nutritional quality. *American Journal of Plant Nutrition and fertilization technology* 2011;1(1):1-22.

7. Sanberg AS, Andersson H. Effect of dietary phytase on the digestion of phytate in the stomach and small intestines of humans. *J Nutr* 1988;118:469-73.

8. Selle P H, Ravindran V, Caldwell RA, Bryden WL. Phytate and phytase: Consequences for protein utilization. *Nutr Res Rev* 2000;13:255-78.

9. Hagerman AE, Butler LG. Determination of protein in tannin-protein precipitates. *Journal of Agricultural and Food Chemistry* 1980;28:944-7.

10. Hagerman AE, Butler LG. Condensed tannin purification and characterization of tannin associated proteins. *Journal of Agricultural and Food Chemistry* 1980;28:947-52.

11. Cousins BW, Tanksley TD Jr, Knabe DA, Zebrowska T. Nutrient digestibility and performance of pigs fed sorghums varying in tannin concentration. *J Anim Sci* 1981:53:1524.

12. Goldstein JL, Swain T. The inhibition of enzymes by tannins. *Phytochemistry* 1965;4:185-92.

13. Tamir M, Alumot E. Inhibition of digestive enzymes by condensed tannins from green and ripe carobs. *Journal of the Science of Food and Agriculture* 1969;20:199-202.

14. Griffiths DW, Moseley G. The effect of diets containing field beans of high or low polyphenolic content on the activity of digestive enzymes in the intestines of rats. *Journal of Agricultural and Food Chemistry* 1980;26:255-49.

15. Gong YY, Cardwell K, Hounsa A, Egal S, Turner PC, Hall AJ, Wild, CP. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. *BMJ* 2002;325:20-1.

16. Hendrickse RG. Kwashiorkor: the hypothesis that incriminates aflatoxins. *Pediatrics* 1991;88(2):376-9.

17. Council for Agricultural Science and Technology Task Force. *Mycotoxins: economic and health risks*. Ames, USA: CAST; 1989.

18. Wedad WH, Abdelhaleem H, Abdullahi El Tinay I, Abdelmoneim M, Elfadil Babiker E. Effect of fermentation, malt-pretreatment and cooking on antinutritional factors and protein digestibility of sorghum cultivars. *Pak J Nutr* 2008;7:335-41.

19. Mohammed NA, Ahmed IAM, Babiker EE. Nutritional evaluation of sorghum flour (Sorghumbicolor L. Moench) during processing of Injera. *Int J Biolog Life Sci* 2010;6:35-9.

20. Abdel-Rahman IE. *Microbiological and biochemical change during fermentation of sorghum*. [M.Sc.Thesis]. Faculty of Agriculture. University of Khartoum, Sudan; 2000.

21. Mahgoub SEO, Elhag SA. Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. *Food Chemistry* 1998;61:77-80.

22. Henderson HM, Ankrah SA. The relationship of endogenous phytase, phytic acid and moisture uptake with cooking time in Vicia faba minor cv. Aladin. *Food Chemistry* 1985;17:1-11.

23. Elkhalil EAI, El Tinay AH, Mohammed BE, Elsheikh EAE. Note on the effect of malt pretreatment on tannin content of sorghum flour. *Univ of Khartoum J Agric Sci* 2003;11(1):144-8.

24. Abdalla AA, El Tinay AH, Mohamed BE, Abdalla AH, Effect of traditional process on phytate and mineral content of pearl millet. *Food Chemistry* 1998;63:79-84.

25. Leenhardt F, Levrat Verny MA, Chanliaud E, Remesy C. Moderate decrease of pH by sourdough fermentation is sufficient to reduce phytate content of whole wheat flour through endogenous phytase activity. *J Agric Food Chem* 2005;53(1):98-102.

26. Towo E, Matuschek E, Svanberg U. Fermentation and enzyme treatment of tannin sorghum gruels: effect of phenolic compounds, phytate and *in vitro* accessible iron. *Food Chemistry* 2006; 94:369-76.

27. Mukuru SZ, Butler LG, Rogler JC, Kirleis AW, Ejeta G, Axtell JD, Mertz ET. Traditional processing of high-tannin sorghum grain in Uganda and its effect on tannin, protein digestibility, and rat growth. *J Agric Food Chem* 1992;40:1172-5.

28. Ravinder Reddy CH, Rajashekhar Reddy, Ashok SA, Farid Waliyar, CLL Gowda, Belum VS Reddy, Rai KN. Mycotoxins in sorghum and pearl millet: effects on human and animal health and their management [poster]. In: *Global theme on crop improvement, 2007*. Available from http://www.icrisat.org/feedcrops/publications/Mycotoxins.pdf; last visited 09/11/2012.

29. Magan N, Aldred D. Post-harvest control strategies: Minimizing mycotoxins in the food chain. Mycotoxins from the Field to the Table, 20 October 2007. *International Journal of Food Microbiology* 2007;119:131-9.

30. Bhat RV, Shetty HPK, Vasanthi S. Human and animal health significance of mycotoxins in sorghum with special reference to fumonisinsin. In: Chandrashekar A, Bandyopadhyay R, Hall AJ (Ed.). *Technical and institutional options for sorghum grain mold management: proceedings of an international consultation*. Andhra Pradesh, India: ICRISAT: 2000. p. 107-15.

31. Galvano F, Piva A, Ritieni A, Galvano G. Dietary strategies to counteract the effects of mycotoxins: a review. *Journal of Food Protection* 2001;64(1):120-31.

32. Frazzoli C, Petrini C, Mantovani A. Sustainable development and next generation's health: a longterm perspective about the consequences of today's activities for food safety. *Ann Ist Super Sanita* 2009;45(1):65-75.

Paper II

Modulation of sorghum biological activities by varieties and traditional processing methods: an integrated *in vitro*/modelling approach

I. Proietti[1,2*], S. Tait[2], A. Mantovani[2]

1 Agrisystem, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29122, Piacenza, Italy
2 Food and Veterinary Toxicology Unit, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy

ABSTRACT

Keywords
Biological activities
DLD-1 cell line
Estimated iron bioavailability
Sorghum

Five sorghum cultivars (*Sorghum bicolor* (L.) Moench) from Nigeria, Senegal, Burkina Faso and Italy (two), were characterized for their nutritional values by analysing the modulation of cellular functional markers as well as estimated iron bioavailability, also considering the impact of food processing methods as fermentation and cooking. Glutathione peroxidase activity and total protein content were assayed in DLD-1 cell line following treatment with the different sorghum varieties. The estimated iron bioavailability was calculated through the application of an algorithm. The results revealed that both variety and food processing affect most of the tested parameters. The African varieties, in particular the Senegal one, showed the most promising nutritional profile, whereas the two Italian varieties overall showed a poor nutritional profile. The estimated iron bioavailability was significantly improved by the fermentation process, whereas cooking had no substantial impact. On the other hand, fermentation process had a negative impact on both cellular functional markers while cooking only on the cellular total protein content. The results indicate that selection of traditional varieties and processing methods can have a significant impact on parameters relevant to sorghum nutritional value.

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop after maize, rice, wheat and barley, in terms of production (FAO, 2012). Indeed, total world annual sorghum production is about 60 million tons from 46 million ha cultivated areas, the most important producers being United States, Nigeria, Sudan, Burkina Faso, China, India and Ethiopia (Dicko *et al.*, 2006). Numerous varieties of sorghum exist worldwide (more than 7000 identified; Kangama and Rumei, 2005); however, especially in low-income countries, these are often traditional varieties (or landraces) adapted to local conditions, seldom scientifically characterized (ICRISAT, 1996).

In Africa and Asia, sorghum is grown primarily for human consumption and is an important component in the diet of many people who rely on it as a main source of micro- and macronutrients (Godwin and Gray, 2000). Traditional food preparation of sorghum is pretty varied and include thin and stiff porridge, e.g. bouillie (Africa and Asia) and tô (West Africa), couscous (Africa), injera (Ethiopia), baked products (USA and Africa) and traditional African beers, such as dolo, tchapalo and burukutu (Dicko *et al.*, 2006). The porridge, in particular, is a key component of the traditional diet of adults as well as young children in many low-income countries; in Africa, sorghum porridges are usually prepared by cooking slurry of fermented or unfermented flour in boiling water (Dicko *et al.*, 2006).

Sorghum is a valuable staple food, due to its content of protein, pro-vitamins (carotenoids)

*Corresponding author: Tel.:+39 649902405; fax: +39 649902363
E-mail address: ilaria.proietti@unicatt.it (I. Proietti).

and vitamins, both fat-soluble (D, E and K) and of B group (except for B12), and minerals, such as iron, phosphorus and zinc. Nevertheless, owing to the presence of endogenous anti-nutritional factors, mainly tannins and phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate: IP6), the nutritional value is partly impaired. In fact, both tannins and phytates have the ability to chelate bivalent cations, in particular iron and zinc, to form complexes with macromolecules, such as proteins and digestive enzymes. The binding to essential elements and proteins determines the reduction of their bioavailability in the human body, thus potentially leading to deficiencies in population where sorghum represents a main source of nutrition. Indeed, according to Butler (1990), the amount of chelating factors in some sorghum varieties is sufficient to cause significant anti-nutritional effects in humans, especially if the diet is poor in minerals and/or inadequate in proteins.

In order to estimate mineral bioavailability in food, several modelling approaches have been previously used, mainly consisting in mathematical models taking into account the quantitative assessment of factors able to influence minerals absorption, such as tannins and phytates. Such models include the application of algorithms (Hallberg and Hulthén, 2000). Even though the ability of tannins and phytates to chelate trace minerals is related to negative effects in some circumstances (especially in poor varied diets and in minerals deficiency conditions), it may result beneficial in others. Indeed, chelating agents may also act as antioxidant and scavengers of free radicals, as well as reduce the bioavailability of toxic metals such as lead and cadmium (Shahidi and Wanasundara, 1992; Rimbach and Pallauf, 1997; Scharf *et al.*, 2003; Bohn *et al.*, 2008; Devi *et al.*, 2011). However, the knowledge about the whole effects of sorghum chelating agents is insufficient.

The available literature reports that fermentation may reduce the presence of chelating factors, thus increasing the iron bioavailability, whereas cooking may reduce protein digestibility (Abdelhaleem *et al.,* 2008; Mohammed *et al.,* 2010). Nevertheless, these data were obtained through chemical analyses; the net effect of fermented and/or cooked sorghum as whole food, after *in vitro* gastrointestinal digestion, has never been investigated in biological systems. Moreover, to our best knowledge, no data exist on the integrated and comparative assessment of the role of variety and main traditional processing methods on the sorghum biological effects.

The objective of the present work was to characterize sorghum varieties of different origins (African and Italian) and subjected to traditional processing methods (fermentation and cooking) in order to identify the combination more suitable to improve the nutritional value of sorghum porridges. Therefore, we performed a simulated gastrointestinal digestion of each sample followed by an integrated *in vitro*/modelling approach. As *in vitro* model, a human cell line representative of the intestinal epithelium (DLD-1) was selected; intestinal epithelium is the primary site of exposure to substances, beneficial or detrimental, present in foods. In particular, the DLD-1 cell line is an established *in vitro* model to investigate metabolism and toxic effects in the intestinal epithelium (Raju *et al.*, 2009; Zhang and Chen, 2011). Sorghum varieties and processing methods were evaluated for their ability to modulate the total cellular protein content, indicator of alteration of enzymatic functions and protein synthesis (Zbasnik et al., 2009), as well as the Glutathione Peroxidase (GPx) activity, an important enzyme involved in oxidative responses processes (Cai *et al.*, 2002), in DLD-1 cells. The *in vitro* results were integrated by the iron bioavailability estimate through the use of algorithm, as an indicator of the modulation of sorghum nutritional value. Moreover, the results are discussed in comparison with analytical assays and phytate/iron and phytate/zinc molar ratios as obtained in a previous study (Proietti *et al.*, 2012).

2. Materials and Methods

2.1. Samples and Standard substances

Five sorghum varieties were investigated. Grains of three African traditional varieties were randomly purchased from local markets in Lagos (Nigeria - N), Dakar (Senegal - S) and Ouagadougou (Burkina Faso - B) in February-April 2011. Grains of two Italian varieties were obtained from the Università Cattolica del Sacro Cuore, Piacenza (Italy), namely Aralba (genotype for grain yield- Ar) and ABF 306 (biomass sorghum hybrid selected by the Institute of Genetics, Università Cattolica del Sacro Cuore - ABF), harvested in September 2010. Whole grains of each variety were cleaned from damaged seeds and extraneous materials and then milled with an automatic pestle mill with internal parts made in agate – model RM100 by Retsch GmbH & Co. (Haan, Germany) – was used.

Porcine enzymes: pepsin (EC 3.4.23.1) and pancreatin (EC 3.1.1.3), bile salts and alpha-amylase (EC 3.2.1.1) from *Bacillus subtilis,* as well as phytic

acid and catechin were supplied from Sigma-Aldrich (Steinheim, Germany).

2.2. Treatments

Fermentation and cooking of the porridges in this study were conducted according to traditional method as commonly practiced in African countries (FAO, 1999). For each variety, slurry was obtained by mixing 100 g of the flour with 200 mL of water.

- *Fermentation*

The slurry was covered and kept for 72 hours at room temperature and away from light for spontaneous fermentation to occur. The pH reduction to an average value of 4.2 was a sign of occurred fermentation.

- *Cooking*

The slurry was further added with 100 mL water and cooked with constant stirring for 10 min at approximately 95 °C to obtain porridges.

- *Subdivision of samples according to treatment*

For each variety of sorghum, four samples were so obtained, namely *fc* (fermented and cooked porridge), *c* (unfermented cooked porridge), *f* (fermented slurry), and *s* (slurry). Samples were stored at - 80 °C prior lyophilization.

2.3. *In vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion was performed according to the method of Crews *et al.* (1996) with small modifications. Briefly, 5mL of gastric juice (1% w/v pepsin in 0.15 M NaCl, pH=2) were added to 1g sample and then incubated in a shaking water bath at 37°C for 4 h to allow pepsin digestion. Procedural blanks were run in parallel. Prior the intestinal digestion, the pH of the gastric digest was adjusted to 6.8 by addition of $NaHCO_3$ 2 M. Then, 5 mL of intestinal juice (3% w/v pancreatin, 1.5% w/v amylase, 1% w/v bile salts in 0.15M NaCl) were added to each sample and incubation was continued for further 4 h. Samples were then centrifuged at 8000 × g for 30 min at 4°C and the supernatant was filtered through a 0.45 µm-pore-diam. membrane filters. The supernatants (soluble fraction) were stored at -80 °C until use in cell assays.

2.4. Cell line and culture conditions

The DLD-1 cell line (derived from a human colorectal adenocarcinoma) was kindly obtained from Prof. Maria Marino (Università Roma Tre, Roma, Italy). Cells were cultured in RPMI 1640 medium without phenol red (Gibco, LifeTechnologies, Paisley, UK), supplemented with 10% Fetal Bovine Serum (Gibco), 2mM-glutamine (Gibco), 100 U/ml penicillin and 100g/ml streptomycin (Gibco). Cells were maintained in a humidified Steri-Cult 200 Incubator (Forma Scientific, Marietta, OH, USA) at 37°C and 5% CO_2.

2.4.1. Growth inhibition assay and working concentration determination

As a preliminary step, all samples (*fc, c, f, s*) were tested in order to determine the most suitable concentration to be used on DLD-1 cells, allowing studying anti-nutritional relevant activities in the absence of effects on cell viability.
Standard solutions of 0.5 mg/ml were prepared for each sorghum sample according to a previous study by Rafi *et al.* (2008) using sorghum samples for cell treatments. The maximum concentration to be used in cell cultures and obtainable by filtering with 0.22 µm filters without losing material, was equal to 1:25 of standard solution, that is 20 µg/ml. Such filtered solutions were utilized to prepare other sequential two-fold diluted solutions ranging from 20 to 2.5 µg/ml (1:200 with respect to standard solutions).

Cytotoxicity was evaluated by the MTS assay (Cell Titer 96 Aqueous One Solution assay, Promega, Madison, WI, USA) according to manufacturer's instructions. Briefly, DLD-1 were plated at 5,000 cells per well in RPMI 1640 medium in a 96 flat-bottomed multiwells and incubated overnight at 37 °C to permit cell adhesion. All treatments were performed in triplicate, incubating the cells for 72h at 37 °C.
At the end, 20µl of the MTS reagent were added to each well incubating for 2h at 37 °C. Cell viability was determined by reading absorbance at 490 nm by a Victor 3 Multilabel Reader (PerkinElmer, Waltham, MA, USA).
All concentrations, except for the 2.5 µg/ml one, affected cell proliferation (data not shown). Accordingly, 2.5 µg/ml was selected as working concentration for all samples.

2.4.2. Cell line treatments

DLD-1 cells were treated with 2.5 µg/ml solubilized samples in culture medium, after *in vitro* gastrointestinal digestion,), or with medium alone as control (herewith CTRL), and incubated for 72h at 37 °C. Treatments were performed in triplicate in order to

obtain three biological replicas. After 72h, DLD-1 medium supernatants were collected and stored at -80 °C, whereas DLD-1 monolayers were trypsinized, harvested, counted and stored at -80 °C until use.

2.4.3. Protein quantification and Glutathione Peroxidase Activity Assay

DLD-1 monolayers from each sample treatment were lysed in 100 µl RIPA Buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% nonyl phenoxy polyethoxy ethanol, supplemented with 1 mM phenylmethylsulfonylfluoride) and homogenized with a 2-mL sterile syringe. After 1-hr incubation on ice, lysates were centrifuged at 13,000 rpm, 4°C, for 20 min. Obtained supernatants were assayed for their total protein content by the BCA Protein Assay Kit (Pierce, Thermo, Rockford, IL).

DLD-1 supernatants from each sample treatment were assessed for GPx (EC 1.11.1.9) activity through the Glutathione Peroxidase Activity Assay (Biovision, Mountain View, CA, US) following manufacturer's instruction and measuring absorbance at 340 nm. One unit of GPx activity is defined as the amount of enzyme that causes the oxidation of 1.0 µmol of NADPH to NADP+ per minute at 25°C. GPx activity was obtained by the equation:

$$\text{GPx Activity} = \frac{(B - B0)}{(T2 - T1) \times V} \times \text{Sample dilution (mU/ml)}$$

Where T1 is the time of first reading (in min); T2 is the time of second reading (in min); B is the NADPH amount that was decreased between T1 and T2 (in nmol); B0 is the background change (without Cumene Hydroperoxide substrate) between T1 and T2; V is the sample volume added into the reaction well (in ml).

2.5. Study of algorithms

Dietary iron absorption was predicted applying the algorithm proposed by Hallberg and Hulthén (2000). Among the parameters considered in the algorithm only the parameters contained in sorghum are taken into account in this study, namely phytates, tannins and calcium, according to the formula:

Abs Fe (%) = 22.1 * f IP6 * f tannin * f Ca

where:
f IP6 = 10^ [-0.30*log (1+phytate-P)]
f tannin = 10^ [0.4515-0.715*log tannic acid (mg)
f Ca = 0.4081+[0.5919/(1+10^-(2.022-log(Ca+1))*2.919)]
In the computation of f IP6, 1mg of phytate-P= 3.53mg phytic acid

The study of the algorithm has been reported to 50g of DM as corresponding to about 250g of porridge, assumed as the mean quantity likely to be ingested by an adult in a meal.

2.6. Analytical determinations used in the algorithm study

The analytical methods for the determination of phytate, iron binding phenolic groups and mineral content were described in detail in a previous study (Proietti *et al.*, 2012). Briefly, phytate content was measured by the determination of myo-inositol-hexaphosphate (IP6) using high performance anion-exchange chromatography (Dionex AS50, using an AS-11 pre-column and column kit; Dionex, Sunnyvale, USA) (Lestienne *et al.* (2005). The content of iron binding phenolic groups, i.e. catechol (ortho-dihydroxyl) and galloyl (trihydroxyl) groups was determined with the modified ferric ammonium sulfate (FAS) method (Brune *et al.*, 1991); catechols were expressed as mg of catechin equivalents (CE)/100g DM and galloyls as mg of tannic acid equivalents (TAE)/100g DM. Total calcium content was determined according to (Hama *et al*, 2011), using an Atomic Absorption Spectrophotometer (Perkin-Elmer Analyst 800) with a deuterium background corrector and identified by air–acetylene flame.

2.7. Data analysis and statistics

Results of *in vitro* assays are reported as the mean of triplicate determinations ± SEM.

A distribution-independent non-parametric Wilcoxon Rank Sum test was applied to compare statistical differences among samples. A p-value ≤ 0.05 was set as significance level. Statistical analysis was performed by using the JMP 9.0 statistical software (SAS Institute Inc.).

The results of the study of algorithm are single values to be used in a qualitative comparison among varieties and treatments; thus, no statistical analysis was performed.

3. Results

3.1. In vitro analysis

3.1.1. Quantization of total protein content

Results are summarized in Table 1. Sorghum vari eties significantly influenced the total protein content of exposed DLD-1 colon cells. Overall, cells ex-

posed to the two Italian cultivars (Ar and ABF) showed a significant decrease in total protein content as compared to those exposed to African samples (N, S and B) or to control cells. In particular, DLD-1 exposed to Ar showed the lowest total protein

Table 1.
Effect of variety and food processing methods on total protein content (μg/ml) of DLD-1 cells exposed to sorghum

Treatment		Variety					
	Across varieties	CTRL	N	S	B	Ar	ABF
Across processes		2211.8±211^{A}	2260.1±78.9^{A}	2073.8±167^{A}	1683.3±170^{AB}	729.0±71.6^{C}	1306.8±98.4^{B}
CTRL	2211.8±211^{a}						
s	1674.3±153^{ab}		2300.0±182	1469.0±113	2170.3±116^{a}	951.9±272	1221.4±477
c	1569.2±210^{b}		2078.9±331	2488.7±70	1136.8±22^{b}	548.1±267	1393.2±436
f	1633.6±179^{b}		2428.7±102	1684.8±382	2237.5±242^{a}	707.3±148	1316.5±342
fc	1470.4±194^{b}		2303.6±316	1803.8±599	1188.5±453^{b}	708.7±215	1296.1±287

Mean values ± SEM of three replicates. Values followed by different small letters in the same column, and capital letter in the same row, are significantly different ($p \leq 0.05$).

content, significantly different from cells exposed to all other varieties. Besides, ABF induced a reduction in protein content significantly different with respect to control cells and to DLD-1 exposed to N and S samples.

Across varieties, either fermentation and/or cooking of sorghum samples significantly reduced the protein content of exposed cells as compared to control cells. Analysis of processing effects within the same sorghum variety showed that only DLD-1 exposed to B samples were significantly different in total protein content among treatments. In particular, cells exposed to cooked porridge, with (*fc*) and without fermentation (*c*), had total protein content significantly lower than slurry (*s*) and fermented slurry (*f*). On the other hand, comparisons among different varieties across the same treatment did not reveal any statistical difference.

3.1.2. GPx activity

Results are summarized in Table 2. Exposure to the different sorghum varieties did not significantly influence the GPx activity in DLD-1 cells. On the contrary, when analysing for food processing effects across varieties, fermented samples (*f*) significantly reduced GPx activity in DLD-1 with respect to cooked samples (*c, fc*), slurries (*s*) and control cells. When considering the effect of processes on GPx activity in DLD-1 cells within the same sorghum variety, only ABF fermented slurry (*f*) induced a significant reduction in GPx activity, being almost null, with respect to cooked porridges (both *c* and *fc*). On the other hand, no statistical difference was found among DLD-1 cells exposed to different varieties across the same treatment.

Table 2.
Effect of variety and food processing methods on GPx activity (mU/ml) of DLD-1 cells exposed to sorghum

Treatment		Varieties					
	Across varieties	CTRL	N	S	B	Ar	ABF
Across processes		3.6 ± 0.8	2.5 ± 0.5	3.1 ± 0.7	2.1 ± 0.4	4.0 ± 0.8	3.0 ± 0.8
CTRL	3.6 ± 0.8[a]						
s	3.1 ± 0.6[a]		3.6 ± 2.3	1.2 ± 1.1	2.9 ± 1.5	4.8 ± 3.8	2.5 ± 0.6[ab]
c	3.6 ± 0.5[a]		3.8 ± 0.9	4.3 ± 1.1	3.3 ± 1.5	2.0 ± 3.1	5.0 ± 2.5[a]
f	1.5 ± 0.6[b]		0.9 ± 1.0	2.5 ± 3.1	0.7 ± 0.3	4.2 ± 1.7	0.00 ± 1.9[b]
fc	3.4 ± 0.5[a]		1.6 ± 0.6	4.3 ± 2.1	1.6 ± 0.9	5.1 ± 1.9	4.6 ± 1.6[a]

Mean values ± SEM of three replicates. Values followed by different small letters in the same column are significantly different ($p \leq 0.05$).

3.2. *Study of algorithms*

Results from the algorithm, expressed as percentage, showed little difference in iron bioavailability among varieties, except for S (Table 3). The variety from Senegal showed, indeed, an approximate 3-fold higher iron bioavailability in all processed samples, with respect to other varieties.

Among food processes, fermentation, alone (*f*) or in combination with cooking (*fc*), improved the estimated bioavailability of iron with respect to slurries (*s*), whereas cooking had no susbstantial impact.

In particular, the increase of estimated bioavailability in fermented samples (*f*), with respect to corresponding slurries (*s*), ranged among varieties from 34% to 77%, with S showing the lowest increase and N the highest (Figure 1). Also fermented and cooked porridges (*fc*) showed a comparable increase of the estimated iron bioavailability with respect to corresponding slurries (*s*), ranging from 18% (S) to 81% (N).

Table 3.
Percentage (%) of bioavailable iron in 50g of DM of sorghum varieties undergoing different treatments[A]

Variety	*fc*	*c*	*f*	*s*
N	1,1	0,5	1,1	0,6
B	0,7	0,6	0,9	0,6
S	3,2	3,0	3,6	2,7
Ar	1,0	0,7	1,0	0,6
ABF	0,7	0,5	0,7	0,4

[A] treatments: *fc* (fermented and cooked porridge), *c* (cooked porridge), *f* (fermented slurry) and *s* (slurry).

4. Discussion

The present study evidenced how variety and processing methods may affect parameters relevant to sorghum nutritional value, including selected markers in DLD-1 cell line, used as model of intestinal epithelium, as well as bioavailability calculations. Moreover, the present study applied a simulated gastrointestinal digestion, coupled to food processing methods, to mimic human digestion and traditional food preparation, respectively, with the aim of improving characterization of sorghum as "whole food".

The two Italian sorghum varieties, namely ABF and Ar, showed the worst profiles as they induced a significant decrease in the cellular protein content of DLD-1 colon cells with respect to control cells, at concentrations devoid of cytotoxicity, as assessed by MTS assay.

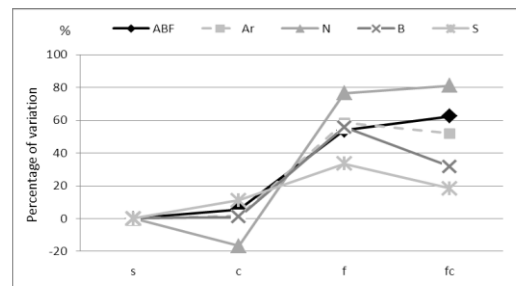


Figure 1. Percentage of variation of bioavailable iron in sorghum varieties undergoing different treatments[A]. Slurry (*s*) of each variety is taken as a reference value; the percentages of variation of bioavailable iron in each treatment were calculated with respect to (*s*).

The decrease of total protein content in cells exposed to sorghum samples can be linked to a reduced bioaccessibility of proteins from the culture medium, due to the antinutrient effect

of tannins (Butler, 1990) as well as of phytates (Morales *et al.*, 2011). On the other hand, it cannot be excluded a direct effect of specific sorghum components on the synthesis and metabolism of cell cycle proteins, which might result in a modulation of cellular protein levels (see, e.g., Vucenik *et al.*, 2005). Pamplona and Barja (2006) found that a methionine decrease reduced mitochondrial oxidative stress. In the present study no difference was exerted by the tested sorghum varieties on DLD-1 GPx activity; thus, the protein content decrease observed is unlikely connected to a change in the oxidative stress response. In a previous study, Ar cultivar also displayed the highest phytate to minerals (both Zn and Fe) molar ratio, corresponding to significantly lower amount of bioavailable elements for the human organism following food intake, and ABF the highest content of chelating factors (both phytates and iron binding phenolic groups), lending further support to the poor nutritional profile of these Italian cultivars (Proietti *et al*., 2012).

On the contrary, S variety (from Senegal) presented the best characteristics since it did not alter the total protein content and the GPx activity in DLD-1 colon cells, moreover showing an approximate 3-fold higher percentage of estimated iron bioavailability in all treatments than the other varieties, which showed similar values. Previous evidence also showed that S had a very low content of chelating factors and phytate to minerals ratios, thus confirming the potential interest of this landrace (Proietti *et al*., 2012).

The overall better profile of African varieties compared to the Italian ones and the differences among African varieties from diverse areas suggests that both genotype and pedoclimatic factors may influence parameters relevant to sorghum nutritional value. Indeed, many nutritional characteristics of plants are dependent by local agro-climatic conditions (Soetan and Oyewole, 2009) which, therefore, influence the intrinsic characteristic of each variety adapted to the local environment (landraces).

Findings showed that also processing was able to modulate the selected parameters. In particular, the fermentation process determined a two-way effect: i) overall, a negative impact was observed on physiological cellular functions, with a significant decrease in both DLD-1 total protein content and GPx activity with respect to control cells; ii) a positive impact on the estimated iron bioavailability was observed, as fermentation increase the percentage of estimated iron bioavailability of 34-77%, as resulted by the algorithm. Moreover, the effect of fermentation resulted to be also modulated by the variety.

A link between the glutathione-dependent redox status of the cells and iron homeostasis has been suggested; in particular, GPx activity was decreased in the presence of excess iron (Auchère *et al.,* 2008). It might be noteworthy the relationship between the increase of bioavailable iron and the significant decrease of GPx in fermented samples, even though an excess iron uptake was highly unlikely in our cells. Alternatively, reduced GPx activity in cells might be attributed to the diminished oxidative stress in cells or to the reduced GPx enzymatic functions. In this case, the interpretation of results would not be straightforward, as fermented samples also showed a significant reduction of cells total protein content. Fermentation, indeed, was found to increase the iron bioavailability; in a previous study on the same sorghum varieties (Proietti *et al*., 2012), fermentation increased also the bioavailability of zinc as well as reduced the content of chelating factors. Indeed, GPx decrease in overall fermented samples was likely due to the marked decrease in ABF variety. Considering that this variety showed a general poor profile, it cannot be excluded that the inhibition of GPx activity of cells exposed to ABF might be due to a particularly enhanced production of fermentation by-products acting as GPx inhibitors.

On the other hand, the increase of estimated iron bioavailability is in accordance with previous studies which show the beneficial effects of fermentation in reducing the content and activity of chelating factors, thus increasing the bioavailability of iron (Lestienne *et al*., 2005; Abdelhaleem *et al.,* 2008).

Similarly to fermentation, also cooking determined an overall a decrease in DLD-1 total protein content, alone or in combination with fermentation; cooked sorghum samples

did affect neither cellular GPx activity nor the estimated iron bioavailability.

It cannot be excluded that the different results observed *in vitro* and by modelling, are linked to the different investigation approaches. The biological effects observed *in vitro* models are correlated to the net effects of food components, including the bioaccessibility of anti-nutritional factors and the production of by-products, while the algorithm method produces mathematical values derived from analytical data.

As regards the interaction between variety and treatment, some effects were noteworthy. Interestingly, S, the variety showing the highest estimated bioavailable iron, exhibited the lowest increase of estimated bioavailable iron by fermentation. This may be due to a lower effect of food processing in varieties displaying good iron bioavailability.

The *in vitro* assays showed that the interaction between variety and treatment differs among varieties. Significant GPx activity decrease was observed only in fermented ABF samples, with respect to cooked samples, thus lending further support to the poor nutritional profile of this Italian cultivar. Moreover, analysis of processing effects on total protein content within the same sorghum variety showed that only DLD-1 exposed to B cooked samples displayed a significant reduction in total protein content.

In order to explain food processing effects *in vitro*, it might be hypothesized that fermentation and/or cooking induce the formation of tannin and/or phytate degradation products, and/or other process by-products, which may inhibit protein synthesis. Net effects of processed whole foods are an up-to-date issue, in particular for foods of vegetable origin that are rich of bioactive substances: one recent example is the antioxidant/anti-inflammatory action elicited by a polyphenol-rich beverage (Nemzer *et al.*, 2011). Therefore, according to our proof-of-concept study, it appears worthwhile to make detailed studies on the products formed during sorghum fermentation and cooking as well as to their effects *in vitro* and on the potential interference with sorghum nutritional profile of main sorghum contaminants, such as arsenic, cadmium and mycotoxins (Baldi and Mantovani, 2008).

5. Conclusions

The present results show that both variety and food processing methods are able to influence biological responses in a model of gut epithelial cells *in vitro* as well estimated iron bioavailability, with likely consequences on sorghum nutritional profile.

The variety from Senegal showed the most promising characteristics. On the other hand, the two Italian varieties (ABF and Ar) overall showed a poor profile.

Among food processes, fermentation, alone and associated with cooking, showed a markedly greater impact than cooking alone. Fermentation process showed a double effect on sorghum biological activities: reduction of *in vitro* parameters and increase of bioavailable iron. Finally, most tested parameters showed further modulation effects of food processing on the variety.

The results outlined the importance of sorghum landraces selection in order to minimize the biological impact of anti-nutrients and to increase the bioavailability of essential trace elements in sorghum. Furthermore, results showed that processes are also important to improve the nutritional characteristics of a sorghum meal; nevertheless, the effects of a “whole food” analysis may be complex and deserve further investigation.

Acknowledgements

This study was supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy). The authors wish to thank T. Djenaba (Senegal), E. Atoe (Nigeria) and S. Amaducci (Italy) for providing the sorghum varieties and to A. Raggi and F. Aureli for assistance with the in vitro gastrointestinal digestion. The authors also thank C. Mouquet, J.P. Guyot and K. Baye for their expert technical advices.

References

Abdelhaleem, W. H., El Tinay, A. H., Mustafa, A.I., Babiker, E.E., 2008. Effect of fermenta-

tion, malt-pretreatment and cooking on antinutritional factors and protein digestibility of sorghum cultivars. Pakistan Journal of Nutrition **7,** 335-341.

Auchère, F., Santos, R., Planamente, S., Lesuisse, E., Camadro, J.M., 2008. Glutathione-dependent redox status of frataxin-deficient cells in a yeast model of Friedreich's ataxia. Human Molecular Genetics 17, 2790–2802.

Baldi, F, Mantovani, A., 2008. A new database for food safety: EDID (Endocrine disrupting chemicals - Diet Interaction Database). Annali Istituto Superiore di Sanita 44: 57-63.

Brune, M., Halberg, L., Skanberg, A. B., 1991. Determination of iron-binding phenolic groups in foods. Journal of Food Science 56, 128-131.

Butler, G. L., 1990. Tannins and other phenols: Effects on sorghum production and utilization. INTSORMIL Annual Report 140-144. A technical research report of the grain sorghum/pearl millet collaborative research support program (CRSP).

Cai, W., Gao, Q.D., Zhu, L., Peppa, M., He, C., Vlassara, H., 2002. Oxidative Stress-Inducing Carbonyl Compounds from Common Foods: Novel Mediators of Cellular Dysfunction Molecular Medicine 8(7), 337–346.

Crews, H.M., Clarke, P.A, Lewis, D.J, Owen, L.M., Strutt, P.R. and Izquierdo, A., 1996. Investigation of selenium speciation in in-vitro gastrointestinal extracts of cooked cod by high performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray mass spectrometry. Journal of Analytical Atomic Spectrometry 11, 1177–1182.

Devi, P.S., Saravanakumar, M., Mohandas, S., 2011. Identification of 3- deoxyanthocyanins from red sorghum (*Sorghum bicolor*) bran and its biological properties. African Journal of Pure and Applied Chemistry 5, 181-193.

Dicko, M. H., Gruppen, H., Traoré, A. S., Voragen, A. G. J., van Berkel, W. J. H., 2006. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. African Journal of Biotechnology 5, 384-395.

FAO, 1999. Fermented cereals a global perspective. FAO agricultural services bulletin No. 138. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO, 2012. 2010 FAO statistics. Food and Agriculture Organization of the United Nations, Rome, Italy: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Accessed 10/10/2012.

Godwin, I. D, Gray, S. J., 2000. Overcoming productivity and quality constraints in sorghum: the role for genetic engineering. In: O'Brien L. & Henry R. J. (Eds.), Transgenic cereals. AACC, St Paul Minnesota USA, pp.153-177.

Hallberg L and Hulthén L, 2000. Prediction of dietary iron absorption: an algorithm for calculating absorption and bioavailability of dietary iron. American Journal of Clinical Nutrition 71(5), 1147-1160.

Hama, F., Icard-Vernière, C., Guyot, J.P., Picq, C., Diawara, B., Mouquet-Rivier, C., 2011. Changes in micro- and macronutrient composition of pearl millet and white sorghum during in field versus laboratory decortication. Journal of Cereal Science 54, 425-433.

ICRISAT, 1996. The World Sorghum and Millet Economies. International Crops Research Institute for the Semi-Arid Tropics and Food and Agriculture Organization of the United Nations. Patancheru, India and FAO, Rome.

Kangama, C.O., Rumei, X., 2005. Introduction of sorghum (*Sorghum bicolor* (L.) Moench) into China*.* African Journal of Biotechnology 4, 575-579.

Lestienne, I., Icard-Vernière, C. , Mouquet, C. , Picq, C. , Trèche, S., 2005. Effects of soaking whole cereal and legume seeds on iron, zinc and phytate contents. Food Chemistry 89, 421-425.

Morales G.A., Moyano, F.J., Marquez, L., 2011. *In vitro* assessment of the effects of phytate and phytase on nitrogen and phosphorus bioaccessibility within fish digestive tract. Animal Feed Science and Technology 170, 209–221.

Nemzer BV, Rodriguez LC, Hammond L, Disilvestro R, Hunter JM, Pietrzkowski Z., 2011. Acute reduction of serum 8-iso-PGF2-alpha and advanced oxidation protein products in vivo by a polyphenol-rich beverage; a pilot clinical study with phytochemical and in vitro antioxidant characterization. Nutrition Journal 10:67.

Pamplona, R., Barja, G., 2006. Mitochondrial oxidative stress, aging and caloric restriction: The protein and methionine connection. Biochimica et Biophysica Acta (BBA) - Bioenergetics 1757, 496-508.

Proietti, I., Mantovani, A., Rivier, C.M., Guyot, J.P., 2012. Modulation of chelating factors, trace minerals and their estimated bioavailability in Italian and African sorghum (*Sorghum bicolor* (L.) Moench) porridges. International Journal of Food Science and Technology (accepted).

Rafi, M.M., Hartman, T.G., Das, Y.T., Shafaie Y., Hargreaves J., 2008. Methods and compositions for preventing or treating various diseases or conditions in a patient with an alcohol ex-

tractable fraction of Sorghum bicolour grain. US Patent Application Publication. Pub. No.: US 2008/0241282 A1.

Raju, J., Bielecki, A., Caldwell, D., Lok, E., Taylor, M., Kapal, K., Curran, I., Cooke, G.M., Bird, R.P., Mehta, R., 2009. Soy isoflavones modulate azoxymethane-induced rat colon carcinogenesis exposed pre- and postnatally and inhibit growth of DLD-1 human colon adenocarcinoma cells by increasing the expression of estrogen receptor-beta. Journal of Nutrition 139(3), 474-81.

Rimbach, G., Pallauf, J., 1997. Cadmium accumulation, zinc status, and mineral bioavailability of growing rats fed diets high in zinc with increasing amounts of phytic acid. Biological Trace Element Research 57, 59–70.

Scharf, G., Prustomersky, S., Knasmuller, S., Schulte-Hermann, R., Huber, W.W., 2003. Enhancement of glutathione and gamma-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, by chemoprotective plant-derived food and beverage components in the human hepatoma cell line HepG2. Nutrition and Cancer-An International Journal 45, 74–83.

Shahidi, F., Wanasundara, P.K.J.P.D., 1992. Phenolic antioxidants. Critical Reviews in Food Science and Nutrition 32, 67–103.

Soetan, K.O., Oyewole, O.E, 2009. The need for adequate processing to reduce the antinutritional factors in plants used as human foods and animal feeds: A review. African Journal of Food Science 3, 223-232.

Vucenik I., Ramakrishna G., Tantivejkul K., Anderson L.M., Ramljak D., 2005. Inositol hexaphosphate (IP6) blocks proliferation of human breast cancer cells through a PKCδ-dependent increase in p27Kip1 and decrease in retinoblastoma protein (pRb) phosphorylation. Breast Cancer Research and Treatment 91, 35-45.

Zbasnik R., Carr T., Weller C., Hwang Kt, Wang L., Cuppett S. and Schlegel V., 2009. Antiproliferation Properties of Grain Sorghum Dry Distiller's Grain Lipids in Caco-2 Cells. Journal of Agricultural and Food Chemistry 57, 10435–10441.

Zhang, Y., Chen, H., 2011. Genistein attenuates WNT signaling by up-regulating sFRP2 in a human colon cancer cell line. Experimental Biology and Medicine (Maywood) 236(6), 714-22

Paper III

Modulation of chelating factors, trace minerals and their estimated bioavailability in Italian and African sorghum (*Sorghum bicolor* (L.) Moench) porridges

Ilaria Proietti[1,2*], Alberto Mantovani[2], Claire Mouquet-Rivier[3] & Jean-Pierre Guyot[3]

1 Agrisystem, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29122, Piacenza, Italy
2 Food and Veterinary Toxicology Unit, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy
3. IRD, UMR 204 « Prévention des Malnutritions et des Pathologies associées » (Nutripass), IRD/Université Montpellier 2/ Université Montpellier 1, BP 64501, 34394 Montpellier Cedex 5, France

Summary Factors able to modulate chelating factors, trace minerals and their bioavailability were investigated in porridges of five sorghum (*Sorghum bicolor* (L.) Moench) varieties: from Nigeria, Senegal, Burkina Faso and two from Italy. Effects of variety and traditional fermentation and cooking were assessed on iron-binding phenolic groups, phytates and iron and zinc content and bioavailability. Chelating factors, trace elements as well as the effect of processing (mainly fermentation) were modulated by variety. Fermentation decreased iron-binding phenolic groups until 49% and phytate content until 72% as well as increased phytase activity 3.4 to 16.4 fold, leading to enhancement of iron and zinc estimated bioavailability. Cooking alone had almost no effect. The lowest chelating factors content and the highest trace minerals bioavailability were shown by fermented Senegal landrace, whereas the Italian varieties overall showed the worst results. The results indicate that selection of traditional varieties and appropriate processing methods can improve sorghum nutritional value.

Keywords Bioavailability, Fermentation, Iron, Phytates, Phytase, Sorghum, Tannins, Zinc

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important grain crop in the world agricultural economy after maize, rice, wheat and barley (FAO, 2012). It is mainly grown in semi-arid areas of the world, where climate conditions are less favorable to the production of other cereals. In particular, in Africa, sorghum is grown primarily for human consumption and is an important component of diet (FAO, 1995; Dicko *et al.,* 2006); it is grown also in industrialized countries like Italy, where it is mainly utilized for the

*Correspondent: Fax: + 00 39 (0)6 4990 2363
e-mail: ilaria.proietti@unicatt.it

Food and Veterinary Toxicology Unit, Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy

production of animal feed (forage or grain) or biofuel (Janssen *et al.*, 2010). However, since sorghum is gluten-free, a possible use in celiac diet has been envisaged (Fenster, 2003).
There are numerous varieties in use throughout the world (more than 7000 sorghum varieties have been identified - Kangama and Rumei, 2005); however, especially in developing countries, sorghum lines are often traditional varieties (or landraces) adapted to local conditions, rarely scientifically characterized (ICRISAT, 1996; Nkongolo *et al.*, 2008).
It is estimated that sorghum is part of the staple diet of more than 300 million people in developing countries, representing a major source of en-

ergy and nutrients (Dicko *et al.,* 2006). Sorghum is a valuable staple food, for its content of protein, pro-vitamins (carotenoids) and vitamins, both fat-soluble (D, E and K) and of B group (except for B12), and minerals, such as iron, phosphorus and zinc. Nevertheless, its nutritional value is impaired by the activity of endogenous chelating factors, such as phenolic compounds and phytic acid, which interact negatively with the absorption of essential elements, in particular iron and zinc, and the digestibility of proteins (Sandberg, 2002; Gilani *et al*, 2005). Phenolic compounds containing catechol and galloyl groups are considered those mainly involved in iron and zinc binding (Brune *et al.*, 1991; Slabbert, 1992).

The amount of chelating factors in some sorghum varieties is enough to cause significant antinutritional effects, especially if the diet is poor in minerals and/or inadequate in protein (Butler, 1990).

Main sorghum-based recipes include couscous, porridge and beer. The porridge, in particular, is a staple component of the traditional diet, especially breakfast, for adult as well as young children; indeed it is used as complementary food, i.e. in complement to breast milk, in breastfed children. In Africa, sorghum porridges are usually prepared by cooking slurry of fermented or unfermented flour in boiling water (Rooney *et al.,* 1986; Asante, 1995). Sorghum nutritional characteristics, including the content of some chelating factors, may be related to the variety (Awadelkareem *et al.*, 2009) and/or be modulated by processing methods, such as the reduction of phytate content during fermentation and protein digestibility during cooking (Abdelhaleem *et al.,* 2008; Mohammed *et al.,* 2010). However, only limited data exist on the integrated and comparative assessment of the role of variety and main traditional processing methods on the sorghum nutritional characteristics.

The objective of this work was to determine the factors able to modulate chelating factors and trace minerals in sorghum porridges of five varieties from Italy and West Africa upon different preparation processes; our aim was to identify varieties with appropriate processing methods in order to improve the nutritional value of sorghum porridges.

Materials and methods

Samples and standard substances

Five sorghum varieties were investigated. Grains of three African traditional varieties were randomly purchased from local markets in Lagos (Nigeria - N), Dakar (Senegal - S) and Ouagadougou (Burkina Faso - B) in February-April 2011. Grains of two Italian varieties were obtained from the Università Cattolica del Sacro Cuore, Piacenza (Italy), namely Aralba (genotype for grain yield- Ar) and ABF 306 (biomass sorghum hybrid selected by the Institute of Genetics, Università Cattolica del Sacro Cuore - ABF), harvested in September 2010. Whole grains of each variety were cleaned from damaged seeds and extraneous materials and then milled with an automatic pestle mill with internal parts made in agate – model RM100 by Retsch GmbH & Co. (Haan, Germany) – was used.

Phytic acid (P-8810) and catechin (C-1251) were purchased from Sigma-Aldrich, Stockholm, Sweden and tannic acid (art. nr. 1.00773.0250) was obtained from Merck.

Treatments

Fermentation and cooking of the porridges in this study were conducted according to traditional method as commonly practiced in African countries (FAO, 1999). For each variety, slurry was obtained by mixing 100 g of the flour with 200 mL of water.

- *Fermentation*

The slurry was covered and kept for 72 hours at room temperature and away from light for spontaneous fermentation to occur. The pH reduction to an average value of 4.2 was a sign of occurred fermentation.

- *Cooking*

The slurry was further added with 100 mL water and cooked with constant stirring for 10 min at approximately 95 °C to obtain porridges.

- *Subdivision of samples according to treatment*

For each variety of sorghum, four samples were so obtained, namely *fc* (fermented and cooked porridge), *c* (unfermented cooked porridge), *f* (fermented slurry), and *s* (slurry). Samples were stored at - 80 °C prior lyophilization.

- *Lyophilization*

Frozen samples were lyophilized for 48 hours at –40 ºC in a freeze dryer Model Alpha 1-2 LD plus (Christ, Germany) and the resulted samples used for subsequent analysis.

- *Dry matter*

Dry matter (DM) contents were determined by oven drying at 105 C to constant weight.

Determination of iron binding phenolic groups

The content of iron binding phenolic groups, i.e. catechol (ortho-dihydroxyl) and galloyl (trihydroxyl) groups was determined with the modified ferric ammonium sulfate (FAS) method according to Brune *et al*. (1991) using a solution of 50% dimethylformamide (pH 4.4) as extraction solvent. Catechol and galloyl groups form iron complexes with FAS which can be read spectrophotometrically at two wavelengths: 578 nm and 680 nm, corresponding to the absorbance maxima of iron-galloyl and iron-catechol, respectively. The amount of the catechol and galloyl groups was calculated against standard curves of catechin and tannic acid, respectively, at the two wavelengths. Catechols are expressed as mg of catechin equivalents (CE)/100g DM and galloyls as mg of tannic acid equivalents (TAE)/100g DM.

Determination of phytate content

The determination of phytate content was performed according to the method of Talamond *et al*. (1998) with modifications: 0.2 g sample was extracted with 10 ml 0.5M HCl during stirring for 6 minutes in boiling water, followed by centrifugation at 4500 *g* for 20 min at 4°C. The supernatant was dried in a Speed-Vac (JOUAN RC 10-10, Saint Herblain, France) vacuum centrifuge at 45°C and the residue was reconstituted in 200 µL of Milli-Q water. After filtration through 0.2 µm pore-diameter filters, the filtrate was diluted 25x and phytate content was estimated by determination of myo-inositol-hexaphosphate (IP6) by high performance anion-exchange chromatography (Dionex AS50, using an AS-11 pre-column and column kit (Dionex, Sunnyvale, USA) according to the method detailed in Lestienne *et al*. (2005a).

Determination of phytase activity

Phytase activity was determined only in the flour and fermented slurries as endogenous phytases are deactivated by heat (Ryden and Selvendran, 1993). The activity was evaluated through spectrophotometry, by determining phosphorous release during incubation of phytase extract in a sodium phytate solution (pH 5.6) at a wavelength of 405 nm. Phytase extraction was performed from 2g of samples according to the method of Konietzny *et al*. (1995) with buffer modification (0.1 M acetate buffer, pH 5.6). Samples were stirred for 2 h at 4°C and centrifuged at 10,000*g* for 30 min at the same temperature. After eliminating phytates and free phosphorus from the supernatants by utilization of an AG 1-X8 anion resin (Bio-Rad Laboratories, Richmond, CA), 3 ml of resulted enzyme extracts were incubated for 1h at 50°C with 2 ml of 2.5 mM sodium phytate solution and 9 ml of 0.1 M acetate buffer at pH 5.6. Phosphorous released by phytase activity was measured according to the method of Heinonen and Lahti, (1981), where 1 phytase unit (PU) is equivalent to the enzymatic activity that liberate 1µM of phosphorus from phytic acid per minute, according to a standard range prepared with 5 mM KH2PO4 solution.

Determination of total iron and zinc content

Total iron and zinc contents were determined according to the method described in Hama *et al*, 2011. The two minerals were extracted with a closed-vessel microwave digestion system (ETHOS-1, Milestone, Italy), from about 0.4 g of flour in a 7:1 nitric acid/hydrogen peroxide

mixture, and digested in a microwave oven at 1200 W power for 30 min.
Iron and zinc content were analysed with an Atomic Absorption Spectrophotometer (Perkin-Elmer Analyst 800) with a deuterium background corrector and identified by air–acetylene flame.
The iron:zinc molar ratio (Fe/Zn) was calculated as relevant indicator of zinc bioavailability (Solomons and Jacob, 1981).

Molar ratios of phytate to iron and zinc

Molar ratios of phytate to iron (Phy/Fe) and phytate to zinc (Phy/Zn) were calculated in order to estimate the bioavailability of these two trace elements (Davies and Olpin, 1979; Saha et al., 1994). The levels of phytic acid, iron and zinc have been converted in mole by using a molecular mass unit of 660 for phytic acid and an atomic mass unit of 56 and 65 for iron and zinc, respectively.

Data analysis and statistics
For all analyses, the results are reported as the mean of triplicate determinations.
All values were calculated per 100 g DM. The approximate DM content in the different treatment samples were as follows: *fc* and *c* = 16%, *f* = 19 % and *r* = 30%.
Data were subjected to analysis of variance (ANOVA) and Tukey's HSD multiple range test was used to compare means at the 5% significance level (Wilkinson, 1990), using the JMP 7.0 statistical software (SAS Institute Inc.).

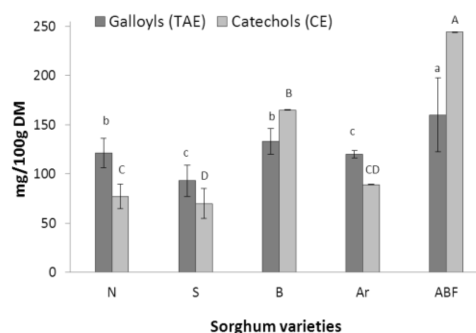


Figure 1. Content of iron binding phenolic groups in porridges of different sorghum varieties

Results and discussion

Content of iron binding phenolic groups

Varieties significantly influenced the amount of catechol and galloyl groups. S showed the lowest content of both iron binding phenolic groups, significantly ($p \leq 0.05$) different from ABF and B (Figure 1). ABF showed the highest content of both catechol and galloyl groups, significantly different from all other groups. The content of catechols in N and Ar was significantly lower than in ABF and B; these two varieties showed also a content of galloyls significantly lower than ABF.
The effect of treatment on catechol and galloyl groups in sorghum varieties are presented in Table 1. Fermentation, associated or not to cooking, significantly ($p \leq 0.05$) reduced the amount of both iron binding phenolic groups. On the contrary, no significant decrease could be seen when porridges were only cooked.

Compared to the slurry, the fermented slurry showed a significant reduction of galloyl groups in all varieties except for S, whereas after cooking, the reduction remained significant in B, Ar and ABF. Catechol groups appeared somewhat less affected by treatment: *f* significantly reduced the content in B only, and *fc* in B and ABF.
The results showed a marked influence of the variety on the amount of phenolic compounds; this finding has been previously reported for other varieties (Tomas-Barberan and Espín, 2001; Dykes *et al.*, 2005). Furthermore, data showed that different variety determined different reduction rates of iron binding phenolic groups during fermentation with and without cooking. Varieties with the highest content of phenolic groups, B and ABF showed also the highest reduction rates. We observed a reduction of phenolic compounds during fermentation (Table 1). On the other hand, cooking alone did not influence the concentrations of galloyl and catechol groups. Re-

duced phenolic content upon fermentation was also observed in previous papers (Towo *et al.*, 2006; Schons *et al.*, 2011): the decrease may be related to microbial activity during fermentation (Dhankher and Chauhan, 1987; El Hag *et al.,* 2002) or to abstraction of hydride ions and rearrangement of the phenolic structures due to the acidic environment (Towo *et al.*, 2006).

Phytate content and phytase activity

Variety significantly ($p \leq 0.05$) influenced also the content of phytates. ABF and S showed the highest and lowest phytate content, respectively, significantly different from all other varieties;

Table 1. Effect of treatments[A] on iron binding phenolic groups in porridges from different sorghum varieties

Galloyls (mg TAE/100g DM)					
Treatments	Varieties				
	N	S	B	Ar	ABF
s	159 ± 13^{a,b}	129 ± 10^{a}	172 ± 6^{a}	137 ± 3^{a}	194 ± 5^{a}
c	186 ± 14^{a}	116 ± 0.3^{a}	168 ± 3^{a}	127 ± 5^{a,b}	182 ± 14^{a,b}
f	121 ± 6^{c}	93 ± 5.0^{a}	133 ± 14^{b}	120 ± 6^{b,c}	160 ± 15^{b,c}
fc	126 ± 14^{b,c}	106 ± 26^{a}	133 ± 0.3^{b}	111 ± 8^{c}	131 ± 14^{c}
Catechols (mg CE/100g DM)					
Treatments	Varieties				
	N	S	B	Ar	ABF
s	151 ± 60^{a}	81 ± 11^{a,b}	221 ± 24^{a}	94 ± 9^{a,b}	254 ± 7^{a}
c	179 ± 41^{a}	56 ± 13^{c}	209 ± 11^{a,b}	104 ± 19^{a}	227 ± 16^{a,b}
f	77 ± 7.0^{a}	70 ± 8^{a,b,c}	165 ± 17^{b,c}	89 ± 11^{a,b}	244 ± 8^{a,b}
fc	95 ± 23^{a}	62 ± 12^{b,c}	149 ± 23^{c}	67 ± 11^{b}	212 ± 23^{b}

Mean values ± SD of three replicates.
Values followed by different letters in the same column significantly differ ($p \leq 0.05$), as assessed by Tukey's test.
[A] Treatments: *fc* (fermented and cooked porridge), *c* (cooked porridge), *f* (fermented slurry) and *s* (slurry).

indeed, the phytate content in S was 50 to 100-fold lower than the other varieties (Table 2).
Fermentation, alone and associated to cooking (*f* and *fc*), significantly ($p \leq 0.05$) reduced the phytate content in all varieties, with the exception of S where the phytate content was already very low in slurry. The extent of reduction differed among variety and ranged from 58 to 72%. In cooked porridge there was no significant difference with respect to slurry, with exception of N which showed a significant increase in phytate content (Table 2). Reduction in the phytate content was consistent with previous reports on other sorghum varieties (Mahgoub and Elhag, 1998; Egli *et al.*, 2003; Kayodé *et al.,* 2006; Towo *et al.,* 2006).
The decrease of phytate content during fermentation is explained by the enzymatic activity of both cereal and microbial phytases (Sandberg and Svanberg, 1991; Abdelhaleem *et al.,* 2008), which hydrolyse phytate (IP6) into inorganic phosphorus (Pi), inositol or *myo*-inositol phosphates (IP5 to IP1) (Lestienne *et al.,* 2005b).
A slight but not significant increase was also observed for B and Ar. Cooking of sorghum was indeed reported to induce a degradation of phytates (Mahgoub and Elhag, 1998; Towo 2006). However, it might be worth noting that Lestienne *et al.* (2005b) found an increase of IP6 in cooked soybean flour; attributed to a possible greater extraction of IP6 molecules during cooking. Thus, the occurrence of similar events in N variety might not be ruled out altogether.
Compared to other cereals, sorghum has low phytase activities (Egli *et al.,* 2003). Our findings showed that also the amount of phytase activity was influenced by the variety. Phytase activity in the flour and in fermented porridge of different

sorghum varieties is reported in Table 2. Flour phytase activity ranged from 0.17 to 0.21 PU/g; N and S showed the highest values, significantly ($p \leq 0.05$) different from the lowest value found in B (Table 2).
In fermented slurries phytase activity ranged from 0.64 to 3.44 PU/g: compared to the flour, a significant increase ($p \leq 0.01$) was found in all varieties. Fermented samples of N and ABF varieties showed the highest values, significantly different ($p \leq 0.05$) from all other varieties, while in S and Ar the phytase activity was significantly lower than in the other varieties (Table 2).
Therefore, S stood out as a variety with low amount of chelating factors and high phytase activity in unprocessed samples. However, S was also weakly susceptible to the increase of phytase activity in fermented porridge. With the exception of S, varieties showed no readily apparent relationship between the concentration of phytates and the phytase activity in the unprocessed samples, as well as between the reduction of phytate content and the increment of phytase activity in fermented slurries.

Table 2. Effect of treatment[A] on phytate content and phytase activity in porridges of different sorghum varieties

Treatment	Varieties				
	ABF	**Ar**	**B**	**N**	**S**
IP6*					
s	1900 ± 0.01^{aA}	1224 ± 0.02^{aB}	1176 ± 0.06^{aB}	1071 ± 0.06^{bB}	5.2 ± 0.000^{aC}
c	1848 ± 0.00^{aA}	1396 ± 0.07^{aB}	1211 ± 0.01^{aC}	1369 ± 0.06^{aBC}	4.3 ± 0.001^{aD}
f	710 ± 0.04^{cA}	361 ± 0.01^{bC}	490 ± 0.01^{cB}	302 ± 0.003^{cC}	2.5 ±0.001^{aD}
fc	962 ± 0.03^{bA}	501 ± 0.02^{bB}	871 ± 0.04^{bA}	252 ± 0.01^{cC}	3.7 ± 0.003^{aD}
Phytase activity**					
flour	0.2 ± 0.006^{aAB}	0.19± 0.015^{aAB}	0.17 ± 0.012^{aB}	0.21 ± 0.002^{aA}	0.2 ± 0.014^{aA}
f	3.18 ± 0.03^{bA}	0.64 ± 0.13^{bC}	2.23 ± 0.598^{bB}	3.44 ± 0.164^{bA}	0.79 ± 0.122^{bC}

Mean values ± SD of three replicates. Values followed by different small letters in the same column, and capital letter in the same row, are significantly different ($p \leq 0.05$).
[A] Treatments: *fc* (fermented and cooked porridge), *c* (cooked porridge), *f* (fermented slurry) and *s* (slurry).
* = expressed as mg/100g DM
** = expressed as PU/g DM

The generally increased activity of phytase upon fermentation, as compared to flour, may be related to an augmentation of enzyme production due to microbial growth (yeasts and lactic acid bacteria): indeed, several yeasts and *Lactobacillus* species are recognized as phytase producers in fermented grain-based products (Omemua *et al.*, 2007).

Iron and zinc content

The iron content in slurries ranged from 3.2 to 9.9 mg/100g DM. S showed the highest content, significantly ($p \leq 0.05$) different compared to all other varieties, and Ar the lowest, three times less than S and significantly ($p \leq 0.05$) different from ABF, Ar and S (Figure 2). Zinc content in slurries ranged from 1.1 to 2.5 mg/100g DM, with a mean value of 1.87 mg/100g DM. With regard to zinc, S showed the lowest content, significantly different ($p \leq 0.05$) compared to ABF, N and B. ABF showed the highest content, more than two times higher than S and significantly ($p \leq 0.05$) different from B, Ar and S (Figure 2).
Processing of the samples had no significant effect ($p \leq 0.05$) neither on the iron nor on the zinc content (data not shown). Therefore, trace mineral content was influenced neither by fermentation, nor by cooking, whereas it showed consistent linkage to variety. Interestingly, Kayodé *et al.*, (2006) pointed out that, with regard to traditional sorghum varieties, iron and zinc content are more influenced by environmental component (e.g. field location) than genetic variation.

Among varieties, the Fe/Zn ratio ranged from 1.6 (N) to 8.9 (S); Ar and ABF showed a 2 ratio and B a 4.4 ratio. Following direct oral administration of inorganic Fe and Zn, a Fe/Zn ratio ≥ 2 significantly inhibited zinc absorption (Solomons and Jacob, 1981) but, on the other hand, no significant negative effect on zinc absorption was found when a Fe/Zn molar ratio up to 10 was provided through a meal (Fairweather-Tait *et al.*, 1995). Thus, the Fe/Zn ratios found in the present varieties as porridge may not impair zinc absorption.

Calculation of phytate ⁄iron and phytate ⁄zinc molar ratios

The calculation of the molar ratios of phytate to iron (IP6/Fe) and phytate to zinc (IP6/Zn) has been proposed by several authors in order to estimate the bioavailability of the two trace elements (Davies and Olpin, 1979; Oberleas and Harland, 1981; Saha *et al.*, 1994).

Results showed that IP6/Fe and IP6/Zn molar ratios significantly differed among sorghum varieties. S variety showed very low molar ratios related to the low phytate content, being significantly different from all other varieties. IP6/Fe and IP6/Zn molar ratios in S samples ranged from 0.02 to 0.05 and from 0.27 to 0.47, respectively (Table 3).

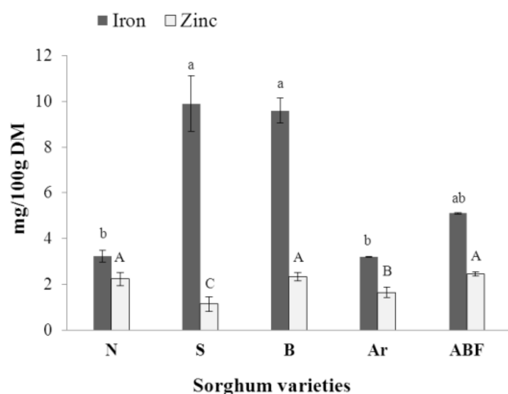


Figure 2. Iron and zinc content in porridges of different sorghum varieties

Since iron and zinc contents were not influenced by processing, molar ratio variation mirrored the influence of sample treatment on IP6. With the exception of S, B showed the lowest IP6/Fe molar ratio in both slurry(*s*) (10.44) and fermented slurry (*f*) (4.48), whereas Ar showed the highest ratios, namely, 32.46 in slurry (*s*) and 12.56 in fermented slurry (*f*). As for IP6/Zn molar ratio, the lowest ratios were observed for N in both slurries (*s*) (51.30) and fermented slurries (*f*) (8.54); again, Ar showed the highest ratios, namely 80.81 in (*s*) and 61.27 in (*f*) (Table 3).

Therefore, both IP6/Fe and IP6/Zn molar ratios were affected by fermentation in all varieties, and the percentage of reduction of fermented slurries (*f*) with respect to slurries (*s*) ranged from 58 to84% and from 24 to 83%, respectively, indicating a potential improvement in iron and zinc bioavailability in these varieties. The cooked porridges (*c*) showed analogous trend in both IP6/Fe and IP6/Zn molar ratios, being Ar the variety showing the highest ratios, and B and N the lowest. Overall, Ar consistently showed the highest ratios, indicating the lowest estimated bioavailability, as regards both IP6/Fe and IP6/Zn in slurries, fermented slurries and cooked porridges; besides S, N and B tended to show lower ratios. An exception was represented by fermented porridge (*fc*), in which B showed the highest IP6/Fe and IP6/Zn ratios, significantly different from all the other varieties. The IP6/Fe and IP6/Zn ratios are estimates influenced by a combination of factors (content of minerals and phytates in the variety, processing effects); their additive effects may explain the rather poor scoring of B in fermented and cooked porridges. Of course, this is an indicative value, suggesting further experimental data.

S showed an optimum IP6/Fe molar ratio in all samples; indeed, according to Hurrell and Egli (2010), the IP6/Fe ratio should be preferably less than 0.4 to significantly improve iron absorption. In composite meals containing ingredients enhancing iron absorption such as vitamin C or animal protein, it is acceptable an IP6/Fe ratio less than 6, as we observed in B and N fermented slurries (*f*) (Table 3).

Regarding the IP6/Zn molar ratio, Gibson (2006) proposed different ranges, based on absorption

studies in humans: a ratio <5 has been associated with high bioavailability, 5 to 15 with moderate bioavailability and >15 with low zinc bioavailability. S showed an optimum IP6/Zn molar ratio in all samples, indicating a high zinc bioavailability. Moreover, fermented slurry (*f*) from N showed a rather good IP6/Zn molar ratio of 8.54. In the other varieties IP6/Zn molar ratio markedly exceeded 15, suggesting a low Zn availability.

Conclusion

Results show that variety strongly influenced the amount of both chelating factors and trace minerals analyzed. Fermentation markedly affected chelating factors content; the effect of fermentation was also modulated by the variety.
Fermentation, with and without cooking, significantly decreased the amount of iron binding phenolic groups and phytates, enhanced phytase activity and improved, consequently, the estimated bioavailability of iron and zinc. On the other hand, cooking alone did not influence chelating factors nor trace mineral content.
There is a clear difference among traditional varieties with regard to chelating factors and trace minerals content. The S (Senegal) variety showed the most promising nutritional profile, according to chelating factors content, IP6/Fe and IP6/Zn molar ratios and bioavailability of trace elements. On the other hand, the two Italian varieties (ABF and Ar) overall showed a poor profile. Another interesting point is the decrease in galloyl and catechol groups during fermentation; their relative contribution compared to phytate would deserve to be investigated further.

Table 3. Effect of treatment and variety on IP6/Fe and IP6/Zn molar ratios

Variety		Treatment			
		s	*c*	*f*	*fc*
Across varieties	IP6/Fe	20.6±4.3^{A}	18.1±5.1^{AB}	6.2±1.4^{C}	10.8±2.4^{BC}
	IP6/Zn	52.4±9.6^{A}	55.4±10^{A}	24.9±7.1^{B}	26.5±6.3^{B}
	Across processes				
IP6/Fe					
Ar	24.3±4.7^{a}	32.46±0.8^{aB}	40.51±1.6^{aA}	12.56±1.1^{aC}	11.72±0.7^{bC}
ABF	21.1±4.0^{ab}	31.69±1.6^{aA}	31.63±0.6^{bA}	9.03±0.005^{bB}	11.94±0.7^{bB}
B	12.0±2.5^{b}	10.44±0.5^{bB}	10.45±0.5^{cB}	4.48±0.2^{cB}	22.46±2.5^{aA}
N	12.2±3.5^{b}	28.15±0.5^{aA}	7.84±0.2^{cB}	4.71±0.1^{cC}	7.98±0.8^{bB}
S	0.03±0.005^{c}	0.05±0.01^{cA}	0.02±0.01^{dA}	0.02±0.01^{dA}	0.03±0.01^{cA}
IP6/Zn					
Ar	66.5±8.0^{a}	80.81±5.5^{aAB}	88.92±3.5^{aA}	61.27±0.9^{aB}	34.96±5.9^{bC}
ABF	48.3±9.7^{ab}	76.58±0.2^{aA}	70.87±2.1^{bA}	21.22±0.2^{cB}	24.51±0.01^{bcB}
B	48.8±3.4^{ab}	53.01±1.3^{bA}	52.87±2.2^{cA}	33.44±0.2^{bB}	55.9±1.4^{aA}
N	35.1±8.8^{b}	51.30±1.5^{bB}	64.00±3.1^{bcA}	8.54±0.2^{dC}	16.78±1.2^{cC}
S	0.4±0.1^{c}	0.47±0.01^{cA}	0.36±0.06^{dA}	0.47±0.2^{eA}	0.27±0.12^{dA}

Mean values ± SD of three replicates. Values followed by different small letters in the same column, and capital letter in the same row, within the same molar ratio, are significantly different ($p \leq 0.05$). Values expressed in mole by using a molecular mass unit of 660 for phytic acid and an atomic mass unit of 56 and 65 for iron and zinc, respectively.

The results outline the importance of sorghum landraces characterization for assessing the impact of traditional processes on the content and the activity of chelating factors.

Acknowledgements

This study was supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy). The authors wish to thank T. Djenaba (Senegal), E. Atoe (Nigeria) and S. Amaducci (Italy) for providing the sorghum varieties and to C. Picq and I. Rochette for technical assistance with the determination of iron and zinc contents and phytate analysis, respectively.

References

Abdelhaleem, W. H., El Tinay, A. H., Mustafa A.I. & Babiker, E.E. (2008). Effect of fermentation, malt-pretreatment and cooking on antinutritional factors and protein digestibility of sorghum cultivars. *Pakistan Journal of Nutrition,* **7,** 335-341.

Asante, S. A. (1995)**.** Sorghum quality and utilization. *African Crop Science Journal,* **3**, 231-240.

Awadelkareem, A. M., Muralikrishna, G., EL Tinay, A.H. & Mustafa, A.I. (2009). Characterization of Tannin and Study of in vitro Protein Digestibility and Mineral Profile of Sudanese and Indian Sorghum Cultivars. *Pakistan Journal of Nutrition,* **8**, 469-476.

Brune M., Halberg, L. & Skanberg, A. B. (1991). Determination of iron-binding phenolic groups in foods. *Journal of Food Science,* **56**, 128-131.

Butler, G. L. (1990). Tannins and other phenols: Effects on sorghum production and utilization. Pp 140-144. *INTSORMIL Annual Report.* A technical research report of the grain sorghum/pearl millet collaborative research support program (CRSP).

Davies, N. T. and Olpin, S. (1979). Studies on the phytate:zinc molar content in diet as a determinant of Zn availability to young rats. *British Journal of Nutrition,* **41**, 591−603.

Dhankher, N., Chauhan, B.M. (1987). Effect of temperature and fermentation time on phytic acid and polyphenol content of rabadi, a fermented pearl millet food. *Journal of Food Science*, **52,** 828–829.

Dicko, M. H., Gruppen, H., Traoré, A. S., Voragen, A. G. J. & van Berkel, W. J. H. (2006). Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *African Journal of Biotechnology*, **5**, 384-395.

Dykes, L., Rooney, L. W., Waniska, R. D., & Rooney, W. L. (2005). Phenolic Compounds and Antioxidant Activity of Sorghum Grains of Varying Genotypes. *Journal of Agricultural and Food Chemistry*, **53**, 6813-6818.

Egli, I., Davidsson, L., Juillerat, M.-A., Barclay, D., Hurrell R. (2003). Phytic acid degradation in complementary foods using phytase naturally occurring in whole grain cereals. *Journal of Food Science,* **68**, 1855–1859.

El Hag, M.E., El Tinay, A.H. & Yousif, N.E. (2002). Effect of fermentation and dehulling on starch, total polyphenols, phytic acid content and in vitro protein digestibility of pearl millet. *Food Chemistry*, **77**, 193–196.

Fairweather-Tait, S. J., Wharf, S. G. & Fox, T. E. (1995). Zinc absorption in infants fed iron-fortified weaning food. *American Journal of Clinical Nutrition*, **62**, 785-789.

FAO (1995). Sorghum and millets in human nutrition. Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO (1999). Fermented cereals. a global perspective. *FAO agricultural services bulletin No. 138.* Food and Agriculture Organization of the United Nations, Rome, Italy.

FAO (2012). 2010 FAO statistics. Food and Agriculture Organization of the United Nations, Rome, Italy:http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor. Accessed 10/07/2012.

Fenster, C. (2003). White food sorghum in the American diet. In US Grains Council 43rd Board of Delegates' Meeting July 2003 Minneapolis.

Gilani, G. S., Cockell, K. A. & Sepehr, E. (2005). Effects of antinutritional factors on protein digestibility and amino acid availability in foods. *Journal of AOAC International,* **88**, 967-8.

Gibson, R.S. (2006). Zinc: The missing link in combating micronutrient malnutrition in developing countries. *Proceedings of the Nutrition Society*, **65**, 51–60.

Godwin, I. D and Gray, S. J. (2000). Overcoming productivity and quality constraints in sorghum: the role for genetic engineering. In: *Transgenic cereals* (edited by L. O'Brien & R. J. Henry). Pp.153-177. AACC St Paul Minnesota USA.

Hama F, Icard-Vernière C, Guyot J-P, Picq C, Diawara B & Mouquet-Rivier C (2011) Changes in micro- and macronutrient composition of pearl millet and white sorghum during in field versus laboratory decortication. *Journal of Cereal Science* **54**, 425-433.

Heinonen, J. K. and Lahti, R. J. (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. *Analytical Chemistry,* **113**, 313–317.

Hurrell, R. and Egli, I. (2010). Iron bioavailability and dietary reference values. *American Journal of Clinical Nutrition*, **91**, 1461S–1467S.

ICRISAT (1996). The World Sorghum and Millet Economies. International Crops Research Institute for the Semi-Arid Tropics and Food and Agriculture Organization of the United Nations. Patancheru, India and FAO, Rome.

Janssen, R., Rutz D., Braconnier S., Reddy B., Rao S., Schaffert R., Parella R., Zaccharias A., Rettenmaier N., Reinhardt G., Monti A., Amaducci S., Marocco A., Snijman W., Terblanche H. & Zavala-Garcia F. (2010). Sweet Sorghum - An Alternative Energy Crop. Proceedings of the 18th European Biomass Conference and Exhibition; ISBN 978-88-89407-56-5.

Kangama, C.O. and Rumei, X. (2005). Introduction of sorghum (*Sorghum bicolor* (L.) Moench) into China. *African Journal of Biotechnology,* **4**, 575-579.

Kayodé, A. P, Linnemann, A. R, Hounhouigan, J. D., Nout, M. J. & van Boekel, M. A. (2006). Genetic and environmental impact on iron, zinc, and phytate in food sorghum grown in Benin. *Journal of Agricultural and Food Chemistry*, **54**, 256-62.

Konietzny, U., Greiner, R., & Jany, K. L. D. (1995). Purification and characterization of a phytase from spelt. *Journal of Food Biochemistry*, **18**, 165–183.

Lestienne, I., Icard-Vernière, C. , Mouquet, C. , Picq, C. , Trèche, S. (2005a). Effects of soaking whole cereal and legume seeds on iron, zinc and phytate contents. *Food Chemistry*, **89**, 421-425.

Lestienne I., Mouquet-Rivier C., Icard-Verniere C., Rochette I. & Treche S. (2005b). The effects of soaking of whole, dehulled and ground millet and soybean seeds on phytate degradation and Phy/Fe and Phy/Zn molar ratios. *International Journal of Food Science and Technology*, **40**, 391–399.

Mahgoub, S.E.O. and Elhag, S.A. (1998). Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. *Food Chemistry,* **61***,* 77–80.

Mohammed, N. A., Mohamed I. A. & Babiker, E. E (2010). Nutritional Evaluation of Sorghum Flour (Sorghum bicolor L. Moench) During Processing of Injera. *International Journal of Biological and Life Sciences*, **6**, 35-39.

Nkongolo K. K., Chinthu K. K. L., Malusi M. & Vokhiwa Z. (2008). Participatory variety selection and characterization of Sorghum (*Sorghum bicolor* (L.) Moench) elite accessions from Malawian gene pool using farmer and breeder knowledge. *African Journal of Agricultural Research*, **3**, 273-283.

Oberleas, D. and Harland, B. F. (1981**).** Phytate content of foods: effect on dietary Zn bioavailability. *Journal* of the *American Dietetic Association*, **79**, 433−436.

Omemua, A.M., Oyewole, O.B. & Bankole, M.O. (2007). Significance of yeasts in the fermentation of maize.for ogi production. *Food Microbiology,* **24**: 571-576.

Ryden, P. and Selvendran, R. R. (1993). Phytic acid: properties and determination. In *Encyclopaedia of food science, food technology and nutrition* (edited by R. Macrae, R. K. Robinson & M. J. Sadler). Pp. 3582–3587 . London: Academic Press.

Rooney, L. W., Kirleis, A. W. & Murty, D. S. (1986). Traditional foods from sorghum: their production, evaluation and nutritional value. In: *Advances in Cereal Science and Technology* (edited by Y. Pomeranz). Pp. 317-353. ICRISAT, American Association of Cereal Chemistry, St. Paul, MN, USA.

Saha, P. R., Weaver, C. M. & Mason, A. C (1994). Mineral bioavailability in rats from intrinsically labeled whole wheat flour of various phytate levels. *Journal of Agricultural and Food Chemistry*, **42**, 2531−2535.

Sandberg, A. S. and Svanberg, U. (1991). Phytate hydrolysis by phytase in cereals; effects on in vitro estimation of iron availability. *Journal of Food Science, **56**, 1330–1333.*

Sandberg, A. S. (2002). Bioavailability of minerals in legumes. *British Journal of Nutrition*, **88**,
281-285.

Schons, P. F., Ries, E. F., Battestin, V. and Macedo, G. A. (2011), Effect of enzymatic treatment on tannins and phytate in sorghum (*Sorghum bicolor*) and its nutritional study in rats. *International Journal of Food Science & Technology*, **46**, 1253–1258.

Slabbert, N. (1992). Complexation of condensed tannins with metal ions. In *Plant Polyphenols – Synthesis, properties, significance* (edited by R. W. Hemingway, P. E. Laks). Pp. 421-436. Plenum Press: New York.

Solomons, N. W. and Jacob, R. A. (1981). Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. *American Journal of Clinical Nutrition,* **34**, 475-482

Talamond, P., Gallon, G., & Trèche, S. (1998). Rapid and sensitive liquid chromatographic method using a conductivity detector for the determination of phytic acid in food. *Journal of Chromatography A*, **805**, 143–147.

Tomas-Barberan, F., Espín, J. C. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *Journal of the Science of Food and Agriculture,* **81**, 853−876.

Towo, E., Matuschek, E. *&* Svanberg, U. (2006). Fermentation and enzyme treatment of tannin sorghum gruels: effects on phenolic compounds, phytate and *in vitro* accessible iron. *Food Chemistry,* **94***,* 369–376.

Wilkinson, L. (1990). SYSTAT : The system for statistics. IL, Evanton : SYSTAT Inc.