DIPARTIMENTO DI SCIENZE AGRARIE ALIMENTARI E AMBIENTALI

CORSO DI LAUREA IN: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

EXPLOITATION OF INNOVATIVE COMMON BEAN (*Phaseolus vulgaris* L.) FLOURS TO PRODUCE NOVEL FORTIFIED FOODS

TIPO TESI: Sperimentale

Studente:
SILVIA GIOFRÈ

Relatore:
PROF.SSA ELENA BITOCCHI

Correlatore:
DOTT.SSA FRANCESCA SPARVOLI

ANNO ACCADEMICO 2019-2020

A mia Mamma.
Che ama la scienza e la natura,
che si meraviglia guardando un fiore sbocciare.
A Lei,
che ha creduto in me ogni giorno,
come se il mio sogno fosse il suo stesso sogno.

LIST OF TABLES

LIST OF FIGURES

ACRONYMS AND ABBREVIATIONS

ADP	Adenosine-5'-Diphospate
AI	Amylase Inhibitor
AIU	Amylase Inhibitory activity Unit
ANC	Anti-Nutritional Compound
AOAC	Association of Official Analytical Collaboration
ATP	Adenosine-5'-Triphosphate
AUC	Area Under the Curve
BBI	Bowman-Birk Inhibitors
BSA	Bovine Serum Albumin
CGIAR	Consultative Group for International Agricultural Research
CIAT	International Center for Tropical Agriculture
EA	Emulsion Activity
ES	Emulsion Stability
EtOH	Ethanol
FC	Foaming Capacity
FS	Foaming Stability
GF	Gluten-Free
GOPOD	Glucose Oxidase/Peroxidase reagent
GOS	Galactooligosaccharide
GWP	Global Warming Potential
G6P	Glucose-6-Phosphate
G6PD	Glucose-6-Phosphate Dehydrogenase

HIB	High Iron Beans
HK	Hexokinase
IBE	Institute for BioEconomy
ICP	Inductive Coupled Plasma
InsP	myo-Inositol Phosphate
$InsP_6$	myo-Inositol HexakisPhosphate
LC-PUFA	Long-Chain Polyunsaturated Fatty Acid
lec/lec lec-	PHA-L and PHA-E deficiency
lf	PHA-E deficiency
LGC	Least Gelling Capacity
lpa	Low phytic acid
MES-TRIS	Morpholino Ethanesulfonic acid-Tris(hydroxymethyl)aminomethane solution
$NADP^+$	Nicotinamide-Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide-Adenine Dinucleotide Phosphate
NDO	Non-Digestible Oligosaccharide
NPU	Net Protein Utilization
NUA	Nutritional Improvement of Andean beans
OAC	Oil Absorption Capacity
PA	Phytic Acid
PBS	Phosphate Buffered Saline
PEF	Pulsed Electric Field
pGI	Predicted Glycemic Index
PGI	Phosphoglucose Isomerase
PHA	Phytohemagglutinins
PTFE	Polytetrafluoroethylene

RDS	Rapidly Digested Starch
RFO	Raffinosaccharide
RPV	Relative Protein Value
RS	Resistant Starch
SDS	Slowly Digested Starch
TDF	Total Dietary Fiber
TDS	Temporal Dominance of Sensation
TIU	Trypsin Inhibitor Unit
WAC	Water Absorption Capacity

ITALIAN SUMMARY

Il fagiolo (*Phaseolus vulgaris* L.) è un legume molto diffuso e considerato un'importante fonte di proteine e di diversi macro e micronutrienti essenziali. È noto per i suoi effetti benefici sulla salute umana, sulla prevenzione di patologie croniche, incluse quelle cardiovascolari, il diabete mellito e diversi tipi di cancro e sul controllo di alcune funzioni metaboliche. Grazie alle loro caratteristiche, i fagioli possono essere usati nelle diete gluten-free, vegetariane e vegane, come ottimi sostituti delle proteine animali. Inoltre, rappresentano un alimento di base essenziale e completo in molti paesi in via di sviluppo, dove il problema della "hidden hunger" è una delle maggiori criticità che le popolazioni più povere devono affrontare. Nonostante i loro effetti benefici, i fagioli contengono anche molti composti antinutrizionali, come l'acido fitico e le lectine, che possono interferire con l'assorbimento dei minerali essenziali e con la digeribilità di alcune molecole, riducendo così il loro potenzialmente elevato valore nutritivo. Questo è il motivo per cui la biofortificazione è importante per il miglioramento genetico di tali legumi. Negli ultimi decenni la maggior parte degli studi ha cercato, da un lato, di sviluppare approcci volti a ridurre i composti indesiderati nei semi e a migliorarne le eccellenti proprietà e, dall'altro, di trovare metodi utili a sensibilizzare i consumatori al loro regolare consumo. A tale scopo le metodologie genetiche, basate sulla biofortificazione convenzionale e/o transgenica, sono sempre più impiegate. Inoltre, l'impiego della farina di fagiolo nella formulazione di prodotti industriali molto diffusi e apprezzati può essere utile ad aumentare il suo uso all'interno di più svariati prodotti alimentari, in modo che tutti i gruppi di consumatori possano beneficiare degli effetti positivi di questo legume.

Tale progetto di tesi ha avuto l'obiettivo di sviluppare nuovi prodotti biofortificati, caratterizzati da una migliore qualità nutritiva e da una ridotta frazione antinutrizionale che fossero allo stesso tempo apprezzati dai consumatori. Abbiamo cercato di ottenere l'approvazione dei consumatori attraverso la produzione di prodotti da forno tradizionali che fossero ampiamente diffusi nelle abitudini alimentari della maggior parte delle persone. Tali prodotti contenevano quantità relativamente elevate di farine di fagiolo biofortificate, prodotte sulla base di precedenti studi relativi a semi di fagiolo mutanti geneticamente migliorati. Si ritiene che l'acido fitico, un forte chelante di minerali, e le lectine (in particolare le

fitoemoagglutinine), altamente tossiche per l'apparato gastro-intestinale, abbiano i peggiori effetti antinutrizionali. Per tale motivo il nostro progetto è stato incentrato sullo studio di due genotipi di fagiolo, *lpa* e *lpa/lec-*, caratterizzati da assenza di lectine e/o da un basso contenuto di acido fitico, usati per la produzione di farine. Con queste farine sono stati ottenuti dei crackers, dei biscotti e una crema. Tali prodotti sono stati analizzati, utilizzando gli stessi alimenti prodotti usando una farina di fagiolo non modificato come controllo, per valutarne le proprietà biochimiche e sensoriali e per capire se i cambiamenti genetici applicati al seme avessero effettivamente esercitato l'effetto atteso, e in quale misura, anche dopo la sua lavorazione.

Tramite le analisi abbiamo dimostrato l'importanza della tecnologia alimentare nello sviluppo di nuovi alimenti e ingredienti funzionali. Abbiamo inoltre potuto evidenziare l'efficacia della biofortificazione nell'ottenimento di un prodotto primario migliorato e nutrizionalmente prezioso. Lo sviluppo di prodotti a base di fagiolo è stato possibile grazie alle adeguate proprietà funzionali delle farine, le quali hanno dimostrato di conservare totalmente o parzialmente le proprie proprietà nutritive dopo la cottura. Entrambi i genotipi *lpa* e *lpa/lec-* hanno mostrato la loro completa funzionalità nel prodotto finito, ulteriormente potenziata dalle proprietà intrinseche del legume, principalmente legate al suo contenuto proteico e al suo positivo coinvolgimento nella modulazione dell'indice glicemico.

La nostra ricerca ha dimostrato la possibilità di sviluppare in modo efficiente snacks a base di fagiolo con tutti gli attributi richiesti. In particolare, il biscotto di pasta frolla è risultato quello avente la migliore formulazione che l'ha reso il migliore anche dal punto di vista nutrizionale, biochimico e sensoriale. Questo prodotto ha ricevuto una valutazione sensoriale simile al campione di controllo ed è stato il più apprezzato dai giudici. Questo biscotto è inoltre caratterizzato da un'interessante composizione, la quale potrebbe essere ulteriormente migliorata riducendo la frazione lipidica (derivante dall'utilizzo di una significativa quantità di burro) in modo da esaltarne maggiormente il valore nutritivo. Inoltre, la sua formulazione, caratterizzata da una quantità limitata di zuccheri aggiunti (rispetto al biscotto di farina integrale e grano saraceno), ha contribuito a renderne il prodotto con il più basso indice glicemico. È stato dimostrato che la farina *lpa/lec-* è la più utile per la produzione di un biscotto biofortificato. Infatti, il biscotto di pasta frolla ottenuto con la farina *lpa/lec-* non è solo caratterizzato dall'assenza di lectine tossiche, ma anche dalla presenza di una notevole quantità di ferro biodisponibile.

Questo alimento biofortificato potrebbe essere oggetto di futuri studi più approfonditi, finalizzati ad ottenere una migliore formulazione e proprietà nutrizionali e sensoriali

ulteriormente potenziate. Il consumo e la commercializzazione di questo snack potrebbero essere proposti in modo efficiente a varie classi di consumatori, compresi i bambini e le persone affette da diabete, obesità, celiachia o carenze minerali, ma anche a tutte le persone che fanno della sana alimentazione un vero e proprio stile di vita.

AIM OF THE STUDY

Common bean (*Phaseolus vulgaris* L.) is considered an important source of proteins and many macro and micronutrients. It is known for its beneficial effects on human health and related pathologies, such as the reduction of cardiovascular diseases and diabetes mellitus, the prevention of different types of cancer and the control of some metabolic functions. Because of their characteristics, beans can be introduced in gluten-free, vegetarian and vegan diets, as optimal source of proteins. Moreover, they are an essential and complete staple food in many developing countries, where 'hidden hunger' is a critical challenge and where legumes represent one of the most common crops. Despite all their positive characteristics, beans also contain many antinutritional compounds, such as phytic acid and lectins, that may interfere with the absorption of essential minerals and the digestibility of some molecules, thus reducing their potentially elevated nutritional value. This is the reason why biofortification is important for the genetic improvement of beans. In the last decades most research tried, on the one hand, to develop approaches aimed at reducing seed antinutrients and improving their excellent properties and, on the other, to find possible ways to sensitize consumers to their regular consumption. For this purpose, not only well-tested technological methods (such as germination, extrusion and heat treatments) exist, but even genetic approaches, based on conventional and/or transgenic biofortification, are always more employed. Moreover, the introduction of beans into industrial popular products, especially snacks, may be useful to attract people and increase the use of legumes within different food formulations, so that all groups of consumers can benefit from the positive effects of common beans.

This work had the aim of developing new biofortified products, characterized by an improved nutritional quality, reduced antinutritional fraction and appreciated by consumers. We tried to gain consumer's acceptance through the production of traditional baked foods that are widely spread in the dietary habits of most people. These innovative products contained relatively high amounts of biofortified bean flours, produced on the basis of previous studies on genetically improved bean mutants. Phytic acid, a strong mineral binder, and toxic lectins (especially phytohemagglutinins) are considered to have the worst negative effects among all antinutritive compounds. For this reason, two common bean genotypes (*lpa* and *lpa/lec*-),

characterized by absence of lectins and/or low phytic acid content, were used to obtain biofortified flours. Different baked products, crackers and biscuits, and a cream, were produced with these flours. They were analyzed to assess their sensory and biochemical properties and whether the genetic changes applied on the seed exerted their expected effect, and in which dimension, even after processing.

CHAPTER 1
INTRODUCTION

1.1 **Legumes: a general overview on their global importance**

Legumes species belong to the *Fabaceae* family and have a fruit typically called pod, which contains seeds in variable number depending on the species. Several species, such as soybean, beans, faba bean, pea, chickpea, lentil, peanut, lupine pigeon pea, mung bean, peanut were domesticated worldwide by humans and many of them have a relevant importance in human and animal nutrition. Food legumes are normally consumed as dry seeds and in some cases as immature seeds or pods (De Ron, 2015).

Several grain legume crops (also known as pulses) are key elements of global agriculture and nutrition, since they are major sources of plant protein. Furthermore, pulses contribute to the sustainable development of the environment, due to their ability of biological nitrogen fixation and their effects on soil, and yield of the next crop when crop rotation is applied. It is estimated that the legume-rhizobia symbioses fix some 21 tons nitrogen annually with up to 1/3 nitrogen returning to soils (Chen et al., 2018). Considering the emerging urgent challenge of climate change, legumes play a crucial role in the diversification and sustainable intensification of agriculture. The reduction in manufacture of inorganic nitrogen fertilizers will result in decreasing the emission of greenhouse gas: in fact, nitrous oxide (N_2O) has an enormous global warming potential (GWP) if compared to carbon dioxide (CO_2). Agricultural systems involving legumes represent a sustainable alternative to conventional practices, allowing a more efficient and reduced use of industrially produced nitrogen fertilizers (De Ron, 2015). It has also been proven that legume crops provide additional benefits to both the soil and other crops grown in combination with them or following them in a rotation.

Despite the legumes trade remains a relatively small market compared to other food commodities (such as cereals and oil crops), according to FAOSTAT data the global pulse production has almost doubled in the past 18 years, ranging from 56 053 814 tons in 2000 to 92 277 859 tons in 2018 (Figure 1-1). Moreover, there has been a change in the consumption of pulses in several developed countries where they are increasingly considered as healthy

foods. In these places the increase in the growth rate of pulses is mainly related to the cultivation area expansion, especially in countries like Canada. Among developed countries, Canada, United States and Australia have emerged as major exporters of pulses (Akibode & Maredia, 2012).

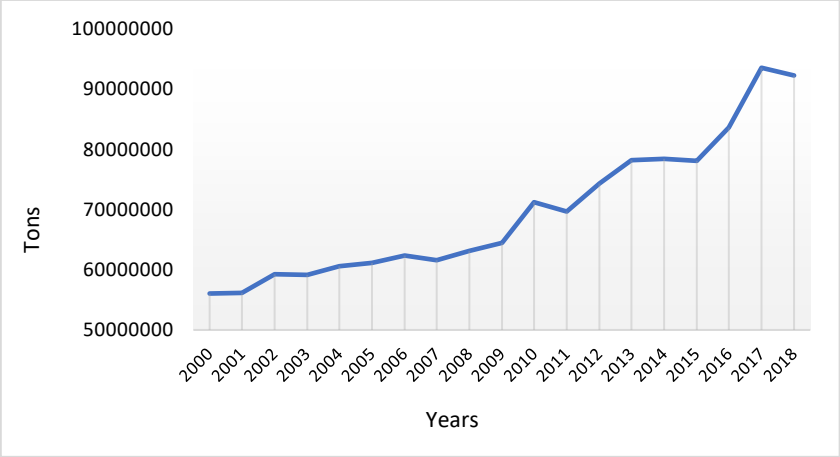

Figure 1-1: Global increase of pulses production from 2000 to 2018 in million tons (FAOSTAT, 2020)

Due to their low-cost production, high nutritional value, and contribution to a sustainable agriculture, food legumes are ideal crops for simultaneously achieving the three CGIAR's[1] developmental goals in targeted populations: reducing poverty, improving human health and nutrition and enhancing ecosystem resilience. In particular, they play an important and diverse role in farming systems and in the diets of poor people in the world. On average, legumes have a contribution of about 3% of total calories consumed in developing countries (4% in Sub Saharan Africa) (Akibode & Maredia, 2012). This is a small percentage if compared to cereal crops. However, in some countries of Sub Saharan Africa, such as Niger, Burundi and Rwanda, pulses provide more than 10% of total calories consumption per day. Pulses contribute relatively more towards total protein intake than calories consumption. On average legume crops contribute as 7.5% of total protein intake in developing countries against 2.5% in developed countries (Akibode & Maredia, 2012).

[1]CGIAR, the Consultative Group for International Agricultural Research, is the world's largest global agricultural innovation network. It unites international organizations engaged in research for a food-secured future. CGIAR research is dedicated to reducing rural poverty, increasing food ~~security~~, improving human health and nutrition, and ensuring sustainable management of natural resources.

While pulses are produced all over the world, South Asia and Sub Saharan Africa together account for about half of global production. Sub Saharan Africa, Latin America, Caribbean and Southeast Asia are the main producers of dry beans, while South Africa alone contributes for 74% of the global production of chickpea. Dry pea is primarily produced in North America and Europe and cowpea, which is specific to arid regions, is mainly grown in Sub Saharan Africa. North America is the largest producer of lentils (Rawal & Navarro, 2019). The following table shows the regional distribution of production of different types of pulses in the years 2012-2014:

Table 1-1: Share of different regions of the world in production of major pulses, 2012-2014 in percentage (Rawal & Navarro, 2019)

Region	Dry bean	Chickpea	Dry pea	Lentil	Pigeon pea	Faba bean	All pulses
East Asia	6.1	0.1	13.4	3.0	0.0	37.3	6.4
Southeast Asia	17.9	2.8	0.6	0.0	12.8	0.0	7.7
South Asia	17.3	74.3	7.6	30.2	67.8	0.1	27
West Asia	0.9	4.8	0.1	10.5	0.0	1.5	2.1
Caucasus and Central Asia	0.5	0.1	0.8	0.1	0.0	0.3	0.4
Oceania	0.3	5.9	2.8	7.7	0.0	7.8	4.3
Europe	2.3	1.2	29.1	1.8	0.0	14.2	8.4
North Africa	0.4	0.8	0.5	0.8	0.0	13.1	1.4
Sub Saharan Africa	24.0	4.7	5.5	3.1	16.7	21.1	22.8
North America	6.4	2.3	37.9	42.4	0.0	0.0	10.7
Latin America and Caribbean	24.0	2.1	1.7	0.3	2.7	4.7	8.9

1.1.1 *Nutritional value and beneficial effects*

It is claimed that the regular consumption of legumes promotes a better overall health, and it may be associated to the prevention of cardiovascular diseases, obesity, diabetes mellitus and some types of cancer (Venter & van Eyssen, 2001). It is recognized that grain legumes are optimal nutritional sources, especially for those people suffering from 'hidden hunger'[2], typically affecting women and children from the lower-income groups of developing countries, but also people in developed world which don't seem hungry (Sparvoli et al., 2015).

[2] The 'hidden hunger' is a chronic lack of vitamins and minerals deriving from a poor dietary quality, which may have a negative impact on health, cognition, function, survival and economic development.

The principal characteristic of legumes is their high seed protein content and amino acids composition. According to this property, if assumed in adequate amounts, legumes can be considered valid meat substitutes, especially for people of poor countries, where protein foods are scarce or their absence in diet is self-imposed due to religious or cultural habits, and for people that follow vegetarian or vegan diets. Most legume seed proteins have a storage role and they range from 16% (dry weight) in cowpea, pigeon pea and chickpea to as much as about 40-50% in lupin and soybean (Hedley, 2001; Chibbar et al., 2010; Burstin et al., 2011). The most abundant storage proteins in legumes are oligomeric globulins and albumins, which constitute about 70% and 10-20% of the total protein, respectively. Among globulins, legumins (11S globulins) and vicilins (7S globulins)[3] represent the major part, legumins being normally more abundant than vicilins (Vitale & Bollini, 1995). Storage proteins are characterized by an aminoacidic profile poor of essential sulfur-containing amino acids (such as methionine and cysteine) and tryptophan, while lysine, another essential amino acid, is quite abundant (Sarwar & Peace, 1986). It is usually recommended to eat legumes in association with cereals (usually rich in sulfur amino acids and poor in lysine), since their protein fractions can be considered nutritionally complementary. The effect of the combined consumption of cereals and legumes may contribute to the reduction of mutual deficiencies and ensures a balanced diet.

Even if legumes are mainly known and appreciated for their protein content, the fiber and starch composition is also involved in the definition of the seed nutritional value and health effects. Generally, the total carbohydrate content constitutes the 45-66% of pulse dry weight and starch represents the largest part of the carbohydrate fraction. It ranges from 20 to 52% of seed dry weight (apart from soybean and lupin) (Guillon & Champ, 2002). Legumes are a source of resistant starch (RS) that, differently from rapidly digested starch (RDS) and slowly digested starch (SDS), is not hydrolyzed in the small intestine but is fermented by colonic bacteria living in the large intestine. Most starches from legumes have a high amylose content (30-40%) compared to starches from cereals; this property may lead to the increase of the resistant starch fraction after processing, determining beneficial effects on human physiology (Aller et al., 2011). Dietary fiber in legumes varies depending on the species, variety and processing of seeds. In most grain legumes consumed by humans, the content ranges from 8 to 27.5%, with soluble fiber in the range of 3.3-13.8%. Studies on soybean and pea fiber tried to explain its physiological effects by its fermentation pattern and discovered that it may

[3] 11S and 7S represent the sedimentation coefficients of legumin and vicilin respectively.

provide a broad range of positive effects, both physiological and metabolic, at least in subjects suffering from disorders. These effects are related to the source of fiber (cotyledon fibers and hull fiber are degraded to a different extent), are dose related and depend on the form in which the fiber is ingested (Guillon & Champ, 2002). Among pulses carbohydrates even oligosaccharides play an important role. They are mainly in the form of undigestible α-galactosides, in the amount of 2-10% of the dry matter, and stachyose is the prevalent oligosaccharide in most pulses. Their fermentation in the gut can have a double effect on human organism: on the one side, it produces excessive amounts of gas causing flatulence, on the other, it allows the development of small metabolites which provide additional energy to the body (Aller et al., 2011).

Legume seeds are an excellent source of essential minerals and phenolic compounds. Among minerals, iron, zinc and calcium are the most abundant (Campos-Vega et al., 2010). The highest levels of iron can be found in common bean, faba bean, mung bean and lentil. High zinc contents have been reported for *Lupinus* spp., lentil and chickpea, while the highest calcium content is found in seeds of common bean, lupin, faba bean and chickpea (Sparvoli et al., 2015). Polyphenols range between 0.4 and 1.1% of the dry weight and consist mainly in tannins, phenolic acids and flavonoids. Pulses with the highest phenolic content, meaning dark, highly pigmented varieties (such as black gram, lentil, red kidney and black bean), exert the best antioxidant capacity (Campos-Vega et al., 2010).

Considering all their properties, grain legumes are considered as 'functional foods', meaning foods that have proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond their basic nutritional functions. The regular consumption of pulses is highly recommended by governments and health organizations all over the world.

1.2 **Common bean (*Phaseolus vulgaris* L.)**

Among legume species, common bean (*Phaseolus vulgaris* L.) is the most important grain legume for direct human consumption worldwide (Broughton et al., 2003). Beans were domesticated independently in Mesoamerica and Andes about 8000 years ago. These two independent domestication events gave origin to two major domesticated gene pools: the Mesoamerican, diffused between northern Mexico and Colombia, and the Andean between Peru and Argentina. Thus, common beans are one of the most ancient crops of the New World and have been (but in some cases still are) a dominant staple in the Americas for millennia.

Among the main food crops, *P. vulgaris* has the greatest variation in growth habit, seed characteristics (size, shape and color) and maturation time. This variability enables its production in a wide range of agricultural systems and environments as diverse as the Americas, Africa, Middle East, China and Europe (Blair et al., 2010a). The consumption of *P. vulgaris* is also common in countries that surrounds the Mediterranean basin and fundamental part of the Mediterranean diet. Even in United States the consumption of dry beans has been increasing due the increasingly diffused interest in 'ethnic' and healthy foods (Blair & Izquierdo, 2012). The global production of these pulses is one of the highest: FAO estimates that the global bean production (covering not only the common bean), has risen from 17.03 million tons in 1997-99 up to 29.30 mt in 2015-2017. This significant growth is caused by the increase of both cultivation area and yields over the past 20 years, with the Americas and Asia as the most important producing regions (Table 1-2).

Data obtained from household surveys show that beans consumption is high in traditional beans consuming countries. In Brazil, for example, the per-capita consumption is about 20 kg per year. In eastern and southern Africa, where beans are a major staple, their consumption per year is as high or higher than in Latin America, reaching up to 66 kg per person in some areas of Kenia and 40 kg per person in both Rwanda and Burundi (Broughton et al., 2003).

Table 1-2: Estimated global dry beans production, 1997-2017 in million tons (FAOSTAT, 2019)

Region	1997-99	2000-02	2003-05	2006-08	2009-11	2012-14	2015-17
Asia	7.17	8.07	8.97	9.67	10.57	11.20	14.03
Americas	6.46	6.79	6.86	7.43	7.23	7.39	7.50
Africa	2.72	3.33	3.41	4.08	5.27	6.21	6.70
Europe	0.63	0.57	0.47	0.38	0.44	0.56	1.01
Oceania	0.05	0.05	0.05	0.04	0.05	0.04	0.03
World	17.03	18.82	19.77	21.60	23.26	25.40	29.27

Note: Data on dry beans are aggregated and include different species: the common bean (Phaseolus vulgaris), other bean species (Phaseolus spp.) and, for some countries, some Vigna species.

1.2.1 *Seed composition*

Beans are an excellent source of dietary proteins that complement other foods, like cereals, in human nutrition. There are some countries, such as Mexico and Brazil, in which beans are the primary source of protein in human diets (Broughton et al., 2003). In fact, the protein

content is being equal to that of meat, raging 20-30%, on average 25% of the dry weight (OECD, 2019). In beans globulins constitute the major seed protein fraction, consisting of 50-70% of total proteins and, contrary to other legumes, vicilins are more abundant than legumins (Derbyshire et al., 1976). The second most abundant class of storage proteins is represented by albumins, comprising lectins and lectin-related proteins (phytohemagglutinins, α-amylase inhibitor and arcelin) representing about 10% of the total seed proteins, and trypsin inhibitors. Lectins and lectin-related proteins are defined as APA proteins, since they are coded by the single Mendelian APA locus (Lioi et al., 2003). Like other pulses, bean proteins contain great amounts of some essential amino acids (mainly leucine, lysine, phenylalanine and arginine) while are deficient in sulfur-containing amino acids.

Carbohydrates are the major components of beans, accounting up to 68-70% of the dry matter (OECD, 2019). The carbohydrate fraction is mainly composed by starch, which accounts for 30-50% of the dry seed as average, and non-starch polysaccharides, along with considerable amounts of carbohydrate derivatives, like oligosaccharides. Starch is predominantly in the form of RS (Henningson et al., 2001), while total sugars (monosaccharides and oligosaccharides) represent only a small percentage of total carbohydrate in legume seeds. However, common bean contains more oligosaccharides than the average (Reddy & Pierson, 1984), being galactooligosaccharides the most abundant in bean seeds, accounting for 3.1-5.7%, fraction mainly composed of raffinose, stachyose and verbascose (Da Silva Fialho et al., 2006). Beans also contain considerable amounts of fiber, mainly cellulose, hemicellulose, pectin and lignin. One-half cup of beans provides between 5.2 and 7.8 g of total fiber compared with 1.7-4 g of fiber per one half-cup serving of whole grain (Messina, 2014). They are also an optimal source of soluble fiber: one-half cup of beans provides between 0.6 and 2.4 g of soluble fiber (Anderson, 1990).

The lipid content in *P. vulgaris* cultivars is low, on average 1.4%. The major lipid components in beans are phospholipids and triacylglycerols, but lower quantities of diacylglycerols, monoacylglycerols hydrocarbons, stearyl esters and free esters may also be found in the seed (Mabaleha & Yeboah, 2004).

Beans are also an essential source of micronutrients, like vitamins and mineral salts. They have one of the highest levels of mineral content compared to other legumes. They contain big amounts of iron, phosphorus, magnesium, manganese and, in lesser degree, zinc, copper and calcium. Together with lentils, beans may have the highest iron and zinc content, up to 122 mg/g and 44 mg/g, respectively (Campos-Vega et al., 2010). Common bean seeds contain a high amount of folate (400-600 mg/g), representing 95% of daily requirements, and a good

source of tocopherols, thiamine, riboflavin, niacin, biotin and pyridoxamine (Campos-Vega et al., 2010).

Bean seeds also contain many phytochemicals, especially phenolic compounds such as flavonoids, anthocyanins, flavonol, proanthocyanidins, tannins, glycosides, as well as wide range of phenolic acids (Cardador-Martínez et al., 2002a).

Table 1-3: Composition of some common bean varieties in percentage on dry weight (OECD, 2019)

Source	Protein	Carbohydrate	Lipid	Dietary fiber	Ash	Moisture
Black beans	24.28	70.70	1.60	17.42	4.05	11.02
Cranberry beans	26.29	68.53	1.40	28.20	3.78	12.39
Kidney beans, all varieties	26.72	68.00	0.94	28.21	4.34	11.75
Navy beans	25.40	69.11	1.71	17.41	3.78	12.10
Pink beans	23.30	71.37	1.26	14.12	4.07	10.06
Pinto beans	24.16	70.55	1.39	17.50	3.90	11.30
Small white beans	23.91	70.50	1.34	28.20	4.25	11.71
Pérola, Carioca	24.96	68.61	1.78	21.94	4.65	13.07

1.2.2 *Nutritional value*

1.2.2.1 Protein digestibility

Net protein utilization (NPU) and relative protein value (RPV) are the two main parameters used to assess the protein nutritional quality and they are ascribed to several factors, including the aminoacidic composition, the amino acids bioavailability and the protein digestibility. In common bean, and generally in all legumes, the low amount of sulfur amino acids, the compact structure of proteins and the presence of antinutritional compounds, which can impair digestion, have been proposed to be responsible for the apparent low protein value (Evans & Bauer, 1978; Chang & Satterlee, 1981; Sarwar & Peace, 1986; Jansman et al., 1998). In particular, bean storage proteins have a low digestibility (65-80%) when they are unprocessed or untreated (Shimelis & Rakshit, 2007) and in some cases even after cooking.

Phaseolin, like other tetrameric 7S globulin, has low NPU and RPV (Carbonaro, 2006). Raw phaseolin is highly resistant to both *in vitro* and *in vivo* digestion due to its high glycosylation level and its rigid and compact structure rich in β-sheets (Deshpande &

Damodaran, 1989). 7S globulins are characterized by high amounts of negatively charged amino acids, such as aspartic acid and glutamic acid, that affect the solubility behavior of proteins. According to Carbonaro results (2006), both electrostatic interactions and hydrophobic forces play a role in the subunit association of 7S globulins, leading to the formation of protein aggregates characterized by a low solubility, even after cooking. Such aggregation may be one reason for the low digestibility of legume proteins, before and after heat treatments (Carbonaro et al., 1997).

Interferences with gut functions by lectins, which are present in large amount in raw bean seeds, as well as the reduction of the proteolytic activity by trypsin-inhibitors, are considered to be another primary factor responsible for the low digestibility of bean proteins (Carbonaro et al., 2000). *P. vulgaris* lectins are themselves characterized by a high resistance to breakdown by proteolytic enzymes and not hydrolyzed during the passage in the gastro-intestinal tract (Pusztai et al., 1991; Vasconcelos & Oliveira, 2004). It is demonstrated that also protease inhibitors present in bean seeds, including trypsin inhibitors, can pass through the stomach in their unaltered form as they are stable against pepsin and low pH, contributing to the low digestibility of raw seeds (Weder & Kahley, 2003).

1.2.2.2 Carbohydrates assimilation and digestibility

Carbohydrates (starch, soluble and insoluble dietary fiber and oligosaccharides) play an important role in the nutritional value of common beans. The global digestibility of bean starch is low if compared to that of cereals and, like in other pulses, the carbohydrate fraction is mostly in the form of RS. A study based on a 3 hours digestion period of beans showed a starch hydrolysis of 49.5% as compared to 75% starch hydrolysis of wheat, corn and rice (Socorro et al., 1989). Starch digestibility of beans is affected by several factors, among which the amylose/amylopectin ratio plays an important role. High amylose content in bean starch results in high RS and frequent protein-starch interactions, which may account for the decreased digestibility. Carbohydrate digestibility, thus RS levels and fiber content, strongly correlates to the glycemic index: compared to other carbohydrate sources, beans have a low glycemic index, varying from 26 to 42% relative to glucose (Foster-Powell & Miller, 1995).

Foods characterized by a low glycemic index are involved in the prevention of hyperglycemia (high glucose concentration in the blood) and hyperinsulinemia (high insulin concentration in the blood), which are hallmark features of diabetes and obesity. Regular beans consumption may help in the treatment of these chronic diseases (Bennik, 2005; Obiro et al., 2008), even thanks to the presence in such legumes of high levels of phaseolamin, a proteinaceous α-amylase inhibitor (α-AI) belonging to lectin the lectin family of seed proteins.

It can be considered as a 'starch-blocker' because of its interference activity in the breakdown of starch (Figure 1-2) and its important role in the further reduction of the glycemic index. Since the early 1980s, the α-AI, together with many other starch-blockers, was commercialized as dietary supplement for the control of weight. In particular, it may promote weight loss by inhibiting the breakdown of complex carbohydrates, thereby reducing, or at least slowing, starch digestion (Layer et al., 1985). Studies on α-AI showed that its inhibitory activity in several dietary supplements containing *Phaseolus vulgaris* ranges from 400 to 611 AIU/g[4], although it is much slower than those of raw beans, thus showing a partial inactivation after processing (Boniglia et al., 2008). However, recent developments, with improved extraction, fractionations and heat treatment have led to demonstrate the high efficiency of bean α-AI on humans and its implication in the treatment of obesity and diabetes mellitus (Obiro et al., 2008). In addition, it has been proven that starch-blockers from common bean at least cause subtle weight loss (Obiro et al., 2008), which has been shown to have advantages relative to drastic weight loss (Goldstein, 1992). Natural α-AIs are extracted from many vegetable sources, but the *P. vulgaris* α-AI has relatively wide potential because common beans are grown widely all over the world. Moreover, its pure form is not associated to negative effects, such as asthma and dermatitis, which are typical consequences of cereals α-AI humans (Sanchez-Monge et al., 1997; Kusaba-Nakayama et al., 2000).

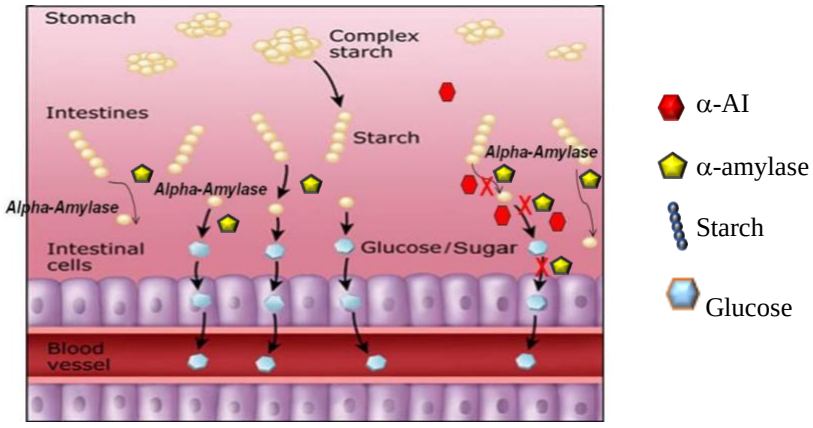

Figure 1-2: Mechanism of action of α-amylase inhibitor in the intestine

Oligosaccharides can be divided into digestible and non-digestible oligosaccharides (NDOs) on the basis of their physicochemical and physiological properties. Galactooligosaccharides (GOSs) belong to the NDOs group and represent the main fraction of

[4] Units of amylase inhibitory activity per gram of sample.

oligosaccharides in *P. vulgaris*. Like many other carbohydrate compounds in beans, GOSs remain undigested by humans and monogastric animals, as they do not possess the enzyme α-1,6-galactosidase in the intestinal mucosa (De Lumen, 1992). Despite their proven negative effect in causing flatulence (De Lumen, 1992; Rackis, 1981; Price et al., 1988), GOSs may bring some benefits and lead to the rebalancing of the bacterial ecology (Da Silva Fialho et al., 2006; Nie et al., 2020). Moreover, clinical trials on overweight and obese diabetic individuals based on the supplementation of diets with 5.5 or 10 g/day of non-digestible GOSs, showed positive effects on the composition of the gut microbiota and enhancement of gastrointestinal health. Therefore, GOS-induced improvement of gut microflora could alleviate metabolic syndrome, lipid homeostasis and low-grade systemic-inflammation on obesity (Nie et al., 2020).

A consequence of the low bean carbohydrate digestibility is linked to the bacterial fermentation of RS, soluble and insoluble dietary fiber and non-digestible oligosaccharides, in the large intestine (Henningson et al., 2001). Once non-digested molecules reach the intestine, they are metabolized by the endogenous microflora into short chain fatty-acids (such as acetic, butyric and propionic acids), which have been suggested to have beneficial physiological effects. Butyric acid is the main source of energy for the colonocytes and it may play a role in the prevention and treatment of several colonic diseases: studies demonstrated that in human colonic tumor cell lines, butyrate inhibits growth and differentiation and induces apoptosis (Whitehead et al., 1986; Hague & Paraskeva, 1995), whereas propionic acid was considered to be involved in the reduction of plasma cholesterol in rats through the inhibition hepatic cholesterologenesis (Chen et al., 1984). Even soluble fiber, a significant fraction of common bean carbohydrates (Anderson, 1990), plays an important role in the balance of cholesterol levels: according to the National Cholesterol Education Program, 5-10 g of soluble fiber reduces LDL cholesterol by about 5%.

1.2.2.3 Fatty acids

The reported fatty acids profiles of the seed oils of *P. vulgaris* suggest that they may be more valuable than can be inferred from their very low oil content. Common bean seeds are a source of unsaturated fatty acids, especially palmitic, oleic and linoleic acids (representing 61% of the total fatty acids), with linolenic at the first position (43.1% of the total) (Grela & Günter, 1995). The predominance of these fatty acids, which are the precursors of the long-chain polyunsaturated essential fatty acids (LC-PUFA), gives an additional value to the

nutritional composition and possible health benefits of *P. vulgaris* beans (Mabaleha & Yeboah, 2004).

1.2.2.4 Phenolic compounds

Common beans have been recently proposed as nutraceutical food given their content in phenolic compounds which are known for their antimutagenic, anticarcinogenic and antioxidant activities. These compounds can inhibit mutagenic agents, such as polycyclic aromatic hydrocarbons, nitrosamines and mycotoxins, responsible for genotoxicity and carcinogenesis (Kun-Young et al., 2004; Bhattacharya, 2011). Studies showed that antimutagenic activity involves the formation of complexes between phenolic compounds and mutagens and it may apparently be mediated by the scavenging activity of the phenolics (Cardador-Martínez et al., 2002a and 2002b). Dry beans contain significant amounts of quercetin, the most important flavonol, and many other flavonoids, whose antioxidant activity results in the prevention of cancer.

1.2.2.5 Micronutrients

Common beans are considered an optimal source of micronutrients, including minerals and vitamins. These compounds are very important for all the populations in poor countries which suffer for diet deficiencies, including iron deficiency. Common beans are rich in iron, even if a significant percentage is not bioavailable. However, the growing development of technologies aimed at increasing iron availability in beans, could make beans a solution to iron deficiency in low-income developing countries.

On the other hand, a substantial portion of diets in developed countries, results to be lacking in folate (vitamin B9), which represents the most deficient vitamin in these populations. Folates are essential cofactors in metabolic pathways that involve methylation reactions and DNA biosynthesis, and their deficiency has a role in the promotion of atherosclerotic cardiovascular disease, neuropsychiatric disorders, congenital defects and carcinogenesis (Stanger, 2002). Common beans are an excellent source of folates: they contain 400-600 μg/100 g (on dry weight) of folates, representing 95% of daily requirements (Campos-Vega et al., 2010).

Knowing the vary and complete composition of beans and the beneficial properties of all their components, it is easy to understand their excellent nutritional value and their important role in human nutrition. Some of the mechanisms involved in the positive effects on human health subsequent to the consumption of beans are summarized in the following schemes (Hayat et al., 2014):

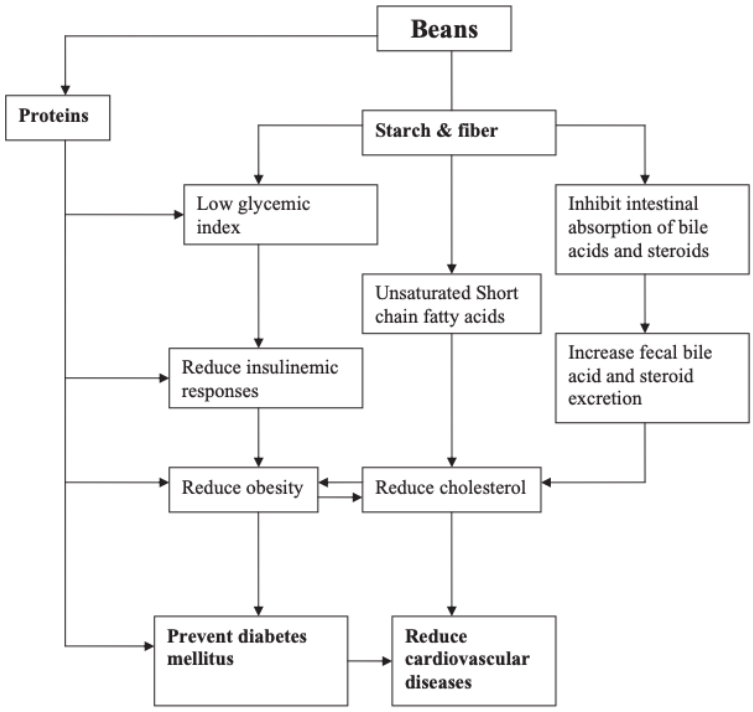

Figure 1-3: Mechanism involving the reduction of cardiovascular diseases, obesity and diabetes mellitus by beans (Hayat et al., 2014)

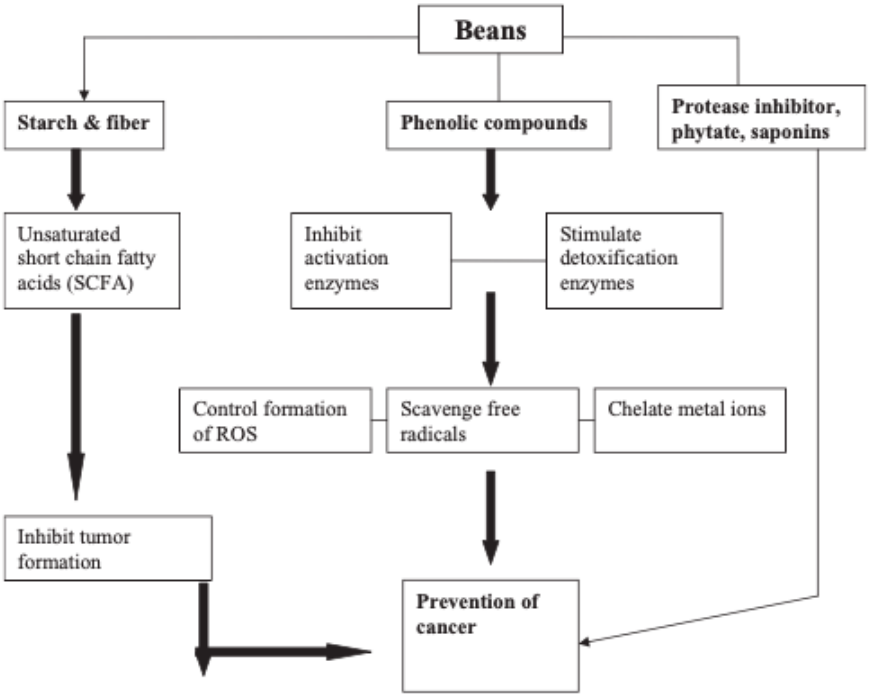

Figure 1-4: Mechanism involving the prevention of cancer by beans (Hayat et al., 2014)

1.2.3 *Antinutritional compounds*

Despite their many positive nutritional properties, common beans, as all legume seeds, contain a number of bioactive substances, defined as antinutritional compounds (ANCs), that play a defensive role against pests and animal predators, and they exert metabolic effects on people that consume these food forms. These effects, that are generally observed if legumes are consumed with a regular frequency, are regarded as negative for human health.

The most common ANCs are lectins, enzyme inhibitors, phytates, oxalates, saponins, oligosaccharides, phenolic compounds and cyanogenic glycosides. Many of them affect the digestive system, with the inhibition of digestive enzymes (e.g. protease inhibitors and α-AIs), the imbalance of hydrolytic functions and transport at enterocyte sites (lectins) and the production of gases in the colon (oligosaccharides). Effects of these compounds are mainly related to the limitation of carbohydrates, proteins and other nutrients absorption (Champ, 2002; Krupa, 2008; Thakur et al., 2019). Some antinutritional factors, like lectins, α-AI, protease inhibitors and cyanogenic glycosides, perform their activity only in the raw seed and are partially or completely inactivated by heat treatments. Unfortunately, many others, such as phytic acid, condensed tannins, alkaloids and saponins are not destroyed by high temperatures.

1.2.3.1 Phytic acid and oxalic acid

Although legume seeds have abundant amounts of essential minerals, they also accumulate compounds, such as phytic acid (myo-inositol-1,2,3,4,5,6-hexakiphosphate, PA, $InsP_6$) and oxalic acid, that reduce their nutritional value by lowering minerals bioavailability. Significant amounts of PA are stored in bean seeds and the average content is 5.79 mg/g, with a variation ranging from 4.26 mg/g to 8.33 mg/g (expressed as phytic acid phosphorous, Doria et al., 2012).

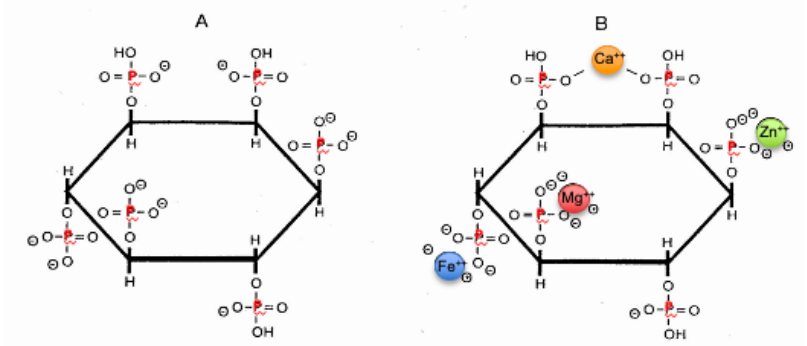

Figure 1-5: Structure of phytic acid (A) and phytic acid chelate (B)

During seed maturation PA accumulates as phytate salts in seeds, where it represents the primary storage form of both phosphorous and inositol for the plant (Champ, 2002). After

germination, phosphorous becomes available for the developing seedlings, due to the activity of phytases, enzymes that hydrolyze PA into inorganic phosphorous and lower myo-inositol phosphates (InsPs). Phytases are present in ruminant, but absent within the digestive tract of monogastric organisms (Thakur et al., 2019), in which phytate degradation in the stomach and the small intestine by food phytases is very limited. PA strongly binds to minerals and trace elements under the acidic conditions of the gastric chyme and forms soluble complexes (Figure 1-5). During the passage from the stomach to the small intestine, and with increasing pH, they precipitate. Thus, PA is not destroyed and fully performs its chelating activity reducing minerals and trace elements absorption and leading to their inevitable loss. PA fulfills this function through the presence of reactive phosphate groups attached to the inositol ring which is involved in its structure; its capacity to bind polycations is a function of the number of phosphate groups (Kumar et al., 2010). Metal-PA complexes are known as phytates and they are insoluble at physiological pH values and essentially non-absorbable in the absence of the phytase enzymatic activity. The presence of PA and, consequently, the lack of most minerals and phosphorous bioavailability in beans, is inevitably responsible for the reduction of seed nutritional value.

PA interferes not only with mineral absorption, but it may also affect protein and starch digestibility. Phytate can negatively influence the activity of digestive enzymes through the chelation of mineral cofactors which are normally involved in the enzymatic activity. For example, some digestive enzymes, such as α-amylase and trypsin, require calcium ions for full activity (Kumar et al., 2010). The reduction of protein digestibility can be caused by the slowdown of enzymatic proteolytic activity, but it may be also affected by the entrapment of protein molecules by phytate, which leads to the production of phytate-proteins complexes. Phytate can form complexes with proteins and amino acids both at alkaline and acidic pH (Cheryan & Rackis, 1980) and these interactions may determine changes in protein structure that decrease enzymatic activity, protein solubility and proteolytic digestibility; the consequence is a reduction of amino acid availability and absorption (Nissar et al., 2017).

P. vulgaris seeds are also rich in oxalic acid, a compound that, like PA, is able to chelate mineral ions forming oxalates, and reduce their bioavailability. Toxic effects of oxalic acid and oxalates are related to the corrosion of the mouth and gastrointestinal tract, gastric hemorrhage, severe kidney stones with renal failure and hematuria, as well as the reduction of plasma calcium, which may cause convulsions (Noonan & Savage, 1999). Raw and boiled seeds of kidney bean contain about 95 mg/100 g and 38 mg/100g of oxalates, respectively (Judprasong et al., 2006), proving that a proper cooking allows their reduction to a safety level.

1.2.3.2 Phytohemagglutinins

Lectins are defined as ‘proteins or glycoproteins of non-immune origin with one or more binding sites per subunit, which can reversibly bind to a specific mono- or oligosaccharide’ (Lis & Sharon, 1998; Peumans & Van Damme, 1995). *P. vulgaris* lectins, better known as phytohemagglutinins (PHA), show a remarkable sequence homology with other legume lectins, however they are among the most toxic (Grant et al., 1983). PHA is a tetramer composed by two types of polypeptide chains, E and L, having a preferential binding to erythrocytes and leukocytes, respectively; thus, five possible tetrameric isolectins (E_4, E_3L_1, E_2L_2, E_1L_3, and L_4) can be formed randomly (Weber, 1969). While PHA-L agglutinates only leucocytes, PHA-E is able to agglutinate both leucocytes and erythrocytes; analyses conducted on several varieties and market classes of dry beans showed a high variability in haemagglutinin activity (Barampama & Simard, 1993), indicating a certain degree of variability among bean PHAs.

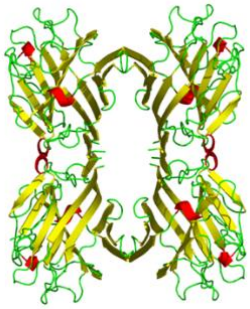

Figure 1-6: Tridimensional structure of PHA-L tetramer

The PHAs tetrameric structure is characterized by a high compactness, which is at the basis of the lectin resistance to high temperatures. Treatments consisting of dry or moisty heating of seeds at 70°C for several hours have little or no effect on their lectin activity and processing at higher temperatures is needed to inactivate the biological and antinutritional effects of legume lectins (Pusztai, 1991; Grant & Van Driessche, 1993). While the resistance to heat is linked to the lectin structure itself, the high stability against enzymatic attack by pancreatic, intestinal and bacterial proteases, has been associated to the lectin ability of avidly binding carbohydrates (including glycoproteins and glycolipids) of the gut epithelial membrane. The formation of compact lectin-carbohydrate complexes causes the molecules to be found intact in human feces (Pusztai et al., 1991). It has been found that even Ca^{2+} metal ions contribute to strengthen PHAs conformation by stabilizing lectin domain and inhibiting trypsin proteolysis (Morari et al., 2008; Gabius et al., 2011). Therefore, calcium may contribute to the enhancement of PHAs thermal resistance: cooking of bean seeds in the presence of increasing

concentrations of $CaCl_2$ has a strong effect mainly on PHA-L stability (Cominelli et al., 2020). Cominelli and colleagues have come to the conclusion that PHAs and PA are mutually related: the reduced level of PA in common bean (for example, after a long storage period or in mutant lines) is responsible for the accumulation of higher amount of free calcium ions in the seed. The growing availability of Ca^{2+} is sufficient to better stabilize the PHAs structure. In such conditions, PHA-L becomes more strongly resistant to thermal degradation, being still detectable after 5 hours cooking at 95 and 100°C. On the contrary, the PA reduction seems not to affect the PHA-E, that is more easily degraded by heat. The study suggests that PHA-L (structure shown in Figure 1-6) is generally more stable than PHA-E, and its stability is further enhanced by the presence of Ca^{2+} cations (Cominelli et al., 2020).

The high stability of lectins and their resistance to degradation can be deleterious for the human organism: if ingested raw or not properly cooked, lectins, and in particular PHAs, show a toxic effect that can be displayed both at oral and gastro-intestinal level. Usually, the oral acute toxicity of common bean lectins manifests as nausea, vomiting, bloating and diarrhea in humans (Burbano et al., 1999; Vasconcelos & Oliveira, 2004). The ability of PHAs to selectively bind to different types of blood cells has been proposed has mean to predict their oral toxicity. It has been suggested that PHAs which are able to agglutinate a wide range of different red blood cells should have a high oral toxicity, whereas those agglutinating only rabbit and/or enzyme-treated rat erythrocytes should not (Grant et al., 1983). However, results did not provide evidences able to demonstrate this hypothesis and the doubts about mechanisms involved in the oral toxicity still remain.

On the contrary, the mechanism of action of lectins on the digestive trait is well known. The surface epithelium of the gut is characterized by the presence of membrane proteins (e.g. hormone, growth factor receptors, and transport proteins), lipids and gangliosides. PHAs reach the gastro-intestinal tract in their intact form and, as surface membrane receptors of cells are largely glycosylated, PHAs perform a mimesis activity, replacing endogenous growth factors, hormones and cytokines in all types of cells and leading to changes in cell's metabolism (Pusztai, 1991). In particular, undigested lectins may induce modifications in digestive, absorptive, protective and secretory functions and affect cellular proliferation and turnover. In the worst case, lectins, which are avidly bound to the gut epithelial surface, are readily endocytosed by enterocytes, thus determining the disruption of brush borders, with the consequent lesion of tissues and global malfunction of the organ (Thompson et al., 1986; Sasaki et al., 2002). The lectin-induced disruption of the intestinal mucosa may allow the entrance of bacteria and their endotoxins to the blood stream and cause toxic response. Since

lectins may be also internalized through endocytosis and transported within the body, their effects do not just occur locally but can be systemic. Systematically, PHAs can disrupt lipid, carbohydrate and protein metabolism, promote enlargement and/or atrophy of key internal organs and tissues and alter the hormonal and immunological status (Vasconcelos & Oliveira, 2004).

1.2.3.3 Protease inhibitors

Pulses contain two general classes of protease inhibitors: Kunitz and Bowman-Birk Inhibitors (BBI). In common bean the BBI family has been identified and characterized. BBIs are serine protease inhibitors, characterized by a high percentage of cysteine residues, in which the residue toward the amino-terminal side of the scissile bond determines the specificity of inhibition (Prakash et al., 1996). Among them, trypsin inhibitors are the most abundant: on average *P. vulgaris* seeds contain 9.6 TIU/mg[5] of dry matter (Campos-Vega et al., 2010). The antinutritional effect of trypsin inhibitors is related to their antitryptic and antichymotryptic activity, which significantly reduces the bioavailability of dietary proteins and may cause intestinal disfunctions. When beans are consumed raw, or without being properly cooked, they upset digestive functions and may cause diarrhea or excessive gas (Thakur et al., 2019). The regular consumption of trypsin inhibitors determines the formation of irreversible enzyme-trypsin inhibitors complexes which are undigestible even in the presence of high amounts of digestive enzymes.

1.2.3.4 Saponins

Saponins are compounds consisting of a lipid-soluble center, having either a steroid or a triterpenoid aglycone structure, with one or more lateral chains of water-soluble carbohydrates characterized by variable size and complexity (Burbano et al., 1999). The structural complexity of saponins results in a number of physical, chemical and biological properties. Many saponins are bitter and are considered antinutrients because of their toxicity and hemolytic activity: erythrocytes are disrupted in saponins solutions due to interactions with cholesterol in the erythrocyte membrane (Birk & Peri, 1980). Moreover, saponins are able to reduce the bioavailability of some nutrients and decrease enzymatic activity. In particular, they may affect protein digestibility by inhibiting digestive enzymes, including trypsin and chymotrypsin (Gemede & Ratta, 2014). However, there are many types of saponins,

[5] Units of trypsin inhibitory activity per milligram of dry matter.

characterized by different forms and properties and, depending on their structure, only few of them are toxic.

1.2.3.5 Phenolic compounds and tannins

Bean seeds may contain large amounts of phenolic compounds. For example, dark red kidney beans are among those with the highest phenolic fraction, containing 4,98 mg/g[6] of phenolic compounds. Among them 3.85 mg/g[7] are condensed tannins (Campos-Vega et al., 2010). Despite polyphenols are mainly known for their antioxidant activity, they may be also classified as antinutrients for several reasons. Condensed tannins are polyphenolic bioflavonoids with an antinutritional activity mainly related to the ability of forming complexes with some nutrients and precipitating compounds, including proteins and amino acids. Tannins-protein complexes involve both hydrogen bonding and hydrophobic interactions and their precipitation is affected by pH, ionic strength and molecular size of tannins (Akande et al., 2010). Because tannins are able to form complexes with proteins, it is not surprising that they also bind to enzymes. Studies reported that the activity of trypsin, chymotrypsin and α-amylase are reduced in simple-stomached animals after the ingestion of tannin-containing extracts (Jansman, 1993). These interactions have a significant negative effect on protein digestibility and amino acid bioavailability and may cause global disfunctions in the gastrointestinal trait. In addition, it has been largely demonstrated that polyphenols have an inhibiting effect on iron absorption. The capability of complex formation with iron in the intestine, and thereby the reduction of iron uptake into the body, depends on their quantity, quality, source and structure (Hurrel et al., 1999; Tuntipopipat et al., 2006). Studies show that the administration of reference meals containing 50 and 200 mg of bean hull polyphenols cause an iron absorption reduction of 14 and 45%, respectively, in young healthy women. It seems that the combined action of PA and polyphenols does not have an additive effect on the absorption of iron, but rather that modulating one without major changes in the other will have only modest effects on iron absorption (Petry et al., 2013).

1.2.3.6 Oligosaccharides

Despite their beneficial effects already described, GOSs are listed in the group of antinutrients due to the effect of their fermentation in the intestinal tract. The content of GOSs in the dry seed of common bean ranges from 18.6 to 34.7 mg/g. This fraction is composed by raffinosaccharides (RFOs): raffinose (0.9-5.6 mg/g), stachyose (18.3-29.3 mg/g) and

[6] Milligrams of gallic acid equivalents g^{-1}
[7] Milligrams of catechin equivalents g^{-1}

verbascose (0.4-2-7 mg/g), considered α-D-galactosides of sucrose (Burbano et al., 1999). Once these compounds reach the intestinal tract, they are fermented into carbon dioxide, hydrogen and methane, invasive gases that accumulate in big amounts causing undesirable flatulence effects. RFOs are considered the main components involved in the production of gas and the major responsible for the resulting discomfort (De Lumen, 1992). In addition to flatulence, the excessive consumption of these undigestible nutrients may cause diarrhea and severe gastrointestinal disorders (Da Silva Fialho et al., 2006).

1.3 **Legumes nutritional improvement**

1.3.1 *Technological approaches*

During the past 30 years, many studies have tried to find treatment methods which lead to the elimination/reduction of ANCs in legumes, without the deterioration of their nutritive value. Germination is one of the most effective methods employed for the increase of legumes nutritive value and reduction of antinutritional substances. During the germination process, seed enzymatic systems are activated and protease activity increases and, therefore, peptides, polypeptides and non-protein amino acids increase, improving protein quality. At the same time, the content of minerals and vitamins is enhanced. Phytic acid decreases during this process, since naturally occurring phytates are activated and phytate is gradually degraded, leading to the decrease of its mineral binding strength and to an improved minerals bioavailability. Germination also seems to reduce protease and α-amylase inhibitors, as well as the hemagglutination activity, probably due proteolysis during this process (Gulewicz et al., 2014). Heat treatment may be also applied for the reduction of protease inhibitors and phytates, but it has the disadvantage of reducing other important micronutrients, like vitamins. Legume seeds can be also fermented in order to improve their sensory characteristics and to increase the amount and availability of nutrients. This can be obtained by the degradation of ANCs by the hydrolysis of some food components and the synthesis of some nutritious promoters (Gulewicz et al., 2014).

The development of treatments, like germination, fermentation, but also cooking, soaking and toasting is a tradition typically linked to the consume of potentially toxic foods. Today the treatments for the reduction of antinutritional substances in foods are based on more scientific approaches and the availability of new technologies allows their exploitation at industrial level for the enhancement of food nutritional quality. Extrusion cooking, for example, shows its effects on legume-based products at a cost lower than other heating systems due to a more

efficient use of energy and better process control with greater production capacities (Reimerdes, 1990). Extrusion is the best method for the elimination of trypsin and chymotrypsin inhibitors and hemagglutinins activity without modifying protein content. Generally, all the antinutrients are significantly reduced during this process (Alonso et al., 1998 and 2000). Even the combination of high temperatures and pressure can be exploited: autoclaving (heating at 121°C under pressure) causes significant reduction in trypsin inhibitor and tannins and complete reduction in hemagglutinins (Abbas & Ahamad, 2018). Various chemical processes have been employed in order to improve the nutritional value of legumes. For example, Great Northern beans soaked in acidic or alkaline solutions, showed a substantial leaching of trypsin inhibitors, while jackbeans were almost completely detoxified using potassium bicarbonate (Akande & Fabiyi, 2010). Microwave cooking has a shorter processing time, so it destroys antinutritional molecules without affecting other nutritional qualities, allowing, for example, vitamin retention (El-Adawy, 2002). This treatment has also been proven effective in enhancing protein digestibility in many legumes (El Beltagy, 1996). Even the application of pulsed electric field (PEF) for the treatment of vegetable matrices is becoming more and more popular. PEF treatment is based on the selective disintegration of cells and is a valuable tool that can improve functionality, extractability, and recovery of nutritionally valuable compounds as well as the bioavailability of micronutrients and components. In addition, PEF has minimal costs in term of energy consumption, and it is non-thermal, thus it allows the retention of compounds that otherwise would have been destroyed by heat (Barba et al., 2015). This method has recently been tested on several protein sources, including pea seed. This technology applied on a protein sample is suitable to inactivate vegetative microbial cells and thereby to achieve food safety and increase shelf-life, by maintaining unaffected the protein structure (Baier, 2016).

1.3.2 *Genetic approaches*

The current availability of new genome and genetic technologies, including the next generation sequencing, have allowed the screening of many legumes' germplasm and, moreover, the development of potent tools to assist, improve and speed up the genetic improvement. The exploitation of genetic diversity of legumes (including that generated by mutagenesis) is an alternative approach to identify genotypes with optimal nutrient composition and low antinutrient content in order to obtain biofortified crops. Biofortification is the development of crops that by harvest accumulate higher concentrations of specific micronutrients than standard crops. Biofortification techniques are applied on a wide number of cultures all over the world, but its highest application and importance are related to the

increase of micronutrients and nutritional quality of staple crops in developing countries (Lockyer et al., 2018). Conventional biofortification programs, based on the selection and crossbreeding of plants that naturally contain high amounts of micronutrients, are in many cases aimed at addressing the issue of loss of diversity. Genetic variation in relation to seed mineral content has been extensively studied in pea, soybean, lentil, pigeon pea, cowpea, chickpea and peanut (White & Broadley, 2005; Pfeiffer & McClafferty, 2007) and breeding programs have allowed to enhance, in both cereals and pulses, the content of the seven mineral elements most frequently lacking in human diets: iron, zinc, copper, calcium, magnesium, selenium and iodine (White & Broadley, 2009).

Biofortification programs may also benefit of transgenic approaches, based on the insertion of genes for the accumulation of substances which would not exist in the standard crop. Moreover, genetic modifications, including the use of mutagenesis, should be exploited to increase the expression of genes related to mineral acquisition and to the abundance of minerals in a biologically accessible form. Further strategies involving these approaches may be related to the manipulation of genes that inhibit nutrients uptake, such as those that modulate the mineral-binding activity of phytic acid. Over the past decades, mutations that significantly reduce the levels of seed phytic acid have been identified in several main legume crops, including soybean (Wilcox et al., 2000; Hitz et al., 2002; Yuan et al., 2007), pea (Warkentin et al., 2012; Shunmugam et al., 2015) and common bean (Campion et al., 2009 and 2012; Cominelli et al., 2018). Many of them have been proven effective in providing more bioavailable iron and in enhancing its uptake into the body.

The bioavailability of minerals, especially iron, in foods is commonly assessed using the Caco-2 cells model, that is cheaper and more affordable than clinical studies. Derived from the human colon carcinoma, Caco-2 cell monolayer adds a living component to the model system that in theory should reflect the key step in iron bioavailability (Glahn, 2009). Iron uptake by the cell monolayers is assessed by measuring ferritin concentrations in the cells. Its formation in Caco-2 cells occurs in response to iron uptake at concentrations of the available mineral greater than that of the culture media to which the cells have been adapted (Glahn et al., 1998).

1.3.3 *Programs for P. vulgaris biofortification*

Since our knowledge of all the bioactive compounds in bean seeds is gradually advancing, programs of genetic improvement may be undertaken to decrease the level of ANCs, increase the content of essential minerals, like iron, and the quantity of health-supporting metabolites in bean cultivars. In the specific case of *P. vulgaris*, breeding programs are usually applied in

order to meet consumers preferences related to commercial aspects, such as size, shape, color, and pattern of the seed, or to improve some agronomic characteristics of the plant, including the increase of yield potential and disease resistance, and improvement of grain quality. In addition, several programs of genetic improvement have been considered in order to make the global seed nutritional quality more valuable through the decrease of antinutrients, such as lectins and phytic acid, as well as the increase of essential minerals content and protein quality.

Despite all the efforts for the removal or reduction of antinutrients in *P. vulgaris* seeds, it is important to take in consideration that a complete elimination of ANCs could be a problem, since many of them have also beneficial properties on human health. For example, it has been demonstrated that phytates could be involved in the prevention of some diseases, such as cancer and diabetes mellitus (Nissar et al., 2017), as well as polyphenols, which have optimal antioxidant properties, are considered important antimutagenic and anticarcinogenic compounds (Kun-Young et al., 2004; Bhattacharya, 2011). Toxic PHAs are the only compounds that should be eliminated since they seem to have no significant beneficial effect. Giuberti and colleagues have taken into account these aspects during the development of their breeding program, started with the elimination of lectins from the bean seeds (lectin-free line, *lf*) and continued with the introgression of different traits. The result was the development of several mutant lines characterized by the combination of different mutated traits. In particular, *lpa*, *lf* and *wsc* (white seed coat, characterized by the addition of 'reduced tannins') genotypes were combined. They found that the genetic removal/reduction of the three antinutrients (main lectins, phytic acid and condensed tannins) led to a reduction of other antinutrients such as lignin and saponins, and to a strong increase of nutrients, including crude proteins, total zinc (30% each) and free inorganic phosphorous (600%). In addition, the *in vitro* iron bioavailability resulted on average twelve times higher in the '*lf* + *lpa* + *wsc*' bean seeds than in the wild type (*wt*) colored parents (Giuberti et al., 2019).

1.3.3.1 Development of *low phytic acid (lpa)* mutants

It is estimated that the *P. vulgaris* content of iron is high, on average 50.4 μg/g. However, less than 2% of this fraction is absorbed (Donangelo et al., 2003). Iron is only one of the several minerals that are chelated by phytic acid, which is responsible for their bioavailability reduction. This is the reason why the development of biofortified bean cultivars with the aim of increasing iron or reducing PA content, can be considered an important goal in the improvement of nutritional quality of this legume. In the last years, common bean was chosen as one of the target species to be iron biofortified through the HarvestPlus program developed by CGIAR and started in 2003. The main approach to beans biofortification (2003-2008) has

been to produce varieties with 80% more iron content through the application of breeding programs in the most interested areas (Africa, Latin America and Caribbean, and Asia). The first result was obtained on the Andean gene pool of common beans by the production of Nutritional Improved Andean (NUA) lines, developed by the International Center of Tropical Agriculture (CIAT) in Cali (Colombia). NUA35 and NUA56 were developed to be high in seed iron and zinc content, by the cross of the G14519 high seed accession from CIAT with a CAL96 commercial cultivar in Uganda and Colombia. NUA35 and NUA56 resulted in 81 μg/g and 76 μg/g of iron content, respectively (Blair et al., 2010b). In the following years (2009-2013), many other High Iron Beans (HIB) lines were developed, tested and officially released in 2016 under HarvestPlus in Rwanda, Democratic Republic of Congo, and Uganda (Mulambu et al., 2017).

The other approach to iron biofortification of bean seeds concerns the development of *lpa* (low phytic acid) mutant lines, characterized by an increased iron content and a global improved mineral availability. Breeding programs aimed at obtaining *lpa* crops have been developed and were firstly based on the isolation of two allelic mutants affecting the *PvMRP1* gene, coding for the phytic acid transporter: $lpa^{1\text{-}1}$ and $lpa^{1\text{-}2}$, characterized by a 75-90% reduction in PA seed concentration (Campion et al., 2009; Panzeri et al., 2011; Cominelli et al., 2018). $lpa^{1\text{-}1}$ was also characterized by a 25% reduction of RFOs and a seven-fold increase of free iron cations was shown (Panzeri et al., 2011). The decrease of RFOs is linked to the fact that myo-inositol is a precursor in the synthesis of these sugars: the decrease of PA leads to the reduction of myo-inositol and, consequently, RFOs.

Despite these positive properties of mutated seeds, generally many *lpa* mutants in different crops showed negative pleiotropic effects, including reduced germination, reduced tolerance to stress, stunted growth, thus a very limited potential of these mutant lines for use in breeding (Sparvoli & Cominelli, 2015). However, differently from other cereals or legumes *lpa* mutations, the common bean *lpa*-280-10 ($lpa^{1\text{-}1}$) mutant did not cause macroscopic negative effects influencing seed germination, plant growth, seed yield and other traits of agronomic relevance (Campion et al., 2009 and 2012). Subsequent studies allowed the isolation of other 28 putative *lpa* mutants, all characterized by an increased free inorganic phosphorus concentration (between 51 and 176% compared to the *wt* concentration) (Cominelli et al., 2018).

1.3.3.2 Development of lectin null mutants

Another approach aimed at improving common bean nutritional quality is based on the elimination of antinutritional proteins, in particular lectins, that are considered the most

deleterious in the group of APA proteins (PHA is the most dangerous for mammalian health). Since a residual lectin activity, causing reduced protein digestibility and toxicity, can be detected even after cooking (Bender & Reaidi, 1982), the genetic elimination of lectins has been proposed to improve grain nutritional quality especially in those cases when bean seeds cannot be properly cooked. Lines of the bean cv. 'Taylor's Horticultural' devoid of active PHA were obtained by genetically introducing in the seed the lectin null (*lec/lec* or *lec*-) character from two null genotypes, 'Pinto UI 111' and 'Heidi' (Confalonieri et al., 1992). The influence of PHA on nutritional value of seed proteins of two breeding lines, one lacking and the second with the presence of this lectins, was evaluated by Bollini et al. (1999). Results showed that true protein digestibility was higher for raw and cooked lectin-null beans compared to the parent 'Taylor's Horticultural', raising from 17.55 to 40.13% and from 66.77 to 72.23%, respectively (Bollini et al., 1999).

1.4 **Legumes applications in the food industry**

Research bodies on legume health benefits are growing and are always more promoting the introduction of grain legumes in human diets. In the same way the consumer awareness of a healthy and balanced diet is increasing, especially in more developed countries, as well as the number of intolerant or allergic people which require specific diets. Pulses are well-recognized functional foods and their use as ingredients for food formulations is rapidly increasing, even in the field of gluten-free industry.

1.4.1 *Composite flours with enhanced properties*

Legumes are usually used in the form of flour inside wheat or other cereal-based foods. Composite flours are blends of several flours, which are obtained from different vegetal sources, such as roots, tubers, cereals, and legumes, with or without the addition of wheat flour. The production of composite flours has normally the aim of getting a product that is better than the individual components, meaning with improved properties or performances, or in some cases, improved economies. Moreover, these flours encourage the use of some uncommon and little-known plant sources, which however have a potential high nutritional value. In most cases of composite flours production, cereal flours are enriched in their protein content by the addition of complementary legume flours. Similarly, the nutritional value of root and tuber flours, that have a low protein content, can be sufficiently improved by the addition of pulses as well. For example, the introduction of legume sources to a commercial wheat flour significantly affects the chemical composition of the final composite flour: several

blends prepared by mixing wheat flour with lentil and chickpea flours in different proportion where characterized by increasing content of crude protein, fat, and fiber, as well as a significant variation in phytic acid content (Shahzadi et al., 2005).

The use of pulses in the formulation of composite flours allows not only the enhancement of the nutritional value, but also the modification and/or improvement of some functional properties. Functional properties, including water and oil absorption capacity (WAC and OAC), emulsion activity and stability (EA and ES), foaming capacity and stability (FC and FS), and least gelation capacity (LGC) determine the performance of pulse flour as a food ingredient, thereby affecting the end-product characteristics and the consumer acceptance. Moreover, functional properties constitute the major basis of criteria for the adoption and acceptability of proteins in food systems (Kaur & Singh, 2005). The most relevant functional properties of legume proteins in food matrices are related to their solubility, gel forming, emulsifying, water-/fat-binding, and foaming capabilities (Foschia et al., 2017). Legumes also positively influence rheological properties, indicating a better stability of the suspensions based on pulse flours compared to the same control (Patrascu et al., 2017). Studies demonstrated that, compared with the reference commercial wheat flour, all treated pulse flours (boiled, roasted and germinated) had significant increased WAC, EA, ES and LGC and only slightly decreased emulsion and foaming properties (Ferawati et al., 2019).

1.4.2 *Legume flours in baked goods*

Functional properties of legumes are affected by the treatment to which flours are subjected for the development of the end-product. Pulse flours in several baked products (bread, cake, and muffin) showed positive effects in enhancing resistance to dough expansion, providing good crumb, structure and loaf volume, increasing emulsification, foaming and gelling properties, and preventing coalescence (Foschia et al., 2017). The development of biofortified baked products has the main purpose of extending the knowledge of highly nutritional foods and healthy diets through the consumption of popular foods, which are commonly appreciated by the major part of the population. Crackers and other baked products, like cookies, represent a good way to propose composite flours and to reach all segments of population, since they are characterized by low manufacturing costs, convenience and long shelf-life. Chickpea flour, for example, was used in different proportion for the preparation of wheat-based crackers, leading to a significative increase of protein, dietary fiber, lipid, RS content, density and thickness, thus enhancing both the nutritional value and the functional properties of the final product (Kohajdová et al., 2011). Biscuits with acceptable physical characteristics were produced by partially substitute wheat flour with pulse flour. In particular, composite flour

cookie containing 10% chickpea flour, 10% pigeon pea flour, 10% moong bean flour, 10% cowpea flour, and 60% wheat flour, had maximum proteins (13.42%), fats (22.90%), and total energy (503.83 kcal), considerable free radical scavenging activity, and total phenol content (Thongram et al., 2016).

1.4.3 *Legume flours in gluten-free products*

Another important application of legumes is in the development of gluten-free (GF) products. As the demand for GF products is continuously growing, there is the need to find GF ingredients that can deliver enhanced end-product quality at affordable prices. Generally, GF products tend to have poor nutrient content, poor texture quality and shortened shelf-life (O'Neil, 2010). Consequently, numerous studies on the application of legume sources in GF products have been performed with the aim of enriching their formulation and improving their functional properties. Legume protein supplements have been found to be efficient not only in increasing the nutritional quality (Giuberti et al., 2015; Giuberti & Gallo, 2018; Rocchetti et al., 2019) but also in improving GF dough/batter functionality, sensory characteristics, general acceptance and shelf-life (Ryan et al., 2002; Sciarini et al., 2010). In GF products containing pulse ingredients the final stability of the dough is guaranteed by the presence of polysaccharides hydrocolloids and proteins of various origin. Recently, the use of non-gluten protein sources, such as pea, soybean, lupine, and carob, has been applied in developing GF dough with the desired viscoelastic properties (Foschia et al., 2017).

1.4.4 *Consumers acceptance of legume-based products*

Successful performance of legume flours as food ingredients not only depends on their nutritional and functional characteristics, but also on the sensory quality that they impart to the end-product. Normally, the consumer chooses legume-based products for the benefits that they provide and not for their attractive appearance and organoleptic quality. The main limiting factor in the commercialization of pulse-based goods is to meet consumers expectations as regard sensory aspects, primarily because these products result far from taste and texture of the more traditional cereal-based products. Typically, legumes develop a flavor profile characterized by beany and bitter notes (depending on the type of pulses) that is greatly responsible for the reduction of their acceptability and thus their consumption (Bresciani & Marti, 2019). Moreover, it was observed that the baking times influenced the sensory attributes in terms of aroma: longer exposure to heat treatment caused a higher development of volatile compounds from pulse fraction, responsible for unwanted off-flavors (Setyaningsih et al., 2019). Because of these negative effects on the end-product, it is preferable that pulse flours

do not become predominant in processed foods and they must always be used in combination with other flours.

1.4.5 *Bean-based food products*

Despite their well proven nutritional properties, consumption of dry beans has been relatively flat over the past decades and their regular use in traditional recipes is really appreciated only in some localized areas. In Nigeria and other African countries, for example, fried bean balls, known as '*akara*', are commonly consumed together with other typical dishes as a part of family breakfast (Asif et al., 2013). The creation of new bean products, that appear more attractive and result more tasty than simple boiled seeds, may increase the consumption of common bean also in other countries and allow more consumers to obtain health benefits. In the past decades, common bean has been processed in several ways and used in a wide variety of products, mainly as component of extruded composite flours. Blends of cereals and bean extruded flours always result to have an enhanced nutritional quality, coming from the presence of the legume source and improved through the extrusion process. Cereal-based extruded, fortified with bean flour, are characterized by a reduction of phytates and a complete elimination of active lectins (Arribas et al., 2019) and, on the other side, by an increase of the protein fraction, dietary fiber, mineral content and phenolic compounds (Félix-Medina et al., 2020). The aminoacidic fraction is also affected and it has been demonstrated that the lysine limitation of an extruded cereal flour, as well as the limiting sulfur amino acid content of an extruded bean flour, can be completely offset in a blend of these two ingredients (Nosworthy et al., 2017).

1.4.5.1 Common bean in traditional snacks

Since the snack food market is currently demanding healthier products, some technological innovations are necessary to allow the development of product recipes characterized by less carbohydrates, saturated fats and energy density, and improved nutraceutical quality. Cereals, particularly corn, are the most appropriate for the production of second-generation (expanded) snacks, due to their excellent expansion and texture properties (Meng et al., 2010; Estrada-Girón et al., 2015). Blends of corn and common bean flours have been used for the development of biofortified second-generation snacks, characterized by a good expansion ratio, bulk density and hardness (which are quality indicators for traditional expanded snacks). Results also showed an increase in the protein content, dietary fraction and phenolic compounds (responsible for the improved antioxidant activity), together with a reduction in

the glycemic index of biofortified snacks if compared to expanded commercial snacks (Anton et al., 2009; Espinoza-Moreno et al., 2016; Félix-Medina et al., 2020).

1.4.5.2 Common bean in bakery products

Common bean flour is employed also in the bakery sector and several attempts of biofortification were performed on biscuits. Despite gluten network plays a fundamental role in the structure and texture of baked products, it has to be only slightly developed in biscuits in order to obtain a cohesive but not too elastic dough. The structure of cookies does not depend on protein/starch structure, but primarily on starch gelatinization (Di Cairano et al., 2018). For this reason, biscuits are extremely suitable to be biofortified with non-wheat flours, including bean flour, and to develop GF products intended for celiac people. Bean flours characterized by a low antinutrient content: low phytic acid (*lpa*), lectin absence (*lec*-), and active α-AI, were employed to assess their contribution to biscuit nutritional quality by Sparvoli et al. (2016). Different formulations of composite flours were used in combination with wheat, maize, or with both. These biscuits resulted nutritionally enhanced compared to the control, having a better amino acid score, higher fiber amount and lower starch content and predicted glycemic index (Sparvoli et al., 2016). *P. vulgaris* has also been exploited in the form of extruded flour for the production of reduced-fat biscuits. The high fraction of RS in common bean resulted optimal to counterbalance texture defects by giving crumblier and less hard biscuits. Bean-based biscuits showed characteristic nearly comparable to a traditional reduced-fat product but with improved nutritional profile, having an interesting potential in the reduction glycemic impact or in low-fat product development for diabetic and obese individuals (Moriano et al., 2019).

As for all the other legume-based products, also for bean-based products the consumer acceptability is the main challenge. In many of the already mentioned studies it has been demonstrated that biofortified products resulted on average acceptable, but the level of consumer satisfaction gradually decreased as bean flour percentage in the product increased (Hooper et al., 2019; Sparvoli et al., 2016., Di Cairano et al., 2018).

CHAPTER 2
MATERIALS AND METHODS

2.1 **Materials**

Flours were obtained from three different *P. vulgaris* genotypes, all belonging to the bean market class Borlotto. Genotypes differed for their composition as regards lectins and phytic acid content: (i) control genotype (*wild type, wt*): cv. Mercato; (ii) *lpa* $BC2F_4$ genotype: derived from a cross between cv. Mercato and the original *lpa*-280-10 mutant line (Campion et al., 2009), characterized by low phytic acid content (*lpa*); (iii) *lpa/lec*- BC1F5: derived from a cross between the *lpa* BC2F2 and the Lady Joy genotype, characterized by absence of active lectins (*lec*-).

Composite flours, containing different percentages of the bean flours, wheat flour type 2, whole wheat flour and buckwheat flour were used to prepare biscuits, crackers and a cream. Recipes were developed with the technical assistance of the backers and pastry chefs Mr. Matteo Consolo and Mr. Ferruccio Farioli at ENAIP Lombardia, Busto Arsizio (VA). Two types of crackers, two types of biscuits and a cream were produced to be tested in this study compared to their control product. Recipes are reported in Table 2-1.

Table 2-1: Products recipes

Product	Ingredients	Quantity (g)	% total	% bean flour	
				Total flour	**Whole product**
Cracker 1	Wheat flour type 2	600	38.10	**40**	**26**
	Bean flour	400	25.40		
	Water	550	34.92		
	Yeast	10	0.63		
	Salt	15	0.95		
Cracker 2	Wheat flour type 2	400	24.62	**60**	**38**
	Bean flour	600	36.92		
	Water	600	36.92		
	Salt	15	0.92		
	Yeast	10	0.62		
Biscuit 1 (shortbread)	Butter	500	30.30	**50**	**20**
	Milk	350	21.21		
	Wheat flour type 2	325	19.70		
	Bean flour	325	19.70		
	Vanilla icing sugar	150	9.09		
Biscuit 2 (buckwheat biscuit)	Sugar	350	27.08	**33**	**12**
	Eggs	250	19.34		
	Butter	200	15.47		
	Whole wheat flour	160	12.38		
	Buckwheat flour	160	12.38		
	Bean flour	160	12.38		
	Baking	12.5	0.97		
Cream	Almond milk	1000	28.41	**100**	**9**
	Sugar	300	8.52		
	Egg yolks	300	8.52		
	Bean flour	150	4.26		
	Lemon peel	10	10		

2.2 **Methods**

Crackers and biscuits were both prepared in four variants, using the flours from the three different bean genotypes (*wt*, *lpa/lec*- and *lpa*) or replacing the common bean flour with an equivalent amount of wheat flour type 2 or whole wheat flour (biscuit 2). The cream was prepared in two variants, one containing the *lpa* bean genotype flour and the control one containing rice flour.

For crackers preparation the dough was produced by mixing wheat flour type 2, bean flour, 95% of water. The dough was left to rest in order to activate the autolysis process. After half an hour, yeast was added and also the remaining 5% of water was gradually added to the dough. The dough was divided in pieces of same weight and each loaf was reduced to a thin sheet. Each sheet was put in a baking tin, previously oiled, brushed with olive oil and sprinkled with salt to taste. Sheets were pierced and cut in regular pieces. They were cooked in oven at 190° for about 8 minutes.

Type 1 biscuits, or shortbread biscuits, were prepared by mixing sugar with butter in a planetary mixer until they resulted completely homogenized. Then the other ingredients were added. The dough resulted soft and biscuits were formed using the sac a posh. Biscuits were cooked in oven at 190°C for 14-18 minutes.

Buckwheat biscuits (type 2) were prepared by mixing all the ingredients together in order to produce the dough. Once all the ingredients were completely homogenized, the dough was worked by the 'cylinder processing' and formed. Before cooking, biscuits were sprinkled with a mixture of sugar or salt and cornmeal. They were cooked in oven at 190°C for 14-18 minutes.

For the cream the ingredients were mixed together and cooked about 15 minutes at low temperature (65°C) to pasteurize the egg yolks. The final cream was stored in the fridge at about 4°C.

2.2.1 *Samples preparation*

Bakery products were firstly left in oven at 37 °C overnight in order to dry them. Then they were manually grinded into flour using mortar and pestle.

For some analyses the elimination of lipids was essential. 2 g of flour were mixed with 2 ml of hexane in Corex tubes. They were shaken for 30 minutes on a magnetic stirrer under hood and then centrifuged at 8500 rpm for 20 minutes at room temperature, the supernatant was discarded, and the pellet was kept. The procedure was repeated three times for each sample. After the complete elimination of hexane, defatted samples were left under vacuum inside a diaphragm pump overnight to allow the complete solvent evaporation. At the end of

this process samples were completely dried, and they were transferred into 50 ml Falcon tubes and stored at -18°C until use.

2.2.2 *Hemagglutination test*

This analysis is based on the ability of PHA-E to agglutinate blood erythrocytes and it was used to determine the presence or absence of this lectin activity in the different samples. Hemagglutinating activity in the extracts was determined by a serial dilution method using a human type A erythrocytes suspension. Drops of blood were drawn from the donor finger and collected inside 1.5 ml Eppendorf tube: about 150 µl of blood were collected. Erythrocytes were separated from the serum by washing with about 10 volumes (1.5 ml) of phosphate buffered saline (PBS) (10 mM $KHPO_4$, 15 mM NaCl, pH 7.4) followed by a centrifugation for 2 minutes at 3000 rpm. This step was repeated for three times. The serum was eliminated, and the erythrocytes were stored at 4°C in 10 volumes of PBS. At the time of use erythrocytes were diluted 1:10 with PBS.

100 mg of each sample (biscuits flours were previously defatted) were extracted with 20 volumes (2 ml) of PBS, left under agitation for one hour and centrifuged at 13 000 rpm for 5 minutes at room temperature. The supernatant was transferred into a 2 ml Eppendorf tube and used to perform the hemagglutination test. For each sample (PBS samples both from flours and defatted products), serial dilutions in PBS, ranging from 1:2 to 1:256, were assayed. Agglutination was visually determined after 4 h incubation at room temperature. For this test a microwell plate was used. Each line corresponded to a different sample and each column corresponded to a different dilution of a specific sample. 25 µl of erythrocytes suspension and 25 µl of sample/diluted were mixed inside each well.

The percentage of residual agglutinating activity was calculated considering the amount of bean flour contained in defatted samples, 1/3 (29%) in biscuit 1 and 1/6 (14%) in biscuit 2, and that the shift of one serial dilution corresponds to 1/4 of the product agglutinating activity.

2.2.3 *Assay of α-amylase inhibitor activity*

The analysis was based on a protocol that measures the inhibitory activity of the sample against human salivary α-amylase (EC 3.2.1.1; Type IX-A) by the increase of iodine staining after the action of the salivary α-amylase with soluble starch (Altabella & Chrispeels, 1990).

The incubation solution was composed by 12 µl bovine serum albumin (BSA) solution (10 mg/ml in PBS), different volumes of sample extracts (5, 10, 15 and 25 µl), 100 µl iodine reagent or KI solution (stock solution prepared dissolving 6 g of KI and 0.6 g of I_2 in 100 ml

of distilled water; working solution prepared dissolving 600 µl of stock solution in 5 ml of 0.5 N HCl and 45 ml of distilled water), human saliva α-amylase type IX-A (E.C. 3.2.1.1, 1,000-3,000 units/mg) (use conditions: 0.01 U/µl in PBS, prepared at the time of use from a 2 U/µl stock solution), 0.15% potato starch solution and PBS (50 mM KH_2PO_4 and 10 mM $CaCl_2$, pH 4.4) in a final volume reaction of 200 µl.

Before setting up the assay, it is necessary to determine the amount of starch and α-amylase to be used during the analysis. To this purpose, calibration curves were prepared using increasing amounts of starch and/or α-amylase in a 200 µl final volume. Starch solution (0.15%) was calibrated first to identify the optimal volume able to give an OD_{620} value of about 1.0 (the selected volume was 45 µl). Then, different volumes of α-amylase (0.01 U/µl in PBS) were assayed in order to identify the amount needed to degrade he starch bringing to about 0.4 the OD_{620} of the reaction (selected volume was 15 µl). For each sample the absorbance was read at 620 nm.

After the preparation of the calibration curves, samples could be evaluated: 50 mg of ground defatted products and defatted bean flours were extracted with 20 volumes (1 ml) of PBS. Samples were left under agitation for one hour and centrifuged for 5 minutes at 13 000 rpm. Each supernatant was transferred into a 2 ml Eppendorf tube and stored at -18°C until use. At the time of use, products and flour extracts were diluted with PBS, 1:200 and 1:4000, respectively. The assay was performed in a microplate with the aid of an Infinite® 200PRO – Tecan analyzer (Figure 2-1).

Figure 2-1: Microplate reader Infinite® 200PRO – Tecan analyzer

Samples were first incubated with the α-amylase for 30 minutes at room temperature. During this time the α-amylase was able to react with the α-AI present in the samples. Then, the potato starch solution was added and after 5 min at room temperature the reaction was stopped by adding 100 µl of iodine reagent. In this way it was possible to determine the

residual α-amylase activity after the interaction with the α-AI. Three blanks were prepared: (i) analytical blank: containing only BSA solution, PBS and iodine staining; (ii) blank for α-amylase: containing all the solutions except the samples; (iii) blank for starch: containing all the solutions except the samples and the α-amylase solution. For each sample, including blanks, the absorbance was measured at 620 nm at the end of the reaction. The protocol used is summarized in Table 2-2 (the same scheme was repeated for all samples).

Table 2-2: Scheme used to assay the α-amylase inhibitor activity of samples

Added sample		**BSA (µl)**	**PS sol. (µl)**	**Sample (µl)**	**Amylase (µl)**	**5' 20°C**	**Starch (µl)**	**30' 20°C**	**KI (µl)**	**Total volume (µl)**
Control flour	*wt*	12	23	5	15		45		100	200
		12	18	10	15		45		100	200
		12	13	15	15		45		100	200
		12	3	25	15		45		100	200
	lpa	12	23	5	15		45		100	200
		12	18	10	15		45		100	200
		12	13	15	15		45		100	200
		12	3	25	15		45		100	200
	lpa/lec-	12	23	5	15		45		100	200
		12	18	10	15		45		100	200
		12	13	15	15		45		100	200
		12	3	25	15		45		100	200
Blanks	Analytical	12	88	-	-		-		100	200
	α-amylase	12	28	-	15		45		100	200
	Starch	12	43	-	-		45		100	200

Results were expressed as units of α-amylase inhibited per 100 mg of flour, where one unit of inhibitor activity is the amount which will bring about 50% inhibition of the α-amylase in 30 min under the above conditions. The percentage of residual activity was calculated comparing the expected U of α-AI/100 mg of flour to the measured ones.

2.2.4 *Water content determination*

5 g of each ground sample were transferred into aluminum melting pots previously calibrated in stove at 105°C to evaporate all the residual humidity. Samples were left in a stove at 105°C for 24 hours, cooled and weighted to determine the loss of water.

2.2.5 *Crude protein determination*

For this analysis, samples were assayed according to the AOAC standard Kjeldahl method for the total nitrogen quantitative determination (AOAC, 2000). This method is based on three main steps:

- Mineralization or digestion of the sample;
- Distillation of ammonia;
- Titration for the quantitative determination of the produced ammonia.

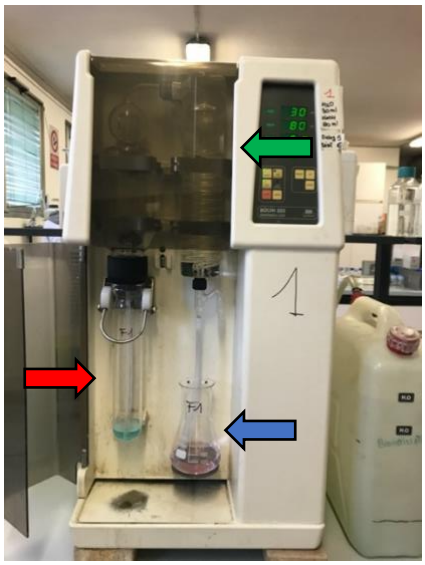

Figure 2-2: Kjeldahl distillation equipment

Red arrow: 400 ml glass tube containing the mineralized sample; blue arrow: flask containing H_3BO_3; green arrow: condenser.

During the digestion 1 g of sample was mixed with 20 ml of pure sulfuric acid (H_2SO_4) inside a 400 ml glass tube (red arrow in Figure 2-2) and it was heated at 400°C for one hour. During this step sodium sulphate (Na_2SO_4) was added as catalyzer in order to elevate the boiling point of sulfuric acid and accelerate the mineralization step. An antifoaming agent was also added to avoid the formation of bubbles. During the mineralization all the protein nitrogen bound the sulfuric acid, producing ammonium sulfate ($(NH_4)_2SO_4$); the organic material was

converted into carbonium dioxide (CO_2) and water (that evaporated at high temperatures) and all the salts into sulphates. The solution continued boiling until the brown color and all visible particles disappeared, leaving a distinct clear and greenish liquid. Concentrated sodium hydroxide (NaOH) was added to the mineralized sample in order to neutralize the sulfuric acid in excess and convert ammonium into free ammonia. A known amount (about 100 ml) of boric acid (H_3BO_3) in excess to ammonia was added in a flask (blue arrow in Figure 2-2). The distillation was performed with an automatic equipment, shown in Figure 2-2. During the distillation the free ammonia boiled off the sample and condensed. The end of the condenser (green arrow in Figure 2-2) was dipped into the flask containing boric acid: here, the acid bound ammonia that was reconverted into ammonium salts.

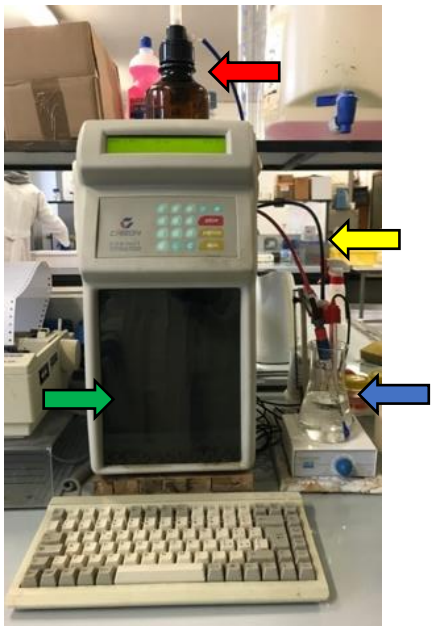

Figure 2-3: Automatic titration equipment

Red arrow: bottle containing HCl; green arrow: syringe (not visible due to the black plastic coverage); yellow arrow: black plastic pipe that connect the syringe to the flask; blue arrow: flask containing the distilled solution.

Ammonium ion concentration in the acid solution, and thus the amount of nitrogen in the sample, was quantified through an acid-base titration. An automatic system was used to perform the titration, using hydrogen chloride (HCl) as titrant and Tashiro's reagent as pH indicator. HCl was contained in a bottle (red arrow in Figure 2-3) and used to automatically fill a syringe inside the instrument (green arrow in Figure 2-3) at the beginning of each titration. The syringe was connected to the flask, containing the solution coming from the

distillation, by a black plastic pipe (yellow arrow in Figure 2-3). HCl was automatically drained from the syringe into the flask (blue arrow in Figure 2-3) through the plastic pipe. At the end of the titration the system automatically calculated the amount of nitrogen that was contained in the sample. The determination of crude proteins was obtained by multiplying the amount of total nitrogen by the conversion factor, 6.25.

2.2.6 *Crude lipid determination*

For this analysis, the official Soxhlet method for lipids extraction was employed (AOAC, 2000). Normally, the Soxhlet equipment is composed by three components, the distillation flask, the extractor, and the condenser. Our extractor (Figure 2-4) was designed to analyze four samples at a time and it is composed by:

- Four distillation glasses at the bottom;
- Four extractors in the middle;
- Four condensers at the top.

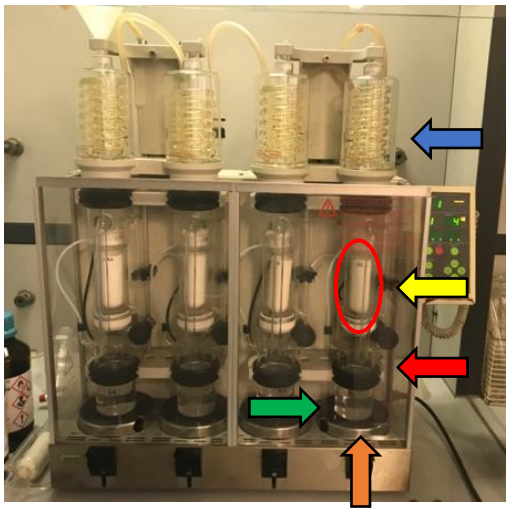

Figure 2-4: Soxhlet extractor equipment

Red arrow: lower chamber in contact with the distillation glass; green arrow: distillation glass filled with the extraction solvent; yellow arrow: extraction chamber containing the thimble filter with the sample; blue arrow: condenser in contact with the extraction chamber; red circle: thimble filter containing the sample; orange arrow: heating plate.

Each separated extraction system is composed by two overlapping and separated chambers communicating through two connectors: one channel for the solvent in the vapor state and a siphon for the extract discharge. The lower chamber (red arrow in Figure 2-4) is in contact

with the distillation glass (green arrow in Figure 2-4), while the central chamber, or extraction chamber (yellow arrow in Figure 2-4) is in contact with a condenser (blue arrow in Figure 2-4) in order to have a continuous recirculation of fresh solvent.

5 g of grinded sample (not previously defatted), in duplicate, were placed in the extraction chamber inside white thimble filters (red circle in Figure 2-4), composed by filter paper and permeable to the solvent. The distillation glasses were filled with ethyl ether, used as solvent. Then it was performed a simple classic extraction procedure: the solvent, in contact with a heating plate (orange arrow in Figure 2-4), was headed to its boiling point, evaporated and was sent to the extractor and condenser. It flowed as condensed in the extraction chamber, where it was charged of solute (sample lipids) and dragged it through the filter paper. Solvent and solute were poured together inside the glass, where the solute remained as precipitate and was recovered at the end. The ethyl ether was removed from the solvent-solute mixture using a rotary evaporator.

2.2.7 *Total starch determination*

For this analysis the AOAC Method 996.11 for the total starch assay procedure (amyloglucosidase/α-amylase method) was used (Megazyme, 2019). 100 mg of grinded sample were placed, in duplicate, in polytetrafluoroethylene (PTFE) tubes, including one tube in duplicate for the blank sample used as control (it will not contain the enzymes). Each sample was moistened with 0.2 ml of 80% v/v aqueous ethanol (EtOH) and tubes were stirred on a vortex mixer to completely wet and disperse the sample: this step is important because it aids the complete dissolution of samples with a high starch content. Two ml of cold 1.7 M sodium hydroxide solution were added to each sample, and tubes were stirred on the vortex mixer for 15 seconds. Tubes were placed in a rack over magnetic stirrer inside cold water and stirred for 15 minutes, in order to reach the complete dissolution of the samples. This step was fundamental to prepare the sample for the enzymatic digestion of the starch. 8 ml of 600 mM sodium acetate buffer (pH 3.8) were added to each sample in order to obtain a final pH around 5.0, and tubes were stirred on the vortex mixer. Immediately, 0.1 ml of undiluted thermostable α-amylase from *Bacillus* sp. (EC 3.2.1.1; 3.000 U/ml) and 0.1 ml of amyloglucosidase (EC 3.2.1.3; 3.300 U/ml) were added in the sample tube. 0.2 ml of 100 mM sodium acetate buffer (pH 5.0) were added to the blank control tubes, instead of enzymes. Tubes were mixed on the vortex mixer and samples were incubated at 50°C for 30 minutes (time required for the enzymatic reaction on starch). Subsequently tubes were cooled and 2 ml from each sample (including the blank control) were transferred to microfuge tubes and centrifuged at 13 000

rpm for 5 minutes. 1 ml aliquot of each supernatant was transferred to 12x120 mm tubes containing 4 ml of 100 mM acetate buffer (pH 5) and mixed. Duplicate aliquots of 0.1 ml for each sample (including the blank control) were accurately transferred to the bottoms of 16x120 mm glass test tubes; also, a single 0.1 ml aliquot of sample blanks was transferred to a 16x120 mm glass test tube. After the addition of 3.0 ml of glucose oxidase/peroxidase reagent (GOPOD), each sample was incubated at 50°C for 20 minutes to allow the reagent to react. This reagent contains two enzymes, a glucose oxidase and a peroxidase, together with a 4-aminoantipyrine and a p-hydroxybenzoic acid. Glucose oxidase catalyzes the oxidation of D-glucose (produced by α-amylase and amyloglucosidase from starch) converting it into D-gluconate and producing hydrogen peroxide. Hydrogen peroxide reacts with the 4-aminoantipyrine and the p-hydroxybenzoic acid releasing a quinone (quinonimine dye) and water. The quinone is a chromophore, thus it is possible to quantify the amount of glucose by measuring the absorbance at 510 nm of the solution against the blank solution. The amount of quinone produced is directly proportional to the amount of glucose and thus to the amount of starch.

The total starch in solid samples was calculated as it follows:

$$\text{Starch \%} = \Delta A \times F \times (EV/0.1) \times D \times (1/1\ 000) \times (162/180)$$

$$= \Delta A \times F \times EV \times (D/W) \times 0.90$$

where:

ΔA = absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against the reagent blank (only where a sample blank is determined).

F = factor to convert absorbance values to mg glucose (100 mg glucose divided by the GOPOD absorbance value obtained for 100 mg of glucose).

EV = sample extraction volume.

0.1 = volume of sample analyzed.

D = further dilution of sample solution.

1/1 000 = conversion from mg to g.

100/W = conversion to 100 mg sample; W = sample weight in mg.

162/180 = factor to convert from free glucose, as determined, to anhydroglucose, as occurs in starch.

2.2.8 *Total sugars determination*

This analysis was performed by using the sucrose, D-fructose and D-glucose assay procedure (Megazyme, 2018). The D-glucose concentration was determined before and after hydrolysis of sucrose by β-fructosidase (invertase), while the D-fructose content was determined subsequent to the determination of D-glucose, after isomerization by phosphoglucose isomerase (PGI). At pH 7.6, hexokinase (HK) catalyzes the phosphorylation of D-glucose by adenosine-5'-triphosphate (ATP) to glucose-6-phospate (G6P) with the simultaneous formation of adenosine-5'-diphosphate (ADP). In the presence of the enzyme glucose 6-phosphate dehydrogenase (G6PD), G6P is oxidized by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. The NADPH was measured by the increase in absorbance at 340 nm.

HK also catalyzes the phosphorylation of D-fructose to fructose-6-phosphate (F6P) by adenosine-5'-triphosphate (ATP). G6P reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance. NADPH again was measured by the increase in absorbance at 340 nm.

At pH 4.6 sucrose is hydrolyzed by β-fructosidase to D-glucose and D-fructose. The D-glucose in the sample following hydrolysis of sucrose (total D-glucose) was determined as mentioned above. The sucrose content was calculated from the difference in D-glucose concentration before and after hydrolysis of β-fructosidase.

Plastic 1 cm light path cuvettes were used. Grinded and defatted samples were analyzed in duplicate as it follows:

Table 2-3: Scheme of samples preparation for the total sugar determination

Pipetted into cuvettes	**Blank sucrose sample**	**Sucrose sample**	**Blank D-glucose/D-fructose sample**	**D-glucose/D-fructose sample**
Solution 6	0.20 ml	0.20 ml	-	-
Sample solution	-	0.10 ml	-	0.10 ml
Mixed and incubated for 5 minutes. Then added:				
Distilled water (at 25°C)	2.00 ml	1.90 ml	2.20 ml	2.10 ml
Solution 1	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Solution 2	0.10 ml	0.10 m	0.10 ml	0.10 ml

Mixed and incubated for 3 minutes. The absorbance of the solutions (A_1) was read at 340 nm. Then added:				
Suspension 3	0.02 ml	0.02 ml	0.02 ml	0.02 ml
Mixed and incubated for 5 minutes. The absorbance of the solutions (A_2) was read at 340 nm. Then added:				
Suspension 4	-	-	0.02 ml	0.02 ml
Mixed and incubated for 10 minutes. The absorbance of the solutions (A_3) was read at 340 nm.				

At the end calculations were applied to determine the amount of free D-glucose, sucrose and free D-fructose.

The absorbance differences (A_2-A_1) and (A_3-A_2) were determined for both blank samples and values of $\Delta A_{D\text{-glucose}}$, $\Delta A_{sucrose}$ and $\Delta A_{D\text{-fructose}}$ were calculated as described below:

- Determination of free D-glucose: $\Delta A_{D\text{-glucose}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$ (from the D-glucose/D-fructose sample);
- Determination of sucrose: the difference between $\Delta A_{total\ D\text{-glucose}}$ and $\Delta A_{D\text{-glucose}}$ (from the D-glucose/D-fructose sample) yields $\Delta A_{sucrose}$ ($\Delta A_{total\ D\text{-glucose}} = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)$ (blank from the sucrose sample));
- Determination of free D-fructose: the absorbance difference (A_3-A_2) was determined for both blank and sample (D-glucose/D-fructose sample only). Subtracting the absorbance difference of the blank from the absorbance difference of the sample $\Delta A_{fructose}$ was obtained.

The concentration (g/l) of D-glucose, sucrose and D-fructose were calculated as it follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A$$

where:

V = final volume (ml)

MW = molecular weight of the substance assayed (g/mol)

e = extinction coefficient of NADPH at 340 nm, 6300 (l m mol^{-1} x cm^{-1})

d = light path (cm)

v = sample volume (ml)

2.2.9 *Total dietary fiber determination*

The total dietary fiber (TDF) was determined using the AOAC Method 991.43 (Megazyme, 2017) based on the sequential enzymatic digestion of 1 g of sample by heat-stable α-amylase, protease and amyloglucosidase. According to this method, all the non-fiber fractions have to

be removed from samples and, only what remains at the end is quantified as TDF. The starch fraction of samples is first subjected to gelatinization, hydrolysis and depolymerization by being cooked at about 100°C with heat stable α-amylase. Samples are then incubated at 60° with protease (to solubilize and depolymerize proteins) and amyloglucosidase (to hydrolyze starch fragments to glucose). The digested residue is filtered, washed, dried and weighted.

1 g of each dehydrated and defatted sample was weighted in duplicate into 400 ml tall-form beakers. Two blank controls, containing no enzymes, were also prepared for each sample. 40 ml of MES-TRIS buffer solution (pH 8.2), prepared dissolving 19.2 g of 2(N-morpholino) ethanesulfonic acid (MES) and 12.2 g of tris(hydroxymethyl)aminomethane (TRIS) in 1.7 deionized water, were added to each beaker. Samples were agitated on magnetic stirrer and completely solubilized in order to prevent the formation of lumps, which would make sample inaccessible to enzymes. After this preliminary preparation, samples were subjected to the enzymatic digestion, performed in continuous agitation as it follows:

Table 2-4: Scheme of samples digestion for the total dietary fiber determination

Solution pipetted into samples (duplicates)	**Pipetted volume**
Heat stable α-amylase solution[a]	50 µl
Covered with aluminum foil squares and incubated in a water bath at 98-100°C for 30 minutes. Then cooled at 60° and added:	
Protease solution[b]	100 µl
Covered with aluminum foil squares and incubated at 60°C for 30 minutes. Then added:	
HCl (0.56 N)	5 ml
pH checked (optimal between 4.1-4.8) and adjusted, if necessary, with 5% NaOH solution or 5% HCl solution. Then added:	
Amyloglucosidase solution[c]	200 µl
Covered with aluminum foil squares and incubated in a water bath at 60°C for 30 minutes.	

[a]About 3 U/ml; Megazyme cat. No. E-BLAAM (Megazyme Interntional, Wicklow, Ireland).
[b]50 mg/ml, about 350 tyrosine U/ml; Megazyme cat. No. E-BSPRT (Megazyme International, Wicklow, Ireland).
[c]3.3 U/ml on soluble starch; Megazyme cat. No. E-AMGDF (Megazyme International, Wicklow, Ireland).

After the digestion process, TDF was quantified by applying in succession precipitation, filtration, washing and drying of both digested samples and blanks. The dietary fiber in

solution was precipitated adding to each beaker 225 ml of 95% EtOH, pre-heated to 60°C. The EtOH volume was measured after heating in order to verify that the ratio of EtOH volume to sample was 4:1 (if EtOH had been accidentally overheated, an increase of volume would have occurred because of alcohol expansion). Beakers were covered with large sheets of aluminum foil and samples were allowed to precipitate after 60 minutes at room temperature. The filtration of each precipitate was performed by using previously calibrated crucibles containing a bed of Celite. The beaker was rinsed in order to collect all the precipitate particles, using a wash bottle with 78% EtOH and a rubber spatula. This liquid was also filtered. The filter residue was washed using a vacuum with two 15 ml portions of 78% EtOH, 95% EtOH and acetone. The crucible containing the residue was dried overnight in oven at 103°C and once cooled, left in desiccator for approximately one hour. At this point, the gross weight (crucible and dried residue) was determined and the net weight of the dried residue was calculated by subtracting the tare weight to the gross weight.

TDF had to be corrected for protein and ash content. For this purpose, one sample residue was analyzed for proteins and the second residue of the duplicate was analyzed for ash. The same procedure has been also applied on blank controls. The protein analysis was performed using the Kjeldahl method, described in the paragraph 2.2.5. For ash analysis, the second residue was incinerated for 5 hours at 525°C, cooled in desiccator and the ash net weigh was calculated.

Calculations have been performed to determine the percentage of TDF in the sample:

$$\text{TDF}\ (\%) = \frac{\frac{\text{R1} + \text{R2}}{2} - \text{P} - \text{A} - \text{B}}{\frac{\text{m1} + \text{m2}}{2}} \times 100$$

where:

m1 = sample weight 1

m2 = sample weight 2

R1 = residue weight from m1

R2 = residue weight from m2

A = ash weight from m1

P = protein weight from R2

B = blank

$$\text{Blank} = \frac{\text{BR1} + \text{BR2}}{2} - \text{BP} - \text{BA}$$

where:

BR = blank residue

BA = blank ash from BR2

BP = blank protein from BR1

The complete method used for TDF is summarized in the scheme below:

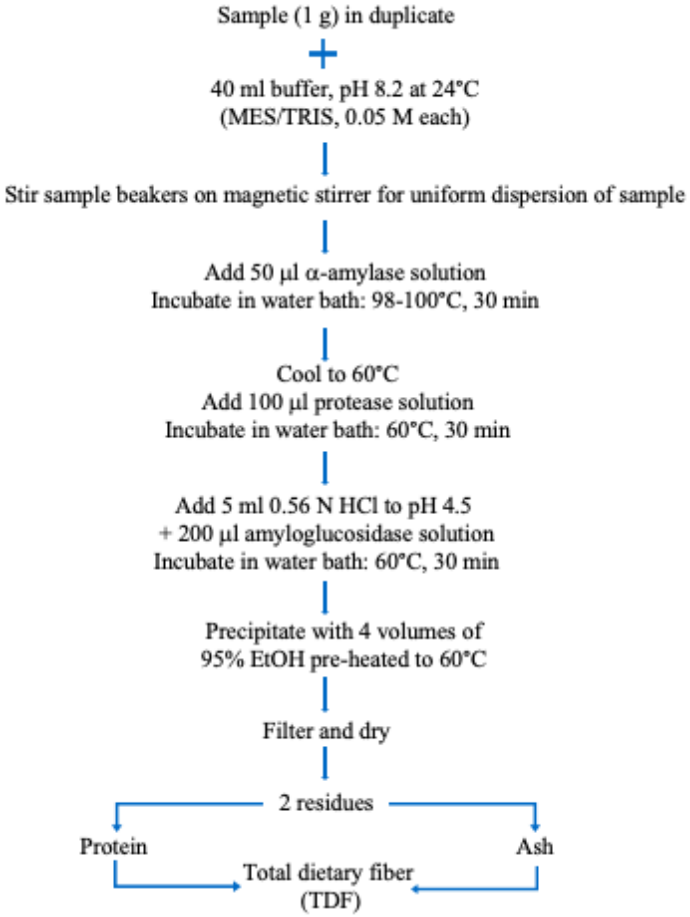

Figure 2-5: Analytical scheme for the total dietary fiber determination procedure

2.2.10 *Iron content determination*

The iron quantification in bean flours and baked products was performed as in Campion et al. 2009 in order to determine iron bioavailability among the three bean genotypes differing in their content of PA. Only biscuit 1, characterized by a higher amount of bean flour, was tested. This analysis was based on the sample mineralization after the iron extraction with different concentrations of hydrogen chloride (0.03 M HCl and 0.1 M HCl). 150 g of bean flours and 450 g of defatted samples were weighted and placed inside Eppendorf tubes. Different concentrations of HCl were added to obtain a final volume of 450 ml for bean flours and 1350 ml for biscuits. The iron extraction is summarized in the following table:

Table 2-5: Scheme of samples extraction for the iron content determination

Genotype	Product	Sample weight (g)	Added HCl volume (ml)
wt	Flour	150	450 (0.03 M) 450 (0.1 M)
	Biscuit	450	1350 (0.03 M) 1350 (0.1 M)
lpa	Flour	150	450 (0.03 M) 450 (0.1 M)
	Biscuit	450	1350 (0.03 M) 1350 (0.1 M)
lpa/lec-	Flour	150	450 (0.03 M) 450 (0.1 M)
	Biscuit	450	1350 (0.03 M) 1350 (0.1 M)

The extraction was carried out overnight at 8°C under agitation and subsequently samples were centrifuged at 13000 rpm for 10 minutes. The supernatant was recovered, transferred into glass tubes and mineralized at 100°C. This step allowed to collect the mineral component after the elimination of the organic fraction. All samples were then resuspended with nitric acid (70% HNO_3) and mineralized in a microwave oven in order to completely eliminate any organic residue. Samples were then read in an optical ICP (Inductive Coupled Plasma) Spectrometer Perkin Elmer Optima 2100 DV.

2.2.11 *In vitro predicted glycemic index*

The analysis was based on the simulation of a human digestion process (Englyst et al., 1992), consisting of three steps: oral phase, gastric phase and intestinal phase. At the end the absorbance of samples was read at 510 nm to determine the amount of glucose produced from the enzymatic digestion. Commercial fresh white bread was used as reference. According to this method, the following reagents were prepared:

- Salivary solution (for the oral phase): dissolved in distilled water 58.5 mg of sodium chloride (NaCl), 74.5 mg of potassium chloride (KCl), 1.05 g of sodium bicarbonate ($NaHCO_3$), 0.2 g of urea and 1.06 g of α-amylase from porcine pancreas (Type VI-B). pH corrected with hydrogen chloride or sodium hydroxide until 6.8;

- Acid solution (for the gastric phase): 375 mg of pepsin from porcine gastric mucosa (5 mg/ml) dissolved in 75 ml of hydrogen chloride 0.05 N;
- Final enzymatic solution (for the intestinal phase): three enzymatic solutions were mixed in different proportions: 6 ml of enzymatic solution 1, 1.5 ml of enzymatic solution 2 and 4 ml of enzymatic solution 3.

  Enzymatic solution 1: 4 ml of amyloglucosidase from *Aspergillus niger* (about 300 U/ml) diluted in 8 ml of distilled water (prepared at the moment of use).

  Enzymatic solution 2: 12 g of pancreatin from porcine pancreas (about 7500 FIP-U/g) dissolved in 80 ml of distilled water, divided in four aliquots in centrifuge Teflon tubes and centrifuged at 5000 rpm for 5 minutes at room temperature (prepared at the moment of use).

  Enzymatic solution 3: 36 mg of invertase from *Saccharomyces cerevisiae* (about 300 U/ml) dissolved in 4 ml of distilled water (prepared at the moment of use).

1 g of grinded sample was weighted in duplicate and was analyzed following the three digestion steps. Each sample was compared with its blank control, the sample not treated with enzymes. For each step different solutions and different periods of incubation at body temperature were necessary in order to simulate the human digestion process, according to the following scheme:

Table 2-6: Scheme of samples preparation for the glycemic index analysis

Digestion step	**Pipetted solutions**	**Samples (duplicate)**	**Blank control**
Oral phase	Salivary solution	5 ml	-
	Distilled water	-	5 ml
	Incubation for 5 minutes at 37°C in orbital incubator at 200 rpm.		
Gastric phase	Acid solution	5 ml	-
	HCl	-	5 ml
	Incubation for 30 minutes at 37°C in orbital incubator at 200 rpm.		
Intestinal phase	Acetate buffer	20 ml	20 ml
	Final enzymatic solution	5 ml	-
	Distilled water	-	5 ml
	Incubation for 180 minutes at 37°C in orbital incubator at 200 rpm.		

In order to monitor the digestion process, several withdrawals were performed from the digested solutions during the analysis. After the incubation period of the gastric phase a first 500 ml aliquot from the duplicate sample and the blank was placed in a plastic tube with 20 ml of 96% EtOH (used to block the enzymatic reaction). This was considered the time zero, from which to start calculating the digestion process. During the intestinal phase four different sampling were done (500 ml from the duplicate sample and the blank were mixed with 20 ml of EtOH for each sampling) at four different times: 30, 60, 120 and 180 minutes. After each collection, samples and blank were rapidly stirred on vortex mixer.

For each solution (digested sample + EtOH and blank + EtOH) 150 ml were placed in a cuvette with 3 ml of GOPOD reactive and incubated at 40°C for 20 minutes. The absorbance was read at 510 nm against the blank (150 ml of EtOH + 3 ml of GOPOD).

For the determination of the glycemic index a digestion curve was created for each sample and compared with the digestion curve of white bread: the predicted glycemic index (pGI) was calculated as the incremental area under the blood glucose response curve (AUC) divided by the incremental area under the blood response curve of the standard food (white bread) and multiplied by 100:

$$\mathrm{pGI} = \frac{\mathrm{AUC\ (test\ food)}}{\mathrm{AUC\ (white\ bread)}} \times 100$$

2.2.12 *Sensorial evaluation*

The sensorial analysis for the evaluation of the customer's acceptance was performed in collaboration with Dr. Stefano Predieri and its team at the Institute for BioEconomy (IBE), CNR of Bologna. For this analysis, products without bean flour (control products) were compared to those containing different amounts of the legume flour: two types of shortbread biscuits (0% and 20% of bean flour), two types of buckwheat biscuits (0% and 12% of bean flour), three types of crackers (0%, 26% and 38% of bean flour) and two types of cream (0% and 9% of bean flour) were analyzed. Evaluated products are shown in Figure 2-6. The test was carried out by a panel composed of 10 expert judges, trained and selected in accordance with the standard UNI EN ISO 8585-2:2008 ('Sensorial analysis – General guide for the selection, training and periodic verification of judges – Part 2: judges expert in sensorial analysis'). These judges were also familiar with the sensorial software used for the creation of specific programs for the execution of sensorial tests (Fizz-Biosystemès, France). For each sample, it was asked to evaluate all the descriptors required by the descriptive analysis,

following the standard ISO 13299:2016 ('Sensory analysis – Methodology – General guidance for establishing a sensory profile'), and the dominance by the Temporal Dominance of Sensation (TDS). Tests were performed inside individual cabins and each judge executed two replicas, for a total of 20 evaluations of each reference. Data were registered in notebooks with the specific sensorial software.

Figure 2-6: Samples used for the sensorial evaluation

On the top, from left to right: control cracker, cracker with 26% of bean flour and cracker with 38% of bean flour. In the middle, from left to right: control buckwheat biscuit and buckwheat biscuit with 12% of bean flour. On the bottom, from left to right: control shortbread biscuit and shortbread biscuit with 20% of bean flour. On the right, from left to right: control cream and cream with 9% of bean flour

Before to start the session, the judges received by the leader panel the detailed instructions on the attributes to be evaluated and on the test methodology. Samples were distributed to the judges inside plastic dishes (biscuits and crackers) and plastic cups (cream), coded with three-digit numbers and presented randomly. Mineral water was distributed to the judges to clean their mouth between one sample and another. Each judge was delivered one product at a time and asked to evaluate, according to its experience, all the attributes using a nine-points intensity scale (1 = hardly perceptible; 9 = very intense). All the products had some descriptors in common, including the odor and flavor of legumes (for the olfactory/aromatic aspect), graininess (for the texture) and bitterness, salinity, umami and astringency (for the gustatory/chemesthetic aspect). Baked products, instead, had in common the whole meal/bran odor and flavor and part of the texture (consistency, crunchiness, friability and adhesiveness).

The humidity was considered for the shortbread and cracker and the chewiness only for the latter. The sweetness was considered for all the products except for the cracker, since it had little impact and it did not discriminate the product. The odor and flavor of butter were evaluated for the biscuits and, in particular, the odor and flavor of biscuit for the buckwheat biscuit and the odor and flavor of shortcrust pastry for the shortbread biscuit. Only for the cracker the initial snapping was considered to evaluate the intensity of the characteristic sound produced by breaking the product in two pieces, and also the typical odor of cracker and the flavor of wheat were evaluated. The cream was the product that, for its preparation method and consistency, has differentiated the most in descriptive terms. Indeed, in addition to the aforementioned attributes, it was evaluated for the odor and flavor of eggs, cream, lemon and almond, the texture of creaminess, viscosity and flouriness. At the end of each evaluation it was required an overall rating of the product.

To complete the descriptive evaluation of the sensorial attributes, a TDS test was performed. This method describes the evolution of the dominant sensorial perceptions during the product tasting (60 seconds). It is a sensorial test based on a 'dynamic tasting', in which several attributes are presented, and the judge is asked to indicate and evaluate the dominant one until the end of the sensorial perception. According to this analysis, a list of attributes to be assessed was presented to each judge and he had to continuously choose between them throughout the tasting process in order to describe the multidimensionality of the sensorial perception. The test begun by clicking on the 'start' button suddenly after taking the sample to the mouth. During the evaluation each judge selected the attribute perceived as dominant, moving among different attributes whenever the perceived stimulus changed. At the end of the sensorial perception, the test was ended by clicking on the 'stop' button. For these products a TDS taste/flavor was performed, considering only the attributes linked to the gustative/aromatic aspect. The flavor of legume was in common to all the products. Regarding the biscuits, the flavor of butter, whole meal/bran, the sweetness and umami were also evaluated, while the flavors of biscuit and shortcrust pastry were considered only for the buckwheat biscuit and shortbread biscuit, respectively. For the crackers, the flavor of wheat, whole meal/bran, the salty and umami were added to the flavor of legumes. For what concern the cream, the flavor of lemon, almond, eggs, cram, the sweetness and umami were evaluated in addition to the flavor of legumes.

Finally, results were elaborated by a statistical analysis using the software SAS® (SAS 9.4, SAS Institute Inc., Cary, NC, USA). The sensorial profiles were analyzed through the ANOVA and post hoc test (Turkey's HSD) and displayed in a visual plot. For the TDS, the proportion

of runs for which each attribute was considered as dominant was calculated for each point in time. These proportions were traced over time using the SAS® TRANSREG procedure and named ‘TDS curves’.

CHAPTER 3
RESULTS

3.1 **Proximate composition of bean-based products**

Crackers and biscuits in all their variants were analyzed for their proximate composition, in comparison to the three bean-flours, in order to give an estimation of their nutritional value (Table 3-1).

Results show that the protein content is relatively high in all the baked products, due to the contribution of the bean flour. The highest protein fraction is found in cracker 2 (20.5 g/100g), also characterized by the highest bean flour content (38% on the total product). As expected, biscuits contain the lowest protein amount (11.4 g/100g), because of the low percentage of the bean flour in their formulation (20% and 12% on the total in biscuit 1 and biscuit 2, respectively). However, in biscuits the protein content is increased by the presence of milk in biscuit 1 and eggs in biscuit 2.

The lipid content is high in biscuit 1 (32.5 g/100g) and is significant in biscuit 2 (15.0 g/100g), since, in both cases, butter gives its contribution. On the contrary, crackers, which are prepared with no added fats (except for the olive oil used to sprinkle the pan), show a low lipid content (3.2 g/100g), almost completely deriving from wheat and bean flours. In these products the increase of bean flour percentage seems to not affect the lipid fraction. Compared to control biscuits, the addition of bean flour increased protein content, as well as ashes and crude fiber.

The carbohydrate fraction appears variable between baked products. The starch content is high in cracker 1 (45.9 g/100g), but it decreases in cracker 2 (37.5 g/100g), where the bean flour is present in a higher proportion. On the contrary, in biscuits as the bean flour content increases, even the starch fraction increases, being higher in biscuit 1 than biscuit 2 (20.3 g/100g and 16.9 g/100g, respectively). This difference might be explained by the composition of biscuit 2, where the presence of whole wheat and buckwheat flours in the recipe contribute to the lower starch fraction. On the other hand, these ingredients are responsible for the slight increase of dietary fiber (16.8 g/100g in biscuit 1 and 17.4 g/100 g in biscuit 2). The dietary fiber constitutes a significant fraction also in crackers and it increases as the bean flour in the product increases, being higher in crackers 2 than in cracker 1 (20.9 g/100 g against 16.4

g/100g). In crackers the total sugars are negligible, while, as regard biscuits, they seem to be relevant only in the saccharose fraction (glucose and fructose are almost absent). This difference between products is simply linked to the recipe: biscuits are characterized by the presence of added sugar, almost three times higher in biscuit 2 than in biscuit 1 (30.9 g/100 g and 11.6 g/100g, respectively), mainly composed by saccharose. Therefore, it is possible to deduce that bean flour does not give any contribution to the sugar percentage in the final product.

Finally, the ash content is significant in crackers (6.6 g/100 g in cracker 1 and 8.0 g/100g in cracker 2), and probably it derives from the salt sprinkled on the products before cooking. It is observed a small increase of the ash content in cracker 2, parallel to the increase of common bean flour. For what concern biscuits, biscuit 2 has a slightly higher ash fraction (1.8 g/100 g), that can be explained by the presence of less refined flours in the recipe.

Comparing the three flour genotypes, it seems not to emerge any particular difference regarding the nutrient fractions composing the final product. The only exception is related to the protein content: the presence of a higher protein content in the *lpa/lec*- genotype seems to be a common trend in all the product groups.

Table 3-1: Proximate composition of bean-based products in g/100 g

Sample		Water	Crude protein	Crude lipid	Total carbohydrates					Ash
					Starch	Saccharose	Glucose	Fructose	Dietary fiber	
Bean flour	*wt*	10.6	23.1	1.1	44.9	-	-	-	-	3.8
	lpa	10.3	27.9	0.7	34.5	-	-	-	-	3.7
	lpa/lec-	10.6	27.7	0.6	36.0	-	-	-	-	3.5
Cracker 1	*wt*	6.3	17.0	3.1	46.3	< LOQ	0.9	< LOQ	17.9	6.7
	lpa	7.4	18.8	3.7	46.7	< LOQ	0.9	< LOQ	14.6	6.7
	lpa/lec-	7.3	19.1	3.3	44.6	< LOQ	0.9	< LOQ	16.7	6.3
Cracker 2	*wt*	6.9	18.5	2.2	38.5	< LOQ	1.1	0.5	22.8	7.8
	lpa	7.7	21.2	3.4	37.5	< LOQ	0.5	1.2	18.3	8.6
	lpa/lec-	6.5	21.7	3.4	36.6	< LOQ	< LOQ	1.0	21.7	7.5
Biscuit 1	*wt*	5.0	11.0	32.6	21.2	12.7	0,0	0.0	14.8	1.5
	lpa	4.3	11.5	32.9	20.5	10.7	0.0	< LOQ	17.3	1.3
	lpa/lec	4.6	11.7	32.0	19.3	11.3	0.0	< LOQ	18.4	1.3
Biscuit 2	*wt*	4.5	11.0	15.0	18.6	28.5	0.0	< LOQ	17.7	1.8
	lpa	5.0	11.6	15.0	17.2	32.0	0.0	< LOQ	16.2	1.8
	lpa/lec-	4.6	11.6	15.4	14.8	32.3	0.1	< LOQ	18.2	1.8

3.2 **Evaluation of hemagglutinating activity**

Bean seeds accumulate high amounts of toxic PHAs, and the excessive consumption of raw or unproperly cooked beans may cause poisoning. Therefore, since the aim of this work is to exploit the use of biofortified bean flours to obtain novel fortified foods, it is important to determine any residual lectin activity in the bean-based products (crackers and biscuits in their variants). To quantify the amount of lectins in the different bean-based products, equal amounts of extracts were compared with equal amounts of extracts from flours of the *wt* genotype as control, and *lpa* and *lpa/lec-* genotypes, with and without PHAs respectively (Figure 3-1 A and B). Blood erythrocytes were gently stirred inside the microplate in order to understand the level of hemagglutination: agglutinated erythrocytes formed a film, while the non-agglutinated ones could be easily resuspended in solution.

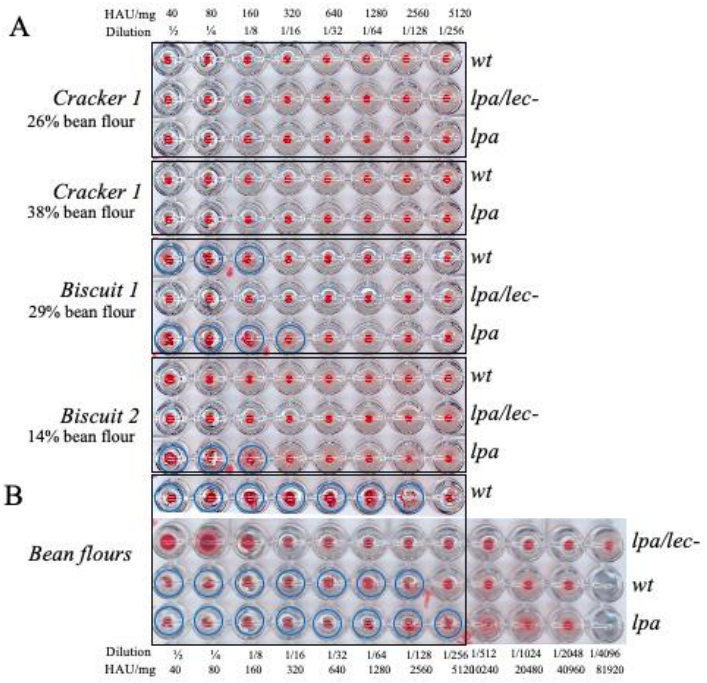

Figure 3-1: Hemagglutinating activity of bean-based products and bean flours extracts

Serial dilutions of equal amounts of bean-based products (A) or seed flour extracts (B) obtained from three different genotypes (wt, lpa and lpa/lec-) are compared. Blue circles indicate sample dilutions able to agglutinate red blood cells. The percentage of bean flour in biscuits is adjusted on the weight of defatted samples.

Results on flour extracts (Figure 3-1 B) show that, as expected, the flour extract characterized by the absence of active PHAs (*lpa/lec*- genotype) is not able to agglutinate blood erythrocytes at any dilution. On the contrary, flours extracts containing PHAs are able to agglutinate blood erythrocytes after a serial dilution until 1/128 for the *wt* genotype and 1/256 for the *lpa* genotype.

The hemagglutination test on defatted products (Figure 3-1 A) indicates the presence of residual lectin activity only in biscuit 1 and biscuit 2, containing 29% and 14% of bean flour. As expected, none of the *lpa/lec*- genotypes report a residual hemagglutinating activity. Cracker 1 and cracker 2, containing 26% and 38% of bean flour on the total product, respectively, are not able to agglutinate blood erythrocytes. The bean flour *lpa* genotype has a higher hemagglutinating activity than the *wt* and this behavior is reflected in biscuits: biscuit 1, made with flours of *wt* and *lpa* genotypes show a residual hemagglutinating activity until the dilution 1/8 and 1/16, respectively, while for biscuit 2 the agglutinating activity is present until the dilution 1/8 and it is detected only on the sample made with flour of the *lpa* genotype.

Comparing these agglutination results with those made on corresponding amounts of unprocessed bean fours, and considering that biscuit 1 and 2 contain about 1/3 and 1/6 of bean flour, that means a shift of one serial dilution, it is possible to quantify the residual PHA activity in processed biscuits, resulting in about 10-12% in biscuit 1 *wt* genotype, 20-25% in biscuit 1 *lpa* genotype, 0% and 20-25% in biscuit 2 *wt* and *lpa* genotypes, respectively.

3.3 **Evaluation of α-amylase inhibitor activity**

α-AI plays an important role in lowering the starch digestion and its effect may be reflected on the glycemic index reduction. Processed products characterized by a total, or at least partial, α-AI activity retention could be introduced in diets for people who require a low blood-glucose level (including diabetic people) for the maintenance of a good health status. In order to verify the role of α-AI, its activity was assessed in defatted extracts of crackers, biscuits and lyophilized cream, and compared to the α-AI of control raw flours. We performed the analysis on two sets of products: set A and set B. Set A was composed by control flours, cracker 1, cracker 2, biscuit 1, biscuit 2 and cream. Biscuits and cracker 1 were produced in three variants using the three genotypes of bean flour (*wt*, *lpa* and *lpa/lec*-), cracker 2 was produced with *wt* and *lpa* bean genotypes and the cream was produced only with the *lpa/lec*- bean flour. In order to analyze cracker 2 made with *lpa/lec*- genotype (lacking in set A) we prepared another set of products (set B), composed by control flours and crackers, both produced with the three

different bean flour genotypes (with the exception of cracker 2 that was available only with *lpa* and *lpa/lec-* bean flours).

Results of α-AI activity tested in the two sets of products tested in the α-AI assay are reported in Table 3-2. Looking at set A, an insignificant residual inhibitory activity is detected in cracker 1 made with *wt* and *lpa* genotypes, and in cracker 2 made with *wt* genotype, while a higher percentage of residual activity is reported for cracker 1 made with *lpa/lec-* genotype (16.63%) and cracker 2 made with *lpa* genotype (32.34%). Biscuit 1 is characterized by a limited retention of α-AI activity (about 0.6%) regarding the two genotypes containing lectins, while in biscuits made with the flour from the *lpa/lec-* genotype a higher inhibitory activity (51.19%) is observed. Biscuit 2 in general is characterized by a high residual activity, especially when *lpa* and *lpa/lec-* genotypes are considered, in which an almost full (71.01% and 84.17%, respectively) inhibitory activity is maintained. The cream is characterized by a retention of 27.28% of α-AI activity.

Looking at set B, control flours show a little higher activity (around 1.3 folds) than corresponding samples of set A. However, crackers 1 and 2 are characterized by a different inhibitory activity if compared to crackers of set A and, differently from the latter, crackers of set B are similar to each other, having a residual activity that ranges from 28% to 36% in cracker 1 and from 33% to 40% in cracker 2. These data indicate that absolute values of α-AI activity of the same products obtained in different moments are not easy to compare, most likely, major differences may be due to small different cooking conditions (the two sets were produced with different ovens). Furthermore, the α-AI assay requires a very high standardization technically and only a limited number of samples can be handled. A higher number of replicates would have been helpful to obtain more reliable results.

Despite this, some conclusions can be drawn. First of all, the *lpa/lec-* genotype shows the highest retention activity in all the food categories (except for cracker 2 of set B, in which the difference between *lpa* and *lpa/lec-* genotypes is not so relevant, but the residual α-AI activity is around 36%). In addition, in set A biscuits retain a higher percentage of inhibitory activity, probably due to the slower heat penetration in the food matrix during the baking process, caused by their more complex composition. Differently, crackers are the products with the lowest α-AI retention (even if they were left in oven for only 8 minutes), probably because the fast heat penetration in their simple matrix allowed a very efficient inactivation of the inhibitor. Furthermore, results suggest that the level of α-AI activity did not correlate with the amount of bean flour in the different products: the highest value was recorded in biscuit 2, in which it

represents only 12%, while cracker 2 of set 1, containing the highest amount of bean flour (38%) is characterized by a low inhibitory activity.

Table 3-2: α-amylase inhibitor activity (%) in defatted bean-based products extracts

Set of products	Sample		% bean flour on the total sample	Expected U α-AI/100 mg flour	Measured U α-AI/100 mg flour	% of residual α-AI activity
Set A	*Control flours*	*wt*	100	-	1552.33	-
		lpa		-	1067.25	-
		lpa/lec-		-	1253.10	-
	Cracker 1	*wt*	26	620.93	4.23	0.68
		lpa		426.90	2.76	0.65
		lpa/lec-		501.24	83.86	16.63
	Cracker 2	*wt*	38	931.40	2.20	0.24
		lpa		640.35	207.09	32.34
	Biscuit 1	*wt*	29[a]	494.73	101.11	20.44
		lpa		340.13	47.42	13.94
		lpa/lec-		399.36	204.43	51.19
	Biscuit 2	*wt*	14[b]	232.38	87.77	37.77
		lpa		159.78	113.45	71.01
		lpa/lec-		187.60	157.89	84.17
	Cream	*lpa/lec-*	24[c]	304.50	83.06	27.28
Set B	*Control flours*	*wt*	100	-	1985.94	-
		lpa		-	1460.28	-
		lpa/lec-		-	1724.66	-
	Cracker 1	*wt*	26	794.38	224.03	28.20
		lpa		584.11	174.84	29.93
		lpa/lec-		689.87	245.78	35.63
	Cracker 2	*lpa*	38	876.17	347.06	39.61
		lpa/lec-		1034.80	343.05	33.15

[a-b] percentage adjusted on the weight of defatted samples
[c] percentage adjusted on the weight of the lyophilized sample

3.4 **Iron content determination**

In order to verify whether the use of biofortified bean flour (carrying the *lpa* mutation) had a positive effect on iron bioavailability in bean-based products, the content of iron was measured by extracting bean and biscuit 1 flours (both for the three bean genotypes) with HCl solutions at different concentrations. Results, referred to different concentrations of HCl, are reported in Table 3-3.

Table 3-3: Iron content of bean flours and bean-based biscuits in percentage

Genotype	Sample	Added acid (M)	Iron content (%)
wt	Flour	0.03 HCl	10.51
		0.1 HCl	10.63
		HNO_3	44.77
	Biscuit	0.03 HCl	0.83
		0.1 HCl	7.62
		HNO_3	34.18
lpa	Flour	0.03 HCl	6.07
		0.1 HCl	29.83
		HNO_3	26.83
	Biscuit	0.03 HCl	8.39
		0.1 HCl	18.09
		HNO_3	28.17
lpa/lec-	Flour	0.03 HCl	26.13
		0.1 HCl	28.11
		HNO_3	27.82
	Biscuit	0.03 HCl	1.11
		0.1 HCl	15.24
		HNO_3	28.17

It is expected that low-concentration of HCl (0.03 N) would extract only or mainly free or weakly bound organic iron (not complexed with PA), and a more concentrated HCl (0.1 N) solution should increase, beside of free iron, the extractability of weakly bound organic iron

(Campion et al., 2009). Total iron (including that in the form of PA salts) is extracted by mineralization in 70% nitric acid.

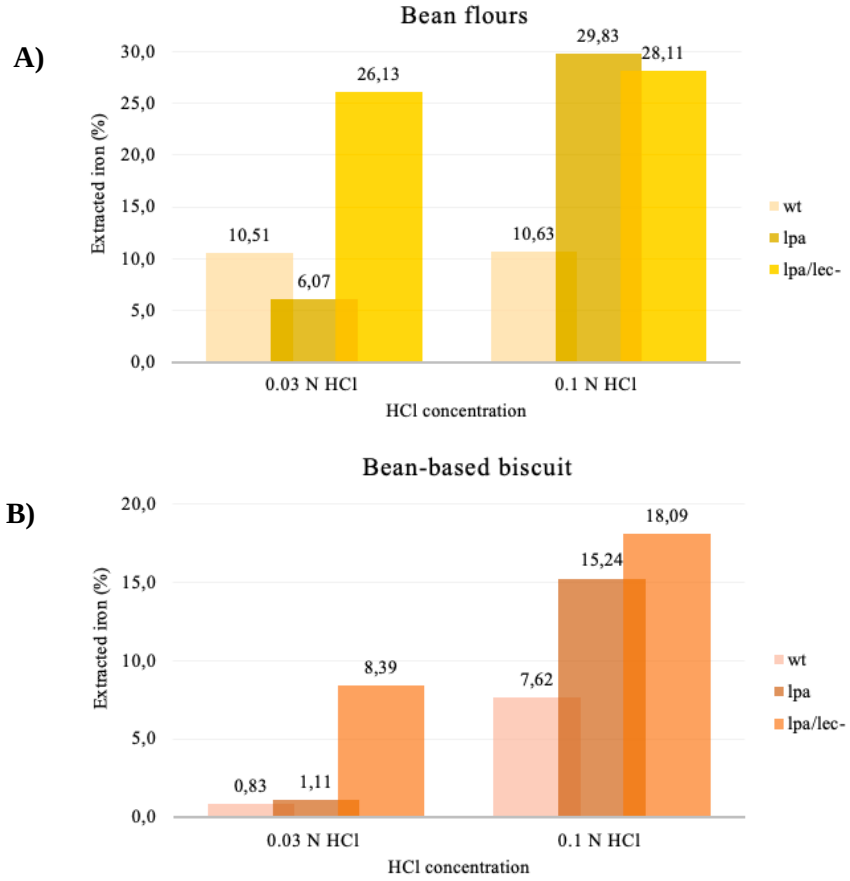

Figure 3-2: Percentage of iron extracted from flours (A) and biscuits (B)

Data in Figure 3-2, clearly show that, as expected, for seed flours (Figure 3-2, A) iron is poorly extracted from *wt* samples, both with 0.03 and 0.1 HCl (10.51% and 10.63%, respectively). With 0.03 N HCl, a higher iron amount is extracted from the *lpa/lec-* genotype (26.13%), while for the *lpa* genotype only 6.07% of iron is detected. However, the last flour extracted with 0.1 HCl shows a significantly higher iron content (29.83%), which is similar to the one detected in the *lpa/lec-* genotype (28.11%) using the same HCl concentration.

When biscuit samples are considered (Figure 3-2, B) a similar trend is observed, although at 0.1 N HCl an increase in extractable iron is detected in all biscuit flours that is more significant for *lpa* genotypes (7.62% of iron is extracted from the *wt* genotype, while 15.24% and 18.09% of iron is extracted from *lpa* and *lpa/lec-* genotypes, respectively). *wt* and *lpa* genotypes resulted poorly extractable with 0.03 N HCl (0.83% and 1.11%, respectively), while the *lpa/lec-* genotype showed the best iron extractability with both HCl concentrations (8.39%

with 0.03 N HCl and 18.09% with 0.1 N HCl). However, comparing the iron contents of biscuits, it is particularly evident (more than in flours) the effect of using different HCl concentrations: 0.1 N HCl solution allows to extract far more iron than the less acidic one.

3.5 *In vitro* predicted glycemic index

The glycemic index was predicted *in vitro* on crackers and biscuits using white bread as control. Results are reported in Table 3-4, where pGI is compared to the carbohydrate fractions of each sample.

Table 3-4: In vitro predicted glycemic index of bean-based products

Sample		% of bean flour on the total sample	Total carbohydrates				Dietary fiber	pGI[a]
			Starch	Saccharose	Glucose	Fructose		
Cracker 1	*wt*	26	46.3	< LOQ	0.9	< LOQ	17.9	76.6
	lpa		46.7	< LOQ	0.9	< LOQ	14.6	81.1
	lpa/lec-		44.6	< LOQ	0.9	< LOQ	16.7	71.4
	Average		**45.9**	**< LOQ**	**0.9**	**< LOQ**	**16.4**	**76.4**
Cracker 2	*wt*	38	38.5	< LOQ	1.1	0.5	22.8	61.6
	lpa		37.5	< LOQ	0.5	1.2	18.3	58.3
	lpa/lec-		36.6	< LOQ	< LOQ	1.0	21.7	55.4
	Average		**37.5**	**< LOQ**	**0.5**	**0.9**	**20.9**	**58.4**
Biscuit 1	*wt*	29[b]	21.2	12.7	0,0	0.0	14.8	39.8
	lpa		20.5	10.7	0.0	< LOQ	17.3	44.8
	lpa/lec		19.3	11.3	0.0	< LOQ	18.4	42.9
	Average		**20.3**	**15.6**	**0.0**	**0.0**	**16.8**	**42.5**
Biscuit 2	*wt*	14[c]	18.6	28.5	0.0	< LOQ	17.7	50.4
	lpa		17.2	32.0	0.0	< LOQ	16.2	51.8
	lpa/lec-		14.8	32.3	0.1	< LOQ	18.2	45.0
	Average		**16.9**	**30.9**	**0.0**	**< LOQ**	**17.4**	**49.1**

[a] white bread as control (pGI = 70)
[b-c] percentage adjusted on the weight of defatted samples
LOQ = Limit Of Quantification

Cracker 1 has the highest glycemic index (76.4), which is directly linked to its high percentage of starch (primarily deriving from wheat flour). On the contrary, in cracker 2, the high amount of bean flour contributes to the reduction of the starch fraction and the increase

of the dietary fiber, responsible for a lower pGI (58.4). In crackers sugars represent an insignificant fraction and they probably do not directly affect the pGI.

Biscuits have a more complex composition and a similar pGI (42.5 for biscuit 1 and 49.1 for biscuit 2). The starch fraction is higher in biscuit 1, while sugars, consisting almost completely in saccharose, are doubled in biscuit 2. Saccharose is primarily responsible for the higher pGI of biscuit 2. Biscuit 2 is also characterized by the presence of whole wheat and buckwheat flours that counterbalance the effect of sugars by increasing the fiber fraction, which keeps the pGI relatively low. However, biscuit 1 has a higher content of bean flour and the half of sugars of biscuit 2, which lead to the slight reduction of pGI.

Looking at bean flour genotypes, no significant trends have been found in relation to the glycemic index.

3.6 **Consumer test**

The sensorial profiles obtained from the descriptive test allowed to determine the sensorial attributes and the consumer's acceptance of these products. Data are displayed in graphic representations, where the sensory characteristics of the ideal product of reference (without bean flour) are compared to the sensory profiles of bean-based samples. The TDS taste was useful to understand the changes in consumer's perception during the tasting experience. From this analysis, curves representing the evolution of the dominance index of an attribute during time were created.

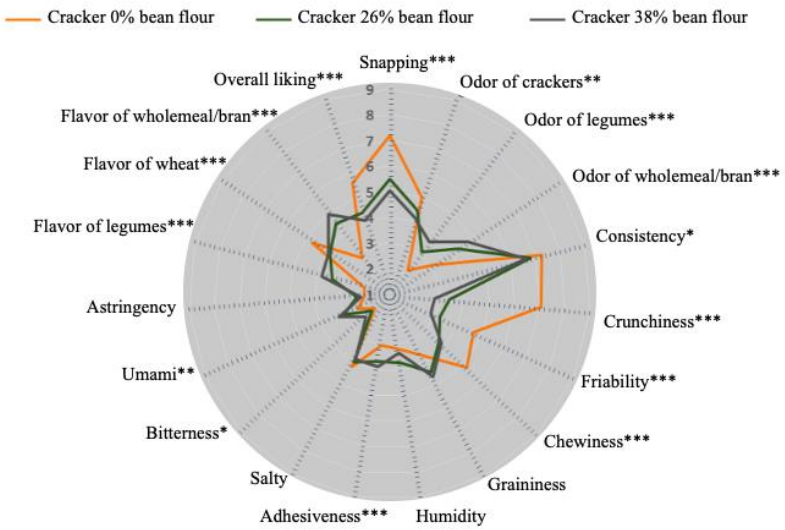

Figure 3-3: Sensorial profile of crackers with and without bean flour

The sensorial profile of crackers is reported in Figure 3-3. The comparison between crackers shows several significant differences. In particular, the control cracker (0% bean flour) has a better snapping and an easier chewiness. In fact, it results more consistent, crunchy and friable. According to odors and flavor, it has a typic cracker odor and a more intense wheat flavor. It is also the most appreciated product. Crackers with 26% and 38% of bean flour have, indeed, a strong odor and flavor of legumes and wholemeal/bran, they are more adhesive in the mouth, slightly more bitter and with a more accentuated umami taste.

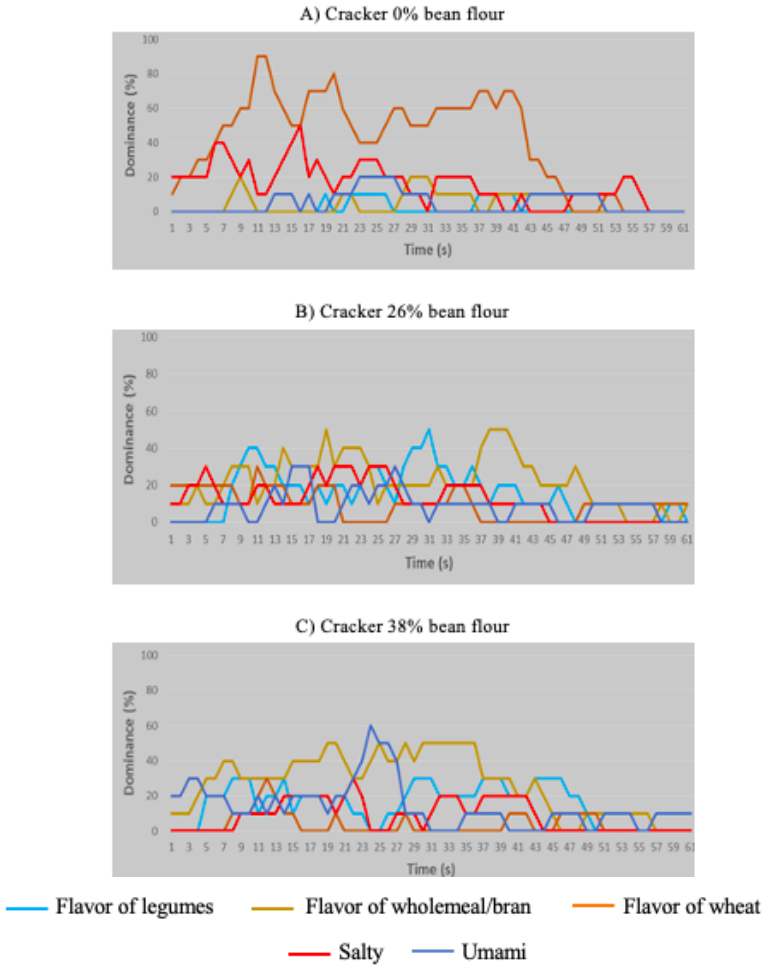

Figure 3-4: TDS flavor of crackers with and without bean flour

Regarding the TDS flavor (Figure 3-4), in the cracker 0% bean flour (Figure 3-4 A) the flavor of wheat predominantly dominates during the whole tasting, partially covering the salty that prevales only during the initial phase. The addition of bean flour affects the aromatic percepton. In particular, adding the 38% of bean flour (Figure 3-4 C) the flavor of wholemeal/bran becomes dominant, but it is disturbed by a sensation of sapidity given by the umami. The flavor of legumes is perceived only at the end. On the contrary, adding 26% of bean flour (Figure 3-4 B), the flavor of legumes is more perceived, recording clear peaks at the beginning and in the middle of the tasting. Salty and the flavor of wholemeal/bran alternate to the flavor of legumes.

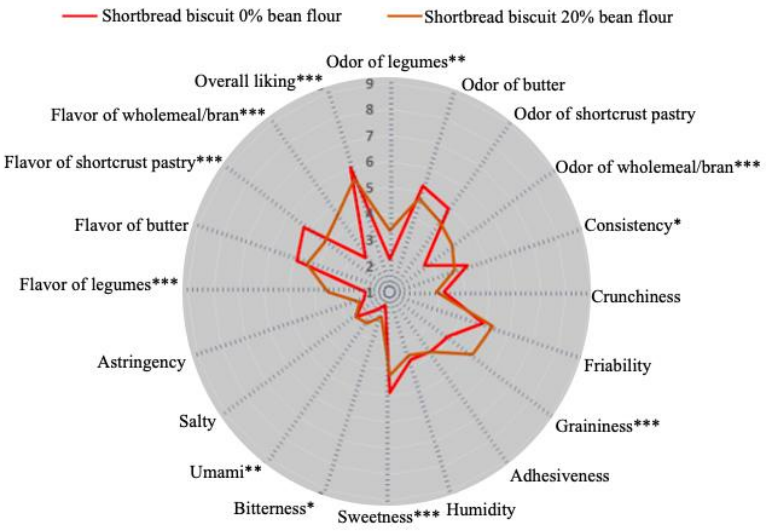

Figure 3-5: Sensorial profile of shortbread biscuits with and without bean flour

The sensorial profiles of shortbread biscuits, with and without bean flour, present several significative differences, which are shown in Figure 3-5. In particular, the biscuit with 20% of bean flour has a more intense odor of wholemeal/bran and legumes and, from a tactile point of view, it is characterized by a greater graininess. It is slightly more bitter and it has a more accentuated umami taste. As regards the aroma, it has a higher flavor of wholemeal/bran and legumes. The shortbread biscuit without bean flour has a better consistency, it is sweeter and it has a stronger shortcrust pastry flavor.

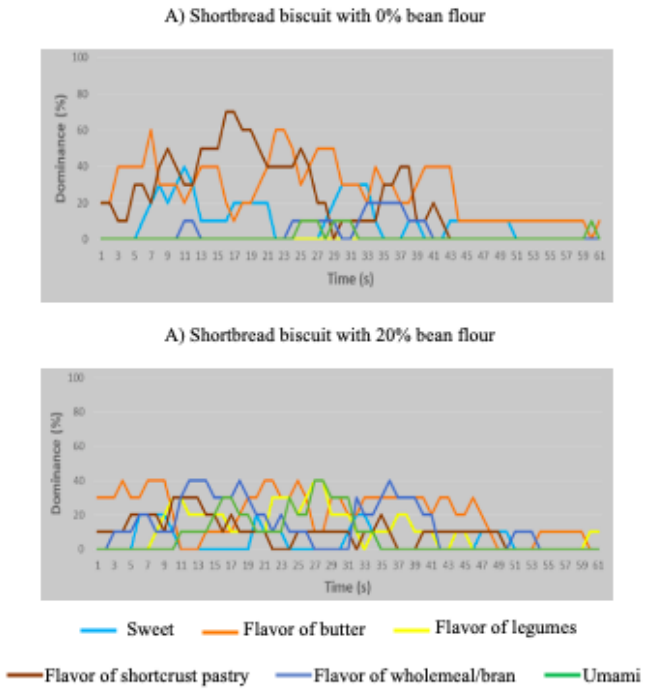

Figure 3-6: TDS flavor of shortbread biscuits with and without bean flour

The TDS flavor of the shortbread biscuit with 0% bean flour (Figure 3-6 A) shows a prevalence of flavor of shortcrust pastry and butter during the whole tasting. There is only a peak of sweetness at about 11 seconds from the beginning. In the biscuit with 20% bean flour (Figure 3-6 B), indeed, the flavor of wholemeal/bran and butter alternate during the tasting (the latter with a lower dominance than in the biscuit without bean flour). This alternance is interrupted after 25 seconds by the flavor of legumes and the umami taste.

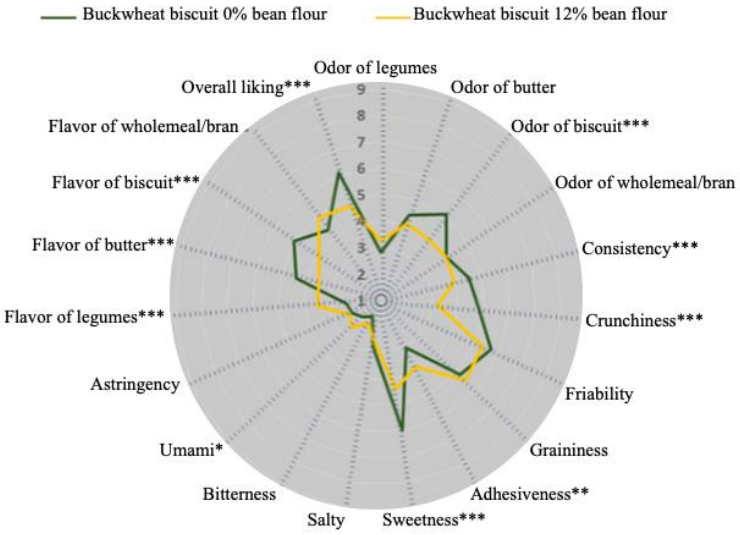

Figure 3-7: Sensorial profile of buckwheat biscuits with and without bean flour

Figure 3-7 shows the sensorial profile of buckwheat biscuits with and without bean flour. It is possible to observe several significant differences among the two biscuits. The biscuit with 12% bean flour has, from a tactile point of view, a higher adhesiveness. It is perceived with a more intense umami taste, so more sapid, and it has a stronger legumes flavor. The biscuit without bean flour is more appreciated and it is characterized by a typic biscuit odor and a better consistency and crunchiness. It is perceived sweeter and with a more intense flavor of butter and biscuit.

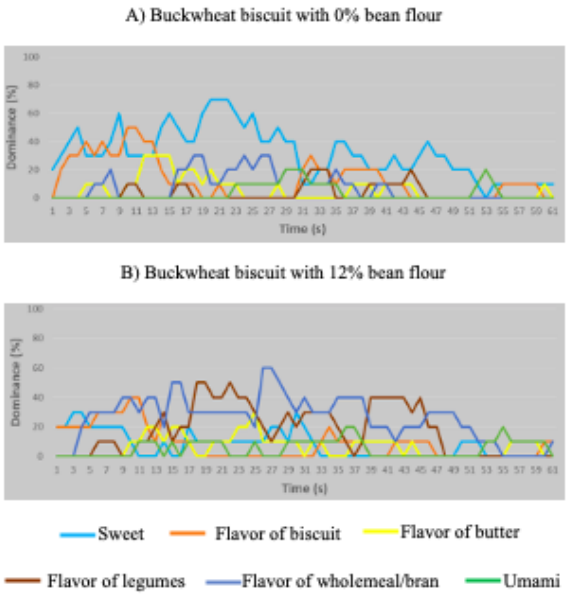

Figure 3-8: TDS flavor of buckwheat biscuits with and without bean flour

Regarding the TDS flavor of buckwheat biscuits (Figure 3-8), in the biscuit without bean flour the sweetness dominates during the whole tasting. Between 5 and 13 seconds. The typic flavor of biscuits alternates with sweetness. On the contrary, in the biscuit with 12% bean flour the sweetness is only perceptible at the beginning, followed by a dominance of flavor of wholemeal/bran and legumes that alternate during the tasting. A slight umami peak is recorded at the end of the tasting (55 seconds).

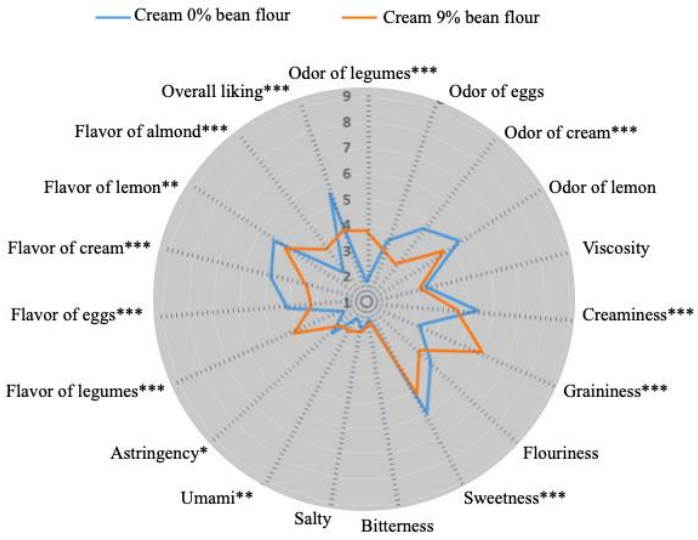

Figure 3-9: Sensorial profile of creams with and without bean flour

The comparison between the sensorial profile of creams with and without bean flour is shown in Figure 3-9, where it is possible to observe several significant differences among the two products. In particular, the cream with 9% bean flour has a strong legumes olfactive intensity, it results grainier and more sapid (high umami perception). From an aromatic point of view, it has a higher flavor of legumes and almond. The cream without bean flour is more appreciated and it is characterized by a typic cream odor, better creaminess and higher sweetness. It is perceived slightly more astringent, with a more intense flavor of eggs, cream and lemon.

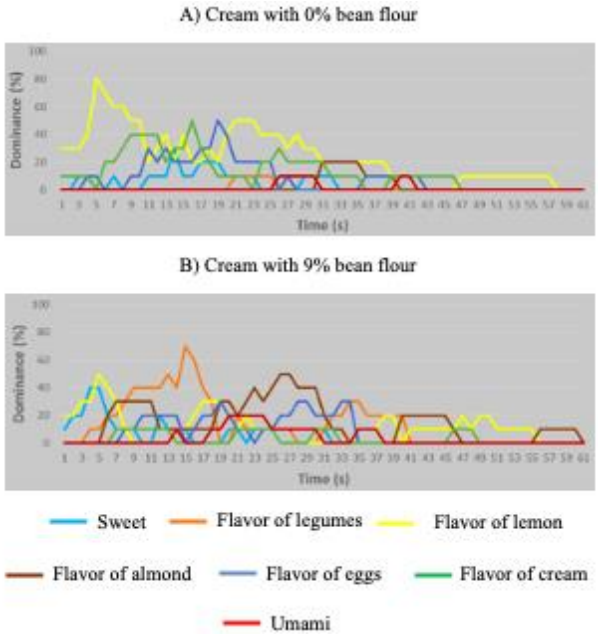

Figure 3-10: TDS flavor of creams with and without bean flour

The TDS taste of creams in Figure 3-10 shows a dominant peak of lemon flavor for the cream without bean flour at the beginning of the tasting (around 11 seconds), followed by the flavor of cream and eggs. The impact of lemon is remarkable, since it is a sensation that dominates during the whole tasting. The cream with 9% bean flour is characterized by a lower lemon flavor, which is substituted by the flavor of legumes and, later, almond. In the middle of the tasting there is a peak related to the flavor of eggs, followed again by the one of legumes, almond and lemon.

CHAPTER 4
DISCUSSION

4.1 **Importance of the use of bean flour in baked snacks**

The nutritional quality of traditional snacks is considered low due to their high sugar and fat content and their protein deficiency. However, for many people they constitute a considerable fraction of daily calories, representing a fast and convenient food source that can be consumed without preparation. Snacks occupy a huge slice of the ready-to-eat food market and are appreciated by consumers at all ages, eaten during work-break by adults and at school recreation by children and adolescents. In some cases, snacks have become real meal substitutes: the tendency of consuming low-value snacks instead of having a complete and balanced breakfast is frequent and common, mainly among teenagers. This issue has been correlated with an increase in the body mass index and poor academic performance of students (Dhurandhar et al., 2014). The increased consumption of energy-dense, nutrient-poor snacks is one of the major and growing concerns associated to the alarming trend of overweight, obesity and metabolic disorders worldwide. This trend is also related to undesirable changes in snack-food patterns, including an increase in portion sizes, added sugars, total fats, saturated and trans-fats (Kerr et al., 2008). For this reason, the snack food market is currently searching for healthier products, characterized by an enhanced nutritional value, a lower caloric content and a more balanced composition, in order to satisfy the changed habits of adults and children and to prevent from the development of chronic diseases. Because of their high nutritional value and their positive performances in heat-treated products, legume flours are ideal ingredients to be used in baked goods and bean flour seems to have a positive response in the formulation of ready-to-eat snacks.

This thesis project is completely located in the context described above, having the purpose of evaluating the properties of bean flour as nutritionally valuable ingredient, as well as the effect of specific genetic characteristics that affects seed composition on the final nutritional value of processed bean-based products.

4.1.1 *Improvement of the protein content and amino acid score*

It is well known that pulses are characterized by a high and qualitative protein fraction. Our results highlight the effect that increasing amounts of bean flour have on the protein content of baked products. Crackers are characterized by a significant content of proteins that increases from 18.3% of cracker 1 (containing 26% of bean flour) to 20.5% of cracker 2 (containing 38% bean flour). As expected, biscuits have a lower protein content (11-12%), that, moreover, partially derive from other ingredients used for their formulation. Our results are consistent with those of a previous study carried out by Sparvoli et al. (2016), where the protein content increased from 7.07% of control biscuit (without bean flour) to 8.07% of biscuit B12 (containing 12% of bean flour), until 10.3% of biscuit B29 (containing 29% of bean flour).

In addition, our data demonstrate that the protein content changes depending on the bean genotype: it is lower in the *wt* while it increases in the *lpa* and even more in the *lpa/lec-*, both for bean flours and baked products (mainly in crackers and biscuit 1), reflecting the fact that *wt* flour contains less crude protein compared to *lpa* and *lpa/lec-* flours. This trend is particularly unusual, since the *lpa/lec-* genotype is characterized by the lack of a consistent protein fraction, the lectins, which most likely has been largely compensated by an increase in the other seed proteins, as their absence doesn't negatively affect the global protein content.

Sparvoli et al. (2016) also demonstrated that the nutritional improvement of the protein fraction of bean-containing products is also related to the amino acid composition. In fact, the addition of bean flour in biscuits enhanced the content of limiting essential amino acids without lowering the content of sulfur-containing amino acids and leading to an improved amino acid score. In particular, the highest effect was on the content of lysine, tryptophane and arginine, which compered to control biscuits raised one to two-fold for lysine (118-275%), 39-134% for tryptophane and 84-108% for arginine. The average increase for all the remaining amino acids was ranging from about 30% (leucine) up to 53% (phenylalanine). Having biscuits with an analogous percentage of bean flour, we can hypothesize a similar improvement of their amino acid fraction. Crackers are characterized by an even higher content of bean flour, so it is legitimate to think that they have a further enhanced amino acid score.

4.1.2 *Reduced rapidly digested carbohydrates and glycemic index*

The addition of bean flour to cereal-based snacks is also responsible for the reduction of rapidly digested carbohydrates (simple sugars and RDS), the main responsible for the development of diabetes and obesity in people. Together with the reduction of these components, bean flour brings a significant increase to the dietary fiber fraction. Looking at

our products, the starch content of crackers is reduced from 46% of cracker 1 (containing 26% of bean flour) to 37.5% of cracker 2 (containing 38% of bean flour). Simple sugars, including saccharose, glucose and fructose are not detected in these products, that are composed by only flour and water. At the same time, cracker 2 is characterized by an improved fiber content (21% against 16.4% of cracker 1) proving that the higher percentage of bean flour in its composition is responsible for such enhancement. In accordance to our results, it is demonstrated that legume-based crackers have a double fiber content if compared to traditional cereal-based ones (Malcolmson et al., 2013).

Differently from crackers, biscuits show a significantly higher sugar content (mainly saccharose) deriving exclusively from the presence of added sugar in their recipes. The starch fraction correlates with the amount of bean flour: from 17% of biscuit 2 (12% of bean flour) to 20.3% of biscuit 1 (20% of bean flour). In this case the contribution of bean flour is not as visible as in crackers, probably due to the more complex composition of biscuits. In fact, unlike the former, the latter show a raise in the starch fraction parallel to the increase of the bean flour in the recipe. This can be explained by the presence of buckwheat and whole wheat flour in biscuit 2, which probably contribute to improve the fiber content at the expenses of starch. On the contrary, biscuit 1 only contains type 2 wheat flour, richer in starch and poorer in fiber than other flours. The fiber content is slightly higher in biscuit 2 (17.4%) than in biscuit 1 (16.8%), proving this hypothesis.

The effect of bean flour on the nutritional composition of biscuits was also demonstrated by Sparvoli et al. (2016), according to which the starch content decreased from 31% of biscuit B12 to 26.4% of biscuit B29 parallel to the increase of the bean flour percentage. At the same time the reduction of the starch fraction was accompanied by a slight increase of dietary fiber.

The effect of bean flour is strictly associated to the improvement of snacks glycemic index. It is possible to observe a significant reduction of this parameter in crackers, contemporary to the increase of the bean flour content: the average pGI shifts from 76.4 from cracker 1 to 58.4 of cracker two. In this case it is easy to associate this difference to the added bean flour, since crackers are composed by few ingredients, all referred to a carbohydrate source. On the contrary, it is more difficult to assess the effect of bean flour on the glycemic index of biscuits due to their formulation. However, they have an average lower pGI than crackers (42.5 in biscuit 1 and 49.1 in biscuit 2), probably derived from the presence of a reduced carbohydrate fraction over the global composition. It is worthy to mention that the pGI of biscuit 1 and 2 is lower that observed in the previous study of Sparvoli et al (2016) that was around 70% for B14 and B22 biscuits, having a percentage of bean flour comparable to that of biscuit 1 and

2 (20% and 12%, respectively). This difference most likely is due to the use of buckwheat and type 2 wheat flours instead of maize and type1 flours.

Theoretically, foods can be categorized into low (<55), medium (55–69), and high glycemic index (>70) (Foster-Powell et al., 2002). Accordingly, our biscuits and crackers are classified as low and medium glycemic index snacks, respectively (except for cracker 1). The presence of an active α-AI, typical of raw common bean seeds, and still active in baked products, is considered to be involved in the glycemic index reduction. In their study, Sparvoli et al. demonstrated that biscuits characterized by the highest α-AI activity were those with the lowest pGI (Sparvoli et al., 2016). We detected the presence of a residual inhibitory activity in baked samples, but unfortunately with no specific connection with their pGI.

4.2 **Importance of seed nutrient genetic modulation for foods quality**

Since common bean is characterized by the presence of several ANCs, in many cases it is difficult to exploit its intersting properties. In paticular, the presence of unwanted components in common bean seeds highly limites their employment beyond the traditional processing and uses and, even worse, it can cause undesired effects if consumed in excessive amounts. Biofortification is optimal to obtain a seed with improved properties, avoiding problems caused by industrial processes (including thermal treatments, extrusion, cooking and dehitration), that, despite their positive effect on the reduction of ANCs, are a source of additional costs and can cause the loss of nutritional value. In our work we assessed the behavior of bean lines characterized by different genetic composition in antinutritional factors in order to evaluate their effects on the final product. Our purpose was to obtain a functional snack in which, the absence of the most common ANCs (lectins and phytates) was responsible for the enhancement of the product nutritional value and for the reduction of its possible negative effects on human health.

4.2.1 *Effect of the use of lpa mutants on the product nutritional value*

Iron deficiency is a serious issue in many areas of the world. Nearly two billion people are currently iron-deficient, especially resource-poor women, infants, and children in developing countries (Mason & Garcia, 1993; World Bank, 1994). The program of biofortification aimed at increasing the amount of iron in the seed is mainly important to guarantee a balanced diet for people in these countries, where legumes, mainly beans, highly contribute to the daily caloric intake. It has been demonstrated that *lpa* bean mutants are able to provide more iron

and, in particular, the reduction of PA by more than 90% significantly increases iron absorption from 60% to 163% (Petry et al., 2013).

However, no one has never verified the effect of the *lpa* genotype on the iron bioavailability inside processed snacks. Our results show that the iron extractability of biscuits containing *lpa* bean genotypes is higher than that of biscuits obtained with the *wt* flour, and these data have been confirmed by an assay of iron bioavailability done with Caco2 cells (Mauro Rossi, ISA-CNR personal communication). Considering the treatment with 0.03 N HCl, it is already possible to observe that the amount of iron obtained from the *lpa/lec-* genotype is much higher than that extracted from the *wt* genotype. This is much more evident considering the 0.1 N HCl treatment, since the amount of iron obtained from *lpa* and *lpa/lec-* genotypes is more than double the percentage extracted from the *wt* genotype. Even the type 2 wheat flour contributes to the total iron content of biscuits. However, this fraction is evidently bound to PA and it cannot be extracted.

4.2.2 *Effect of the use of lpa/lec- mutants on the product nutritional value*

Lectins, and in particular PHAs, may exert a very toxic acion on the consumer if not properly heat inactivated as shown by Petry et al. (2016), who observed unexpected adverse gastrointestinal symptoms in Rwandese women with low iron status participating to a clinical trilal aimed at evaluating the biofortification potential of *lpa* beans. In a very recent work, Cominelli et al. (2020) demonstated that these unwanted effects were due to the contribution of the *lpa* mutation to the thermal stability of PHA-L, while significant effects were observed on PHA-E or PHA-E+PHA-L oligomers. The role of baking on PHA activity was assessed by Sparvoly et al. (2016), which demonstrated that, after baking, samples extracts were still partially able to agglutinate erythrocites, but this activity was significantly decreased with only three minutes of overbaking. Their biscuits retained about 5-10% PHA activity. Our reults confirm the previous finding, showing that no agglutination activity is detected in *lpa/lec-* bean flour and its derivative products. On the contrary, a significant residual agglutinating activity is reported in bean-based baked products containing active PHA. This activity is higher in the *lpa* genotypes for both types of biscuits (about 20-25%) than in *wt* biscuit 1 (about 10-12%). In both biscuits the *lpa* genotype retains a more pronounced activity that correlate to a higher agglutinating activity of the seed flour. Crackers do not show any residual agglutination activity for all the genotypes, probably due to the more efficient heat penetration that allowed the complete PHA inactivation. For this reason, our data confirm that a proper heat treatment is necessary to obtain a partial or complete inactivation of the toxic protein fraction in the PHA containing genotypes.

Crackers and biscuits are typical snacks that can be consumed frequently and in consistent amounts, especially by children. In this case, even the presence of a residual PHA activity can be a problem for the consumer health. For this reason, we recommend the consumption of products obtained through bean mutant lines (*lec*-) and by a proper backing process, in order to avoid any possible risk of lectin poisoning due to frequent and abundant consumption.

4.3 **Consumers acceptability and commercialization of bean-based snacks**

The first step toward the expansion of the legume-based snacks market to more specific consumers sectors is to ensure their sensorial appreciation by target people. In sensory science, the common practice is to conduct hedonic and descriptive analysis separately. Hedonic analysis is performed by consumers who evaluate the products in terms of liking, whereas descriptive analysis is carried out with expert, or trained, panelists who generate sensory profiles of the products. Despite our test was more focused on the latter, we also asked panelists to give a global evaluation to the product, based on their subjective perception. All the bean-based products demonstrate to have an intense umami taste, that is considered a positive attribute associated to these products. However, legume-based snacks are globally less appreciated than traditional ones. In fact, the latter received a higher score for the overall liking and for all the textural attributes, including crunchiness, consistency and friability, which are decisive to attest the quality and the consumer acceptability of extruded products. The less appreciated characteristic of bean-based crackers is not only their lack of friability and crunchiness, typical of traditional ones, but also the presence of an excessive humidity, adhesiveness and chewiness. Even in in the case of creams, the one containing bean flour is characterized by a worse texture, consisting in a higher graininess and a poor creaminess. On the contrary, among bean-based products, biscuits seem to have the best textural attributes and a consistency that is comparable to that of traditional ones. Moreover, judges agree in underlying the dominant flavor and taste of legumes in bean-based crackers (especially in cracker 2), which is intense, even if in a lower extent, also in biscuits and cream. This property may be not appreciated by common consumers, even because it is always accompanied by a pronounced astringency and bitterness.

Our results agree with those of other studies. In Sparvoli et al. (2016) liking scores of bean-based biscuits decreased with the increase of the bean flour content. Moderate to low texture scores were recorded in a research based on the sensorial evaluation of bean-based nutrient-enriched puffed snacks by Natabirwa et al. (2020), according to which this characteristic could be attributed to the protein-starch interactions or the starch-fiber interactions that tend to limit

expansion of extrudates. Regarding flavor attributes, Nyombaire et al. (2011) and Siddiq et al. (2013) also associated low flavor scores to the inherent beany flavors, not desired by consumers.

The acceptance of these new products by the consumer is important in order to embrace the possibility to extend their market to a more diverse and wider group of people. Beyond their interesting use in children feeding, they can be successfully employed as efficient substitutes in diets for people suffering from celiac disease, diabetes and obesity. It is quite common that products characterized by the presence of unusual ingredients, including whole wheat, buckwheat and bean flours, tend to have a lower degree of acceptability. However, usually their perception from consumers changes when these products are related to a detailed description of their health effects. People are more likely to appreciate uncommon foods when they are sure to receive a positive effect on the body from their regular consumption. For this reason, it could be useful to perform a deeper analysis in which the evaluation is also influenced by this aspect, in order to understand the real willingness to buy these products by target consumers. Once we get results in this context, it is possible to modify the products formulation (especially that of crackers, that requires a significant enhancement) to improve their technological, functional and sensorial properties. Moreover, if we want to expand the consume of our snacks to young people, we should be aware about the fact that children have a different approach to products from adults. Even teenagers have a different perception of product quality from their parents (Bech-Larsen & Jensen, 2011) and this will ultimately lead to different hedonic evaluations and different optimal product formulation.

CONCLUSIONS

This study demonstrated the importance of food technology in the development of novel foods and food ingredients. It also highlighted the efficiency of biofortification through genetic breeding in the obtainment of an improved and nutritionally valuable primary product. The feasibility of making nutritionally improved bean-based products was proven by the suitable functional properties of bean flours and verified by the possibility to have a total or partial retention of their nutritional attributes after baking. Both *lpa* and *lpa/lec*- genotypes showed their complete functionality in the final product, further enhanced by the *P. vulgaris* intrinsic properties, mainly related to its protein content and its positive involvement in the glycemic index modulation.

The major criticisms of this study are related to the vary products composition: the presence of several ingredients could divert the attention from the main goal, the bean flour valorization, interfering with the final result. Among them, the impossibility to demonstrate a direct relationship between the α-AI activity and the glycemic index reduction was a partial failure, since it would have been an interesting starting point for the development of bean-based commercially available starch blockers. Moreover, the negative opinion on the sensorial attributes of bean-based products would have been better explained (and maybe partially denied) by a more in-depth consumers analysis based on a hedonistic evaluation.

Despite these few gaps, the study demonstrated the possibility of efficiently developing a bean-based snack having all the required attributes. In particular, biscuit 1 received a sensorial evaluation similar to the control sample and it was the most appreciated by judges among bean-based products. It is characterized by an interesting composition that should be further improved by reducing the lipidic fraction in order to enhance its nutritional value. Additionally, its formulation is characterized by a limited amount of added sugar (if compared to biscuit 2), resulting in the product with the lowest glycemic index. The *lpa/lec*- genotype could be considered the most useful for the development of a biofortified biscuit. In fact, *lpa/lec*- biscuit 1 is not only characterized by the absence of toxic lectins, but also by the presence of a significant amount of bioavailable iron, easily extracted also with a poorly acid

solution. It also showed one of the highest α-AI retained activity: more than half of the activity detected in the raw *lpa/lec*- bean flour was performed by the baked product.

This biscuit could be the object of future in-depth studies, aimed at obtaining a better formulation and further enhanced nutritional and sensorial properties. The consumption and commercialization of this snack can be efficiently proposed among target consumers, including children and people suffering from diabetes, obesity, celiac disease or mineral deficiencies, but also people who make healthy food a real lifestyle.

BIBLIOGRAPHY

Abbas, Y., & Ahmad, A. (2018). Impact of processing on nutritional and antinutritional factors o legumes: a review. *Food Science and Technology*, 19, 199-215.

Akande, K. E., Doma, U. D., Agu, H. O., & Adamu, H. M. (2010). Major antinutrients found in plant protein sources: their effect on nutrition. *Pakistan Journal of Nutrition*, 9(8), 827-832.

Akande, K. E., & Fabiyi, E. F. (2010). Effect of processing methods on some antinutritional factors in legume seeds for poultry feeding. *International Journal of Poultry Science*, 9(10), 996-1001.

Akibode, C. S., & Maredia, M. (2012). *Global and regional trends in production, trade and consumption of food legume crops* (No. 1099-2016-89132).

Aller, E., Abete, I., Astrup, A., Martinez, J. A., & van Baak, M. A. (2011). Starches, sugars and obesity. *Nutrients*, 3, 41-369.

Alonso, R., Orue, E., & Marzo, F. (1998). Effects of extrusion and conventional processing methods on protein and antinutritional factor contents in pea seeds. *Food Chemistry*, 63, 505-12.

Alonso, R., Aguirre, A., & Marzo, F. (2000). Effects of extrusion and traditional processing methods on antinutrients and *in vitro* digestibility of protein and starch in faba and kidney beans. *Food Chemistry*, 68, 159-165.

Altabella, T., & Chrispeels, M. J. (1990). Tobacco plants transformed with the bean α-AI gene express an inhibitor of insect α-amylase in their seeds. *Plant Physiology*, 93(2), 805-810.

Anderson, J. W. (1990). Plant fiber in foods. Lexington, KY: HCF Nutrition Research Foundation, Inc.

Anton, A. A., Fulcher, R. G., & Arntfield, S. D. (2009). Physical and nutritional impact of fortification of corn starch-based extruded snacks with common bean (*Phaseolus vulgaris* L.) flour: effects of bean addition and extrusion cooking. *Food chemistry*, 113, 989-996.

AOAC (2000). *Official Method of Analysis, 7th Edn*. Washington, DC: Association of Official Analytical Chemists.

Arribas, C., Cabellos, C., Cuadrado, C., Guillamón, E., & Pedrosa, M. M. (2019). Bioactive compounds, antioxidant activity, and sensory analysis of rice-based extruded snacks-like fortified with bean and carob fruit flours. *Foods*, 8, 831.

Asif, M., Rooney, L. W., Ali, R., & Riaz, M. N. (2013). Application and opportunities of pulses in food system: a review. *Critical Review in Food Science and Nutrition*, 53, 1168-1179.

Baier, A. K. (2016). Potential of high isostatic pressure and pulsed electric fields for the processing of potato and pea proteins: structural and techno-functional characterization in model solutions and plant tissue. Doctoral Dissertation, Technische Universität, Berlin, Germany.

Barampama, Z., & Simard, R. E. (1993). Nutrient composition, protein quality and antinutritional factors of some varieties of dry beans (*Phaseolus vulgaris*) grown in Burundi. *Food Chemistry*, 47(2), 159-167.

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, 77, 773-798.

Bech-Larsen, T., & Jensen, B. B. (2011). Food quality assessment in parent-child dyads – A hall-test of healthier in-between meals for adolescents. *Food quality and preference*, 22(7), 614-619.

Bender, A. E., & Reaidi, G. B. (1982). Toxicity of kidney beans (*Phaseolus vulgaris*) with particular reference to lectins. *Journal of Plant Foods*, 4, 15-22.

Bennik, M. (2005). Eat beans for good health. *Annual Report of the Bean Improvement Cooperative*, 48, 1-5.

Bhattacharya, S. (2011). Natural antimutagenes: a review. *Research Journal of Medicinal Plant*, 5(2), 116-126.

Birk, Y., & Peri, I. (1980). Saponins. *In Toxic Constituents of Plant Foodstuffs* (pp. 161-182). Elsevier Accademic Press, New York.

Blair, M. W., González, L. F., Kimani, P. M., & Butare, L. (2010a). Genetic diversity, inter-gene pool introgression and nutritional quality of common beans (*Phaseolus vulgaris* L.) from Central Africa. *Theory of Applied Genetics*, 121, 237-248.

Blair, M. W., Monserrate, F., Beebe, S. E., Restrepo, J., & Flores, J. O. (2010b). Registration of high mineral common bean germplasm lines NUA35 and NUA56 from the red-mottled seed class. *Journal of Plant Registrations*, 4, 55-59.

Blair, M. W., & Izquierdo, P. (2012). Use of the advanced backcross-QTL method to transfer seed mineral accumulation nutrition traits from wild to Andean cultivated common beans. *Theory of Applied Genetics* 125, 1015-1031.

Bollini, R., Carnovale, E., & Campion, B. (1999). Removal of antinutritional factors from bean (*Phaseolus vulgaris* L.) seeds. *Biotechnology, Agronomy, Society, and Environment*, 4, 219-219.

Boniglia, C., Carratù, B., Di Stefano, S., Giammarioli, S., Mosca, M., & Sanzini, E. (2008). Lectins, trypsin and α-amylase inhibitors in dietary supplements containing *Phaseolus vulgaris*. *European Food Research and Technology*, 227, 689-693.

Bresciani, A., & Marti, A. (2019). Using pulses in baked products: lights, shadows, and potential solutions. *Foods*, 8(10), 451.

Broughton, W. J., Hernández, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (*Phaseolus* spp.)–model food legumes. *Plant and Soil*, 252, 55-128.

Burbano, C., Muzquiz, M., Ayet, G., Cuadrado, C., & Pedrosa, M. M. (1999). Evaluation of antinutritional factors of selected varieties of *Phaseolus vulgaris*. *Journal of the Science of Food and Agriculture*, 79(11), 1468-1472.

Burstin, J., Gallardo, K., Mir, R. R., Varshney, R. K., & Duc, G. (2011). Improving Protein Content and Nutrition Quality. *Biology and breeding of food legumes*, 314.

Campion, B., Sparvoli, F., Doria, E., Tagliabue, G., Galasso, I., Fileppi, M., Bollini, R., & Nielsen, E. (2009). Isolation and characterisation of an *lpa* (low phytic acid) mutant in common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics*, 118, 1211-1221.

Campion, B., Glahn, R., Tava, A., Perrone, D., Doria, E., Sparvoli, F., Cecotti, R., Dani, V., Nielsen, E. (2012) Genetic reduction of antinutrients in common bean (*Phaseolus vulgaris* L.) seed, increases nutrients and *in vitro* iron bioavailability without depressing main agronomic traits. *Field Crops Research* 141, 27-37.

Campos-Vega, R., Loarca-Piña, G., & Ooman, B. (2010). Minor components of pulses and their potential impact on human health. *Food Research International*, 43, 461-482.

Carbonaro, M., Cappelloni, M., Nicoli, S., Lucarini, M., & Carnovale, E. (1997). Solubility-digestibility relationship of legume proteins. *Journal of Agricultural and Food Chemistry*, 45(9), 3387-3394.

Carbonaro, M., Grant, G., Cappelloni, M., & Pusztai, A. (2000). Perspectives into factors limiting *in vivo* digestion of legume proteins: antinutritional compounds or storage proteins? *Journal of Agriculture and Food Chemistry*, 48, 742-749.

Carbonaro, M. (2006). 7S globulins from *Phaseolus vulgaris* L.: impact of structural aspects on the nutritional quality. *Bioscience, Biotechnology and Biochemistry*, 70(11), 2620-2626.

Cardador-Martínez, A., Loarca Piña, G., & Oomah, B. D. (2002a). Antioxidant activity of common beans (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, 50, 6975-6980.

Cardador-Martínez, A., Castano-Tostado, E., & Loarca Piña, G. (2002b). Antimutagenic activity of natural phenolic compounds present in the common bean (*Phaseolus* V*ulgaris*) against aflatoxin B1. *Food Additives and Contaminants*, 19, 62-69.

Champ, M. M.-J. (2002). Non-nutrient bioactive substances of pulses. *British Journal of Nutition*, 88 (Suppl. 3), S307-S319.

Chang, K. C., & Satterlee, L. D. (1981). Isolation and characterization of the major protein from Great Northern beans (*Phaseolus vulgaris*). *Journal of Food Science*, 46(5), 1368-1373.

Chen, W. J., Anderson, J. W., & Jennings, D. (1984). Propionate may mediate the hypocholesterolemic effects of certain soluble plant fibers in cholesterol-fed rats. *Proceedings of the Society for Experimental Biology and Medicine*, 175, 215–218

Chen, Y., Djalovic, I., & Siddique, K. H. M. (2018). Advances in understanding grain legume physiology: understanding root architecture, nutrient uptake and response to abiotic stress. In *Achieving sustainable cultivation of grain legumes: Advances in Breeding and Cultivation Techniques* (pp. 11-27). Burleigh Dodds Science Publishing, Cambridge.

Cheryan, M., & Rackis, J. J. (1980). Phytic acid interactions in food systems. *Critical Reviews in Food Science & Nutrition*, 13(4), 297-335.

Chibbar, R., Ambigaipalan, P., Hoover, R. (2010). Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. *Cereal Chemistry*, 7, 342-352.

Cominelli, E., Confalonieri, M., Carlessi, M., Cortinovis, G., Daminati, M. G., Porch, T. G., Losa, A., & Sparvoli, F. (2018). Phytic acid transport in *Phaseolus vulgaris*: a new low phytic acid mutant in the PvMRP1 gene and study of the PvMRPs promoters in two different plant systems. *Plant science*, 270, 1-12.

Cominelli, E., Rodiño, A. P., De Ron, A. M., & Sparvoli, F. (2019). Genetic approaches to improve common bean nutritional quality: current knowledge and future perspectives. *In Quality Breeding in Field Crops* (pp. 109-138). Springer, Cham.

Cominelli, E., Galimberti, M., Pongrac, P., Landoni, M., Losa, A., Paolo, D., Daminati, M.

G., Bollini, R., Cichy, K. A., Vogel-Mikus, K., & Sparvoli, F. (2020). Calcium redistribution contributes to the hard-to-cook phenotype and increases PHA-L lectin thermal stability in common bean low phytic acid 1 mutant seeds. *Food Chemistry*, 126680.

Confalonieri, M., Bollini, R., Berardo, N., Vitale, A., & Allavena, A. (1992). Influence of phytohemagglutinin on the agronomic performance of beans (*Phaseolus vulgaris* L). *Plant breeding*, 109(4), 329-334.

Da Silva Fialho, L., Guimarães, V. M., De Barros, E. G., Moreira, M. A., Dos Santos Dias, L. A., De Almeida Oliveira, M. G., José, I. C., & De Rezende, S. T. (2006). Biochemical composition and indigestible oligosaccharides in *Paseolus vulgaris* L. seeds. *Plant Foods for Human Nutrition*, 61, 87-89.

De Lumen, B. O. (1992). Molecular strategies to improve protein quality and reduce flatulence in legumes: a review. *Food Structure*, 11(1), 4.

De Ron, A. M. (ed.). (2015). *Grain legumes* (Vol. 10). Springer.

Derbyshire, E., Wright, D. J., & Boulter, D. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry*, 15, 3-24.

Deshpande, S. S., & Damodaran, S. (1989). Structure-digestibility relationship of legume 7S proteins. *Journal of Food Science*, 54(1), 108-113.

Dhurandhar, E. J., Dawson, J., Alcorn, A., Larsen, L. H., Thomas, E. A., Cardel, M., Bourland, A. C., Astrup, A., St-Onge, P., Hill, J. O., Apovian, C. M. Shikany, J. M., & Allison, D. B. (2014). The effectiveness of breakfast recommendations on weight loss: a randomized controlled trial. *The American journal of clinical nutrition*, 100(2), 507-513.

Di Cairano, M., Galgano, F., Tolve, R., Caruso, M. C., & Condelli, N. (2018). Focus on gluten free biscuits: ingedients and issues. *Trends in Food Science & Technology*, 81, 203-212.

Donangelo, C. M., Woodhouse, L. R., King, S. M., Toffolo, G., Shames, D. M., Viteri, F. E., Cheng, Z., Welch, R. M., & King, J. C. (2003). Iron and zinc absorption from two bean (*Phaseolus vulgaris* L.) genotypes in young women. *Journal of agricultural and food chemistry*, 51(17), 5137-5143.

Doria, E., Campion, B., Sparvoli, F., Tava, A., & Nielsen, E. (2012). Antinutrient components and metabolites with health implications in seeds of 10 common bean (*Phaseolus vulgaris* L. and *Phaseolus lunatus* L.) landraces cultivated in southern Italy. J*ournal of Food Composition and Analysis*, 26, 72-80.

El-Adawy, T. A. (2002). Nutritional composition and antinutritional factors of chickpeas (*Cicer arietinum* L.) undergoing different cooking methods and germination. *Plant*

Foods for Human Nutrition, 57, 83-97.

El Beltagy, A. E. M. (1996). Effect of home traditional methods on quality aspects of some legumes. MS Thesis, Menofya University, Shibin El-Kom, Egypt.

Englyst, H. N., Kingman, S. M., & Cummings, J. H. (1992). Classification and measurement of nutritionally important starch fractions. *European journal of clinical nutrition*, 46, S33-50.

Espinoza-Moreno, R. J., Reyes-Moreno, C., Milán-Carrillo, J., López-Venezuela, J. A., Paredes-López, O., & Gutiérrez-Dorado, R. (2016). Healthy ready-to-heat expanded snack with high nutritional and antioxidant value produced fromwhole amarantin transgenic maize and black common bean. *Plant Foods and Human Nutrition*, 71, 218-224.

Estrada-Girón, Y., Martínez-Preciado, A. H., Michel, C. R., & Soltero, J. F. A. (2015). Characterization of extruded blends of corn and beans (*Phaseolus vulgaris*) cultivars: Peruano and black-querétaro under different extrusion conditions. *International Journal of Food Properties*, 18(12), 2638-2651.

Evans, R. J., & Bauer, D. H. (1978). Studies of the poor utilization by the rat of methionine and cystine in heated dry bean seed (*Phaseolus vulgaris*). *Journal of agricultural and food chemistry*, 26(4), 779-784.

FAOSTAT/FAO. http://www.fao.org/faostat/en/#data/QC, accessed March 2020.

Félix-Medina, J. V., Montes-Ávila, J., Reyes-Moreno, C., Perales-Sánchez, J. X. K., Gómez-Favela, M. A., Aguilar-Palazuelos, E., & Gutiérrez-Dorado, R. (2020). Second-generation snacks with high nutritional and antioxidant value produced by an optimized extrusion process from corn/common bean flours mixtures. *LWT*, 124, 109172.

Ferawati, F., Hefni, M., & Witthöft, C. (2019). Flours from Swedish pulses: effects of treatment on functional properties and nutrient content. *Food Science and Nutrition*, 7, 4116-4126.

Foschia, M., Horstmann, S. W., Arendt, E. K., & Zannini, E. (2017). Legumes as functional ingredients in gluten-free bakery and pasta products. *Annual Review of Food Science and Technology* 8, 75-96.

Foster-Powell, K., & Miller, J. B. (1995). International tables of glycemic index. *The American journal of clinical nutrition*, 62(4), 871S-890S.

Foster-Powell, K., Holt, S. H., & Brand-Miller, J. C. (2002). International table of glycemic index and glycemic load values: 2002. *The American journal of clinical nutrition*, 76(1), 5-56.

Gabius, H. J., André, S., Jiménez-Barbero, J., Romero, A., & Solís, D. (2011). From lectin structure to functional glycomics: principles of the sugar code. *Trends in biochemical sciences*, 36(6), 298-313.

Gemede, H. F., & Ratta, N. (2014). Antinutritional factors in plant foods: benefits and adverse effects. *International Journal of Nutrition and Food Sciences*, 3(4), 284-289.

Giuberti, G., Gallo, A., Cerioli, C., Fortunati, P., & Masoero, F. (2015). Cooking quality and starch digestibility of gluten free pasta using new bean flour. *Food Chemistry*, 175,43-49.

Giuberti, G., & Gallo, A. (2018). Reducing the glycaemic index and increasing the slowly digestible starch content in gluten-free cereal-based foods: a review. *International Journal of Food Science and Technology*, 53, 50-60.

Giuberti, G., Tava, A., Mennella, G., Pecetti, L., Masoero, F., Sparvoli, F., Lo Fiego, A., & Campion, B. (2019). Nutrients' and antinutrients' seed content in common bean (*Phaseolus vulgaris* L.) lines carrying mutations affecting seed composition. *Agronomy*, 9, 317.

Glahn, R. P., Lee, O. A., Yeung, A., Goldman, M. I., & Miller, D. D. (1998). Caco-2 cell ferritin formation predicts nonradiolabeled food iron availability in an *in vitro* digestion/Caco-2 cell culture model. *The Journal of nutrition*, 128(9), 1555-1561.

Glahn, R. (2009). The use of Caco-2 cells in defining nutrient bioavailability: Application to iron bioavailability of foods. In *Designing Functional Foods* (pp. 340-361). Woodhead Publishing, UK.

Goldstein, D. J. (1992). Beneficial health effects of modest weight loss. *International Journal of Obesesity and Related Metabolic Disorders*, 16, 397-415.

Grant, G., More, L. J., McKenzie, N. H., Stewart, J. C., & Pusztai, A. (1983). Survey on the nutritional and hemagglutination properties of legume seeds generally available in the UK. *British Journal of Nutrition*, 9(20), 7-214

Grant, G., & Van Driescche, E. (1993). Legume lectins: physicochemical and antinutritional properties. In *Recent advances of research in antinutritional factors in legume seeds.* Wageningen Pers, WUR, Netherlands.

Grela, E. R., & Günter, K. D. (1995). Fatty acid composition and tocopherol content of some legume seeds. *Animal Feed Science and Technology* 52, 325-331.

Guillon, F., & Champ, M. M. (2002). Carbohydrate fractions of legumes: uses in human nutrition and potential for health. *British Journal of Nutriton 88* (Suppl. 3), S293-S306.

Gulewicz, P., Martinez-Villaluenga, C., Kasprowicz-Potocka, M., & Frias, J. (2014). Non-

nutritive compounds in Fabaceae family seeds and the improvement of their nutritional quality by traditional processing-a review. *Food Nutriton and Science*, 64, 65-89.

Hague, A., & Paraskeva, C. (1995.) The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. *European Journal of Cancel Prevention*, 4, 359-364.

Hayat, I., Ahmad, A., Masud, T., Ahmed, A., & Bashir, S. (2014). Nutritional and health perspectives of beans (*Phaseolus vulgaris* L.): an overview. *Critical reviews in Food Science and Nutrition*, 54, 580-592.

Hedley, C. L. (ed.) (2001). *Carbohydrates in grain legume seeds: improving nutritional quality and agronomic characteristics*. CABI, New York.

Henningson, A. M., Nyman, E. M., & Bjorck, I. M. (2001). Content of short-chain fatty acids in the hindgut of rats fed processed bean (*Paseolus vulgaris*) flours varying in distribution and content of indigestible carbohydrates. *British Journal of Nutrition*, 86, 379-89.

Hitz, W. D., Carlson, T. J., Kerr, P. S., & Sebastian, S. A. (2002). Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. *Plant physiology*, 128(2), 650-660.

Hooper, S. D., Glahn, R. P., & Cichy, K. A. (2019). Single varietal dry bean (*Phaseolus vulgaris* L.) pastas: nutritional profile and consumer acceptability. *Plant Foods for Human Nutrition*, 74(3), 342-349.

Hurrell, R. F., Reddy, M., & Cook, J. D. (1999). Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *British Journal of Nutrition*, 81(4), 289-295.

Jansman, A. J. M. (1993). Tannins in feedstuffs for simple-stomached animals. *Nutrition Research Reviews*, 6, 209-236.

Jansman, A. J. M., Hill, G. D., Huisman, J., & van der Poel, A. F. B. (eds.) (1998). *Recent advances of research in antinutritional factors in legume seeds and rapeseed.* EAAP Publication, Netherlands.

Judprasong, K., Charoenkiatkul, S., Sungpuag, P., Vasanachitt, K., & Nakjamanong, Y. (2006). Total and soluble oxalate contents in Thai vegetables, cereal grains and legume seeds and their changes after cooking. *Journal of Food Composition and Analysis*, 19(4), 340-347.

Kaur, M., & Singh, N. (2005). Studies on functional, thermal and pasting properties of flours from different chickpea (*Cicer arietinum* L.) cultivars. *Food Chemistry*, 91, 403-411.

Kerr, M. A., Rennie, K. L., McCaffrey, T. A., Wallace, J. M., Hannon-Fletcher, M. P., & Livingstone, M. B. E. (2008). Snacking patterns among adolescents: a comparison of

type, frequency and portion size between Britain in 1997 and Northern Ireland in 2005. *British Journal of Nutrition*, 101(1), 122-131.

Kohajdová, Z., Karovičová, J., & Magala, M. (2011). Utilisation of chickpea flour for crackers production. *Acta Chimica Slovaca*, 4, 2, 98-107.

Krupa, U. (2008). Main nutritional and antinutritional compounds of bean seeds–a review. *Polish Journal of Food and Nutrition Science*, 58, 149-155.

Kumar, V., Sinha, A. K., Makkar, H. P., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food chemistry*, 120(4), 945-959.

Kun-Young, P., Geun-Ok, J., Kyung-Tae, L., Jongwon, C., Moo-Young, C., Gab-Tae, K., Hyun-Ju, J., & Hee-Juhn, P. (2004). Antimutagenic activity of flavonoids from the heartwood of *Rhus verniciflua*. *Journal of Ethnopharmacology*, 90, 73-79.

Kusaba-Nakayama, M., Ki, M., Iwamoto, M., Shibata, R., Sato, M., & Imaizumi, K. (2000). CM3, one of the wheat α-amylase inhibitor subunits, and binding of IgE in sera from Japanese with atopic dermatitis related to wheat. *Food Chemistry and Toxicology*, 38, 179-185.

Layer, P., Carlson, G. L., & Di Magno, E. P. (1985). Partially purified white bean amylase inhibitor reduces starch digestion *in vitro* and inactivates intraduodenal amylase in humans. *Gastoenterology*, 88, 1895-1902.

Lioi, L., Sparvoli, F., Galasso, I., Lanave, C., & Bollini, R. (2003). Lectin-related resistance factors against bruchids evolved through a number of duplication events. *Theoretical and Applied Genetics*, 107(5), 814-822.

Lis, H., & Sharon, N. (1998). Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chemical Reviews*, 98, 637-674.

Lockyer, S., White, A., & Buttriss, J. L. (2018). Biofortified crops for tackling micronutrient deficiencies–what impact are these having in developing countries and could they be of relevance within Europe? *Nutrition bullettin*, 43, 319-357.

Mabaleha, M. B., & Yeboah, S. O. (2004). Characterization and compositional studies of the oils from some legume cultivars, *Phaseolus vulgaris*, grown in Southern Africa. *Journal of the American Oil Chemists' Society*, 81,361-364.

Malcolmson, L., Boux, G., Bellido, A. S., & Frohlich, P. (2013). Use of pulse ingredients to develop healthier baked products. *Cereal Foods World*, 58(1), 27-32.

Mason, J. B., & Garcia, M. (1993). Micronutrient deficiency–the global situation. *Science News*, 9, 11-16.

Megazyme (2017). Assay kit K-TDFR, Megazyme international, Wicklow, Ireland.

Megazyme (2018). Assay kit K-SUFRG, Megazyme International, Wicklow, Ireland.

Megazyme (2019). Assay kit K-TSTA, Megazyme International, Wicklow, Ireland.

Meng, X., Threinen, D., Hansen, M., & Driedger, D. (2010). Effects of extrusion conditions on system parameters and physical properties of a chickpea flour-based snack. *Food Research International*, 43(2), 650–658.

Messina, V. (2014). Nutritional and health benefits of dried beans. *American Journal of Clinical Nutrition*, 100 (suppl), 437S-442S.

Morari, D., Stepurina, T., & Rotari, V. I. (2008). Calcium ions make phytohemagglutinin resistant to trypsin proteolysis. *Journal of Agricultural and Food Chemistry*, 56(10), 3764–3771.

Moriano, M. E., Ceppa, C., Casiraghi, M. C., Ciappellano, S., Romano, A., Torri, L., & Alamprese, C. (2019). Reduced-fat biscuits: interplay among structure, nutritional properties and sensory acceptability. *Food Science and Technology*, 109, 467-474.

Mulambu, J., Andersson, M., Palenberg, M., Pfeiffer, W., Saltzman, A., Birol, E., Oparinde, A., Boy, E., Asare-Marfo, D., Lubobo, A., Mukankusi, C., & Nkalubo, S. (2017). Iron beans in Rwanda: crop development and delivery experience. *African journal of food, agriculture, nutrition and development*, 17(2), 12026-12050.

Natabirwa, H., Nakimbugwe, D., Lung'aho, M., Tumwesigye, K. S., & Muyonga, J. H. (2020). Bean-based nutrient-enriched puffed snacks: Formulation design, functional evaluation, and optimization. *Food Science & Nutrition*, 1-10.

Nie, Q., Chen, H., Hu, J., Tan, H., Nie, S., & Xie, M. (2020). Effects of non-digestible oligosaccharides on obesity. *Annual Review of Food Science and Technology*, 11.

Nissar, L., Ahad, T., Naik, H. R., & Hussian, S. Z. (2017). A review phytic acid: as antinutrient or nutraceutical. *Journal of Pharmacognosy and Phytochemistry*, 6(6), 1554-1560.

Noonan, S. C., & Savage, G. P. (1999). Oxalate content of foods and its effect on humans. *Asia Pacific Journal of Clinical Nutrition*, 8(1), 64-74.

Nosworthy, M. G., Franczyk, A., Zimoch-Korzycka, A., Appah, P., Utioh, A., Neufeld, J., & House, J. D. (2017). Impact of processing on the protein quality of pinto bean (*Phaseolus vulgaris*) and buckwheat (*Fagopyrum esculentum* Moench) flours and blends, as determined by *in vitro* and *in vivo* methodologies. *Journal of agricultural and food chemistry*, 65(19), 3919-3925.

Nyombaire, G., Siddiq, M., & Dolan, K. D. (2011). Physico-chemical and sensory quality of extruded light red kidney bean (*Phaseolus vulgaris L*.) porridge. *LWT-Food Science and Technology*, 44(7), 1597-1602.

O'Neil, J. O. (2010). Gluten-free foods: Trends, challenges, and solutions. *Cereal Foods World*, 55, 220.

Obiro, W. C., Zhang, T., & Jiang, B. (2008). The nutraceutical role of *Phaseolus vulgaris* α-amylase inhibitor. *British Journal of Nutrition*, 100, 1-12.

OECD (2019). Safety assessment of foods and feeds derived from transgenic crops, *Volume 3: Common bean, rice, cowpea and apple compositional considerations, Novel Food and Feed Safety*. OECD Publishing, Paris, https://doi.org/10.1787/f04f3c98-en.

Panzeri, D., Cassani, E., Doria, E., Tagliabue, G., Forti, L., Campion, B., Bollini, R., Brearley. C. A., Pilu, R., Nielsen, E., & Sparvoli, F. (2011). A defective ABC transporter of the MRP family, responsible for the bean *lpa1* mutation, affects the regulation of the phytic acid pathway, reduces seed myo-inositol and alters ABA sensitivity. *New Phytologist*, 191(1), 70-83.

Patrascu, L., Vasilean, I., Banu, I., & Aprodu, I. (2017). Functional properties of pulse flours and their opportunities in spreadable food products. *Quality Assurance and Safety Crops & Foods*, 09(1), 67-78.

Petry, N., Egli, I., Campion, B., Nielsen, E., & Hurrell, R. (2013). Genetic reduction of phytate in common bean (*Phaseolus vulgaris* L.) seeds increases iron absorption in young women. *Journal of Nutrition*, 143, 1219-1224.

Petry, N., Rohner, F., Gahutu, J. B., Campion, B., Boy, E., Tugirimana, P. L., Zimmermann, M. B., Zwahlen, C., Wirth, J. P., & Moretti, D. (2016). In Rwandese women with low iron status, iron absorption from low-phytic acid beans and biofortified beans is comparable, but low-phytic acid beans cause adverse gastrointestinal symptoms. *The Journal of nutrition*, 146(5), 970-975.

Peumans, W. J., & Van Damme, J. M. (1995). Lectins as plant defense proteins. *Plant Physiology*, 109, 347-352.

Pfeiffer, W. H., & McClafferty, B. (2007). HarvestPlus: breeding crops for better nutrition. *Crop Science*, 47(Supplement 3), S88-S105.

Prakash, B., Selvaraj, S., Murthy, M. R. N., Sreerama, Y. N., Rao, D. R., & Gowda, L. R. (1996). Analysis of the amino acid sequences of plant Bowman-Birk inhibitors. *Journal of Molecular Evolution*, 42(5), 560-569.

Price, K. R., Lewis, J., Wyatt, G. M., & Fenwick, G. R. (1988). Review article Flatulence–Causes, relation to diet and remedies. *Food/Nahrung*, 32(6), 609-626.

Pusztai, A. (1991). *Plant lectins*. Cambridge University Press, Cambridge, UK.

Pusztai, A., Begbie, R., Grant, G., Ewen, S. W. B., & Bardocz, S. (1991). Indirect effects of

food antinutrients on protein digestibility and nutritional value of diets. In *In vitro digestion for pig and poultry* (pp 45-61). CAB international, Wallingford, Oxon, UK.

Rackis, J. J. (1981). Flatulence caused by soya and its control through processing. *Journal of the American Oil Chemists' Society*, 58(3), 503-509.

Rawal, V., & Navarro, D. K. (eds.). (2019). *The global economy of pulses*. FAO, Rome.

Reddy, N. R., & Pierson, M. D. (1984). Chemical, nutritional and physiological aspects of dry bean carbohydrates–a review. *Food Chemistry*, 13, 25-68.

Reimerdes, E. H. (1990). New impacts for food science and food industry–view from outside. In *Processing and Quality of Foods, Vol 1* (pp. 14-111). Elsevier Applied Science, London, UK.

Rocchetti, G., Lucini, L., Rodrigues, J. M. L., Barba, F. J., & Giuberti, G. (2019). Gluten-free flours from cereals, pseudocereals and legumes: Penolic fingerprints and *in vitro* antioxidant properties. *Food Chemistry*, 271, 157-164.

Ryan, K. J., Homco-Ryan, C. L., Jenson, J., Robbins, K. L., Prestat, C., & Brewer, M. S. (2002). Lipid extraction process on texturized soy flour and wheat gluten protein-protein interactions in a dough matrix. *Cereal Chemistry*, 79, 434–38

Sanchez-Monge, R., Garcia-Casado, G., Lopez-Otin, C., Armentia, A., & Salcedo, G. (1997). Wheat flour peroxidase is a prominent allergen associated with baker's asthma. *Clinical and Experimental Allergy*, 27, 1130-1137.

Sarwar, G., & Peace, R. W. (1986). Comparison between true digestibility of total nitrogen and limiting amino acids in vegetable proteins fed to rats. *Journal of Nutrition*, 116, 1172-1184.

Sasaki, M., Fitzgerald, A. J., Grant, G., Ghatei, M. A., Wright, N. A., & Goodlad, R. A. (2002). Lectins can reverse the distal intestinal atrophy associated with elemental diets in mice. *Alimentary pharmacology & therapeutics*, 16(3), 633-642.

Sciarini, L. S., Ribotta, P. D., Leon, A. E., & Perez, G. T. (2010). Influence of gluten-free flours and their mixtures on batter properties and bread quality. *Food and Bioprocess Technology*, 3, 577–85

Setyaningsih, D. N., Fathonah, S., Putri, R. D. A., Auda, A. K., & Solekah, N. (2019). The influence of baking duration on the sensory quality and the nutrient content of mung bean biscuits. *Food Research*, 777-82.

Shahzadi, N., Butt, M. S., Rehman, S. U., & Sharif, K. (2005). Chemical characteristics of various composite flours. *International Journal of Agriculture & Biology*, 7(1), 105-108.

Shimelis, E. A., & Rakshit, S. K. (2007). Effect of processing on antinutrients and *in vitro*

protein digestibility of kidney bean (*Phaseolus vulgaris* L.) varieties grown in East Africa. *Food Chemistry*, 103, 161-172.

Shunmugam, A. S. K., Liu, X., Stonehouse, R., Tar'An, B., Bett, K. E., Sharpe, A. G., & Warkentin, T. D. (2015). Mapping seed phytic acid concentration and iron bioavailability in a pea recombinant inbred line population. *Crop Science*, 55(2), 828-836.

Siddiq, M., Kelkar, S., Harte, J. B., Dolan, K. D., & Nyombaire, G. (2013). Functional properties of flour from low-temperature extruded navy and pinto beans (*Phaseolus vulgaris* L.). *LWT-Food Science and Technology*, 50(1), 215-219.

Socorro, M., Levy-Benshimol, A., & Tovar, J. (1989). *In vitro* digestibility of cereals and legumes (*Phaseolus vulgaris*) starches by bovine pancreas and human pancreatic α-amylase. *Starch*, 41, 69-71.

Sparvoli, F., Bollini, R., & Cominelli, E. (2015). Nutritional value. In *Handbook of plant breeding. Journal of Chemical Information and Modeling* (pp. 291-325). Springer, New York.

Sparvoli, F., & Cominelli, E. (2015). Seed biofortification and phytic acid reduction: a conflict of interest for the plant? *Plants*, 4(4), 728-755.

Sparvoli, F., Laureati, M., Pilu, R., Pagliarini, E., Toschi, I., Giuberti, G., Fortunati, P., Daminati, M. G., Cominelli, E., & Bollini, R. (2016). Exploitation of common bean flour with low antinutrient content for making nutritionally enhanced biscuits. *Frontiers in Plant Science*, 7, 928.

Stanger, O. (2002). Physiology of folic acid in health and disease. *Current Drug Metabolism*, 3(2), 211-223.

Thakur, A., Sharma, V., & Thakur, A. (2019). An overview of antinutritional factors in food. *International Journal of Conservaion Science*, 7(1), 2472-2479.

Thompson, L. U., Tenebaum, A. V., & Hui, H. (1986). Effect of lectins and the mixing of proteins on rate of protein digestibility. *Journal of Food Science*, 51(1), 150-152.

Thongram, S., Tanwar, B., Chauhlan, A., & Kumar V. (2016). Physicochemical and organoleptic properties of cookies incorporated with legume flours. *Cogent Food & Agriculture*, 2.

Tuntipopipat, S., Judprasong, K., Zeder, C., Wasantwisut, E., Winichagoon, P., Charoenkiatkul, S., Hurrell, R., & Walczyk, T. (2006). Chili, but not turmeric, inhibits iron absorption in young women from an iron-fortified composite meal. *The Journal of nutrition*, 136(12), 2970-2974.

Venter, C. S., & van Eyssen, E. (2001). More legumes for better overall health. *South Africa*

Journal of Clinical Nutrition, Magnesium (mg), 172, 280.

Vitale, A., & Bollini, R. (1995). Legume storage proteins. In *Seed Development and Germination* (pp. 73-102). Dekker, New York.

Warkentin, T. D., Delgerjav, O., Arganosa, G., Rehman, A. U., Bett, K. E., Anbessa, Y., Rossnagel, B., & Raboy, V. (2012). Development and characterization of low-phytate pea. *Crop science*, *52*(1), 74-78.

Weber, T. H. (1969). Isolation and characterization of a lymphocyte-stimulating leucoagglutinin from red kidney beans (*Phaseolus vulgaris*). *Scandinavian journal of clinical and laboratory investigation. Supplementum*, 111, 1-80.

Weder, J. K. P., & Kahley, R. (2003). Reaction of lentil trypsin-chymotrypsin inhibitors with human and bovine proteinases. *Journal of Agriculture and Food Chemistry*, 51, 8045-8050.

White, P. J., & Broadley, M. R. (2005). Biofortifying crops with essential mineral elements. *Trends in plant science*, 10(12), 586-593.

White, P. J., & Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets–Iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist*, 182, 49-84.

Whitehead, R.H., Young, G. P., & Bhathal, P. S. (1986). Effects of short chain fatty acids on a new human colon carcinoma cell line (LIM1215). *Gut*, 27, 1457–1463.

Wilcox, J. R., Premachandra, G. S., Young, K. A., & Raboy, V. (2000). Isolation of high seed inorganic P, low-phytate soybean mutants. *Crop Science*, 40(6), 1601-1605.

World Bank (1994). The challenge fo dietary deficiencies of vitamins and minerals. In *Enriching lives: Overcoming Vitamin and Mineral Malnutrition in Developing Countries* (pp 6-13). World Bank, Washington, DC.

Yuan, F. J., Zhao, H. J., Ren, X. L., Zhu, S. L., Fu, X. J., & Shu, Q. Y. (2007). Generation and characterization of two novel low phytate mutations in soybean (*Glycine max* L. Merr.). *Theoretical and Applied Genetics*, 115(7), 945-957.