Development of innovative probiotic finger millet- and amaranth-based weaning products

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Dedicated to

My Beloved Parents, Husband,

& Sibling

Abstract

Millet and amaranth are two nutritious gluten-free cereal and pseudo-cereal grains, respectively. They have short growing seasons and are resistant to drought and unfertile soils. They are appropriate sources of protein, carbohydrate, energy, vitamins, and minerals with exceptional essential amino acid profiles. Although, their productions have been restricted in the past few decades, currently, they are receiving a growing attention due to their high functional and nutritional characteristics.

The principal aim of the present study was to explore the suitability of these grains to be utilized in the weaning food industry as alternatives for the current counterparts, wheat and rice grains. This objective was achieved by understanding beneficial impacts of three important and common food processing stages; malting, fermentation, and drying.

For the malting process, the goal was to optimize its main involved design parameters, germination duration and temperature, in a way that nutritional characteristics of the processed flour be improved. This was achieved by a corresponding reduction in the involved anti-nutrient contents, mainly phytic acid, tannin, and oxalate. The central composite design was selected for the experimental design with the mentioned two design factors; germination duration with three levels, 24, 36, and 48 hr, and germination temperature with three levels, 22, 26, and 30 °C. Using ANOVA, the response surfaces were constructed for the desired nutrient and anti-nutrient components. Results declared germinating for 48 hr at 30 and 26 °C were optimum conditions for finger millet and amaranth, respectively.

Protein digestibly of finger millet and amaranth was optimized following the above performed experimental design. Possessing high proteins, both quantitatively and qualitatively, is

a key for the development of weaning products. Along with protein, all of the nutrient elements of interest were monitored and the effects of germination treatments were explored. Furthermore, in order to evaluate the potential of malted finger millet and amaranth in the incorporation of value–added products, total phenol and antioxidant properties of these grains were investigated as well.

Positive effects of probiotic compounds on the infants' immature gastrointestinal flora has been highlighted in recent studies. As a result, there is a growing tendency toward inclusion of gram-positive bacteria in weaning food products. To follow this goal, in the next step, the malted flours were fermented using three health-beneficial gut bacteria; *Lactobacillus rhamnosus*, *Bifidobacterium longum,* and *Bifidobacterium infantis*. Fermentation was conducted at 37 °C for 48 hr and bacterial populations were monitored periodically every 6 hr. Although both flours were found to act as good substrates for the bacterial growth, slightly better growth was observed for finger millet. For all design combinations, bacterial populations were maximized approximately at 18 to 24 hr. In addition, a notable reduction in phytic acid and oxalate contents were observed.

The impact of freeze drying on the viability of inoculated bacteria and efficacy of flours as appropriate carriers for the investigated bacterial species were evaluated. Finally, the effects of adding extra protectant, skim milk and maltodextrin, on the enhancement of bacterial survival rate throughout freeze-drying and the subsequent storage stage were assessed.

The results presented in this study are useful for the development of nutritious gluten free millet- and amaranth-based weaning food products. Preserving around 8 log (CFU)/g of viable bacteria at the consumption stage confirmed the probiotic properties of the developed nutritionally enhanced functional flours.

Résumé

Les grains de millet et d'amaranthe sont des céréales et des pseudo-céréales sans gluten. Ces grains ont de courtes saisons de croissance et sont résistants à la sécheresse et aux terres infertiles. Ces grains sont de bonnes sources de protéines, de glucides, d'énergie, de vitamines, et de minéraux avec de bons profils d'acides aminés. Malgré le fait que leur production a décliné aux cours des dernières années, ces grains reçoivent maintenant un intérêt grandissant de par leurs caractéristiques fonctionnelles et nutritives.

L'objectif principal de cette étude a été d'explorer l'utilité de ces grains dans la préparation de d'aliments de sevrage en remplacement des grains de riz et de blé. Cet objectif fut atteint en comprenant les effets de trois importants procédés de transformations: la germination, la fermentation et la déshydratation.

Pour le procédé de fermentation, le but était d'optimiser la durée et la température du procédé, de façon à ce que les valeurs nutritives des farines qui en découlent soient améliorées. Ceci fut atteint et accompagné d'une réduction des composés anti-nutritionnels, principalement l'acide phytique, les tannins et l'oxalate. Un plan expérimental composite central a été utilisé se basant sur deux facteurs soit la durée de la germination à trois niveaux, 24, 36 et 48 h, et la température de germination à trois niveaux 22, 26 et 30°C. Grace à une analyse ANOVA, des surfaces de réponses ont été construites pour les composantes désirées et les éléments anti-nutritionnels. Les résultats favorisent une germination de 48 h à 30 et 26°C pour les grains de millet et d'amaranthe respectivement.

La digestibilité de la protéine du millet et de l'amaranthe a été optimisée lors du plan expérimental. Une bonne quantité et une bonne qualité de protéines sont requises dans le

développement d'aliments de sevrage de même qu'un équilibre des éléments nutritifs. Afin d'évaluer le potentiel de valeur-ajoutée de la germination des grains de millet et d'amaranthe, les phénols totaux et les propriétés anti-oxydantes ont également été étudiés.

De récentes études ont démontré les effets positifs des cultures probiotiques sur la flore intestinale des poupons. De par ce fait, il existe une tendance croissante à intégrer ces organismes dans les aliments de sevrage qui leurs sont offerts. Dans ce but, les farines de grains germés ont été fermentées à l'aide de trois cultures probiotiques bénéfiques; *Lactobacillus rhamnosus*, *Bifidobacterium longum,* et *Bifidobacterium infantis*. La fermentation fut effectuée à 37 °C pour 48 h et les populations de bactéries ont été suivies à toutes les 6 h. Les deux farines se sont avérées être de bons substrats pour la croissance des cultures avec une meilleure performance pour la farine de millet. Pour toutes les combinaisons testées, les populations bactériennes ont été optimisées après une période de 18 à 24 h. De plus une réduction notable de l'acide phytique et de la concentration en oxalate a été observée.

L'impact de la déshydratation par lyophilisation sur la viabilité des bactéries et l'efficacité des farines à titre de matière porteuse ont été évalués. L'effet de l'ajout d'une matière protectrice, telle le lait écrémé ou la maltodextrine sur le taux de survie des bactéries a été étudié pendant le séchage et une période d'entreposage.

Les résultats de cette étude sont utiles pour le développement d'aliments de sevrage sans gluten à base de millet et d'amaranthe. Ayant conservé 8 log (CFU)/g de culture viable, les farines proposées confirment leur potentiel probiotique.

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Contribution of Authors

In accordance with the guidelines of the Faculty of Graduate Studies and Research of McGill University "Guidelines for a Manuscript Based Thesis Preparation", the prepared manuscripts and contribution of authors are presented below.

Manuscripts presented in this thesis are prepared by the principle author, **Sara Najdi Hejazi,** and co-authored by **Dr. Valérie Orsat** at the Department of Bioresource Engineering, Macdonald Campus, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada. Principle author was responsible for preparing the experimental design, developing the methods, conducting the laboratory work, analyzing data, and the documentation. The second author, **Dr. Valérie Orsat**, is the thesis supervisor, who provided the technical, financial and moral guidance, proofreading of all manuscripts, and providing valued criticisms and suggestions towards the research. **Dr. Stan Kubow**, Department of Dietetics and Human Nutrition, Macdonald Campus, McGill University, and his laboratory assistant, **Mr. Behnam Azadi**, co-authored the third manuscript (chapter 5). They provided the laboratory facilities and required instructions in performing the protein digestibility experiments. **Miss Usha Chandrakant,** co-authored the fifth manuscript (chapter 7). She assisted the first author to perform some parts of the laboratory work. **Dr. Darwin Lyew,** co-authored the fifth manuscript (chapter 7) by providing the required instructions for the performed experiments.

The list of manuscripts included in this thesis:

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5- Najdi Hejazi S, Orsat V, Chandrakant U, Lyew D, "Development of millet and amaranth-based probiotic weaning products," **International Journal of Food Microbiology.** (Submitted).

Table of Content

List of Figures

List of Tables

List of symbols and abbreviations

α	Alpha
β	Beta
μ	Micron
λ	Wavelength
±	Plus or Minus
%	Percent
Log	Logarithm
IU	International Unit
ISO	International Organization for Standardization
m	Meter
g	Gram
kg	Kilogram
MoL	Mole
L	Liter
cm	Centimeter
nm	Nanometer
mm	Millimeter
mg	Milligram
mM	Millimole
mL	Milliliter
μg	Microgram
μm	Micrometer
μMol	Micromole
μL	Microliter
Kcal	Kilocalorie
kDa	Kilo Daltons
ha	Hectare
V	Volume
W	Weight
w/v	Weight in Volume
°C	Degree Celsius
hr	Hours
min	Minute
ANOVA	Analysis Of Variance
CCD	Central Composite Design
FFD	Full Factorial Design
SAS	Statistical Analysis System
SD	Standard Deviation
R^2	R-squared; Coefficient of Determination
db	Dry Basis
DM	Dry Matter
CE	Catechin Equivalent
CGA	Chlorogenic Acid Equivalent
GAE	Galic Acid Equivalent
TE	Trolox Equivalent
TE	Tocopherol Equivalent

Ca	Calcium
Fe	Iron
Cu	Copper
Zn	Zinc
Mn	Manganese
P	Phosphorus
P_i	Inorganic Phosphorus
H_2	Hydrogen
ddH_2O	Double Distilled Water
H_2O_2	Hydrogen Peroxide
HCl	HydroChloric Acid
KOH	Potassium Hydroxide
ABTS	2,2'-Azino-Bis(3-ethylbenzothiazoline-6-Sulphonic Acid)
ALP	Alkaline Phosphatase
AMG	Amyloglucosidase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
GOPOD	Glucose Oxidase-Peroxidase
Leu-Gly	Leucine-Glycine
OPA	O-Phtaldialdehyde
SDS	Sodium Dodecyl Sulfate
OD	Optical Density
rpm	Revolutions Per Minute
x g	Gravitational Force
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
CFU	Colony Forming Counts
GI	Gastro Intestinal
GIT	Gastro Intestinal Tract
IVPD	In Vitro Protein Digestibility
LAB	Lactic Acid Bacteria
pH	Potential of Hydrogen
AACC	American Association of Cereal Chemists
AOAC	Association of Analytical Communities
ARS	Agricultural Research Services
FAO	Food and Agricultural Organization
USDA	United States Department of Agriculture
GA	Germinated Amaranth
GM	Germinated Millet
RS	Resistant Starch
Vs.	Versus
DS	Digestible Starch
TS	Total Starch
GE	Gross Energy
et al.	And others
etc.	et cetera
RE	Rational Equivalent

1 Introduction

Early childhood nutrition is crucial, since it constitutes the foundation of adult productivity. Although exclusive breastfeeding is highly recommended for infants up to four to six months of age, afterwards breast milk alone cannot provide adequate energy and nutrient requirements for the growing body (Sajilata et al., 2002). Therefore, in order to sustain normal growth and development, introduction of supplementary products, such as weaning foods is necessary (Anigo et al., 2009; Desikachar, 1982; Lombard & Labuschagne, 2013; Sajilata et al., 2002). However, as the first infants' semisolid staple, nutrients may have short-term effects throughout the infancy and long-term consequences on the adulthood health, weaning foods should possess some special characteristics and be formulated with care (Pearce & Langley-Evans, 2013a; Sajilata et al., 2002).

Infants' weaning products should be rich in calories and good-quality protein, vitamins, and minerals (Sajilata et al., 2002). According to the guidelines of the Protein Advisory Group, based on the dry weight basis, a weaning food is desired to have a protein content of 15 to 20%, around 10% of fat content, carbohydrate content of 60 to 70%, less than 5% of total ash, and around 5 to 10% of moisture (Kumkum et al., 2013; Sajilata et al., 2002). Furthermore, it is recommended that a weaning food provides 15% of its calories from protein, 55% from carbohydrates, and 30% from fat (Kumkum et al., 2013). Being free from anti-nutritional factors is desired as well (Sajilata et al., 2002).

Weaning products should fulfill protein and nutritional requirements of the body by means of small feeding portions (Desikachar, 1980). The restriction on the serving size is due to the limited capacity of a baby's stomach. Therefore, the calorie density of the weaning food is important and should be appropriately high (Mosha & Svanberg, 1983; Sajilata et al., 2002). In

order to minimize the preparation steps prior to feeding, it is desired that the food be precooked, processed, and easy to digest (Desikachar, 1982). Besides, it is important for weaning food formulations to have appropriately low dietary bulks and when stirred with cold or warm water or milk result in soft slurries, to ease the swallowing action (Ljungqvist et al., 1981).

According to recent health guidelines, it is strongly recommended that gluten-free cereals be preferably used for weaning of infants younger than six months old (Fasano & Catassi, 2011; Guandalini, 2007; Omary et al., 2012; Saleh et al., 2013). Especially, in the case of a family history of celiac disease, the restriction for consumption of gluten containing foods may be extended (Fasano & Catassi, 2011; Guandalini, 2007). Therefore, wheat-based products, barley, oat, and multigrain cereals are not advised for infants before six months, while alternatively, gluten-free cereals, like rice, millet, amaranth, buckwheat, quinoa, corn, and sorghum are preferably suggested (Fasano & Catassi, 2011; Guandalini, 2007; Omary et al., 2012).

In addition to the chance of having gluten intolerance, infants are at higher risk of developing food allergies, especially in the first few months of life (Sajilata et al., 2002). This potential is higher when there is a family history of atopic diseases, such as asthma and eczema (Sajilata et al., 2002). Therefore, it is recommended that in the formulation of weaning products, potential food allergens like egg, cow's milk, nuts, citrus fruits, and wheat be avoided (Sajilata et al., 2002).

Throughout the weaning period, significant changes occur in the gastrointestinal flora, which may result in pathological processes (Baldeon et al., 2008). These changes are mostly due to drastic alterations in nutritional and physiological conditions, as babies grow and their feeding habits change from breastmilk to weaning food (Baldeon et al., 2008). Gastrointestinal microbiota is a complex ecosystem and plays important nutritional and immunological functions; to name a few, maintenance of mucus barrier, enhancement of nutrients' absorption, protection against infectious

agents, and stimulation of the immune system (Baldeon et al., 2008). Conditions that alter the composition of normal microbiota, for instance the use of antibiotics, stimulate pathological processes such as diarrheic syndromes (Baldeon et al., 2008; Shobana et al., 2012). Recently, it is proposed that usage of probiotics, particularly during weaning period, may strengthen the immature infant's flora against gastrointestinal diseases, especially diarrhea and constipation (Arora et al., 2011; Baldeon et al., 2008; du Toit et al., 2013; Farooq et al., 2013; Heller, 2001).

Till now, the weaning food industry has mostly focused on wheat- and rice-based products, which are the usual main ingredients across the developed countries (Sajilata et al., 2002). However, in the recent decade, due to a drastic increase in the world population, climate change, world spread drought conditions and lack of water resources, which all threaten the food security and restrict the present agricultural products, introduction of new food sources or the re-introduction of traditional foods seems crucial (Joshi et al., 2008; Rai et al., 2008). Furthermore, the popular standardized weaning food brands are expensive and usually non-affordable for the lower socio-economic families. Therefore, for these families, babies are usually fed either with the same food that is prepared for the rest of the household, or stay a while longer being exclusively fed with mother's breastmilk. In either case, the infants would not obtain adequate nutrients for appropriate growth. Consequently, in the weaning industry, there is a growing demand to utilize a selection of appropriate alternative cereals for the replacement of current main ingredients (Rai et al., 2008).

Through the present research study, utilization of processed finger millet, as the main ingredient cereal in the formulation of infants' weaning food is investigated. In addition, to upgrade the overall protein quality of the food, an appropriately processed supplementary pseudo-cereal, amaranth, is proposed to be adequately added to the formulated product. The food

processing stages are designed to improve the bio-availability and digestibility of nutrients, decrease the anti-nutrient components, improve the synbiotic (pre and probiotic) properties of the food, and to introduce a strong and affordable competitor for the present industrial weaning products.

Millet is one of the most important drought-resistant crops, which is widely grown in arid and semiarid tropics of Asian and African countries. The crop is well known for being gluten-free, and having a short growing season and good productivity in dry and high temperature climates (Saleh et al., 2013). It is a major source of carbohydrate, protein, and minerals, and is mostly utilized in the economically poor countries of Africa and Asia (Anigo et al., 2009; Anigo, 2010; Chandrashekar, 2010). Despite its great nutritional potential, in north America, millet is mostly used to feed livestock and birds (Saleh et al., 2013). Millet varieties can be categorized in six main species, pearl millet, finger millet, proso millet, foxtail millet, kodo millet, and little millet (Saleh et al., 2013). Among them, finger millet (*Eleusine coracana*) is traditionally used for the preparation of weaning food, especially in India, where the food is usually prepared at home instead of at the industrial scale (Dahiya & Kapoor, 1993; Thathola & Srivastava, 2002). It has been shown that some of these traditional millet-based weaning gruels were not sufficiently rich in protein and had low calorie densities (Gupta & Sehgal, 1992a; Mosha et al., 2000; Solomon & Ubom, 2007). The importance of food processing approaches and suitable complementarity of ingredients in preparation of the millet-based weaning food has been highlighted through recent investigations (Saleh et al., 2013; Solomon & Ubom, 2007).

Amaranth is a pseudo-cereal, which has been cultivated for more than 8000 years worldwide (Kauffman & Weber, 1990). The grain is mostly well known for its high level of good quality protein, and well-balanced amino acid profile, especially lysine, comparing to other grains

(Rastogi & Shukla, 2013). It is appropriately rich in vitamins and minerals, especially calcium, iron, and magnesium (Colmenares de Ruiz & Bressani, 1990; Kauffman & Weber, 1990; Rastogi & Shukla, 2013). In recent years, the USA has been the leading commercial producer of amaranth grain (Rastogi & Shukla, 2013). Like millet, amaranth is categorized in the gluten-free grains group, making it a suitable pseudo-cereal to be consumed by infants suffering from gluten allergies (Alvarez-Jubete et al., 2010a; Saturni et al., 2010).

1.1 Problem statement

Based on the recent studies, utilization of gluten-free cereals in the formulation of weaning products for infants younger than six months old is highly recommended. However, the current weaning industry has mostly focused on wheat and multigrain cereals that contain gluten in their protein storage. Therefore, introduction of new, economical, and nutritious gluten-free cereals and pseudo-cereals is crucial. According to the literature, finger millet and amaranth could be appreciable raw materials for processing value added products.

Although finger millet and amaranth possess many essential nutrients, these grains contain considerable amounts of anti-nutrient compounds such as phytic acid, oxalate, and tannin (Chauhan et a1. 1986; Mahajan and Chauhan 1987). These anti-nutritive compounds are known to affect mineral availability (Maga 1982, Reddy et a1. 1982) and digestibility of carbohydrates (Deshpande and Salunkhe 1982; Thompson and Yoon 1984) and proteins (Knuckles et a1. 1985) in various plant foods. Also, dietary phytate is known to inhibit proteolytic and amylolytic enzymes activities (Sutardi and Buckle 1983). Relatively high concentrations of these anti-nutrients in finger millet and amaranth may account for lower availability of their nutrients (Dhankher and Chauhan 1987a).

1.2 Hypothesis

It is hypothesized that minor cereals and pseudo-cereals, like finger millet and amaranth, may be considered as the main ingredients in the formulation of infants' weaning products, following appropriate processing steps to ensure digestibility of their nutrients and inactivation of their included anti-nutrients.

1.3 Objectives

Considering the problem statement and hypothesis, to achieve the following principal objective of the thesis, a number of specific objectives were planned and the corresponding experimental studies were conducted.

1.3.1 Principal objective

The principal objective of the present research study was to formulate and characterize highly nutritious millet- and amaranth-based infants' weaning products as alternatives/competitors for the present wheat- and rice-based counterparts.

1.3.2 Specific objectives

1. To employ and optimize the malting process for anti-nutrient reduction and protein digestibility enhancement in finger millet (**Chapter 3)** and amaranth (**Chapter 4**) grains.
2. To employ and optimize the malting process for improvement of the nutrients bio-availability and energy density of finger millet and amaranth grains (**Chapter 5)**.
3. To evaluate the total phenolic content and antioxidant activity of the extracts prepared from malted finger millet and amaranth grains (**Chapter 6)**.
4. To develop, evaluate, and optimize a probiotic fermentation process using three probiotic bacteria; *Lactobacillus rhamnosus, Bifidobacterium longum,* and *Bifidobacterium infantis* (**Chapter 7)**.

5. To investigate and optimize the fermentation duration to achieve high probiotic concentrations in the final fermented flours (**Chapter 7)**.
6. To evaluate capabilities of finger millet and amaranth as carriers and substrates for preserving probiotics during freeze drying (**Chapter 7)**.
7. To investigate the need to employ a variety of protectant combinations throughout the freeze drying step to preserve the viability of inoculated probiotics within the fermented finger millet and amaranth slurries (**Chapter 7)**.
8. To monitor viability and vitality of the inoculated probiotic cultures throughout the freeze drying and storage stages (**Chapter 7)**.

2 Literature review

In the present chapter, the previous investigations on the infants' weaning products including their desired nutritional profile are explained. Two cereal and pseudo-cereal grains, finger millet and amaranth, are introduced and their potential to substitute wheat and rice in current infants' weaning products are assessed. Finally, effects of the most influential food processing steps, malting, probiotic fermentation, and drying, on the improvement of the nutritional characteristics of these grains are presented based on the previously performed scientific research investigations throughout the past few decades.

2.1 Infants nutritional requirements

As infants grow, breastmilk alone would not be sufficient to fulfill all nutritional requirements of the body. Therefore, the necessary extra calories and nutrients should be provided through complementary products, called weaning food. Weaning food is a semisolid staple food modified through appropriate processing of the ingredients, so that its digestion is facilitated for infants (Sajilata et al., 2002). The weaning period refers to the gradual changes in the infants diet from exclusive breastfeeding and infants' formulas toward a regular diet (Sajilata et al., 2002). Through this transitional period, infants not only gain the required nutrition and calories, but also learn how to bite and chew solid products, which correlate their mouth and tongue movements, as an essential step in speech development (Sajilata et al., 2002).

It is suggested that the weaning stage begins at three to four months of age, and not later than six months (Wright et al., 2004). The introduction time depends on the physiological development factors and infant tendency toward the food as well as the family wealth and knowledge (Sajilata et al., 2002). In economically poor societies, the weaning stage starts around six months, while for the developed countries it mostly begins around four months (Wright et al., 2004).

The weaning period is an important stage in the human life. It is well established that the diet in infancy clearly affects health throughout childhood and adulthood (Guardamagna et al., 2012; Pearce & Langley-Evans, 2013b; Stettler, 2011). According to Barker's hypothesis (1995), reduced fetal growth and malnutrition during infancy may cause a number of chronic conditions, like coronary heart disease, stroke, diabetes, and hypertension, later in life. Therefore, special attention should be given to the quality of weaning products.

Table 2-1 presents the amount of required daily intake energy from breastmilk and complementary foods for infants up to 23 months of age (Brown et al., 1995). The percentages of energy coming from these two food sources are also graphically represented in Figure 2-1. The figure clearly illustrates how breastmilk should be gradually replaced by quality weaning foods as a baby grows.

Table 2-1: The required energy intake of babies from 6 months to 2 years (Brown et al., 1995).

	Age group (month)		
	6-8	**9-11**	**12-23**
Recommended energy intake (kcal/day)	783	948	1,170
Amount of breastmilk consumed (g/day)	673	592	538
Energy intake from breastmilk (kcal/day)	437	387	350
Energy required from complementary foods (kcal/day)	346	561	820

Table 2-2 presents the daily dietary allowance for infants in their first year of life, as recommended by the National Academy of Sciences of the United States (1989). Industrial weaning foods are prepared based on these required nutrients and the number of feedings per day. Here, two important concepts should be noticed; calorie density, and dietary bulk. The amount of calories in a quantity or volume of a prepared food is called its calorie density. It is a very important concept in the formulation and production of weaning foods. Due to the limited capacity of the babies' stomachs, the amount of food in one feeding is restricted (Anigo, 2010). Furthermore, since babies would only be fed 2 to 3 times a day, the weaning food should have sufficiently high

calorie density to fulfill the daily requirements of energy and nutrition for the healthy development of the body (Table 2-2).

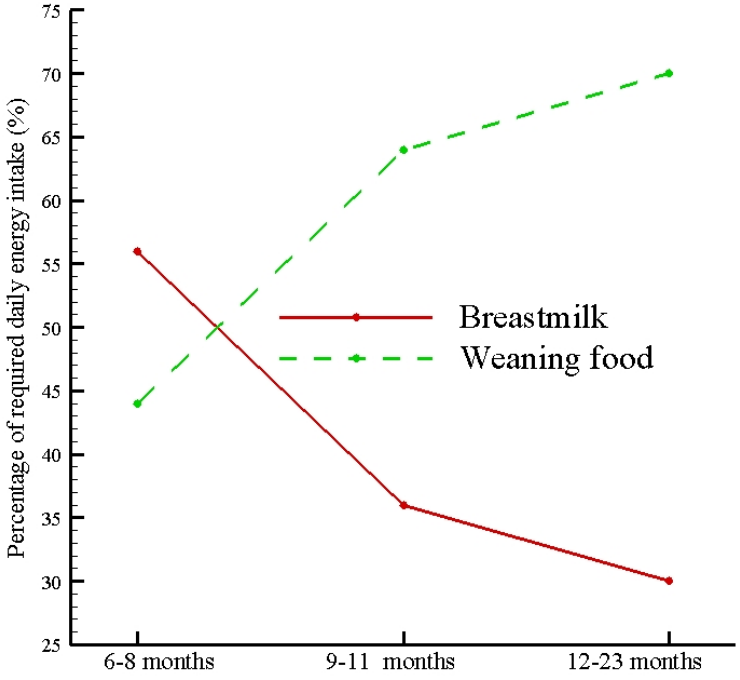

Figure 2-1: Percentage of the required daily energy intake (%) from breastmilk and weaning foods.

Table 2-2: Recommended daily dietary allowances for infants (National Academy of Science, USA (1989)).

Averaged Values	0-6 months	6-12 months
Weight (kg)	6	9
Height (cm)	60	71
Energy (kcal/kg)	108	98
Protein (g/kg)	2.2	1.6
Vitamin A (µg RE)a	375	375
Vitamin D (µg)b	7.5	10
Vitamin E (mg αTE)c	3	4
Vitamin K (µg)	5	10
Vitamin C (mg)	30	35
Thiamine (mg)	0.3	0.4
Riboflavin (mg)	0.4	0.5
Vitamin B6 (mg)	0.3	0.6
Vitamin B12 (mg)	0.3	0.5
Calcium (mg)	400	600
Phosphorus (mg)	300	500
Magnesium (mg)	40	60
Iron (mg)	6	10
Zinc (mg)	5	5
Iodine (µg)	40	50
Selenium (µg)	10	15

a) Retinol Equivalents 1 RE = 1 µg retinol/6 µg β-carotene.
b) 10 µg cholecalciferol = 400 International Units (IU) vitamin D.
c) α-Tocopherol equivalents (TE). 1 µg *d*-α-tocopherol = 1 αTE.

On the other hand, the dietary bulk of weaning food should be adequately low (Sajilata et al., 2002). Usually, the first infants'supplementary foods are based on cereals and pseudo-cereals, containing a high amount of starch. Starch granules become gelatinized as they are mixed with water, which increases the viscosity of the prepared product and makes it difficult to swallow or less palatable for the infants being fed (Anigo, 2010). This results into either the food rejection by the baby, or the necessity of adding extra water to smoothen the slurry. Both situations may lead to inadequate satisfaction of the infants' nutritional requirements.

Table 2-3 presents the nutritional properties of two commercial weaning products from Nestlé Company, wheat- and rice-based Cerelacs (Al-Othman et al., 1997) compared with four millet species, wheat, rice, and amaranth (Shobana et al., 2012), as well as the FAO reference nutrient pattern (FAOSTAT, 2014). The values are based on 100 g of the flours. To nutritionally enrich Cerelac's products, skimmed milk, oil, and a variety of vitamins and minerals are supplemented (Treche, 1999). This can be observed by comparing the nutritional values of crude wheat or rice with the corresponding wheat- and rice-based Cerelac cereals.

Table 2-3: Nutritional facts for wheat- and rice-based Nestlé Cerelac cereals (Al-Othman et al., 1997), seven selected grains (Shobana et al., 2012), and FAO reference pattern (FAOSTAT, 2014).

	Cerelac (wheat)	Cerelac (Rice)	Wheat	Rice	Finger Millet	Pearl Millet	Proso Millet	Foxtail Millet	Amaranth	FAO Pattern
Moisture (%)	2.5	2.5	12.8	13.7	13.1	12.4	11.9	11.2	-	-
Energy (Kcal)	408	407	346	345	328	361	341	331	374	-
Protein (g)	16.3	16.2	11.8	6.8	7.3	11.6	12.5	12.3	15	-
Fat (g)	8.5	8.7	1.5	0.5	1.3	5.0	1.1	4.3	6.5	-
Carbohydrate (g)	70.8	71.2	71.2	78.2	72.0	67.5	70.4	60.9	66.2	-
Dietary Fiber (g)	1.5	1.0	12.5	4.1	11.5	11.3	-	2.4	7	-
Minerals (g)	2.3	2.9	1.5	0.6	2.7	2.3	1.9	3.3	-	-
Essential Amino Acids (mg/g N)										
Arginine	-	-	290	480	300	300	290	220	556	-
Histidine	-	-	130	130	130	140	110	130	158	-
Lysine	-	-	170	230	220	190	190	140	364	270
Tryptophan	-	-	70	80	100	110	50	60	86	90
Phenylalanine	-	-	280	280	310	290	310	420	238	180
Tyrosine	-	-	180	290	220	200	-	-	205	180
Methionine	-	-	90	150	210	150	160	180	148	-
Cystine	-	-	140	90	140	110	-	100	116	270
Threonine	-	-	180	230	240	240	150	190	230	180
Leucine	-	-	410	500	690	750	760	1040	349	306
Isoleucine	-	-	220	300	400	260	410	480	218	270
Valine	-	-	280	380	480	330	410	430	264	270

The main objective of the present research is to investigate the possibility of the replacement of wheat and rice with finger millet and amaranth. Among the four millet species presented in Table 2-3, finger millet is traditionally used for homemade infants' weaning (Dahiya & Kapoor, 1993; Thathola & Srivastava, 2002). Although total protein of finger millet is lower compared to other millet varieties (Table 2-3), finger millet is mostly well known for having good protein quality in terms of the completeness of its amino acid profile. This is confirmed by comparing the

essential amino acids, where finger millet stands in a much better position compared to other millet varieties, especially for its lysine content (Table 2-3). Most cereals are limited in essential amino acids such as threonine, lysine, and tryptophan, making their protein quality poorer compared to animal protein. This is the case with Cerelac, where skimmed milk is added to improve the protein quality (Al-Othman et al., 1997; Mosha et al., 2000; Treche, 1999). Since cow's milk may potentially lead to allergies in infants in the proposed weaning product, we would try to avoid its utilization.

Protein deficiency of the finger millet could be improved by adding other supplementary cereal ingredients. Here, amaranth is chosen for this purpose. Amaranth is a pseudo-cereal mostly recognized for its high level of crude protein and especially well-balanced essential amino acid profile. It is notably rich in lysine compared to all other cereals (Rastogi & Shukla, 2013). As for the minerals, finger millet and amaranth have appreciable contents of calcium and magnesium (Kauffman & Weber, 1990), which is an important positive factor for these cereals to be used in the formulation of weaning products (Kumkum et al., 2013).

In order to improve the nutritional characteristics of the ingredient cereals, various food-processing stages are proposed. Among them malting, fermentation, and drying are of greater importance (Badau et al., 2006; Desikachar, 1980; Desikachar, 1982).

2.2 Millet

"Millet" term is used to describe a number of small seeded cereals, which were domesticated about 10000 years ago in tropical and sub-tropical regions of the world, especially Africa and Asia (Lu et al., 2009). It is considered as an important reserve crop for famine-prone areas due to its capability to be stored for long periods (Rengalakshmi, 2005) and its resistance to pests and diseases (Devi et al., 2011b). It is fast growing, early maturing, and ready to harvest plant in as

little as 65 days under a harsh and drought environment (Chaturvedi et al., 2011; Devi et al., 2011b).

Millet belongs to the grass sub-family of *Panicoidea*, and family *Poaceae*. Based on the seed size, millet is classified into two categories; **major millets**; pearl millet *(Pennisetum glaucum)* and sorghum, and **minor millets**; Brown top millet *(Brachiaria ramosa)*, Japanese Barnyard millet *(Echinochloa frumentacea)*, Finger millet *(Eleusine coracana)*, Little millet *(Panicum sumatrense)*, Kodo millet *(Paspalum scrobiculatum)*, Foxtail millet *(Setaria Italica)*, Proso millet *(Panicum miliaceum)*, and Fonio *(Digitaria exilis)*. Although various additional millet species exist throughout the world, only these varieties, with an approximate total production of thirty million tons per year, are applied in the food industry (FAOSTAT, 2014). It should be mentioned that during the last three decades, cultivation of minor millets has been declining while food application of major millets has been increasing (Rengalakshmi, 2005). In the following sub-sections some historical, nutritional, morphological, and application characteristics of our selected millet species, finger millet, are briefly explained.

2.3 Finger millet

"Finger millet" is known as African millet, bird's foot, and coracana in English, is also named dagusha in Ethiopia and Eritrea, wimbi in Swahili in east Africa, bulo in Uganda, tamba and pwana in Nigeria, and ragi in India (Taylor, 2007). Finger millet plant height is from 0.4 to 1 m and the length of its panicles is between 3 to 13 cm (Bavec & Bavec, 2006). This species of millet is widely adapted to varied soil characteristics and tolerates some degrees of alkalinity. The plant is well adapted to dry climatic conditions, but its cultivation requires slightly more water than other millets. It has the highest yield among millets, where values up to 1000 to 1200 kg/ha have been reported (Taylor, 2007).

2.3.1 History

The primary genetic center of finger millet is Uganda. However, India began producing finger millet more than 3000 years ago. Today, finger millet is one of the staple foods and important cereal in Africa and India (Taylor, 2007). Its production spreads to the western parts of southwest India, to the hills under the Himalayas, and to Nepal and along the hills of east China and southern Asia. It is also the most important cereal grain in Uganda and Zambia. Production of finger millet is ranked sixth after wheat, rice, maize, sorghum and bajra (pearl millet) in India (Devi et al., 2011b). The world annual total production of finger millet is approximately 4.5 million tons, where India produces 2.5 million and Africa about 2 million tons (Jayasinghe et al., 2013).

2.3.2 Application as a food source

Finger millet is one of the staple foods of millions of people in India, Asia, and Africa. It is majorly consumed as traditional foods such as; roti (unleavened breads or pancake), mudde (dumpling), and ambali (thin porridge) (Devi et al., 2011b). Possessing superior nutritional fact enables numerous potential industrial food usage for finger millet, especially in weaning foods, snack foods, dietary products, millet nutrition powders, and beer applications. It is well established that the presence of phenolic compounds in significant levels in finger millet makes it a potential nutraceutical and functional food ingredient which can be used in promoting health by reduction of certain chronic diseases (Saleh et al., 2013). Although, finger millet has an excellent nutritional profile, it is not involved in mass production of processed foods such as fermented, extruded, and bakery products. Thus further investigations may be required to fully understand its benefits on humans' health by carrying out *in-vitro* and clinical studies.

Finger millet grain has high social value in eastern and northern Uganda. People of Uganda traditionally hold celebrations for the new harvest, and serve finger millet bread or hot porridge

with either sugar or banana juice (Kannan, 2010). In India, Himalayas, and Nepal, until recently, finger millet has been a principal cereal for the rural poor and the farming classes (Kannan, 2010). It is also consumed as a flavoured drink in festivals in India (Devi et al., 2011b). Commonly, finger millet is malted, grinded into flour, and mixed with other cereal flours in the preparation of different traditional foods, cakes, breads, baked products, puddings, and pancakes (Taylor 2007). Malted finger millet flour is utilized in India as an infant food and also for patients having diabetes (Hilu & De Wet, 1976). The flour itself can also be consumed after being mixed with milk, boiled water or yogurt (Obilana, 2003b). In addition to helping to address the nutritional security of the rural and urban poor, this grain could also be an appropriate choice for the elite who will benefit from its functional and health enhancing properties (Kannan, 2010).

2.3.3 Morphology

Finger millet seeds are small spherical grains with diameters in the range of 1 to 2 mm. The weight of 1000 kernel is approximately 2.5 g. Finger millet color varies in a spectrum ranging from white to orange-red to brown to almost black (FAO, 1995b; Hulse et al., 1980). As it is presented in Figure 2-2, similar to the other cereals, the kernel of finger millet grain is composed of three main components: (1) the coat layers or bran, (2) the embryo or germ, and (3) the endosperm (FAO, 1995a). The bran includes; the fruit coat, which consists of three layers of pericarps (epicarp, mesocarp, and endocarp) and the seed coat that is a five-layered testa. Finger millet seeds is utricle type, where the pericarp forms a sac and is loosely (only one point) attached to the endosperm (McDonough et al., 2000). The pericarp is of little nutritional significance. The embryo in finger millet is around five percent of the total seed weight and has essentially all the grain fat (Belton & Taylor, 2002a). The endosperm represents the largest portion of the grain. It consists of an aleurone layer, and three distinct starchy sections, peripheral, corneous, and floury

endosperm. The peripheral endosperm contains small and tightly packed cells, with a large number of protein bodies that are embedded in protein matrices and are associated with compound and simple starch granules (8-16.5 μm in diameter). Corneous is the largest portion of the endosperm, containing cells of different sizes. Predominantly, the corneous endosperm consists of compound starch granules (3-19 μm), where patches of protein matrices are associated. Finally, the floury endosperm is made up of compound starch granules (11-21 μm), where protein bodies and protein matrices rarely exist (Belton & Taylor, 2002a). Depending on the variety, the composition of each component may be slightly different.

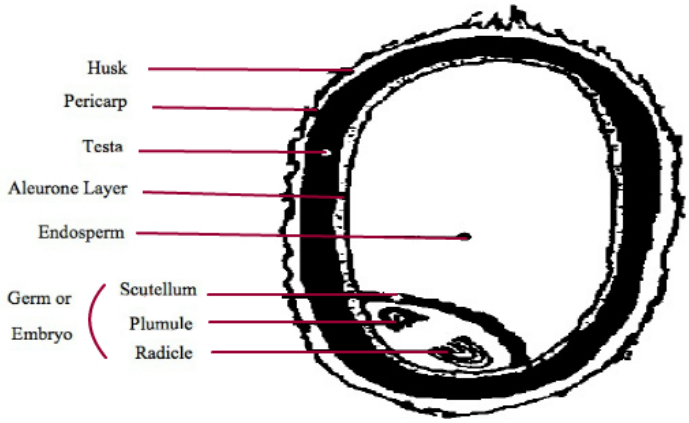

Figure 2-2: Finger millet grain morphology (Ramashia, 2015).

2.3.4 Nutritional value

Finger millet is an excellent source of protein, fat, and minerals (calcium, iron, and phosphorous), relative to rice, corn, and sorghum (Taylor, 2007). In the following sub-sections, the main nutrients of finger millet are presented.

2.3.4.1 Protein

The protein content of finger millet grain varies from 6 to 14%, where the average widely reported value is 7.3% (Mbithi-Mwikya et al., 2002a). The variations are due to the genotype, soil fertility, temperature, environmental conditions, and water availability. Although the total protein

content of finger millet is comparable to that of other cereals and is limiting in lysine, it has high amounts of sulfur-containing amino acids at an equal level of milk protein (Mbithi-Mwikya et al., 2002a). Cereal protein could be categorized in different ways. Traditionally, Osborne's classification has been used, where protein is divided into four groups based on their extraction in a series of solvents: water (albumins), dilute salt solutions (globulins), alcohol/water mixtures (prolamins), and dilute alkali (glutelins) (Mbithi-Mwikya et al., 2002a). Recently, proteins classification is mostly according to their functional and structural roles. In cereals, most of the proteins are storage proteins, although they are also used in cell matrices and enzymes structures (Mbithi-Mwikya et al., 2002a). Based on Osborne's classification, prolamins are the major fractions of finger millet protein, followed by glutelins. They are mainly found in the starchy endosperm. Prolamins fractions are mostly located within the protein bodies, while the protein matrices consist of glutelins (Dharmaraj & Malleshi, 2011). Based on the weight fractions of the total protein, prolamin (19%) and prolamin like (28%) are the main protein fractions (47%). Glutelin like (41%) is another major fraction of finger millet, while albumins (8%) and globulins (4%) are the minorities (Dharmaraj & Malleshi, 2011).

Consumption of protein is vital for the body. Proteins are involved in almost all cell functions. A specific role could be assigned to each protein. For instance, generally, albumin fraction is the main protein of human plasma, where its main function is to regulate the colloidal osmotic pressure of blood (Hulse et al., 1980). Proteins may act as antibodies to defend the body from germs. Proteins as enzymes speed up chemical reactions. Contractile proteins are responsible for movement, and finally storage proteins would store amino acids for a later need in the human body (Hulse et al., 1980).

Amino acids are the building blocks of the protein polymers. Usually twenty of the more common or standard amino acids are investigated, where among them, nine amino acids are called "essential" for human beings, since they cannot be produced from other compounds by the human body. Therefore, these amino acids must be consumed as food. In Table 2-4, the amino acids composition of finger millet is presented for the total protein content of the native grain, as well as prolamin, glutelin, and albumin-globulin protein fractions (Ramachandra et al., 1978). The essential amino acids are highlighted. Finger millet is appropriately high in methionine, composing around 5% of the protein, while, as with other cereals, lysine and tryptophan are the main limiting amino acids. The prolamin fraction contains higher amounts of glutamic acid, proline, valine, alanine, isoleucine, leucine, and phenylalanine comparing to other fractions. However, lysine, arginine, and glycine of prolamins are lower (Ramachandra et al., 1978).

Table 2-4: Amino acids composition of the finger millet proteins (Ramachandra et al., 1978).

g/100 g protein	Total Protein	Prolamin Fraction	Glutelin Fraction	Albumin-Globulin Fraction
Lysine	3.1	0.66	7.69	5.56
Histidine	2.8	3.08	3.88	2.21
Threonine	5.2	5.23	4.85	5.16
Valine	8.2	7.56	5.32	5.35
Methionine	4.5	3.01	1.64	0.76
Isoleucine	5.2	5.13	4.17	3.24
Leucine	11.7	12.24	7.98	6.40
Tryptophan	----	4.65	3.26	3.05
Phenylalanine	6.1	7.55	4.44	2.79
Arginine	4.9	2.08	8.67	8.96
Aspartic acid	7.2	4.38	7.53	9.01
Serine	6.6	6.30	5.60	6.45
Glutamic acid	24.2	32.24	19.02	16.70
Proline	7.6	10.40	6.85	5.35
Glycine	4.5	1.65	4.29	6.20
Alanine	7.2	6.76	6.46	8.34

Finger millet grain has good amounts of tryptophan, cysteine, methionine, and total aromatic amino acids, which are vital for human health and growth and are often deficient in most other cereal grains (Belton & Taylor, 2002a).

Using the SDS-PAGE approach, the Molecular weight (Mw) bands of each protein fraction can be determined. Prolamins are generally 15-30 kDa in size, although traces as low as 6 kDa and as high as 50 kDa are observed (Ramachandra et al., 1978). It is claimed that the α-prolamin is the major type in prolamin fraction, with Molecular weight (Mw) bands of 25.0 and 22.5 kDa. The glutelin protein fraction pattern covers 10 to 11 bands in the Mw ranging from 6-50 kDa, where the low Mw polypeptide components (6-8.5 kDa) are quantitatively the most important fractions (Ramachandra et al., 1978).

In the SDS-PAGE pattern of the albumin-globulin fraction, protein bands with Mw ranging from 8-50 kDa are observed. Low Mw components quantitatively are the major fraction as it is observed from the intensity of their bands, while higher Mw components are more numerous and distinct (Ramachandra et al., 1978). The chemical score, which is a measure of a protein's quality with respect to a reference protein, for finger millet protein is 52 (Shobana et al., 2012).

2.3.4.2 Carbohydrate

Finger millet is a rich source of carbohydrates with estimated values of 70 to 76% (Obilana & Manyasa, 2002), based on the whole grain. Among millet saccharides, similar to the other cereals, starch (56 to 65%) is the major fraction. Starch consists of two types of molecules, amylose and amylopectin. For millet, it is reported that 18 to 30% of the starch is the linear and helical amylose, while the rest is the branched amylopectin (Obilana & Manyasa, 2002). The remaining fraction of the finger millet carbohydrates includes free sugars, mono and di-saccharides (around 1%), oligosaccharides, and non-starchy polysaccharides or dietary fibers (10 to 18%). Among these polysaccharides, pentosans (5.5 to 7.2%), cellulose (1.4 to 1.8%), and resistant starch (1%) are of more importance (Shobana et al., 2012). The dietary fiber content of finger millet is comparable to wheat and much higher than rice and other millet species. The large discrepancy in

some of the the reported values is due to the complexity of the dietary fiber determination and varietal differences (Devi et al., 2011a).

For the native finger millet flour, from 70% ethanol-extractable sugars, sucrose (31 to 35%) is the main fraction followed by, glucose (9.9 to 15%), fructose (8.6 to 15%), maltose (9 to11.0%), raffinose (8.6 to 12%), maltotriose (5 to 6.1%), xylose (1.5 to 4.3%), and higher oligosaccharides (5 to 10%) (Shobana et al., 2012). Besides, the water-soluble gums of the finger millet contain arabinose and xylose as the major sugar components together with minor amounts of mannose, galactose, and glucose. In finger millet, hemicellulose A is found to consist mainly of glucans (especially β-glucan) containing arabinose and xylose in minor amounts. Hemicellulose B contains arabinose and xylose with smaller amounts of glucose and galactose (Shobana et al., 2012). The high dietary fiber content of the millet may serve as an appropriate prebiotic source.

The gelatinization temperature of the native finger millet starch is reported from 62 to 70 °C. Size of the native finger millet starch granule varies from 4 to 7 μm (39%), 8 to 12 μm (42%), and 14 to 22 μm (19%) (Shobana et al., 2012). The starch content of finger millet has a high peak and set back viscosities and large break down comparing to most cereals. This means that finger millet starch granules swell-absorb more water and break down more easily under shear stress. Besides, among non-starchy polysaccharides, pentosans are known to influence dough rheological characteristics and also to protect protein molecules against thermal disruption. The enthalpy of gelatinization, level of crystallinity, and resistance to digestion by α-amylase enzyme of finger millet starch is higher than wheat and rice grains (Belton & Taylor, 2002a).

2.3.4.3 Fat

Although the fat content of finger millet is low, it is highly enriched in polyunsaturated fatty acids. Finger millet contains various lipids, including phospholipids, glycolipids, triglycerides,

phytosterols, tocopherols, and carotenoids (Shobana et al., 2012). These lipids can be categorized as free, bound, or structural lipids. The main free lipids in finger millet are triglycerides. The total lipid content of finger millet grain is approximately 1.3%, where palmitic (20 to 35%), oleic (46 to 62%), and linoleic acids (8 to 27%) are the main constituents (Shobana et al., 2012). The low fat content, which is probably due to the small germ of the finger millet grain, is one of the factors contributing to the better storage properties of finger millet comparing to other cereals. Finger millet may be stored up to 50 years, which makes it a very good reserve staple against famine (Shobana et al., 2012).

2.3.4.4 Minerals

Finger millet is a rich source of minerals, particularly calcium. The calcium content of finger millet (0.26 to 0.43% of the grain weight) is 5 to 30 times more than in most cereals (0.01 to 0.06%) (Sripriya et al., 1997). About half of the total calcium content exists in the husk. The total ash content of finger millet is 2.25 to 4%, where 1.58 to 2.8% are minerals. The pericarp, aleurone layer, and germ are rich source of minerals. In addition to calcium, finger millet has high levels of potassium (408 mg/100 g), iron (3.9 mg/100 g), magnesium (137 mg/100 g), copper (0.47 mg/100 g), sodium (11 mg/100 g), and phosphorus (283 mg/100 g) (Mbithi-Mwikya et al., 2002a).

2.3.4.5 Vitamins

Finger millet contains both types of water-soluble and lipo-soluble vitamins. It is appropriately rich in thiamine (0.2 mg/100 g), riboflavin (0.1 mg/100 g), and niacin (0.1 mg/100 g). The water-soluble B vitamins of finger millet are concentrated in the aleurone layer and germ, while lipo-soluble vitamins are mostly located in the germ (Shobana et al., 2012).

2.3.4.6 Anti-nutrients

Finger millet has nutrient binding components, generally known as anti-nutrients, such as phytate, tannins, oxalate, and enzyme inhibitors, like trypsin inhibitor (Antony & Chandra, 1998). These anti-nutrients restrict the maximum utilization of the included nutrients of finger millet. While tannins complex with proteins and enzyme inhibitors, phytates bind essential minerals and proteins. As a result, the digestibility of proteins and bio-availability of minerals would be negatively affected.

2.3.4.7 Total phenolic compounds

Phytochemicals are known to have pharmacological effects and are found in small amounts in plants (Belton & Taylor, 2002a). In the past, food processing was aimed at removing these substances since they were perceived to have negative effects on human nutrition (considered to be anti-nutrients). However, through recent studies, it has been revealed that most phytochemicals have many positive health benefits, such as prevention of cardiovascular and Parkinson diseases and having anti-tumor, anti-inflammatory, and anti-cancer effects (Becker et al., 1981; Shahidi & Chandrasekara, 2013). In plants, phenolic compounds are the major source of natural antioxidants. Antioxidants are molecules that prevent oxidation of other components by being oxidized themselves (Viswanath et al., 2009). In fact, antioxidants are known as reducing agents that terminate the chain reactions through the removal of free radical intermediates and inhibit excessive oxidation reactions.

Finger millet has plentiful amounts of phytochemicals, such that it is recognized as a "nutri-cereal" (Singh & Raghuvanshi, 2012). Depending on the finger millet genotype, white or brown, different phenolic contents were reported in the literature (Banerjee et al., 2012; Hegde et al., 2005; Pushparaj & Urooj, 2014; Siwela et al., 2007; Sreeramulu et al., 2009). Usually white finger millets have lower phenolic compounds (below 0.09 mg GAE/100 mg), while for brown species, much

higher values in the range of 0.34 to 1.84 mg GAE/100 mg have been reported (Siwela et al., 2007). GAE stands for Gallic Acid Equivalent. A similar seed color dependency was observed by Chethan and Malleshi (2007), who reported a polyphenol content of finger millet ranging between 0.3 to 0.5% (GAE) in white, versus 1.2 to 2.3 % in brown seeds.

2.4 Amaranth

"Amaranth" is a common name for more than sixty different species of *Amaranthus*. From a botanical point of view, amaranth is allocated to the class *Dicotyledoneae*, subclass *Caryophyllidae*, order *Caryophyllales*, family *Amaranthaceae*, sub-family *Amaranthoideae*, genus *Amaranthus*, which is assigned to the monocotyledonous grasses (Bressani et al., 1993). Most of its species are relatively tall plants (with a height of 91 to 274 cm and stem diameter of 2.54 to 15 cm) with green leaves and purple, red, or gold flowers, which are mostly consumed as leafy vegetables (Kauffman & Weber, 1990). The plant is a C4 annual herbaceous, with the potential of photosynthesis by means of carbon dioxide under extensive range of temperature (from 25 to 40 °C), high light intensity, and moisture stress environmental conditions. It fixes carbon dioxide in the chloroplasts of specialised cells surrounding the leaf's vascular bundles, which is responsible for lower water losses by transpiration through the stomata (Caselato & Amaya, 2012). Amaranth also has the capacity to photosynthesize at high rates and at high temperatures, through osmotic adjustments, which allows the plant to tolerate lack of water to some extent without wilting (Bressani et al., 1993). It has a short growing season and is mostly cultivated in tropical regions. These characteristics make amaranth an appropriate crop, which can adapt itself to climate changes and ensure food security.

The main species that are grown as vegetables are A. hybridus, A. creuntus, A. tricolor, A. dubius, A. lividus, A. palmeri, A.blitum, A.ascendense, A.viridis, and A.gangeticus while A.

hypochondriacus, A. cruentus and A. caudatus are the main species mostly cultivated for their grain (Teutonico & Knorr, 1985). In the United States, amaranth is almost exclusively grown for its grain (Kauffman & Weber, 1990). Amaranth has a relatively high grain yield efficiency, where values up to 5,000 kg/ha have been reported (Stallknecht & Schulz-Schaeffer, 1993). Amaranth is also known as atoco and sangoracha in Ecuador, achos, achita, achis, incajataco, kiwicha, and comi in Peru, and millmi and coimi in Bolivia (Belton & Taylor, 2002b). Since most of amaranth's grain usage is similar to that of cereal grains, it is often referred to as a pseudo-cereal. Because there is no gluten in amaranth's protein, it can substitute wheat in the diet of those suffering from gluten allergy. Currently, the largest sector for amaranth usage is the health food industry (Belton & Taylor, 2002b).

2.4.1 History

Amaranth has a rich history dating back 8000 years, when it was initially cultivated in Mesoamerica. It has been domesticated in Mexico and Peru around 6000 to 8000 years ago (Sauer, 1950). For the ancient Aztec emperor, amaranth was the main staple food in a way that it was known as "super grain of the Aztecs" (Caselato & Amaya, 2012).

After the Spanish conquistadors landed in the "new word" in the sixteen century, growth and consumption of amaranth was banned mostly due to the fact that amaranth was vastly consumed as food and beverage in Aztecs religious rituals, as the "golden grain of the gods". Spanish conquistadors wanted to convert the Aztecs to Christianity, hence, handed sever punishment to anyone found growing or possessing amaranth. However, complete eradication of this fast growing, culturally important, and widely spread plant proved to be impossible (Sauer, 1950).

Amaranth may adapt itself to grow in any elevation, temperature, and soil. This amazing characteristics has spread its cultivation in most parts of the world from Nepal, China, Russia, and Thailand, to Nigeria, Mexico, south and north America, India, and Africa (Sauer, 1950).

2.4.2 Application as a food source

As it was previously mentioned, currently, the main sector for amaranth usage is through purchase at health food shops. This is mostly due to the appropriate contents of potential health promoting compounds in amaranth, such as rutin and nicotiflorin with antioxidant benefits. Due to appreciable amounts of these components, amaranth is usually recognized as a "natural biopharmaceutical" plant that may positively influence human health. It should be noted that the main purpose of amaranth application as a cereal is to obtain gluten-free products. However, in addition to having no gluten, these products are nutritionally enriched due to fantastic nutrient characteristics of amaranth grains. Amaranth grain may be cooked, roasted, popped, flaked, or extruded for human consumption. Amaranth is vastly combined with traditional cereal grains in breakfast foods, breads, multigrain crackers, and pastas (Bressani et al., 1993). Depending on the thermal conditions of their processing, amaranth proteins form supporting gels that could be applied in different gel-like products (Avanza et al., 2005).

Traditionally, amaranth was consumed in Mexico as a candy called alegría (means happiness in Spanish). In north India, amaranth is popped and mixed with honey. Amaranth popped grain is also mixed with melted jaggery to make iron and energy rich "laddus" (Sanz-Penella et al., 2012). Laddus is a popular food provided at the Mid-day Meal Program in municipal schools in India. Recently, there are many commercial uses of the nutrition amaranth extracted oil, especially in cosmetic applications (Bruni et al., 2001).

2.4.3 Morphology

Amaranth grain shape is approximately spherical with an average diameter of 1 mm, approximately 30 to 70 times smaller than a typical wheat grain. One thousand grains of amaranth weigh only 0.5 to 1.2 g with seed coat colour varying from black to brown and yellow to white. Similar classification as finger millet could be explained for the amaranth kernel (Figure 2-3). However, the embryo is much larger (25% of the grain weight) than finger millet grain (5%) and surrounds a starch-rich tissue (perisperm) in the form of a ring and lies curved in the inside of the seed coat (Palombini et al., 2013). The nutrient reserves of the amaranth seed are mainly discrete cellular structures including lipid, protein, carbohydrate, and organic and inorganic compounds. These reserves are not uniformly stored throughout the seed tissues. Storage proteins are within membrane-bound protein bodies in the embryo and endosperm cells. Lipid bodies are located in both embryo and endosperm cells, where they usually cover the protein bodies. Carbohydrates are stored as starch in the perisperm cells. There is no starch in the embryo or in the endosperm cells. Carbohydrates are also present throughout the entire seed kernel functioning as cell wall polysaccharides (Palombini et al., 2013).

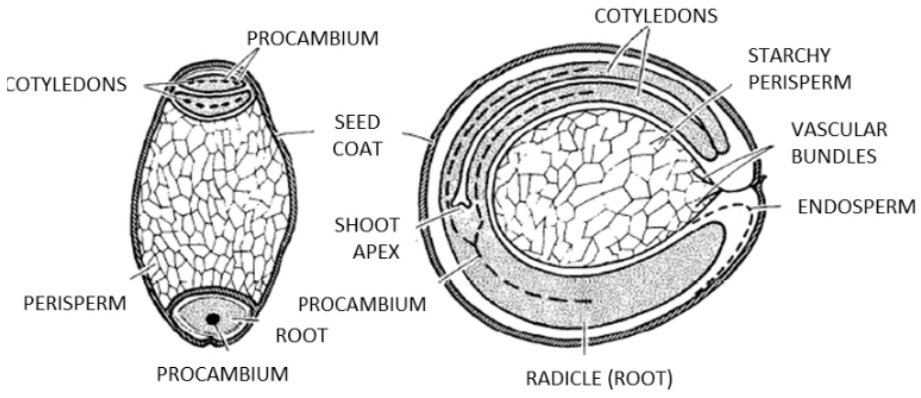

Figure 2-3: Amaranth grain morphology (a) cross-section view (b) longitudinal section view (Irving et al., 1981).

2.4.4 Nutritional value

Similar to millet, amaranth grain is an excellent source of protein, fat, and minerals. In the following sub-sections, the main included nutrients of amaranth grain are thoroughly presented.

2.4.4.1 Protein

Amaranth grain has a high content of protein with a range of 11.7 to 18.4%. The protein is mainly found in the germ and seed coat (65%), and in the starch-rich perisperm (35%) (Palombini et al., 2013). Similar to finger millet grain, amaranth does not contain gluten; therefore, is suitable for persons suffering from celiac disease. According to Osborne's protein classification, 40% of the amaranth proteins are albumins, 20% are globulins, 25 to 30% are glutelins, and 2 to 3% are prolamins (Antony & Chandra, 1998). Therefore, unlike finger millet, where prolamin was the major fraction, it is only found in small amounts in amaranth. Using the SDS-PAGE and according to the sedimentation coefficient, two main classes of globulin can be differentiated, namely 7S (conamaranthin) and 11S (amaranthin) globulins (Sripriya et al., 1997). The corresponding molecular weights of the 7S and 11S globlulin fractions are 30 to 37 kDa, and 18 to 27 kDa, respectively. These are classified as storage globulins. In addition to the high nutritional value of amaranth globulins, they also possess some potential functional properties, like emulsification. The functional properties of amaranth globulin isolate are considerably much better than soybean isolate, especially in the vicinity of its isoelectric point (Belton & Taylor, 2002a).

The composition of amaranth amino acids is outstanding. While lysine is a limiting amino acid in cereals compared to the reference protein of the FAO, it vastly exists in amaranth. The lysine content of amaranth is comparable with that of soybeans, which has one of the highest quality plant protein (Bressani & Garcia-Vela, 1990). Amaranth has a biological value of 75 (mostly due to high lysine content), which is similar to milk protein (Sripriya et al., 1997). In

addition, appreciable amounts of arginine and histidine, which are essential amino acids for infants, make amaranth grain valuable for infants' nutrition (Belton & Taylor, 2002a). The limiting amino acid of amaranth is leucine. The amino acids composition of native amaranth grain, as well as its albumin, glutelin, and prolamin protein fractions are presented in Table 2-5.

Table 2-5: Amino acids composition of the amaranth proteins (Bressani & Garcia-Vela, 1990).

g/100 g protein	Total Protein	Albumin Fraction	Glutelin Fraction	Prolamin Fraction
Lysine	7	7.3	6.2	6.0
Histidine	4.3	2.	2.2	2.5
Threonine	3.7	4.4	2.7	5.1
Valine	5.7	5.4	3.2	5.5
Methionine	2.8	2.0	3.6	0.6
Isoleucine	3.1	3.8	2.9	4.3
Leucine	5.5	5.9	4.4	9.0
Tryptophan	1.3	1.6	1	---
Phenylalanine	4.5	4.5	5.6	4.6
Arginine	13.4	7.1	12.0	6.6
Aspartic acid	11.9	9.4	9.8	8.7
Serine	4.9	4.7	3.4	6.2
Glutamic acid	21.5	18.8	28.9	15.6
Glycine	9.6	4.1	4.9	7.2
Alanine	7.7	3.7	3.4	4.6

Comparing Table 2-4 and Table 2-5 illustrates that finger millet and amaranth proteins in combination may serve as an appropriate complete protein source, where amaranth compensates the finger millet lysine deficit, while finger millet high leucine content covers the lack of this limited essential amino acid in amaranth.

2.4.4.2 Carbohydrate

Amaranth's carbohydrate content is comparably lower than other cereals like wheat, rice, and millet. Its total carbohydrate is around 65%. Amaranth starch is not located in the endosperm, but it is in the perisperm (Palombini et al., 2013). The amylose content of amaranth starch is much lower than that in other cereal starches, where its values vary from 0.1 to 11.1%. Amylopectin content is found to be composed of short-chain branched glucans with an average molecular weight

of 11.8×10^6 g/mol (Palombini et al., 2013). The small size of the starch granule, besides its high amylopectin content, results into distinguished physical properties for the amaranth starch. Comparing to other cereals, the starch obtained from amaranth grain has an excellent freeze-thaw and retrogradation stability. It has higher gelatinization temperature and viscosity, higher water-binding capacity, higher sorption capacity at higher water activity values, higher solubility, and higher swelling power and enzyme susceptibility (Palombini et al., 2013). Amaranth starch content may decrease by heat treatment, while in contrast, the dietary fiber increases slightly. This is mostly due to the formation of resistant starch (RS) from the contained starch (Palombini et al., 2013). Like dietary fiber, RS is not susceptible to human digestive enzymes; hence, reaches the colon, where it is fully or partially fermented by the bacterial microbiota. The RS/total starch proportion of native amaranth is around 2%. Crops containing more than 4.5% RS are usually considered to be a good source of prebiotics (Palombini et al., 2013). Resistant starch may act, as a carrier and preserve the viability of probiotics throughout food processing stages, like drying, and through the gastrointestinal tract. Similar to finger millet, mono- and disaccharides are in small amounts in amaranth, where the total sugar content is in the range of 3 to 5% of the total carbohydrate content. Among the sugars, sucrose (1.08-2.26%) is the major fraction (Palombini et al., 2013).

2.4.4.3 Fat

The fat content of amaranth is comparably higher than other cereals (8 to 10%) (Hulse et al., 1980). This is mostly due to the large germ size of the amaranth, where most of the fat of cereals is located. The fat is characterized by a high amount of unsaturated fatty acids, with a very high content of linoleic acid (more than 50% of the total fatty acids). Oleic acid content (20%) and

palmitic acid (20%) are the next major fractions in the composition of amaranth's fatty acids (Arendt & Dal Bello, 2011).

Among oils extracted from cereal grains, amaranth has the highest level of squalene (3 to 7%), which makes it an appropriate choice in pharmaceutical and cosmetic industries as well as for applications as an oxidation-resistant industrial lubricant (Bruni et al., 2001; Escudero et al., 2004).

2.4.4.4 Minerals

Similar to finger millet, total ash content of the amaranth (3.5%) is about two times higher than other cereals. Amaranth is rich in minerals particularly calcium, magnesium, iron, potassium, and zinc (Palombini et al., 2013). Calcium in amaranth is reported to be as high as 308 mg/100 g; therefore, the grain has been proposed to be used as a weaning food ingredient (Gélinas & Seguin, 2007).

2.4.4.5 Vitamins

Generally, amaranth is not an important source of vitamins. However, the thiamin content of amaranth is higher than in wheat. Besides, amaranth contains more riboflavin (vitamin B_2) and ascorbic acid (vitamin C) than most other cereals. Amaranth is a good source of vitamin E as well (Belton & Taylor, 2002a).

2.4.4.6 Anti-nutrients

Although they affect nutritional bio-availability, most anti-nutrients that have been studied to date in grain amaranth appear to be non-problematic for human health. Levels of tannin and phytic acid are comparable to those observed in other cereals, though trypsin and chymotrypsin activity were reported to be lower. Amaranth contains 0.3 to 0.6% phytic acid. It has been proven that phytic acid and tannins have inhibitory effects on the digestibility of starch (Belton & Taylor,

2002a; Gélinas & Seguin, 2007). The tannin content of the amaranth grain is 0.104 to 0.116% (Gélinas & Seguin, 2007). Phytic acid may also influence protein digestion and absorption processes through forming complexes with protein and digestive enzymes.

Amaranth grain contains protease inhibitors in very small amounts, less than most conventional pseudo-cereals and cereals. These inhibitors competitively inhibit the activity of proteolytic enzymes by forming compounds with them (Belton & Taylor, 2002b). Grain amaranth does not contain an appreciate amount of saponins (less than 0.1% in aescin equivalent). Saponins can cause intense foaming activity in aqueous solutions, by making complexes with proteins and lipids (Belton & Taylor, 2002b). It is concluded that due to the low content of saponins in amaranth seeds and their relatively low toxicity, the amaranth-derived products create no significant hazard for consumers (Belton & Taylor, 2002b). Other anti-nutrient factors like oxalates and nitrates are present mostly in amaranth leave and stem; hence, they would be important in foraging applications (Rastogi & Shukla, 2013).

2.4.4.7 Total phenolic compounds

A study performed on two varieties of *Caudatus* amaranth, Centenario and Oscar Blanco, demonstrated total phenolic contents of 98.7 and 112.9 mg/100 g, respectively, using Gallic acid (Folin-Ciocalteu reagent) as the reference (Repo-Carrasco-Valencia et al., 2009). Czerwinski et al. (2004) determined total phenolics expressed as gallic acid equivalent in two amaranth samples. The amount of polyphenols in the amaranth samples was in the range of 14.72 to 14.91 mg/100 g.

2.5 Food processing steps

In this section, three important food processing steps; malting, fermentation, and drying are introduced. In addition, effects of these processing stages on nutritional characteristics of finger millet and amaranth grains are thoroughly presented.

2.5.1 Malting

Malting is a low-cost traditional processing approach to improve nutritional quality of cereals through some biochemical modifications of the grains (Saleh et al., 2013). It is mainly used to lower the dietary bulk of cereals by conversion of significant amounts of starch to sugars and short-chain oligosaccharides (Arora et al., 2011; Saleh et al., 2013).

Starch is principally responsible for the viscosity in cereal gruels (Desikachar, 1980; Treche, 1999), which, within the malting process, is hydrolyzed by the malt enzymes (α and β amylases) (Malleshi & Desikachar, 1986b). This would reduce the slurry's viscosity, thus improving texture, and the reduced starch would consequently improve digestibility and calorie uptake. For that reason, malted finger millet is traditionally used for infants' weaning foods (Malleshi, 1999).

The complete process of malting mainly consists of four stages; soaking, germination, roasting, and milling, where the most desired physico–chemical changes occur during the soaking and germination stages (Swami et al., 2013). Among the various tropical cereals, finger millet shows good malting characteristics (Malleshi & Desikachar, 1986a). Variations in the free sugar and non-starch polysaccharides have been observed and reported throughout the soaking and germination of finger millet (Omary et al., 2012). Omary et al. (2012) reported a 23% reduction in the starch content of finger millet following malting. An increase in the amount of glucose, fructose, maltose, and sucrose in malted finger millet has been reported (Charalampopoulos et al., 2002).

Malted cereals contain highly digestible carbohydrates. The number of small starch granules (4–7 μm) increase during germination, therefore, the apparent viscosity has been shown to decrease from 1.52 (Pa.s) to 1.35 and 1.18 after 24 and 96 hr germination of finger millet (Shobana et al., 2012). Besides, the peak viscosity of malted starch is lower than the one obtained for native

finger millet. The swelling power in water at 45 °C increased from 0.5 to 0.7 g/g, while at 85 °C, it would be 9.0 g/g (Shobana et al., 2012).

Malting improves protein bio-availability and digestibility in the cereal grains as well. Throughout the malting process, the cell walls surrounding starch granules are broken-down and proteinaceous matrices are converted into soluble peptides and amino acids to provide substrates for protein synthesis (Eneje et al., 2013). In addition, the extractability of proteins and the free amino acid contents significantly increase during germination (Mbithi-Mwikya et al., 2000a). Therefore, germination has been traditionally used in the development of weaning foods with improved protein digestibility.

Crude finger millet grain has a high content of anti-nutrients, such as phytate, and phytic acid that bind divalent cations, and tannins that complex proteins and carbohydrates (Omary et al., 2012). The presence of these anti-nutrients and enzyme inhibitors restricts the bio-availability and digestibility of the nutrients present in finger millet (Ramachandra et al., 1977; Ravindran, 1991). It is shown that a well designed soaking stage significantly decreases the phytate and tannin content to undetectable levels (Mbithi-Mwikya et al., 2000b). Nirmala et al. (2000) reported on the beneficial changes in carbohydrate, essential amino acids, organic acids and phytate levels during the malting of finger millet. Furthermore, soaking and germination are found to be very effective in increasing the extractability of trace elements like Ca, Fe, Cu, Zn and Mn (Rateesh et al., 2012). Phytate forms complex matrices with these essential elements and reduces their bio-availability (Rateesh et al., 2012). Degradation of phytate would thus enhance the availability of these minerals (Rateesh et al., 2012). According to Sripriya et al. (1997), finger millet is the richest source of calcium among all cereals (340 mg/100 g); however, due to the presence of anti-nutrients, only 162 mg/100 g is available for uptake in the crude grains. It was shown that malting enhanced this

value up to 227 mg/100 g (Sripriya et al., 1997). Similar improvement was observed for iron and other minerals (Omary et al., 2012).

Similarly, the nutritional characteristics of amaranth grain could be improved through malting. These improvements are associated with analogous biochemical mechanisms as experienced by finger millet, and generally most cereals (Omary et al., 2012). Therefore, total and reducing sugars and reduced starch values increase in grains that are malted (Omary et al., 2012). As for the anti-nutrients, tannin, phytate content, and phytic acid are drastically reduced through soaking and germination of amaranth seeds (Colmenares de Ruiz & Bressani, 1990). The content reduction of these anti-nutrients results into a corresponding increase in the bio-availability of nutrients and minerals (Colmenares de Ruiz & Bressani, 1990).

The effects of temperature and duration of the malting stage on the nutritional improvement of cereal grains have been studied by many researchers (Chiba et al., 2012; Saleh et al., 2013; Swami et al., 2013). Swami et al. (2013) stated that as the germination time increased, the protein availability of finger millet increased. Indeed, in their study, the protein content of samples increased from 14 to 17.5% as the germination time increased from 8 to 24 hr. Malleshi and Desikachar (1986a) stated that as malting progressed, finger millet produced adequate amount of α-amylase with "agreeable flavor and acceptable taste", which was comparable to wheat. Amylases are the main malt enzymes that hydrolyze the starch contents of the grains.

Although amaranth grain is famous for having a high quantity of lysine, this limiting amino acid may further increase throughout the malting process. Balasubramaninan and Sadastivam (2002) claimed a 31% increase in the lysine content following 24 hr of germination. Besides, their study stated an increase in the percentage of water-soluble protein from 15.6 to 21.6% after 24 hr of germination. On the other hand, Colmenares de Ruiz and Bressani's (1990) study illustrated

that there was no significant difference in total protein, crude fiber, and ash content of amaranth during germination, while a decrease in the level of lipid and phytic acid was observed. Aligned with findings of other researchers, they observed an increase in reducing sugars, total sugars, and damaged starch as a function of increasing germination time. Therefore, for both millet and amaranth grains, malting is a simple way to promote the amino acids profile, especially the lysine content.

Germination of amaranth increases the crude protein contents as well as free amino acid components. Similar to Balasubramaninan and Sadastivam (2002), a 30% increase in the lysine content of amaranth after 24 hr of germination was observed by Arendt and Dal Bello (2011). Lipid and phytic acid content of the grain decreased as germination progressed. Furthermore, it has been observed that the germinated seeds show a pinky color, which appears to be very attractive for various food uses. This coloring is probably due to the synthesis of amaranthine pigments during germination (Palombini et al., 2013).

Effects of the germination duration, up to 192 hr, on α- and β- amylases activities, starch, sugar, protein, and lysine contents of amaranth grain have been investigated (Omary et al., 2012). The highest α-amylase activity was observed initially, while β-amylase reached its peak activity after 72 hr of germination. The joint action of both amylases resulted into a uniform decrease in the starch content. Furthermore, an increase in the total sugars content during the initial period of germination was reported (Omary et al., 2012). Kanensi et al. (2012) suggested that the optimum soaking and germination times for amaranth grain are 5 and 24 hr, respectively.

Towo et al. (2003) investigated the effect of germination process on the total phenolic content of finger millet. Grains were germinated in darkness at 25 °C. Germination duration was not specified in their paper. Total phenol decreased from 4.2 to 3.3 mg/g in Catechin Equivalents

(CE). Sripriya et al. (1996) reported a similar decrease in phenolic content during germination of finger millet. In their study, total phenol decreased from 102 to 67 mg/100 g in Chlorogenic Acid Equivalent (CGA). Again, malting parameters, including duration, were not specified.

In another study, the effect of germination on the phenolic content and DPPH radical scavenging activity was investigated for *Cruentus* amaranth (Alvarez-Jubete et al., 2010b). Grains were germinated for 98 hr at 10 °C. Total phenol content increased from 21.2 up to 82.2 mg GAE/100g after germination. A slight decrease in the DPPH activities from 28.4 to 27.1 mg Trolox/100 g was observed.

In summary, reduction of anti-nutrient factors, alteration in phenolic compounds and antioxidant activities, enhancement of protein, starch, and mineral availability and digestibility, improvement of the texture and taste of the resulted flour, and improvement of calorie density through the viscosity reduction are the most important outcomes of the finger millet and amaranth malting. This process could be used to produce nutrient rich flours, which can be applied in weaning food production. However, millet and amaranth are not currently utilized at the industrial scale and the use of malting processes in the food industry are still very limited. To further employ malting processes in industry for millet and amaranth grains, it will require process optimization along with large-scale germinators with state of art control systems. Scaling-up to produce malted millet and amaranth products would increase product uniformity and quality, which ultimately would result in consumer acceptability and satisfaction.

2.5.2 Probiotic fermentation

Over the past centuries, fermentation has been widely employed for preservation of a broad range of foods and improvement of their flavor, taste, and palatability (Nout, 1994; Poutanen et al., 2009; Sajilata et al., 2002). Fermentation is known to lower the anti-nutrient contents of cereals;

hence, it can significantly improve products nutritive values (Poutanen et al., 2009). Saleh et al. (2013) showed that fermentation processes drastically decreased the anti-nutrient components, phytates, and tannins in finger millet. As a result, an enhancement was observed in the mineral availability, soluble protein, and *in-vitro* protein and starch digestibility (Saleh et al., 2013). Studies showed that fermentation of finger millet for 24 hr using endogenous grain microflora significantly reduced the amount of anti-nutrients activities; phytate (20%), tannins (52%) and trypsin inhibitor (32%). Consequently, fermentation increased HCl mineral extractability; Ca (20%), p (26%), Fe (27%), Zn (26%), Cu (78%), and Mn (10%) (Shobana et al., 2012).

Fermentation also improved the calorie density by dropping the product's viscosity or dietary bulk (Sajilata et al., 2002). Singhal and Kulkarni (1988) observed an improvement in the nutritive properties of amaranth grain following a fermentation stage. Similar results have been reported by other researchers on the positive effects of fermentation on finger millet (Antony & Chandra, 1997; Mbithi-Mwikya et al., 2002b; Shobana et al., 2012) and amaranth grains (Busolo, 1992).

Sripriya et al. (1997) studied the effects of germination and fermentation stages on carbohydrate, protein, and minerals in finger millet. They germinated the grains for 24 hr and then fermented them for 48 hr. They observed major biochemical changes during the first 8 hr of fermentation, where the pH decreased from 5.8 to 3.8 and total sugar, reducing sugar, and free amino acid increased by 2, 12, and 10 folds, respectively. The phytate content decreased (60%), and consequently the mineral HCl extractability increased (47%) (Sripriya et al., 1997).

Traditionally, lactic acid fermentation has been used for amaranth grain. Fermentation breaks down the starch and non-starchy polysaccharides to smaller components like sugars and oligosaccharides, and protein polymers to amino acids; hence it improves the digestibility of starch

and protein. Besides, by reducing the anti-nutrient factors, the bio-availability of minerals would be improved throughout the fermentation stage (Palombini et al., 2013).

If fermentation is accomplished with probiotic organisms, it can bring specific added advantages apart from the nutritional improvement. Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer health benefits on the host" (Sanders, 2003). Specific bacteria, especially the species of lactobacilli compose the majority of recommended probiotics (Goldin & Gorbach, 1992). In addition to their nutritional benefits during fermentation, probiotic organisms have positive effects on metabolism improvement, constipation reduction, and cholesterol level reduction (Fukushima & Nakano, 1996; Sindhu & Khetarpaul, 2001).

In the case of infants' weaning products, supplementing them with probiotics can improve the balance of gastrointestinal microbiota, which are normally damaged through the consumption of antibiotics (Baldeon et al., 2008). Antibiotics kill both harmful and beneficial bacteria. Therefore, as the number of beneficial bacteria reduces, the probability of digestive problems, such as diarrhea, yeast, and urinary tract infections increases (Baldeon et al., 2008).

As it was mentioned in the definition of probiotics, they are live microorganisms and should remain viable in an adequate amount before reaching their desired destination, the gastrointestinal tract (GI). Therefore, they should be appropriately maintained from the fermentation step throughout all food processing stages, storage, and shelf-life until reaching the GI for sustained growth and adhesion. The most well-known and appropriate nutrient source for probiotic bacteria, throughout these steps, are prebiotics (Crittenden & Playne, 1999).

Prebiotics are defined as non-digestible food components that when they are administered in sufficient amount can selectively stimulate the growth and activity of one or a number of microbes

in the colon (Crittenden & Playne, 1999). Therefore, prebiotics are necessary for viability and vitality of the probiotics. The mixture of probiotics and prebiotics results into a new family of functional foods, called synbiotic products (Roberfroid, 1998; Schrezenmeir & de Vrese, 2001). Farooq et al. (2013) showed that the *in-vitro* synthesis of short chain fatty acids was enhanced through fermentation of millet dietary fiber by human fecal probiotic bacteria; *Lactobacillus rhamnosus*, *Lactobacillus acidophilus, Bifidobacterium longum,* and *Bifido-bacterium bifidus*. Therefore, the fiber content of finger millet may be used as an appropriate prebiotic source for our selected bacteria; *Lactobacillus rhamnosus, Bifidobacterium longum, and Bifidobacterium infantis*.

Bifidobacteria were first isolated from a breast-fed infant by Henry Tissier (1990). He found that these bacteria are dominant in the gut flora of infants. He noticed that when the bacteria existed in their intestinal flora, the baby suffered less from gastrointestinal disorders. Therefore, he recommended that these bacteria be administered to babies diagnosed with diarrhea (Tissier, 1990).

Naturally, mother's breastmilk contains high contents of lactose. In the infant's gastrointestinal tract, when the milk is fermented by *Lactobacillus rhamnosus* and *Bifidobacterium longum*, the lactose content of the milk is converted to lactic acid. This drops the pH of the medium, which would consequently provide a harsh environment for the gram-negative bacteria (pathogens) to grow; hence, provides health benefits for the host.

Cereals, such as finger millet and amaranth, can be utilized as fermentable substrates for the growth of probiotic microorganisms (Charalampopoulos et al., 2002; Heller, 2001). In addition, cereals are appropriate sources of non-digestible carbohydrates that not only promote several valuable physiological effects, but also stimulate the growth of lactobacilli and bifidobacteria;

hence, act as prebiotics (Anekella, 2011; Crittenden & Playne, 1999; Samanta et al., 2011). Cereals contain water-soluble fiber, oligosaccharides, and resistant starch that have been recognized to act as prebiotic agents (Charalampopoulos et al., 2002; Crittenden & Playne, 1999). However, most of the lactic acid bacteria cannot ferment starch directly; therefore, it is usually necessary to have an amylolytic preparation for that purpose. This can be mostly favoured through a germination or malting stage (section 2.5.1), which then enhances the fermentation process (Hansen et al., 1989; Lorri & Svanberg, 1993).

2.5.3 Drying

The survival of probiotics in a food system, especially during the storage period, is strongly affected by the moisture content or water activity of the product; the lower the moisture level, the higher the survival of probiotics (Champagne et al., 2012; Morgan et al., 2006; Reddy, 2007). Dried conditions keep bacteria in the quiescent state or stationary phase; hence, the probiotic viability can be maintained at an acceptable level even if the product is stored at ambient temperature for long periods (Chávez & Ledeboer, 2007; Higl et al., 2007; Reddy, 2007).

Throughout the food processing stages, if the temperature exceeds certain limits, 45 to 50 °C, the probiotics' viability would be negatively affected. Higher temperatures result into shorter probiotics' lifetimes (Chávez & Ledeboer, 2007; Reddy, 2007). Therefore, it is recommended to add probiotics downstream of the heating, cooking, and pasteurization stages in the food manufacture to avoid the imposed high temperature of these processes (Chávez & Ledeboer, 2007; Reddy, 2007). Since, in the present research study, the probiotics are used in the fermentation stage, and are desired to remain alive throughout the drying stage, traditional high temperature drying approaches should be avoided (Morgan et al., 2006; Reddy, 2007). For instance, forced-air drying is a very common drying approach in industry for moisture reduction (Hall, 1980). The

convection heat transfer mechanism enhances the drying rate. As the moisture content of the solid samples falls, they heat up and the resulted higher temperature speed up diffusion of water from the interior of the samples to the surface (Hall, 1980). However, since usually high temperature air is employed, the method is not well established for thermolabile probiotic products (Reddy, 2007).

Among different drying approaches, spray drying and freeze drying are more common downstream processes used for preparation of dried probiotic cultures (Madhu et al., 2011; Reddy, 2007). Despite the fact that spray drying is less costly than freeze drying (Reddy, 2007), it has not been widely commercially developed for viable cultures (Daemen & Stege, 1982; Reddy, 2007). This is mainly due to the low survival rates of the probiotics in tolerating its high temperature conditions, and difficulties in rehydrating the product at the consumption stage (Daemen & Stege, 1982; Phadtare & Inouye, 2001).

Alternatively, freeze-drying, also known as lyophilisation or cryodesiccation, is the most satisfactory method for the long term preservation of viable cultures (Champagne et al., 1991). It is usually employed to preserve LAB bacterial starter cultures in the dairy and food fermentation industries (Kearney et al., 1990). The basic idea is to freeze the fermented slurry and then reduce the surrounding pressure to allow the frozen water to sublime directly from the solid phase to the gas phase (Champagne et al., 1991). Usually a secondary drying process (desorption) is employed to drop further the moisture level to a value that will no longer support biological activity or chemical reactions (Reddy, 2007). Therefore, not only the inoculated probiotics are conserved viable, the approach also causes less damage to the food structure, like shrinkage or toughening, compared to other drying approaches using high temperatures (Reddy, 2007). Besides, the flavor, smell and nutritional content generally remain unchanged using this approach (Champagne et al.,

1991). Freeze-drying is commonly used for drying of fermented cereals both at laboratory and industrial scales (Gimbi & Kitabatake, 2002; Omary et al., 2012).

The percentage of probiotic survival through drying approaches has been reported to increase by increasing their initial concentration up to 10^{11} CFU/mL (Reddy, 2007). In addition, bacterial cells at the stationary stage are more resistant to the drying conditions (Reddy, 2007). It is recommended that probiotic products should contain at least 10^7 CFU live microorganisms per gram or per mL (Ishibashi & Shimamura, 1993) at the time of consumption in order to adequately benefit the consumer.

Connecting Statement to Chapter 3

In **Chapter 2,** the current commonly employed food-processing techniques for nutritional improvement of the cereal grains flours were presented. According to the performed literature survey, malting process has been traditionally used for enhancing the nutrient bio-availability in cereal grains, especially in infants' weaning food. In Chapter 3, optimization of the germination/malting process for anti-nutrient reduction and consequently protein digestibility enhancement in finger millet grain has been investigated. To pursue this goal, effects of the two most influential design parameters in malting process, germination duration and temperature, were studied. For each temperature-duration combination, the involved anti-nutrients and protein contents were assessed. An ANOVA study was performed on the obtained data to assess possible correlations between the design factors and the monitored quantities. The current chapter has been written in the manuscript style and is accepted to be published in **Journal of food science and technology** as,

"**Malting process optimization for protein digestibility enhancement in finger millet grain**"

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Contributions made by the different authors are as follows;

- The first author, **Sara Najdi Hejazi**, is the Ph.D. student, who prepared the experimental design, conducted the laboratory work, analyzed the results, and prepared the manuscript.

- The second author, **Valérie Orsat**, is the thesis supervisor, who initiated and stimulated ideas, provided the technical advice, suggestions, experiment facilities, and guided the first author in writing, correcting, and preparing the manuscript throughout the study.

Furthermore, parts of the obtained data and performed analyses in **Chapter 3** have been presented in two conference presentations, as follows;

1. Sara Najdi Hejazi and Valérie Orsat, "**Effect of Germination Process on the Protein Digestibility of Finger Millet and Amaranth"** Northeast Agricultural and Biological Engineering Conference, Oral presentation, July 12-15, 2015, Newark, Delaware, USA.
2. Sara Najdi Hejazi and Valérie Orsat, "**Effects of Germination on Finger Millet and Amaranth Grains**" Northeast Agricultural and Biological Engineering Conference, Oral presentation, July 27-30, 2014, Kemptville, Ontario, Canada.

3 Malting process optimization for protein digestibility enhancement in finger millet grain

3.1 Abstract

Finger millet (*Eleusine coracana*) is a nutritious, gluten-free, and drought resistant cereal containing high amounts of protein, carbohydrate, and minerals. However, bio-availability of these nutrients is restricted due to the presence of an excessive level of anti-nutrient components, mainly phytic acid, tannin, and oxalate. It has been shown that a well-designed malting/germination process can significantly reduce these anti-nutrients and consequently enhance the nutrient availability. In the present study, the effects of two important germination factors, duration and temperature, on the enhancement of *in-vitro* protein digestibility of finger millet is thoroughly investigated and optimized. Based on a central composite design, the grains were germinated for 24, 36, and 48 hr at 22, 26, and 30 °C. For all factor combinations, the protein, peptide, phytic acid, tannin, and oxalate contents were evaluated and digestibility was assessed. It was shown that during the malting/germinating process, both temperature and duration factors significantly influenced the investigated parameters. Germination of finger millet for 48 hr at 30 °C increased protein digestibility from 74% (for native grain) up to 91%. Besides, it notably decreased phytic acid, tannin, and oxalate contents by 45%, 46%, and 29%, respectively. Linear correlations between protein digestibility and these anti-nutrients were observed.

Keywords: *finger millet, germination process, in-vitro protein digestibility, anti-nutrient components*

3.2 Introduction

According to FAO, in 2014, the worldwide production of millet grains was 30 million tons, which made it the 6th most important agricultural cereal crop (FAOSTAT, 2014). Millet is one of the most drought-resistance grains; hence, it is widely grown in arid and semi-arid areas of Asia and Africa (Saleh et al., 2013). Furthermore, millet grain is gluten-free, has a short growing season, has low production costs, and possesses a good nutritional profile, which is comparable or better than the other vastly consumed cereals (Omary et al., 2012; Saleh et al., 2013).

Based on their annual production volumes, four millet varieties are more common: Pearl millet (*Pennisetum typhoideum*), Foxtail millet (*Setaria italica*), Proso millet (*Panicum miliaceum*), and Finger millet (*Eleusine coracana*). Pearl millet is more widely produced and consumed worldwide and therefore, it has been the subject of more research investigations (Abdelrahaman et al., 2007; Omary et al., 2012). Nevertheless, finger millet is attracting increasing interest since it contains higher amounts of essential amino acids, especially lysine, threonine, and valine (Ravindran, 1991). This makes finger millet superior comparing to other millets and even other cereals, particularly in its use as functional food staples, like weaning products. Finger millet typically has a carbohydrate content of 72%, protein 7.3%, fat 1.3%, dietary fiber 11.5%, and mineral 2.7% (FAOSTAT, 2014).

Despite the appropriate nutrition profile of finger millet, similar to other cereals, the bio-availability of its nutrients is restricted due to the presence of high levels of anti-nutrient components and enzyme inhibitors. Anti-nutrients like phytate and oxalate, that bind essential minerals and proteins, along with tannins that complex proteins and inhibiting enzymes, all negatively affect the nutrient bio-availability of finger millet and reduce its digestibility (Pushparaj & Urooj, 2014; Ravindran, 1991).

Among millet varieties, finger millet has been reported to contain higher amounts of tannin (0.04 to 3.74% of catechin equivalents) (Antony & Chandra, 1999; Rao, 1994). Besides, the grain has considerable amounts of phytic acid (500-600 mg/100g) (Antony & Chandra, 1999). Studies have shown that the *in-vitro* protein digestibility is negatively associated with the tannin (Mbithi-Mwikya et al., 2000b; Ramachandra et al., 1977) and phytic acid (Mbithi-Mwikya et al., 2000b) contents in finger millet. Surprisingly, very few studies have been performed on the assessment of the oxalate content of finger millet. Ravindran (1991) reported that finger millet contains 29 mg/100 g of oxalate. A more recent study claims that oxalate in raw finger millet is 11.3 mg/100 g (Amalraj & Pius, 2015). In both studies, it was shown that an appropriate soaking step may significantly reduce the oxalate content of the grains. This is due to the fact that in millet, oxalate mostly exists in its water-soluble form (Amalraj & Pius, 2015).

It is recognized that simple traditional food processing treatments, like soaking and malting/germination may significantly reduce the anti-nutrient contents of cereal grains and improve their nutrients' bio-availabilities. The increased phytase activity during the germination step is the reason for the reduction of phytic acid in sprouts, since phytase hydrolyzes phytic acid. Khetarpaul and Chauhan (1989) reported a 40% reduction in phytate level after 24 hr germination of pearl millet. Chavan et al. (1989) reported an overall trend of phytic acid reduction in sprouts. Similarly, germination decreased the tannin content by 54% in finger millet (Rao, 1994). In addition, soaking and germination are found to be very effective in increasing the extractability of trace elements, like Ca, Fe, Cu, Zn, and Mn. This is due to the fact that phytates form complex matrices with these essential elements, which would be broken throughout the food processing steps (Rateesh et al., 2012). In recent investigations, it has been confirmed that well-designed

soaking and germination stages may significantly decrease the phytate and tannin contents in millet grains (Abdelrahaman et al., 2007; Omary et al., 2012).

A study on 32 varieties of finger millet showed relatively low *In-Vitro* Protein Digestibility (IVPD) ranging from 55.4 to 88.1% (Ramachandra et al., 1977). Antony and Chandra (1999) reported slightly lower values for IVPD of finger millet ranging from 50 to 65%. Besides, the authors observed a negative correlation between IVPD and phytates and tannins contents. Studies have shown that the IVPD of millet increased after a malting/germination process (Omary et al., 2012). Germination decreased the tannin content in millets and consequently improved their protein bio-availability (Mbithi-Mwikya et al., 2000b). Khetarpaul and Chauhan's (1990) experiments showed a notable increase (51%) in IVPD of pearl millet grains after soaking for 12 hr and germinating for 24 hr at 30 °C. Similarly, a 59% increase was observed by Chaturvedi and Sarojini (1996) for 72 hr germinated pearl millet.

Effect of germination temperature and duration on the nutritional improvement of cereal grains has been studied by a few researchers (Saleh et al., 2013; Swami et al., 2013). Due to different soaking practices, germination duration and temperature, and other influential factors, a few contradictory results were reported in the literature (Malleshi & Klopfenstein, 1998; Mbithi-Mwikya et al., 2000b; Sripriya et al., 1997). However, regarding protein content and IVPD, the majority of the studied millet species have shown a direct positive correlation with germination duration (Saleh et al., 2013; Swami et al., 2013). Swami et al. (2013) stated that as the germination time increases, the protein availability of finger millet increases. Indeed, in their study, the protein content of samples increased from 14 to 17.5%, when the germination time increased from 8 to 24 hr.

Crude protein of Proso millet showed 16% increase in seeds that were germinated up to 8 days at room temperature (Parameswaran & Sadasivam, 1994). Mbithi-Mwikya et al. (2000b) observed 30% increase in total protein content of finger millet after 96 hr of germination at 30 °C. In addition, they observed significant decreases in the anti-nutrient factors, where tannins and phytates decreased to undetectable levels at the end of treatment. On the other hand, their study reported a 13.3% loss of the seeds' dry matter throughout the germination process. Since the most significant nutrient changes occurred in the first 48 hr of germination, it was concluded that germination of finger millet beyond 48 hr was not necessary (Mbithi-Mwikya et al., 2000b). Abdelrahman et al. (2007) investigated the effect of germination duration (up to 6 days) on the anti-nutrient elements of pearl millet. They showed that the phytic acid content decreased significantly ($P<0.01$) within the first 2 days of germination. Thereafter, the drop-rate was considerably reduced.

Unlike for the duration, effects of temperature have not been well investigated for the germination of finger millet grains. In the past studies, germination was usually conducted at room temperature (Omary et al., 2012). Since temperature directly affects the enzyme activities during sprouting, it is important to precisely monitor its effect. In the present study, the two main important factors in the germination of finger millet, duration and temperature, were thoroughly investigated. As the main anti-nutrient components in finger millet, phytate, tannin, and oxalate contents were monitored. Proteins and peptides were evaluated for each germination treatment and after digestion by pepsin and pancreatin enzymes. Finally, the protein digestibility was obtained and optimized for the selected germination factors and over their specified ranges.

3.3 Materials and methods

3.3.1 Materials

The sproutable finger millet was obtained from University of Agricultural Sciences (Dharwad, India). All the chemicals used in the study were of analytical grades. HPLC graded water (double distilled water (ddH_2O)) was supplied using Simplicity^{TM} water purification system (Millipore, USA). Hydrochloric acid, pepsin, pancreatin, o-phthaldialdehyde (OPA), sodium tetraborate, β-mercaptoethanol, ascorbic acid, and leucine-glycine (Leu-Gly) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, sodium phosphate, vanillin, ammonium molybdate, sulphuric acid, and methanol were provided by Fisher Scientific (Fair Lawn, NJ, USA). Sodium Dodecyl Sulfate (SDS) was obtained from Bio-Rad. Ethanol was purchased from Commercial Alcohols (Industrial and Beverage Alcohol Division of Green-field Ethanol Inc., Brampton, Ontario, Canada).

3.3.2 Malting process

Grains seeds were steeped in water at a ratio of (1:5 w/v) for 24 hr at room temperature. Water was drained off and the soaked seeds were germinated for three different durations (24, 36, and 48 hr), each at three different temperatures (22, 26, and 30 °C). The sprouted and native seeds were freeze-dried and ground to flour for further analysis.

3.3.3 Statistical design and analysis

The Face-centered Central Composite Design (CCD) was used for the experimental design in the current study. Two factors, malting temperature (Temp), and duration (Time), each at three levels, (22, 26, 30 °C) and (24, 36, 48 hr), respectively, were used for preparing the experimental design with a total of 12 treatment combinations. This consists of 4 factorial (GM1, GM3, GM7, and GM9), 4 axial (GM2, GM4, GM6, and GM8), and 4 central (GM5.1, GM5.2, GM5.3, and

GM5.4) point combinations (Table 3-1 and Table 3-2). Based on the literature, due to the loss of seeds' dry matter and decrease in process efficiency, the germination duration in the present study was restricted to 48 hr (Mbithi-Mwikya et al., 2000b; Vidyavathi et al., 1983). In addition, the temperature range was chosen close to room temperature to avoid the increased cost due to heating requirements. JMP software version 11 (SAS Institute Inc., Cary, NC, USA) was used for the experimental design and analysis. Using ANOVA and regression models, the individual (linear), quadratic, and combined (bi-linear) effects of the factors were evaluated. The resulting response surface for each investigated quantity, Z, is expressed as follows;

$$\mathrm{Z} = \mathrm{Intercept} + \beta_1 \mathrm{X}_1 + \beta_2 \mathrm{X}_2 + \beta_{11} \mathrm{X}_1^2 + \beta_{22} \mathrm{X}_2^2 + \beta_{12} \mathrm{X}_1 \mathrm{X}_2 \qquad (3\text{-}1)$$

In this equation, X_1 =temperature and X_2 =duration are the design factors. β_1 and β_2 represent the regression coefficients of the linear, β_{11} and β_{22} are the coefficients of the quadratic, and β_{12} expresses the interactive or bilinear effects. These coefficients were assessed using ANOVA, while their significance were evaluated statistically based on the F-values at the probabilities; $p<0.0001$, $p<0.0005$, $p<0.001$, $p<0.005$, $p<0.01$, and $p<0.05$.

3.3.4 Protein content determination

Protein content was quantified using the Bradford method according to the instructions of the kit manufacturer (#500-0006, Bio-Rad, Mississauga, Ontario) (Bradford, 1976). The assay was based on the ability of proteins to bind Coomassie brilliant blue G 250 and shift the color from red to blue. Briefly, 20 μL of the sample was mixed with 980 μL of dye reagent (1 part Bradford reagent in 4 parts ddH_20) and incubated for 5 min at room temperature. The resulting color intensity was directly proportional to the concentration of protein and was determined spectrophotometrically (Ultraspec 1000, Amersham Pharmacia Biotech, NJ, USA) at the wavelength of 595 nm. The protein concentrations were compared to a standard curve of bovine

serum albumin (0–2 mg/mL). Proteins with molecular weight of more than 3-5 kDa were detected using this approach.

3.3.5 *In-vitro* enzymatic protein digestion

In-vitro protein digestibility was assessed by employing the pepsin and pancreatin digestion method of Vilela et al. (2006) with small modifications. The undigested protein residues before pepsin digestion, after pepsin digestion, and after pancreatin digestion were evaluated using the Bradford method (section 3.3.4). The *in-vitro* protein digestion percentage was calculated by subtracting the undigested protein from the initial total protein of the sample as follows;

$$\text{IVPD}(\%) = \frac{\text{Initial protein} - \text{final undigested protein}}{\text{Initial Protein}} \times 100 \qquad (3\text{-}2)$$

3.3.6 Peptide content determination

The peptide content was determined by modification of the Church et al. (1983) method. The method is based on binding of the released α-amino groups with OPA reagent, causing an invisible color change, which can be detected spectrophotometrically.

Initially, 50 mL of OPA stock solution (25 mL of 100 mM sodium tetraborate solution, 2.5 mL of 20% w/w SDS, 40 mg o-phthaldialdehyde in 1 mL ethanol, 100 μL of β-mercaptoethanol and adjusted volume to 100 mL using ddH_20) was prepared. Subsequently, 20 μL aliquots of each sample were collected before and after digestion with pepsin and pancreatin and added to 980 μL of OPA solution and incubated precisely for two minutes. The resulting color intensity was directly proportional to the concentration of peptide and was determined spectrophotometrically at the wavelength of 340 nm. The peptide concentration was compared to a standard curve of Leu-Gly (0-8 mg/mL).

3.3.7 Oxalate content determination

Total oxalate was quantified with an enzymatic kit (procedure 591, Trinity Biotech, Newark, NJ) according to the method of Horner et al. (2005). The kit principle is based on the oxidation of oxalate in the presence of oxalate oxidase (Chiriboga, 1963). The hydrogen peroxide (H_2O_2) that is produced during the peroxidase-catalyzed reaction is measured spectrophotometrically at 590 nm.

Briefly, 100 mg malted finger millet flour was weighed in 15 mL falcon tube and autoclaved for 20 min. Next, 4mL sample diluent (EDTA) was added to each tube, vortexed, and sonicated in a sonicator bath (Branson 5510, Kell-Strom, Wethersfield, CT) for 6 min. The respective tubes were centrifuged at 2000 rpm or 1500 ×g for 5 min after 24 hr incubation at 55 °C. Finally, 1mL oxalate reagent A (3-methyl-2-benzothiazolinone hyrazone, 3-dimethylamino benzoic acid), 50 μL supernatant, and 100 μL oxalate reagent B (oxalate oxidase; 3000 μ/L, peroxidase; 100 μ/L) were added into each tube and immediately mixed by gentle inversion. The intensity of the resulted indamine dye was proportional to the concentration of oxalate and was detected by spectro-photometer at 590 nm after 5 min of incubation at room temperature.

3.3.8 Phytic acid content determination

Phytic acid content was determined using Megazyme phytic acid (Phytate/Total Phosphorous) assay kit (#K-PHYT, Megazyme International, Ireland). Principle of the kit is based on the hydrolysis of phytic acid into myo-inositol $(phosphate)_n$ and inorganic phosphate (Pi) in the presence of phytase and alkaline phosphatase (ALP) (Loewus & Murthy, 2000). Ammonium molybdate and Pi react to form 12-molybdophosphoric acid, which is subsequently reduced under acidic conditions to molybdenum blue (Fiske & Subbarow, 1925). The absorbance of molybdenum

blue, which is proportional to the amount of Pi in the sample, is measured spectrophotometrically at 655nm.

Briefly, sample extraction was carried out with addition of 20 mL hydrochloric acid (0.66 M) into 2.5 g of sample with vigorous stirring overnight. The extract was centrifuged at 13,000 rpm for 10 min. Thereafter, 20 μL of phytase was added to 50 μL of sample extract, vortexed and incubated in a water bath at 40 °C for 10 min. Subsequently, 20 μL of ALP was added, vortexed, and incubated at 40 °C for 15 min. Sample tube was re-centrifuged at 13,000 rpm for more than 10 min. Colorimetric determination of phosphorous was performed using color reagent (1 part ammonium molybdate (5% w/v) into 5 parts ascorbic acid (10% w/v)/sulphuric acid (1 M)) at 655 nm against a phosphorus calibration curve.

3.3.9 Tannin content determination

Tannin content was estimated using the vanillin hydrochloride method, developed by Price et al. (1978). The principle of the method is based on the reaction of vanillin reagent with any phenol that has an un-substituted resorcinol or phloroglucinol nucleus (Siwela et al., 2007). The intensity of the resulted color is proportional to the amount of tannin and is measured using catechin (CE) as a standard curve (10-100 μg/mL). To prepare the extract, mixture of 1 g of sample in 50 mL 100% methanol was shaken for 24 hr. The sample was centrifuged at 1200 ×g for 10 min at 25 °C and supernatant was collected. About 1 mL of extract was mixed with 5 mL of vanillin hydrochloride reagent (8% hydrochloric acid in methanol and 4% vanillin in methanol) and incubated at 30 °C for 20 min. Absorbance was measured at 500 nm and data were expressed as g CE/100 g db.

3.4 Results and discussions

In the present study, ANOVA was employed to investigate the combined effects of germination design factors on the protein and anti-nutrient contents of finger millet grain. The goal was to optimize the *in-vitro* protein digestibility of the finger millet malt by selecting the best combination of the involved design factors. The experiments were developed using a face-centered Central Composite Design (CCD) with two factors, germination duration (Time) and temperature (Temp). For each factor, three levels were selected and the data were analyzed using the response surface methodology. All the experiments were conducted in triplicate for each design combination. The averaged data as well as their corresponding Standard Deviation (SD) errors are presented in Table 3-1 and Table 3-2.

Table 3-1: Protein and peptide content (g/100g db) before and after pepsin and pancreatin enzymes digestion for the designed germination treatments.

EXP	Germination factors & levels		Protein Content (g/100g db)			IVPD (%)	Peptide Content (g/100g db)		
	Temp.	Time	Before Digestion	After Pepsin Digestion	After Pancreatin Digestion		Before Digestion	After Pepsin Digestion	After Pancreatin Digestion
GM0	Control		6.42 ± 0.21	1.73 ±0.02	1.65 ± 0.04	74.37 ± 2.49	0.79 ± 0.03	4.80 ± 0.21	5.23 ± 0.26
GM1	22	24	6.52 ± 0.09	1.39 ± 0.09	1.25 ± 0.12	80.87 ± 2.07	1.48 ± 0.06	5.50 ± 0.20	6.11 ± 0.32
GM2	22	36	6.6 0 ± 0.05	0.88 ± 0.02	0.84 ± 0.01	87.33 ± 0.64	1.55 ±0 .05	7.01 ± 0.16	7.42 ± 0.36
GM3	22	48	6.75 ± 0.13	0.65 ± 0.02	0.65 ± 0.03	90.44 ± 1.56	2.64 ± 0.11	8.46 ± 0.14	8.51 ± 0.28
GM4	26	24	6.58 ± 0.08	1.05 ±0.03	0.89 ± 0.04	86.49 ± 1.17	1.64 ± 0.04	5.51 ± 0.14	6.34 ± 0.31
GM5.1	26	36	7.05 ± 0.21	0.78 ±0.09	0.76 ± 0.04	89.25 ± 2.45	2.13 ± 0.05	7.80 ± 0.19	8.59 ± 0.41
GM5.2	26	36	6.91 ± 0.08	0.80 ±0.04	0.81 ± 0.06	88.28 ± 1.38	2.07 ± 0.04	8.12 ± 0.32	8.41 ± 0.28
GM5.3	26	36	7.02 ± 0.13	0.78 ±0.09	0.80 ± 0.06	88.60 ± 1.98	1.93 ± 0.05	8.01 ± 0.40	8.48 ± 0.45
GM5.4	26	36	7.12 ± 0.26	0.84 ±0.05	0.78 ± 0.01	89.04 ± 2.72	2.14 ± 0.07	7.79 ± 0.42	8.60 ± 0.30
GM6	26	48	7.19 ± 0.06	0.73 ±0.04	0.62 ± 0.02	91.38 ± 0.77	3.36 ± 0.10	8.28 ± 0.45	8.91 ± 0.32
GM7	30	24	7.02 ± 0.13	0.80 ±0.05	0.74 ± 0.08	89.46 ± 2.07	1.95 ± 0.06	5.78 ± 0.18	6.52 ± 0.31
GM8	30	36	7.29 ± 0.20	0.80 ±0.05	0.72 ± 0.04	90.09 ± 2.35	2.88 ± 0.14	8.33 ± 0.07	8.43 ± 0.14
GM9	30	48	7.32 ± 0.13	0.70 ±0.01	0.61 ± 0.03	91.67 ± 1.57	3.74 ± 0.19	8.82 ± 0.13	9.18 ± 0.21

First, the crude protein contents were evaluated for the selected design combinations as described in section 3.3.4. The obtained results are presented in Table 3-1. While for the control sample (GM0, native grain), protein was estimated at 6.42 g/100 g db, this value increased for GM9 sample up to 7.32 g/100 g db. This means that germination of finger millet for 48 hr and at

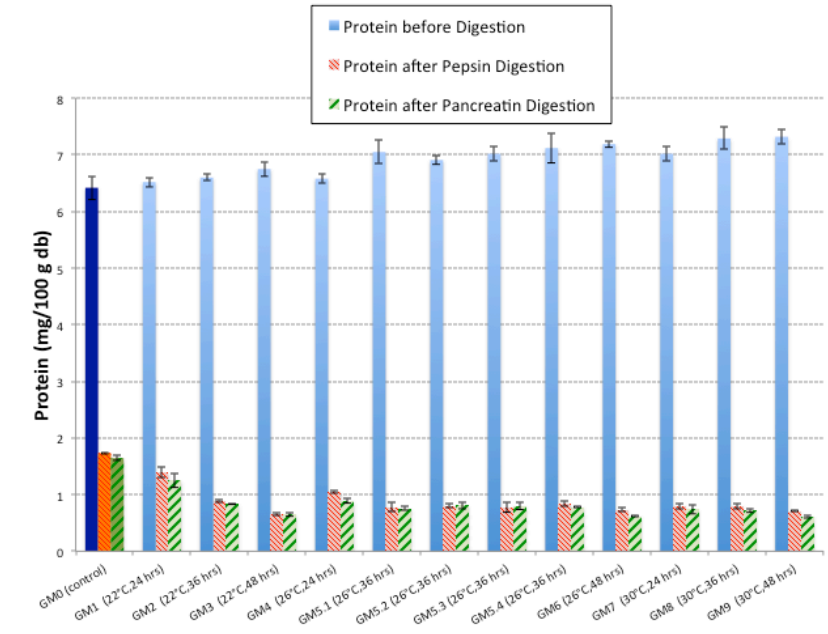
30 °C increased the crude protein content by 14%. These data are graphically presented in Figure 3-1 (a) as well.

Table 3-2: Phytic acid (g/100g db), tannin (g/100g db), and oxalate (mg/100 g db) contents for the designed germination treatments.

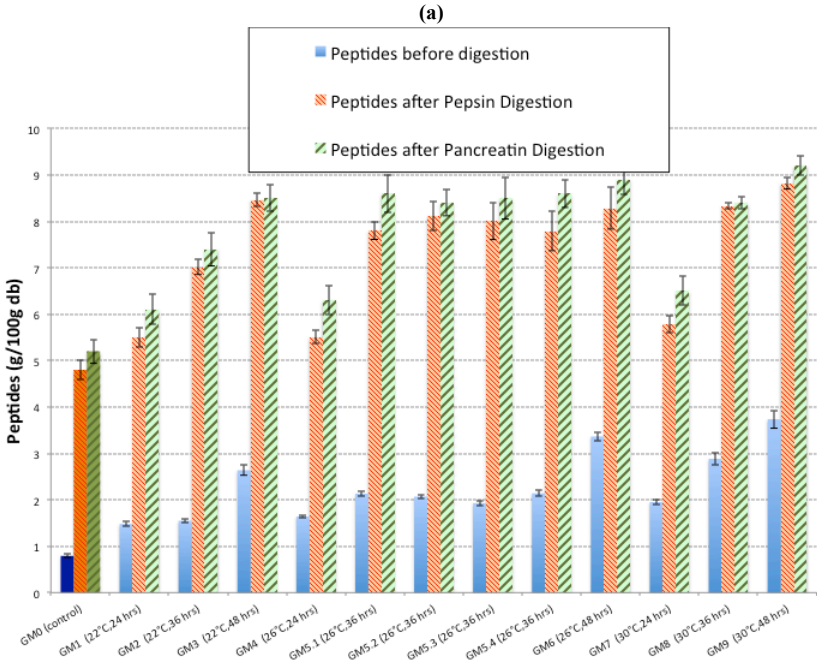
EXP	Germination factors and their levels		Anti-Nutrient Content		
	Temp.	Time	Phytic Acid (g/100g db)	Tannin (g CE/100g db)	Oxalate (mg/100g db)
GM0	**Control**		0.77 ± 0.04	3.24 ± 0.04	44.77 ± 0.74
GM1	**22**	**24**	0.61 ± 0.02	2.31 ± 0.04	39.46 ± 0.92
GM2	**22**	**36**	0.48 ±0.02	1.64 ± 0.05	37.19 ± 1.05
GM3	**22**	**48**	0.44 ± 0.03	1.53 ± 0.03	36.39 ± 0.90
GM4	**26**	**24**	0.60 ± 0.03	1.98 ± 0.03	37.96 ± 0.42
GM5.1	**26**	**36**	0.48 ± 0.02	1.73 ± 0.04	35.51 ± 0.63
GM5.2	**26**	**36**	0.48 ± 0.03	1.82 ± 0.04	36.12 ±0.11
GM5.3	**26**	**36**	0.47 ±0.02	1.78 ± 0.07	35.49 ±0.92
GM5.4	**26**	**36**	0.46 ± 0.02	1.76 ± 0.04	34.83 ± 0.67
GM6	**26**	**48**	0.41 ± 0.04	1.63 ± 0.02	32.96 ± 0.52
GM7	**30**	**24**	0.62 ± 0.01	2.11 ± 0.05	37.41 ± 0.81
GM8	**30**	**36**	0.46 ± 0.03	1.83 ± 0.06	33.84 ± 0.55
GM9	**30**	**48**	0.42 ± 0.02	1.75 ± 0.07	31.36 ± 0.65

From the figure, it is observed that the crude protein content was significantly influenced by both germination duration and temperature. These results were aligned with findings of other researchers (Mbithi-Mwikya et al., 2000b; Singh & Raghuvanshi, 2012). Despite a broad range in the reported finger millet protein content (5 to 13%), the average mostly mentioned value is 7% (Singh & Raghuvanshi, 2012).

Mbithi-Mwikya et al. (2000b) observed around 30% increase in the available protein content of finger millet after four days of germination at room temperature. Swami et al. (2013) reported a linear increase in the available protein content of finger millet grains that were germinated up to 24 hr. In their study, protein availability enhanced from the initial 14% up to the final 17.5%, indicating a 25% increase. In the present study, a similar positive relationship between total crude protein availability and germination duration was observed.



(a)



(b)

Figure 3-1: The measured (a) protein and (b) peptides before and after pepsin and pancreatin digestion for the selected experimental design factor combinations.

The physical mechanisms and bio-chemical reactions that cause the observed increase in bio-availability of protein content during germination process are strongly associated with the morphology of the finger millet seed. In finger millet, the endosperm represents the largest portion of the grain, which consists of an aleurone layer, and three distinct starchy sections, peripheral,

corneous, and floury endosperms. Peripheral endosperm contains small and tightly packed cells, with a large number of protein bodies that are embedded in fiber-starch-protein matrices. Corneous is the largest portion of the endosperm containing predominantly protein matrices. Finally, the floury endosperm is made up of compound starch granules, where protein bodies and protein matrices rarely exist (Belton & Taylor, 2002a). The observed increase in the crude protein level during germination was the outcome of an enhancement in the plant amylolytic activity (plant α-amylase) (Traoré et al., 2004). The activity of this enzyme resulted into the starch granules breakdown, which led to the release of the protein from the packed cells and consequently, increased its bio-availability.

Peptide content of the samples before the digestion process was assessed (as described in section 3.3.6) and the results are presented in Table 3-1and Figure 3-1 (b). While the initial peptide level of the raw finger millet (GM0) was around 0.79 g/100 g db, germination of the seeds for 48 hr and at 30 °C (GM9) increased it up to 3.74 g/100 g db.

Hamad and Fields (1979) showed that the total free amino acids notably increased by about 4.5 fold during germination of finger millet. This increase may be associated with the activation of proteases enzyme during germination, which consequently enhances protein hydrolysis. Protease breaks down the protein into smaller polypeptides and amino-acid groups and facilitates the digestion procedure. Because of that, germination is known as a natural pre-digestion step. Vidyavathi et al. (1983) showed that the protease activity in finger millet increased with germination time, maximized on the third day, and decreased afterward. They reported that protease inhibitors disappeared during the germination process. It is proposed that degradation of prolamins (the main fraction of finger millet protein) into lower peptides and free amino acids supplies α-amino groups, which may be used through transamination in the synthesis of lysine and

other essential amino acids (Mbithi-Mwikya et al., 2000a). The observed elevation in peptide content is favorable as the protein quality of a food not only depends on its protein level, but also on the availability of its peptides.

In the next step, the samples were digested with pepsin enzyme as described in section 3.3.5 and their protein and peptide contents were monitored. The results are numerically presented in Table 3-1 and graphically in Figure 3-1. It was noticed that the enzymatic digestion with pepsin remarkably dropped the protein content, with a proportional increase in the peptides. In fact, a major fraction of protein was digested in this step. The importance of pepsin digestion is more easily highlighted if it is compared with pancreatin digestion, where only a little further reduction in protein (Table 3-1 and Figure 3-1(a)) and slight increase in the peptide levels (Table 3-1 and Figure 3-1(b)) are observed. Therefore, in the employed *in-vitro* digestion simulation, although protein digestion was improved after using pancreatin enzyme, the enhancement was not as notable as with pepsin enzyme.

The IVPDs were calculated following equation (3-2) and the results are presented in Table 3-1. Again, a relatively low digestion value was obtained for the control (≈74%), while for case GM9, the IVPD improved up to 92%. Mittal (2002) reported 62.94% for the IVPD of native finger millet flour. The increase in IVPD with respect to germination duration has been frequently reported in the literature (Chaturvedi & Sarojini, 1996; Desai et al., 2010; Khetarpaul & Chauhan, 1990). Germination of cereals involves complex enzymatic reactions that decompose macromolecules (e.g. proteins) into smaller units (e.g. peptides) and make them more digestible. Partial solubilization and some proteolysis, which usually occurs during germination, improves the IVPD (Mbithi-Mwikya et al., 2000b). Further ANOVA of the obtained IVPD values will be presented shortly.

To quantify the major anti-nutrients and investigate any possible correlation between their levels and the IVPD, phytic acid, tannin, and oxalate contents were measured for all experimental design combinations. Unlike oxalate, phytic acid and tannin have previously been the subject of many investigations. The obtained results are presented in Table 3-2. It is observed that all of these anti-nutrients were significantly reduced throughout the germination process. Comparing the values between the control sample (GM0) and the sample that was germinated for 48 hr at 30 °C (GM9) shows that phytic acid, tannin, and oxalate levels decreased by 45%, 46%, and 29%, respectively.

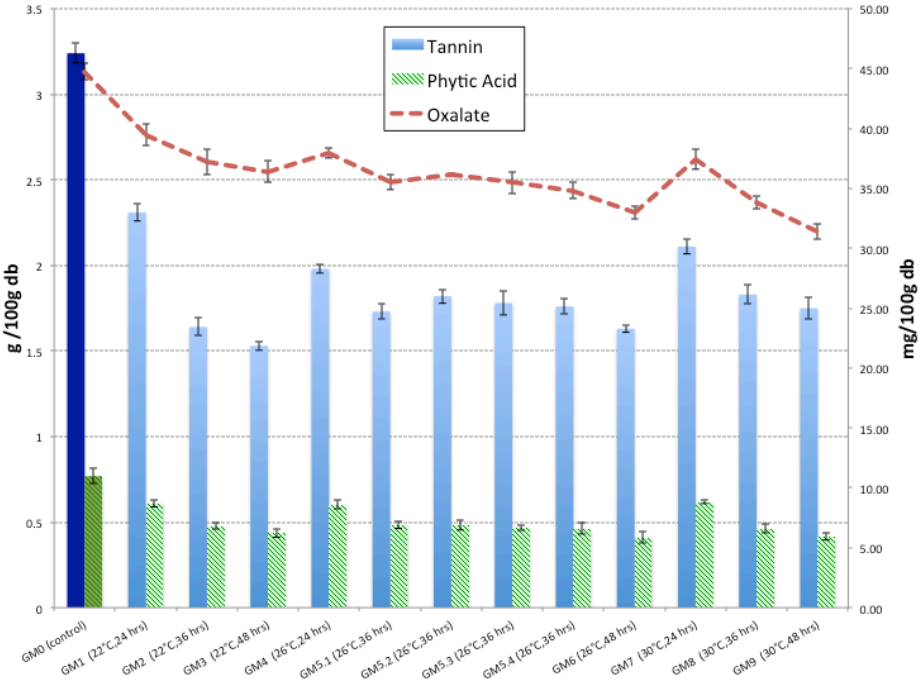


Figure 3-2: Measured anti-nutrient contents; left vertical axis presents phytic acid (g/100 g db) and tannin (g CE/100g db) and the right axis quantifies the oxalate content (mg/100 g db).

In Figure 3-2, the obtained anti-nutrient contents are graphically presented. It should be noted that for phytic acid (g/100 g db) and tannin (g CE equivalent/100 g db), the left vertical axis and for oxalate (mg/100 g db), the right axis should be used as the reference scale. From the figure, it is observed that while germination duration was important and influential for all the investigated

quantities, temperature was more influential in the case of oxalate not tannin and phytic acid. This will be further verified through ANOVA.

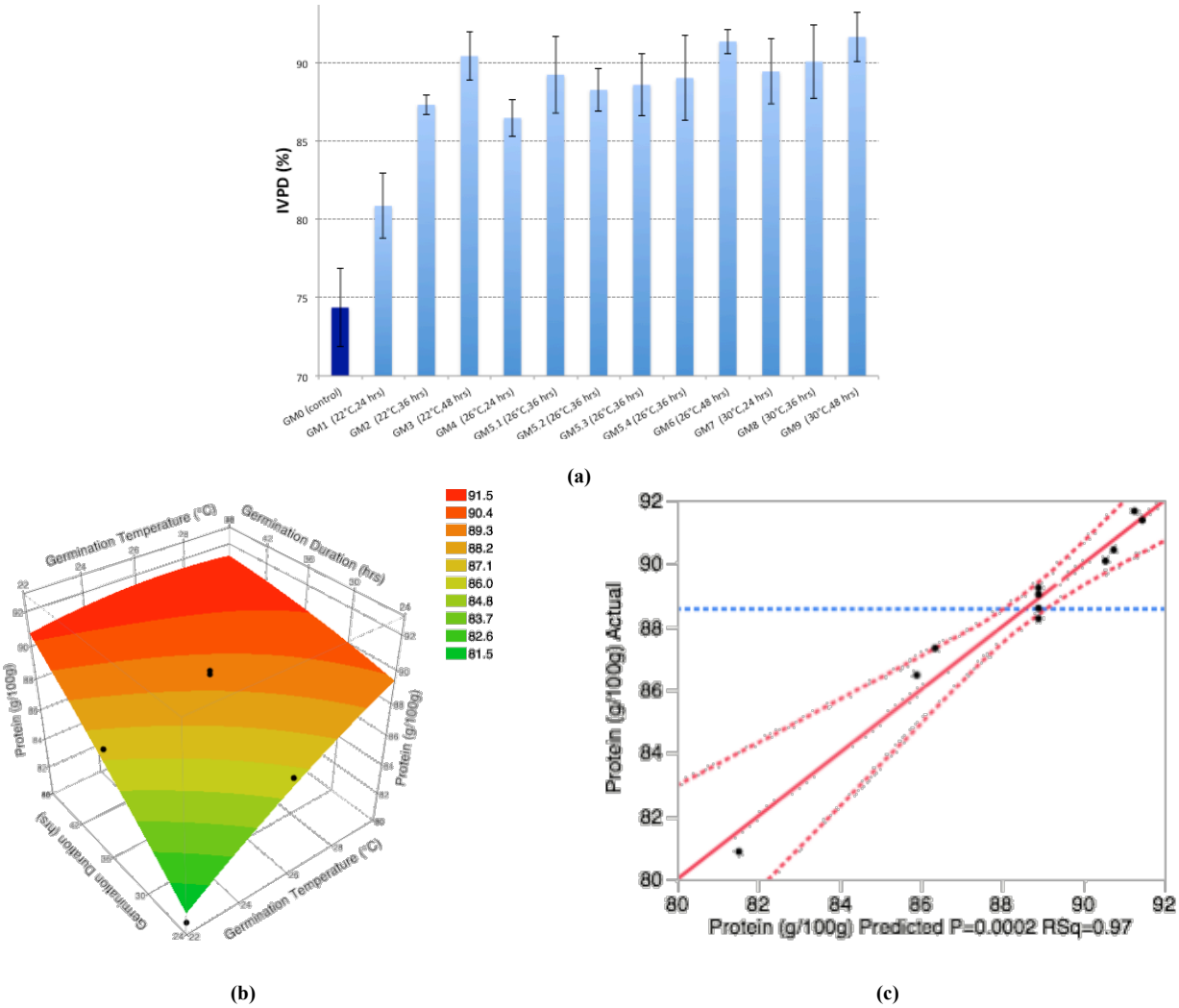


Figure 3-3: (a) IVPD (%) evaluated for all design factor combinations, (b) the response surface for the IVPD (%), and (c) the leverage plot based on the ANOVA of IVPD.

The reduction of tannin and oxalate contents during soaking and germination stages has been mostly attributed to their leaching into the sprouting medium (Mbithi-Mwikya et al., 2000b). Tannin reduction may also be due to the high activity of polyphenol oxidase and other catabolic enzymes during germination (Mbithi-Mwikya et al., 2000b). Sripriya et al. (1997) reported a reduction in tannin content during germination, which occurred by its hydrolysis into lower molecular weight phenols. Germination significantly reduces the phytate content, due to the

increment in endogenous phytases action, which degrades the phytate into inorganic phosphorous and inositol (Traoré et al., 2004). The observed decrease in phytate content in the present study (45%) was lower compared to that reported by Traoré et al. (2004) (67%). The difference is probably due to having shorter germination duration in this investigation (48 hr) comparing to their study (62 hr).

In Figure 3-3 (a), the obtained IVPD data (Table 3-1) are graphically presented. The figure shows how digestibility of protein has been improved from below 75% (native grains) to values greater than 90%. Furthermore, it was observed that at 22 °C, germination duration was more influential on the IVPD, while this dependency decreased at 26 and 30 °C. At 22 °C, the IVPD increased around 10% as germination duration was increased from 24 to 48 hr, while this value was 5% and 2% for germination at 26 and 30 °C, respectively.

The obtained IVPD data were analyzed using response surface methodology. The results are summarized in Table 3-3. Based on the obtained data, both temperature and duration were significant elements with $p<0.0005$ and $p<0.0001$, respectively. Their positive coefficients indicated a direct relation between the linear terms of the design factors and IVPD. The cross product term, duration×temperature, was significant as well ($p<0.005$). The obtained negative coefficient of this bi-linear term illustrated its adverse influence on the IVPD, when duration and temperature simultaneously were changed. Therefore, a maximum or saddle point in the IVPD response surface would be expected. It was observed that effects of quadratic terms were not significant. Based on the obtained results, IVPD was related to the selected design factors, X_1 and X_2, as follows;

$$\text{IVPD (\%)} = 88.906 + 2.09623\ X_1 + 2.78008\ X_2 - 0.44866\ X_1^2 - 0.22667\ X_2^2 - 1.84090\ X_1X_2 \quad (3\text{-}3)$$

This response surface is plotted in 3D format in Figure 3-3 (b). The surface has a saddle point at 31.6 °C and 41.5 hr, which is slightly outside of the selected domain of operation. The predicted value at this point is IVPD=91%. The corresponding leverage plot is presented in Figure 3-3 (c), which clarifies an appropriate prediction of the obtained experimental data by using the proposed response surface. Since the confidence curves (dashed-line curves) crossed the horizontal line, the correlation of design factors is significant ($p<0.05$). According to the obtained response surface, the local IVPD maximum in the investigated range of design factors was roughly around 26 °C and 48 hr. Although based on the original experimental data, germination at 30 °C and 48 hr provided slightly better IVPD, the difference was negligible.

Table 3-3: Summary of the ANOVA of the responses for IVPD (%) and the corresponding parameter estimates of the different terms.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	88.9063492	0.3115380364	285.38	**<.0001**
Temperature	2.09622640	0.2786480908	7.52	**0.0003**
Duration	2.78008463	0.2786480908	9.98	**<.0001**
Duration×Temperature	-1.84089555	0.3412728202	-5.39	**0.0017**
Temperature×Temperature	-0.44866013	0.4179721362	-1.07	0.3243
Duration×Duration	-0.22667274	0.4179721362	-0.54	0.6071

Similar procedures were performed for other investigated quantities. The response surfaces were constructed and the linear, quadratic, and bi-linear effects of the germination factors were analyzed. The results are presented in Table 3-4.

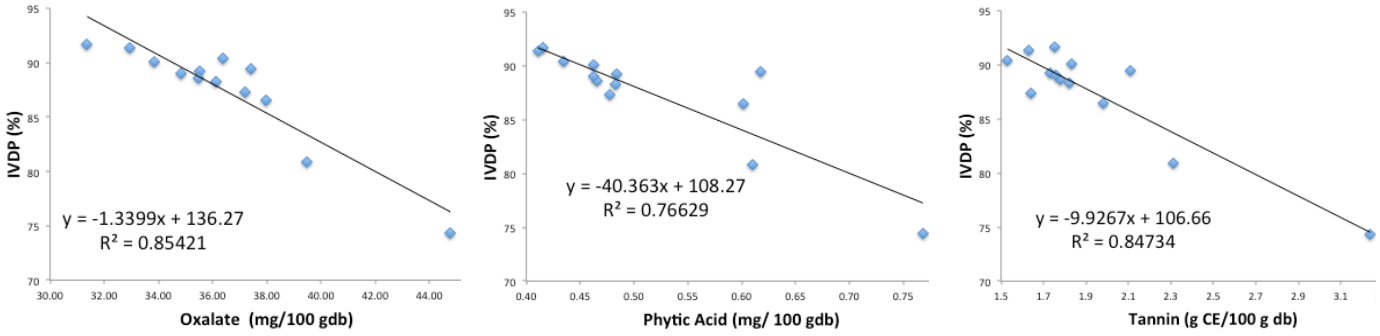
In this table, the factors that were significant are highlighted in bold character. While germination duration was notably influential on all investigated parameters, germination temperature was not significant for phytic acid and tannin contents. Besides, similar to the IVPD, the bi-linear term was important in the assessments of oxalate and protein after pepsin and pancreatin digestions. Finally, only the quadratic term of germination duration was important in the case of peptide and phytic acid. All surfaces had an acceptable R^2 and RMS Error values.

Table 3-4: Summary of the ANOVA of the responses for all protein, peptides, and anti-nutrient contents.

	Protein content			Peptides content			Anti-nutrient Content		
	Before Digestion	After Pepsin Digestion	After Pancreatin Digestion	Before Digestion	After Pepsin Digestion	After Pancreatin Digestion	Phytic Acid	Tannin	Oxalate
Intercept	**7.0088**[a]	**0.8077**[a]	**0.7763**[a]	**2.1000**[a]	**7.8458**[a]	**8.4283**[a]	**0.4692**[a]	**1.7463**[a]	**35.3763**[a]
Temp	**0.2933**[c]	**-0.1028**[d]	**-0.1094**[d]	**0.4833**[b]	**0.3267**[f]	**0.3483**[c]	-0.0050	0.0350	**-1.7383**[a]
Time	**0.1900**[c]	**-0.1913**[b]	**-0.1669**[b]	**0.7783**[a]	**1.4617**[a]	**1.2717**[a]	**-0.0933**[a]	**-0.2483**[b]	**-2.3533**[a]
Time×Temp	0.0175	**0.1602**[d]	**0.1181**[d]	0.1575	0.0200	0.0650	-0.0075	0.1050	**-0.7450**[f]
Temp×Temp	-0.0313	0.0177	0.0242	0.0500	-0.0075	-0.3200	0.0075	0.0413	0.3613
Time×Time	-0.0913	0.0670	-0.0005	**0.3350**[f]	**-0.7825**[e]	**-0.6200**[d]	**0.0425**[b]	0.1113	0.3063
R^2	0.909	0.955	0.950	0.971	0.962	0.976	0.989	0.908	0.978
R^2 adj	0.833	0.918	0.908	0.944	0.930	0.956	0.981	0.832	0.960
RMS	0.113	0.056	0.051	0.169	0.316	0.224	0.010	0.089	0.448

a: p<0.0001, b: p <0.0005, c: p<0.001, d: p<0.005, e: p<0.01, f: p<0.05

To investigate the existence of any correlation between the IVPD and anti-nutrient component levels, the obtained results are graphically presented as scattered data in Figure 3-4 (a), (b), and (c). In these figures, for each sample, IVPD values are presented with respect to phytic acid, tannin, and oxalate contents. Based on the plotted linear regression curves, it was concluded that there may be a linear correlation between the level of these anti-nutrients and the desired investigated IVPD. Generally, for all selected anti-nutrient factors, IVPD increased as the level of anti-nutrient decreased.



(a) **(b)** **(c)**

Figure 3-4: Linear regression curves representing the possible correlations between the IVPD (%) and the anti-nutrient contents, (a) oxalate (b) phytic acid, and (c) tannin.

3.5 Conclusion

Protein quality determination is an important factor in the manufacture of nutritionally improved cereal products. The nutritive value of protein primarily depends on its capacity to

supply needs of nitrogen and essential amino acids. Protein quality improves as the biological availability of its building block components, amino acids, increases. The availability of amino acids and peptides depends upon the extent of digestibility of proteins by the proteolytic enzymes of the alimentary tract. Malting/germination is a simple and low-cost traditional food processing approach, which improves the nutritional quality of cereals through its involved biochemical modifications. Germination decreases anti-nutrient contents and enzyme inhibitors; hence, increases the protein digestibility. In the present study, the *in-vitro* protein digestibility of finger millet was enhanced by 17% after germinating the seeds for 48 hr at 30 °C. In addition, phytic acid, tannin, and oxalate contents notably decreased by 45%, 46%, and 29%, respectively. In addition, negative correlations between the protein digestibility and these anti-nutrient components were observed.

3.6 Acknowledgements

The authors acknowledge financial support from the Natural Sciences and Engineering Research Council of Canada.

Connecting Statement to Chapter 4

In **Chapter 3,** optimization of the germination/malting process for anti-nutrient reduction and consequently protein digestibility enhancement in finger millet grain was investigated. Seventeen percent increase in the protein availability of finger millet grains, which were germinated for 48 hours at 30 °C, was observed comparing to the flour obtained from native grains. In Chapter 4, a similar study has been performed on amaranth grain. As it was mentioned in **Chapter 1**, due to some protein deficiency in finger millet comparing to the standard requirements for weaning products, amaranth, which is a highly nutritious and gluten-free pseudo-cereal grain, would be appropriately added as a supplemental ingredient.

As the outcome of the present chapter, the optimum germination duration and temperature were obtained at 48 hr and 28 °C, respectively. For these conditions, the *in-vitro* protein digestibility of amaranth was enhanced from its initial 76.03% for the native grain up to 83.58% for the optimized malted sprouts.

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Improvement of the *in-vitro* protein digestibility of amaranth grain through optimization of the malting process

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Contributions made by the different authors are as follows;

- The first author, **Sara Najdi Hejazi**, is the Ph.D. student, who prepared the experimental design, conducted the laboratory work, analyzed the results, and prepared the manuscript.
- The second author, **Valérie Orsat**, is the thesis supervisor, who initiated and stimulated ideas, provided the technical advice, suggestions, experiment facilities, and guided the first author in writing, correcting, and preparing the manuscript throughout the study.
- The third, **Behnam Azadi**, and fourth, **Stan Kubow**, authors provided the laboratory facilities and required instructions for the performed experiments.

Furthermore, parts of the obtained data and performed analyses in **Chapter 4** have been presented in two conference presentations, as follows;

1. Sara Najdi Hejazi and Valérie Orsat, "**Effect of Germination Process on the Protein Digestibility of Finger Millet and Amaranth"** Northeast Agricultural and Biological Engineering Conference, Oral presentation, July 12-15, 2015, Newark, Delaware, USA.
2. Sara Najdi Hejazi and Valérie Orsat, "**Effects of Germination on Finger Millet and Amaranth Grains**" Northeast Agricultural and Biological Engineering Conference, Oral presentation, July 27-30, 2014, Kemptville, USA.

4 Improvement of the *in-vitro* protein digestibility of amaranth grain through optimization of the malting process

4.1 Abstract

In recent decades, amaranth grain has been extensively studied for its remarkable nutritional profile and agricultural characteristics, e.g., having a short cultivation period and being drought resistant. Despite possessing a high nutritional quality, amaranth also contains several anti-nutrient compounds that decrease the bio-availability of its nutrients, especially its protein content. Malting/germination can be employed as an appropriate pre-treatment to improve the nutritional properties of native cereal grains. Therefore, in the present study, effects of malting including its two important design factors, duration (24, 36, and 48 hr) and temperature (22, 26, and 30 °C), on the *in-vitro* protein digestibility and anti-nutrient components of amaranth grain were investigated and optimized. The germinated seeds yielded higher protein content (14.86 g/100 g db) and digestibility (83.58%) when compared to the native grains, 13.76 g/100 g db and 76.03%, respectively. Significant reductions in phytic acid (30%) and oxalate (38%) and increase in tannin content (47%) were observed. Both germination duration and temperature were found to be significant factors on the measured properties. Over the selected range of variables, it was found that germinating amaranth for 48 hr at 28 °C provides the maximum protein digestibility. Finally, correlations between the anti-nutrient content and protein digestibility were observed.

Keywords: *amaranth, germination/malting, in-vitro protein digestibility, tannin, phytic acid, oxalate*

4.2 Introduction

In a study performed by the U.S. National Academy of Sciences in 1975, amaranth was elected among the top 36 of the world's most promising crops (Evans & Ayensu, 1975). Since then, this grain has been the subject of extensive research. Comparing to other grains, amaranth has the highest amount of protein with a range of 11 to 20%, with twice the content of lysine as an essential amino acid and 5 to 20 times the content of calcium and iron (Mustafa et al., 2011; Rastogi & Shukla, 2013). In addition, amaranth does not contain gluten making it a suitable choice for people suffering from gluten intolerance and celiac disease (Rastogi & Shukla, 2013).

Besides having an appreciably high protein content, the amino acids composition of amaranth is outstanding relative to other cereal grains. While lysine is a limiting amino acid in most cereals, compared to the reference protein of the Food and Agricultural Organization (FAO), it can be adequately found in amaranth (Rastogi & Shukla, 2013). The lysine content of amaranth is comparable with that of soybeans, which has one of the highest quality plant proteins (Bressani & Garcia-Vela, 1990). Moreover, the high content of arginine and histidine, which are essential amino acids for infants, also makes amaranth valuable for infant nutrition (Berghofer & Schoenlechner, 2002). Leucine is the limiting amino acid of amaranth (Rastogi & Shukla, 2013).

Despite presenting a high and excellent quality protein content, similar to other cereal and pseudo-cereal grains, the presence of anti-nutrient components restricts the nutrient bio-availability of amaranth, especially protein. Phytochemicals are the main anti-nutrient components in the cereals (Amalraj & Pius, 2015). Most anti-nutrients that have been studied to date in grain amaranth appear to be non-problematic (Venskutonis & Kraujalis, 2013).

Levels of tannin and phytic acid are comparable to those observed in other cereal grains, though trypsin and chymotrypsin activity were reported to be lower (Rastogi & Shukla, 2013). Phytic acid content of amaranth has been reported within the range of 0.3 to 2.25% (Egli et al., 2002; Rastogi & Shukla, 2013). It has been proven that phytic acid has inhibitory effects on the digestion of protein and starch (Berghofer & Schoenlechner, 2002). Similar to phytic acid, tannin influences the digestion and absorption processes, through forming complexes with protein that inhibit the digestibility of the available protein. A range of values from 0.04 up to 0.8 mg /100 g catechin equivalent have been reported for the tannin content of amaranth (Okoth et al., 2011). Amaranth grain is considered as a high oxalate source, where values of 178 to 1650 mg/100 g were reported (Gélinas & Seguin, 2007). Similar to tannin and phytic acid, oxalate may reduce nutrient and mineral availability in cereals (Gélinas & Seguin, 2007).

Many studies have shown that germination can significantly improve grain nutrient profile, namely protein content. During germination, the crude protein content may increase due to the absorption of nitrates, which facilitates the metabolism of nitrogenous compounds from carbohydrate reserves (Morgan et al., 1992). Additionally, germination improves protein quality due to an increase in the grain's proteolytic activity. This enhancement leads to hydrolysis of storage proteins mainly prolamin into albumins and globulins and consequently conversion of glutamic and proline amino acids into the limiting amino acids such as lysine (Chavan et al., 1989).

Sprouting of amaranth increases the availability of proteins as well as the free amino acid components. A 30% increase in the lysine content of amaranth after 24 hr of germination was reported by Paredes-López and Mora-Escobedo (1989). Besides, they reported 41% and

22% enhancements in crude protein and true protein of amaranth, respectively, after soaking for 10 min and germinating for 72 hr at 35 °C. On the other hand, Gamel et al. (2006a) found a reduction trend in both the crude (2% and 3%) and true protein (31% and 19%) content of two species of amaranth, when seeds were soaked in distilled water for 5 hr and germinated at 32 °C for 48 hr. The observed opposite responses in the protein content upon germination between the above two studies may be attributed to differences in germination methods. Regarding protein content, the majority of species have shown an increase with respect to germination duration (Colmenares de Ruiz & Bressani, 1990).

Anti-nutrient content has been reported to decrease among most germinated cereals and pseudocereals (Gamel et al., 2006b). Chavan and Kadam (1989) reviewed the changes in anti-nutrients as a result of the germination process and noticed an overall trend of anti-nutrient reduction in sprouts. Phytic acid decreased in sprouting cereals (Colmenares de Ruiz & Bressani, 1990). Germination has been shown to decrease the tannin content in cereals. It seems that the observed decrease in tannins may be the outcome of the reduction in both hydrogen bonding and nonpolar hydrophobic interactions of tannins and proteins (Asquith & Butler, 1986).

In the present study, the effects of two important germination parameters, duration and temperature, on the *In-Vitro* Protein Digestibility (IVPD) of amaranth grain were investigated and optimized. In addition to protein, peptides and IVPD, the anti-nutrients phytic acid, tannin, and oxalate were monitored and any significant relationship between their levels and IVDP was explored.

4.3 Materials and methods

4.3.1 Materials

The sproutable amaranth grain (*Amaranthus caudatus L.* (*love-lies-bleeding*)) was purchased from Bulk Barn (Qc., Canada). Pepsin, pancreatin, *o*-phthaldialdehyde (OPA), hydrochloric acid, sodium tetraborate, β-mercaptoethanol, ascorbic acid, and leucine-glycine (Leu-Gly) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Vanillin, sodium hydroxide, sodium phosphate, ammonium molybdate, sulphuric acid, and methanol were provided by Fisher Scientific (Fair Lawn, NJ, USA). Sodium dodecyl sulfate (SDS) was procured from Bio-Rad (kit # 500-006, Bio-Rad, Mississauga, Ontario, Canada). Ethanol was purchased from Commercial Alcohols (Industrial and Beverage Alcohol Division of Greenfield Ethanol Inc., Brampton, Ontario, Canada).

4.3.2 Malting/Germination process

Amaranth seeds were steeped in water at a ratio of (1:5 w/v) for 24 hr at room temperature. Water was drained off and the soaked seeds were germinated in quart jars at three different temperatures (22, 26, and 30 °C) and in each case incubated for three different durations (24, 36, and 48 hr). The sprouted and native seeds were freeze-dried and ground to flour for further analysis.

4.3.3 Chemical analysis

4.3.3.1 Total protein

The total protein content was measured using the Bradford method (kit # 500-0006, Bio-Rad, Mississauga, Ontario) (Bradford, 1976).

4.3.3.2 *In-vitro* protein digestibility

In-vitro protein digestibility was assessed by employing the pepsin and pancreatin digestion method of Vilela et al. (2006) after a few modifications. The undigested protein residues before digestion, after pepsin digestion, and after complete digestion (pepsin and pancreatin) were evaluated using the Bradford method (subsection 4.3.3.1). The *in-vitro* protein digestion percentage was calculated by subtracting the undigested protein from the initial total protein of the sample as follows;

$$\text{IVPD}(\%) = \frac{\text{Initial protein} - \text{final undigested protein}}{\text{Initial Protein}} \times 100 \quad (4\text{-}1)$$

The produced peptide content before and after pepsin and pancreatin digestions was determined spectrophotometrically by modification of the Church et al. (1983) method. The absorbance was detected at a wavelength of 340 nm and the data was expressed against a Leu-Gly standard curve.

4.3.3.3 Oxalate

Total oxalate content was measured using an enzymatic kit (procedure #591, Trinity Biotech, Newark, NJ) according to the method of Horner et al. (2005). The sample absorbance was read at 590 nm and data was expressed as mg/100 g db.

4.3.3.4 Phytic acid

The phytic acid content was evaluated using a kit (#K-PHYT, Megazyme phytic acid (Phytate/Total Phosphorous), Megazyme International, Ireland). Colorimetric determination of the phosphorous was performed at 655 nm against a phosphorus calibration curve. Data were expressed in g/100g db.

4.3.3.5 Tannin

Tannin content was estimated spectrophotometrically using the vanillin hydrochloride method of Price et al. (1978). Catechin (10-100 μg/mL) was used as a standard curve. The absorbance of the sample was read at 500 nm and data was expressed as g Catechin Equivalent (CE) /100 g db.

4.3.3.6 Statistical design and analysis

In the present study, a Central Composite Design (CCD) was employed as the experimental design and the data were statistically analyzed using JMP (SAS Institute Inc., Cary, NC, USA). Malting/germination was applied with two independent factors, each with three levels (time; 24, 36, and 48 hr, temperature; 22, 26, and 30 °C). There were 12 combinations in total (4 factorial, 4 axial, and 4 central points). The germination duration range was selected such that the grains' dry matter loss be restricted and the efficiency of the malting step be considered. Malting temperature range was also specified considering industrial energy saving requirements and proper grain enzymatic activities. Using ANOVA, second-order response surfaces were constructed for each monitored quantity (Z) and the significance of each involved term was evaluated statistically based on the F-values.

$$Z = \text{Intercept} + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (4\text{-}2)$$

4.4 Results and discussion

Based on the designed germination steps, the interested quantities were monitored and presented in the following subsections.

4.4.1 Protein digestibility and peptide content

Following the instructions in section 4.3.3.2, the protein digestibility of the native and malted samples were evaluated (Table 4-1). For the native grains (GM0), the digestible protein content of the studied amaranth grain was found to be 13.76 g/100 g db. This amount

slightly increased throughout germination and reached a maximum in the case of GM5.1, where it was established at 14.88 g/100 g db. This latter result indicated approximately 8% increase in the availability of amaranth protein content after 36 hr of germination at 26 °C. These results are graphically presented in Figure 4-1 (a). The comparison among GM1, GM2, and GM3 or GM7, GM8, and GM9 clearly indicated that as germination duration increased, protein availability also increased. This behavior supported the existence of a positive dependency between digestible protein content and germination duration. In addition, the relatively higher values for the samples germinated at 26 °C (GM4, GM5, and GM6) highlighted the possibility of a quadratic dependency of protein availability with germination temperature.

Table 4-1: Protein and peptide content (g/100g db) before and after pepsin and pancreatin enzymes digestion for the designed germination treatments.

EXP	Germination factors & levels		Protein Content (g/100g db)			IVPD (%)	Peptide Content (g/100g db)		
	Temp.	Time	Before Digestion	After Pepsin Digestion	After Pancreatin Digestion		Before Digestion	After Pepsin Digestion	After Pancreatin Digestion
GM0	Control		13.76 ± 0.07	7.5 ± 0.07	3.30 ± 0.07	76.03 ± 1.35	0.83 ± 0.04	4.31 ± 0.60	10.46 ± 1.21
GM1	22	24	14.33 ± 0.21	6.8 ± 0.56	2.72 ± 0.56	81.03 ± 1.54	1.57 ± 0.08	6.48 ± 0.32	12.10 ± 1.60
GM2	22	36	14.53 ± 0.11	5.70 ± 0.13	2.69 ± 0.13	81.50 ± 0.91	2.01 ±0 .06	7.12 ± 0.11	13.36 ± 0.16
GM3	22	48	14.75 ± 0.07	4.89 ± 0.21	2.50 ± 0.21	83.04 ± 0.53	3.43 ± 0.14	8.28 ± 0.20	13.13 ± 0.18
GM4	26	24	14.52 ± 0.38	5.93 ± 0.10	2.65 ± 0.10	81.77 ± 2.09	2.12 ± 0.05	7.04 ± 0.16	11.98 ± 0.43
GM5.1	26	36	14.88 ± 0.08	5.79 ± 0.05	2.55 ± 0.05	82.86 ± 0.81	2.80 ± 0.07	7.45 ± 0.67	14.26 ± 0.82
GM5.2	26	36	14.78 ± 0.01	5.41 ± 0.02	2.60 ± 0.02	82.43 ± 0.18	2.69 ± 0.06	8.84 ± 0.26	13.81 ± 0.21
GM5.3	26	36	14.74 ± 0.45	5.55 ± 0.40	2.57 ± 0.40	82.58 ± 2.89	2.51 ± 0.07	8.18 ± 0.51	13.70 ± 0.06
GM5.4	26	36	14.86 ± 0.03	5.46 ± 0.19	2.59 ± 0.19	82.58 ± 0.75	2.78 ± 0.09	8.31 ± 0.11	13.49 ± 0.08
GM6	26	48	14.74 ± 0.16	5.00 ± 0.22	2.45 ± 0.22	83.38 ± 1.35	4.37 ± 0.13	10.09 ± 0.64	13.54 ± 0.47
GM7	30	24	13.97 ± 0.18	5.92 ± 0.63	2.62 ± 0.63	81.23 ± 1.64	2.54 ± 0.08	8.45 ± 0.18	11.58 ± 0.74
GM8	30	36	14.55 ± 0.43	5.50 ± 0.22	2.53 ± 0.22	82.61 ± 2.55	3.75 ± 0.18	8.82 ± 0.07	13.39 ± 0.20
GM9	30	48	14.74 ± 0.35	3.73 ± 0.26	2.42 ± 0.26	83.58 ± 2.24	4.86 ± 0.25	9.12 ± 0.08	13.89 ± 0.16

Colmenares de Ruiz and Bressani (1990) studied the effect of germination duration on three varieties of amaranth grains, where grains were germinated for 24, 48, and 72 hr. A slight increase was observed on the protein content relative to native grains. The protein content before and after 72 hr of germination was assessed for *A. hypochondriacus* (16.1 to

16.8%), *A. cruentus* (14.9 to 15.1%), and *A. caudatus* (16.3 to 16.9%) (Colmenares de Ruiz & Bressani, 1990). In a more recent study, Okoth et al. (2011) performed a range of germination treatments and monitored the protein content. The native grain protein content was assessed to be 15. 4 g/100 g db, whereas for those samples steeped for 24 hr and germinated for 24, 48, and 72 hr, protein values of 10.9, 10.9, and 20.0 g/100 g db were obtained.

The observed increase in the protein digestibility of the malted amaranth grains may be associated to an increase in the amylolytic activities of the grain throughout germination. It is possible that enhancement of the α-amylase activity breaks down the starch granules in the seed structure, thus releasing the embedded packed protein.

Significant effects of germination on peptide content were observed from Table 4-1 and Figure 4-1 (b). The peptide levels before digestion showed a six-fold increase versus control native sample (GM0) when sprouting grains for 48 hr at 30 °C (GM9). Increased enzymatic and proteolytic activities during germination are likely the main mechanisms associated with this observation. Figure 4-1 (b) (blue columns) highlights the importance of the studied variables, germination duration and temperature, on the peptides enhancement.

4.4.2 Pepsin and pancreatin digestion

The *in-vitro* protein digestions of samples included two sequential digestion steps of pepsin and pancreatin digestion to simulate the *in-vivo* digestion in the stomach and small intestine, respectively. For each set of germination factor combinations, samples were digested following the above designated steps and the protein and peptide content were assessed (Table 4-1). As expected, after each digestion step, the protein content decreased with a corresponding increase in peptide levels as pepsin and pancreatin enzymes broke down

protein into peptides and amino acids. The obtained results are graphically presented in Figure 4-1 (a) for protein content and Figure 4-1 (b) for peptide content after each digestion

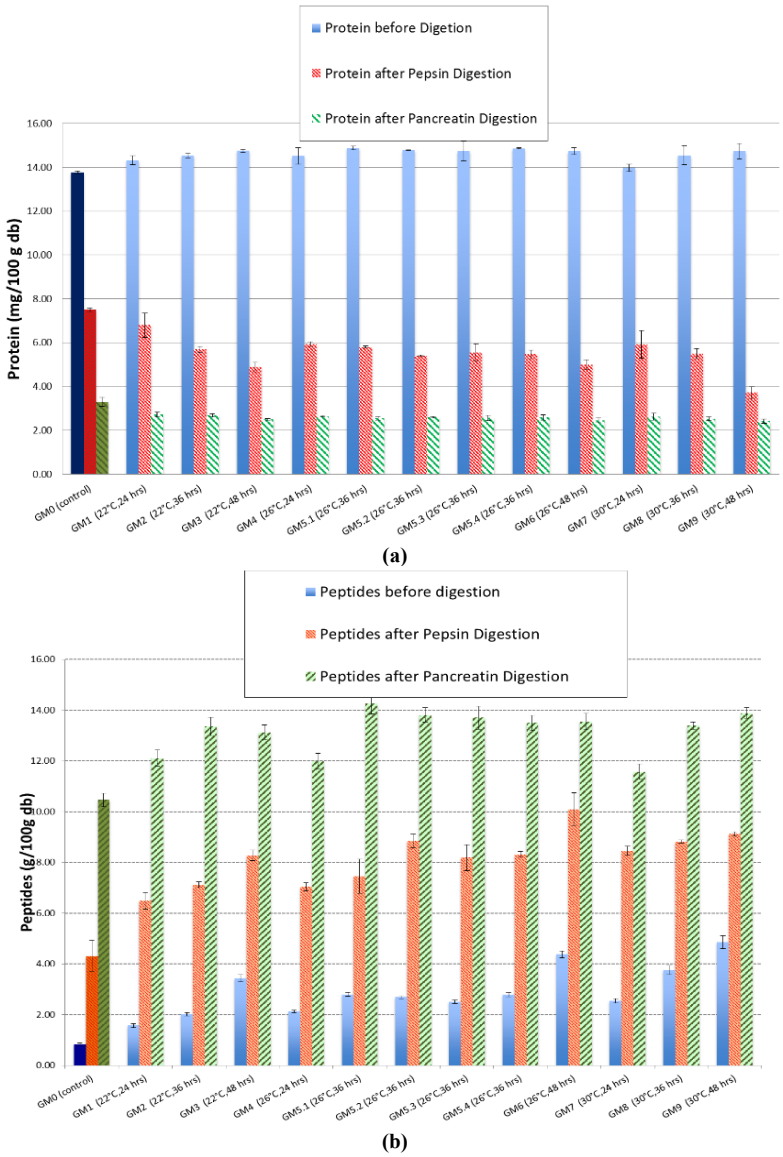


Figure 4-1: The measured (a) protein and (b) peptides before and after pepsin and pancreatin digestion for the selected experimental design factor combinations.

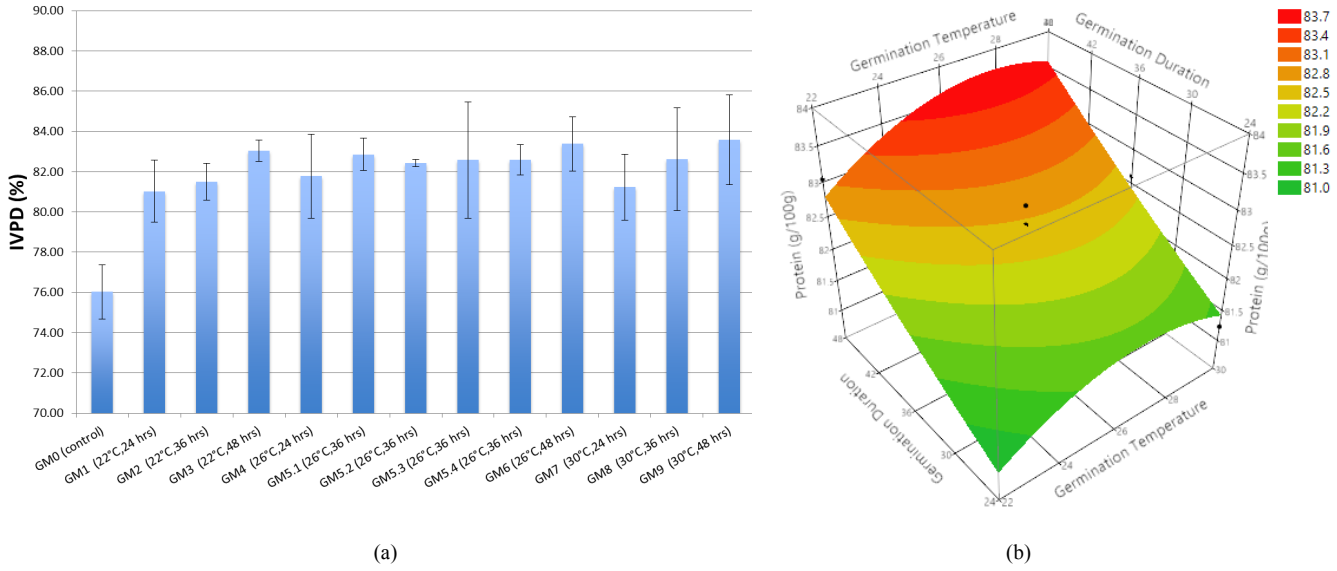
step. The comparison of the dashed green and red columns in Figure 4-1 (a) indicates that most proteins were digested in the first pepsin digestion with further reduction in protein content after pancreatin digestion. A similar but reverse pattern was observed in the peptide content, whose levels increased after each digestion step. These figures illustrate that both

germination duration and temperature were significant factors on the monitored quantities of protein and peptides.

4.4.3 *In-vitro* protein digestibility

Protein digestibility of the studied samples was evaluated based on the initial (before digestion) and final (after the full digestion) total protein values for each germination treatment using equation (4-1). Results are provided numerically in Table 4-1 and graphically in Figure 4-2 (a). While for the raw sample, IVDP was assessed around 76%, this value was increased up to around 84% after germination indicating an approximate 8% elevation in the protein digestibility. From this figure, it is clear that digestibility was strongly correlated with the germination duration. To quantitatively investigate effects of selected independent variables, temperature and duration, on the dependent variable, IVDP, data were analyzed using ANOVA. The obtained response surface that acts as a second-order interpolator over the studied range of operation is presented in Figure 4-2 (b). Based on the performed statistical analysis, germination duration, temperature and temperature square were found to be significant factors (Table 4-3). On the constructed response surface, a local maximum for IVPD was found at 48 hr and 28 °C.

The effect of germination on the *in-vitro* protein digestibility of amaranth grain has been studied by a few researchers (Gamel et al., 2005; Paredes-Lopez & Mora-Escobedo, 1989). An investigation performed by Paredes-Lopez and Mora-Escobedo (1989) showed that, while the true protein content increased from 14.4 to 17.6% after 72 hr of germination, IVDP was not significantly altered (80.6 to 79.2%). In another study, while the IVDP was evaluated to be 77.6% for native amaranth grains, this value increased up to 97.8% for germination of grains up to 72 hr (Okoth et al., 2011).



(a) (b)

Figure 4-2: (a) IVPD evaluated for all design factor combinations and (b) its corresponding response surface.

4.4.4 Anti-nutrient content

Phytic acid, tannin, and oxalate contents were monitored for the designed malting stages (Table 4-2). While phytic acid and oxalate contents decreased after germination, an increase in the tannin level was observed. Phytic acid decreased to the greatest extent for GM9 (30%), where grains were germinated for 48 hr at 30 °C. Similarly, GM9 displayed the lowest level of oxalate, which showed a 38% reduction in comparison with the control sample. On the other hand, tannin followed a reverse trend by increasing throughout germination and reaching a maximum value for GM6 (47% increase vs. control). These data are graphically presented in Figure 4-3 (a) indicating that both temperature and duration were important factors.

Effect of germination on the tannin content was studied by Kanensi et al. (2012), where unlike the present study, tannin decreased from its initial value to non-detectable amounts after 72 hr of germination of the amaranth grain. Nevertheless, a general increase in phenolic

compounds of amaranth grain throughout germination process has been reported (Alvarez-Jubete et al., 2010b; Pasko et al., 2009). A study on two varieties of amaranth, *A. caudatus* and *A. cruentus* showed a reduction in phytic acid content as germination duration increased (Colmenares de Ruiz & Bressani, 1990). For *A. caudatus*, phytic acid decreased from its initial 0.44% value for native grain to 0.31, 0.20, and 0.14% after 24, 48, and 72 hr of germination, respectively. Similarly, for *A. cruentus*, phytic acid decreased from 0.32% to non-detectable levels after 72 hr of germination (Colmenares de Ruiz & Bressani, 1990).

Table 4-2: Phytic acid (g/100g db), tannin (g/100g db), and oxalate (mg/100 g db) contents for the tested germination treatments.

EXP	Germination factors and their levels		Anti-Nutrient Content		
	Temp.	Time.	Phytic Acid (g/100g db)	Tannin (g CE/100g db)	Oxalate (mg/100g db)
GM0	Control		0.70 ± 0.01	0.19 ± 0.02	218.19 ± 3.98
GM1	22	24	0.63 ± 0.02	0.23 ± 0.02	163.27 ± 3.82
GM2	22	36	0.60 ±0.01	0.21 ± 0.01	148.83 ± 2.16
GM3	22	48	0.57± 0.01	0.25 ± 0.02	146.28 ± 3.34
GM4	26	24	0.61 ± 0.03	0.24 ± 0.01	161.51 ± 2.09
GM5.1	26	36	0.56 ± 0.02	0.26 ± 0.03	153.02 ± 2.47
GM5.2	26	36	0.55 ± 0.01	0.25 ± 0.01	146.20 ± 3.52
GM5.3	26	36	0.53 ±0.01	0.24 ± 0.02	149.52 ± 2.45
GM5.4	26	36	0.53 ± 0.02	0.26 ± 0.01	151.01 ± 4.25
GM6	26	48	0.50 ± 0.01	0.28 ± 0.02	143.59 ± 4.14
GM7	30	24	0.60 ± 0.04	0.23 ± 0.02	158.12 ± 2.99
GM8	30	36	0.55 ± 0.01	0.26 ± 0.01	139.17 ± 4.06
GM9	30	48	0.49 ± 0.02	0.26 ± 0.03	134.54 ± 3.10

The similar (tannin) and inverse (phytic acid and oxalate) trends between anti-nutrient content and IVDP levels may indicate possible correlations between these quantities. To further explore that relationship, the results are presented as scattered data in Figure 4-3 (b), (c), and (d) for IVPD versus oxalate, phytic acid, and tannin, respectively. Based on the obtained linear regression curves and their corresponding R^2 values, the proposed hypothesis was strengthened. A general statement regarding increasing IVPD after decreasing anti-nutrient content has been reported by others (Ravindran, 1992). It is not probable that the increase in IVDP was due to the higher tannin content as protein digestibility is more likely

related to a decrease in the phytic acid and oxalate components. This latter observation is based on the assumption that the other anti-nutrient factors remained constant or followed a similar increasing behavior as tannin.

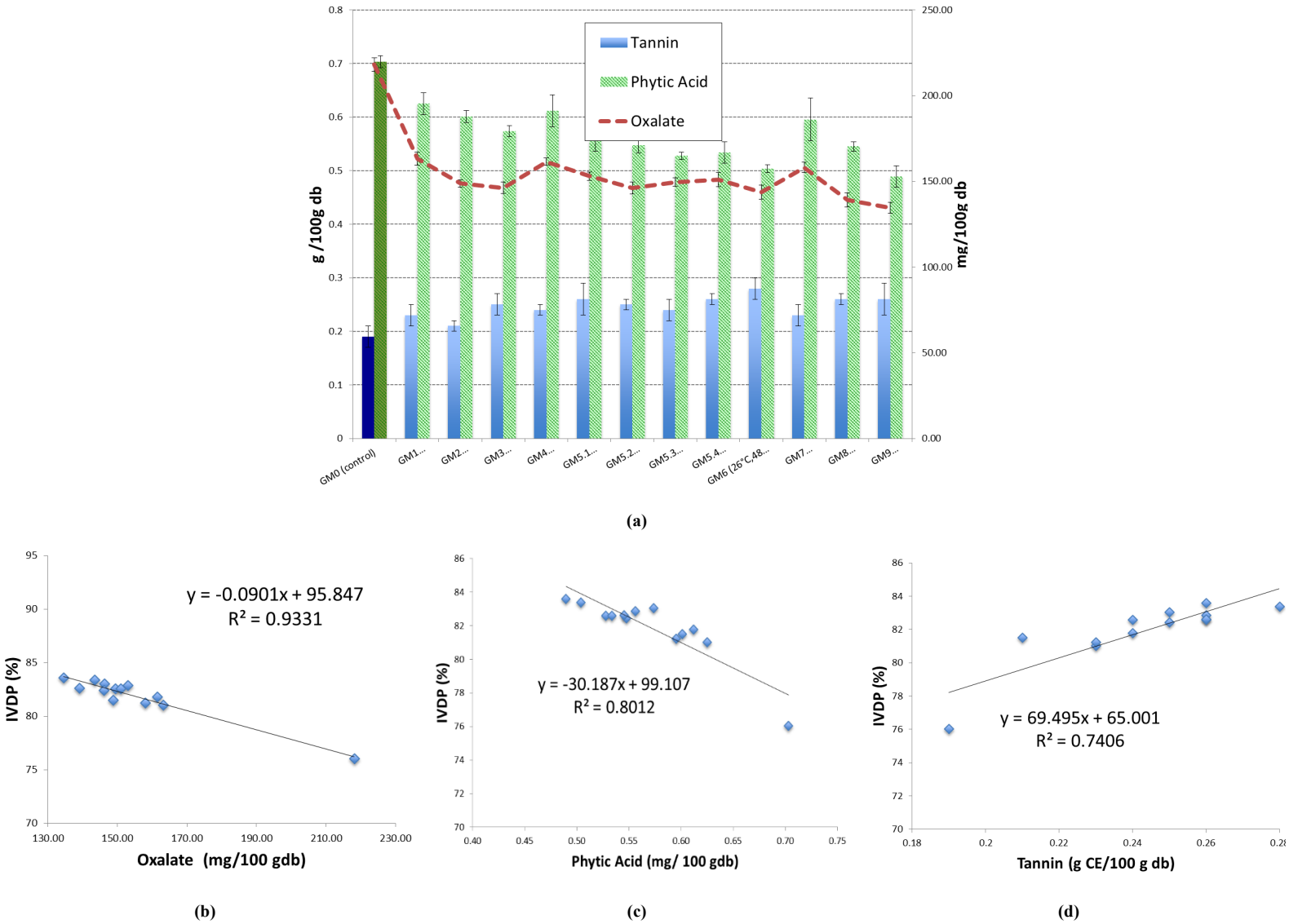


Figure 4-3: (a) The measured anti-nutrient contents; left vertical axis presents phytic acid (g/100 g db) and tannin (g CE/100g db) and the right axis quantifies the oxalate content (mg/100 g db) and the linear regression curves representing the possible correlations between the IVPD (%) and the anti-nutrient contents, (b) oxalate (c) phytic acid, and (d) tannin.

4.4.5 ANOVA for monitored quantities

Similar to the IVPD, the obtained data for proteins, peptides, and anti-nutrients for all performed studies have been analyzed using ANOVA. The response surfaces were

constructed and linear, quadratic, and bi-linear terms for the germination factors were obtained.

Table 4-3: Summary of the ANOVA of the responses for protein, peptides, IVDP, and anti-nutrient contents.

	Protein content			IVPD (%)	Peptides content			Anti-nutrient Content		
	Before Digestion	After Pepsin Digestion	After Pancreatin Digestion		Before Digestion	After Pepsin Digestion	After Pancreatin Digestion	Phytic Acid	Tannin	Oxalate
Intercept	**14.799** [a]	**5.582** [a]	**2.580** [a]	**82.578** [a]	**2.750** [a]	**8.238** [a]	**13.756** [a]	**0.545** [a]	**0.253** [a]	**149.281** [a]
Temp	-0.058	**-0.373** [p]	**-0.056** [d]	**0.308** [f]	**0.690** [a]	**0.752** [f]	0.0450	**-0.027** [d]	0.010	**-4.425** [d]
Time	**0.235** [d]	**-0.838** [d]	**-0.103** [a]	**0.995** [a]	**1.072** [a]	**0.920** [e]	**0.817** [b]	**-0.047** [a]	**0.015** [f]	**-9.748** [a]
Time×Temp	0.088	-0.070	0.005	0.085	0.115	-0.283	**0.320** [f]	-0.013	0.003	-1.648
Temp×Temp	**-0.229** [p]	-0.041	0.024	**-0.456** [f]	0.020	-0.354	-0.263	**0.025** [f]	-0.018	**-3.968** [f]
Time×Time	-0.139	-0.176	**-0.036** [f]	0.064	**0.385** [f]	0.241	**-0.878** [d]	0.005	0.008	**4.583** [f]
R^2	0.892	0.874	0.963	0.945	0.978	0.809	0.948	0.951	0.732	0.954
R^2 adj	0.802	0.769	0.932	0.900	0.960	0.651	0.904	0.910	0.509	0.915
RMS	0.115	0.353	0.023	0.263	0.196	0.599	0.260	0.013	0.013	2.500

a: $p<0.0001$, b: $p<0.0005$, c: $p<0.001$, d: $p<0.005$, e: $p<0.01$, f: $p<0.05$

Results are presented in Table 4-3, in which significant factors were indicated in bold with their corresponding probabilities. It was observed that while germination duration was notably influential on all investigated parameters, germination temperature was not significant for protein content before digestion, peptide levels after pancreatin digestion, and tannin levels. Similar to IVPD, the quadratic term for germination temperature was significant in the case of protein before digestion, phytic acid, and oxalate. The quadratic term for germination duration was significant for protein and peptide after digestion, peptide levels before digestion and oxalate. The bi-linear term was only weakly significant for peptide levels after digestion ($p<0.05$).

4.5 Conclusion

The aim of the present study was to evaluate the effects of malting process on the *in-vitro* protein digestibility of an ancient and nutritious pseudo-cereal, amaranth. The obtained results showed that malting/germination of amaranth may be considered as an inexpensive

and appropriate pre-treatment to produce higher nutrient quality and more digestible amaranth food products. Main germination design parameters, temperature and duration, were extensively studied and optimized. Based on the performed ANOVA and constructed second-order response surface, germinating at 28 °C and 48 hr resulted into the most favorable protein digestibility (84%), when comparing with the native grains (76%). In addition, phytic acid and oxalate content decreased during the process, while tannin showed an increase. Linear correlations between IVDP and the investigated anti-nutrient contents were proposed.

4.6 Acknowledgements

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Connecting Statement to Chapter 5

In **Chapter 3** and **Chapter 4,** protein bio-availability and its *in-vitro* digestibility has been notably enhanced for finger millet and amaranth grains by optimizing the two main malting design parameters, germination duration and temperature. For finger millet, 17% increase in the *in-vitro* protein digestibility was observed for the grains that were germinated for 48 hr at 30 °C **(Chapter 3)**. For amaranth, the optimized malting condition was found to be 48 hr and 28 °C, where protein digestibility enhanced by 8% **(Chapter 4)**. In Chapter 5, similar optimization study was performed for both finger millet and amaranth grains, over all their important nutritional components. Therefore, in addition to protein content, other main nutritional components including; digestive, resistant, and total starch, fat, fatty acids, and their composition, fiber, ash, and energy were monitored and optimized. Based on the obtained nutritionally enriched flours and considering the infants' nutritional requirements, various weaning products may be formulated.

The current chapter is written in the manuscript style and is submitted to the **International Journal of Food Sciences and Nutrition** as follows,

Optimization of the malting process for nutritional improvement of finger millet and amaranth flours in the infants' weaning food industry

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Contributions made by the different authors are as follows;

- The first author, **Sara Najdi Hejazi**, is the Ph.D. student, who prepared the experimental design, conducted the laboratory work, analyzed the results, and prepared the manuscript.
- The second author, **Valérie Orsat**, is the thesis supervisor, who initiated and stimulated ideas, provided the technical advice, suggestions, experiment facilities and guided the first author in writing, correcting and preparing the manuscript throughout the study.

Furthermore, parts of the obtained data and performed analyses in **Chapter 4** were presented in two conference presentations, as follows;

1. Sara Najdi Hejazi and Valérie Orsat, "**Weaning Infant Foods from Minor Millets- Effect of Germination on Total Phenol Contents and Anti-Nutrient Compounds of Finger Millet and Amaranth"** American Society of Agricultural and Biological Engineers, Emerging Technologies in Food Processing Session, July 13-16, 2014, Montreal, Canada.
2. Sara Najdi Hejazi and Valérie Orsat, "**Weaning Infant Foods from Minor Millets"** Northeast Agricultural and Biological Engineering Conference, Oral presentation, June 16-19, 2013, Altoona, PA, USA.

5 Optimization of malting process for nutritional improvement of finger millet and amaranth flours in the infants' weaning food industry

5.1 Abstract

Malting, as a simple and low cost food-processing step, could be an efficient and beneficial approach to improve the nutritional value of cereal grains used in infant preparations. Malted finger millet and amaranth grains might be considered as good gluten-free alternatives for common wheat-based infants' weaning products, especially in case of those suffering from celiac disease. In the present study, the effect of germination temperature and duration on the main nutrients of malted finger millet and amaranth grains were evaluated and optimized based on a central composite design. Grains were germinated for 24, 36, and 48 hr at 22, 26 and 30 °C. In the case of finger millet, germinating for 48 hr at 30 °C resulted into an overall 17% increase in protein availability, 10% increase in total energy, and 60% reduction in resistant starch contents. For amaranth, germinating for 48 hr at 26 °C was preferable, resulting in 8% increase in protein, 11% increase in total energy, 70% reduction in resistant starch, and a 10% increase in the linoleic acid content. Based on the obtained results, an optimized malting process may be employed as an effective food processing approach for improving the nutritional characteristics of finger millet and amaranth grains.

Keywords: *finger millet, amaranth, germination/malting, nutrient components, weaning food*

5.2 Introduction

Early childhood nutrition is crucial, since it constitutes the foundation of adult productivity. Although exclusive breastfeeding is highly recommended for infants up to four months of age, afterwards breast milk alone cannot provide adequate energy and nutrient requirements for the growing body (Sajilata et al., 2002). Therefore, in order to sustain normal growth and development, introduction of supplementary products, such as weaning foods is necessary (Anigo et al., 2009; Lombard & Labuschagne, 2013; Sajilata et al., 2002; Shobana et al., 2012). However, as the first infants' semisolid staple nutrients may have short-term effects throughout the infancy and long-term consequences on the adulthood health, the weaning foods should possess some special characteristics and be formulated with care (Najdi Hejazi, 2012; Pearce & Langley-Evans, 2013a ; Sajilata et al., 2002).

Infants' weaning products should be rich in calories and provide good-quality protein, vitamins, and minerals (Sajilata et al., 2002). According to the guidelines of the Protein Advisory Group, based on dry weight basis, weaning products are desired to have protein content of 15 to 20%, fat content around 10%, carbohydrate content of 60 to 70%, and total ash content of less than 5% (Kumkum et al., 2013; Sajilata et al., 2002). In addition, according to recent health guidelines, it is strongly recommended that gluten-free cereals be preferably used for weaning of infants younger than six months old (Fasano & Catassi, 2011; Guandalini, 2007; Omary et al., 2012; Saleh et al., 2013). Especially, in the case of a family history of celiac disease, the restriction for consumption of gluten containing foods may be extended (Fasano & Catassi, 2011; Guandalini, 2007). Therefore, wheat-based products, barley, oat cereals, and multigrain cereals are not advised for infants before six months, while alternatively, gluten-free cereals, like rice, millet, amaranth, buckwheat, corn, and sorghum

are preferably suggested (Fasano & Catassi, 2011; Guandalini, 2007; Omary et al., 2012). To date, the weaning food industry has mostly focused on wheat- and rice- based products (Desikachar, 1982; Sajilata et al., 2002). In the present study, utilization of malted/germinated finger millet and amaranth grains in the formulation of new infants' weaning products has been investigated.

Millet is one of the most important drought-resistant crops, which is widely grown in arid and semiarid tropics of Asian and African countries. The crop is well known for being gluten-free, having a short growing season, and good productivity in dry and high temperature climates (Saleh et al., 2013). It is a major source of carbohydrate and minerals (Anigo et al., 2009; Anigo, 2010; Chandrashekar, 2010). Among different millet varieties, finger millet (*Eleusine coracana*) is traditionally used for the preparation of weaning food, especially in India (Dahiya & Kapoor, 1993; Thathola & Srivastava, 2002). The importance of the food processing approaches and suitable complementarity of ingredients in the preparation of millet-based weaning food has been highlighted in recent investigations (Dahiya & Kapoor, 1993; Gupta & Sehgal, 1992a; Gupta & Sehgal, 1992b). This is mostly due to the lower protein content of native finger millet grain (around 7%) (Singh and Raghuvanshi, 2012) compared to the required standards in weaning products (15 to 20%) (Kumkum et al., 2013). Protein deficiency of the finger millet could be improved by adding other supplementary cereal ingredients. Here, amaranth is chosen for this purpose. The protein content of raw amaranth grain is around 14.5% (Rastogi & Shukla, 2013).

In the last few decades, amaranth grain has been gaining attention in the scientific research community as one of the most promising pseudo-cereals as it contains appreciable amounts of high-quality protein, lipid, carbohydrates, and dietary fibers (Rastogi & Shukla,

2013). Similar to finger millet and unlike other cereals, amaranth is rich in lysine, which is an essential amino acid, especially in case of weaning products (Rastogi & Shukla, 2013). Besides, amaranth has low levels of saturated fatty acids and is recognized as a hypocholesterolemic grain (Berger et al., 2003). Like millet, amaranth is categorized in the gluten-free grain group, making it a suitable pseudo-cereal to be consumed by infants suffering from gluten allergies (Alvarez-Jubete et al., 2010a; Saturni et al., 2010).

Malting/germination is a simple traditional food processing operation that can improve the nutrient availability of cereal grains (Hejazi & Orsat, 2015; Subba Rao & Muralikrishna, 2002). Through this *in-vivo* biotransformation process, a drastic enhancement in the hydrolytic enzymes occurs, which improves the bio-availability of proteins, carbohydrates, vitamins, and minerals (Hejazi et al., 2015; Malleshi & Klopfenstein, 1998). In the presence of boiled water or milk, the included amylases in the malted flour hydrolyze starch into sugars which results into a good taste, low bulk, and nutrient-dense slurry that can be used as an infant weaning food (Malleshi & Desikachar, 1982).

Germination duration and temperature are the two main factors to be monitored in the malting process. Effects of these factors on the nutritional improvement of cereal grains have been studied in a number of researches (Chiba et al., 2012; Saleh et al., 2013; Swami et al., 2013). Swami et al. (2013) stated that as the germination time increased, the protein availability of finger millet increased. In other studies, it was observed that as malting progressed, finger millet produced adequate amount of α-amylase with "agreeable flavor and acceptable taste", that was comparable with wheat (Malleshi & Desikachar, 1982; Malleshi & Desikachar, 1986a). Amylases are the main malt enzymes that hydrolyze the starch content of the grains. Similarly, malting improves amaranth's nutrient characteristics (Gamel

et al., 2005; Paredes-Lopez & Mora-Escobedo, 1989; Rastogi & Shukla, 2013; Venskutonis & Kraujalis, 2013). It was reported that the availability of lysine in amaranth was increased by 31% following 24 hr of germination (Balasubramanian & Sadasivam, 1989). Aligned with lysine enhancement, water-soluble protein content increased from 15.6 to 21.6% during the performed germination treatment. On the other hand, a decrease in the level of lipid and phytic acid for malted amaranth grain has been observed. Similar to malted finger millet, an increase in reducing and total sugars as a function of germination time was reported (Colmenares de Ruiz & Bressani, 1990).

In the present study, a thorough investigation of the effects of temperature and duration on the malting process of finger millet and amaranth grains has been performed. Following the outcomes, the selected design factors were optimized to enhance the nutrient characteristics of the processed flours. Based on the resulted nutritional profiles, finger millet and amaranth flours are found as good substitutes for wheat and rice that are currently the main ingredient in the weaning food industry.

5.3 Materials and methods

5.3.1 Materials

The sproutable finger millet (*Eleusine coracana*) seeds were procured from University of Agricultural Sciences (Dharwad, Karnataka, India) and amaranth (*Amaranthus caudatus* L. (love-lies-bleeding)) grain was purchased from a local market (Bulk Barn, Qc., Canada). Tridecanoic acid (C13:0) was provided by Nu-Chek Prep Inc. (Elysian, MN, USA). Ethanol was purchased from Commercial Alcohols (Industrial and Beverage Alcohol Division of Green-field Ethanol Inc., Brampton, Ontario, Canada). All other used chemicals were of

analytical grade and were obtained from Fisher Scientific (Ottawa, Canada), Sigma-Aldrich (St. Louis, MO, USA), and Megazyme International (Wicklow, Ireland).

5.3.2 Malting/Germination process

Finger millet and amaranth grains were cleaned and soaked in distilled water for 24 hr. The grains were kept on a germination cloth at 22, 26, and 30 °C for 24, 36, and 48 hr until the rootlets appeared. Germinated seeds were freeze-dried (FreeZone® 2.5 l Freeze Dry System, Labonco Corporation, Kansas City, MO, USA), ground in an electric grinding mill (Bodum 10903, PRC, Intertek, USA), and stored at 4 °C in airtight bags until further analysis.

5.4 Methods

The interested nutrient components were monitored according to the following methods.

5.4.1 Dry matter

Dry matter (DM) was determined using the AACC protocol (AACC, 1999).

5.4.2 Ash content

Ash content was analyzed applying the standard AOAC protocol (Helrich, 1990).

5.4.3 Fiber content

Fiber content was determined based on the Van Soest et al. (1991) method using an Ankom Fiber Analyzer (Ankom Technology Corp., Macedon, NY).

5.4.4 Total protein

Total protein was quantified by the Bradford method based on the kit (# 500-0006, Bio-Rad, Mississauga, Ontario) structure (Bradford, 1976).

5.4.5 Total fat

Total fat content of samples was analyzed using ether extracts following the standard AOAC procedure (Helrich, 1990).

5.4.6 Fatty acids

Fatty acids were made volatile by converting them into methyl esters. The conversion of fatty acids to methyl esters was carried out directly by trans esterification i.e., from glycerol to ester to methyl ester. The esters were identified and quantified by injection into a Gas Chromatograph (GC) and by comparing with a set of standard esters.

Briefly, the fat content of malted finger millet and amaranth samples was extracted by centrifugation at 15,000 ×g for 25 min and 0.5 g of fat was used for fatty acid methyl ester synthesis (O'Fallon et al., 2007). The tridecanoic acid (C13:0) was used as an internal standard. Fatty acid composition of the fatty acid methyl esters was determined by capillary gas chromatography (Varian model 3900 equipped with flame-ionization detector at 260 °C and model 1177 auto injector; Varian, Palo Alto, CA) fitted with a fused silica capillary column (CP7489, 100 m×0.25 mm; Varian). The carrier gas was H_2 and the flow rate was 0.8 mL/min. Injector and detector temperatures were 260 °C, and the split ratio was 50:1. Column temperature was set to 70 °C for 4 min, and then increased to 130 °C at a rate of 120 °C/min and was maintained for 3 min. It was then increased to 175 °C at a rate of 4 °C/min and was maintained for 27 min. Finally, the temperature was increased to 214 °C at a rate of 4 °C /min and maintained for 11 min and increased to 225 °C at a rate of 4 °C /min and held for 5.5 min. Therefore, total run time was 79.25 min. Fatty acids were identified by comparing their retention times with fatty acid methyl standards (Nu-Chek Prep Inc.) (O'Fallon et al., 2007).

5.4.7 Resistant, digestible, and total starch

Total starch content is composed of two portions; resistant starch (RS) and digestible starch (DS). RS is the portion of starch that is not broken down by human enzymes in the small intestine, while it is partially or wholly fermented by the gut bacteria in the large intestine. Resistant starch is mainly recognized as an important part of dietary fiber, which functions as a prebiotic. The rest of total starch is considered as the soluble or digestible starch. The starch contents of the samples were determined using Megazyme Starch Assay Kit (K-RSTAR, Megazyme Bray, Co. Wicklow, Irland) (Odenigbo et al., 2014).

Briefly, milled and freeze dried samples (100 $\pm$ 0.5 mg) were hydrolyzed using pancreatic α-amylase (10 mg/ml) solution containing amyloglucosidase (AMG) and incubated for 16 hr at 37 °C with vigorous agitation. Thereafter, samples were centrifuged and washed three times with ethanol (two times with 99% and one time with 50% v/v). The resulted pellets from supernatants were digested with KOH (2M). Digested pellets and supernatants were incubated with AMG, separately. The absorbance of the released D-glucose was measured using glucose oxidase-peroxidase (GOPOD) as the working reagent at the wavelength of 510 nm. The glucose contents of the supernatant and digested pellet were used in the calculation of the digestible starch (DS) and resistant starch (RS) respectively by applying the factor of 0.9. Total starch (TS) content was reported as the sum of DS and RS in g/100g db.

5.4.8 Gross energy

Gross energy (GE) of the processed finger millet and amaranth flour samples was determined using an oxygen bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) (Bakari & Ngadi, 2013).

5.5 Statistical analysis

A Central Composite Design (CCD) was employed to investigate the optimal malting conditions of finger millet and amaranth grains. The approach determines the effects and interactions between different study design factors on a particular response. The CCD was performed using JMP software (Version 11, SAS Institute Inc. Cary, NC, USA). Malting temperature and duration for finger millet and amaranth were taken as the independent factors at three different levels. Preliminary experiments were conducted to get a range of values for the temperature and duration. Samples were assessed at twelve combinations of three temperatures (22, 26, and 30 °C) and three duration levels (24, 36, and 48 hr) including four central, four factorials, and four axial points. The resulting response surface for each investigated quantity, Z, was constructed as follows;

$$Z = \text{Intercept} + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \qquad (5\text{-}1)$$

5.6 Results

Based on the designed germination steps, the nutrients of interest were monitored for finger millet and amaranth sprouts. The obtained results are presented in the following subsections.

5.6.1 Digestive, resistant, and total starch

Total starch content along with its digestible and resistant components were evaluated following the methodological approach presented in section 5.4.7. The results are provided in Table 5-1. For greater clarity, cases are categorized in a colourful manner as a function of the germination temperature (blue for 22 °C, green for 26 °C, and red for 30 °C) and germination duration (light colors for 24 hr, medium colors for 36 hr, and dark colors for

48 hr). Values are presented as their averages (from triplicate measurements) and the corresponding Standard Deviation (SD). Based on the selected unit, g/100 g db, numbers indicate the mass fraction of each component. Results can be compared with those of the native grains (GM0).

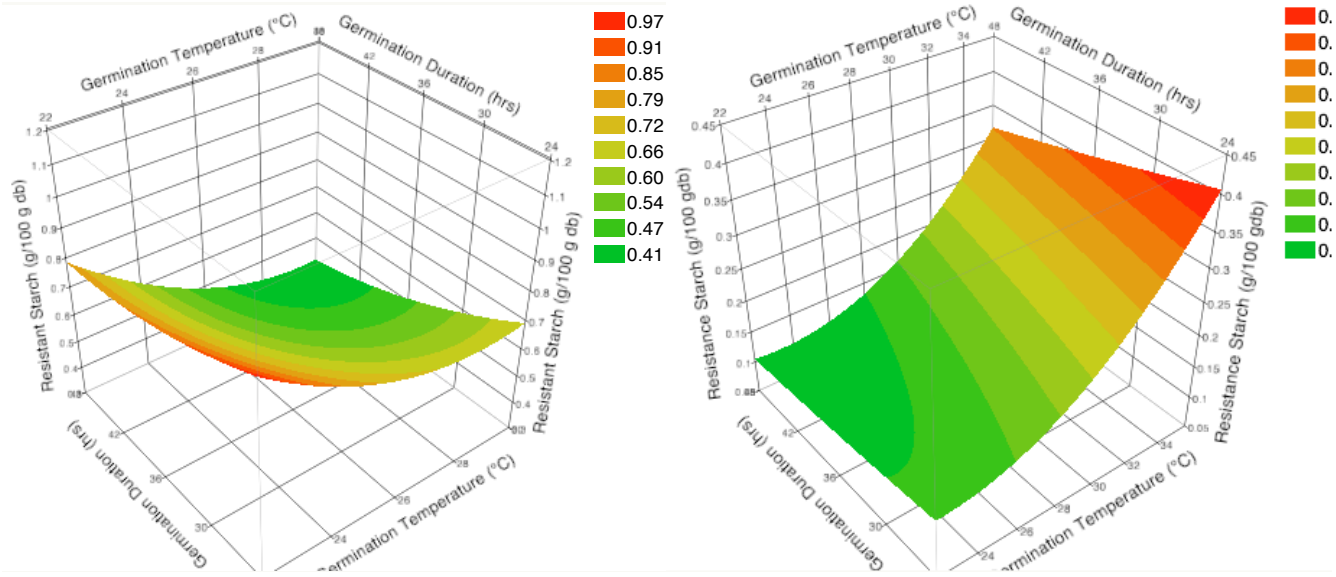
In order to highlight the importance of the germination process on the starch fractions, a second averaging step was performed over all of the germination treatments (Table 5-1). By comparing the obtained SD with averaged magnitudes, it was observed that germination treatments significantly altered the resistant starch of finger millet (SD/average = 94%), and both the resistant (27%) and digestible starch (7%) contents of amaranth grains.

To quantitatively evaluate these dependencies, ANOVA studies were performed on all monitored starch quantities and second-order response surfaces were constructed (equation (5-1)). As an example, the constructed response surfaces for resistant starch contents during the performed malting treatments are presented in Figure 5-1 (a) and Figure 5-1 (b) for finger millet and amaranth grains, respectively. Obtaining relatively high R^2 values emphasized the appropriate prediction of the experimental data by the constructed response surfaces (Table 5-1). For finger millet, starch contents linearly depended on duration and both linearly and quadratically on temperature. Similar dependencies were observed for amaranth sprouts. However, unlike millet, the linear temperature term was not influential on the digestive starch and consequently total starch contents.

Table 5-1: Digestible, resistant, and total starch contents (g/100 g db) of finger millet and amaranth grains for the designed germination treatments.

EXP	Germination factors & levels		Finger millet			Amaranth		
	Temp.	Time	Digestible Starch	Resistant Starch	Total Starch	Digestible Starch	Resistant Starch	Total Starch
GM0	Control		73.37 ± 0.06	1.42 ±0.05	74.79 ± 0.08	63.26 ± 1.37	0.24 ± 0.02	63.50 ± 1.37
GM1	22	24	72.98 ± 0.66	0.92 ± 0.03	73.90 ± 0.66	62.10 ± 4.57	0.14 ± 0.01	62.24 ± 4.57
GM2	22	36	73.17 ± 0.55	0.87 ± 0.05	74.04 ± 0.55	52.12 ±2.22	0.12± 0.01	52.24 ±2.22
GM3	22	48	73.70 ± 1.39	0.81 ± 0.03	74.51 ± 1.39	51.11 ± 2.48	0.11± 0.01	51.22 ± 2.48
GM4	26	24	72.81 ± 1.34	0.84 ± 0.01	73.65 ± 1.34	62.32 ± 0.93	0.17 ± 0.02	62.49 ± 0.93
GM5.1	26	36	73.64 ± 2.26	0.54 ± 0.01	74.18 ± 2.26	57.29 ± 2.92	0.14 ± 0.02	57.43 ± 2.92
GM5.2	26	36	73.52 ± 2.44	0.55 ± 0.01	74.07 ± 2.44	58.39 ± 1.56	0.13 ± 0.01	58.52 ± 1.56
GM5.3	26	36	73.46 ± 2.53	0.58 ± 0.01	74.04 ± 2.53	59.22 ± 0.70	0.15 ± 0.01	59.37 ± 0.70
GM5.4	26	36	73.79 ± 0.65	0.53 ± 0.05	74.32 ± 0.65	60.62 ± 1.69	0.13 ± 0.01	60.75 ± 1.69
GM6	26	48	74.01 ± 1.17	0.45 ± 0.03	74.46 ± 1.17	56.75 ± 4.85	0.10± 0.01	56.85 ± 4.85
GM7	30	24	73.80 ± 0.62	0.63 ± 0.01	74.43 ± 0.62	61.84 ± 0.17	0.23 ± 0.04	62.07 ± 0.17
GM8	30	36	74.82 ± 2.10	0.55 ± 0.01	75.37 ± 2.10	57.28 ± 1.82	0.19 ± 0.01	57.47 ± 1.82
GM9	30	48	75.14 ± 1.45	0.43 ± 0.01	75.57 ± 1.45	53.28 ± 0.69	0.17 ± 0.02	53.45 ± 0.69
Average ± SD			73.71 ± 0.08	0.70 ± 0.66	74.41 ± 0.56	58.12 ± 4.03	0.15 ± 0.04	58.28 ± 4.03
R^2			0.97	0.91	0.96	0.88	0.96	0.88
Intercept			**73.585** [a]	**0.568** [a]	**74.153** [a]	**58.592** [a]	**0.136** [a]	**58.728** [a]
Temp			**0.652** [a]	**-0.165** [c]	**0.487** [b]	1.178	**0.037** [a]	1.215
Duration			**0.543** [b]	**-0.117** [d]	**0.427** [b]	**-4.187** [e]	**-0.027** [b]	**-4.213** [e]
Temp*Duration			0.155	-0.023	0.133	0.608	-0.008	0.600
$Temp^2$			**0.444** [d]	**0.106** [f]	**0.550** [d]	**-3.316** [f]	**0.023** [e]	**-3.294** [f]
$Duration^2$			-0.141	0.041	-0.100	1.519	0.003	1.521

a: p<0.0001, b: p< 0.0005, c: p<0.001, d: p<0.005, e: p<0.01, f: p<0.05



(a) (b)

Figure 5-1: Surface plots for resistant starch content over the investigated range of design parameters (temperature 22 to 30 °C) and (germination duration 24 to 48 hr) for (a) finger millet grain (b) amaranth grain.

5.6.2 Protein, fat, and fatty acids

The obtained results for protein, fat, and fatty acids contents of finger millet and amaranth native grains and sprouts are presented in Table 5-2. Again, having relatively high SD values after averaging among all native and germinated cases (around 10%) indicated that the germination treatments altered these nutrient quantities. Based on the ANOVA study, while germination duration was a significant term for protein content in both millet and amaranth grains, temperature showed a different behaviour. For millet, the protein content was linearly governed by temperature, while this dependency was quadratic for amaranth. Quadratic temperature terms were significant in case of fat and fatty acids in finger millet as well. For amaranth grain, despite 5 to 10% changes in fat and fatty acids, no particular association between these quantities and the selected germination factors were concluded (low R^2 values).

Table 5-2: Protein, fat, and fatty acids contents (g/100 g db) of finger millet and amaranth grains for the designed germination treatments.

EXP	Germination factors & levels		Finger millet			Amaranth		
	Temp.	Time	Protein	Fat	Fatty Acids	Protein	Fat	Fatty Acids
GM0	Control		6.42 ± 0.21	1.14 ±0.07	1.11	13.76 ± 0.07	7.21 ± 0.61	6.74
GM1	22	24	6.52 ± 0.09	0.92 ± 0.07	0.90	14.33 ± 0.21	7.09 ± 0.09	6.61
GM2	22	36	6.6 0 ± 0.05	0.85 ± 0.02	0.82	14.53 ± 0.11	7.21 ± 0.15	7.08
GM3	22	48	6.75 ± 0.13	0.94 ± 0.01	0.89	14.75 ± 0.07	7.28 ± 0.29	7.03
GM4	26	24	6.58 ± 0.08	1.03 ± 0.08	1.01	14.52 ± 0.38	7.31 ± 0.03	6.88
GM5.1	26	36	7.05 ± 0.21	1.01 ± 0.09	0.98	14.88 ± 0.08	6.48 ± 0.32	6.09
GM5.2	26	36	6.91 ± 0.08	0.99 ± 0.01	0.96	14.78 ± 0.01	6.61 ± 0.06	6.31
GM5.3	26	36	7.02 ± 0.13	0.98 ± 0.07	0.95	14.74 ± 0.45	6.52 ± 0.48	6.10
GM5.4	26	36	7.12 ± 0.26	1.02 ± 0.08	0.97	14.86 ± 0.03	6.46 ± 0.19	6.08
GM6	26	48	7.19 ± 0.06	1.05 ± 0.08	1.00	14.74 ± 0.16	7.32 ± 0.54	6.99
GM7	30	24	7.02 ± 0.13	0.89 ± 0.04	0.85	13.97 ± 0.18	7.53 ± 0.03	7.21
GM8	30	36	7.29 ± 0.20	0.96 ± 0.04	0.91	14.55 ± 0.43	7.34 ± 0.13	6.89
GM9	30	48	7.32 ± 0.13	0.92 ± 0.05	0.88	14.74 ± 0.35	6.95 ± 0.05	6.52
Average ± SD			6.91±0.30	0.98±0.08	0.94±0.08	14.55±0.34	7.02±0.38	6.66±0.40
R^2			0.91	0.81	0.84	0.89	0.69	0.67
Intercept			**7.009**[a]	**1.005**[a]	**0.968**[a]	**14.79958**[a]	**6.661**[a]	**6.301**[a]
Temp			**0.293**[c]	0.010	0.005	-0.05833	0.040	-0.017
Duration			**0.190**[e]	0.012	0.000	**0.235**[d]	-0.063	-0.025
Temp*Duration			0.018	0.003	0.009	0.0875	-0.193	-0.279
$Temp^2$			-0.031	**-0.109**[d]	**-0.111**[d]	**-0.22875**[f]	0.328	0.374
$Duration^2$			-0.091	0.026	0.027	-0.13875	0.368	0.324

5.6.3 Fiber, ash and energy

Similar studies were carried out for fiber and ash contents as well as energy level (Table 5-3). Although all linear and quadratic terms appeared to be important in the constructed response surface for the fiber content in finger millet, in case of amaranth grain, only the quadratic term of temperature was found to be significant. The ash content was notably decreased throughout germination with a strong dependency on both temperature and duration. In case of amaranth, bi-linear terms were influential as well. Generally, the energy content increased during germination with a direct dependency on germination duration. Effect of temperature on the assessed energy was important in a linear and quadratic fashion for millet and amaranth sprouts, respectively.

Table 5-3: Fiber (g/100 g db), ash (g/100 g db), and energy (kcal/100 g db) contents of finger millet and amaranth grains for the designed germination treatments.

EXP	Germination factors & levels		Finger millet			Amaranth		
	Temp.	Time	Fiber	Ash	Energy	Fiber	Ash	Energy
GM0	Control		11.11 ± 0.05	2.30 ±0.06	383.23 ± 10.84	9.34 ± 0.43	2.35 ± 0.02	407.14 ± 8.14
GM1	22	24	11.02 ± 0.05	2.21 ± 0.11	380.63 ± 19.88	8.78 ± 0.49	2.34 ± 0.06	418.83 ± 12.57
GM2	22	36	11.39 ± 0.22	2.12 ± 0.01	393.60 ± 18.58	9.13 ± 0.19	2.27 ± 0.03	427.22 ±4.27
GM3	22	48	12.12 ± 0.18	2.02 ± 0.06	399.31 ± 2.52	8.90 ± 0.12	2.18 ± 0.03	446.65 ± 17.87
GM4	26	24	11.68 ± 0.22	2.07 ± 0.04	387.12 ± 8.64	9.29 ± 0.47	2.31 ± 0.04	435.66 ± 13.07
GM5.1	26	36	11.51 ± 0.21	2.00 ± 0.08	404.12 ± 17.40	9.97 ± 0.13	2.19 ± 0.01	442.08 ± 17.68
GM5.2	26	36	11.71 ± 0.06	1.99 ± 0.04	396.96 ± 2.17	9.83 ± 0.40	2.21 ± 0.01	441.86 ± 22.09
GM5.3	26	36	11.61 ± 0.17	2.08 ± 0.09	398.30 ± 2.33	9.72 ± 0.60	2.17 ± 0.01	442.07 ± 8.84
GM5.4	26	36	11.68 ± 0.41	2.03 ± 0.07	406.29 ± 3.41	9.79 ± 0.23	2.23 ± 0.01	438.47 ± 17.54
GM6	26	48	11.98 ± 0.11	1.99 ± 0.02	413.09 ± 4.25	9.98 ± 0.08	2.04 ± 0.09	450.55 ± 27.03
GM7	30	24	11.62 ± 0.13	2.08 ± 0.09	407.74 ± 18.90	8.86 ± 0.70	2.29 ± 0.03	427.72 ± 8.55
GM8	30	36	11.05 ± 0.19	2.01 ± 0.01	415.08 ± 2.89	8.81 ± 0.25	2.13 ± 0.04	438.59 ± 4.39
GM9	30	48	11.02 ± 0.18	1.99 ± 0.09	423.11 ± 7.36	8.76 ± 0.46	1.97 ± 0.08	442.99 ± 13.29
Average ± SD			11.50 ± 0.36	2.07 ± 0.10	400.66 ± 12.64	9.32 ± 0.48	2.21 ± 0.11	435.37 ± 12.12
R^2			0.98	0.87	0.94	0.92	0.98	0.94
Intercept			**11.624**[a]	**2.024**[a]	**401.473**[a]	**9.820**[a]	**2.197**[a]	**441.260**[a]
Temp			**-0.140**[d]	**-0.045**[f]	**12.065**[b]	-0.063	**-0.067**[a]	2.767
Duration			**0.133**[c]	**-0.060**[d]	**10.003**[c]	0.118	**-0.125**[a]	**9.663**[b]
Temp*Duration			**-0.425**[c]	0.025	-0.828	-0.055	**-0.040**[e]	-3.138
$Temp^2$			**-0.396**[c]	0.043	2.758	**-0.834**[b]	0.010	**-8.636**[d]
$Duration^2$			**0.214**[d]	0.008	-1.478	-0.169	-0.015	1.564

a: p<0.0001, b: p <0.0005, c: p< 0.001, d: p<0. 005, e: p<0.01, f: p<0.05

5.6.4 Fatty acids composition

Fatty acid compositions were determined for the designed germination treatments based on the approach explained in section 5.4.6 and the results are presented in Table 5-4 and Table 5-5 for finger millet and amaranth grains, respectively. For finger millet, oleic acid methyl ester (C18:1N9C) was the main fraction with 45.42% of total fatty acids in the native grains. This fraction slightly decreased during germination treatments, where a minimum value of 43.59% was observed for GM9 case. Following oleic acid methyl ester, palmitic acid (C16:0) with 28.28% and linoleic acid methyl ester (C18:2N6C) with 20.72% were the main fatty acid fractions in the native finger millet grains. During germination, these fatty acids slightly increased. Linolenic acid methyl ester (C18:3N3), stearic acid (C18:0), and palmitoleic acid (C16:1) possessed lower fractions with 3.45%, 1.74%, and 0.39%, respectively.

Table 5-4: Fatty acid compositions of finger millet grain for the designed germination treatments and the results of their corresponding ANOVA.

			C16:0	C16:1	C18:0	C18:1N9C	C18:2N6C	C18:3N3
GM0	Control		28.28	0.39	1.74	45.42	20.72	3.45
GM1	22	24	29.05	0.40	1.75	44.59	20.66	3.57
GM2	22	36	29.91	0.48	1.72	43.67	20.63	3.60
GM3	22	48	29.29	0.44	1.74	43.74	21.11	3.68
GM4	26	24	28.41	0.41	1.75	44.94	20.89	3.60
GM5.1	26	36	28.58	0.39	1.76	44.38	21.16	3.73
GM5.2	26	36	28.91	0.42	1.74	44.07	21.16	3.70
GM5.3	26	36	28.78	0.44	1.73	43.99	21.31	3.74
GM5.4	26	36	28.95	0.41	1.74	44.17	20.98	3.75
GM6	26	48	28.49	0.40	1.72	44.21	21.43	3.74
GM7	30	24	29.33	0.42	1.62	44.01	20.99	3.61
GM8	30	36	29.03	0.41	1.71	43.88	21.28	3.70
GM9	30	48	29.61	0.44	1.68	43.59	21.02	3.66
Average±SD			28.97±0.48	0.42±0.02	1.72±0.04	44.20±0.52	21.03±0.25	3.66±0.09
R^2			0.74	0.39	0.75	0.87	0.74	0.86
Intercept			**28.769**[a]	**0.418**[a]	**1.743**[a]	**44.188**[a]	**21.157**[a]	**3.725**[a]
Temp			-0.043	-0.010	-0.032	-0.087	0.150	0.020
Duration			0.101	0.007	0.004	**-0.334**[b]	0.170	**0.050**[c]
Temp*Duration			0.010	-0.006	0.016	0.107	-0.106	-0.017
Temp*Temp			**0.768**[b]	0.023	-0.031	**-0.485**[b]	-0.209	**-0.064**[c]
Duration* Duration			-0.250	-0.013	-0.012	**0.315**[c]	0.001	-0.038

a: $p<0.0001$, b: $p<0.01$, c: $p<0.05$

In the case of amaranth grains, more fatty acid compounds were determined to be significant. Unlike finger millet, linoleic acid methyl ester was the main fraction with 45.05% in native grains. Germination increased this acid fraction up to 49.38% for case GM6. Oleic acid methyl ester and palmitic acid with 26.36% and 19.21% were the next main fractions. While oleic acid methyl ester decreased during germination, palmitic acid did not show any significant change. Eicosapentaenoic acid (C20:5N3) with 3.81% and stearic acid with 3.08% were the next important fractions. Eicosapentaenoic acid significantly reduced during germination at most for GM6 case, where 1.45% was obtained. Arachidic acid (C20:0), paullinic acid (C20:1), ginkgolic acid (C17:1), behenic acid (C22:0), margaric acid (C14:0), lignoceric acid (C24:0), and linolenic acid methyl ester were low fraction fatty acid compounds with 0.66%, 0.56%, 0.56%, 0.22%, 0.18%, 0.16%, and 0.16%, respectively. While most of these fatty acid components did not show a notable change during germination, paullinic acid drastically increased from its initial 0.56% value in native grains up to 2.1% in the GM6 case.

Table 5-5: Fatty acid compositions of amaranth grain for the designed germination treatments and the results of their corresponding ANOVA.

			C14:0	C16:0	C17:1	C18:0	C18:1N9C	C18:2N6C	C20:0	C20:1	C18:3N3	C22:0	C20:5N3	C24:0
GM0	Control		0.18	19.21	0.56	3.08	26.36	45.04	0.66	0.56	0.16	0.22	3.81	0.16
GM1	22	24	0.20	19.56	0.53	3.08	25.36	45.96	0.66	0.80	0.15	0.23	3.29	0.18
GM2	22	36	0.19	19.22	0.55	3.09	24.93	46.60	0.67	1.03	0.16	0.24	3.12	0.21
GM3	22	48	0.19	19.31	0.55	3.10	24.57	46.89	0.68	1.16	0.16	0.27	2.88	0.23
GM4	26	24	0.19	19.39	0.56	3.10	25.05	46.40	0.66	0.97	0.16	0.24	3.09	0.20
GM5.1	26	36	0.20	19.54	0.53	3.11	23.02	48.61	0.67	1.83	0.17	0.28	1.81	0.24
GM5.2	26	36	0.19	19.25	0.57	3.11	23.12	48.63	0.69	1.82	0.17	0.30	1.89	0.26
GM5.3	26	36	0.19	19.31	0.57	3.11	23.10	48.59	0.69	1.82	0.17	0.29	1.89	0.27
GM5.4	26	36	0.20	19.52	0.57	3.11	23.07	48.57	0.68	1.82	0.16	0.28	1.80	0.24
GM6	26	48	0.20	19.43	0.58	3.10	22.30	49.38	0.70	2.10	0.17	0.33	1.45	0.28
GM7	30	24	0.19	19.05	0.56	3.09	25.51	45.74	0.68	0.78	0.17	0.24	3.81	0.20
GM8	30	36	0.19	19.30	0.55	3.11	25.15	46.25	0.67	0.99	0.16	0.25	3.17	0.20
GM9	30	48	0.20	19.38	0.57	3.11	23.42	48.25	0.69	1.67	0.16	0.31	1.96	0.27
Average±SD			0.19±0.00	19.34±0.15	0.56±0.01	3.10±0.01	24.23±1.27	47.30±1.4	0.68±0.01	1.33±0.52	0.16±0.00	0.27±0.03	2.61±0.83	0.23±0.04
R^2			0.70	0.59	0.51	0.67	0.91	0.92	0.62	0.92	0.50	0.90	0.95	0.88
Intercept			**0.194**[a]	**0.058**[a]	**0.559**[a]	**3.108**[a]	**23.230**[a]	**48.436**[a]	**0.681**[a]	**1.757**[a]	**0.165**[a]	**0.285**[a]	**1.944**[a]	**0.247**[a]
Temp			0.000	0.052	0.009	0.005	-0.130	0.132	0.004	0.075	0.002	0.010	-0.059	0.009
Duration			0.003	0.052	0.009	0.006	**-0.936**[c]	**1.072**[c]	**0.013**[d]	**0.395**[c]	0.002	**0.034**[c]	**-0.650**[b]	**0.033**[c]
Temp*Duration			**0.004**[d]	0.063	0.000	0.002	-0.325	0.395	-0.004	0.132	-0.002	0.008	**-0.360**[d]	0.006
Temp*Temp			-0.002	0.078	-0.012	-0.010	**1.503**[c]	**-1.676**[c]	-0.008	**-0.621**[c]	-0.003	**-0.031**[c]	**1.007**[b]	**-0.034**[d]
Duration* Duration			0.002	0.078	0.008	-0.004	0.137	-0.216	0.002	-0.097	-0.002	0.004	0.129	0.001

(a: $p<0.0001$, b: $p<0.0005$, c: $p<0.005$, d: $p<0.05$)

5.7 Discussion

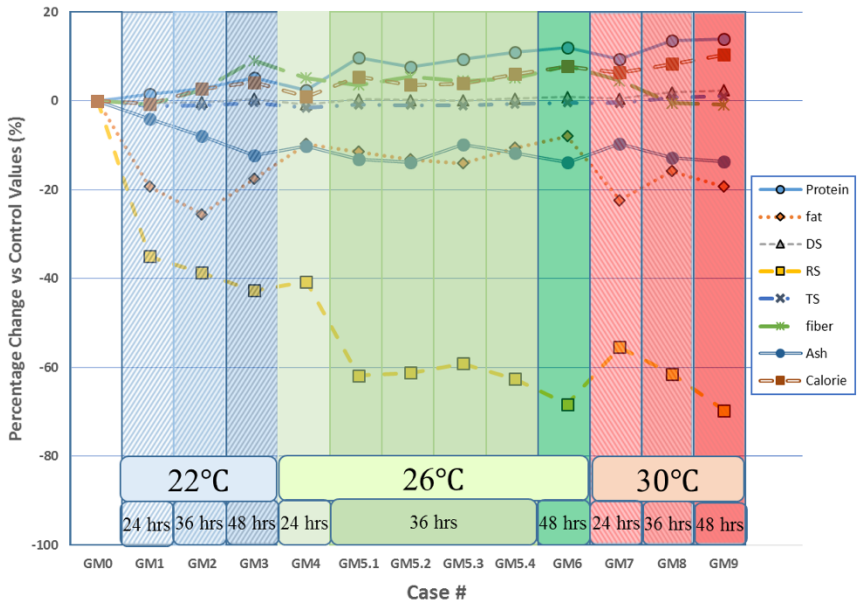
5.7.1 Digestive, resistant, and total starch

For a better comparison, the obtained averaged data for all germination treatments were compared with those of native samples, and the variation of each component (percentage) was evaluated. The results are graphically presented in Figure 5-2 (a) for finger millet and Figure 5-2 (b) for amaranth. Again for better clarity, a similar colourful categorization approach (blue for 22 °C green for 26 °C, and red for 30 °C, light colors for 24 hr, medium colors for 36 hr and dark colors for 48 hr) is employed.

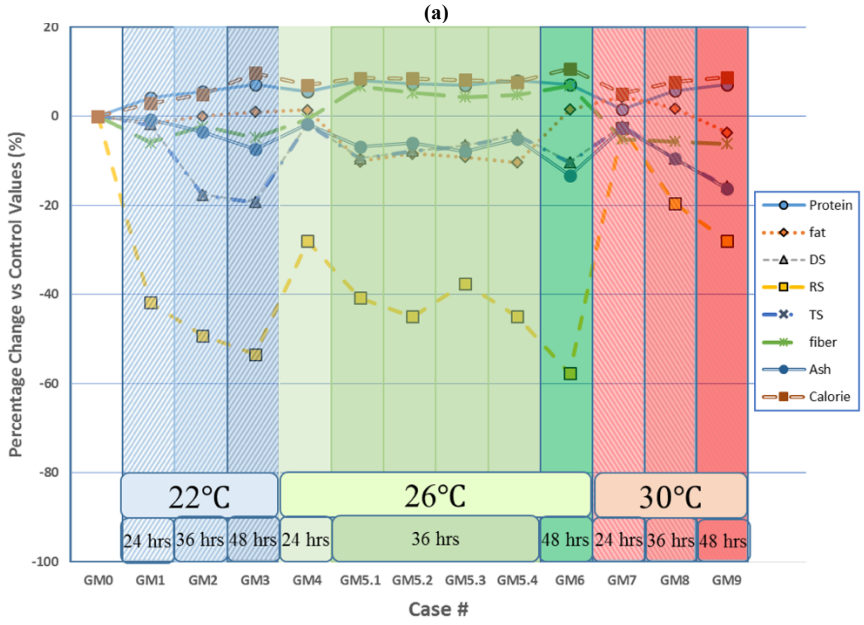
As was mentioned earlier, resistant starch notably changed during germination. Figure 5-2 (a) shows that an approximately 70% reduction in resistant starch content of finger millet was achieved for GM9 case, where grains were germinated for 48 hr at 30 °C. From Figure 5.2, it is clearly observed that this decrease is directly correlated with germination duration and temperature. Similarly, resistant starch is negatively correlated with the germination duration for amaranth grain. This means that one can expect continuous reduction of the resistant starch content as germination duration increases. Minimum resistant starch was observed for GM6 case (48 hr at 26 °C) with a notable 60% reduction.

Although in the performed germination treatments, a particular alteration was not observed in the digestible starch content of finger millet sprouts (Figure 5-2 (a)), a significant reduction was observed (Figure 5-2 (b)) for amaranth grain. For amaranth, digestible starch reduced from its original 63.26% for native grains down to 51.11% for GM3 case (germinated for 48 hr at 22 °C) showing a 19% reduction. As it was mentioned in section 5.6.1 and observed in Table 5-1, this reduction was negatively correlated with germination duration. Besides, a strong negative dependency on the quadratic term of temperature

declared the higher digestible starch values for grains germinated at 26 °C comparing to 22 and 30 °C.



(a)



(b)

Figure 5-2: The percentage change (%) of monitored quantities vs. their corresponding control values for the studied experimental design factor combinations (a) finger millet, (b) amaranth.

During germination, the amylase inhibitory activities decreased. This results into the migration of the amylase enzyme that is synthesized at the scutellum and aleurone layer of the starchy endosperm (Mangala et al., 1999). As a result, starch is partially hydrolized

(Elkhalifa & Bernhardt, 2010). In fact, the observed increase in the reducing and non-reducing sugars are usually associated with the hydrolysis of starch into its shorter polysaccharides chains (Okoth et al., 2011).

Malleshi et al. (1986) assessed the total starch content of native finger millet at 60%. They observed a decrease in this value during the malting process. Sripriya et al. (1997) reported a 12% decrease in the starch content of finger millet after 12 hr of soaking and 24 hr of germination at 30 °C. In another study, a 34% reduction in the total starch content of finger millet sprouts, that were steeped for 24 hr and germinated for 96 hr at 25 °C, was reported (Nirmala et al., 2000). Roopa and Premavalli (2008) reported a 40% reduction in the resistant starch content of finger millet grains soaked for 18 hr and germinated for 48 hr at ambient temperatures (18 to 33 °C). They evaluated the resistant starch fraction of native and germinated finger millet grains at 1% and 0.6%, respectively.

Capriles et al. (2008) reported the digestible, resistant, and total starch contents of native amaranth grains to be 57.50±0.30, 0.50±0.01, and 58.00±0.29 g/100 g db, respectively. Kanensi et al. (2011) observed continuous decrease in the starch content of amaranth sprouts as the germination time increased up to 72 hr. This was followed by a corresponding increase in the reducing and non-reducing sugar contents of amaranth sprouts by 13.1% and 17%, respectively. In another study, amaranth grain starch content continuously decreased during germination of the grains up to 192 hr from their initial 61% to the final 34% contents (Balasubramanian & Sadasivam, 1989).

5.7.2 Protein, fat, and fatty acids

For millet grain, the available protein content of native grains was assessed at 6.42% (Table 5-2). From Figure 5-2 (a), it was observed that as germination duration or temperature

increased the protein content increased. This was further supported by the outputs of the performed ANOVA study presented in Table 5-2, where the significant dependency of protein on duration and temperature was clear. Based on these data, germination of finger millet grains for 48 hr at 30 °C resulted into the maximum protein content (7.32%), showing a 17% increase compared to the native grains.

Amaranth had a higher protein content (13.76%) in its native form when comparing with finger millet (Table 5-2). Similarly, the germination process increased this value up to around 14.8% for sprouts germinated at 26 °C and for 36 hr. From Figure 5-2 (b), it was observed that the protein content was altered with both germination duration and temperature. While the dependency on duration was linear, there existed a significant and negative quadratic dependency between protein content and temperature. This resulted into having a maximum at the mid temperature (26 °C) for the designed germination process.

Despite having a wide range in reported protein contents for finger millet from 5 to 13%, the protein content in millet is usually estimated around 7% (Antony & Chandra, 1998; Ramachandra et al., 1978; Ravindran, 1992; Singh & Raghuvanshi, 2012). Mbithi-Mwikya et al. (2000b) observed an approximate 30% increase in the available protein content of finger millet after four days of germination at room temperature. The increase in protein content through germination has been reported by other authors as well (Parameswaran & Sadasivam, 1994; Swami et al., 2013).

Similarly for amaranth grain species, the majority of studies have shown an increase in protein content during germination (Colmenares de Ruiz & Bressani, 1990; Okoth et al., 2011; Paredes-Lopez & Mora-Escobedo, 1989). Crude protein and true protein contents of amaranth were stated to be increased by 41% and 22%, respectively after 72 hr of

germination at 35 °C (Paredes-Lopez & Mora-Escobedo, 1989). In another study, effects of germination duration on the protein content of three amaranth varieties were investigated, where after 72 hr of germination, slight enhancements were observed for *A. hypochondriacus* (16.1% to 16.8%), *A. cruentus* (14.9% to 15.1%), and *A. caudatus* (16.3% to 16.9%) (Colmenares de Ruiz & Bressani, 1990).

The observed increase in protein content is directly correlated with the reduction of starch content during germination. Within cereal grains, protein granules are surrounded by starchy cells. This structure restricts protein bio-availability of native grains. As it was mentioned, during germination, the amylolytic activity (α-amylase) increases. This enzyme, breaks down the starch granules (Elkhalifa & Bernhardt, 2010), and consequently increases the protein availability (Traoré et al., 2004).

From Figure 5-1 (a) it is observed that the fat content of finger millet slightly decreased during germination, while this reduction was lower for grains germinated at 26 °C and higher for those germinated at 22 and 30 °C. Thus, a negative quadratic temperature term in the constructed response surface (Table 5-2) was the outcome. In the case of amaranth (Figure 5-2 (b)), a reverse behavior was observed, where fat content was lower for the grains germinated at 26 °C when compared with those germinated at 22 and 30 °C. The observed reduction in lipid content was mainly due to the enhanced activity of the lipase enzyme and lipid metabolism during grain sprouting (Vasishtha & Srivastava, 2012).

The total fat of finger millet has been assessed to be around 1.3% (Obilana, 2003a). The low fat content of finger millet, which is usually associated with its small germ (McDonough et al., 2000) results into its superior storage capabilities due to limited lipid oxidation and associated lower tendency for rancidity.

The total fatty acids content of finger millet was reported at 0.87%, where oleic acid and palmitic acid were assessed to be the major non-essential fatty acid components with contents of 44% and 27%, respectively. Linoleic acid (C18:2N6) and alpha-linolenic acid (C18:3N3) contents were evaluated to be 20.2% and 3.74% respectively. The ratio of these two essential fatty acids was 5.4:1 which was within the FAO/WHO recommended range of 10:1 to 5:1 (World-Health-Organization, 1993). Mahadevappa et al. (1978) assessed the total lipids in seven cultivars of finger millet, where values in the range of 1.85 to 2.10% were obtained. Among the fatty acids, oleic acid (49 to 50%), palmitic acid (25 to 30%), and linoleic acid (8 to 12%) were the most important fractions. In another study on Nigerian finger millet, total fatty acid was assessed to be 1 to 2%, where oleic acid at 42% was the main fraction. After oleic acid, linoleic acid (25%), palmitic acid (21%), and linolenic acid (4%) were the other main fractions (Chandrashekar, 2010).

For amaranth, the oil content has been reported in the range of 5.83 to 7.13%, where linoleic acid was the main fraction with 33.52 to 43.88% followed by oleic acid (20.26 to 32.01%), palmitic acid (17.06 to 21.35%), and stearic acid (3.05 to 3.80%) (Berganza et al., 2003). In a study on 104 genotypes from 30 species of amaranth grain, the overall average oil content was assessed at 5.0%, ranging from 1.9 to 8.7% (He & Corke, 2003). The reported average contents of the three major fatty acids in amaranth grain, which are linoleic, oleic, and palmitic, were 44.6, 29.1, and 22.2%, respectively (He & Corke, 2003).

The obtained fatty acid fractions in the present study were aligned with the reported values in the literature. As an interesting conclusion, it was observed that linoleic acid (C18:2N6C), which is an essential fatty acid, was increased during the performed germination treatments for both finger millet and amaranth grains. This fatty acid may

convert to arachidonic acid, which is the most abundant fatty acids in the brain and is involved in infants' neurological development.

5.7.3 Fiber, ash and energy

According to Figure 5-2 (a) and (b), the fiber contents of both amaranth and millet grains increased for samples that were germinated at 26 °C, while lower values were obtained for those at 22 and 30 °C.

A decrease in the fiber content of cereals during the malting procedure has been reported (Desai, 2004). This was mostly associated to the cell wall degradation during sprouting processes. Most of the reports on the fiber content of finger millet and amaranth grains in the literature are based on crude fiber, dietary fiber, and soluble and insoluble portions of total dietary fiber contents.

For finger millet, despite reporting high dietary fiber values (19.1%), crude fiber is comparably low (3.6%) (Serna-Saldivar, 2010). In a study by Malleshi and Klopfenstein (1998), the malt and rootlets of finger millet grain were analyzed to establish the effects of germination duration (up to 96 hr) on the crude fiber and dietary fiber contents. While crude and dietary fiber contents increased from their initial value for the native grains (3.0 and 15.2%) up to 5.8 and 21.7% for the malts, a decrease was observed in the rootlets for the crude fiber from 13.4 down to 11.6% (Malleshi & Klopfenstein, 1998).

Tosri et al. (2001) reported a14.2% content for total dietary fiber in amaranth grain, where 8.1 and 6.1% were accounted for soluble and insoluble fractions, respectively. In a similar study, total, soluble and insoluble dietary fiber contents of two genotypes of amaranth grain were reported to be 14.5, 6.3, and 8.2% and 14.2, 6.1, and 8.1%, respectively

(Czerwiński et al., 2004). A more recent study on two varieties of *A. caudatus* grains, "Oscar Blanco" and "Centenario" claimed 13.8 and 16.4% of total dietary fiber, respectively (Repo-Carrasco-Valencia et al., 2009). In order to determine the effects of the germination process on the crude fiber content of amaranth grain, a comprehensive study on three varieties of amaranth grain, *caudatus*, *cruentus*, and *hypochondriacus* was performed (Colmenares de Ruiz & Bressani, 1990). For *A. caudatus*, crude fiber contents were assessed at 2.5% (db) for native and 2.4, 2.3, and 2.4% for sprouts after 24, 48, and 72 hr of germination. Similarly, for *A. cruentus*, these values were 2.5, 2.4, 2.6, and 2.5% and for *A. hypochondriacus*, 2.3, 2.9, 2.6, and 2.4% (Colmenares de Ruiz & Bressani, 1990). In another study, the crude fiber of amaranth grains after 10 hr of steeping and 24, 48, and 72 hr of germination was reported at 5, 4.4, and 4%, respectively (Okoth et al., 2011).

From Figure 5-2, it was observed that both germination duration and temperature notably influenced the reduction of ash content during germination. The reduction was mostly due to leakage of minerals, anti-nutrients, and other soluble compounds during the soaking and germination stages.

The ash content of amaranth grains, which were steeped for 10 hr and germinated for 24, 48, and 72 hr were reported to be 2, 1.8, and 1.3%, respectively (Okoth et al., 2011). Similarly, germination decreased the ash content in a study on three varieties of amaranth grain (Colmenares de Ruiz & Bressani, 1990). The authors showed that for *A. caudatus*, ash decreased from 2.8% in native grains down to 0.5% after 72 hr of germination. Similar reducing patterns were observed for *A. cruentus* (3.0 to 0.5%) and *A. hypochondriacus* (3.2 to 0.4%).

Generally, the energy content of the malted grains increased during germination with a direct dependency on the germination duration. Effect of temperature on the assessed energy was important in a linear and quadratic fashion for millet and amaranth sprouts, respectively.

5.8 Conclusion

In the present study, malting of finger millet and amaranth grains improved starch digestibility and protein availability, which are critical nutrient components for infants' growth. It also enhanced energy content and essential fatty acids, especially the linoleic acid content which is required for infants' optimal mental development. As for the main nutrient components, protein increased by 17% in finger millet after germinating for 48 hr at 30 °C, while an 8% increase in amaranth protein content was observed for germinated grains at 26 °C for 36 hr. Linoleic acid was maximized when amaranth and finger millet grains were germinated at 26 °C for 48 hr. Resistant starch decreased by 70% in finger millet sprouts germinated for 48 hr at 30 °C. Similarly, 60% reduction in resistant starch content was observed for amaranth grain, germinated at 26 °C and for 48 hr. Energy content roughly increased by 10% after germinating for 48 hr in both grains (maximized at 26 and 30 °C for amaranth and finger millet, respectively). Based on the obtained data, germination of amaranth and finger millet for 48 hr was optimized. As for temperature, while 30 °C should be preferably used for finger millet germination, this value was slightly lower (26 °C) for amaranth grain.

Connecting Statement to Chapter 6

In **Chapter 5,** the nutritional profile of finger millet and amaranth grains were optimized through a designed malting experiment for infants' weaning food application. It was concluded that germination of these grains for 48 hr resulted into the best nutrient combinations. As for the temperature of these treatments, 30 °C and 26 °C were suggested for finger millet and amaranth, respectively. As it was stated in **Chapter 1,** the selected grains, especially finger millet, have appreciable amounts of phenolic compounds, with numerous health benefits. These components act as natural antioxidant in the body and prevent many cell malfunctions, especially during infancy, when the body organs are developing. In the present chapter, effects of the performed malting stage on the total phenol content and antioxidant activities of the processed flours has been investigated. The current chapter is written in the manuscript style and is published in **Journal of Food Research** as follows,

Malting process effects on antioxidant activity of finger millet and amaranth grains

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Contributions made by the different authors are as follows;

- The first author, **Sara Najdi Hejazi**, is the Ph.D. student, who prepared the experimental design, conducted the laboratory work, analyzed the results, and prepared the manuscript.

- The second author, **Valérie Orsat**, is the thesis supervisor, who initiated and stimulated ideas, provided the technical advice, suggestions, experiment facilities and guided the first author in writing, correcting, and preparing the manuscript throughout the study.

Furthermore, parts of the obtained data and performed analyses in **Chapter 6** were presented in the following conference presentation;

1. Sara Najdi Hejazi and Valérie Orsat, "**Weaning Infant Foods from Minor Millets-Effect of Germination on Total Phenol Contents and Anti-Nutrient Compounds of Finger Millet and Amaranth"** American Society of Agricultural and Biological Engineers, Emerging Technologies in Food Processing Session, July 13-16, 2014, Montreal, Canada.

6 Malting process effects on antioxidant activity of finger millet and amaranth grains

6.1 Abstract

Finger millet (*Eleusine coracana*) and amaranth (*Amaranthus caudatus*) are two nutritious and gluten-free grains with important contents of phenolic compounds. Phenols are known as the main source of natural antioxidants, with numerous health benefits. Being rich in phenol makes these grains good choices for the functional food industry. In this study, the effects of malting/germination factors, duration and temperature, on the phenolic content and antioxidant activities of these grains were thoroughly investigated and optimized. Based on a central composite design, the grains were germinated for 24, 36, and 48 hr at 22, 26, and 30 °C. Both temperature and duration factors were found to be significantly influential on the monitored quantities. While malting of amaranth grains for 48 hr at 26 °C increased the total phenol content by four times, in the case of millet, a 25% reduction was observed. Linear correlations between the phenol content and antioxidant activities in terms of DPPH and ABTS scavenging activities were observed.

Keywords: *Amaranth, finger millet, malting/germination process, phenol content, ABTS, DPPH, colorimetric properties*

6.2 Introduction

Even though oxidation reactions are important for cellular metabolism, they may threaten cells' normal functions, especially in the case of chain reactions (Banerjee et al., 2012). When a chain oxidation reaction initiates, excessive amounts of free radicals are produced that may cause serious damage or death to the cell. These excessive free radicals may cause oxidative stress (Hegde et al., 2005). Oxidative stress plays a significant role in many human body malfunctions, most importantly coronary heart disease and cancer (Najdi Hejazi, 2012; Saleh et al., 2013).

Antioxidants are molecules that prevent oxidation of other components by being oxidized themselves (Viswanath et al., 2009). In fact, antioxidants are known as reducing agents that terminate the chain reactions by removing the free radical intermediates and inhibiting excessive oxidation reactions. In the food industry, antioxidants act as natural preservatives in food products by preventing degradation of their lipid and protein contents (Venskutonis & Kraujalis, 2013). In cereal grains, polyphenolic compounds are assumed to be the main source of antioxidants (Towo et al., 2003). Naturally, polyphenols are responsible in the defense of plants against ultraviolet radiations and pathogens aggression (Banerjee et al., 2012). These compounds are referred to as the secondary metabolites of the plant cells. Epidemiological studies have proven that long term consumption of whole cereal grains, which contain appreciable amounts of phytochemicals, may significantly reduce oxidative stress in the body and keep the desired balance between oxidants and antioxidants levels (Hegde et al., 2005).

Recently, with increasing concerns about worldwide food security, increasing attention is given on the cultivation of drought-resistant crops that can be grown in arid and semi-arid

regions (Malleshi & Desikachar, 1986a). Among them, millet and amaranth grains are gaining significant interest and appreciation. The facts that these grains have good sensory qualities, a short growing season, and appropriate nutrient profiles, besides being gluten-free, are the main factors for these attentions (Belton & Taylor, 2002a; Charalampopoulos et al., 2002). The high phenolic contents of these seeds could represent a good source of antioxidant, particularly in the arid regions, where other commercial crops cannot be grown (de la Rosa et al., 2009; Shobana et al., 2012). Among a vast variety of amaranth grains, three of them are of greatest importance, *Amaranthus caudatus, Amaranthus cruentus,* and *Amaranthus hypochondriacus* (Venskutonis & Kraujalis, 2013). Millets are categorized into two groups; major millets, which include species that are most widely cultivated, and minor millets (Issoufou et al., 2013). While sorghum and pearl millet belong to the first category, finger millet, foxtail millet, little millet, barnyard millet, proso millet, and kodo millet are mostly recognized as minor millets (Obilana & Manyasa, 2002). In this study, total phenolic content and antioxidant activities of Finger millet (*Eleusine coracana*) and Amaranth (*Amaranthus caudatus*) were investigated. In addition, the effects of malting/germination process parameters on the resulting phenolic content and antioxidant activities were explored.

A reported study performed on two varieties of *caudatus* amaranth, *centenario* and *oscar blanco*, demonstrated that their total phenolic contents were 98.7 and 112.9 mg/100 g, respectively, using Gallic acid as the reference (Repo-Carrasco-Valencia et al., 2009). In addition, the authors reported that antioxidant activities using DPPH method were 410 and 398.1 µMol Trolox/g for *centenario* and *oscar blanco* varieties respectively. For these varieties, the ABTS activities were reported as 827.6 and 670.1 µMol Trolox/g, respectively.

Pasko et al. (2009) studied the phenolic contents and antioxidant activity of two varieties, Aztec and Rawa, of *cruentus* amaranth. They demonstrated that for the native seeds, average total phenols were 2.95 and 3.0 mg GAE/kg, average DPPH activities were 4.42 and 3.15 mMol Trolox/kg, and average ABTS activities were 12.71 and 11.42 mMol Trolox/kg for Aztec and Rawa seeds, respectively. Furthermore, in the performed study, the effects of germination duration on these quantities were also investigated. Grains were germinated at room temperature between four to seven days in daylight or darkness. It was found that the sprouts' antioxidant activities depended on the growth duration, where maximum values were observed at the 4^{th} day. A significant increase in the DPPH and ABTS scavenging activities and a slight reduction in the total phenolic content were also reported. In addition, the effects of light on the antioxidant activities during germination were claimed to be significant. For Rawa seed, ABTS was in the range of 112.9 to 151.3 mMol Trolox/kg for the grains that were germinated in daylight and 78.8 to 176.1 for those germinated in darkness. ABTS values for Aztec were in the range of 133.1 to 222.1 and 99.5 to 17.5 mMol Trolox/kg for sprouts grown in daylight and darkness, respectively. Finally, significant linear correlations between ABTS and DPPH radical scavenging activities (R^2=0.87), as well as between total phenol content and ABTS and DPPH (R^2=0.98) were obtained (Pasko et al., 2009). However, the present authors believe that these conclusions could be argued, if data on the native grains were included. This is due to the fact that the phenol content did not significantly change after germination, while the antioxidant activities remarkably (more than 10 times) increased throughout the germination process.

In another recent study, effect of germination on the phenolic content and DPPH radical scavenging activity was investigated for *cruentus* amaranth (Alvarez-Jubete et al., 2010b).

Grains were germinated for 98 hr at 10 °C. Total phenol content increased from 21.2 up to 82.2 mg GAE/100 g after germination. A slight decrease in the DPPH activities from 28.4 to 27.1 mg Trolox/100 g was observed.

For finger millet, Siwela et al. (2007) reported a low phenolic content for white varieties with values below 0.09 mg GAE/100 mg. Comparably higher amounts were obtained for brown finger millets, ranging from 0.34 to 1.84 mg GAE/100 mg. These data were aligned with findings of Ramachandra et al. (1977), who detected phenolic contents of 0.06 to 0.1 mg/100 mg, in Chlorogenic acid equivalent (CGA), for white and 0.34 to 2.44 mg/100 mg for brown varieties. A similar seed color dependency was observed by Chethan and Malleshi (2007), who claimed polyphenol content of finger millet ranging from 0.3 to 0.5 % (GAE) in white, versus 1.2 to 2.3 % in brown seeds. Antioxidant activity of white finger millets were reported to be 37.5 to 75.9 mM Trolox/kg, while these values for the dark varieties increased up to 117.1 to 195.4 (Siwela et al., 2007). Sreeramulu et al. (2009) evaluated the DPPH activity of finger millet at 1.73 mg Trolox/g. They reported 373.15± 70.07 mg GAE/100 g for the phenolic content of finger millet (Sreeramulu et al., 2009).

Towo et al. (2003) investigated the effect of a germination process on the total phenolic content of finger millet. Grains were germinated in darkness at 25 °C. Germination duration was not specified in their paper. Total phenol content decreased from 4.2 to 3.3 mg/g in Catechin Equivalents (CE). Sripriya et al. (1996) reported a similar reduction in phenolic content during germination of finger millet. In their study, total phenol decreased from 102 to 67 mg CGA/100 g. Again, germination parameters including duration were not specified.

The observed diversity in the phenolic contents and antioxidant properties of amaranth and millet grains in the literature is due to differences in employed extraction approaches and

sample preparation. Besides, having different evaluation procedures as well as different reference calibrated curves in expressing the results are other main factors for these discrepancies.

It has been shown that many food processing steps (soaking, malting, etc.) may alter the phenolic contents of cereal and pseudo-cereal grains (Kunyanga et al., 2012; Queiroz et al., 2009; Saleh et al., 2013; Shobana et al., 2012). One of the simplest and widely employed pre-treatments is the malting/germination process, where grains are soaked, germinated, dried, and ground to a flour (Najdi Hejazi et al., 2015; Swami et al., 2013; Traoré et al., 2004). In the present study, effects of malting/germination parameters, duration and temperature, on the total phenol content and antioxidant activities of finger millet and amaranth grains were investigated.

6.3 Material and methods

6.3.1 Materials

Brown finger millet (*Eleusine coracana*) was procured from University of Agricultural Sciences (Dharwad, India) and amaranth (*Amaranthus caudatus* L. (love-lies-bleeding)) seeds were purchased from a local market (Bulk Barn store, Qc., Canada).

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol was obtained from Commercial Alcohols (Industrial and Beverage Alcohol Division of Green-field Ethanol Inc., Ontario, Canada). Double distilled

water (ddH_2O) was prepared using Simplicity TM water purification system (Millipore, USA). All other reagents were of analytical and HPLC grades (Teow et al., 2007).

6.3.2 Malting/Germination process

Finger millet and amaranth seeds were cleaned thoroughly with sterile water and surface-air dried by airflow. The seeds were steeped (seed/water ratio of 1:5 (w/v)) overnight, and sprouted at 22, 26, and 30 °C in a B.O.D incubator (Benchmark Incu-Shaker Mini) for 24, 36, and 48 hr. Germinated seeds were freeze-dried (FreeZone® 2.5 l Freeze Dry System, Labonco Corporation, MO, USA) for a week to reach constant dry weight. Native and germinated seeds were ground in an electric grinder (Bodum 10903, PRC, Intertek, USA) for further assays. Samples were stored in hermetic plastic containers at 4 °C.

6.3.3 Statistical analysis

The experimental design used in the present study was a Central Composite Design (CCD) with two independent factors, germination duration and temperature, each at three levels, (24, 36, 48 hours) and (22, 26, 30 °C). All the experiments were performed in triplicate and the data were presented as mean align with their standard deviations. This design had 4 factorial, 4 axial, and 4 central points. For analyzing the data, JMP software version 11 (SAS Institute Inc., Cary, NC, USA) was used. The linear, quadratic, and combined effects of each factor were investigated using ANOVA and regression models, and expressed as follows;

$$Y = \text{Intercept} + \beta_1 \times \text{Time} + \beta_2 \times \text{Temp} + \beta_{11} \times \text{Time}^2 + \beta_{22} \times \text{Temp}^2 + \beta_{12} \times \text{Time} \times \text{Temp} \quad (6\text{-}1)$$

In this equation, β_1 and β_2 represented the regression coefficients of the linear, β_{11} and β_{22} were those of the quadratic, and β_{12} indicated the interactive or bilinear effects. Time

and Temp represented the deviation of independent germination variables, germination duration and temperature with respect to their centroids values, 36 hr and 26 °C, respectively.

6.4 Dry matter

Dry matters of flours were determined using AACC Method 44 – 15.02 (AACC, 1999).

6.4.1 Bioactive compounds and antioxidant activity determination

To determine the total phenol content and antioxidant activities, the phenolic compounds were initially extracted and their radical scavenging activities using ABTS and DPPH approaches were evaluated as follows:

6.4.1.1 Phenolic compounds extraction

Methanolic crude extracts were obtained from native and malted samples (Makkar, 2003). Briefly, 100 mg of fine flour sample was weighed in 1.5 ml eppendorf and 900 µL of methanol (90%) was added. The mixture was sonicated in a dark cold room for 30 min, and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was recovered and the pellet was re-centrifuged with addition of 600 µL methanol (90%) under the same conditions. Finally, the two supernatants were pooled and used for further total phenolic content and antioxidant scavenging activity assays.

6.4.1.2 Total phenol content

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Initially, 2 ml ddH_2O was added to 100 µL of methanolic sample extract. Subsequently, 200 µL of Folin-Ciocalteu reagent (2N) was added to the mixture with vigorous vortexing. After 30 min of incubation in the dark at room temperature, 1 ml of aqueous sodium carbonate solution (7.5%) was added and vortexed. After one hour of

incubation at ambient temperature, absorbance of samples was read at 765 nm (Ultraspec1000, Amersham Pharmacia Biotech, NJ, USA) against methanol as the blank. Using chlorogenic acid for the standard curve, total phenol content was expressed in mg CGA/100g db.

6.4.1.3 DPPH antioxidant scavenging activity

The approach to assess antioxidant activity using the DPPH free radical scavenging assay was adapted from the method of Martinez-Valverda et al. (2002). To perform this assay, a fresh DPPH stock solution (1mM) was made and appropriately diluted with absolute methanol to reach into an absorbance range of 0.5 to 0.9 unit. Briefly, 1.5 ml of the prepared DPPH solution was added to 100 µL of sample extracts, vortexed and incubated at room temperature for 30 min. The absorbance of the resulting solution was read at 517 nm against air as the blank. The free radical scavenging activity was estimated using a standard curve of Trolox in different concentrations (0-500 mM) with R^2=0.992. Additional dilution was required if the absorbance was over the linear range of the standard. In this regard, for the finger millet samples, a dilution factor of 10 was required, while no additional dilution was needed for amaranth samples. The final results were expressed as mg Trolox equivalent per 100 g on a dry basis (mg TE/100 g db).

6.4.1.4 ABTS antioxidant scavenging activity

ABTS free radical scavenging was measured using the method of Re et al. (1999) after some modifications. Initially, an ABTS (7 mM) stock solution and a potassium persulfate (2.45 mM) solution were prepared. The working reagent was obtained by mixing the two stock solutions in equal quantities and left to be incubated in the dark for 12 hr at room temperature. The procedure resulted into the production of radical cations of ABTS (ABT^+).

ABTS radical solution was diluted with 95% ethanol to obtain an absorbance of 0.7 $\pm$ 0.05 unit at 734 nm.

To assess the ABTS activities, 1.2 ml of the prepared working solution was added to 100 µL of the sample extracts. The absorbance of the mixture was determined within 1 to 3 min at 734 nm against air as the blank. For finger millet, a dilution factor of 10 was used to fit the absorbance within the standard curve. This dilution was not required for amaranth samples. The antioxidant activity was estimated using standard curve of Trolox in different concentrations (0-500 mM with R^2=0.99) and results were reported as mg TE/ 100g db.

6.4.2 Color determination

Colorimetric properties of native and germinated samples were assessed using CIE L*a*b* (CIELAB) approach (Leon et al., 2006) by chromameter (Model- CR300, Konica-Minolta®, USA). The measurement was done by covering a 5 g sample of flour with plastic food wrap with gentle spreading so that the flour was fitting perfectly the diameter of the chromameter's testing window. The flour sample was placed on a black material background to prevent unwanted interference from ambient light and scattering of light from the source.

The values for L* (0 = black, 100 = white), a* (+ values = redness, - values = green), and b* (+ values = yellowness, -values=blueness) were obtained for each germination treatment. Total color difference ($\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$) is evaluated versus the control sample (native grain).

6.5 Results

The obtained results are presented for amaranth and finger millet in the following subsections.

6.5.1 Amaranth

Total phenol contents were assessed for the selected design germination combinations as described in section 6.4.1.2 and are presented in Table 6-1. From the table, it was observed that the germination process significantly increased the phenolic compounds in amaranth sprouts when comparing to the initial value (71.55 ± 1.76 mg CGA/ 100 g db) of native grains (GM0). A four-time increase was observed for some of the germination treatments (e.g. GM5 cases). A similar increase (from initial 21.2 to final 82.2 mg GAE/100 g) was reported by Alvarez-Jubete et al. (2010b) after 96 hr of germination at 10 °C. DPPH and ABTS scavenging activities for the studied cases are presented in Table 6-1 as well. Again, similar to the phenol content, an increasing trend in the antioxidant activities throughout germination was observed.

Table 6-1: Total phenol (mg CGA/100 g db) content and DPPH (mg TE/100 g db), and ABTS (mg TE/100 g db) activities of amaranth grain for the tested germination treatments.

Exp. #	Germination factors and their levels		Total Phenol	DPPH	ABTS
	Temperature	Duration	(mg CGA/100 g db)	mg Trolox/100 g db	mg Trolox/100 g db
GM0	Control		71.55 ± 1.76	43.16 ± 1.10	52.27 ± 2.13
GM1	22	24	126.80 ± 3.59	56.71 ± 4.39	90.67 ± 1.79
GM2	22	36	187.54 ± 14.00	59.84 ± 2.77	78.18 ± 3.99
GM3	22	48	220.56 ± 7.47	75.21 ± 4.19	117.23 ± 8.51
GM4	26	24	144.01 ± 1.47	70.11 ± 4.27	91.97 ± 3.74
GM5.1	26	36	262.52 ± 9.53	108.02 ± 6.24	129.82 ± 4.51
GM5.2	26	36	264.68 ± 0.75	108.62 ± 6.30	118.84 ± 7.06
GM5.3	26	36	259.82 ± 16.00	109.00 ± 5.30	121.45 ± 8.21
GM5.4	26	36	261.75 ± 7.17	110.67 ± 1.30	118.94 ± 5.60
GM6	26	48	250.01 ± 5.89	91.89 ± 4.82	127.63 ± 8.92
GM7	30	24	118.82 ± 0.67	60.39 ± 3.15	80.96 ± 1.28
GM8	30	36	160.84 ± 1.97	69.18 ± 3.40	90.88 ± 3.15
GM9	30	48	269.15 ± 0.25	107.74 ± 2.37	126.07 ± 9.22

The obtained phenol and antioxidant activities are graphically presented in Figure 6-1. From this figure, the following conclusions may be drawn. Firstly, the germination process increased the bulk phenol content when comparing with the control sample (GM0). Secondly, the change in phenol content was a strong function of the germination duration. Thirdly, since

there was a phenol content increase followed by a decrease in the averaged phenol contents as a function of the germination temperature, it confirmed the second order dependency of the malting temperature. Lastly, DPPH and ABTS activities followed a similar trend as the total phenol content indicating strong correlations between these variables.

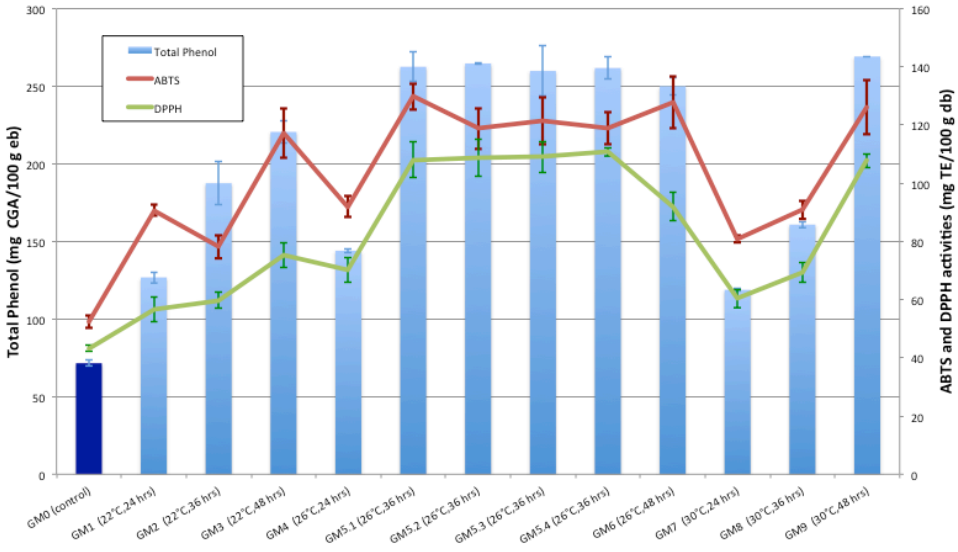


Figure 6-1: Total phenol, ABTS, and DPPH activities of amaranth grain for the selected design factor combinations.

Table 6-2 presents reported data from the literature for the studied parameters of phenol content, ABTS, and DPPH antioxidant activities. For comparison, all the data were converted to be presented in mg/100g. Furthermore, data were categorized based on the different employed calibration curves; Gallic acid, Chlorogenic acid, and Trolox equivalents. Excluding a few exemptions, our results were aligned with other reported data. Having different phenolic extraction approaches and different reading durations for ABTS and DPPH assays significantly influenced the results, which was clearly observed from the reported data.

The obtained phenolic contents were analyzed using response surface methodology. Table 6-3 presents a summary of the obtained results for the performed calculations. The

proposed response surface based on the selected design factors, X1 = (temperature – 26) and X2 = (duration – 36) was obtained as follows,

$$TP = 249.7216 + 2.3183\ X1 + 58.3483\ X2 - 50.59X_1^2 - 27.77\ X_2^2 + 14.1425\ X1X2 \quad (6\text{-}2)$$

Table 6-2: Total phenol content, DPPH, and ABTS antioxidant activities (mg/100 g db) of amaranth grain.

Amaranth Variety	Total Phenol (mg/100 g)	DPPH (mg/100 g)	ABTS (mg/100 g)
	Gallic acid equivalent	**Trolox equivalent**	**Trolox equivalent**
A. caudatus centenario (Repo-Carrasco-Valencia et al., 2009)	98.7	10261	20698
A. caudatus Oscar Blanco (Repo-Carrasco-Valencia et al., 2009)	112.9	9961	16772
A. cruentus (raw) (Alvarez-Jubete et al., 2010b)	21.2 ± 2.3	28.4 ± 1.3	-
A. cruentus (germinated)(Alvarez-Jubete et al., 2010b)	82.2 ± 4.6	27.1 ± 2.7	-
A. caudatus (Klimczak et al., 2002)	39.17	-	-
A. paniculatus (Klimczak et al., 2002)	56.12	-	-
Amaranth (Mošovská et al., 2010)	104.1 ± 2.2	-	-
A. cruentus var. Aztec (raw) (Pasko et al., 2009)	295 ± 7	110.6 ± 12.5	302.85 ± 27.53
A. cruentus var. Aztec (sprout) (Pasko et al., 2009)	160 to 300	-	1972 to 4407
A. cruentus var. Rawa (raw) (Pasko et al., 2009)	300 ± 42	78.8 ± 7.5	285.83 ± 30.03
A. cruentus var. Rawa (sprout) (Pasko et al., 2009)	150 to 250	-	2490 to 5560
A. cruentus var. Aztec seeds (Pasko et al., 2007)	-	110.6 ± 12.0	321.37 ± 23.03
A. cruentus var. Rawa seeds (Pasko et al., 2007)	-	78.8 ± 6	290.59 ± 16.27
Amaranth (Czerwiński et al., 2004)	14.72 to 14.91	-	-
Amaranth (Chlopicka et al., 2012)	271 ± 1	90.1 ± 8.5	-
Amaranth (Queiroz et al., 2009)	3170	-	-
		Gallic acid equivalent	
Amaranth (Asao & Watanabe, 2010)	51	22600	-
		Percentage basis	
A. hypochondriacus (López et al., 2011)	57.1 ± 1.0	86.93 ± 1.4%	-
	Chlorogenic acid equivalent		
A. cruentus (Kunyanga et al., 2012)	1080	84.67 ± 1.18%	-
		DPPH	
A. cruentus (Ogrodowska et al., 2012)	27.26 to 61.53	436.11 to 604.49	-
	Tannic acid equivalent		
A. cruentus (raw) (Gamel et al., 2006b)	516 to 524	-	-
A. cruentus (germinated) (Gamel et al., 2006b)	368 to 420	-	-

The analysis stated that germination duration and square of germination temperature were significant terms with $p<0.005$ and $p<0.05$, respectively. A positive dependency of the obtained response surface on the duration indicates that the phenol content increases as germination time increases. Furthermore, having a negative coefficient for the quadratic

term, X_1^2, means that a maximum should be expected in the constructed response surface (Table 6-3).

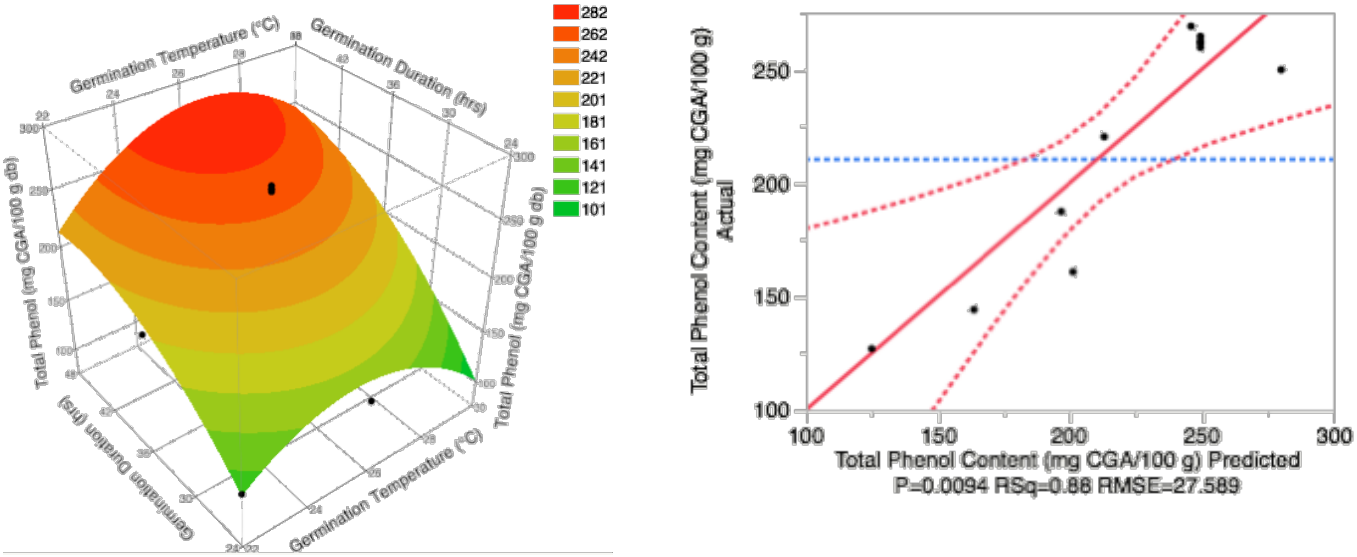
Table 6-3: Summary of the ANOVA of the responses for total phenol content and the corresponding parameter estimates of the different terms for amaranth grain.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	249.7216	12.592	19.83	**<.0001**
Temperature (X_1)	2.3183	11.2632	0.21	0.8437
Duration (X_2)	**58.3483**	11.26312	5.18	**0.0021**
Duration×Temperature (X_1X_2)	14.1425	13.7945	1.03	0.3448
Temperature×Temperature (X_1^2)	**-50.59**	16.8948	-2.99	**0.0242**
Duration×Duration (X_2^2)	-27.77	16.8948	-1.64	0.1513

The obtained response surface (equation (6-2)) is graphically plotted in Figure 6-2(a). As can be pointed out in Figure 6.2, the surface has a maximum at 26.7 °C and 49.14 hr. The predicted maximum value is 281.88 mg CGA/100 g db. This means that based on the conducted germination treatments and experiments, if it is desired to maximize the phenolic content and consequently its potential for higher antioxidant activity of selected amaranth grains, the grains should be germinated approximately for 48 hr at 26 °C. This conclusion is based on the performed ANOVA study and constructed response surface. However, based on the obtained raw experimental data (Table 6-1), the best phenolic content was observed for seeds germinating for 36 hr at 26 °C. The relative difference is below 5% and may be neglected. The leverage plot (Figure 6-2 (b)) emphasized on the appropriate prediction and interpolation of the experimental data by the proposed response surface. Since the confidence curves (dashed-line curves) crossed the horizontal line, the correlation of design factors was significant ($p<0.05$).

Similar procedures were performed for the DPPH and ABTS activities. The response surfaces were constructed and the linear, quadratic, and bi-linear effects of the germination

factors were analyzed. The results are presented in Table 6-4. For all quantities, germination duration (Time) and square of germination temperature (Temp×Temp) were significant.



(a) (b)

Figure 6-2: (a) The response surface for the total phenol content (mg CGA/100 g db) of amaranth grain and (b) the leverage plot based on the ANOVA.

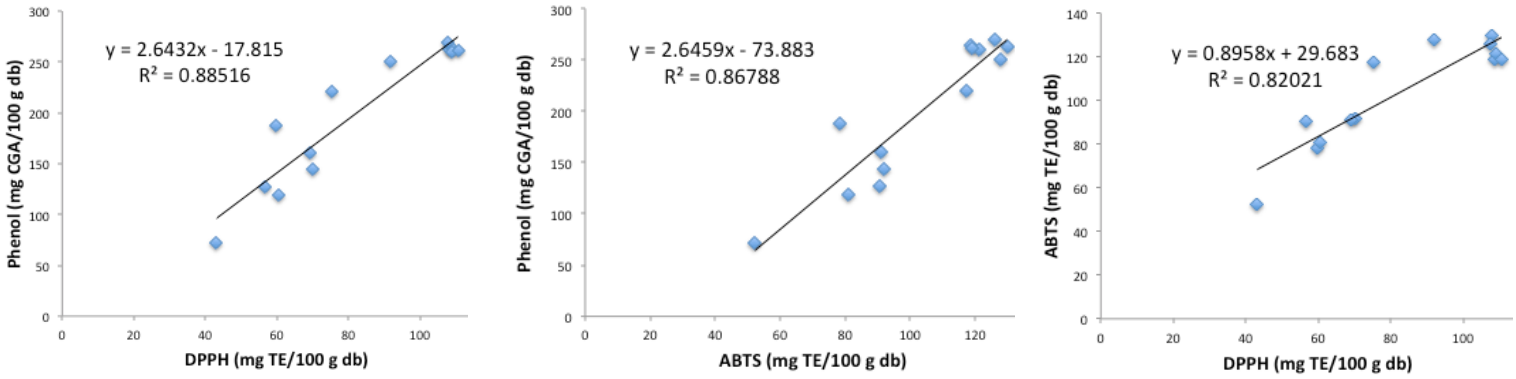
Table 6-4: Summary of the ANOVA for total phenol, DPPH and ABTS of amaranth grain.

	Total Phenol	DPPH	ABTS
Intercept	**249.7216**[a]	**102.6475**[a]	**116.985**[a]
Temp	2.3183	7.5916	1.9716
Time	**58.3483**[d]	**14.605**[f]	**17.8883**[c]
Time×Temp	14.1425	7.2125	4.6375
Temp×Temp	**-50.59**[f]	**-25.2775**[f]	**-21.9**[f]
Time×Time	-27.77	-8.7875	3.37
R^2	0.881	0.806	0.805
R^2adj	0.783	0.645	0.643
RMS	27.59	13.31	11.59

a: p<0.0001, b: p <0.0005, c: p<0.001, d: p<0.005, e: p<0.01, f: p<0.05.

As it was previously stated and clearly observed from Figure 6-1 and Table 6-4, ABTS and DPPH were following similar patterns as for total phenol content. This suggests the presence of a linear correlation between these quantities. Existence of this correlation has been reported in the literature (Chlopicka et al., 2012; Pasko et al., 2007; Pasko et al., 2009). In order to find any correlation, the total phenol versus DPPH and ABTS activities were plotted as scattered data (Figure 6-3). Unlike the data of Pasko et al. (2009), the results of

native seeds were also included. Appropriate linear correlations with acceptable R^2 values were observed.



(a) (b) (c)

Figure 6-3: Linear regression curves representing the linear correlations between total phenol content and the antioxidant activities, DPPH and ABTS (a) phenol vs. DPPH (b) phenol vs. ABTS, and (c) ABTS vs. DPPH of amaranth seeds.

Colorimetric properties of the native and germinated amaranth grains are presented in Table 6-5. A significant color change (ΔE values) was observed in the amaranth grain during germination, where sprouts' color gradually changed to red. This is clearly observed from the increased a* values. The peak of color change occurs in the case of GM6, where grains were germinated for 48 hr at 26 °C. This is the case in which phenol contents and antioxidant activities reached their maximum.

6.5.2 Finger millet

A similar experimental procedure and analyses were performed for the finger millet grains. The obtained data are numerically presented in Table 6-6 and graphically in Figure 6-4. A clear observation from these data was that the germination process decreased the total phenolic content. The highest reduction was observed for the grains germinated at 26 °C for 48 hr (25% reduction in total phenol content). In addition, unlike germination duration, temperature was not significantly influential.

Table 6-5: Colorimetric properties of native and germinated amaranth grain using CIELab approach.

Exp. #	Germination factors and their levels		L*	a*	b*	ΔE
	Temperature	Duration				
GM0	**Control**		86.98 ± 0.53	0.91 ± 0.08	12.95 ± 0.17	0.00 ± 0.56
GM1	**22**	**24**	89.44 ± 0.31	0.61 ± 0.05	11.76 ± 0.12	2.75 ± 0.34
GM2	**22**	**36**	88.16 ± 0.59	1.64 ± 0.07	11.75 ± 0.42	1.84 ± 0.73
GM3	**22**	**48**	88.68 ± 0.51	1.19 ± 0.06	12.20 ± 0.33	1.88 ± 0.62
GM4	**26**	**24**	88.38 ± 0.33	0.83 ± 0.08	12.15 ± 0.16	1.61 ± 0.37
GM5.1	**26**	**36**	86.19 ± 0.45	3.28 ± 0.06	11.21 ± 0.11	3.04 ± 0.46
GM5.2	**26**	**36**	86.60 ± 0.75	3.19 ± 0.06	11.05 ± 0.17	2.99 ± 0.66
GM5.3	**26**	**36**	86.04 ± 0.64	3.32 ± 0.17	11.27 ± 0.49	3.08 ± 0.82
GM5.4	**26**	**36**	86.54 ± 0.64	3.18 ± 0.08	11.16 ± 0.17	2.92 ± 0.52
GM6	**26**	**48**	83.78 ± 0.70	4.34 ± 0.12	11.63 ± 0.44	4.87 ± 0.83
GM7	**30**	**24**	88.59 ± 0.43	0.54 ± 0.09	12.15 ± 0.33	1.84 ± 0.54
GM8	**30**	**36**	88.58 ± 0.61	0.97 ± 0.05	12.21 ± 0.23	1.77 ± 0.66
GM9	**30**	**48**	85.81 ± 0.77	1.99 ± 0.09	13.75 ± 0.27	1.78 ± 0.82

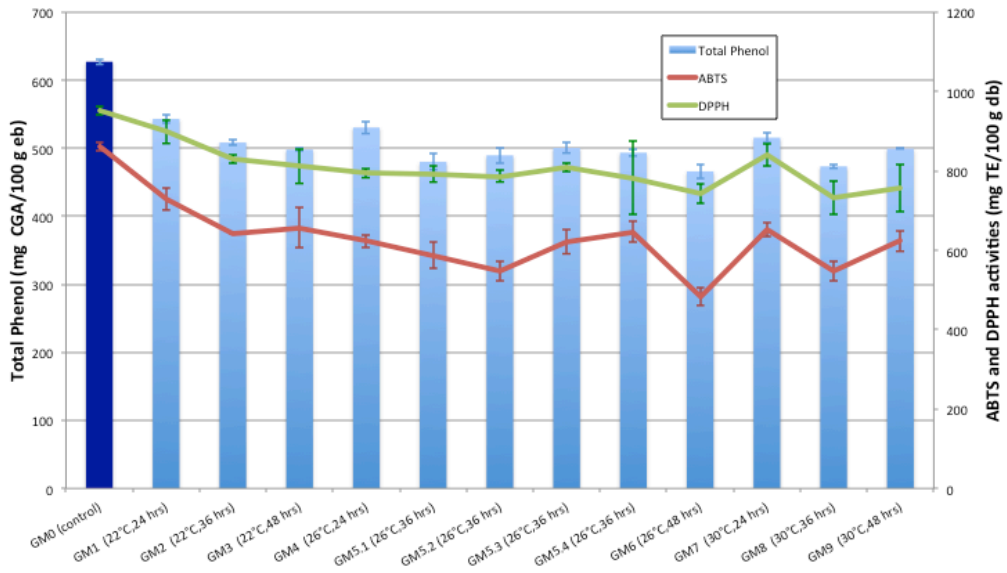


Figure 6-4: Total phenol, ABTS, and DPPH activities of finger millet grain for the selected design factor combinations.

The reported phenol contents and antioxidant activities of finger millet in the literature are summarized in Table 6-7, all in mg/100 g db with respect to different employed equivalents.

It has been reported that the phenol content in finger millet strongly depends on its grain color and its geographical cultivation location (Siwela et al., 2007). As generally

reported in literature, white finger millet has lower phenol contents, while brown finger millet is considerably richer in phenolic compounds. The results of our investigated native Indian brown finger millet were completely in the range as compared to other reports (Towo et al., 2003). In addition, a similar decrease in the finger millet total phenol content throughout the germination process has been pointed out in a few other studies. Towo et al. (2003) reported 21% reduction in total phenol after germination of finger millet. A 34% reduction was reported by Sripriya et al. (1996) in germination of Indian brown finger millet. Abdelrahaman et al. (2007) reported a 35–42% decrease in polyphenol content of millet germinated for six days in several studied varieties.

Table 6-6: Total phenol (mg CGA/100 g db) content and DPPH (mg TE/100 g db), and ABTS (mg TE/100 g db) activities of finger millet grain for the designed germination treatments.

Exp. #	Germination factors and their levels		Total Phenol	DPPH	ABTS
	Temperature	Duration	(mg CGA/100 g db)	(mg TE/100db)	(mg TE/100db)
GM0	**Control**		627.45 ± 3.93	952.39 ± 11.30	861.11 ± 11.10
GM1	**22**	**24**	543.29 ± 5.87	898.89 ± 29.28	729.00 ± 27.29
GM2	**22**	**36**	508.60 ± 3.80	829.70 ± 10.42	643.81 ± 1.13
GM3	**22**	**48**	498.24 ± 1.73	812.50 ± 43.70	657.54 ± 51.60
GM4	**26**	**24**	530.69 ± 8.89	794.72 ± 11.43	623.14 ± 15.69
GM5.1	**26**	**36**	480.21 ± 11.49	793.22 ± 20.98	587.73 ± 33.93
GM5.2	**26**	**36**	489.85 ± 11.59	786.69 ± 14.10	547.57 ± 25.51
GM5.3	**26**	**36**	500.60 ± 7.44	808.86 ± 10.81	621.22 ± 29.74
GM5.4	**26**	**36**	493.76 ± 4.99	783.11 ± 92.87	646.71± 26.63
GM6	**26**	**48**	466.19 ± 10.62	743.11 ± 24.81	483.11 ± 21.88
GM7	**30**	**24**	515.78 ± 7.99	839.79 ± 27.30	651.15 ± 17.53
GM8	**30**	**36**	473.59 ± 2.78	732.36 ± 41.80	547.09 ± 23.80
GM9	**30**	**48**	499.57 ± 0.18	756.54 ± 58.83	623.05 ± 25.28

In the present study, the phenolic contents were analyzed for finger millet using response surface methodology. Results are presented in Table 6-9, where only germination duration was found to be a significant factor. Based on this table the following response surface was derived;

$$TP = 488.4733 - 10.1983\ X_1 - 20.96\ X_2 + 7.885\ X_1^2 + 15.23\ X_2^2 + 7.21\ X_1\ X_2 \quad (6\text{-}3)$$

Table 6-7: Total phenol content, DPPH, and ABTS antioxidant activities (mg/100 g db) of finger millet grain.

Finger millet Variety	Total Phenol (mg/100 g)	DPPH (mg/100 g)	ABTS (mg/100 g)
	Gallic acid equivalent	**Trolox equivalent**	**Trolox equivalent**
Brown finger millet (Siwela et al., 2007)	340 to 1840	-	1734 to 4890
White finger millet (Siwela et al., 2007)	<90	-	<1364
Finger millet (Sreeramulu et al., 2009)	373 ± 70	173 ± 3	-
Brown color (Chethan & Malleshi, 2007; McDonough et al., 1986)	1200 to 2300	-	-
White color (Chethan & Malleshi, 2007)	300 to 500	-	
			Gallic acid equivalent
Millet (Asao & Watanabe, 2010)	360	-	1770
	Catechin equivalent		
Raw Finger millet (Towo et al., 2003)	420 ± 27	-	-
Germinated (Towo et al., 2003)	330 ± 11	-	-
	Chlorogenic acid equivalent	**Percentage basis**	
Finger millet	1050	81.67 ± 2.36%	-
Indian Brown (Shankara, 1991)	60 to 670	-	-
Indian Brown (raw) (Sripriya et al., 1996)	102	-	-
Indian Brown (germinated) (Sripriya et al., 1996)	67	-	-
Indian White (Sripriya et al., 1996)	3.47	-	-
Indian Brown (McDonough et al., 1986)	550 to 590	-	-
Indian White (Geetha et al., 1977)	80 to 90	-	-
Indian Brown (Geetha et al., 1977)	370 to 960	-	-
African Brown (Geetha et al., 1977)	540 to 2440	-	-
Finger millet (Raghavendra Rao et al., 2011)	7200 ± 570	-	-
	Tannic acid equivalent		
Indian Brown (Shankara, 1991)	30 to 570	-	-
	Ferulic acid equivalents	**Ferulic acid equivalents**	
Cooked finger millet (Chandrasekara & Shahidi, 2012)	233 ± 4	314.6 ± 3.5	-

For the case of finger millet, in contrast to amaranth, a negative correlation between phenol content and germination duration existed. Therefore, as germination duration increased, phenol content decreased in the sprouts. Furthermore, having positive coefficients for the quadratic terms indicated the existence of a minimum in the proposed response surface. The constructed surface aligns with its corresponding leverage curves which are presented in Figure 6-5. A minimum point was observed for this surface at 27.49 °C and 43 hr with an interpolated phenol content of 480.28 mg CGA/100 g db. In the performed

experiments, the minimum obtained value (466.19 mg CGA/100 g db) for phenol content belonged to case GM6 (Table 6-6), where the grains were germinated for 48 hr at 26 °C.

Table 6-8: Summary of the ANOVA of the responses for total phenol content and the corresponding parameter estimates of the different terms of finger millet grain.

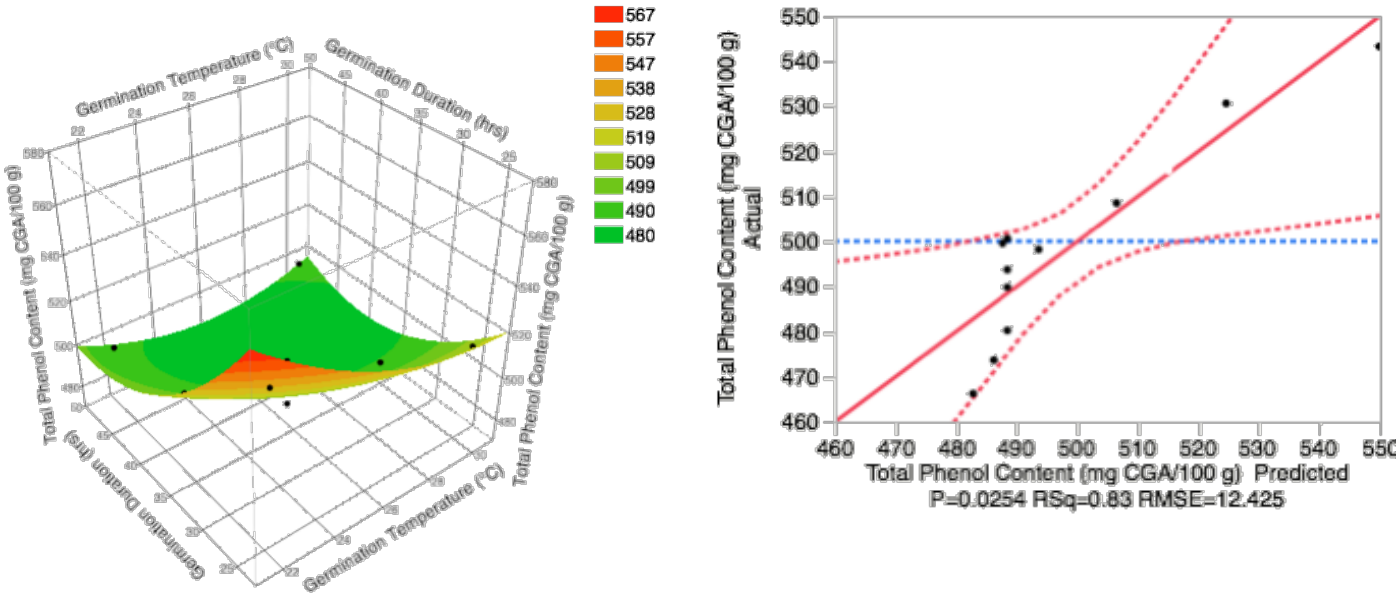
Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	488.4733	5.6711	86.13	**<.0001**
Temperature	-10.1983	5.0724	-2.01	0.0911
Duration	-20.96	5.0724	-4.13	**0.0061**
Duration×Temperature	7.21	6.2124	1.16	0.2899
Temperature×Temperature	7.885	7.6086	1.04	0.3400
Duration×Duration	15.23	7.6086	2.00	0.0922

Table 6-9: Summary of the ANOVA of the responses for total phenol, DPPH and ABTS of finger millet.

	Total Phenol	DPPH	ABTS
Intercept	**488.4733**[a]	**781.3108**[a]	**581.2379**[a]
Temp	-10.1983	**-35.4**[c]	-34.8433
Time	**-20.96**[b]	**-36.875**[c]	-39.9316
Time×Temp	7.21	0.785	10.84
Temp×Temp	7.885	23.0375	53.3513
Time×Time	15.23	10.9225	11.0263
R^2	0.832	0.799	0.604
R^2adj	0.692	0.632	0.274
RMS	12.424	27.530	54.747

a: $p<0.0001$, b: $p<0.01$, c: $p<0.05$.

DPPH and ABTS activities were similarly analyzed. Results are presented in Table 6-9. Unlike for phenol contents, temperature had an influential role for the DPPH activities. In addition, despite the proposed response surface for ABTS activity, none of the factors were significantly influential. This was highlighted in the low obtained R^2 value for the ABTS response surface (Table 6-9).



(a) (b)

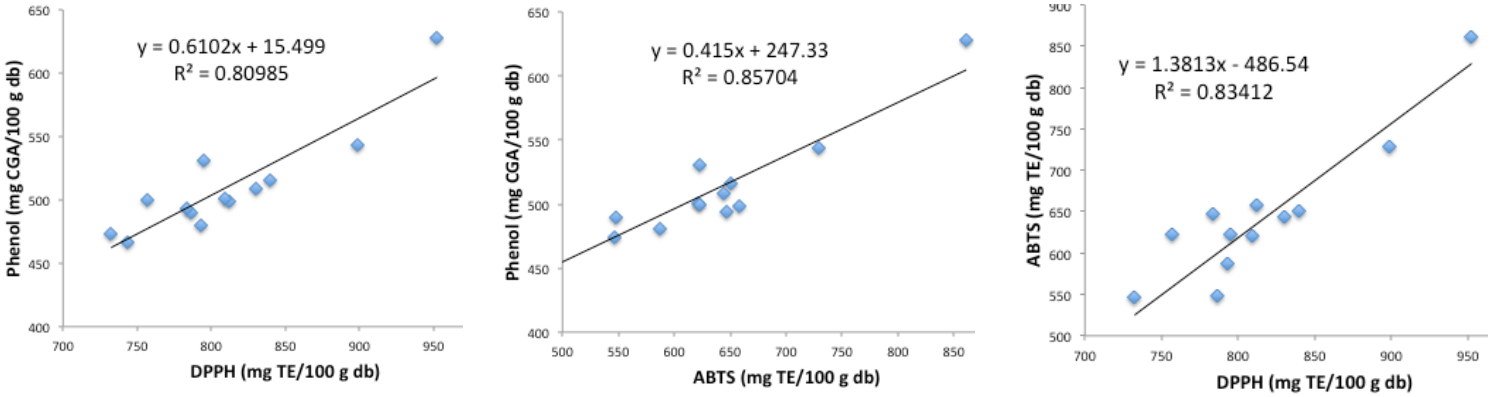
Figure 6-5: (a) The response surface for the total phenol content (mg CGA/100 g db) and (b) the leverage plot based on the ANOVA of finger millet grain.

Table 6-10: Colorimetric properties of native and germinated finger millet grain using CIELab approach.

Exp. #	Germination factors and their levels		L*	a*	b*	E
	Temperature	Duration				
GM0	**Control**		78.31±0.68	2.77± 0.10	7.23± 0.12	0.00±0.70
GM1	**22**	**24**	81.34±0.50	1.82± 0.04	6.50± 0.13	3.26±0.52
GM2	**22**	**36**	82.50± 0.51	1.70± 0.05	6.58± 0.15	4.37±0.53
GM3	**22**	**48**	82.14±0.56	1.71± 0.07	6.58± 0.14	4.03 ±0.58
GM4	**26**	**24**	82.63±0.82	1.73± 0.03	6.39± 0.11	4.52±0.82
GM5.1	**26**	**36**	81.98±0.30	1.69± 0.07	6.74± 0.16	3.86± 0.35
GM5.2	**26**	**36**	81.91±0.43	1.64± 0.07	6.50± 0.13	3.84±0.46
GM5.3	**26**	**36**	81.72± 0.45	1.71± 0.04	6.46± 0.09	3.66±0.46
GM5.4	**26**	**36**	81.66±0.49	1.74± 0.10	6.60± 0.07	3.56± 0.51
GM6	**26**	**48**	82.16±0.44	1.76± 0.09	6.67± 0.16	4.02±0.48
GM7	**30**	**24**	81.47±0.36	1.86± 0.06	6.48± 0.07	3.37±0.37
GM8	**30**	**36**	82.11±0.33	1.64± 0.11	6.46± 0.07	4.04± 0.35
GM9	**30**	**48**	82.69±0.37	1.81± 0.05	6.79± 0.06	4.50±0.37

Similar to amaranth, the possibility of the existence of linear correlations between phenol content and antioxidant activities was investigated. In Figure 6-6, the scattered plots of the phenol contents, DPPH, and ABTS activities versus each other are presented for native

finger millet and its sprouts. Again, acceptable linear correlations were observed, indicating that antioxidant activities decreased as phenol content decreased.



(a) **(b)** **(c)**

Figure 6-6: Linear regression curves representing the linear correlations between total phenol content and the antioxidant activities, DPPH and ABTS (a) phenol vs. DPPH (b) phenol vs. ABTS, and (c) ABTS vs. DPPH of finger millet grain.

Colorimetric values for germinated finger millet grains are presented in Table 6-10, where no significant pattern could be concluded in the color versus germination factors.

6.5.3 Conclusion

Finger millet and amaranth grains were demonstrated as two potentially rich sources of phenolic compounds possessing high antioxidant scavenging activities. Malting of these seeds might be an appropriate pre-treatment in the food industry to optimize the phenolic content quantity and quality. In the present study, effects of two important germinating factors, duration and temperature, on the total phenol contents and radical scavenging activities of finger millet and amaranth seeds were investigated. Using a central composite design with three levels for each design factor enabled us to construct a second order response surface for the total phenol content and ABTS and DPPH antioxidant scavenging activities over the interested range of variation (26 °C <germination temperature<30 °C and 24 hr <germination duration<48 hr). It was shown that germination significantly (up to four times) increased the phenolic content and antioxidant activities of amaranth seeds. In addition, a

significant color change towards pink color was observed in amaranth sprouts, which can increase its consumer acceptability. In the case of finger millet, germination slightly decreased the phenolic content and DPPH and ABTS activities at most by 25%. Nevertheless, the phenolic content of finger millet would still remain appreciably high. For both grains, the extremum point was found to be 26 °C and 48 hr.

Connecting Statement to Chapter 7

In the previous chapters, nutritional profile and functional characteristics of finger millet and amaranth grains were optimized through a designed malting stage. In the current chapter, the obtained malted flours were used as appropriate substrates for a probiotic fermentation stage. Fermentation resulted into further reduction in anti-nutrient contents. Suitable growth of inoculated bacterial strains in this stage brought beneficial probiotic characteristics for the fermented slurries. Furthermore, bacterial vitality throughout freeze-drying and storage stages were monitored and enhanced by using skim milk and maltodextrin as protectant.

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Development of millet and amaranth-based probiotic weaning products

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Contributions made by the different authors are as follows;

- The first author, **Sara Najdi Hejazi**, is the Ph.D. student, who prepared the experimental design, conducted the laboratory work, analyzed the results, and prepared the manuscript.
- The second author, **Valérie Orsat**, is the thesis supervisor, who initiated and stimulated ideas, provided the technical advice, suggestions, experiment facilities and

guided the first author in writing, correcting, and preparing the manuscript throughout the study.

- The third author, **Usha Chandrakant,** is the M.Sc. student, who assisted the first author in performing some parts of the laboratory work.
- The fourth author, **Darwin Lyew,** is the research associate, who provided the microbiological expertise for the study.

Furthermore, parts of the obtained data and performed analyses in **Chapter 7** were presented in the following conference presentation;

1. Sara Najdi Hejazi and Valérie Orsat, "**Effects of Germination and Fermentation Treatments on Finger Millet and Amaranth Grains,"** American Society of Agricultural and Biological Engineers. Poster presentation. Energy Systems Poster Session, Bioprocessing for Value Added Coproducts Subcategory, July 26-29, 2015, New Orleans, Louisiana, USA.

7 Development of millet and amaranth-based probiotic weaning foods

7.1 Abstract

Today, there is a growing demand for the development of gluten-free probiotic weaning food products. This is mostly critical for the infants who suffer from celiac disease. Nevertheless, the industrial weaning products have been mostly based on wheat, which contains gluten in its protein structure. Finger millet (*Eleusine coracana*) and amaranth (*Amaranthus caudatus*) are two nutritious and gluten-free cereal and pseudo-cereal grains that have been traditionally consumed for infant weaning purposes. In the present study, to strengthen the infants' immature gastrointestinal tract flora, probiotic fermentation of these substrate flours, by three suggested bacterial strains, *Lactobacillus rhamnosus, Bifidobacterium longum*, and *Bifidobacterium infantis,* was investigated and optimized. Approximately 4 Log (CFU)/g enhancements in the bacterial population after 24 hr of fermentation at 37 °C were observed, where *Lactobacillus rhamnosus* in finger millet malt grew the best. In order to preserve the bacterial vitality throughout freeze-drying and storage steps, influences of two common cryoprotectants, skim milk and maltodextrin, were monitored. While the need for having extra protectant was minimal for amaranth especially during storage, skim milk notably increased the bacterial vitality for finger millet after drying and during storage stages. Based on the obtained data and performed ANOVA, the probiotic content of the processed flours after four weeks of storage at 4 °C remains above the minimum required levels for probiotic-labeled food products.

Keywords: *finger millet, amaranth, probiotic fermentation, freeze-drying, storage*

7.2 Introduction

The likelihood of a pathogen contacting a host is enhanced during infants' weaning period. This usually results in gastrointestinal infections. Recent studies have confirmed that probiotics consumption, particularly during the weaning period, may strengthen the immature infants' flora against gastrointestinal diseases, especially rotavirus diarrhea and constipation (Arora et al., 2011; Baldeon et al., 2008; du Toit et al., 2013; Farooq et al., 2013; Heller, 2001). Consequently, in the weaning industry, there is a growing desire for the inclusion of probiotic cultures in their food products (Rai et al., 2008).

Although the current weaning foods are mostly based on wheat and rice as the main cereal ingredients, in the recent decades, the demand for availability of new alternative cereals and pseudo-cereals has been growing (Sajilata et al., 2002). This is mostly due to a notable increase in gluten intolerance and celiac diseases among newborns as well as the lack of economical or geographical access to the commonly available commercial cereals (Sajilata et al., 2002). Among the alternative cereals, finger millet (*Eleusine coracana*) and amaranth (*Amaranthus caudatus*) are gaining more interest (Swami et al., 2013).

Finger millet has been traditionally consumed for weaning purposes in households mostly in India and Africa (Guandalini, 2007; Hansen et al., 1989; Kannan, 2010; Mosha & Svanberg, 1983; Sajilata et al., 2002); hence, it could potentially be an appropriate candidate to be employed in the development of weaning foods at the industrial scale. However, due to millet's protein deficiency (in certain amino acids), addition of complementary ingredients that enrich its nutrition profile is essential (Ahmadzadeh Ghavidel & Prakash, 2010). Amaranth, which has one of the highest quantity and quality

of protein storage among cereals, could be an appropriate grain of choice. Absence of gluten in their protein structure makes finger millet and amaranth good substitutes for wheat in the current weaning market for infants, who suffer from celiac disease and/or atopic allergies.

One of the drawbacks in utilization of these cereal crops is their relatively high anti-nutrient contents, mainly phytic acid and oxalate. Phytic acid reduces the bioavailability of nutrients and minerals. Furthermore, accumulation of oxalate in infants' body could increase the renal load of their immature kidney (Abratt & Reid, 2010). Therefore, special pre-treatments, such as germination and fermentation, are required to improve these grains nutritive values by degrading their anti-nutrients (Sajilata et al., 2002).

It is known that nutrition and health benefits of cereals can be greatly improved by fermentation, providing higher sensorial value and extended shelf life. Fermentation can establish optimum pH conditions for the enzymatic degradation of phytate, which exists in cereals by forming complexes with polyvalent cations such as iron, zinc, calcium, magnesium, and proteins. Phytate reduction not only increases the amount of these trace elements, but also improves protein and starch bioavailability and digestibility (Hejazi et al., 2015). Phytic acid reduction is mostly due to enhancement in phytase activity, which is known to be possessed by a wide range of microflora (Sindhu & Khetarpaul, 2001). Improvement in starch digestibility during fermentation can also be associated with the enzymatic properties of fermenting microflora that results into the cleavage of starch oligosaccharides. The enzymes breakdown amylose and amylopectin to maltose and glucose. Reduction in amylase inhibition activity may also be responsible for the

enhancement of starch digestibility. Similarly, improvement in protein digestibility of fermented products is mainly associated with the enhanced proteolytic activity of the fermenting microflora. Their metabolic activities result in the production of short chain fatty acids such as lactic, acetic, butyric, formic, and propionic acids. Acids formed during the fermentation process, lower the pH thus inhibiting the growth of spoilage organisms and pathogens. Most of the Lactic Acid Bacteria (LAB) cannot ferment starch directly; therefore, it is usually necessary to have an amylolytic preparation for that purpose. This can be mostly favoured through a prior germination/malting stage, which enhances the fermentation process (Lorri & Svanberg, 1993).

If fermentation is accomplished with probiotic organisms, it can bring specific added advantages apart from the nutritional improvement. Probiotics are defined as "live microorganisms, which when administered in adequate amounts confer health benefits on the host" (Sanders, 2003). Specific bacteria, especially the species of lactobacilli compose the majority of recommended probiotics (Goldin & Gorbach, 1992). In the current study, two species of Bifidobacteria, *Bifidobacterium longum* and *Bifidobacterium infantis,* as well as one species of Lactic acid bacteria, *Lactobacillus rhamnosus* were investigated.

Bifidobacteria were first isolated from breast-fed infants by Henry Tissier (1990). He found that these bacteria are dominant in the gut flora of infants. He noticed that when the bacteria existed in infants' intestinal flora, they suffered less from gastrointestinal disorders. Therefore, he recommended that these bacteria be administered to babies diagnosed with diarrhea (Tissier, 1990). He proposed that the health and nutritional benefits of *Bifidobacterium* species are, resistance of host to infection, antagonist activity

against pathogens, and antimicrobial properties which contribute to the protection of infants against gut infection.

Lactic acid bacteria are industrially important microbes that are used all over the world in a large variety of industrial food fermentation applications (Ruas-Madiedo et al., 2002). These bacteria are known for having several beneficial physiological effects, including heightening of immune potency, enzyme (lactase) formation, colonization and maintenance of the suitable intestinal microflora, competitive exclusion of undesirable microorganisms, microbial interference and anti-microbial activities, pathogen clearance, immuno-stimulation and modulation, and cholesterol reduction/removal (Kullisaar et al., 2003).

Naturally, mother's breastmilk contains high contents of lactose. In the infant's gastrointestinal tract, when the milk is fermented by *Lactobacillus rhamnosus* and *Bifidobacterium longum* and *infantis*, the lactose content of the milk is converted to lactic acid. This drops the pH of the medium, which would consequently provide a harsh environment for the gram-negative bacteria (pathogens) to grow; hence, providing health benefits for the host (Baldeon et al., 2008).

As it was mentioned in the definition of probiotics, they are live microorganisms and should remain viable in adequate amounts before reaching their desired destination, the gastrointestinal tract (GIT). Therefore, their lively state should be appropriately maintained from the fermentation step throughout all food processing stages, including drying and storage, until reaching the GIT for sustained growth and adhesion. It is recommended that probiotic products should contain at least 10^7 Colony Forming Units (CFU) of live

microorganisms per gram or per mL at the time of consumption in order to adequately benefit the consumer (Goldin & Gorbach, 1992). The survival of probiotics in a food system, especially during the storage period, is strongly affected by the moisture content or water activity of the product; the lower the moisture level, the higher the survival of probiotics (Champagne et al., 2012; Morgan et al., 2006; Reddy, 2007). Dried conditions keep bacteria in the quiescent state or stationary phase; hence, the probiotic viability can be maintained at an acceptable level even if the product is stored at ambient temperature for long periods (Chávez & Ledeboer, 2007; Higl et al., 2007; Reddy, 2007).

Freeze-drying is the most satisfactory method for the long term preservation of viable cultures (Champagne et al., 2012). It is usually employed to preserve LAB bacterial starter cultures in the dairy and fermented foods industries (Kearney et al., 1990). The basic idea is to freeze the fermented slurry and then reduce the surrounding pressure to allow the frozen water to sublime directly from the solid phase to the gas phase (Champagne et al., 2012). The percentage of probiotic survival through drying approaches has been reported to be maintained by increasing their initial concentration up to 10^{11} CFU/mL (Reddy, 2007). Besides, addition of protective agents may enhance the stability of probiotic microorganisms during freeze-drying and storage (Zayed & Roos, 2004). Among many others, skim milk and maltodextrin, were selected in the current study. They are frequently reported as efficient suspending agents for freeze-drying as well as convective drying of probiotic products.

Champagne et al. (2012) investigated and suggested the main parameters that need to be considered in the growth of probiotic microorganisms in cereal substrates. Among

many others, composition and processing of cereal grains and the substrate formulation, growth capability of the starter culture as well as its stability during storage, and the organoleptic properties and nutritional value of the final product were of greatest importance. Studying a large number of cereal and pseudo-cereals, Champagne et al. (2012) reported that most of them, including millet and amaranth grain, appropriately supported the growth of probiotic cultures.

In the present study, the above criteria were assessed for probiotic fermentation of malted finger millet and amaranth flours using *Lactobacillus rhamnosus, Bifidobacterium longum*, and *Bifidobacterium infantis.* The growth capability of these bacteria during fermentation and their stability and vitality throughout drying and storage steps were evaluated and optimized. It was concluded that the studied grains are appropriate substrates for the manufacturing of new gluten-free probiotic infants' weaning products.

7.2.1 Materials and methods

Probiotic strains *Lactobacillus rhamnosus* (NRRL; B-442), *Bifidobacterium longum* (NRRL; B-41409), and *Bifidobacterium infantis* (NRRL; B-41661) were procured from USDA's (United States Department of Agriculture) culture collection of Agricultural Research Services (ARS) at National Center for Agriculture Utilization Research (Peoria, Illinois). These microorganisms were preserved in dormant state in heavy suspension of freeze-dried cells in sterile bovine serum. MRS broth (deMan, Rogosa and Sharpe Medium, Difco™, cat # 69966) was provided by Sigma Aldrich and RCM broth (Reinforced Clostridial Medium, cat # CM 0149) was obtained from Oxoid. Sterile petri plates, cell spreader, and peptone were ordered from Fisher Scientific and gas pak (GasPak™ EZ Gas

Generating Container Systems) and anaerobic indicator strips were purchased from BD (Becton Dickinson, NJ, United States).

7.2.2 Statistical design and analysis

For each step of the performed experiment, individual Full Factorial Design (FFD) approaches were adapted. For the fermentation stage, a design was developed with 54 combinations consisting of one continuous factor: fermentation duration with 9 levels: 0, 6, 12, 18, 24, 30, 36, 42, and 48 hr; and two categorical factors including, grain type: finger millet and amaranth, and bacterial type: *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Bifidobacterium infantis*. For drying, three categorized factors were employed; grain type (2 levels), bacterial type (3 levels), and protectant type (3 levels: no protectant, skim milk, maltodextrin). This resulted into 2×3×3=18 design combinations. For the third experiment, storage duration was selected as a continuous factor with 5 levels: 0, 1, 2, 3, and 4 weeks. Grain, bacterium, and protectant were selected as categorized design factors with 2, 3, and 3 levels, respectively. This resulted into 5×2×3×3=90 design combinations. All the design combinations were triplicated in the sampling process to increase consistency and reliability of the obtained data. The goal in the fermentation stage was to maximize the probiotic populations as well as minimize the studied anti-nutrient compounds; phytic acid and oxalate. For the drying and storage stages, enhancement of the bacterial survival rate was desired. JMP software version 11 (SAS Institute Inc., Cary, NC, USA) was used for the experimental design and analysis. Using ANOVA and regression analysis, linear effects of the design factors as well as their interactions were statistically assessed based on the F-values at the probability $p<0.05$. For the fermentation stage, a non-

linear (third-order) dependency of the constructed response surface on the fermentation duration factor was assumed.

7.2.3 Starter culture preparation

Freeze dried pellets of *Lactobacillus rhamnosus* and *Bifidobacterium* species were reconstituted in 100 ml MRS and RCM broths, respectively. They were grown in an incubator shaker (Model G24, New Brunswick Scientific, NJ, USA) for 18-21 hr at 110 rpm and 37 °C. Subsequently, each culture was sub-inoculated at the level of 1% (v/v) in MRS and RCM broths, and the second revitalized subcultures were obtained after an overnight incubation. Small quantities of each subcultures were stored in sterile glycerol 30% (v/v) at -80 °C (ULT Freezer Model 5698, Thermo Forma®, USA) as the stock culture for further usage. The growth kinetics of the strains were determined by monitoring the changes occurred in the optical density at 600 nm (OD_{600}) using a spectrophotometer (Ultraspec 1000, Amersham Pharmacia Biotech, NJ, USA) and their relative growth curves were obtained (Anekella, 2011). Finger millet and amaranth malted flours were prepared according to the previous studies by the present authors (Hejazi & Orsat, 2015; Hejazi et al., 2015). To obtain the malted flours, grains were germinated for 48 hr at 30 °C, dried, and powdered.

7.2.4 Substrates preparation and probiotic fermentation process

Initially, malted finger millet and amaranth flours were suspended separately in distilled water (40% w/v) to obtain homogeneous slurries. Subsequently, slurries were sterilized in an autoclave for 15 min at 121 °C and cooled down to 37 °C before probiotic fermentation. The grown probiotic curds were appropriately inoculated to each prepared

slurry sample in a way that 10^6 CFU per gram of dried-base flours would be obtained. Fermentation process was performed in incubator at 37 °C for 48 hr. Bacterial counts, pH, phytic acid, and oxalate content were monitored every 6 hr. The fermentation procedure was performed in triplicate to validate the obtained data.

7.2.5 Freeze-drying and storage

The fermented finger millet and amaranth slurries were supplemented with dried skim milk and maltodextrin, as cryoprotective additives. Three different combinations were tested to observe the survival rate of the selected strains after freeze drying and during storage: (1) 10% skim milk (w/v) + fermented slurries; (2) 10% maltodextrin (w/v) + fermented slurries; (3) fermented slurries without any protectant (Celik & O'Sullivan, 2013). Suspensions were maintained for an hour in an ice bath to allow equilibrium between cells and added protectants prior to freezing. Consequently, they were transferred into sterilized petri plates, frozen at -80 °C overnight, and dried in a freeze-dryer (FreeZone® 2.5 l Freeze Dry System, Labconco Corporation, MO, USA). Dried flours were stored in containers at 4 °C in airtight conditions. The viability of the inoculated probiotics after freeze-drying and during storage were monitored weekly up to 4 weeks.

7.2.6 Enumeration of viable probiotics

Probiotics' CFU counts were estimated on MRS agar for *Lactobacillus* and RCM agar for *Bifidobacterium* species using STN ISO 15214 (2002) method at regular intervals during fermentation, freeze-drying, and storage. All the microbial measurements were implemented under a type I biosafety cabinet.

Briefly, ten-fold series of the fermented slurries were prepared using buffered peptone water and 100 μL aliquots of relevant dilutions plated out on solid RCM and MRS agar mediums. Consequently, plates were placed in anaerobic jars containing a gas pak to provide the necessary anaerobic conditions, incubated in a convection incubator (Model 4EG, Precision Scientific, USA) at 37 °C for 48 hr, and survivors were enumerated. The probiotics' CFU of each design combination were assessed by averaging overall fold series and their replicates. The outrange data were laid out in the averaging evaluation. In order to count the survival rate of probiotics after freeze-drying and during storage, 0.5 g of the obtained flours were suspended in 9.5 mL sterile peptone/saline solution, and incubated for an hour. Thereafter, reconstituted cells were serially diluted, pour plated, and incubated at 37 °C for 48 hr under anaerobic conditions. The survival rate of the dried samples was estimated as;

$$\% \text{ Survival} = 100\frac{N_r}{N_f}$$

where, N_r is CFU/g of the rehydrated sample and N_f is CFU/g of the fed solution.

7.2.7 pH measurement

The pH of the substrate and inoculum was measured using a pH meter (CG843, Schott, Mainz, Germany).

7.2.8 Oxalate

Oxalate content was estimated according to the method of Horner et al. (Gélinas & Seguin, 2007; Horner et al., 2005).

7.2.9 Phytic acid

Phytic acid content was quantified using Megazyme phytic acid kit (cat # K-PHYT, (Phytate/Total Phosphorous), Megazyme International, Ireland).

7.3 Results

7.3.1 Probiotic fermentation

A probiotic fermentation process was performed on the amaranth and finger millet flours following the experimental model described in section 7.2.2. Viable bacterial counts, as well as pH, phytic acid, and oxalate contents of the fermented samples were evaluated at the initial state and over fixed six-hour intervals up to 48 hours. Figure 7-1 presents in (a), (c), and (e) the obtained bacterial counts (log (CFU)/g db) and in (b), (d) and (f) the pH trends for the performed fermentation process. The total viable bacterial counts in log (CFU) per gram of dried flours during fermentation are graphically presented for both finger millet (solid lines) and amaranth flours (dashed lines) and all inoculated bacterial strains, *Lactobacillus rhamnosus* (Figure 7-1 (a) and (b)); *Bifidobacterium longum* (Figure 7-1 (c) and (d)) and *Bifidobacterium infantis* (Figure 7-1 (e) and (f)).

The error bars represent the Standard Deviation (SD) of the data among the sample replicates. From Figure 7-1, it was clearly observed that all investigated bacterial strains appropriately grew in both flour substrates. The fermentation process was initiated by around 6 log (CFU)/g db of inoculated bacteria. The population increased approximately by 4 log (CFU)/g db and maximized roughly after 24 hr. Constant or slightly reducing trends were observed afterward. In an opposite behavior, pH decreased during the first 18-24 hr of fermentation to its minimum values and then remained almost constant. Similar patterns and levels for viable bacterial counts and the reduction in pH index have been

reported in previous studies on fermentation of cereal flours (Blandino et al., 2003; Coda et al., 2014; Kocková et al., 2013a; Pelikánová et al., 2015). While most studies have focused on wheat, oath, sorghum, and barley (Helland et al., 2004; Lorri & Svanberg, 1993; Reale et al., 2007; Sindhu & Khetarpaul, 2001; Wakil & Onilude, 2009), a number of studies have been conducted on fermentation of amaranth and millet grains (Antony & Chandra, 1999; Makokha et al., 2002; Sterr et al., 2009; Vogelmann et al., 2009).

Pelikánová et al. (2015) investigated the effects of using milk and water on the growth and metabolic activity of selected Lactobacilli throughout amaranth fermentation for 8 hr at 37 °C. All the studied bacterial strains showed notable growth in the studied cases, where the growth rate ranged from 0.155 to 0.819 log (CFU)/ml hr. The greatest difference in the cell population throughout the fermentation was observed for an amaranth-milk sample, fermented with *Lactobacillus rhamnosus* GG at 8.5 log (CFU)/ml, which showed 3.05 log (CFU)/ml of total increase. Furthermore, pH dropped through the fermentation and 21 days of storage to a pH range between 3.6-5.5 (Pelikánová et al., 2015).

Sterr et al. (Sterr et al., 2009) investigated the effects of fermentation temperature (25, 30, and 35 °C) on the bacterial counts and pH of amaranth flours using a number of bacterial strains, including *Lactobacillus plantarum* RTa12 and *Pediococcus pentosaceus* RTa11. The fermentation process was initiated after an initial inoculation of 10^7 CFU/g of the investigated bacterial species. The bacterial counts increased approximately up to 10^{10} CFU/g and maximized after 12 hr for all samples; although the increase was higher for those that were fermented at 35 °C. For all samples, pH reduced from 6.3 down to values

around 4. The pH reduction slope was high at the initial fermentation step (up to 12 hr), then after remained almost constant.

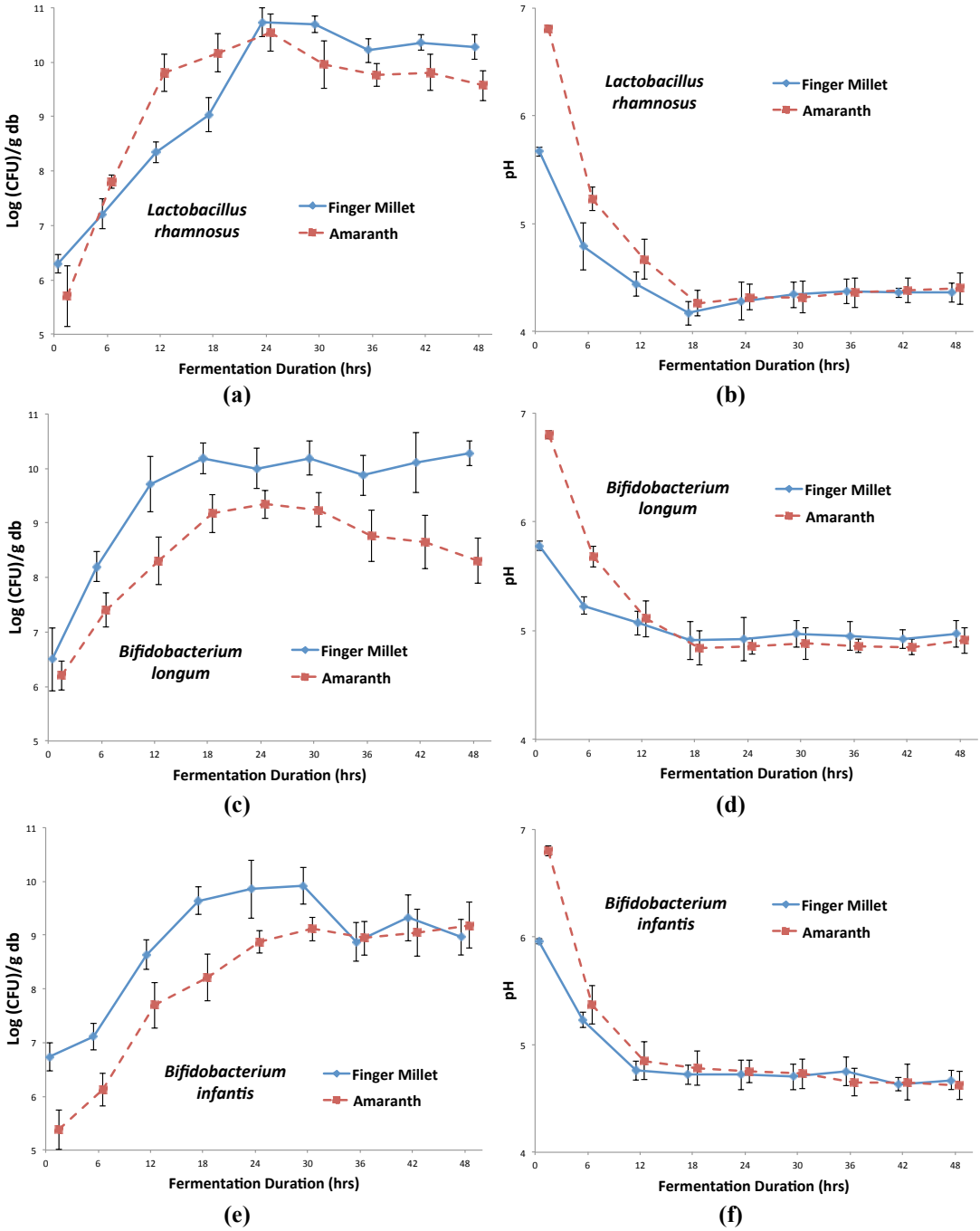

Figure 7-1 (a), (c), and (e) bacterial counts (log(CFU)/g db) and (b), (d), and (f) pH trend for the performed fermentation process.

Kocková, et al. (2013a) investigated the suitability of a number of cereal and pseudo-cereal grains for the development of probiotic products. In a 10-hour probiotic fermentation by *Lactobacillus rhamnosus GG* strain, the bacterial counts in finger millet grain, amaranth grain, and amaranth flour increased from their initial 5.06, 5.36, and 5.23 to final 7.67, 7.96, and 8.80 log (CFU)/g, respectively. Growth rates for millet and amaranth grains and amaranth flour were 0.263, 0.319, and 0.589 log (CFU)/g hr, respectively. While for amaranth flour, pH did not show any significant change (6.10 to 5.99), it dropped from 6 to 5.24 for amaranth and from 5.91 to 5.06 for finger millet. In another study by the same authors (Kocková et al., 2013b), the fermentation duration was extended up to 24 hr. The initial bacterial inoculation was set at 6 log (CFU)/g. For amaranth flour, while the vital bacterial counts were maximized after 24 hr of fermentation (up to ≈ 8.5 log (CFU)/g), their population reduced to ≈ 7.3 log (CFU)/g after 48 hr. On the other hand, for amaranth and millet grains, a constant increasing trend was observed up to 8.3 and 7.8 log (CFU)/g, respectively. In addition, the authors reported a significant reduction in the pH level during the first 24 hr of fermentation process. For amaranth flour, pH dropped from 6 (initial) to 3.5 (after 24 hr) and to 3.4 (after 48 hr), for amaranth grain from 6.2 to 5.5 to 4.9, and for millet grain from 5.8 to 5.5 to 4.8, after 24 and 48 hr, respectively.

Vogelmann et al. (2009) studied the adaptability of Lactic acid bacteria to sourdoughs prepared from a number of cereals and pseudo-cereals. In their study, after 13 days of fermentation, bacterial counts increased up to 8.86-9.23 log (CFU)/g for millet and 9.26-9.66 log (CFU)/g for amaranth grains. Furthermore, the pH dropped to 3.7-3.8 and 3.6-3.8 for amaranth and millet, respectively.

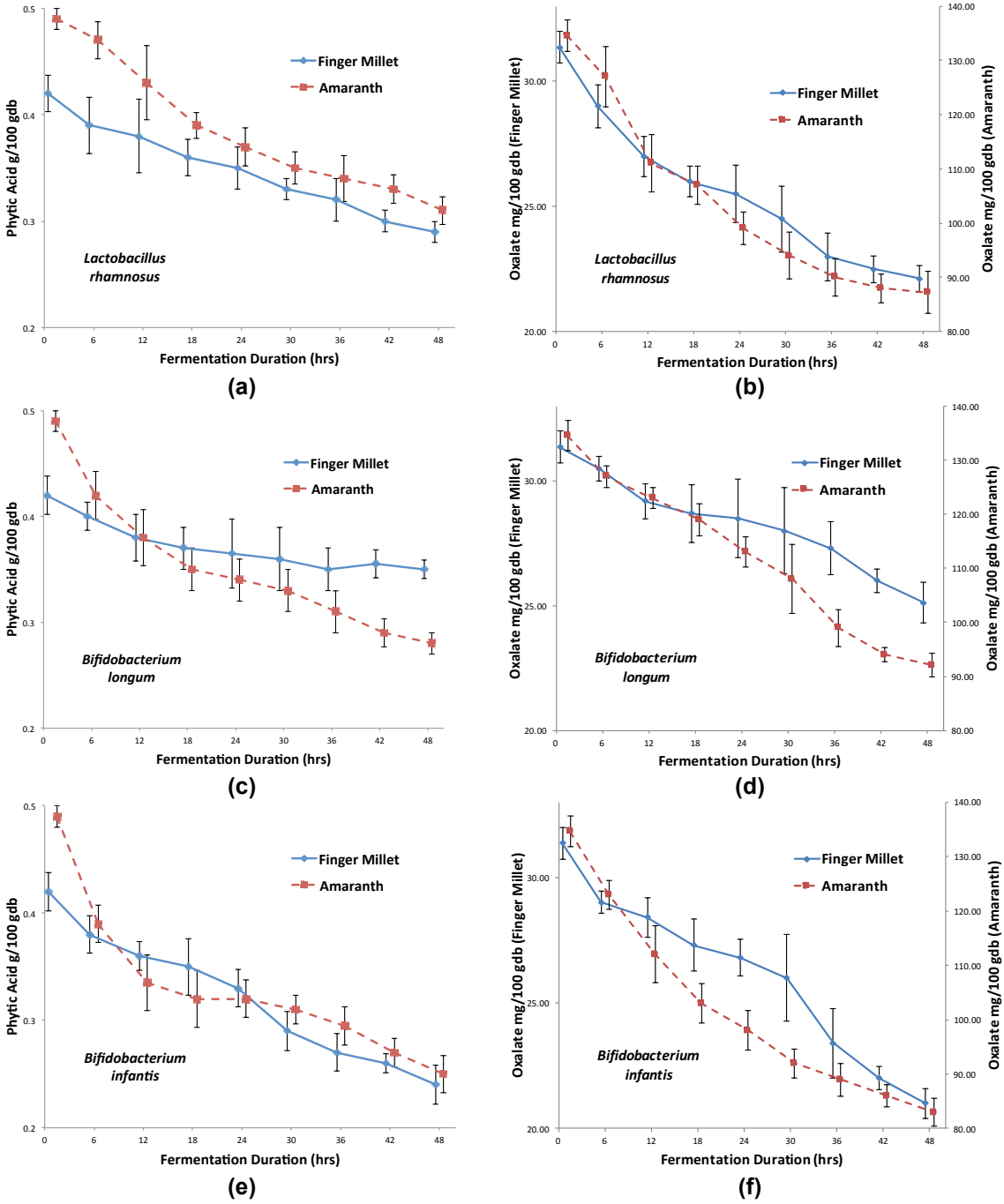

Figure 7-2: (a), (c), and (e) phytic acid (g/100 gdb) and (b), (d), and (f) oxalate (mg/100 gdb) for the performed fermentation process.

Antony and Chandra (1998) studied the natural fermentation of finger millet for 48 hr. In their study, Lactic acid bacteria were found to be dominant throughout fermentation process due to the production of lactic and acetic acids. Production of these acids

consequently reduced the pH and increased titratable activity. The authors reported a consistent increase in microbial population until 18 to 24 hr, where a maximum of 10 to 11 log (CFU)/g was achieved. Furthermore, pH decreased from its initial value of 6.4 to 5.2 and 4.3 after 6 and 48 hr of fermentation, respectively (Antony & Chandra, 1998).

In Figure 7-2, the phytic acid and oxalate contents of the initial and fermented samples are graphically presented. Significant reducing trends were observed for both finger millet and amaranth samples and for all inoculated bacteria. Unlike the observed increase in bacterial population and decrease in pH, the reduction of these anti-nutrient components did not show a cut-off or slow-down behavior after 24 hr.

Positive effects of human gut bacteria, *Bifidobacterium* and Lactic acid bacteria, on degradation of phytate (Haros et al., 2009; Khetarpaul & Chauhan, 1990; Palacios et al., 2008; Shirai et al., 1994) and oxalate (Abratt & Reid, 2010; Azcarate-Peril et al., 2008) have been reported in a number of research studies. Saleh et al. (2013) showed that a fermentation process drastically decreased the anti-nutrient components including phytates and tannins in finger millet. As a result, an enhancement was observed in the mineral availability, soluble protein, and *in-vitro* protein and starch digestibility. Shobana et al. (2012) showed that fermentation of finger millet for 24 hr using endogenous grain microflora significantly reduced the activity of the anti-nutrient compounds; phytate (20%), tannins (52%), and trypsin inhibitor (32%). Consequently, fermentation increased HCl mineral extractability; Ca (20%), P (26%), Fe (27%), Zn (26%), Cu (78%), and Mn (10%).

Singhal and Kulkarni (1988) observed an improvement in the nutritive properties of amaranth grain following a fermentation stage. Similar results have been reported by other researchers on the positive effects of fermentation on finger millet (Antony & Chandra, 1997; Mbithi-Mwikya et al., 2002b; Shobana et al., 2012) and amaranth (Busolo, 1992) grains. Siprya et al. (1997) studied the effects of germination and fermentation stages on carbohydrate, protein, and mineral contents of finger millet. They germinated the grains for 24 hr and then fermented them for 48 hr. They observed major biochemical changes during the first 8 hr of fermentation, where the pH decreased from 5.8 to 3.8 and total sugar, reducing sugar, and free amino acid increased by 2, 12, and 10 folds, respectively. The phytate content decreased (60%), and consequently the mineral HCl extractability increased (47%) (Sripriya et al., 1997). Mokokha et al. (Makokha et al., 2002) claimed 54.3% and 72.3% reduction in phytate content of finger millet after 72 and 96 hr of fermentation, respectively.

7.3.2 Freeze-drying and storage

Bacteria survival rates (%) after the freeze-drying step (Figure 7-3) and throughout the storage period (Figure 7-4) were monitored for the fermented slurries. Effect of using extra protectant, skim milk and maltodextrin, on the preservation of bacterial vitality during these processing steps was investigated as well. In the case when using no extra protectant, the millet and amaranth slurries themselves act as sole protectants for bacterial preservation. For those samples, almost half of viable bacteria were lost during freeze-drying of amaranth slurries. Slightly lower survival rates were observed for the finger millet slurries. The obtained higher rates for amaranth may be related to its higher fat

content (around 8%) when compared with finger millet (less than 1.5%) (Saleh et al., 2013; Venskutonis & Kraujalis, 2013). Fat acts as a protectant during drying steps, thus increasing bacterial vitality (Chávez & Ledeboer, 2007). Between the two employed protectants, skim milk resulted in slightly higher values for amaranth while there were more notable enhancements for the finger millet bacterial survivals. A similar behavior was observed throughout the storage stage, where for finger millet, effects of using protectant, especially skim milk, was more notable (Figure 7-4). For amaranth, employing the extra protectants was not that influential.

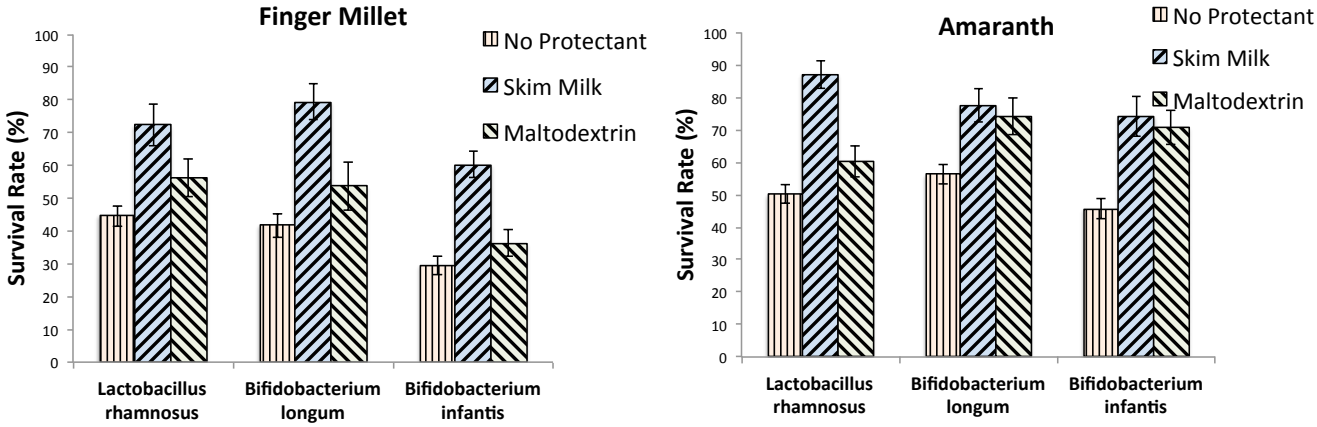

Figure 7-3: Bacterial survival rate (%) after freeze-drying stage (a) finger millet (b) amaranth.

Pelikánová et al. (2015) studied survival of *Lactobacilli* bacteria in a fermented wet amaranth slurry during 3 weeks of cold storage (6 °C). Slurries of amaranth and milk showed a higher bacterial population when compared to those prepared with amaranth and water. Initially, in the milk- and water-based amaranth slurries, there was around 8.8 log (CFU)/ml of *Lactobacillus rhamnosus GG*. This value remains almost unchanged for water-based and dropped to approximately 7.8 log (CFU)/ml for milk-based slurries after the storage period.

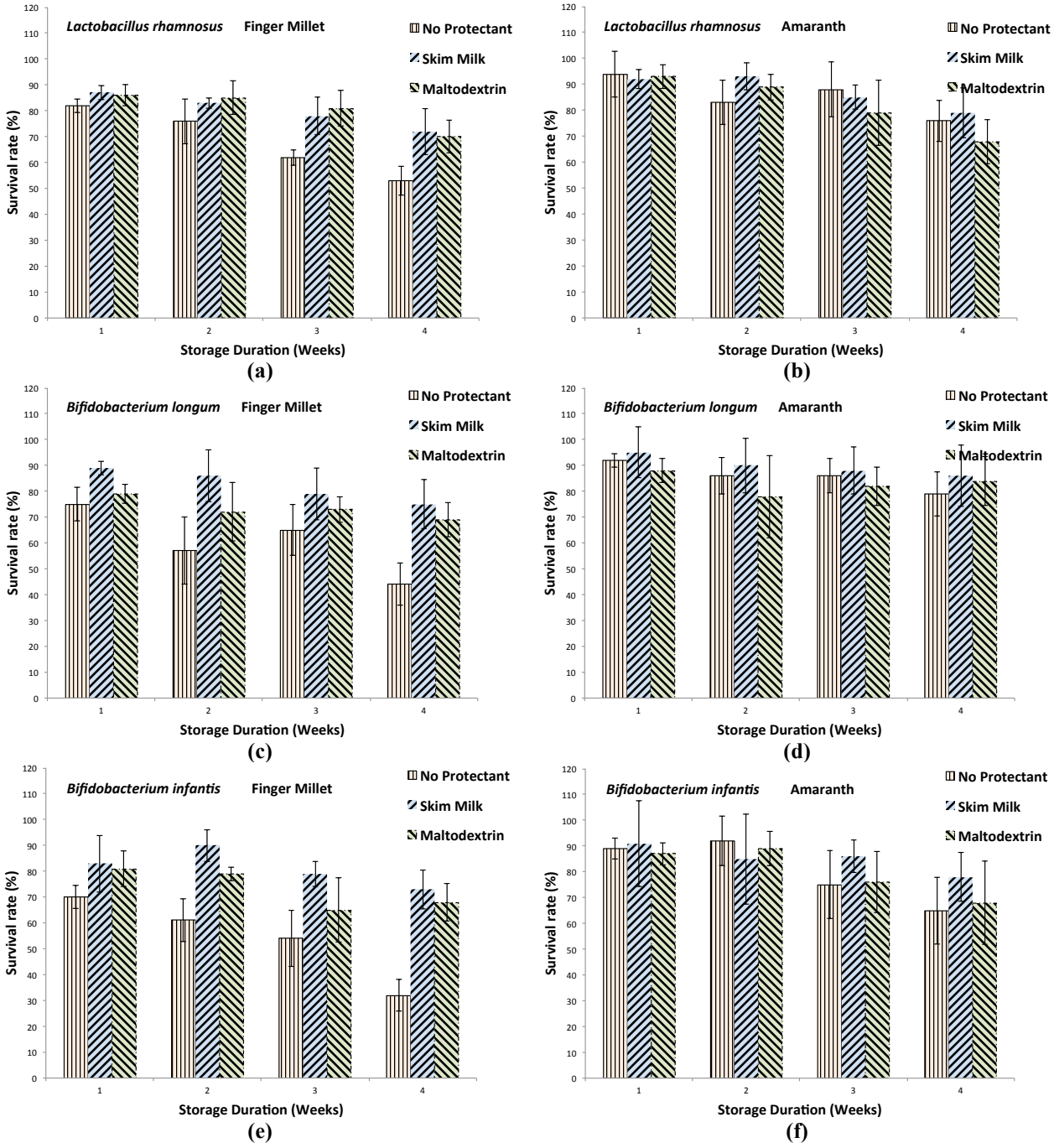

Figure 7-4: Bacterial survival rate (%) during storage: (a), (c), and (e) for finger millet (b), (d), and (f) for amaranth.

In another study, amaranth and millet substrates were fermented using *Lactobacillus rhamnosus GG* for 10 hours at 37 °C. Following the fermentation step, the fermented

slurries were stored for 21 days at 5 °C. The bacterial count dropped from 7.96 to 6.70, 8.02 to 7.79, and 7.83 to 7.60 log (CFU)/g for amaranth flour, amaranth grain, and millet grain, respectively (Kocková et al., 2013a).

7.3.3 Statistical analysis

Following the method described in section 7.2.2, ANOVA was performed on the obtained data. For the fermentation stage, duration was considered as a continuous variable, while grain and bacterial types were categorized or discrete variables. While linear correlations and dependencies were assumed for the selected discrete variables, non-linear dependencies (third order polynomials) were considered for the fermentation duration. This is due to the observed data pattern in Figure 7-1, where the bacterial population increased, maximized, and then remained constant or decreased throughout the fermentation. Based on the performed analysis, t Ratio and p values, grain and bacterium selection were considerably influential ($p<0.0001$). Second and third order dependencies on fermentation duration were significant ($p<0.0001$ and $p<0.005$, respectively) as well as grain×bacteria term ($p<0.001$). Other dependencies were not found to be significant.

In Figure 7-5, the selected results are presented based on the performed analysis. Figure 7-5 indicates that the proposed response surface appropriately predicted the actual raw data ($p<0.0001$). The obtained relatively high $R^2 = 0.9$ reinforced the accuracy of proposed regression curves and constructed response surface. In Figure 7-5 (b), (c), and (d) the correlations between two-selected independent parameters on the dependent variable (bacterial growth) are presented. These interpolator surfaces were obtained by averaging the data over the third independent variable. For instance, in Figure 7-5 (b), correlations

between the grain type and fermentation duration are shown. For each combination (grain and duration), all the available raw data are presented as black circles. Those include the data of three selected bacterial types and the experimental replicates. The constructed response surfaces pass through these data points trying to minimize the defined RMS error. This figure clearly indicates that while the bacterial growth was appropriately performed for both grains, finger millet achieved slightly higher bacterial population after 24 hrs of fermentation when comparing to amaranth.

In Figure 7-5 (c), the bacterial type and fermentation duration were selected as the independent variables and the averaging was over the grain type. It was observed that *Lactobacillus rhamnosus* grown to higher populations when comparing to *Bifidobacterium longum* and *Bifidobacterium infantis*. Finally, in Figure 7-5 (d), the bacterial and grain types were selected as the independent variables and the averaging was over the fermentation duration. Confirming the currently mentioned statements, results clearly illustrated that finger millet was a better substrate compared to amaranth for bacterial growth, where *Lactobacillus rhamnosus* showed the highest attainable CFU/g. On the other hand, the average *Bifidobacterium infantis* growth in the amaranth flour was relatively the lowest obtained population.

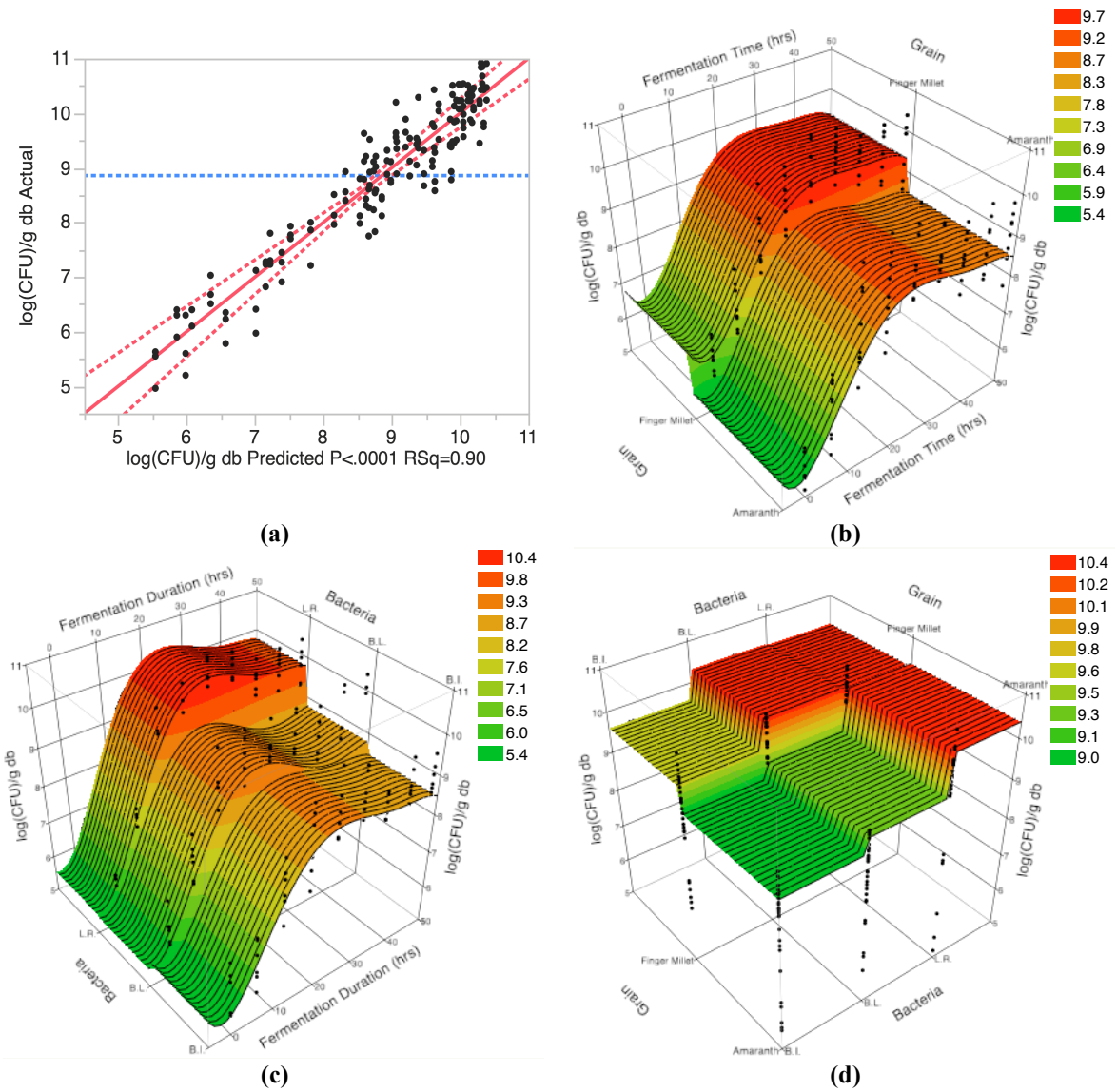

Figure 7-5: ANOVA on data obtained through fermentation stage. (a) actual by predicted plot, bacterial population during fermentation vs grain type (b) and bacterial type (c), and their combination (d).

Similar to the bacterial population, the obtained data for phytic acid and oxalate contents were analyzed (Figure 7-6). Again, obtaining high R^2 values for the constructed interpolation surfaces indicated the good prediction of the actual data. In Figure 7-6 (c) and (d), the independent variables were selected as grain type and fermentation duration and the dependent variables were phytic acid and oxalate, respectively. The averaging was

between the data of the three investigated bacteria multiplied by the sample replicates. It was observed that for both grains, oxalate reduced almost linearly with fermentation duration, where this reduction was higher for amaranth slurries.

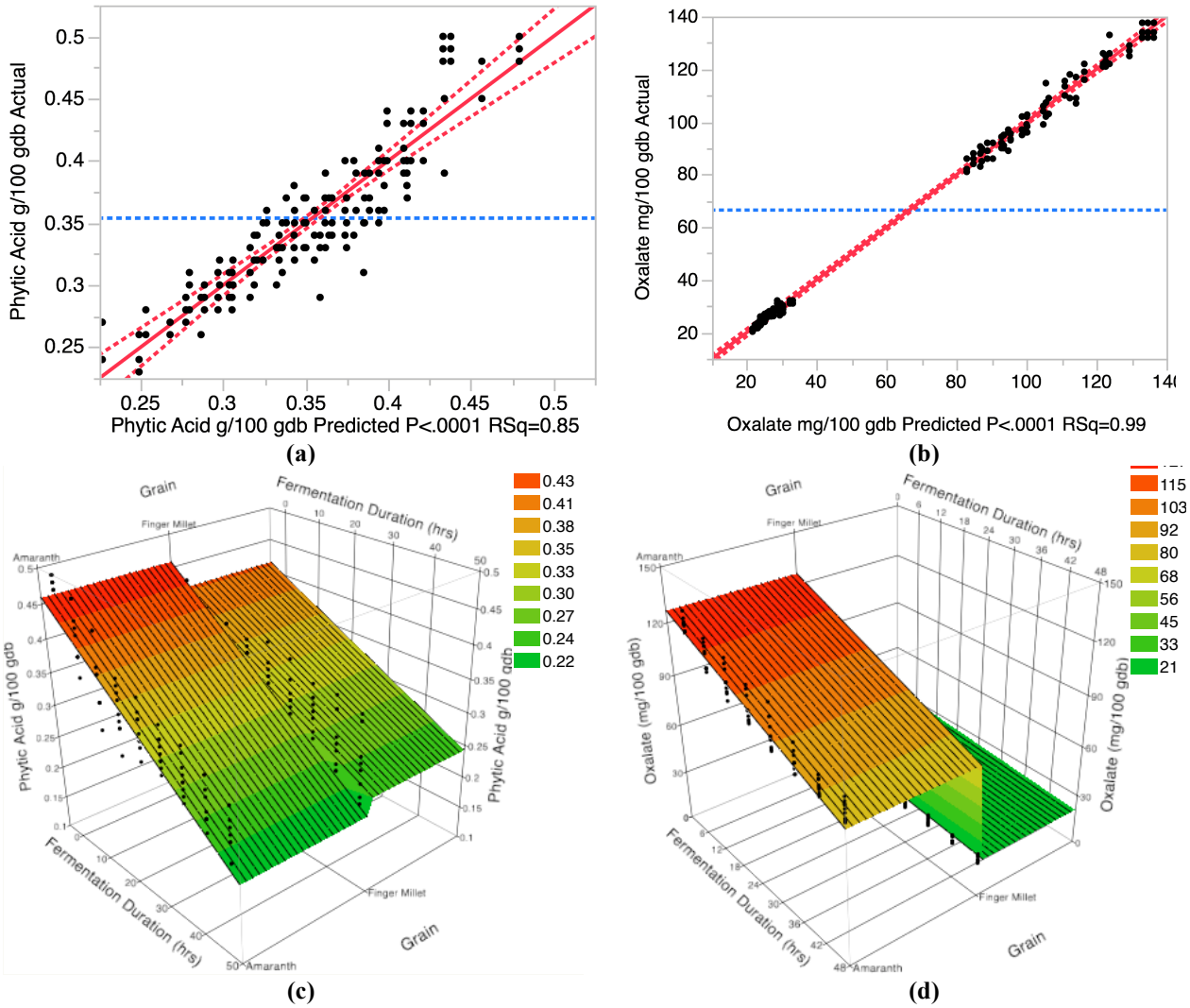

Figure 7-6: ANOVA on data obtained through fermentation stage, actual by predicted plots for phytic acid (a) and oxalate (b), and reduction of phytic acid (c) and oxalate (d) contents during fermentation.

For the freeze-drying stage, possible correlations between the three independent categorized variables (grain, bacteria, and protectant types) and bacterial survival rate (%) were investigated. Based on the performed experimental design and obtained data, the

following conclusions were observed (Figure 7-7). Among the selected protectant, skim milk notably enhanced the bacterial survival rate (Figure 7-7 (b)). As for the grain type, amaranth kept the bacterial vitalities at the higher end when compared to finger millet (Figure 7-7 (c)). *Lactobacillus rhamnosus* and *Bifidobacterium longum* showed a higher survival rate when compared to *Bifidobacterium infantis* (Figure 7-7 (d)).

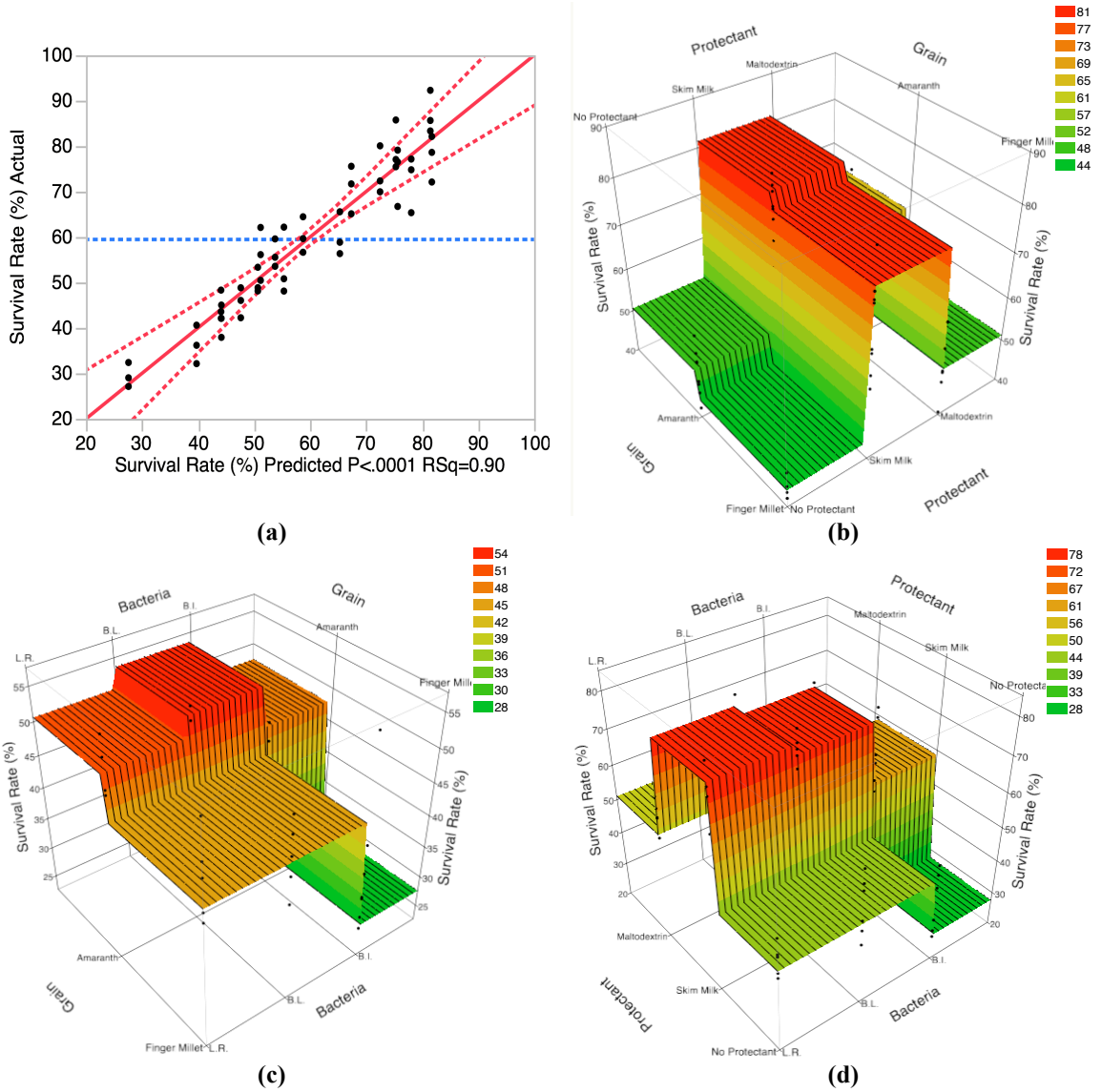

Figure 7-7 ANOVA on data obtained through freeze-drying stage, (a) actual by predicted plot for survival rate (%), and combined effect of (b) protectant-grain (c) bacterium-grain and (d) bacterium-protectant on bacterial survival rate.

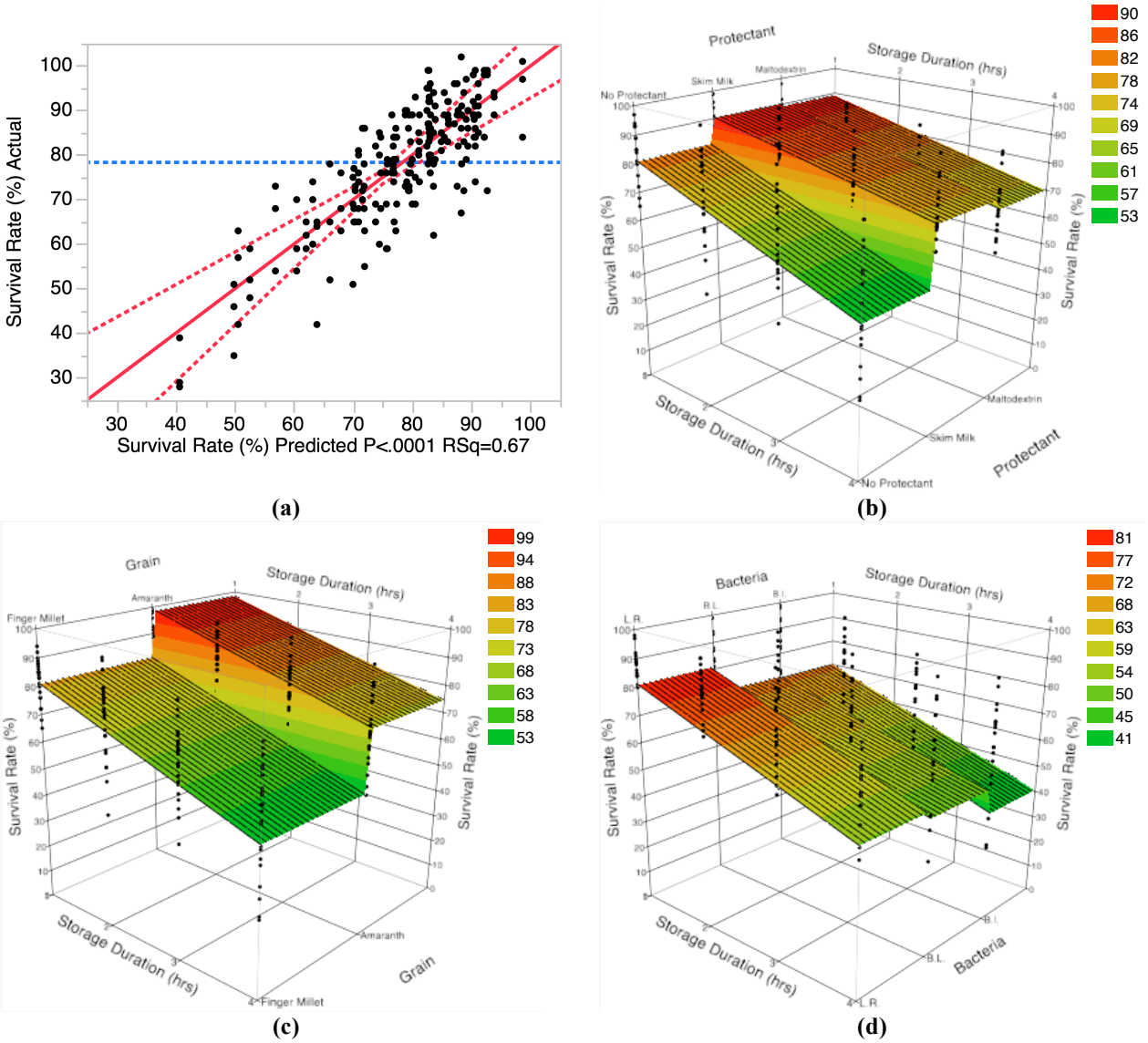

Figure 7-8: ANOVA on data obtained through the storage stage, (a) actual by predicted plot for survival rate (%), effect of (b) protectant (c) grain and (d) bacterium on bacterial survival rate.

As it was mentioned in section 7.2.2, to study the bacterial survival rate (%) during the storage stage, one continuous variable, storage duration, and three discrete or categorized variables, grain, bacteria, and protectant types were considered. Based on the ANOVA study, linear correlations between the independent and dependent variables were

exposed. The results are presented in Figure 7-8 and Figure 7-9. Despite obtaining a relatively low $R^2 = 0.67$ (Figure 7-8 (a)), the following general statements were concluded.

From Figure 7-8 (b) it was concluded that skim milk and maltodextrin improved the bacterial vitality to a higher extent when compared to having the millet and amaranth grains as the sole protectant (no extra protectant). Figure 7-8 (c) indicated that amaranth had a higher survival rate throughout the storage study. Furthermore, *Lactobacillus rhamnosus* and *Bifidobacterium longum* species maintained higher populations when compared with *Bifidobacterium infantis* (Figure 7-8 (d)).

In Figure 7-9, the effects of combined categorized variables are presented, where the averaging was over the samples throughout all storage steps and their replicates. Figure 7-9 (a) indicated that for amaranth, survival rates were generally higher than those for finger millet. In addition, while for amaranth the benefits from having extra protectant (skim milk or maltodextrin) were not significant, a notable enhancement was observed for finger millet slurries that benefitted from the protectant during their freeze-drying and storage steps (Figure 7-9 (b)). Finally, among the protectants, skim milk showed better characteristics in preserving bacterial survival rate when compared with maltodextrin.

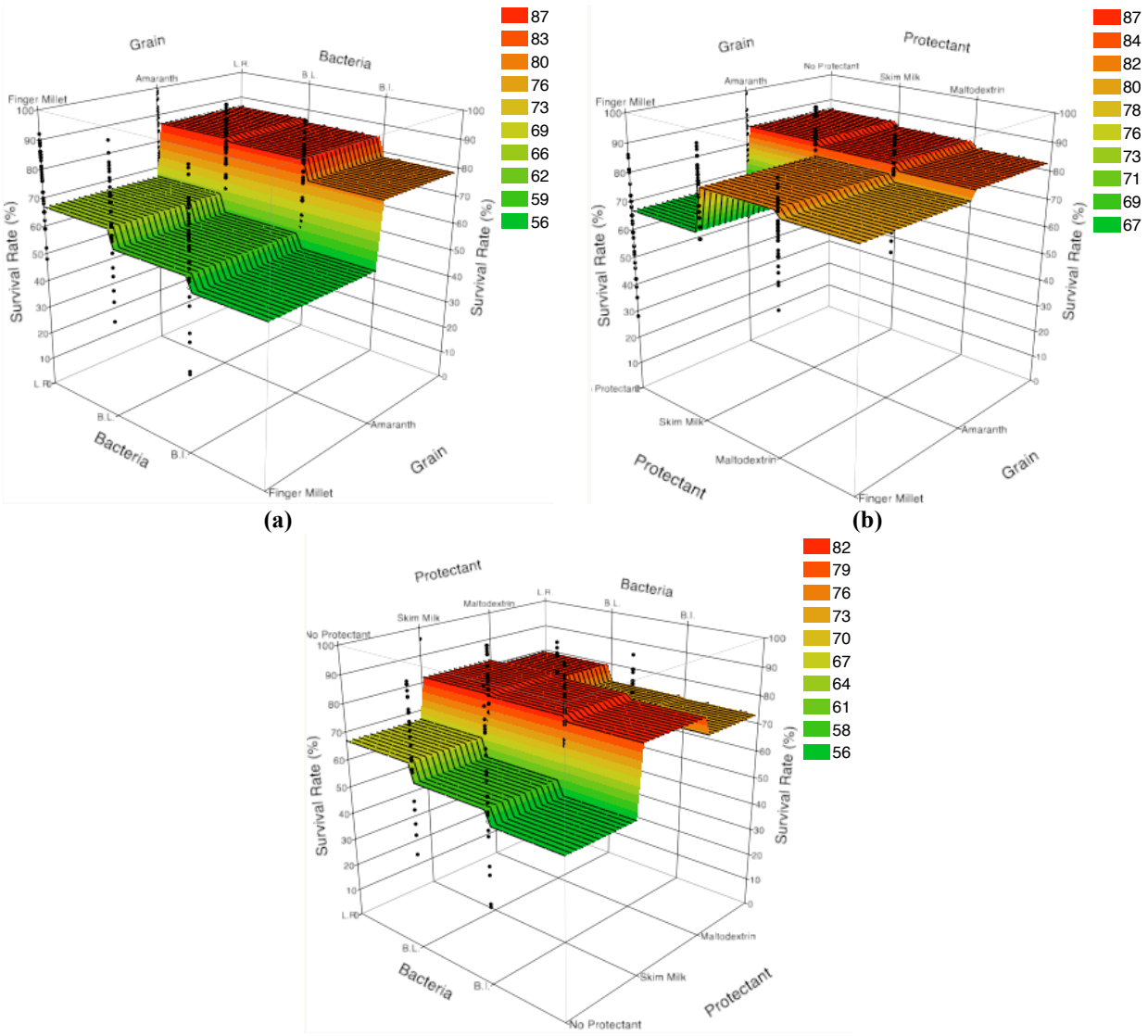

Figure 7-9: Bacterial survival rate (%) during storage: combined effect of (a) bacterium-grain (b) protectant-grain (c) bacterium-protectant.

7.4 Conclusion

Probiotic fermentations of amaranth and finger millet malted flours were performed for 48 hr at 37 °C using three bacterial strains, *Lactobacillus rhamnosus, Bifidobacterium longum*, and *Bifidobacterium infantis*. It was observed that fermentation of the flours for 24 hr maximized bacterial counts. While for both grains, all three bacterial strains appropriately grew, slightly higher CFU values were obtained for finger millet being

fermented with *Lactobacillus rhamnosus*, where approximately 4 Log (CFU)/g increase in the bacterial count was observed. There was almost a 50% loss in the viable bacteria for the fermented slurries after freeze-drying. The loss was slightly higher in the case of finger millet. Potential beneficial effects of two common protectants, skim milk and maltodextrin, on the enhancement of bacterial survival rate were assessed. Skim milk showed a notable influence for bacterial preservation, especially in the case of finger millet slurries, where viable bacteria almost doubled. After 4-weeks of storage at 4 °C the minimum bacterial count among all samples was assessed around 10^8 CFU/g db, which was appropriately higher than the required bacterial counts (10^7 CFU/g or mL) for the manufacture of probiotic food products.

7.5 Acknowledgement

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8 General thesis conclusion

In the present study, the suitability of finger millet and amaranth, to be consumed as the main ingredient flours in the industrial production of weaning foods, has been explored. Enhancement of nutrient contents, especially protein bio-availability and digestibility, reduction of anti-nutrient contents, especially phytic acid, tannin, and oxalate, and inclusion and preservation of probiotic cultures in the final product were the main research objectives. These objectives were successfully met after a thorough parametric study of the main involved food processing operating parameters; malting, probiotic fermentation, freeze-drying, and storage. For each processing stage, based on the involved design parameters, appropriate experimental designs were conducted and corresponding ANOVA was performed.

For the malting process, two main design factors were considered, germination duration and germination temperature. Based on a central composite design, for each factor, three sampling levels were selected; 24, 36, and 48 hr as for duration and 22, 26, and 30 °C for temperature. The ranges were selected in a way that industrial energy saving requirements, proper grain enzymatic activities, and malting efficiency could be considered. Having three sampling levels for each factor enabled a second-order interpolation response surface to be constructed, where optimum conditions (minimum, maximum, or saddle points) may exist within the specified range of factor variations.

Through germination, notable reductions in the monitored anti-nutrient contents were observed. These reductions resulted into a corresponding enhancement in the nutrients bio-availabilities, mainly protein. *In-vitro* protein digestion of finger millet was

enhanced by 17% after 48 hr of germination at 30 °C (chapter 3). Furthermore, protein digestibly enhanced from its initial (76%) to the final (84%) in amaranth after 48 hr of germination at 26 °C (chapter 4). In a similar manner, other nutritional properties such as changes in resistant, soluble, and total starch, total protein, fiber, fat and fatty acid profile, and ash were monitored through the performed malting process (chapter 5). For the same obtained optimum germination conditions, resistance starch decreased by 70% and 60%, for finger millet and amaranth sprouts, respectively. In addition, the calorie content was enhanced roughly by 10% as well. For finger millet, malting at the proposed optimum condition notably decreased phytic acid, tannin, and oxalate contents by 45%, 46%, and 29%, respectively. Likewise, for amaranth, phytic acid and oxalate reduced by 30% and 38%, respectively. However, tannin content enhanced by 47%. Through regression curves, linear correlations between the observed enhancement in the protein digestibility and the monitored anti-nutrient contents were obtained.

Alterations in total phenol concentration, as well as DPPH and ABTS activities of the malted flours as natural functional properties and antioxidant characteristics were evaluated (chapter 6). Both temperature and duration factors were found to be significantly influential on the monitored quantities. While at the optimum treatment point, total phenol content showed a four-time increase for amaranth, a 25% reduction was observed for finger millet. Again, strong linear correlations between the monitored quantities, total phenolic contents and DPPH and ABTS activities, were observed.

For cereal grains, fermentation process was applied to preserve ingredients for longer time, reduce anti-nutrient contents, improve nutrient profiles, and provide more acceptable taste and texture. Therefore, the obtained optimized malted finger millet and

amaranth flours were selected for the next proposed downstream food processing step of probiotic fermentation. As for the experimental design, a full factorial approach was selected, containing one continuous factor, fermentation duration (9 levels; 0 to 48 hr over 6 hr intervals), and two categorized variables, grain type (2 levels; finger millet and amaranth), and probiotic type (3 levels; *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Bifidobacterium infantis)*. Both flours were found as proper substrates for the growth of the inoculated probiotic cultures. Linear correlations and dependencies were assumed among variables, except for fermentation duration, where non-linear dependencies (third order polynomials) were considered. This was due to the fact of having, lag, log, stationary, and death phases in the bacterial growth behavior. After approximately 6 Log (CFU)/g db of initial bacterial inoculation, their counts increased roughly by 4 Log (CFU)/g db between 18 to 24 hr of fermentation. Finger millet showed slightly higher levels for bacterial growth when compared to amaranth. Besides, among studied bacteria, *Lactobacillus rhamnosus* performed better, followed by *Bifidobacterium longum*, and *Bifidobacterium infantis,* respectively. During the fermentation, the observed reduction in pH and enhancement of some enzymatic activities resulted into further reduction of monitored anti-nutrients, phytic acid, and oxalate. These anti-nutrients were approximately reduced by 30%, which means they experienced notable reductions after the combined performed processing treatments of germination and fermentation.

To keep the bacteria in the quiescent or stationary state, the fermented slurries need to be appropriately dried. As long as the moisture content can be reduced, the survival of probiotic cultures, especially during the storage period would be enhanced. Among different drying approaches, freeze-drying has been widely employed for probiotic

cultures. Nevertheless, to further enhance bacterial survival during drying and storage stages, extra-protectants have been usually included in the fermented slurries. In this study, a full factorial design was used for the drying stage with 3 categorized variables; grain type (2 levels), bacterial type (3 levels), and protectant type (3 levels; no extra protectant, skim milk, and maltodextrin). The same methodology was used to design the storage stage, where in addition to above factors and their corresponding levels, storage duration as a continuous factor with 5 levels (0 to 4 weeks) was considered. It was concluded that amaranth showed better drying and storage characteristics in terms of preserving bacterial vitalities. This may be due to its higher fat content when compared to finger millet. Therefore, although the studied protectant enhanced bacterial survival rates, their consumption was not a critical need. On the other hand, protectants, especially skim milk, almost doubled bacterial survivals in finger millet slurries. For all design factor combinations, the bacterial count after the storage stage is appropriately higher than the minimum requirements for a food product to bring probiotic benefits to the consuming host.

Taking into account that applying a number of simple and traditional food processing steps could improve the nutritional quality of cereal grains, functional properties of finger millet and amaranth grains were enhanced to be included in value-added products. As a result, new products based on processed finger millet and amaranth flours like weaning foods could be successfully manufactured at the industrial scale. This would have a positive effect in promoting these forgotten grains as nutritious and applicable raw materials for the food industry.

Contribution to knowledge

The current research study explored thoroughly the suitability of processed finger millet and amaranth, as gluten-free grains, to be applied in industrial probiotic weaning food products. This potential was assessed after employing a number of food processing stages and developing a deeper understanding of their impact on the grains' nutritional improvement. The contributions to knowledge of the present study are summarized as follows:

1- Malting/germination of finger millet and amaranth was studied and optimized for the two main independent factors, germination duration and temperature. This phase of the research highlighted the possibility for enhancement of the nutritional quality and characteristics of the malted flours for an optimized time and temperature combination.

2- The optimized malting/germination of these grains successfully reduced the anti-nutrient contents and improved their *in-vitro* protein digestibility.

3- The obtained data, which illustrated the variation of the phenolic contents and antioxidant activities of the studied grains, finger millet and amaranth, with respect to malting treatments would be beneficial to initiate further investigation for ultimate utilization of these processed flours in value-added functional products. Nowadays, considering the awareness of consumers, these grains could be promoted in functional food sector of the marketplace.

4- The increase in linoleic acid, as an unsaturated fatty acid, in both finger millet and amaranth grains during the proposed optimized malting treatment is a promising aspect. Since linoleic acid is a key fatty acid for mental growth and development of

infants, this outcome could open a new door in production of fat resources that are enriched in natural unsaturated fatty acids, especially in weaning products.

5- The performed probiotic fermentation as an important food process step would be highly beneficial for the development of qualitatively elevated finger millet and amaranth flours. The obtained data, especially the monitored growth patterns of *Lactobacillus rhamnosus*, *Bifidobacterim logum*, and *Bifidobacterim infantis* in these flour substrates were not completely available in the literature. Measuring the optimum fermentation duration for millet and amaranth malts strengthens the suitability of these grains as potentially appropriate carriers for inoculation of probiotic bacteria. The explained processes can open a way for generation of new processed cereal flours in producing probiotic products, especially in weaning industry, where their probiotic characteristics can improve the immature gastrointestinal microflora of infants.

6- Probiotic fermentation was also found as an effective method to reduce the anti-nutrient contents, especially phytic acid and oxalate. By oxalate reduction, the probability of formation of renal stone creation would be reduced. Besides, phytic acid reduction enhanced the overall nutrient availability and mineral extractabilities.

7- Finally, knowledge of effects of using cryoprotectant for bacterial preservation through the drying and storage stages have not been appropriately addressed in the literature for finger millet and amaranth malts. This study brings new knowledge about these industrially important aspects.

Recommendations for future research

Although in the present research study, the suitability of both processed finger millet and amaranth to substitute wheat and rice flours in the current industrial weaning products was examined and confirmed, the commercial production parameters will require additional investigations. Several recommendations for future possible studies are presented here:

1- As the first next step, an animal model design is required to explore the impact of a diet enriched in malted and probiotic fermented finger millet and amaranth, as a source of energy and their short and long term metabolic effects, on the growth, development and physical activities. The animal model should be guinea pigs due to their striking similarities to humans in terms of hepatic cholesterol and lipoprotein metabolism. They respond to dietary factors, physical activity, and metabolic responses mimicking the infants' situation.

2- Consequently, after implementing animal models and *in-vivo* research for the health impact of these flours, clinical studies are required to verify the beneficial effects of probiotics-added-flours in human gut flora and in promoting health in infants.

3- Sensory evaluation of the resulted flours in terms of infants' acceptability and their parents' satisfaction must be investigated.

4- In the present study freeze-drying has been used in drying of probiotic fermented slurries and their survival rate were monitored. Further drying

approaches, especially spray drying as a lower cost operation, is required to verify the potential of these flours as a suitable carrier for inoculated probiotics.

References

AACC. 1999. Approved Methods of the American Association of the Cereal Chemists

Abdelrahaman, S.M., Elmaki, H.B., Idris, W.H., Hassan, A.B., Babiker, E.E., El Tinay, A.H. 2007. Antinutritional factor content and hydrochloric acid extractability of minerals in pearl millet cultivars as affected by germination. *International journal of food sciences and nutrition*, **58**(1), 6-17.

Abratt, V.R., Reid, S.J. 2010. Oxalate-degrading bacteria of the human gut as probiotics in the management of kidney stone disease. *Advances in applied microbiology*, **72**, 63-87.

Ahmadzadeh Ghavidel, R., Prakash, J. 2010. Composite weaning mixes: formulation and quality characteristics. *Food science and technology research*, **16**(1), 65-70.

Al-Othman, A.M., Khan, M.A., Al-Kanhal, M. 1997. Nutritional evaluation of some commercial baby foods consumed in Saudi Arabia. *International journal of food sciences and nutrition*, **48**(4), 229-236.

Alvarez-Jubete, L., Arendt, E.K., Gallagher, E. 2010a. Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. *Trends in food science and technology*, **21**(2), 106-113.

Alvarez-Jubete, L., Wijngaard, H., Arendt, E., Gallagher, E. 2010b. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food chemistry*, **119**(2), 770-778.

Amalraj, A., Pius, A. 2015. Influence of oxalate, phytate, tannin, dietary fiber and cooking on calcium bioavailability of commonly consumed cereals and millets in India. *Cereal chemistry*, **92**(4), 389-394.

Anekella, K. 2011. Microencapsulation of probiotics (Lactobacillus acidophilus and Lactobacillus rhamnosus) in raspberry powder by spray drying: optimization and storage stability studies, McGill University.

Anigo, K., Ameh, D., Ibrahim, S., Danbauchi, S. 2009. Nutrient composition of commonly used complementary foods in North western Nigeria. *African Journal of Biotechnology*, **8**(17).

Anigo, K.M.A., D. A. ; brahim, S. I; Danbauchi, S. S. . 2010. Nutrient composition of complementary food gruels formulated from malted cereals, soybeans and groundnut for use in North-western Nigeria. *African journal of food science*, **4**(3).

Antony, U., Chandra, T. 1998. Antinutrient reduction and enhancement in protein, starch, and mineral availability in fermented flour of finger millet (Eleusine coracana). *Journal of Agricultural and Food Chemistry*, **46**(7), 2578-2582.

Antony, U., Chandra, T. 1999. Enzymatic treatment and use of starters for the nutrient enhancement in fermented flour of red and white varieties of finger millet (Eleusine coracana). *Journal of agricultural and food chemistry*, **47**(5), 2016-2019.

Antony, U., Chandra, T.S. 1997. Microbial population and biochemical changes in fermenting finger millet (Eleusine coracana). *World journal of microbiology and biotechnology*, **13**(5), 533-537.

Arendt, E., Dal Bello, F. 2011. *Gluten-free cereal products and beverages*. Access Online via Elsevier.

Arora, S., Jood, S., Khetarpaul, N. 2011. Effect of germination and probiotic fermentation on nutrient profile of pearl millet based food blends. *British food journal*, **113**(4), 470-481.

Asao, M., Watanabe, K. 2010. Functional and bioactive properties of quinoa and amaranth. *Food science and technology research*, **16**(2), 163-168.

Asquith, T.N., Butler, L.G. 1986. Interactions of condensed tannins with selected proteins. *Phytochemistry*, **25**(7), 1591-1593.

Avanza, M., Puppo, M., Añón, M. 2005. Rheological characterization of amaranth protein gels. *Food hydrocolloids*, **19**(5), 889-898.

Azcarate-Peril, M.A., Altermann, E., Goh, Y.J., Tallon, R., Sanozky-Dawes, R.B., Pfeiler, E.A., O'Flaherty, S., Buck, B.L., Dobson, A., Duong, T. 2008. Analysis of the genome sequence of Lactobacillus gasseri ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism. *Applied and environmental microbiology*, **74**(15), 4610-4625.

Badau, M.H., Jideani, I.A., Nkama, I. 2006. Production, acceptability and microbiological evaluation of weaning food formulations. *Journal of tropical pediatrics*, **52**(3), 166-172.

Bakari, M., Ngadi, M. 2013. Optimization of the energy value determination method for rice husk using bomb calorimeter. in: *ASABE 2013*, American Society of Agricultural and Biological Engineers. St. Joseph, Michigan.

Balasubramanian, T., Sadasivam, S. 1989. Changes in carbohydrate and nitrogenous components and amylase activities during germination of grain amaranth. *Plant foods for human nutrition*, **39**(4), 325-330.

Baldeon, M.E., Naranjo, G., Granja, D. 2008. Effect of infant formula with probiotics on intestinal microbiota. *Arch Latinoam Nutr*, **58**(1), 5-11.

Banerjee, S., Sanjay, K., Chethan, S., Malleshi, N. 2012. Finger millet (Eleusine coracana) polyphenols: Investigation of their antioxidant capacity and antimicrobial activity. *African journal of food science*, **6**(13), 362-374.

Barker, D.J. 1995. Fetal origins of coronary heart disease. *BMJ: British Medical Journal*, **311**(6998), 171.

Bavec, F., Bavec, M. 2006. *Organic production and use of alternative crops*. CRC Press.

Becker, R., Wheeler, E., Lorenz, K., Stafford, A., Grosjean, O., Betschart, A., Saunders, R. 1981. A compositional study of amaranth grain. *Journal of food science*, **46**(4), 1175-1180.

Belton, P.S., Taylor, J.R. 2002a. *Pseudocereals and less common cereals: grain properties and utilization potential*. Springer.

Belton, P.S., Taylor, J.R.N. 2002b. *Pseudocereals and less common cereals: grain properties and utilization potential*. Springer verlag.

Berganza, B.E., Moran, A.W., Rodríguez, G.M., Coto, N.M., Santamaría, M., Bressani, R. 2003. Effect of variety and location on the total fat, fatty acids and squalene content of amaranth. *Plant foods for human nutrition*, **58**(3), 1-6.

Berger, A., Gremaud, G., Baumgartner, M., Rein, D., Monnard, I., Kratky, E., Geiger, W., Burri, J., Dionisi, F., Allan, M. 2003. Cholesterol-lowering properties of amaranth grain and oil in hamsters. *International journal for vitamin and nutrition research*, **73**(1), 39-47.

Berghofer, E., Schoenlechner, R. 2002. Grain amaranth. in: *Pseudocereals and less common cereals*, (Eds.) p. Belton, J. Taylor, Springer, pp. 219-260.

Blandino, A., Al-Aseeri, M., Pandiella, S., Cantero, D., Webb, C. 2003. Cereal-based fermented foods and beverages. *Food research international*, **36**(6), 527-543.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, **72**(1), 248-254.

Bressani, R., De Martell, E., De Godinez, C. 1993. Protein quality evaluation of amaranth in adult humans. *Plant foods for human nutrition*, **43**(2), 123-143.

Bressani, R., Garcia-Vela, L.A. 1990. Protein fractions in amaranth grain and their chemical characterization. *Journal of agricultural and food chemistry*, **38**(5), 1205-1209.

Brown, K.H., Creed-Kanashiro, H., Dewey, K.G. 1995. Optimal complementary feeding practices to prevent childhood malnutrition in developing countries. *Food and Nutrition Bulletin*, **16**(4), 320-339.

Bruni, R., Medici, A., Guerrini, A., Scalia, S., Poli, F., Muzzoli, M., Sacchetti, G. 2001. Wild amaranthus caudatus seed oil, a nutraceutical resource from Ecuadorian flora. *Journal of agricultural and food chemistry*, **49**(11), 5455-5460.

Busolo, D. 1992. Utilization of sorghum, millet and amaranth for the development of high energy weaning foods for Kenyan pre-school children.

Capriles, V., Coelho, K., Guerra-Matias, A., Arêas, J. 2008. Effects of processing methods on amaranth starch digestibility and predicted glycemic index. *Journal of food science*, **73**(7), H160-H164.

Caselato, V.M., Amaya, J. 2012. State of knowledge on amaranth grain: a comprehensive review. *Journal of food science*, **77**(4), R93-R104.

Celik, O., O'Sullivan, D. 2013. Factors influencing the stability of freeze-dried stress-resilient and stress-sensitive strains of bifidobacteria. *Journal of dairy science*, **96**(6), 3506-3516.

Champagne, C., Gardner, N., Brochu, E., Beaulieu, Y. 1991. The Freeze-drying of lactic acid bacterial. A review. *Canadian institute of food science and technology journal*, **24**(3-4), 118-128.

Champagne, C.P., Raymond, Y., Simon, J.P. 2012. Effect of water activity and protective solutes on growth and subsequent survival to air-drying of Lactobacillus and Bifidobacterium cultures. *Applied microbiology and biotechnology*, **95**(3), 745-756.

Chandrasekara, A., Shahidi, F. 2012. Bioaccessibility and antioxidant potential of millet grain phenolics as affected by simulated in vitro digestion and microbial fermentation. *Journal of functional foods*, **4**(1), 226-237.

Chandrashekar, A. 2010. Finger millet: Eleusine coracana. *Advances in food and nutrition research*, **59**, 215-262.

Charalampopoulos, D., Wang, R., Pandiella, S., Webb, C. 2002. Application of cereals and cereal components in functional foods: a review. *International Journal of Food Microbiology*, **79**(1), 131-141.

Chaturvedi, A., Sarojini, G. 1996. Malting of pearl millet (Pennisetum typhoideum): Its effect on starch and protein digestibilities. *Journal of food science and technology*, **33**(4), 342-344.

Chaturvedi, N., Sharma, P., Shukla, K., Singh, R., Yadav, S. 2011. Cereals Nutraceuticals, Health Ennoblement and Diseases Obviation: A Comprehensive Review. *Journal of applied pharmaceutical science*, **1**(07), 06-12.

Chavan, J., Kadam, S., Beuchat, L.R. 1989. Nutritional improvement of cereals by sprouting. *Critical reviews in food science & nutrition*, **28**(5), 401-437.

Chávez, B., Ledeboer, A. 2007. Drying of probiotics: optimization of formulation and process to enhance storage survival. *Drying technology*, **25**(7-8), 1193-1201.

Chethan, S., Malleshi, N. 2007. Finger millet polyphenols: Optimization of extraction and the effect of pH on their stability. *Food chemistry*, **105**(2), 862-870.

Chiba, Y., Bryce, J.H., Goodfellow, V., MacKinlay, J., Agu, R.C., Brosnan, J.M., Bringhurst, T.A., Harrison, B. 2012. Effect of germination temperatures on proteolysis of the gluten-free grains sorghum and millet during malting and mashing. *Journal of agricultural and food chemistry*, **60**(14), 3745-3753.

Chiriboga, J. 1963. Some properties of an oxalic oxidase purified from barley seedlings. *Biochemical and biophysical research communications*, **11**(4), 277-282.

Chlopicka, J., Pasko, P., Gorinstein, S., Jedryas, A., Zagrodzki, P. 2012. Total phenolic and total flavonoid content, antioxidant activity and sensory evaluation of pseudocereal breads. *LWT-food science and technology*, **46**(2), 548-555.

Church, D.C., Kellms, R.O., Stiner, R.J. 1983. Evaluation of feather and hair meals for ruminants. *Anim.Sci*, **57**, 495-5-2.

Coda, R., Di Cagno, R., Gobbetti, M., Rizzello, C.G. 2014. Sourdough lactic acid bacteria: exploration of non-wheat cereal-based fermentation. *Food microbiology*, **37**, 51-58.

Colmenares de Ruiz, A.S., Bressani, R. 1990. Effect of germination on the chemical composition and nutritive value of amaranth grain. *Cereal chemistry*, **67**, 519-522.

Crittenden, R., Playne, M.J. 1999. Prebiotics. *Handbook of probiotics and prebiotics, second edition*, 533-581.

Czerwiński, J., Bartnikowska, E., Leontowicz, H., Lange, E., Leontowicz, M., Katrich, E., Trakhtenberg, S., Gorinstein, S. 2004. Oat (Avena sativa L.) and amaranth (Amaranthus hypochondriacus) meals positively affect plasma lipid profile in rats fed cholesterol-containing diets. *The journal of nutritional biochemistry*, **15**(10), 622-629.

Daemen, A., Stege, H. 1982. The destruction of enzymes and bacteria during the spraydrying of milk and whey, 2: the effect of the drying conditions. *Netherlands milk and dairy journal*, **36**.

Dahiya, S., Kapoor, A.C. 1993. Nutritional evaluation of home-processed weaning foods based on low cost locally available foods. *Food chemistry*, **48**(2), 179-182.

de la Rosa, A.B., Fomsgaard, I.S., Laursen, B., Mortensen, A.G., Olvera-Martínez, L., Silva-Sánchez, C., Mendoza-Herrera, A., González-Castañeda, J., De León-Rodríguez, A. 2009. Amaranth (Amaranthus hypochondriacus) as an alternative crop for sustainable food production: Phenolic acids and flavonoids with potential impact on its nutraceutical quality. *Journal of cereal science*, **49**(1), 117-121.

Desai, A.D., Kulkarni, S.S., Sahoo, A., Ranveer, R., Dandge, P. 2010. Effect of supplementation of malted ragi flour on the nutritional and sensorial quality characteristics of cake. *Advance journal of food science and technology*, **2**(1), 67-71.

Desai, B.B. 2004. *Seeds handbook: processing and storage*. CRC press.

Desikachar, H. 1980. Development of weaning foods with high caloric density and low hot-paste viscosity using traditional technologies. *Food and nutrition bulletin*, **2**(4), 21-23.

Desikachar, H.S.R. 1982. Technology options for formulating weaning foods for the economically weaker segments of populations in developing countries. *Food and Nutrition Bulletin*, **4**(4).

Devi, P.B., Vijayabharathi, R., Sathyabama, S., Malleshi, N.G., Priyadarisini, V.B. 2011a. Health benefits of finger millet (eleusine coracana l.) polyphenols and dietary fiber: a review. *Journal of food science and technology*, 1-20.

Devi, P.B., Vijayabharathi, R., Sathyabama, S., Malleshi, N.G., Priyadarisini, V.B. 2011b. Health benefits of finger millet (eleusine coracana l.) polyphenols and dietary fiber: a review. *journal of food science and technology*.

Dharmaraj, U., Malleshi, N. 2011. Changes in carbohydrates, proteins and lipids of finger millet after hydrothermal processing. *LWT-Food Science and Technology*, **44**(7), 1636-1642.

du Toit, E., Vesterlund, S., Gueimonde, M., Salminen, S. 2013. Assessment of the effect of stress-tolerance acquisition on some basic characteristics of specific probiotics. *International journal of food microbiology*.

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

Elkhalifa, A.E.O., Bernhardt, R. 2010. Influence of grain germination on functional properties of sorghum flour. *Food chemistry*, **121**(2), 387-392.

Eneje, L.O., Aniaku, V.O., Onu, C.O. 2013. Effects of germination time and watering regime on the cold water extract and diastatic power of local cultivars of nigeria millet and sorghum malt. *Asian journal of microbiology, biotechnology and environmental sciences*, **15**(1), 1-4.

Escudero, N., De Arellano, M., Luco, J., Gimenez, M., Mucciarelli, S. 2004. Comparison of the chemical composition and nutritional value of Amaranthus cruentus flour and its protein concentrate. *Plant foods for human nutrition*, **59**(1), 15-21.

Evans, R., Ayensu, E.S. 1975. *Underexploited tropical plants with promising economic value*. National Academy of Sciences, Washington, D.C.

FAO. 1995a. *Sorghum and millets in human nutrition*. FAO.

FAO. 1995b. Sorghum and millets in human nutrition. *Food and nutrition Series*, **27**.

FAOSTAT. 2014. Food and Agriculture Organization of the United Nations, FAO. Rome, Italy.

Farooq, U., Mohsin, M., Liu, X., Zhang, H. 2013. Enhancement of Short Chain Fatty Acid Production from Millet Fibres by Pure Cultures of Probiotic Fermentation. *Tropical journal of pharmaceutical research*, **12**(2), 189-194.

Fasano, A., Catassi, C. 2011. Early feeding practices and their impact on development of celiac disease, Vol. 68, pp. 201-213.

Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *Journal of biological chemistery* **66**(2), 375-400.

Fukushima, M., Nakano, M. 1996. Effects of a mixture of organisms, Lactobacillus acidophilus or Streptococcus faecalis on cholesterol metabolism in rats fed on a fat- and cholesterol-enriched diet. *British journal of nutrition*, **76**(6), 857-868.

Gamel, T.H., Linssen, J.P., Mesallam, A.S., Damir, A.A., Shekib, L.A. 2006a. Effect of seed treatments on the chemical composition of two amaranth species: oil, sugars, fibres, minerals and vitamins. *Journal of the Science of Food and Agriculture*, **86**(1), 82-89.

Gamel, T.H., Linssen, J.P., Mesallam, A.S., Damir, A.A., Shekib, L.A. 2006b. Seed treatments affect functional and antinutritional properties of amaranth flours. *Journal of the Science of Food and Agriculture*, **86**(7), 1095-1102.

Gamel, T.H., Linssen, J.P., Mesallem, A.S., Damir, A.A., Shekib, L.A. 2005. Effect of seed treatments on the chemical composition and properties of two amaranth species: starch and protein. *Journal of the Science of Food and Agriculture*, **85**(2), 319-327.

Geetha, R., Virupaksha, T., Shadaksharaswamy, M. 1977. Relationship between tannin levels and in vitro protein digestibility in finger millet (Eleusine Coracana). *J Agric Food Chem*, **25**, 1101-1104.

Gélinas, B., Seguin, P. 2007. Oxalate in grain amaranth. *Journal of agricultural and food chemistry*, **55**(12), 4789-4794.

Gimbi, D.M., Kitabatake, N. 2002. Changes in alpha-and beta-amylase activities during seed germination of African finger millet. *International journal of food sciences and nutrition*, **53**(6), 481-488.

Goldin, B.R., Gorbach, S.L. 1992. Probiotics for humans. in: *Probiotics*, Springer, pp. 355-376.

Guandalini, S. 2007. The influence of gluten: Weaning recommendations for healthy children and children at risk for celiac disease, Vol. 60, pp. 139-151.

Guardamagna, O., Abello, F., Cagliero, P., Lughetti, L. 2012. Impact of nutrition since early life on cardiovascular prevention. *Italian Journal of Pediatrics*, **38**(1).

Gupta, C., Sehgal, S. 1992a. Protein quality of developed home made weaning foods. *Plant Foods for Human Nutrition*, **42**(3), 239-246.

Gupta, C., Sehgal, S. 1992b. Protein quality of formulated home made weaning foods. *Ecology of Food & Nutrition*, **28**(4), 319-325.

Hall, C.W. 1980. *Drying and storage of agricultural crops*. AVI Publishing Company Inc.

Hamad, A.M., Fields, M.L. 1979. Evaluation of the protein quality and available lysine of germinated and fermented cereals. *Journal of Food Science*, **44**(2), 456-459.

Hansen, M., Pedersen, B., Munck, L., Eggum, B.O. 1989. Weaning foods with improved energy and nutrient density prepared from germinated cereals. 1. Preparation and dietary bulk of gruels based on barley. *Food Nutr Bull*, **11**(2), 40-45.

Haros, M., Carlsson, N.-G., Almgren, A., Larsson-Alminger, M., Sandberg, A.-S., Andlid, T. 2009. Phytate degradation by human gut isolated Bifidobacterium pseudocatenulatum ATCC27919 and its probiotic potential. *International journal of food microbiology*, **135**(1), 7-14.

He, H.-P., Corke, H. 2003. Oil and squalene in amaranthus grain and leaf. *Journal of agricultural and food chemistry*, **51**(27), 7913-7920.

Hegde, P.S., Rajasekaran, N.S., Chandra, T. 2005. Effects of the antioxidant properties of millet species on oxidative stress and glycemic status in alloxan-induced rats. *Nutrition Research*, **25**(12), 1109-1120.

Hejazi, S.N., Orsat, V. 2015. Malting Process Effects on Antioxidant Activity of Finger Millet and Amaranth Grains. *Journal of Food Research*, **5**(1), 1.

Hejazi, S.N., Orsat, V., Azadi, B., Kubow, S. 2015. Improvement of the in vitro protein digestibility of amaranth grain through optimization of the malting process. *Journal of Cereal Science*.

Helland, M.H., Wicklund, T., Narvhus, J.A. 2004. Growth and metabolism of selected strains of probiotic bacteria in milk-and water-based cereal puddings. *International Dairy Journal*, **14**(11), 957-965.

Heller, K.J. 2001. Probiotic bacteria in fermented foods: product characteristics and starter organisms. *The American journal of clinical nutrition*, **73**(2), 374s-379s.

Helrich, K. 1990. *Official methods of Analysis of the AOAC. Volume 2*. Association of Official Analytical Chemists Inc.

Higl, B., Kurtmann, L., Carlsen, C.U., Ratjen, J., Först, P., Skibsted, L.H., Kulozik, U., Risbo, J. 2007. Impact of Water Activity, Temperature, and Physical State on the Storage Stability of Lactobacillus paracasei ssp. paracasei Freeze-Dried in a Lactose Matrix. *Biotechnology progress*, **23**(4), 794-800.

Hilu, K.W., De Wet, J. 1976. Domestication of Eleusine coracana. *Economic botany*, **30**(3), 199-208.

Horner, H.T., Cervantes-Martinez, T., Healy, R., Reddy, M.B., Deardorff, B.L., Bailey, T.B., Al-Wahsh, I., Massey, L.K., Palmer, R.G. 2005. Oxalate and phytate concentrations in seeds of soybean cultivars [Glycine max (L.) Merr.]. *Journal of agricultural and food chemistry*, **53**(20), 7870-7877.

Hulse, J.H., Laing, E.M., Pearson, O.E. 1980. *Sorghum and the millets: their composition and nutritive value*. Academic Press.

Irving, D.W., Betschart, A.A., Saunders, R.M. 1981. Morphological Studies on Amaranthus cruentus. *Journal of Food Science*, **46**(4), 1170-1174.

Ishibashi, N., Shimamura, S. 1993. Bifidobacteria: research and development in Japan. *Food Technology*, **47**.

Issoufou, A., Mahamadou, E.G., Guo-Wei, L. 2013. Millets: Nutritional composition, some health benefits and processing—A review. *Emirates Journal of Food and Agriculture*, **25**(7), doi: 10.9755/ejfa. v25i7. 12045.

Jayasinghe, M., Ekanayake, S., Nugegoda, D. 2013. Effect of different milling methods on glycaemic response of foods made with finger millet (Eucenea coracana) flour. *Ceylon Medical Journal*, **58**(4), 148-152.

Joshi, A., Rawat, K., Karki, B. 2008. Millet as" Religious Offering" for nutritional, ecological, and economical security. *Comprehensive Reviews in Food Science and Food Safety*, **7**(4), 369-372.

Kannan, S. 2010. Finger millet in nutrition transition: an infant weaning food ingredient with chronic disease preventive potential. *British Journal of Nutrition*, **104**(12), 1733-1734.

Kauffman, C.S., Weber, L.E. 1990. Grain amaranth. *Advanced in new crops. Timber Press. Portland, USA*, 127-139.

Kearney, L., Upton, M., Mc Loughlin, A. 1990. Enhancing the viability of Lactobacillus plantarum inoculum by immobilizing the cells in calcium-alginate beads

incorporating cryoprotectants. *Applied and Environmental Microbiology*, **56**(10), 3112-3116.

Khetarpaul, N., Chauhan, B. 1990. Effect of germination and fermentation on in vitro starch and protein digestibility of pearl millet. *Journal of Food Science*, **55**(3), 883-884.

Khetarpaul, N., Chauhan, B. 1989. Effect of germination and pure culture fermentation on HCl-extractability of minerals of pearl millet (Pennisetum typhoideum). *International Journal of Food Science & Technology*, **24**(3), 327-331.

Klimczak, I., Małecka, M., Pachołek, B. 2002. Antioxidant activity of ethanolic extracts of amaranth seeds. *Food/Nahrung*, **46**(3), 184-186.

Kocková, M., Dilongová, M., Hybenová, E. 2013a. Evaluation of Cereals and Pseudocereals Suitability for the Development of New Probiotic Foods. *Journal of Chemistry*, **2013**.

Kocková, M., Mendel, J., Valík, A., Šturdík, E., Valik, L.U. 2013b. Cereals and pseudocereals as substrates for growth and metabolism of a probiotic strain Lactobacillus rhamnosus GG. *Journal of Food and Nutrition Research*, **52**(1), 25-36.

Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T., Zilmer, M. 2003. Antioxidative probiotic fermented goats' milk decreases oxidative stress-mediated atherogenicity in human subjects. *British Journal of Nutrition*, **90**(02), 449-456.

Kumkum, R., Gowda, C., Khanam, A., Swamylingappa, B. 2013. Chemical, Functional and Nutritional Characteristics of Weaning Food Formulations. *Journal of Food Science and Technology Nepal*, **6**, 36-42.

Kunyanga, C.N., Imungi, J.K., Okoth, M.W., Biesalski, H.K., Vadivel, V. 2012. Total phenolic content, antioxidant and antidiabetic properties of methanolic extract of raw and traditionally processed Kenyan indigenous food ingredients. *LWT-Food Science and Technology*, **45**(2), 269-276.

Leon, K., Mery, D., Pedreschi, F., Leon, J. 2006. Color measurement in L∗ a∗ b∗ units from RGB digital images. *Food research international*, **39**(10), 1084-1091.

Ljungqvist, B.G., Mellander, O., Svanberg, U.S. 1981. Dietary bulk as a limiting factor for nutrient intake in pre-school children 1. A problem description. *Journal of Tropical pediatrics*, **27**(2), 68-73.

Loewus, F.A., Murthy, P.P. 2000. myo-Inositol metabolism in plants. *Plant Science*, **150**(1), 1-19.

Lombard, M.J., Labuschagne, I.L. 2013. Introducing solid foods. *South African Family Practice*, **55**(1), 56-58.

López, V.R.L., Razzeto, G.S., Giménez, M.S., Escudero, N.L. 2011. Antioxidant properties of Amaranthus hypochondriacus seeds and their effect on the liver of alcohol-treated rats. *Plant foods for human nutrition*, **66**(2), 157-162.

Lorri, W., Svanberg, U. 1993. Lactic acid-fermented cereal gruels: viscosity and flour concentration. *International journal of food sciences and nutrition*, **44**(3), 207-213.

Lu, H., Zhang, J., Liu, K.-b., Wu, N., Li, Y., Zhou, K., Ye, M., Zhang, T., Zhang, H., Yang, X. 2009. Earliest domestication of common millet (Panicum miliaceum) in East Asia extended to 10,000 years ago. *Proceedings of the National Academy of Sciences*, **106**(18), 7367-7372.

Madhu, A.N., Awasthi, S.P., Reddy, K., Prapulla, S.G. 2011. Impact of Freeze and Spray drying on the retention of probiotic properties of Lactobacillus fermentum: An in vitro evaluation model. *Int J Microbiol Res*, **2**(3), 243-251.

Mahadevappa, V.G., Raina, P.L. 1978. Nature of some Indian legume lipids. *Journal of Agricultural and Food Chemistry*, **26**(5), 1241-1243.

Makkar, H.P. 2003. *Quantification of tannins in tree and shrub foliage: a laboratory manual*. Springer Science & Business Media.

Makokha, A., Oniang'o, R., Njoroge, S., Kamar, O. 2002. Effect of traditional fermentation and malting on phytic acid and mineral availability from sorghum (Sorghum bicolor) and finger millet (Eleusine coracana) grain varieties grown in Kenya. *Food and nutrition bulletin*, **23**(3 Suppl), 241-245.

Malleshi, N., Desikachar, H. 1982. Formulation of a weaning food with low hot-paste viscosity based on malted ragi (Eleusine coracana) and green gram (Phaseolus radiatus). *Journal of Food Science and Technology*, **19**(5), 193-198.

Malleshi, N., Desikachar, H. 1986a. Studies on comparative malting characteristics of some tropical cereals and millets. *Journal of the Institute of Brewing*, **92**(2), 174-176.

Malleshi, N., Desikachar, H., Tharanathan, R. 1986. Physico-chemical Properties of Native and Malted Finger Millet, Pearl Millet and Foxtail Millet Starches. *Starch-Stärke*, **38**(6), 202-205.

Malleshi, N., Klopfenstein, C. 1998. Nutrient composition, amino acid and vitamin contents of malted sorghum, pearl millet, finger millet and their rootlets. *International journal of food sciences and nutrition*, **49**(6), 415-422.

Malleshi, N.G., Desikachar, H.S.R. 1986b. Nutritive value of malted millet flours. *Qualitas Plantarum Plant Foods for Human Nutrition*, **36**(3), 191-196.

Malleshi, N.G.G., S. S. 1999. A process for preparation of Infant food. Patent No. 194999.

Mangala, S.L., Malleshi, N.G., Tharanathan, R.N. 1999. Resistant starch from differently processed rice and ragi (finger millet). *European Food Research and Technology*, **209**(1), 32-37.

Maria Vilela, R., Lands, L.C., Chan, H.M., Azadi, B., Kubow, S. 2006. High hydrostatic pressure enhances whey protein digestibility to generate whey peptides that improve glutathione status in CFTR-deficientlung epithelial cells. *Molecular nutrition & food research* **50**, 1013 – 1029

Martínez-Valverde, I., Periago, M.J., Provan, G., Chesson, A. 2002. Phenolic compounds, lycopene and antioxidant activity in commercial varieties of tomato (Lycopersicum esculentum). *Journal of the Science of Food and Agriculture*, **82**(3), 323-330.

Mbithi-Mwikya, S., Ooghe, W., Van Camp, J., Ngundi, D., Huyghebaert, A. 2000a. Amino acid profiles after sprouting, autoclaving, and lactic acid fermentation of finger millet (Eleusine coracan) and kidney beans (Phaseolus vulgaris L.). *Journal of agricultural and food chemistry*, **48**(8), 3081-3085.

Mbithi-Mwikya, S., Van Camp, J., Mamiro, P.R., Ooghe, W., Kolsteren, P., Huyghebaert, A. 2002a. Evaluation of the nutritional characteristics of a finger millet based complementary food. *Journal of agricultural and food chemistry*, **50**(10), 3030-3036.

Mbithi-Mwikya, S., Van Camp, J., Mamiro, P.R.S., Ooghe, W., Kolsteren, P., Huyghebaert, A. 2002b. Evaluation of the nutritional characteristics of a finger

millet based complementary food. *Journal of Agricultural and Food Chemistry*, **50**(10), 3030-3036.

Mbithi-Mwikya, S., Van Camp, J., Yiru, Y., Huyghebaert, A. 2000b. Nutrient and Antinutrient Changes in Finger Millet (*Eleusine coracan*) During Sprouting. *LWT-Food Science and Technology*, **33**(1), 9-14.

McDonough, C., Rooney, L., Earp, C. 1986. Structural characteristics of Eleusine coracana (finger millet) using scanning electron and fluorescence microscopy. *Food microstructure (USA)*.

McDonough, C.M., Rooney, L.W., Serna-Saldivar, S.O. 2000. The millets. *Food science and technology*, 177-202.

Mittal, M. 2002. Development of finger millet and barnyard millet based convenience mixes for food products and their evaluation for nutritional quality, storage stability and acceptability, Vol. PhD, G.B. Pant Univ. of Agric and Technol, pp. 260.

Morgan, C., Herman, N., White, P., Vesey, G. 2006. Preservation of micro-organisms by drying; a review. *Journal of Microbiological Methods*, **66**(2), 183-193.

Morgan, J., Hunter, R., O'Haire, R. 1992. Limiting factors in hydroponic barley grass production. in: *The 8th International Congress on Soilless Culture*. Hunter's Rest, South Africa.

Mosha, A.C., Svanberg, U. 1983. Preparation of weaning foods with high nutrient density using flour of germinated cereals. *Food Nutr Bull*, **5**(2), 10-14.

Mosha, T.C.E., Laswai, H.S., Tetens, I. 2000. Nutritional composition and micronutrient status of home made and commercial weaning foods consumed in Tanzania. *Plant Foods for Human Nutrition*, **55**(3), 185-205.

Mošovská, S., Mikulášová, M., Brindzová, L., Valík, Ĺ., Mikušova, L. 2010. Genotoxic and antimutagenic activities of extracts from pseudocereals in the Salmonella mutagenicity assay. *Food and Chemical Toxicology*, **48**(6), 1483-1487.

Mustafa, A.F., Seguin, P., Gélinas, B. 2011. Chemical composition, dietary fibre, tannins and minerals of grain amaranth genotypes. *International Journal of Food Sciences and Nutrition*, **62**(7), 750-754.

Najdi Hejazi, S. 2012. Impact of neonatal total parenteral nutrition and early glucose-enriched diet on glucose metabolism and physical phenotypes in Guinea Pig. in: *Nutrition Department*, Vol. M.Sc., University of Montreal.

Najdi Hejazi, S., Orsat, V., Azadi, B., Kubow, S. 2015. Improvement of the in vitro protein digestibility of amaranth grain through optimization of the malting process. *Journal of Cereal Science*.

Nirmala, M., Subba Rao, M., Muralikrishna, G. 2000. Carbohydrates and their degrading enzymes from native and malted finger millet (Ragi, Eleusine coracana, Indaf-15) *Food chemistry*, **69**(2), 175-180.

Nout, M. 1994. Fermented foods and food safety. *Food Research International*, **27**(3), 291-298.

O'Fallon, J., Busboom, J., Nelson, M., Gaskins, C. 2007. A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *Journal of Animal Science*, **85**(6), 1511-1521.

Obilana, A. 2003a. Overview: importance of millets in Africa. *World (all cultivated millet species)*, **38**, 28.38.

Obilana, A. 2003b. Overview: Importance of millets in Africa. -in: AFRIPRO Proceedings of workshop on the Proteins of Sorghum and Millets: Enhancing Nutritional and Functional Properties for Africa. *Pretoria, South African Journal of Agricultural Research*, 26–43.

Obilana, A.B., Manyasa, E. 2002. Millets. in: *Pseudocereals and less common cereals*, Springer, pp. 177-217.

Odenigbo, A.M., Danbaba, N., Ngadi, M. 2014. Nutrient Composition and Predicted Glycemic Index of Rice Varieties from Nigeria. *British Journal of Applied Science & Technology*, **4**(2), 302-318.

Ogrodowska, D., Czaplicki, S., Zadernowski, R., Mattila, P., Hellström, J., Naczk, M. 2012. Phenolic acids in seeds and products obtained from Amaranthus cruentus. *Journal of food and nutrition research*.

Okoth, J.K., Ochola, S., Gikonyo, N.K., Makokha, A. 2011. Optimization of the Period of Steeping and Germination for Amaranth Grain. *Journal of Agriculture Food and Technology*, **1**(6), 101-105.

Okoth Judith Kanensi, J.K., Ochola, S., Gikonyo, N.K., Makokha, A. 2012. Optimization of the Period of Steeping and Germination for Amaranth Grain.

Omary, M.B., Fong, C., Rothschild, J., Finney, P. 2012. REVIEW: Effects of Germination on the Nutritional Profile of Gluten-Free Cereals and Pseudocereals: A Review. *Cereal Chemistry Journal*, **89**(1), 1-14.

Palacios, M.C., Haros, M., Rosell, C.M., Sanz, Y. 2008. Selection of phytate-degrading human bifidobacteria and application in whole wheat dough fermentation. *Food microbiology*, **25**(1), 169-176.

Palombini, S.V., Claus, T., Maruyama, S.A., Gohara, A.K., Souza, A.H.P., Souza, N.E.d., Visentainer, J.V., Gomes, S.T.M., Matsushita, M. 2013. Evaluation of nutritional compounds in new amaranth and quinoa cultivars. *Food Science and Technology (Campinas)*, **33**(2), 339-344.

Parameswaran, K.P., Sadasivam, S. 1994. Changes in the carbohydrates and nitrogenous components during germination of proso millet, Panicum miliaceum. *Plant Foods for Human Nutrition*, **45**(2), 97-102.

Paredes-Lopez, o., Mora-Escobedo, R. 1989. Germination of amaranth seeds: effects on nutrient composition and color. *Journal of Food Science*, **54**(3), 761-762.

Pasko, P., Bartoń, H., Fołta, M. 2007. Evaluation of antioxidant activity of amaranth (Amaranthus cruentus) grain and by-products (flour, popping, cereal). *Rocz. Państw. Zakl. Hig*, **58**, 35-40.

Pasko, P., Bartoń, H., Zagrodzki, P., Gorinstein, S., Fołta, M., Zachwieja, Z. 2009. Anthocyanins, total polyphenols and antioxidant activity in amaranth and quinoa seeds and sprouts during their growth. *Food Chemistry*, **115**(3), 994-998.

Pearce, J., Langley-Evans, S. 2013a. The types of food introduced during complementary feeding and risk of childhood obesity: a systematic review. *International Journal of Obesity*, **37**(4), 477-485.

Pearce, J., Langley-Evans, S.C. 2013b. The types of food introduced during complementary feeding and risk of childhood obesity: A systematic review. *International Journal of Obesity*, **37**(4), 477-485.

Pelikánová , J., Liptáková, D., Valík, L.u. 2015. Suitability of lactic acid bacteria for fermentation of maize and amaranth. *Journal of Food & Nutrition Research*, **54**(4).

Phadtare, S., Inouye, M. 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in Escherichia coli. *Journal of bacteriology*, **183**(4), 1205-1214.

Poutanen, K., Flander, L., Katina, K. 2009. Sourdough and cereal fermentation in a nutritional perspective. *Food Microbiology*, **26**(7), 693-699.

Price, M.L., Van Scoyoc, S., Butler, L.G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agricultural and Food Chemistry*, **26**(5), 1214-1218.

Pushparaj, F.S., Urooj, A. 2014. Antioxidant activity in two pearl millet (Pennisetum typhoideum) cultivars as influenced by processing. *Antioxidants*, **3**(1), 55-66.

Queiroz, Y., Manólio, S.R., Capriles, V.D., Torres, E., Areas, J. 2009. [Effect of processing on the antioxidant activity of amaranth grain]. *Archivos latinoamericanos de nutricion*, **59**(4), 419-424.

Raghavendra Rao, B., Nagasampige, M., Ravikiran, M. 2011. Evaluation of nutraceutical properties of selected small millets. *Journal of Pharmacy and Bioalternative Science*, **3**, 277-279.

Rai, K.N., Gowda, C.L.L., Reddy, B.V.S., Sehgal, S. 2008. Adaptation and potential uses of sorghum and pearl millet in alternative and health foods. *Comprehensive Reviews in Food Science and Food Safety*, **7**(4), 340-352.

Ramachandra, G., Virupaksha, T.K., Shadaksharaswamy, M. 1978. Comparison of the protein fractions of finger millet. *Phytochemistry*, **17**(9), 1487-1490.

Ramachandra, G., Virupaksha, T.K., Shadaksharaswamy, M. 1977. Relation between tannin levels and in vitro protein digestibility in finger millet (Eleusine coracana Gaertn.). *Journal of Agricultural and Food Chemistry*, **25**(5), 1101-1104.

Ramashia, S.E. 2015. Research Gaps on Scientific Investigation of Finger Millet (Eleusine coracana) Grain / Flour. in: *21st SAAFoST Biennial Congress and Exhibition*. Durban.

Rao, P.U. 1994. Evaluation of protein quality of brown and white ragi (Eleusine coracana) before and after malting. *Food chemistry*, **51**(4), 433-436.

Rastogi, A., Shukla, S. 2013. Amaranth: A New Millennium Crop of Nutraceutical Values. *Critical reviews in food science and nutrition*, **53**(2), 109-125.

Rateesh, K., Usha, D., Malleshi, N. 2012. Influence of decortication, popping and malting on bioaccessibility of calcium, iron and zinc in finger millet. *LWT-Food Science and Technology*, 1-6.

Ravindran, G. 1992. Seed protein of millets: amino acid composition, proteinase inhibitors and in-vitro protein digestibility. *Food Chemistry*, **44**(1), 13-17.

Ravindran, G. 1991. Studies on millets: Proximate composition, mineral composition, and phytate and oxalate contents. *Food Chemistry*, **39**(1), 99-107.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*, **26**(9), 1231-1237.

Reale, A., Konietzny, U., Coppola, R., Sorrentino, E., Greiner, R. 2007. The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. *Journal of agricultural and food chemistry*, **55**(8), 2993-2997.

Reddy, K.B.P.K. 2007. Effect o drying processes on viability and selected functional properties of probiotic lactic acid bacteria, Vol. PhD University of Mysore.

Rengalakshmi, R. 2005. Folk biological classification of minor millet species in Kolli Hills, India. *Journal of Ethnobiology*, **25**(1), 59-70.

Repo-Carrasco-Valencia, R., Pena, J., Kallio, H., Salminen, S. 2009. Dietary fiber and other functional components in two varieties of crude and extruded kiwicha (Amaranthus caudatus). *Journal of Cereal Science*, **49**(2), 219-224.

Roberfroid, M. 1998. Prebiotics and synbiotics: concepts and nutritional properties. *The British journal of nutrition*, **80**(4), S197-202.

Roopa, S., Premavalli, K. 2008. Effect of processing on starch fractions in different varieties of finger millet. *Food chemistry*, **106**(3), 875-882.

Ruas-Madiedo, P., Hugenholtz, J., Zoon, P. 2002. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Journal*, **12**(2), 163-171.

Sajilata, G., Singhal, R.S., Kulkarni, P.R. 2002. Weaning foods: a review of the Indian experience. *Food Nutr Bull*, **23**(2), 208-26.

Saleh, A.S., Zhang, Q., Chen, J., Shen, Q. 2013. Millet Grains: Nutritional Quality, Processing, and Potential Health Benefits. *Comprehensive Reviews in Food Science and Food Safety*, **12**(3), 281-295.

Samanta, A.K., Kolte, A.P., Senani, S., Sridhar, M., Jayapal, N. 2011. Prebiotics in ancient Indian diets. *Current Science*, **101**(1).

Sanders, M.E. 2003. Probiotics: considerations for human health. *Nutrition Reviews*, **61**(3), 91-99.

Sanz-Penella, J.M., Laparra, J.M., Sanz, Y., Haros, M. 2012. Bread supplemented with amaranth (Amaranthus cruentus): effect of phytates on in vitro iron absorption. *Plant foods for human nutrition*, **67**(1), 50-56.

Saturni, L., Ferretti, G., Bacchetti, T. 2010. The gluten-free diet: Safety and nutritional quality. *Nutrients*, **2**(1), 16-34.

Sauer, J.D. 1950. The grain amaranths: a survey of their history and classification. *Annals of the Missouri Botanical Garden*, 561-632.

Schrezenmeir, J., de Vrese, M. 2001. Probiotics, prebiotics, and synbiotics—approaching a definition. *The American Journal of Clinical Nutrition*, **73**(2), 361s-364s.

Serna-Saldivar, S.O. 2010. *Cereal grains: properties, processing, and nutritional attributes*. CRC Press.

Shahidi, F., Chandrasekara, A. 2013. Millet grain phenolics and their role in disease risk reduction and health promotion: A review. *Journal of functional foods*, **5**(2), 570-581.

Shankara, P. 1991. Investigations on pre-harvest and post harvest aspects of finger millet, Ph. D. thesis, University of Mysore, India.

Shirai, K., Revah-Moiseev, S., Garcia-Garibay, M., Marshall, V.M. 1994. Ability of some strains of lactic acid bacteria to degrade phytic acid. *Letters in Applied Microbiology*, **19**(5), 366-369.

Shobana, S., Krishnaswamy, K., Sudha, V., Malleshi, N., Anjana, R., Palaniappan, L., Mohan, V. 2012. Finger Millet (Ragi, Eleusine coracana L.): A Review of Its Nutritional Properties, Processing, and Plausible Health Benefits. *Advances in food and nutrition research*, **69**, 1-39.

Sindhu, S.C., Khetarpaul, N. 2001. Probiotic fermentation of indigenous food mixture: effect on antinutrients and digestibility of starch and protein. *Journal of Food Composition and Analysis*, **14**(6), 601-609.

Singh, P., Raghuvanshi, R.S. 2012. Finger millet for food and nutritional security. *African Journal of Food Science*, **6**(4), 77-84.

Singhal, R., Kulkarni, P. 1988. Amaranths–an underutilized resource. *International Journal of Food Science & Technology*, **23**(2), 125-139.

Singleton, V.L., Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, **16**(3), 144-158.

Siwela, M., Taylor, J.R., de Milliano, W.A., Duodu, K.G. 2007. Occurrence and location of tannins in finger millet grain and antioxidant activity of different grain types. *Cereal chemistry*, **84**(2), 169-174.

Solomon, M., Ubom, G. 2007. Proximate Nutrient and Amino Acid Composition of Three Complementary Foods Based on Cereals and Legumes in Jos, Plateau State.

Sreeramulu, D., Reddy, C.V.K., Raghunath, M. 2009. Antioxidant activity of commonly consumed cereals, millets, pulses and legumes in India. *Indian journal of biochemistry & biophysics*, **46**(1), 112.

Sripriya, G., Antony, U., Chandra, T. 1997. Changes in carbohydrate, free amino acids, organic acids, phytate and HCl extractability of minerals during germination and fermentation of finger millet (Eleusine coracana). *Food Chemistry*, **58**(4), 345-350.

Sripriya, G., Chandrasekharan, K., Murty, V., Chandra, T. 1996. ESR spectroscopic studies on free radical quenching action of finger millet (Eleusine coracana). *Food Chemistry*, **57**(4), 537-540.

Stallknecht, G., Schulz-Schaeffer, J. 1993. Amaranth rediscovered. *New Crops. Wiley, New York*, 211-218.

Sterr, Y., Weiss, A., Schmidt, H. 2009. Evaluation of lactic acid bacteria for sourdough fermentation of amaranth. *International journal of food microbiology*, **136**(1), 75-82.

Stettler, N. 2011. Infant feeding practices and subsequent development of adipose tissue, Vol. 68, pp. 215-225.

STN. ISO 15214 (2002) Microbiology of food and animal feeding stuffs. Horizontal method for enumeration of mesophilic lactic acid bacteria. Colony-count technique. Bratislava: Slovak Institute of Technical Normalization.

Subba Rao, M., Muralikrishna, G. 2002. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, Eleusine coracana Indaf-15). *Journal of Agricultural and Food Chemistry*, **50**(4), 889-892.

Swami, S.B., Thakor, N.J., Gurav, H.S. 2013. Effect of soaking and malting on finger millet (*Eleusine Coracana*) grain. *Agricultural Engineering International: CIGR Journal*, **15**(1), 194-200.

Taylor, F.G. 2007. Millet. *Chapter four*

.

Teow, C.C., Truong, V.-D., McFeeters, R.F., Thompson, R.L., Pecota, K.V., Yencho, G.C. 2007. Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. *Food chemistry*, **103**(3), 829-838.

Teutonico, R.A., Knorr, D. 1985. Amaranth: Composition, properties, and applications of a rediscovered food crop. *Food technology (USA)*.

Thathola, A., Srivastava, S. 2002. Physicochemical properties and nutritional traits of millet-based weaning food suitable for infants of the Kumaon hills, Northern India. *Asia Pacific Journal of Clinical Nutrition*, **11**(1), 28-32.

Tissier, H. 1990. Recherches sur la flore intestinale normale et pathologique du nourisson., University of Paris. Paris, France

Tosi, E.A., Ré, E., Lucero, H., Masciarelli, R. 2001. Dietary fiber obtained from amaranth (Amaranthus cruentus) grain by differential milling. *Food Chemistry*, **73**(4), 441-443.

Towo, E.E., Svanberg, U., Ndossi, G.D. 2003. Effect of grain pre-treatment on different extractable phenolic groups in cereals and legumes commonly consumed in Tanzania. *Journal of the Science of Food and Agriculture*, **83**(9), 980-986.

Traoré, T., Mouquet, C., Icard-Vernière, C., Traore, A., Trèche, S. 2004. Changes in nutrient composition, phytate and cyanide contents and α-amylase activity during cereal malting in small production units in Ouagadougou (Burkina Faso). *Food chemistry*, **88**(1), 105-114.

Treche, S. 1999. Viscosity, energy density and osmolality of gruels for infants prepared from locally produced commercial flours in some developing countries. *International journal of food sciences and nutrition*, **50**(2), 117-125.

Van Soest, P.v., Robertson, J., Lewis, B. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of dairy science*, **74**(10), 3583-3597.

Vasishtha, H., Srivastava, R. 2012. Changes in Lipids and Fatty Acids during Soaking and Germination of Chickpea (Cicer arietinum). *Indian Journal of Agricultural Biochemistry*, **25**(1), 14-19.

Venskutonis, P.R., Kraujalis, P. 2013. Nutritional components of amaranth seeds and vegetables: a review on composition, properties, and uses. *Comprehensive Reviews in Food Science and Food Safety*, **12**(4), 381-412.

Vidyavathi, U., Shivaraj, B., Pattabiraman, T. 1983. Proteases in germinating finger millet (Eleusine coracana) seeds. *Journal of Biosciences*, **5**(3), 219-224.

Viswanath, V., Urooj, A., Malleshi, N.G. 2009. Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (Eleusine coracana). *Food Chemistry*, **114**(1), 340-346.

Vogelmann, S.A., Seitter, M., Singer, U., Brandt, M.J., Hertel, C. 2009. Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudocereals and cassava and use of competitive strains as starters. *International journal of food microbiology*, **130**(3), 205-212.

Wakil, S.M., Onilude, A.A. 2009. Microbiological and chemical changes during production of malted and fermented cereal-legume weaning foods. *Adv. Food Sci*, **31**(3), 139-145.

World-Health-Organization. 1993. *Fats and oils in human nutrition: report of a joint expert consultation*. WHO; FAO.

Wright, C., Parkinson, K., Drewett, R. 2004. Why are babies weaned early? Data from a prospective population based cohort study. *Archives of Disease in Childhood*, **89**(9), 813-816.

Zayed, G., Roos, Y.H. 2004. Influence of trehalose and moisture content on survival of Lactobacillus salivarius subjected to freeze-drying and storage. *Process Biochemistry*, **39**(9), 1081-1086.